BioTek
Get a Better Reaction.

Application Note

Kinetic Analysis of β-Galactosidase Activity using the PowerWave™ HT and Gen5™ Data Analysis Software

Basic enzyme kinetic determinations

Paul Held Ph. D. Senior Scientist, Applications Dept., BioTek Instruments, Inc.

The determination of the enzyme kinetic parameters for newly discovered proteins is an important procedure in cellular and molecular biology. Here we describe the use of kinetic reading for the analysis of the bacterial enzyme, β-galactosidase, using o-nitrophenol-β-D-galactoside (ONPG) as the substrate.

Introduction

Most biological processes require an enzyme to act as a catalyst in order for the reaction to take place. With the exception of catalytic RNA, all enzymes are proteins. Most importantly, enzymes are highly effective in catalyzing diverse chemical reactions in a regulated, selective, and specific manner. With a few exceptions, most reactions catalyzed by enzymes can be described satisfactorily by the Michaelis-Menten Equation (Eq. 1),

$$v = V_{max} \frac{[S]}{Km + [S]} \qquad \text{Eq. 1}$$

where v = reaction rate, [S] = substrate concentration, V_{max} = maximal velocity, and K_m is the Michaelis constant. Each enzyme has physical characteristics with regard to substrate specificity, reaction velocity or required cofactors that affect the Michaelis-Menten constants. The determination of these constants involves the performance of kinetic enzymatic activity measurements. Here we utilize an absorbance-based assay for β-galactosidase enzyme activity to demonstrate the capabilities of the PowerWave HT Microplate Spectrophotometer in conjunction with Gen5 Data Analysis Software to perform routine analysis of enzyme kinetics in microplates.

The enzymatic product of the *LacZ* gene, β-galactosidase, catalyses the hydrolysis of β-D-galactosides, such as lactose, into their component sugars by hydrolysis of the terminal nonreducing β-D-galactose residues (Figure 1).

Fortunately the substrate specificity of the enzyme is such that a variety of different substrates, each with a β-D-galactopyranoside moiety, can be acted upon. Investigators have taken advantage of this by synthesizing compounds which when hydrolyzed by β-galactosidase result in a colored product. One such compound is *o*-nitrophenol-β-D-galactoside (ONPG), which when hydrolyzed forms galactose and *o*-nitrophenol (Figure 1).

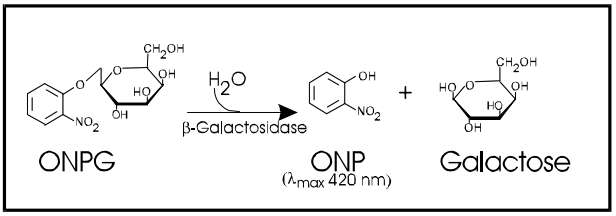

Figure 1. Enzymatic hydrolysis of *o*-nitrophenol-β-D-galactoside (ONPG) to of *o*-nitrophenol (ONP) and galactose by β-galactosidase. β-galactosidase catalyses the hydrolysis of the terminal β-D-galacto-pyranoside moiety resulting in the formation of the yellow (λ_{max} 420 nm) compound ONP.

The compound ONP absorbs light at 420 nm whereas the precursor molecule ONPG does not. Therefore, the increase in light absorbance at 420 nm can be used to monitor β-galactosidase when ONPG is used as a substrate.

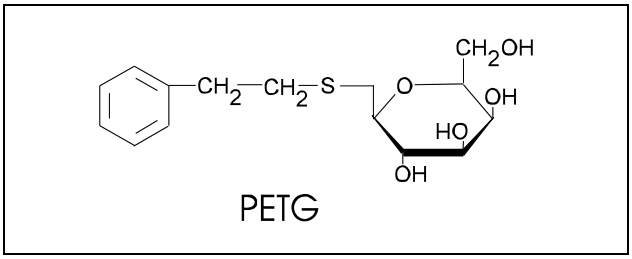

Figure 2. Molecular structure of phenylethyl β-D-thiogalactopyranoside (PETG). Thiol containing compound that cannot be hydrolyzed by β-galactosidase.

