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A simple in vitro assay to measure the activity of geranylgeranyl diphosphate synthase and other short-chain prenyltransferases

M. Victoria Barja and Manuel Rodriguez-Concepcion*

Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, 08193 Barcelona, Spain.

(*) Corresponding author: manuel.rodriguez@cragenomica.es

1. Running head

Determination of GGPP synthase activity
ii. Abstract

Most carotenoids are C40 metabolites produced from C20 geranylgeranyl diphosphate (GGPP). The enzymes that produce this precursor, GGPP synthases (GGPPS), are members of the short-chain prenyltransferase (SC-PT) family. SC-PTs are enzymes that catalyze the sequential head-to-tail addition of one or more C5 molecules of isopentenyl diphosphate (IPP) to dimethylallyl diphosphate (DMAPP) with the concomitant release of pyrophosphate (PPi). SC-PTs produce linear isoprenyl diphosphates of up to C20 (GGPP) that serve as precursors for many groups of isoprenoids with a wide range of essential biological functions in Eucarya, Bacteria and Archaea. Enzymatic analysis of SC-PT activity normally requires complex, laborious and expensive methods such as radioactivity-based assays or liquid chromatography-mass spectrometry (LC-MS). Here we describe a fast and inexpensive spectrophotometric protocol for determining the kinetic parameters of SC-PTs in purified enzyme preparations, using an adapted assay for PPi quantification. We developed the method using the Arabidopsis thaliana GGPPS11 enzyme, which produces geranylgeranyl diphosphate for the synthesis of carotenoids in the chloroplast.

iii. Key Words

Short-chain prenyltransferases, isoprenyl diphosphate synthases, enzymatic activity, kinetic parameters, EnzCheck kit, geranylgeranyl diphosphate synthase, GGPP, isoprenoids, carotenoids.
1. Introduction

Isoprenoids (also known as terpenoids) are a widely diverse group of natural metabolites, and many of them are essential in all-living organisms. Particularly, plant isoprenoids show an enormous structural and functional diversity. Plants produce a myriad of specialized isoprenoids involved in their interaction with the environment, but also a number of essential isoprenoids that participate in photosynthesis (chlorophylls, carotenoids, tocopherols, prenylquinones), respiration (ubiquinone) or growth regulation (cytokinins, brassinosteroids, gibberellins, abscisic acid, strigolactones) [1]. All isoprenoids derive from the C5 units isopentenyl diphosphate (IPP) and its double-bond isomer dimethylallyl diphosphate (DMAPP) (Fig. 1). Condensation of one or several IPP molecules to one DMAPP unit catalyzed by isoprenyl diphosphate synthases (IDSs), also called prenyl transferases (PTs), produces linear isoprenyl diphosphates of different chain length that represent the first intermediates of nearly all isoprenoid groups. IDS enzymes can be classified as trans- or cis-PTs depending on the stereochemistry of the double bonds formed in the synthesized product, forming two evolutionary distinct groups of proteins [2,3]. With some exceptions [4–8], most trans-PTs generate isoprenyl diphosphates of up to C50, whereas cis-PTs synthesize much longer molecules.

Among the trans-PTs, short-chain prenyltransferases (SC-PTs) catalyse the sequential head-to-tail addition of up to three molecules of IPP to one molecule of DMAPP producing C10 geranyl diphosphate (GPP), C15 farnesyl diphosphate (FPP) or C20 geranylgeranyl diphosphate (GGPP) [2,3] (Fig. 1). This enzymatic reaction requires two highly conserved domains, referred to as First Aspartate-Rich Motif (FARM, DDx2-D) and Second Aspartate-Rich Motif (SARM, DDx2-D), that are involved in substrate binding [9–14] and catalysis using Mg^{2+} as cofactor [15]. The sequential addition of IPP molecules to the isoprenyl diphosphate product releases a pyrophosphate (PPi) molecule per IPP unit added. This process takes place in the elongation pocket of the SC-PT, a hydrophobic cavity that controls the hydrocarbon tail length by the size of the side chain of some amino acid residues located on the N-terminal side of the FARM. In most cases, the fifth amino acid upstream to this motif is the responsible of the isoprenyl product chain length [16–22]. However, other residues could also play a role controlling the number of IPP condensations during product elongation [3].

