CHAPTER ONE

Steady-State Kinetic Characterization of Sesquiterpene Synthases by Gas Chromatography–Mass Spectroscopy

Steven R. Garrett*, Richard J. Morris†, Paul E. O'Maille*,‡,1

*Department of Metabolic Biology, John Innes Centre, Norwich Research Park, Norwich, United Kingdom
†Department of Computational and Systems Biology, John Innes Centre, Norwich Research Park, Norwich, United Kingdom
‡Institute of Food Research, Norwich Research Park, Norwich, United Kingdom
1Corresponding author: e-mail address: paul.omaille@jic.ac.uk

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Abstract

Sesquiterpene synthases produce a wide variety of structurally diverse hydrocarbon products from a single substrate: farnesyl pyrophosphate. Each enzyme will often produce a multitude of products for which the kinetic efficiency is traditionally measured using a radioactivity assay. Here, we introduce a gas chromatography–mass spectroscopy-based assay to measure the formation of a single abundant product from which the kinetic parameters of the enzyme in question can be elucidated. We present an accounting of experimental components and considerations, such as solution conditions and instrument parameters, necessary to perform a standardized vial assay experiment. Further, we outline pilot experiments to establish analyte quantification and the linear range of enzyme concentration versus reaction velocity. Finally, we describe a protocol for a steady-state kinetics experiment, and the processing of experimental data to produce a Michaelis–Menten plot enabling one to derive kinetic parameters.

1. INTRODUCTION

Terpene synthases (TPS) are a family of specialized metabolic enzymes, which produce an immense diversity of chemical structures, from linear to multicyclic. For example, the TPS subfamily of sesquiterpene synthases utilizes the 15-carbon farnesyl pyrophosphate (FPP) substrate to generate one or several of the 300 theoretically possible sesquiterpene hydrocarbon skeletons (Cane, 1985). TPS are lyases (EC 4.2.3.47), specializing in the metal-assisted cleavage of a carbon—oxygen bond, resulting in the formation of a carbocation. Following the lyase reaction, many TPS enzymes make cyclic products by promoting intramolecular cyclizations of the carbocation prior to termination (Cane, 1985). Pre-steady-state kinetics experiments have indicated that product release is rate-limiting, and hence the rearrangement chemistry is thought to be rapid by comparison (Cane, Chiu, Liang, & Anderson, 1997; Mathis et al., 1997). Like that of many enzymes, the activity of sesquiterpene synthase has previously been measured using radioactive assays, in this case by measuring tritium incorporation into extractable hydrocarbon products (Vogeli, Freeman, & Chappell, 1990). Here, we present a detailed protocol using the vial assay and gas chromatography–mass spectrometry (GC–MS) to measure the steady-state kinetic activity of this large family of enzymes (O'Maille et al., 2004) as an alternative to radioactive assays.

1.1. The vial assay for enzyme kinetics

The vial assay was developed to enable terpene synthase assays to be conducted in and directly measured from GC vials (Fig. 1.1). Aqueous phase reactions (typically 500 μL) are composed of GC vials overlaid with an organic phase of equal volume. Reactions are quenched by vortexing, which inactivates the enzyme and extracts hydrocarbons into the organic phase. Adjusting the needle-sampling depth of the GC instrument enables the organic phase to be directly sampled and terpene products measured (O'Maille et al., 2004). The principle advantage of using the GC–MS vial assay for kinetics is the ability to quantify the rate of formation of individual reaction products, provided the product is dominant, given the constraints on instrument detection limits and linear range (Section 3). While radioactivity assays only permit the measurement of total hydrocarbon product formation, these methods provide the highest sensitivity, which is particularly useful for measurements at low substrate concentrations near the K_m of the enzyme. Single-ion monitoring (SIM) can extend the linear range of the GC–MS method to enable measurements down to low nanomolar concentrations and has proved to be a useful approach for measuring the kinetic properties of TPSs. Vial assays have a further advantage of not requiring access to radiation areas or training in those methods.