Other compounds that possess a conformation similar to ONPG or lactose (i.e. have a β-D-galactose moiety), can be used in combination with β-galactosidase. There are several compounds, such as 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), that form colored insoluble products after hydrolysis. These compounds are quite useful in screening for bacterial plasmid recombinants (1). Bacterial cells containing the *LacZ* gene (*Lac*$^+$) would hydrolyze X-gal and turn blue, while *Lac*$^-$ cells, unable to hydrolyze the compound would remain white. Alternatively, compounds that cannot be hydrolyzed but retain a similar structure would be expected to act as an inhibitor. Phenylethyl β-D-thiogalactopyranoside (PETG) has a thiol group substituted for the hydroxyl linkage present in compounds that β-galactosidase normally hydrolyzes (Figure 2). PETG when present in a reaction would be expected to occupy the enzyme and thus inhibit its action on hydrolyzable substrates such as ONPG.

Materials and Methods

o-Nitrophenyl β-D-galactopyranoside (ONPG), catalogue number N-8431, and phenylethyl β-D-thiogalactopyranoside (PETG), catalogue number P-1692, were purchased from Invitrogen (Carlsbad, CA). The 96-well microplates, catalogue number 3635, were purchased form Corning Life Science, (Cambridge, Massachusetts). β-galactosidase enzyme cat. # G-6008, sodium phosphate, magnesium chloride and 2-mercaptoethanol were obtained from Sigma-Aldrich Chemical Company (St Louis, Missouri)

The β-galactosidase assay was performed according to Sanbrook et.al (1). 100 µl aliquots of samples or standards diluted in distilled water were placed in each well of a 96-well microplate. The assay was initiated by the addition of 100 µl of 2X assay buffer. Assay buffer (1X) consists of 100 mM sodium phosphate, pH 7.0; 1 mM $MgCl_2$; 50 mM β-mercaptoethanol; and 0.665 mg/ml ONPG in distilled water. Assay buffer was prepared previously as a 2X stock solution and stored frozen at -20°C. Lyophilized β-galactosidase enzyme was reconstituted with distilled water to stock concentration of 500 U/ml. Enzyme dilutions were made fresh daily and stored on ice until assayed. A series of enzyme dilutions ranging from 0 to 5 U/ml of β-galactosidase (β-gal) were then made using distilled water as the diluent. All absorbance determinations were made at 420 nm using a PowerWave™ HT Microplate Spectrophotometer (BioTek Instruments, Winooski, VT) with the reader controlled by Gen5 Data Analysis Software (BioTek Instruments, Winooski, VT). After the assay was initiated by the addition of the 2X assay buffer, kinetic readings were initiated immediately with absorbance determinations made every 30 seconds for a total of 60 minutes at ambient temperature.

In order to examine the effect of the inhibitor PETG on β-gal activity a series of dilutions were made and added to the reaction mixture. Stock solutions (10 mg/ml) of PETG were made in acetonitrile and stored at -20°C until needed. Further dilutions ranging from 0 to 1000 µg/ml were then made using 1X assay buffer as the diluent. These dilutions were added to equivalent β-gal reactions. To each well, 100 µl of 0.25 U/ml β-gal enzyme was added along with 100 µl of the appropriate inhibitor dilution in 1X reaction buffer. As before, the assay was initiated by the addition of 100 µl of 2X reaction buffer containing ONPG as the substrate. Subsequent data reduction was performed using Gen5 software.

