Understanding Enzyme Assays for Lysosomal Storage Disorders

Part IV: The Influence of Substrate Concentration and Incubation Time

Enzyme assays are the backbone of lysosomal storage disorder (LSD) newborn screening, and the key output of these assays is enzyme activity. In this white paper, we will review the factors that determine enzyme activity and reveal how enzyme assays with short incubation times match, or in some cases outperform, those with longer incubation times.

Click Here to Read Part I: Synthetic Substrates

Click Here to Read Part II: How to Measure Product Formation

Click Here to Read Part III: Integration of X-Linked Adrenoleukodystrophy and SEEKER Workflows for Same-Day Referrals

Relevant Definitions

V_{max}: the maximum velocity of the enzyme reaction under ideal assay conditions
K_m: the concentration of substrate at half V_{max}; the affinity of the enzyme to bind its substrate
k_{cat}: the rate of product formation (expressed as molecules of substrate turned over to product per unit time)
Enzyme Activity: the rate of product formation per unit time in a given volume

Lysosomal storage disorders constitute a group of around 50 rare, inherited metabolic disorders that are caused by deficient function of lysosomal enzymes. Both the initial newborn screening test and many of the subsequent diagnostic tests for LSDs use synthetic substrates (see **Part I of this series**) to determine enzyme activity based on the rate of product formation (see **Part II in this series**). A schematic of a typical one-step enzymatic reaction is shown in Figure 1; the endogenous enzyme in the sample is combined with a reaction mixture containing its specific substrate to form enzyme-substrate complexes. After cleavage by the enzyme, product(s) are generated and measured using an appropriate detection technology to determine the activity of the enzyme.

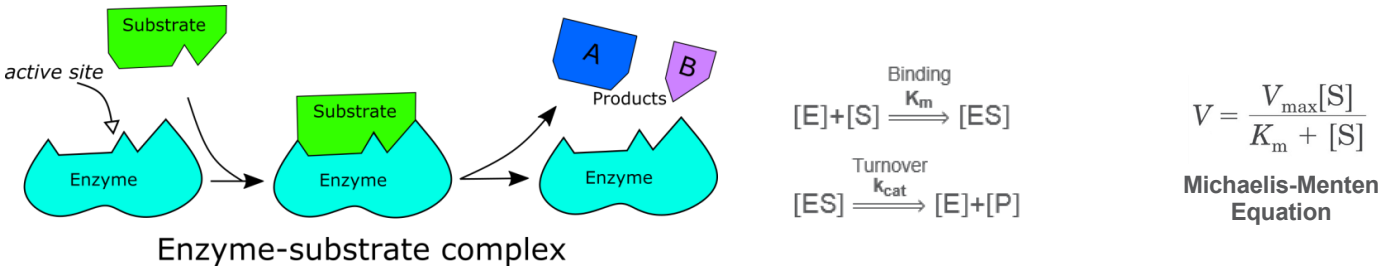

Figure 1. Equation and model of typical single enzyme catalysis. [*E*]=enzyme concentration; [*S*]=substrate concentration; [*ES*]=enzyme substrate complex concentration; [*P*]=product concentration; [*v*]=initial velocity. This model, combined with the law of mass action, can be used to derive the Michaelis-Menten equation.

Enzyme activity is defined as the *rate* of product formation in a reaction volume. Representative curves of measured enzyme activity in normal vs deficient samples are illustrated by the *slopes* in Figure 2. **Changing the incubation time does not change the measured enzyme activity because the slope of the measured product over time is constant.** In Figure 2A the plot of product produced appears to show larger separation between normal and deficient samples with incubation time; **ultimately when enzymatic**

