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Comparative analysis and validation of the malachite green assay for the high throughput biochemical characterization of terpene synthases

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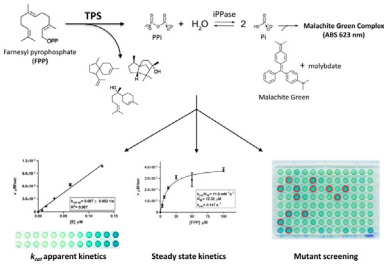
GRAPHICAL ABSTRACT



ABSTRACT

Terpenes are the largest group of natural products with important and diverse biological roles, while of tremendous economic value as fragrances, flavours and pharmaceutical agents. Class-I terpene synthases (TPSs), the dominant type of TPS enzymes, catalyze the conversion of prenyl diphosphates to often structurally diverse bioactive terpene hydrocarbons, and inorganic pyrophosphate (PPi). To measure their kinetic properties, current bio-analytical methods typically rely on the direct detection of hydrocarbon products by radioactivity measurements or gas chromatography–mass spectrometry (GC–MS).

Abbreviations: TPS, terpene synthase; MG, malachite green; FPP, farnesyl diphosphate; PPi, pyrophosphate; Pi, monophosphate.

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In this study we employed an established, rapid colorimetric assay, the pyrophosphate/malachite green assay (MG), as an alternative means for the biochemical characterization of class I TPSs activity.

- We describe the adaptation of the MG assay for turnover and catalytic efficiency measurements of TPSs.
- We validate the method by direct comparison with established assays. The agreement of k_{cat}/K_M among methods makes this adaptation optimal for rapid evaluation of TPSs.
- We demonstrate the application of the MG assay for the high-throughput screening of TPS gene libraries.

ARTICLE INFO
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Method details

Substrate and reagents

Farnesyl diphosphate (FPP) was synthesized following the procedures developed by Meyers [1] and Poulter [2] starting from (2*E*,6*E*)-farnesol; malachite green powder; pure (*E*)-β-farnesene and all other reagents were purchased from commercial vendors.

Protein expression and purification

To evaluate the MG/pyrophosphate assay as a tool for the biochemical characterization of TPSs, we chose three well studied enzymes as exemplars, namely *Artemisia annua* β-farnesene synthase (AaFS), *A. annua* amorpha-4,11-diene synthase (AaADS), and *Nicotiana tabacum* 5-epi-aristolochene synthase (TEAS). Gene constructs of AaFS, AaADS, and TEAS were inserted into the expression vector pH9GW (Gateway cloning system), and introduced into *Escherichia coli* BL21 (DE3) cells. Cell cultures were grown at 37 °C in Terrific Broth (TB) complemented with kanamycin (50 μg/mL). Protein expression was induced, at OD 600 nm ≥ 0.8, with 0.1 mM IPTG. The cultures were incubated with shaking for a further 5 h at 20 °C. Cells were harvested by centrifugation. Cell pellets were re-suspended in 50 mL of buffer A (50 mM Tris–HCl, 50 mM glycine, 5% glycerol, 0.5 M NaCl and 20 mM imidiazole, pH 8), complemented with an EDTA-free protease inhibitor tablet.

The cells were lysed (cell disruptor, 25 kpsi) and the clarified lysate was loaded onto a FPLC apparatus, AKTAxpress, for a two-step purification using a 5 mL Ni^{2+}-immobilized metal ion affinity chromatography column (HisTrap™ HP), equilibrated with buffer A, and an S200 26/60 Sephadex Gel filtration column equilibrated with 20 mM HEPES, 0.15 M NaCl, at pH 7.5. Fractions containing purified protein were measured using the Bradford assay. Protein purity was verified with SDS-PAGE chromatography.

Protein expression and purification (for library screening)

Mutant clones were transformed into BL21 (DE3) and spread onto LB plates with kanamycin. Individual colonies were transferred in 3 mL LB with kanamycin, in 24-well plates, and incubated o/n (37 °C, 230 rpm). 0.5 mL of each o/n culture was diluted to 5 mL with TB containing kanamycin. Growth was sustained at 37 °C in 24-well round bottom plates covered with micro-porous tape, until cultures reached OD600 ≥ 0.8. Protein expression was induced by addition of 0.1 mM IPTG at 20 °C. After 5 h, cells were harvested by centrifugation and stored at −20 °C until further use.

For the protein purification, pellets were re-suspended (25 °C) in 0.8 mL lysis buffer (50 mM Tris–HCl, 500 mM NaCl, 20 mM imidazole, 10% glycerol (v/v), 10 mM β-mercaptoethanol, and 1% (v/v) Tween-20, pH 8) containing lysozyme (1 mg/mL) and 1 mM EDTA for 30 min. Subsequently, 10 μL of benzonase solution (850 mM $MgCl_2$ and 3.78 U/μL benzonase) was added with shaking at 250 rpm for 15 min.