Enzyme kinetic measurements performed on BMG LABTECH´s FLUOstar OPTIMA

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Introduction

Esterases catalyze hydrolysis reactions by converting esters into an acid and an alcohol using water as nucleophil. Therefore, esterases belong to the enzyme group of hydrolases. They are often used as biocatalysts to produce optically pure compounds.[1-2]

Esterases differ in their affinity to specific substrates and this affinity is represented by the Michaelis-Menten constant K_m. It describes the affinity of an enzyme to a substrate. K_m is equivalent to the substrate concentration at which the reaction velocity is half of the maximal velocity. That means that a high affinity to a substrate leads to a small K_m-value and vice-versa.

BMG LABTECH has developed a new evaluation software feature able to calculate the K_m value as well as the maximal velocity (V_{max}) from an enzymatic kinetic measurement. Next to the Michaelis-Menten fit, other linearized fits are also shown and a comparison of the results is given.
As a model reaction, the p-nitrophenyl acetate (pNPA) assay was performed on a FLUOstar OPTIMA microplate reader.

Assay Principle

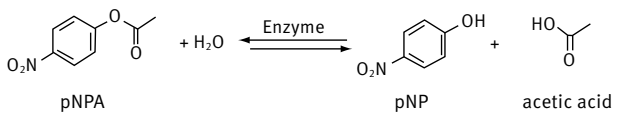

Fig. 1: Scheme of the pNPA assay

The enzyme hydrolyzes the acetate ester with the help of water. The products are acetic acid and p-nitrophenol (pNP), the latter showing an absorption maximum at about 405 nm.

Materials and Methods

Enzyme kinetic measurements
First a 10 mM stock solution of the substrate pNPA in DMSO is prepared. In each well 190 µL phosphate buffer (50 mM, pH 7.4) and 10 µL enzyme preparation is pipetted. 40 µL of substrate (solved in DMSO) at different concentrations is added using the onboard injectors. The final volume per well is 240 µL. The enzymatic reaction starts after adding the substrate. The absorbance is measured at 410 nm for every second for 90 seconds at 37°C. The blank represents the autohydrolysis of pNPA without enzyme. In addition, a pNPA-free negative control (NC) is run that consists only of buffer and enzyme.
The measurement is performed using the well mode (see instrument settings below) that provides the possibility to inject and to measure absorbance at the same time.

Instrument settings
Measurement type: Absorbance, Well Mode
No. of intervals: 90
Kinetic interval time: 1 s
No. of flashes per well: 20
Positioning delay: 0.5 s
Target temperature: 37°C
Filter: 410-10 nm

Injection settings:
Volume of pump 1 and 2: individual
Pump speed: 310 µl/s
Injection start time: 2.0 s

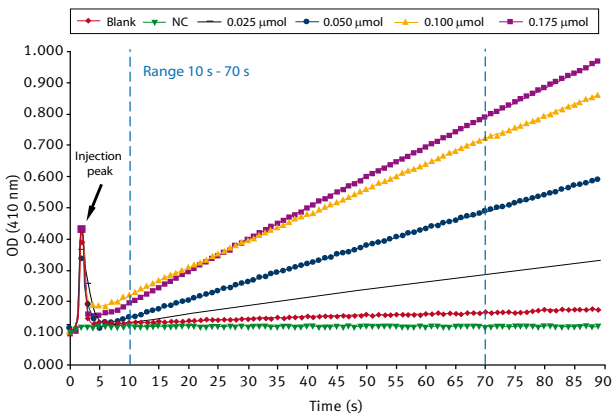

Fig. 2 : Signal curves of esterase-catalyzed reactions using different concentrations of the substrate pNPA (pNPA concentrations range from 0.025 µmol to 0.175 µmol / 240 µL).

pNP standard curve
For the calculation of the initial reaction rates it is necessary to convert the delta OD values per minute into µmol product per minute. Therefore a standard curve of the product using the same reaction conditions as for the kinetic measurements has to be done. From a 10 mM pNP stock solution, solved in phosphate buffer (50 mM, pH 7.4), different dilutions of pNP ranging from 0.0025 µmol to 0.1 µmol were prepared in 200 µL. 40 µL of DMSO were added simulating the DMSO concentration in the kinetic samples. The optical density (OD) is measured using an absorbance 410-10 nm filter.