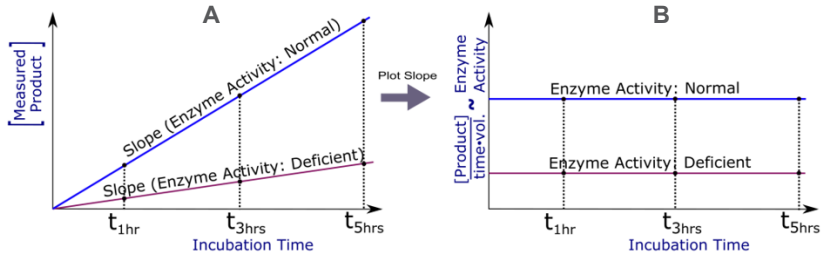

Figure 2. Plot of measured enzyme product over time (**A**) and computed enzyme activity, converted from an endpoint read and incubation time (**B**). The derived slopes of the lines (**A**) represent the enzyme activity, usually represented as µM/hr. Here, changing incubation times (e.g. 1hr, 3hr, 5hr) does not alter the separation of normal/deficient.

activity is reported, this separation does not matter as the slope (enzyme activity) is constant with incubation time as shown in Figure 2B.

Table I. Factors that determine the rate of enzyme reactions
Substrate concentration [S]
Temperature
pH
Substrate structure / affinity for enzyme (K_m)
Enzyme catalysis / turnover rate (k_{cat})
Presence / absence of activators
Presence / absence of inhibitors
Enzyme concentration [E]

When developing an enzyme activity assay for newborn screening, the first goal is to maximize the formation of enzyme-substrate complexes in order to ensure that the enzyme of interest (which is deficient in high risk/ affected LSD samples) is able to readily bind its substrate. The precise conditions necessary to maximize enzyme activity must be determined empirically for each enzyme by optimization of the factors listed in Table I[1]. Note that enzyme concentration contributes to the rate of the reaction, but is the measurand in a clinical enzyme assay and therefore cannot be manipulated.

Using the Michaelis Menten equation (Figure 1), a higher concentration of substrate can increase the overall velocity (slope) of the enzyme, as shown in Figure 3. The increase in velocity occurs relatively linearly with substrate concentration until reaching a concentration indicated as K_m. It then slows and proceeds with a flattened slope toward a maximum enzyme activity (V_{max}). At substrate concentrations much greater than K_m, every enzyme will be bound to a substrate, not waiting for a binding event, with the enzyme activity approaching V_{max}. Under these conditions, velocity is limited only by the inherent turnover rate of the enzyme (k_{cat}), not by the rate of substrate-enzyme binding. Note that for certain enzymes (e.g. β-galactocerebrosidase) a very slow enzyme turnover rate will necessitate longer incubation times even under conditions in which substrate is present in excess[2]. With respect to LSD enzyme assays, an excess substrate concentration will increase the enzyme activity slopes for both normal and deficient samples. **Increased substrate concentration with respect to K_m will reduce incubation times without compromising the separation of normal and deficient samples.**

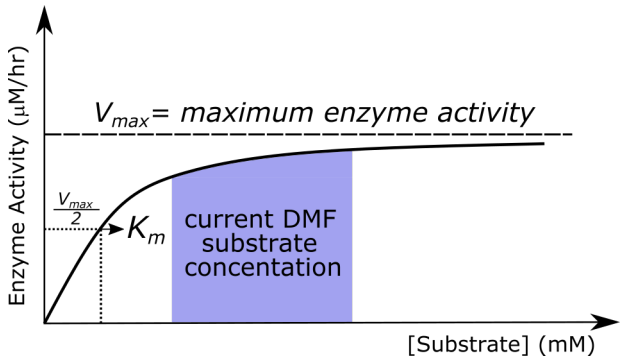

Figure 3. Enzyme activity or velocity as a function of substrate concentration: 1/2 maximum a quantity called K_m.

Figure 4 depicts an example of how a digital microfluidic (DMF) LSD enzyme assay, which uses a substrate concentration far in excess of K_m, compares to an assay in which the substrate concentration is limiting. In this example, the enzyme velocity (rate of product formation over a short incubation time) is maximized by using an excess amount of substrate.