1.2. Steady-state kinetics and the Michaelis–Menten model

This chapter details the use of the vial assay for steady-state kinetics of sesquiterpene synthases. Accordingly, steady-state kinetics relies on a model, most commonly the Michaelis–Menten model (Menten & Michaelis, 1913), expressed as follows:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Provided enzyme reaction rates are measured at a range of appropriate substrate concentrations, the Michaelis–Menten model can be used to derive the kinetic parameters commonly assigned to enzymes: k_{cat}, K_m, and V_{max}. The steady-state approximation assumes a negligible rate of change in the concentration of the enzyme–substrate complex during the course of the reaction. Hence, experiments are performed under conditions of excess substrate with protein concentration linear with respect to reaction velocity. To ensure this, measurements are made during the early phase of the reaction where $< 10\%$ turnover of the substrate has occurred. This is a necessary condition of the Michaelis–Menten equation to calculate the K_m (i.e., $[S] \gg [E]$)

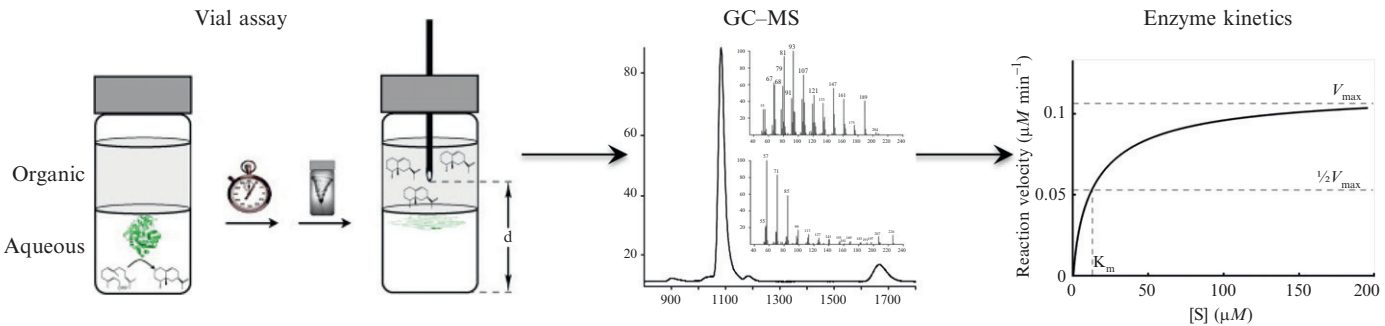

Figure 1.1 *The vial assay for enzyme kinetics*. A 500 μL-vial assay reaction is composed of a GC vial by mixing substrate and enzyme in the aqueous phase and overlaid with an equal volume of organic phase (hexane) containing an internal standard (hexadecane). The vial is vigorously vortexed after a fixed incubation time (e.g., 12 min) to quench the reaction and extract hydrocarbon products. The organic phase is then directly injected into the GC–MS and products are quantified by integrating peak areas of the total ion chromatogram. Data are adjusted using an internal standard and a Michaelis–Menten plot is fitted using kinetics software to elucidate kinetic parameters of interest from the protein under study.

(Laidler, 1955). In addition, it has been found that for the steady-state conditions to remain valid, the following conditions should be met (Schnell & Maini, 2003):

$$[S_0]/[E_0] > 100$$
$$[E_0]/K_m \ll 1$$
$$[E_0]/(K_m + [S_0]) \ll 1$$

Further discussion of Michaelis–Menten kinetics can be found in many excellent textbooks, for instance, Fersht (1999).