Figure 3 shows a pNP standard curve.

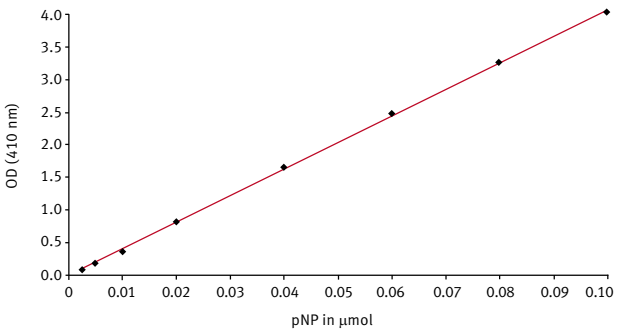

Fig. 3: pNP standard curve using 240 µL volume with 16.6 % DMSO.

The slope of the standard curve stands for the extinction coefficient for pNP under the conditions mentioned. It was determined to be about 41 OD per µmol. The coefficient is used to calculate the initial reaction velocities.

- p-nitrophenol (pNP) and p-nitrophenyl (pNPA) acetate, Sigmaaldrich, Germany
- Clear 96-well microplates, Greiner, Frickenhausen, Germany
- FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany
- MARS Data Analysis Software, Version 1.20 or higher
- Two different enzyme preparations (E1 and E2) were kindly provided by the working group of Prof. Bornscheuer, Greifswald

Results and Discussion

After the enzyme kinetic measurement is finished, the evaluation software provides different plots and presents the corresponding K_m and V_{max} values. The Michaelis-Menten fit shows the reaction velocities depending on the substrate concentration (Figure 4).

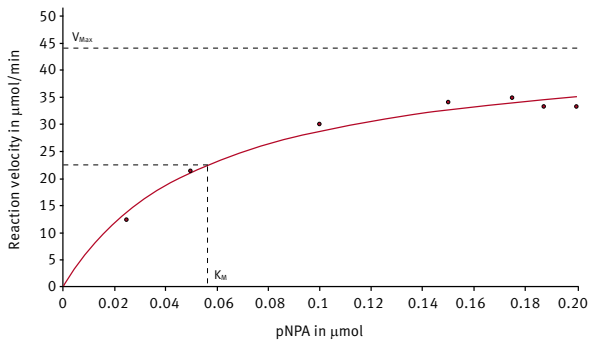

Fig. 4: Michaelis-Menten plot of the reaction velocity, obtained with the E1 enzyme preparation, depending on the substrate concentration. K_m is the substrate concentration corresponding to the half of the maximal velocity.

Next to the Michaelis-Menten fit different linearized plots are available, like Lineweaver-Burk.
The results of further linearized fits are compared to the Michaelis-Menten findings in table 1.

Table 1: K_m and V_{max} comparison obtained from different enzyme kinetic plots.

Plot	E1		E2	
	K_m [µmol]	V_{max} [µmol/min]	K_m [µmol]	V_{max} [µmol/min]
Michaelis-Menten	0.056	44.8	0.12	16.3
Lineweaver-Burk	0.073	49.2	0.08	12.5
Eadie-Hofstee	0.063	46.4	0.07	13.2
Scatchard	0.068	47.9	0.10	15.2
Hanes	0.056	44.4	0.11	15.5

The different methods to determine K_m and V_{max} have all assets and drawbacks. Because of that it is recommended to have a look at different plots for deciding which calculated constants are really correct. With the help of the MARS Data Analysis Software enzyme kinetic evaluation is very simple because every plot is available directly after measurement and calculation.

Conclusion

The new software feature for enzyme kinetic offers fast and easy calculation of K_m and V_{max}.
Available plots are the common Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee, Scatchard and Hanes kinetic fits.

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