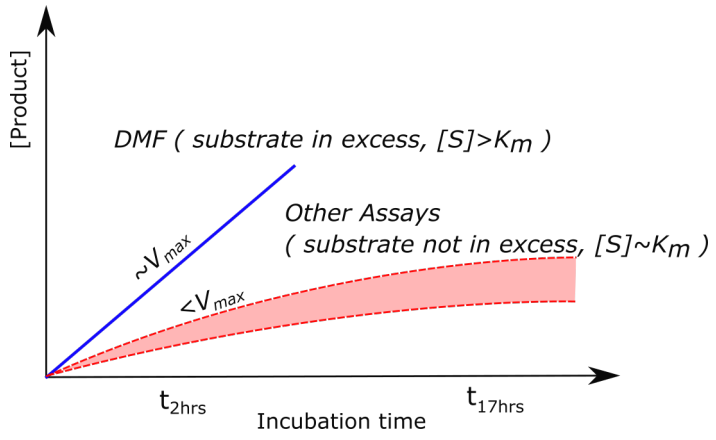

Figure 4. Product vs. time curves as determined by the concentration of substrate. If the enzyme assay reaction conditions are such that [S] is in excess (blue line), as in DMF reactions, then the rate of product formation is maximized ($v \rightarrow V_{max}$) and is directly proportional to enzyme concentration, [E]. However, if [S] is limited (red line), then longer assay incubation times are necessary to produce more product and are susceptible to nonlinearity (bias) and variability[3].

The goal of LSD newborn screening is to differentiate normal samples from those with very low enzyme activity; therefore it is essential to use very pure (i.e. expensive) synthetic substrates. The amount of substrate used is therefore typically kept as low as possible in order to support cost-effective newborn screening. The nanoliter volume reaction droplets used in DMF assays enable high substrate concentrations to be used and fast/linear rate kinetics to be attained without compromising costs; a critical advantage of the DMF technology. **Using this strategy, sufficient separation has been achieved between normal and affected samples with reaction times of ~1 hour**[4]. Reactions with limiting substrate concentrations need to run longer to accumulate sufficient product to compute enzymatic activity (as shown by the red band in Figure 4).

The use of limited amounts of substrate, and consequential long incubation times, introduces several undesirable assay performance issues (red line in Figure 4). As substrate is turned over to product by the enzyme, the substrate concentration decreases and its availability to the enzyme becomes more limited, known as **substrate depletion**. When this occurs over a long period of time, the measured enzyme activity rate is slowing down instead of being constant, leading to the flattening of the red line with time.

Other concerns with longer reaction times include the potential loss of enzyme activity due to enzyme instability (denaturing) over long incubation periods[5] and product inhibition due to excess product concentration relative to substrate (Table II). Most importantly, the use of prolonged reaction times in enzyme reactions with limited substrate has greater potential to lead to erroneous enzyme activity calculation due to the non-linear generation of product over time (red line of Figure 4) when a constant enzyme activity is assumed for the reaction.

Table II.
Potential disadvantages with use of limited amounts of substrate + long incubation

Enzyme activity not constant
Enzyme degradation with time
Underestimate enzyme activity
Product inhibition
Substrate depletion

The DMF LSD enzyme assays all operate under conditions designed to maximize enzyme velocity. These conditions, which include an appropriate concentration of substrate, allow DMF assays to obtain sufficient amount of product to accurately compute enzyme activity without risking degradation of the enzyme. **The nanoliter reaction format enables DMF enzyme assays to perform under desirable (excess) substrate conditions without incurring additional cost or compromising assay performance.**

References

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[4] Millington DS, Bali D. *MGM Reports*. 2017; 11: 72-73.

[5] Banroques J, Gregori C, Schapira F. *Biochim. Biophys. Acta* 1981; 657: 374-382.