2. EXPERIMENTAL COMPONENTS AND CONSIDERATIONS

2.1. Enzyme purification and quantification

Sufficient quantities of highly pure protein are desirable to ensure consistent and reproducible results from kinetic experiments. While there are several options for protein purification (as reviewed elsewhere; Burgess & Deutscher, 2009), we recommend immobilized metal affinity chromatography and Ni^{2+} resin for the purification of His-tagged proteins. Protease cleavage followed by gel filtration can achieve ca. 99% pure protein as judged by SDS-PAGE analysis. The sample should then be concentrated (ca. 10–20 mg mL^{-1}) and the final concentration accurately measured before storage. The stability of proteins may vary widely; the investigator must determine the proper storage and freeze-thaw conditions for each protein. For quantification, we recommend full denaturation in guanidinium hydrochloride followed by A_{280} measurement. The ExPASy Web program ProtParam (Gasteiger et al., 2005) at http://web.expasy.org/protparam/ can be used to calculate a theoretical extinction coefficient from protein sequences. There are other methods for accurately measuring the protein concentration, including the Lowry assay (Lowry, Rosebrough, Farr, & Randall, 1951), the Bradford assay (Bradford, 1976), and the BCA assay (Smith et al., 1985). A thorough review of these techniques and UV spectroscopy can be found in "Quantitation of Protein" by Noble and Bailey (2009). When removing from −80 °C, it is recommended that the aliquot is rapidly defrosted by hand and then placed immediately on ice. Use one aliquot per set of kinetics experiments as multiple freeze-thaw cycles may denature and inactivate enzymes. Ideally, all kinetics experiments should be conducted with enzyme from the same purification experiment.

2.2. Substrates

FPP is commercially available from specialists (Isoprenoids LC, Echelon Biosciences Inc.) and more general suppliers (Sigma). Purchased as a Trisammonium salt of known mass, FPP can be quantitatively resuspended in the necessary volume of water to make a stock solution of 100 m*M* that can be aliquoted and stored at −20 °C. Additionally, substrate analogs have proven useful for establishing reaction mechanisms and used in vial assay kinetics experiments. For example, the fluorinated derivative 6F-FPP enabled interception of a fluorogermacrene intermediate (Faraldos, Zhao, O'Maille, Noel, & Coates, 2007), while (2*Z*,6*E*)-FPP enabled elucidation of a "cisoid" reaction pathway in tobacco 5-*epi*-aristolochene synthase (Faraldos, O'Maille, Dellas, Noel, & Coates, 2010; Noel et al., 2010).

2.3. Buffer and pH

While it is generally assumed that a pH of 7.0 is optimal, given the presumed cytosolic localization of sesquiterpene synthases (Nieuwenhuizen et al., 2009), it may be of interest to measure the optimum pH of an enzyme experimentally and characterize the enzyme's kinetic parameters under these optimal conditions. We recommend measuring the k_{cat} apparent (Section 3.3) over a range of pH values and fixed time. Plotting k_{cat} apparent versus pH will reveal the optimal pH condition to carry forward in subsequent steady-state kinetic experiments. Although many buffers are suitable for enzyme kinetics studies, a single buffering agent rarely covers more than a few pH units. To ensure uniform buffer components and constant ionic strength over a large pH range, we recommend a three-component buffer system (Ellis & Morrison, 1982). Based on these guidelines, we formulated MTC buffer: 25 m*M* 2-(*N*-morpholino) ethanesulfonic acid, 50 m*M* Tris, and 25 m*M* 3-(cyclohexylamino) propanesulfonic acid. The MTC buffer has been found to be particularly suitable for vial assays with a useful pH range of 5–11 (±1 pK_a of each titratable group). When varying buffers, it is important to consider that each buffer may have a different effect on the enzymatic activity.