In order to determine Michaelis-Menten equation values, a series of dilutions of the ONPG substrate were utilized. A 20 mM stock solution of ONPG substrate in 2X reaction buffer was prepared and dilutions from 0 to 20 mM were made using 2X reaction buffer without ONPG as the diluent. Aliquots of 100 µl for each dilution were pipetted into microplate wells in replicates of 8. Reactions were initiated by the addition of 100 µl of 0.25 U/ml β-galactosidase in water to each well and kinetic absorbance reading performed as previously described. In experiments where PETG was used in conjunction with variable ONPG substrate concentrations, reactions were performed in duplicate.

Results

The absorbance of enzyme concentrations ranging from 0 to 5 U/ml were read kinetically. When absorbance values are plotted as a function of time, enzyme concentrations up to 0.1 U/ml are linear for at least 60 minutes (Figure 3). Enzyme concentrations above 0.5 U/ml result in a plateau of absorbance prior to 60 minutes, with the 5 U/ml samples reaching maximal values by 2 minutes. When V_{max} values are calculated from these data and plotted against enzyme concentration using Gen5 a linear relationship is observed (Figure 4). Using a polynomial regression analysis of these data an equation describing this relationship can be used with a high degree of confidence, as the coefficient of determination (R^2) is 0.9998. When data from enzyme concentrations up to 1 U/ml are considered, the V_{max} response is linear. Linear regression analysis of this subset of the data results in a coefficient of determination (R^2) of 0.998 (data not shown).

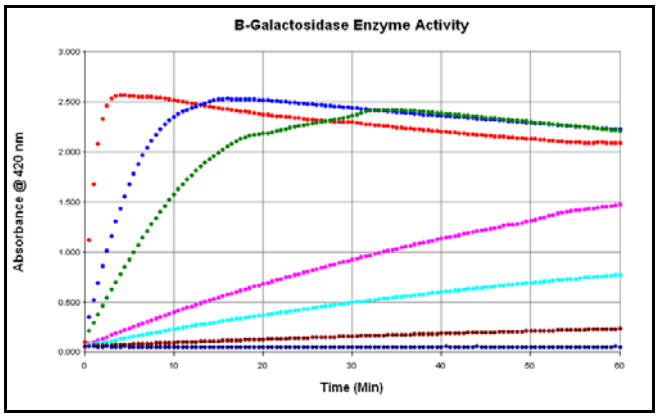

Figure 3. Kinetic reaction of various β-galactosidase enzyme concentrations. The absorbance of samples containing the indicated concentrations of β-galactosidase enzyme were determined in 30 second intervals for a total of 60 minutes. Absorbance data was then plotted versus time using Gen5.

The effect of substrate concentration on velocity was analyzed. As demonstrated in Figure 5, the velocity of the reaction increases dramatically as the ONPG substrate concentration is increased to 2 mM in the presence of 0.25 U/ml of purified β-galactosidase enzyme. ONPG levels above 2 mM do not appreciably increase the reaction rate. An estimation of V_{max} and K_m can be made from these data. Using a 4-Parameter logistic fit of the data to describe the data, V_{max} can be estimated from parameter "d" (theoretical response at infinite concentration) of the equation (2). Using this method, the V_{max} was determined to be 33.4 mOD/min (Figure 5).

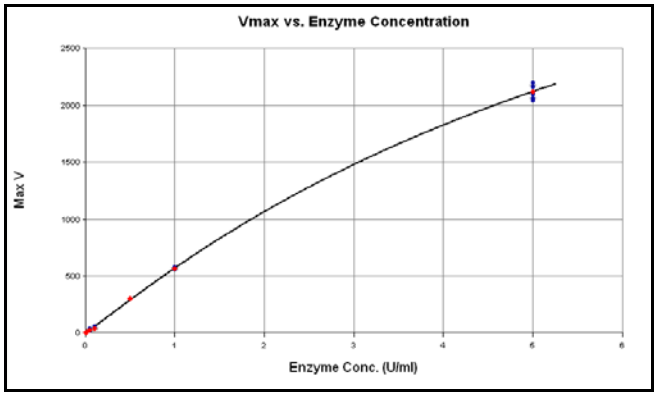

Figure 4. V_{max} vs. β-galactosidase enzyme concentration. The V_{max} values were determined from kinetic data using Gen5 data reduction and plotted against enzyme concentration using a 4-parameter logistic fit.