Despite all the studies highlighting the role of the elongation pocket on final product length, predictions are difficult and highly sensitive analytical methods are still required to determine the actual product of uncharacterized SC-PTs. Radioactive enzymatic assays followed by the hydrolysis of the products require
specialized equipment for the measurements such as radio-gas chromatography (radio-GC), radio-high-performance liquid chromatography (radio-HPLC), thin layer chromatography (TLC), or liquid scintillation counting (LSC) [22–28]. These methods, however, often fail to conclusively demonstrate the chain length (i.e. identity) of the product. Moreover, they are extremely time-consuming when used to calculate the enzymatic kinetics of the tested SC-PT. Nonradioactive methods based on liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) have been developed to accurately determine isoprenyl diphosphate metabolites in a much more precise and faster way [29–31]. However, they are still complex and expensive for the enzymatic characterization of SC-PTs (i.e. for the calculation of their optimal pH or kinetic parameters such as Vmax and Km).

Here we describe an easy and inexpensive spectrophotometric protocol to enzymatically characterize previously identified SC-PTs using purified enzyme preparations. The described method is an adaptation of the commercial EnzCheck Pyrophosphate Assay Kit (E-6645), that allows the detection of the PPi released by a biochemical reaction. The PPi produced in the reaction mix is converted into two molecules of inorganic phosphate (Pi) by an inorganic pyrophosphatase. The Pi then reacts with the substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) by a purine nucleoside phosphorylase (PNP) producing ribose 1-phosphate and 2-amino-6-mercapto-7-methyl-purine, which can be measured at 360 nm, hence allowing the quantification of the PPI released during the enzymatic reaction.

In this article, the method is used to measure the activity of the Arabidopsis thaliana GGPPS11 protein (At4g36810), a GGPP synthase (EC 2.5.1.29) that supplies GGPP for the production of carotenoids and other groups of plastidial isoprenoids [32]. This enzyme has been well characterized in previous studies [21,31,33], which served as a reference to validate the results obtained with the reported assay.

2. Materials

1. Reaction buffer (20X): 1 M Tris-HCl, pH 7.5, 20 mM MgCl2 (see Note 1).
2. Solid Tris Base. Tris(hydroxymethyl)aminomethane.
3. Hydrogen chloride (HCl) 37%
4. Solid magnesium dichloride (MgCl2).
5. 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG): 1 mM in milli-Q water (store at -20 °C) (see Note 2).
6. Purine nucleoside phosphorylase (PNP): 100 U/mL in milli-Q water (store at 4 °C) (see Note 3).
7. Inorganic pyrophosphatase: 3 U/mL in 1X Reaction buffer (store at 4 °C) (see Note 4).
8. PPi standard: 500 µM in milli-Q water (see Note 5).
9. IPP and DMAPP (Echelon Biosciences Inc.): 1 mM in milli-Q water (store at -80 °C) (see Note 6).
10. Eppendorf Safe-Lock Tubes™ of 1.5 and 2 mL capacity.
11. Sterile polystyrene 96 well-plates (costar®) with low evaporation lid and flat bottom.
12. SpectraMax M3 multi-mode microplate reader (Molecular Devices).
13. GraphPad Prism Software

3. Methods

3.1. Standard GGPPS Activity Assay

All 200 µL enzyme reactions should be carried out in 96-well plates (Fig. 2). The empty wells can be reused for subsequent experiments. Always perform three technical replicates per condition and at least two independent experiments.

1. Prepare the reaction mixture (Table 1) by adding 10 µL of 20x reaction buffer (see Note 7), 40 µL of 1 mM MESG substrate, 2 µL of 100 U/mL PNP and 2 µL of the 3 U/mL inorganic pyrophosphatase (see Note 8). Add enough water to reach a final total volume of 200 µL minus the volumes of the enzyme, IPP and DMAPP that will be added later (Table 1).

2. Add 5 µg of the purified enzyme (see Note 9 and Note 10) to the reaction mixture and mix well by up and down pipetting.

3. Pre-incubate at room temperature for 10 min (see Note 11).

4. Add the enzyme substrates IPP and DMAPP as shown in Table 1 for a standard reaction (see Note 12) and mix well by up and down pipetting. It is very important to always perform a control assay in parallel in which solvent buffer will be added instead of the experimental substrates (no-substrate control, see Note 13, Fig. 2).

5. Immediately introduce the plate in the spectrophotometer, shake it and start measuring the absorbance at 360 nm as a function of time during 1 h at 25°C. Reading the absorbance every 2 min should be enough to build the activity curve (see Note 14).