2.4. Metal ions

Sesquiterpene synthases typically require the divalent metal ion Mg^{2+} as a cofactor, with peak activity ranging from 0.01 to 100 m*M*. Other metal ions such as Mn^{2+}, Ni^{2+}, and Co^{2+} can substitute for Mg^{2+} and affect product specificity and kinetics (Picaud, Brodelius, & Brodelius, 2005), though

Mg^{2+} is generally accepted as the physiological cofactor. Other metals such as K^{+} have been shown to profoundly affect the catalytic efficiency of some sesquiterpene synthases (Christianson, 2006; Green, Squire, Nieuwenhuizen, Baker, & Laing, 2009; Vedula, Jiang, Zakharian, Cane, & Christianson, 2008). As a starting point, assays can be conducted using 20 m*M* Mg^{2+} and if desired optimal Mg^{2+} ion concentrations can be determined by establishing the k_{cat} apparent at different metal ion concentrations (from 10 μ*M* to 20 m*M*). More rigorous kinetic analysis (Section 3.3) under alternative conditions may be warranted to measure kinetic parameters and metal-binding constants (Shishova et al., 2008), but this will not be explored further here.

2.5. Internal standard

An internal standard is a compound added in constant amounts to all samples and calibration standards to correct for variability in injection amounts during GC–MS analytical procedures. This allows analyte measurements to be adjusted prior to product quantification (Section 5). An ideal internal standard will be chemically similar to the analyte yet chromatographically resolved from the analyte of interest during the GC–MS run. Hexadecane is a linear hydrocarbon that serves as an excellent internal standard, with a single GC–MS peak that is clearly separable from sesquiterpenes under standard separation conditions (Section 2.5). The concentration of the internal standard should be in the same range as the analyte under investigation (ca. 1 μ*M* is suitable for steady-state experiments).

2.6. Vial assay method

The vial assay provides a standardized methodology for quantitation of sesquiterpene products via GC–MS. Owing to the time constraints of enzyme kinetics experiments, vials should be set up in batches (e.g., nine at a time) to allow sufficient time to compose reactions, overlay organic solvent and cap vials prior to vortexing at fixed times (see below). Therefore, it is important to stagger reaction setup appropriately. The following generic method describes the typical vial assay experiment:

1. Prepare buffer system of choice and metal ion stock solutions (Sections 2.3 and 2.4).
2. Thaw protein aliquot(s) and substrate (FPP) and place on ice.
3. Compose a substrate solution, adding FPP last (see Table 1.1), vortex vigorously.

Table 1.1 Standard vial assay components

	Component		Volume (μL)	Final concentration
	General	Specific		
Protein solution	Buffer	5 × MTC buffer (pH 7)	60	1 ×
	Metal ion	1 *M* $MgCl_2$	10	20 m*M*
	Substrate	FPP (100 m*M*) add last	0.5 μL	100 μ*M*
	Water	H_2O (Millipore)	To make 300 μL	
Enzyme solution	Buffer	5 × MTC buffer (of required pH)	40	1 ×
	Protein	Enzyme ($\sim$10 mg mL^{-1})	0.5 μL	Excess
	Water	H_2O (Millipore)	To make 200 μL	

4. Compose an enzyme solution, adding enzyme last (see Table 1.1), mix gently by inversion or pipetting.
5. Pipette 300 μL of substrate solution into each vial in the first batch.
6. Add 200 μL of enzyme solution to each vial, mix by pipetting ($t = 0$ min) and start the clock.
7. Carefully overlay 500 μL of hexane containing internal standard, ensuring not to disturb the aqueous phase (slowly dispense solvent with the needle resting on the vial wall), and then cap each vial.
8. After a defined time (typically 12 min), vortex each vial vigorously for 10 s.
9. Place all the vials on the GC–MS and run samples (Section 2.7).

2.7. GC–MS instrument and run parameters

GC flame ionization detector (FID) is ideally suited for quantitative studies given that analyte combustion is insensitive to chemical structure. The signal measured on GC–MS instruments derives from compound ionization and may vary for different chemical structures. Therefore, authentic standards are important for instrument calibration and determining the ionization efficiency. While developed for the GC–MS, the methods listed below can readily be adapted for use with a GC-FID.