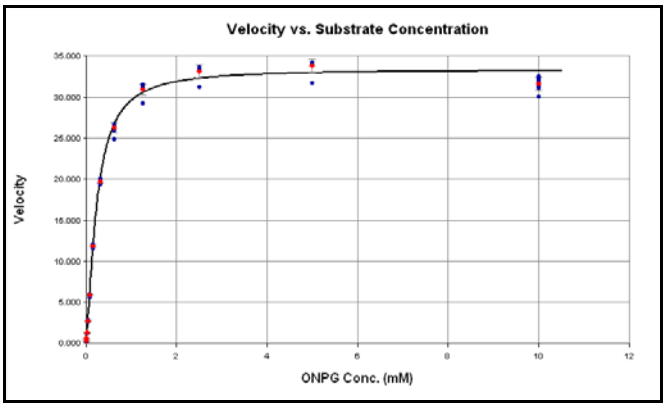

Figure 5. Effect of substrate concentration on enzyme velocity. Increasing concentrations of ONPG were reacted with 0.25 U/ml β-galactosidase enzyme and the absorbance determined kinetically. The average velocity was then plotted against the ONPG substrate concentration using a 4-Parameter logistic fit to describe the data. The Michaelis-Menten equation values for K_m and V_{max} were then calculated using Gen5.

The Michaelis constant or K_m, which represents the substrate concentration, which results in half-maximal velocity, can also be calculated using Gen5 and was determined to be 0.24 mM in this experiment.

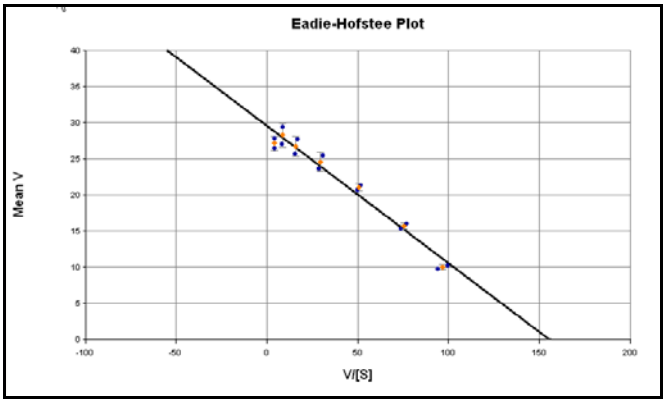

Figure 6. Eadie-Hofstee Transformation of the substrate concentration vs. reaction velocity. Reaction velocity (v) was plotted against velocity divided by substrate concentration (v/[S]) and linear regression analysis performed. Note that the y-intercept "b" defines the V_{max} of the reaction, while the slope "a" defines $-K_m$.

Although the value for K_m is equal to the substrate concentration ([S]) at half-maximal velocity ($V_{max}/2$), direct determination of V_{max} is frequently difficult to obtain experimentally. Michaelis-Menten values for K_m and V_{max} have been estimated from several transformations of the original equation. These transformations include the Eadie-Hofstee and the Lineweaver-Burk plots. The Eadie-Hofstee transformation plots velocity against the velocity divided by the substrate concentration (Eq. 2).

$$V = -K_m\left(\frac{V}{[S]}\right) + V_{Max} \qquad \text{Eq. 2}$$

The Lineweaver-Burk or double reciprocal transformation plots the reciprocal of velocity (1/v) against the reciprocal of substrate concentration (1/[S]) (Eq. 3). These types of plots, which generate linear regressions, allow the investigator to more easily determine K_m and V_{max}. When using the Eadie-Hofstee transformation (Eq. 2) the slope is equal to $-K_m$, while V_{max} is determined from the y-intercept.

