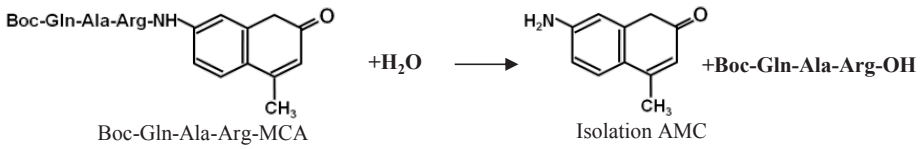
Application Note
FP-0011

Activity Measurement of Trypsin Using a Fluorescence Peptide Substrate

Introduction

In biology, proteolytic reactions occur to regulate enzyme and protein activity for metabolic to signal processing functions. Proteases are responsible for breaking down biological molecules into smaller polypeptide chains through hydrolysis. A hydrolysis reaction adds a water molecule to the location where the peptide bond has been cleaved. Scheme 1 shows the hydrolysis reaction of fluorescent dye, methylcoumarin-amide (MCA), which is bound to a trypsin peptide, Boc-Gln-Ala-Arg. Upon hydrolysis of the substrate, isolated AMC and a water-bound trypsin peptide are produced.

FP-8300
Spectrofluorometer



Boc-Gln-Ala-Arg-MCA　　Isolation AMC

Scheme 1. Hydrolysis of trypsin methylcoumarin-amide (MCA) to form isolated 7-amido-4-methylcoumarin (AMC).

Trypsin is a protease that is commonly used in assays to determine the enzymatic activity of a molecule. After cleavage of the substrate via hydrolysis, the trypsin activity can be measured by monitoring the fluorescence intensity of the isolated product, AMC. This application note demonstrates how to obtain enzyme kinetic data using a FP-8300 and the Kinetics Analysis program.

Keywords

FP-8300, Fluorescence, STR-812 Water thermostatted cell holder, Kinetics, Enzyme activity, VWKN-772 Kinetics Analysis Program

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Experimental

Measurement Conditions			
Fluorescence		**Time Course**	
Excitation Wavelength	360 nm	Excitation Wavelength	360 nm
Emission Wavelength	440 nm	Emission Wavelength	440 nm
Excitation Bandwidth	5 nm	Excitation Bandwidth	5 nm
Emission Bandwidth	10 nm	Emission Bandwidth	10 nm
Data Interval	1 nm	Data Interval	0.1 sec
Response Time	0.5 sec	Response Time	0.1 sec
Sensitivity	200 V	Sensitivity	200 V
Scan Speed	500 nm/min		

The enzyme solution was prepared by adding 10 nmol/L of trypsin bovine pancreas type VIII to a buffer solution containing 50 mmol/L Tris-HCl, 0.15 mol/L NaCl, 1.0 mmol/L $CaCl_2$, and 0.1 mg/mL BSA.

Results

To find the maximum emission wavelength to monitor the fluorescence intensity of AMC after trypsin cleavage, the excitation and emission spectra were measured and are shown in Figure 1. The maximum emission wavelength is found to be 440 nm.

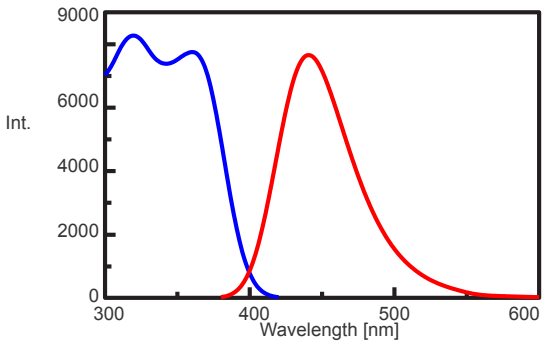

Figure 1. Emission (blue) and fluorescence (red) spectra of 50 µmol/L of AMC.