A generic GC–MS method for sesquiterpene analysis has been developed using a Hewlett–Packard 6890 gas chromatograph (GC) coupled with a 5932 mass selective detector (MSD) outfitted with a 7683B series injector and autosampler and equipped with an HP-5MS capillary column (0.25 mm i.d. × 30 m with 0.25 μm film) (Agilent Technologies). The standard sampling depth places the needle position 3.6 mm above the vial bottom by default (considered position = 0 by the instrument). For these experiments, needle-sampling depth was set to 7 mm (10.6 mm above the vial bottom), placing the needle in the middle of the organic layer (near the 750 μL level in the 2-mL glass vial). This should, however, be validated as this may vary between machines and autosamplers. The GC was operated at a He flow rate of 2 mL min^{-1}, and the MSD was operated at 70 eV. Splitless injections (4 μL) were performed with an injector temperature of 250 °C. The GC was programmed with an initial oven temperature of 80 °C (1 min hold), which was then increased 20 °C min^{-1} up to 140 °C (1 min hold), followed by a 7 °C min^{-1} to 180 °C (2 min hold) and finally an increase of 100 °C min^{-1} until 300 °C (1 min hold). A solvent delay of 6 min was allowed prior to the acquisition of MS data.

2.8. Additional points

It is recommended that the ambient temperature be measured at the start of each experiment. Ground glass syringes should be used for all solvent manipulations to avoid contamination from plasticizers that are extractable from plasticware. Though manual handling of the ground glass syringes may be cumbersome, a Chaney adaptor can be used to create a set point on the syringe (e.g., 500 μL) to improve precision of repeated dispensing maneuvers. Hexane is a widely used solvent for extracting sesquiterpene synthase products, though other solvents, such as ethyl acetate and diethyl ether, are especially useful for extracting terpene alcohols and polar products. Prior to pilot experiments, it is important to ensure that solvent controls are run on the GC–MS under the experimental settings and that instrument maintenance is performed to evaluate and minimize the baseline. Running solvent blanks after vortexing will establish if there are any extractable components from the vials or caps that are potential sources of contamination. Of note, following on from a successful experiment or calibration with standard, vials can be recapped and stored at −20 °C to be run at a later date (i.e., use of calibration standards at the beginning of each GC–MS experiment).

3. PILOT EXPERIMENTS

Pilot experiments are necessary to determine the instrument parameters and reaction conditions for the enzyme system to be studied. This will ensure both valid and relevant results are achieved in the steady-state kinetic experiments. The pilot experiments also provide practice in the experimental technique; rehearsal is recommended for time-critical methods and provides the investigator with greater confidence and precision in executing the main experimental program.

3.1. Analyte detection and quantification

Initial GC–MS runs are typically done using total ion monitoring (TIM) mode to examine the total ions resulting from compound ionization. This produces a full mass spectral fingerprint for the compound that is useful for compound identification, especially when coupled with relative retention time (Hochmuth, 2011). This also allows identification of the most abundant ions in the mass spectra for SIM mode experiments. Tuning the mass detector to select ions of interest in SIM mode greatly reduces the background and increases the limits of detection by 10-fold or greater, while extending the linear range of quantification down to the low nanomolar range. The dynamic range of the GC–MS can extend from $\sim$1 nm to $>$100 μM, encompassing analyte concentrations of relevance to kinetic measurements. Based on the full mass spectrum from TIM mode, the mass spectra can be tabulated to identify ions that constitute $>$50% of the total instrument signal for the analyte of interest and the internal standard (Fig. 1.2). The most abundant ions for both the product (calibration standard) and the internal standard are used when setting up a GC–MS method for SIM mode.