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}}\right)\left(\frac{1}{[S]}\right) + \frac{1}{V_{max}} \qquad \text{Eq. 3}$$

Using this transformation we determined an apparent K_m of 0.1556 mM for the enzyme and a V_{max} of 34.89 mOD/min (Figure 6). Using a Lineweaver-Burk plot, V_{max}, which can be calculated from the reciprocal of the y-intercept, was determined to be 39.1 mOD/min (Figure 7). The apparent K_m, which can be calculated from the slope of the line (slope = K_m/V_{max}), was determined to be 0.3449 mM (Figure 7).

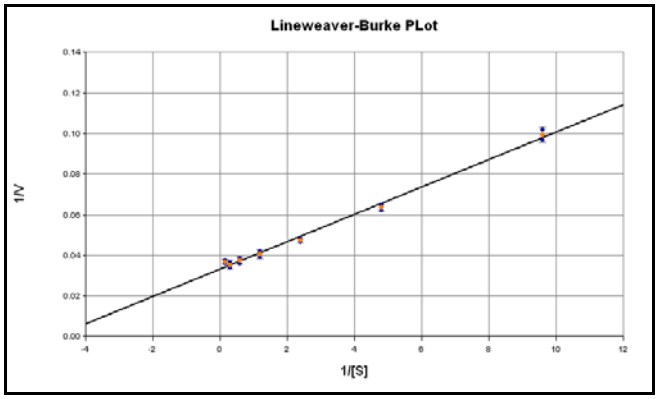

Figure 7. Lineweaver-Burk or double reciprocal plot of velocity versus substrate concentration. Reciprocals of experimentally determined reaction velocity (1/v) were plotted against the reciprocal of substrate concentration (1/[S]) and linear regression analysis performed. The y-intercept "b" defines the reciprocal of V_{max}, while the slope "a" is equal to K_m divided by V_{max}.

The effect of PETG on the β-galactosidase enzyme activity was investigated. β-galactosidase reactions containing equal amounts of both enzyme and ONPG substrate were read kinetically in the presence of increasing amounts of the non-hydrolyzable compound PETG. Increasing amounts of PETG result in a sigmoidal decrease in the V_{max} values for β-galactosidase (Figure 8). Inhibitor concentrations above 1000 μg/ml result in virtually no enzyme activity, while concentrations below 0.15 μg/ml demonstrate very little inhibition.

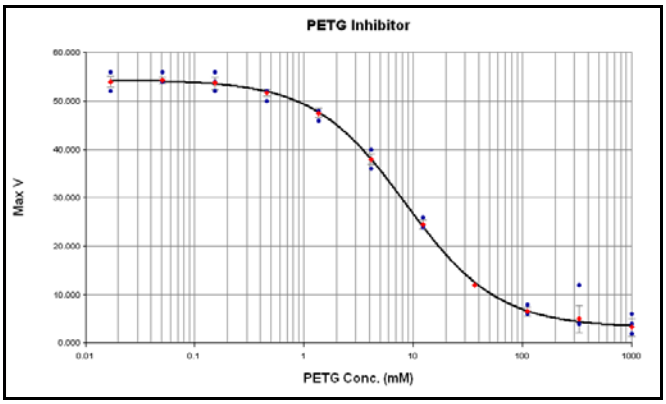

Figure 8. Effect of PETG inhibitor concentration on β-galactosidase V_{max}. Increasing concentrations of PETG were added to samples containing 0.25 U/ml β-galactosidase enzyme and the samples, kinetic readings at 420 nm every 30 seconds taken. V_{max} values were calculated for each inhibitor concentration and plotted using a 4-parameter logistic fit. Note that the ordinate axis (PETG concentration) is expressed in a log scale.