6. When analyzing the data, remember subtracting the no-substrate control values from the corresponding experimental samples (see Note 15).

7. Calculate the enzyme activity obtained for each condition as the increase of PPi production per minute
and µg of enzyme (µM PPI min⁻¹ µg⁻¹) (see Note 16). Use them to build a pH curve (see Note 17) or to obtain the kinetic parameters of the enzyme (Vmax and Km) from the Michaelis-Menten curve using the GraphPad Prism software (see Note 18).

Table 1. Reagents and volumes to prepare a standard 200 µL single GGPPS enzyme reaction

| Reagent                  | Stock concentration | Assay concentration | Vol. added |
|--------------------------|---------------------|---------------------|------------|
| *20X Reaction buffer     | 1 M Tris-HCL, 20 mM MgCl₂, pH 7.5 | 50 mM Tris-HCL, 1 mM MgCl₂, pH 7.5 | 10 µL |
| MESG                     | 1 mM                | 0.2 mM              | 40 µL      |
| PNP                      | 100 U/mL            | 1 U/mL              | 2 µL       |
| Inorganic pyrophosphatase| 3 U/mL              | 0.03 U/mL           | 2 µL       |
| bIPP                     | 1 mM, 150 µM        | 150 µM              | 18 µL      |
| bDMAPP                   | 1 mM, 50 µM         | 50 µM               | 9 µL       |
| cEnzyme                  | 3-5 µg              |                     |            |
| Water                    |                     |                     | Up to 200 µL |

*Volume of Reaction buffer used depends on the stock solution concentration (see Note 7)
*bVolume of IPP and DMAPP will change when performing the assays for the determination of the kinetic parameters (see Note 12)
*cVolume of enzyme will depend on the concentration of the purified enzyme

3.2. Standard Curve for PPI Quantification

The linear range for the quantification of PPI using the EnzChek Kit is from 1 µM to 75 µM.

1. Follow the standard reaction (Table 1) adding increasing amounts of the 500 µM PPI standard solution (see Note 19) and omitting the volumes of the substrates (IPP and DMAPP) and the experimental enzyme.

2. Prepare a no-PPI control without PPI (0 µM point, see Note 19). Also, include a no-pyrophosphatase control as a blank with no PPI and no inorganic pyrophosphatase (see Note 11).

3. After mixing all the reagents, incubate the plate at 25 °C for 60 min (see Note 20).

4. Measure the absorbance at 360 nm.

5. Subtract the no-pyrophosphatase control absorbance value from each experimental reaction and build the PPI standard curve plotting the absorbance at 360 nm as a function of PPI concentration.

4. Notes

1. The 20X Reaction buffer provided in the EnzChek Kit contains 2 mM of sodium azide, that acts as a preservative. The Kit allows to perform the activity assay over a pH range of 6.5 to 8.5, but in this case the buffer must be prepared with the pH of interest (here, sodium azide can be omitted). To
generate a pH curve, we recommend to prepare a 20x stock of Reaction buffer at pH 9.5. Then, distribute it in 5 ml aliquots (as many as pH values to be tested). Adjust the pH of every aliquot to the desired value with 37% HCl and then add milli-Q water up to 10 mL to get 10X aliquots of each pH. Remember that the volume of these 10X aliquots added per activity reaction must be doubled (as the recipe in Table 1 contains a 20X Reaction buffer).

2. The Kit includes 6.3 mg (20 µmoles) of MESG. Add 20 mL of milli-Q water directly to the container to prepare a 1 mM stock. Immediately, aliquot the homogenized MESG solution and store the aliquots at -20 ºC. As each individual reaction requires 40 µL of MESG substrate, and normally triplicates will be performed per condition, we recommend to prepare both 200 µL and 500 µL aliquots. Immediately before use, thaw the required aliquot of MESG at 37 ºC (no more than 5 min), vortex strongly and place on ice. If more than one aliquot is needed, we recommend to mix all of them together and use the same solution for all reactions. As described in the manufacturer’s instructions, MESG solution is stable on ice at least for 4h at pH 7.5. Be aware that the half-life of this substrate may change in different conditions. It is not recommended to freeze and reuse MESG leftovers.

3. The Kit provides two vials of 50 U of freeze-dried PNP. To prepare a 100 U/mL stock solution add 0.5 mL of milli-Q water to one of the vials. This solution can be stored at 4 ºC for at least one month. The non-reconstituted PNP vial may be stored at -20 ºC.