3.2. Instrument calibration

The linear range of the GC–MS defines the analyte concentrations that can be accurately quantified in kinetics experiments and must be empirically determined for each product to be measured. If an authentic standard is available, then serial dilutions of known concentration can be prepared (ranging from 100 μM to 100 pM) and the analyte quantified from SIM mode measurements. The resulting data can be plotted as instrument response [total ion current (TIC) from integrated peak area] versus analyte

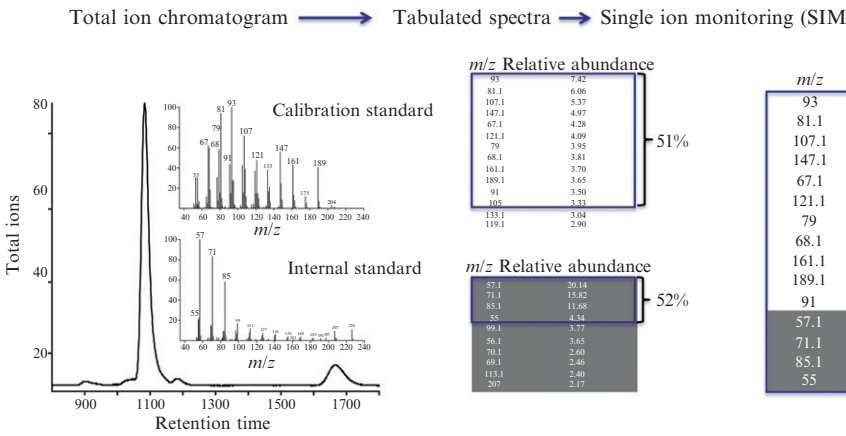

Figure 1.2 *Identifying ions from TIM mode for use in SIM mode GC–MS analysis*. A typical vial assay experiment will produce a gas chromatogram and mass spectrum of the calibration standard and internal standard. Mass spectra are tabulated using Agilent Chemstation software and the most abundant *m/z* values from both the calibration and internal standard are chosen for SIM mode.

concentration. Regression analysis of the resulting plot will reveal the linear range of the instrument and the slope of the line can be subsequently used to calculate product concentration for kinetics experiments. It is recommended that the calibration standards be run with each kinetics experiment to ensure consistent instrumentation performance and comparability between separate GC–MS runs.

Pure authentic standard for GC–MS instrument calibration is desirable. However, in the absence of a commercially available authentic standard, chemical synthesis or large-scale enzyme incubations can be performed followed by product purification on silica columns or preparative TLC plates (Zhao, Schenk, Takahashi, Chappell, & Coates, 2004). Alternatively, vial assays can be used to generate standard solutions for direct measurement to produce a calibration curve. For these experiments, reactions are conducted with excess enzyme and known substrate concentrations for prolonged incubations (overnight) to drive the reaction to completion. Also, calibrations should be performed using SIM mode based on parameters (ion features of the analyte and internal standard) determined from TIM mode GC–MS runs (Fig. 1.2). Importantly, most sesquiterpene synthases produce minor products in addition to a dominant product, so the molar concentration of the product can be approximated based on its relative

amount in the product spectrum, assuming 100% substrate conversion. A sample protocol is as follows:

1. Make a series of substrate solutions using serial dilution of FPP substrate (minimum of six concentrations ranging from 100 μ*M* to 1 n*M*).
2. Add enzyme solution containing an excess enzyme (to give ~10 μ*M* final concentration) in triplicate.
3. Carefully overlay 500 μL of hexane containing internal standard before capping the vial and incubate overnight.
4. Vortex vials to extract products for GC–MS quantification.
5. Analyze samples by GC–MS using SIM mode.
6. Plot instrument response (corrected for the fraction of product) versus product concentration to produce a calibration curve.

To calculate product concentration, adjust the TIC readings from the GC–MS, taking the original product TIC and multiplying by the fraction of the total product and then scaling with the internal standard.