The influence of inhibitor on the substrate concentration ([S]) reaction velocity (v) relationship was investigated. As demonstrated in Figure 8, when a constant amount of enzyme is incubated with increasing amounts of substrate in the presence of PETG, the ability to reach the maximal reaction rate (V_{max}) is diminished.

When the substrate is in large excess relative to the inhibitor, V_{max} is achieved (Figure 9, red line). However, when a relatively large amount of inhibitor relative to substrate is present (Figure 9, green line) then V_{max} cannot be achieved. When Lineweaver-Burk plots are made using these data, regression lines with different slopes are observed, suggesting that the inhibitor influences the K_m of the reaction (Figure 10). The linear regressions all relatively have the same y-intercept point indicating that all of the reactions have the same V_{max}. When the V_{max} values are calculated using Gen5 from the y-intercepts of the plots values of 30.94, 30.65, and 26.07 mOD/min are obtained for samples containing 0, 0.167, and 1.67 mM PETG respectively.

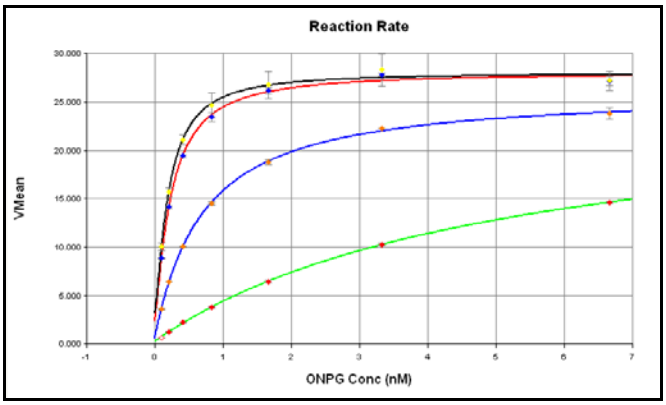

Figure 9. Effect of PETG on reaction velocity with increasing substrate concentration. PETG was added to samples at 0 mM (black); 0.167 mM (red); 1.67 mM (blue); and 16.7 mM (green) final concentration to samples with various concentrations of ONPG substrate in duplicate. Four individual concentrations curves were plotted using a 4-parameter fit and overlaid using Gen5.

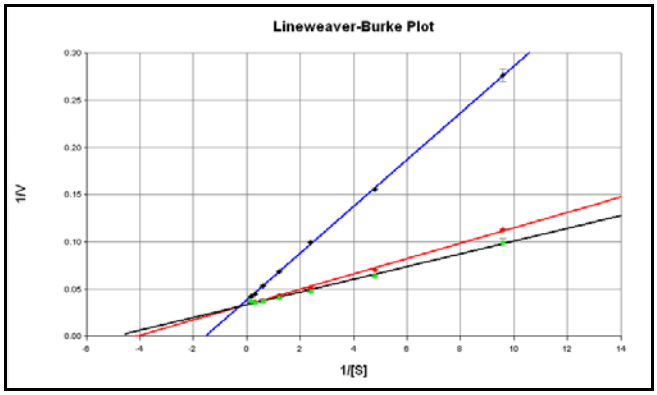

Figure 10. Lineweaver-Burk plot of β-galactosidase activity in the presence of PETG inhibitor. Double reciprocal plot of the data presented in Figure 9. The black line represents samples with 0 mM PETG, while the red line plots 0.167 mM PETG and the blue line plots 1.67 mM PETG. Linear regression of the three data sets were plotted and overlaid using Gen5.

Discussion

Initial experiments demonstrate the importance of reaction concentrations. Maintaining an appropriate relationship between the catalytic enzyme concentration, substrates, and incubation time is paramount in obtaining appropriate linearity in the assay. For example, the plateau of absorbance seen with the higher enzyme concentrations indicates that either shorter incubation times (e.g. 2 to 5 minutes) would provide superior linearity for these enzyme concentrations. Lower enzyme concentrations can be used to maintain linearity for 30 or 60 minute incubations. Increasing the ONPG substrate concentration is limited by the solubility of the compound in the aqueous reaction buffer and by the microplate reader absorbance limits.