In order to standardize the measured fluorescence intensity of the enzyme solution to the concentration of isolated AMC, a titration was performed and the fluorescence measured. 0.5 mL aliquots of a 50 µmol/L AMC solution was added to a 2.5 mL enzyme solution and the initial and final concentrations of isolated AMC are summarized in Table 1.

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Initial Concentration [µmol/L]	3	6	15	30	60	120	240
Final Concentration [µmol/L]	0,5	1	2.5	5	10	20	40

Table 1. Isolated AMC concentrations before and after fluorescence intensity standardization.

The kinetics of the trypsin activity were then obtained by measuring the fluorescence intensity of isolated AMC upon cleavage of trypsin from the substrate. Figure 2 shows the time course measurement after 0.5 mL of varying concentrations of the Boc-Gln-Ala-Arg-MCA substrate solution were added to 2.5 mL of the enzyme solution.

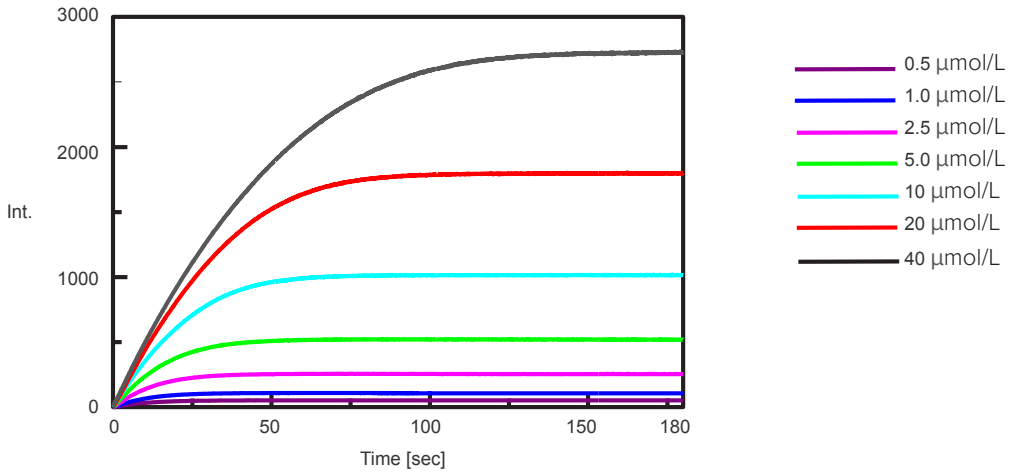

Figure 2. Fluorescence intensity of varying substrate concentrations added to the enzyme solution. The substrate concentrations are the final concentrations seen in Table 1.

Using the Kinetics Analysis program, a Lineweaver-Burk graph (Figure 3) was plotted from the fluorescence time course measurements in Figure 2. The Lineweaver-Burk plot provides enzyme kinetic parameters such as the maximum rate of the reaction, V_{max}, and the Michaelis-Menten constant, K_m. V_{max} describes the rate of the reaction when the enzyme is saturated with substrate while K_m indicates the amount of substrate to reach the maximum reaction velocity. V_{max} was 35,270 nmol/L•min^{-1} and K_m was 5.99. The equation of the line was $1/v = 0.000270 \cdot 1/[S] + 0.0000284$.

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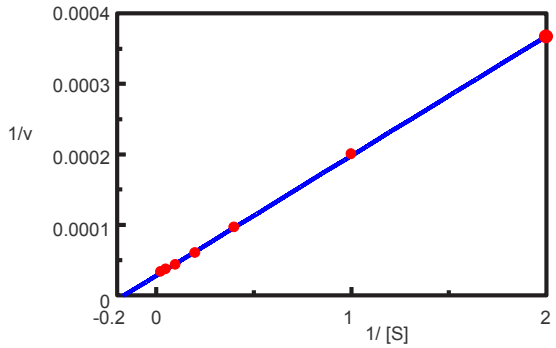

Figure 3. Lineweaver-Burk plot.

Conclusion

This application note demonstrates the measurement reproducibility of the One Drop microsampling accessory by obtaining a calibration curve with good linearity over a wide concentration range.

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