3.3. Linear range of protein concentration: Measuring k_{cat} apparent

Steady-state experiments require that the enzyme concentration falls on the linear range of activity; that is, the reaction velocity (product per unit time) is linear with respect to enzyme concentration. To establish the linear range of protein concentration, vial assays are conducted using a serial dilution of enzyme against a fixed (and excess) substrate concentration (100 μ*M*) for a set time interval. This experiment will initially be used to ensure that the reaction time and enzyme concentrations used are within the linear range (less than 10% turnover). From this experiment, a plot of reaction velocity versus enzyme concentration enables the k_{cat} apparent to be calculated from $V_0 = k_0\,[E_0]$, where k_0 is the k_{cat} apparent. Measurement of k_{cat} apparent is convenient for identifying optimal conditions where the enzyme is working "most efficiently" (pH, metal ion concentration, etc.) and for analyzing large numbers of enzymes from libraries (O'Maille et al., 2008). A series of time intervals can be examined (8, 12, and 16 min) based on the known kinetic parameters of most sesquiterpene synthases. Reactions are then composed for a series of enzyme concentrations at each time point (typically 0.1 and 100 n*M*). GC–MS runs in SIM mode are then used to analyze samples, and product formation can be calculated from calibration curves. Plotting the velocity (μ*M* product min^{-1}) versus enzyme concentration will reveal conditions where reaction velocity is linear with respect to enzyme concentration. A sample protocol is as follows:

1. Dilute an enzyme stock in 1 × MTC buffer and then serially dilute (e.g., 100, 50, 25, 10, 5, 2.5, 1.25, and 0.625 n*M*).
2. Make a substrate solution of fixed FPP concentration (100 μ*M* in 1 × MTC buffer, pH 7).
3. Set up vial assay (Section 2.5) with three vials per enzyme concentration point and with three time courses, 8, 12, and 16 min.
4. Quench by vortexing at indicated times.
5. Run samples on the GC–MS (Section 2.6).
6. Integrate product peak areas, scale against internal standard, and calculate μ*M* product concentration per minute.
7. Plot velocity (μ*M* product min^{-1}) against enzyme concentration (μ*M*).

To calculate the k_{cat} apparent, the reaction velocity is divided by the enzyme concentration to produce a turnover per minute (the slope of the linear portion of the plot). With this value, it is possible to calculate the amount of time in which a given enzyme concentration will produce 10% substrate turnover. Finally, this experiment will reveal the suitable protein concentrations that can be used in full in Section 4.

4. STEADY-STATE KINETIC EXPERIMENTS

After determination of the critical experimental parameters from pilot experiments (linear range of enzyme concentrations, instrument parameters, etc.), a full steady-state kinetics experiment can be conducted. To satisfy the conditions of the Michaelis–Menten equation, this experiment involves using a fixed enzyme concentration against variable substrate concentrations (where $[S_o]/[E_o] > 100$) to derive velocity versus substrate concentration plot from which K_m and V_{max} can be derived.

4.1. Reaction velocity versus substrate concentration

As previously stated, it is important to accurately measure substrate turnover at concentrations 10-fold below the K_m. Given that the K_m of most sesquiterpene synthases ranges from 1 to 5 μ*M*, the lowest substrate concentration should be 100 nm. Therefore, to measure the first 10% of substrate turnover entails quantifying 10 n*M* product, which approaches the end of the linear range of the instrument. This makes investigating enzymes with low K_m technically challenging, depending on the limit of detection that can be achieved on the GC–MS. Using the lowest enzyme concentration that is still in the linear range will ensure excess substrate is achievable, which is necessary for the steady-state approximation. On the other extreme, it

is worth noting that FPP may form micelles at high concentrations ($>300\ \mu M$) in the presence of Mg^{2+} and other divalent metal cations. Micelles will be seen frequently when the FPP is added to the substrate mix, so care must be taken to mix the substrate well, particularly at high substrate concentrations, to disperse micelles.