The reaction kinetics for this enzyme would be expected to be linear with regard to V_{max}. and enzyme concentration. Because the monomeric protein interacts with one substrate at a time, allosteric binding effects would not be observed. The tailing off of the V_{max} seen in Figure 4 at the highest enzyme concentration tested is most likely due to rapid depletion of ONPG substrate within 60 seconds (two time points). This is corroborated by the data in Figure 3, which demonstrates the rapidity of reaching maximal absorbance for high concentrations of enzyme.

When reaction velocity is plotted against substrate concentration maximal velocity V_{max} and K_m can be determined. With a 4-parameter logistic fit an estimate of maximal velocity is made using parameter "d". The Michaelis constant can then be calculated by entering in the value "$V_{max}/2$" using the interpolation function of Gen5. The resultant concentration returned is the K_m. Because of the difficulty of estimating the V_{max} prior to the advent of computers, several methods were developed to determine these values using linear regression. Two such examples are the Eadie-Hofstee transformation and Lineweaver-Burk plots. Similar results were obtained for V_{max} regardless of the method. Interestingly, the average of these two means of determining K_m (0.25 mM) results in a value very close to that determined from the original velocity vs. substrate concentration plot (Figure 5) which was 0.24 mM. Although the calculated K_m for ONPG is higher than reported values for β-galactosidase using lactose as the substrate (3), it is not remarkably greater.

The inhibitor PETG is a non-hydrolyzable substrate for β-gal. The substitution of a thiol linkage for a hydroxyl group eliminates hydrolysis (Figure 3). Experiments with PETG suggest that the inhibitor has an equal affinity for the enzyme as the substrate ONPG. The molar concentration at which PETG begins to affect enzyme activity is very close to the concentration of the ONPG substrate indicating similar affinities. If the enzyme had a greater affinity for ONPG than PETG then inhibition would have required proportionally more of the inhibitor to elicit the same response.

These data presented also indicate that PETG is a competitive rather than a non-competitive inhibitor of β-galactosidase. Measurements of the reaction rates at different concentrations of substrate and inhibitor serve to distinguish between competitive and non-competitive inhibition. Competitive inhibitors compete for enzyme binding sites and as such, a large excess of substrate to inhibitor will result in reaction rates similar to samples that do not contain inhibitor. This would result in V_{max} values that are equivalent to that of enzyme without inhibitors present (Figure 10). The discrepancy between the calculated V_{max} for the 1.67 mM PETG samples and controls (0 mM PETG) is the result of ONPG not being in sufficient excess. Noncompetitive inhibitors would show different V_{max} values regardless of the substrate concentration (3). PETG also presents chemical structure very similar to ONPG, suggesting that it will bind to the active binding site of the β-galactosidase enzyme.

In the past such kinetic determinations have been performed using a conventional spectrophotometer, which usually entails the use of matched cuvettes to perform the analysis, resulting in a very low throughput. The ability to use the PowerWave™ HT Microplate Reader to perform this analysis allows this routine procedure to be performed on 96 samples in a matter of seconds leading to a tremendous increase in productivity and throughput.

These data presented in this report also demonstrate the utility of Gen5 Data Analysis Software to perform routine enzyme kinetic studies. Automatic determination of values such as V_{max} and K_m allows the end user flexibility in regards to data reduction of kinetic assays.

References

(1) Sanbrook J. E.F. Fritsch, and T. Maniatis (1989) Molecular Cloning: A Laboratory Manual 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.
(2) BioTek Instruments, Application Note "BioTek's 4-Parameter Logistic Fit", Winooski, Vermont.
(3) Stryer, L (1988) Biochemistry 3rd edition, W.H. Freeman and Co., New York, NY, p. 190.