4. The Kit contains one vial of 6 U of lyophilized inorganic pyrophosphatase. Add 200 µL of milli-Q water to the vial to prepare a stock of 30 U/mL and store it at 4 ºC. Before performing the activity assay, prepare a 3 U/mL aliquot by diluting 10-fold the 30 U/mL stock into 1X Reaction buffer (previously diluted from 20X Reaction buffer). Never dilute the inorganic pyrophosphatase into 20X reaction buffer. Leftover 3 U/mL solution can be stored at 4ºC for one week.

5. The Kit provides 500 µL of 50 mM Na$_4$P$_2$O$_7$ (with 2 mM sodium azide) as a source of PPI. Prepare a 500 µM working solution of PPI standard by diluting 100-fold a portion of the given stock in milli-Q water. Preparing 500 µL of working solution should be enough for a regular standard curve (see Note 19).

6. IPP and DMAPP are provided by Echelon Biosciences Inc. as Tris-ammonium salts. Prepare 4 mM stock solutions in milli-Q water, dispense in 50 µL aliquots and store at -80 ºC. Prior to use, dilute an aliquot of these substrates in water to prepare 1 mM working solutions. Leftover 1mM solutions
can be frozen again and stored at -80 ºC.

7. This volume can change when testing different pH reaction buffers. If 10X buffers are used instead of 20X, 20 µL should be added.

8. Depending on the number of reactions, a mix of the first four reagents can be prepared.

9. The amount of purified enzyme added to the reaction mixture should be determined empirically. It is important that the release of PPI by the enzyme tested does not surpass the activity of the inorganic pyrophosphatase. In our case, 3 µg of the Arabidopsis GGPPS11 enzyme also worked well in the assays.

10. Arabidopsis GGPPS11 was purified using the pET-G11 construct [31], which harbors a version of the enzyme lacking the plastid targeting peptide and fused to an N-terminal 6x-histidine tag. The recombinant protein was produced in the E. coli Rosetta 2 (DE) strain (Novagen, Merck KGaA, Darmstadt, Germany). The cells were grown at 37°C in 100 mL of 2xYT medium supplemented with the corresponding antibiotics. When they reached an OD₆₀₀ of 0.5-0.8, 1 mM IPTG was added. After the induction, the culture was incubated overnight at 18 ºC and bacterial cells were then recovered by centrifugation. The pellet was resuspended in 10 mL of buffer A (100 mM Tris-HCl pH7.5, 100 mM NaCl, 10% glicerol) supplemented with 1 mM DTT, 1 mg/mL lysozyme and one tablet of complete protease inhibitor cocktail (Roche), and incubated in ice for 20 min. After a brief sonication (five pulses of 30 s, 17%), the cell lysate was centrifuged at 12,500 rpm at 4°C for 20 min. Then 2 µg/ml DNase I and 10 µg/ml RNase A were added directly to the tube and gently mixed. The mixture was incubated 20 min in ice and then centrifuged again at 17,500 rpm at 4 ºC for 50 min. The supernatant was filtered using a 0.2 µm filter and incubated for 2 h with 2 mL of nickel-nitrilotriacetic acid (Ni-NTA) beads (Qiagen) previously equilibrated with washing buffer (buffer A + 20 mM imidazole). After the incubation, the mixture was placed into a filter column where the flowthrough is discarded and the Ni-NTA beads are stacked with the recombinant protein attached. To remove the unspecific proteins retained in the column five washes of 1 mL of washing buffer were performed, and finally the protein was recovered in one tube by five elutions with 150 µL of elution buffer (buffer A + 150 mM imidazole). The eluted sample was desalted by Thermo Scientific™ Zeba™ Desalting Columns using buffer A as exchange buffer and the purified protein was quantified, aliquoted with glycerol 40% and finally stored at -20 ºC.

11. Reagents and enzymes may be contaminated with Pi which will interfere with the measurements.
The reagents should be tested in a standard reaction (Table 1) with no inorganic pyrophosphatase (no-pyrophosphatase control). If present, contaminating Pi should be subtracted out from the experimental reaction measurements. In addition to the reagents, the purified enzyme may also show Pi contamination that could prevent the obtainment of conclusive data. To reduce the contaminating Pi, the enzyme sample can be pre-incubated for 10 min before the addition of the substrates (e.g. IPP and DMAPP). In this pre-incubation step, the reaction catalysed by the PNP can act as a “Pi mop” lowering the contaminating Pi to submicromolar levels. It is recommended to always carry out this step to avoid any possible Pi interference.