4.2. Steady-state kinetics experiment

Set up the vial assay according to the generic method described above, using the desired (or optimal) pH and metal ions, with fixed protein and variable substrate concentrations. A typical experiment involves setting up reactions in batches (three substrate concentrations in triplicate = nine vials). Importantly, compose the enzyme and substrate solution fresh just prior to starting the experiment and record ambient temperatures. A sample protocol as follows:

1. Thaw protein aliquot(s) and substrate (FPP) and place on ice.
2. Compose a substrate solution, adding FPP last (see Table 1.1), and vortex vigorously.
3. Compose an enzyme solution, adding enzyme last (see Table 1.1), mix gently by inversion or pipetting.
4. Add 300 μL of substrate solution to each vial.
5. Add 200 μL of enzyme solution to each vial, mix by pipetting ($t = 0$ min) and start the clock.
6. Carefully overlay 500 μL of hexane containing internal standard ensuring not to disturb the aqueous phase (slowly dispense solvent with the needle resting on the vial wall), then cap each vial.
7. Vortex each vial at the appropriate time to quench the reaction.
8. Repeat steps 2 through 6, for batch 2, and so on.
9. Run all reaction vials and calibration standards on the GC–MS.

5. DATA HANDLING/PROCESSING

Following the GC–MS experiment, the data is processed and a Michaelis–Menten curve plotted to derive kinetic parameters (Fig. 1.3). In brief, products (and internal standards) are quantified by integrating peak areas (TIC). The internal standard is then used to scale all data. After scaling, the ion-counts for the products of interest can be converted to concentrations using the calibration curve. Once a product concentration has been calculated, this can be divided by the reaction time (usually 12 min) to produce a velocity (i.e., 0.05 $\mu M\ min^{-1}$).

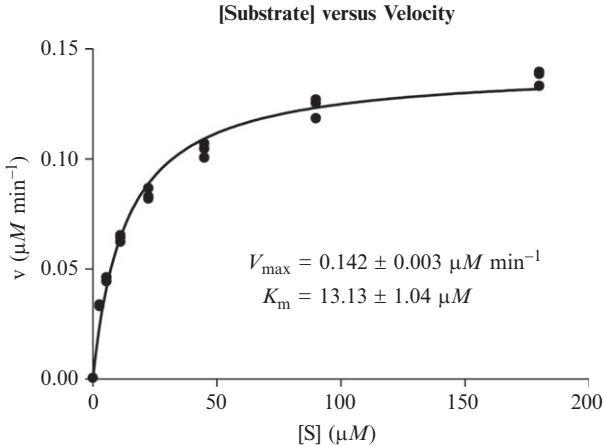

Figure 1.3 *Example of the Michaelis–Menten curve for a sesquiterpene synthase.* Statistical programs with an enzyme kinetics plugin will calculate the V_{max} and K_m while k_{cat} can be calculated: $k_{cat} = V_{max}/[E]$, although like V_{max} and K_m most statistics programs will also calculate this.

The resulting velocities can then be plotted against the substrate concentration and a Michaelis–Menten curve (Fig. 1.3) can be plotted with the data using a statistical package of choice. Most graphing programs have templates for enzyme kinetics, with *R* being a free open-source option and Sigmaplot and Graphpad being two popular commercial software packages. This will calculate a V_{max}, a K_m, and a k_{cat} ($V_{max}/[E]$) while providing a measure of fit to the Michaelis–Menten curve.

6. SUMMARY

This chapter presented a general method for the steady-state kinetic analysis of sesquiterpene synthases using the vial assay. This protocol is adaptable to analyze kinetic function under a variety of conditions (enzyme inhibitors, temperature dependence) with native substrates FPP or with substrate analogs as has been reported (Faraldos et al., 2007; Noel et al., 2010). Further, the vial assay kinetic protocol can in principle be adapted to examine mono- and diterpene synthases. It is possible (and indeed recommended) to adapt the protocols and pilot studies to the enzymatic system of interest, particularly when anomalous behavior is observed (substrate and/or product inhibition), which would prevent the enzyme in question fitting the Michaelis–Menten model, as has been reported for

mono- and diterpene synthases (Lucker et al., 2002; Peters et al., 2000). With this in mind, we would encourage the use of this general method for kinetic characterization of a wide variety of TPS with only minor alterations, or indeed as a set of standard conditions for larger scale experiments.

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