12. The concentration of IPP and DMAPP was empirically calculated to observe an increase of enzymatic activity throughout the time using 3 µg of enzyme. We add 3-fold more IPP than DMAPP since every GGPP molecules is formed from 3 molecules of IPP and only one of DMAPP.

The concentration and proportion of the substrates may be determined empirically for other SC-PTs. For the generation of pH curves we used the amount of substrates specified in Table 1.

For kinetic parameters determination assays, the concentration of the substrates will change and so will their required volumes. We calculated the basic kinetic parameters (Vmax and Km) for each substrate separately, designing the plate to test different concentrations of one of the substrates fixing the other one in 100 µM (Fig. 2). The range of concentrations used to calculate the kinetics of the enzyme for each substrate was: 5, 10, 20, 50, 75, 100 and 200 µM.

13. The no-substrate control must be done whenever a reagent of the Standard Reaction change. For example, when testing the activity of the experimental enzyme in the presence of different pH media, a no-substrate control must be included for each pH (column 4 of the Fig. 2, light grey). However, for kinetic parameters determination assays, only one no-substrate control is needed (well A4 of the Fig. 2) since only the volume of the substrates changes.

14. The assay may require to set up the measuring time points to stablish the linearity region of PPi production versus time.

15. The no-substrate control absorbance value should not increase during the one-hour assay, as an indication that there is not too much Pi or PPi contamination that could be transformed after the 10 min “Pi-mop” pre-incubation. When the absorbance of the no-substrate control is stable, the mean of the values obtained in each time point can be calculated and subtracted from the values of the experimental reactions.
16. Use the linear equation from a PPI standard curve (see Section 3.2) to quantify the PPI produced in each time point and, after plotting it, select the linear range of PPI production to calculate to calculate the enzyme activity (µM PPI min⁻¹ µg⁻¹) for each condition.

17. To build the pH curve represent the enzyme activities versus the pH. Relative values can be calculated after giving a 100% to the optimal performance.

18. Other softwares may be used to build the Michaelis-Menten curve and calculate the enzyme kinetics. Using Prism, we created an XY table selecting “Enzyme kinetics – Michaelis-Menten” as sample data and added the triplicates of the enzyme activities obtained for each substrate concentration. To obtain the regression curve and the kinetic parameters from the generated graph we performed a “Nonlinear regression” analysis selecting “Enzyme Kinetics – Substrate vs Velocity” and “Michaelis-Menten equation”. As a result of the analysis, the regression curve is superimposed on the graph and a table with the values of Vmax and Km together with the statistical parameters is retrieved.

19. A standard curve with 9 different PPI concentrations (0, 5, 10, 20, 30, 40, 50, 60, 75 µM) is enough to obtain the linear regression equation with a square of the Pearson correlation coefficient (R²) > 0.99. Prepare at least three replicates per PPI concentration to build the standard curve.

20. The manufacturer’s instructions indicate an incubation of 30-60 min, enough time to transform all the PPI in the reaction mixture into 2-amino-6-mercapto-7-methylpurine. We incubated the Standard Curve 60 min adjusting the time to the Standard Reaction for GGPPS activity measurement. Around an hour is needed to (1) synthesize the GGPP and release the PPI molecules and then to (2) consume the PPI by the kit enzymes.

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Figure 1. Biosynthesis of short-chain prenyl diphosphates by short-chain prenyltransferases. Each IPP condensation involves the elongation of the prenyl diphosphate molecule with the subsequent release of one PPI molecule (dashed circle). Solid arrows represent one enzymatic step and dashed arrows indicate multiple enzymatic steps. Abbreviations: DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; PPI, inorganic pyrophosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPPS, geranyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase.

Figure 2. Experimental design in a 96-well plate format. Black triangle indicates increasing pH or substrate concentration from top to bottom. Dark grey squares (columns 1 to 3) represent standard enzymatic reactions in triplicates per condition. Light grey squares (column 4) indicate no-substrate control. Perform a no-substrate control whenever a reagent is changed among
conditions. If only the concentration of a reagent changes but the reagent solution is the same, only one no-substrate control is needed (dashed square).