Application Note

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Monitoring Protein Dehydrogenase Activity with Filter-based Absorbance Spectroscopy

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Abstract

Enzyme kinetics of alcohol dehydrogenase were determined using a microplate spectrophotometer. The alcohol substrates ethanol and 2-propanol demonstrated K_m of 9.7 and 210 mM and V_{max} of 50 and 33 µmoles min^{-1}, respectively.

Introduction

Absorbance spectroscopy is an analytical method with a long history of use in a wide range of fields. Assays such as ELISA, and those relying on UV-absorption and colorimetric absorbance are performed in a variety of laboratory settings including basic research in academia and clinical laboratory testing. Many of these assays have evolved to include higher throughput formats that require the use of microplates and dedicated instrumentation. Instrumentation comes in a variety of configurations ranging from ultra-high sensitivity, single-wavelength light source instruments to monochromator-based platforms for broad range wavelength selection. With increased complexity comes increased costs and training requirements. Many applications can be accomplished with cost-effective instrumentation using a selection of installed bandpass filters that provide wavelength selection as well as excellent sensitivity. This application note shows the use of a filter-based absorbance microplate reader for the enzymatic analysis of a member of the dehydrogenase family of proteins.

A long studied enzyme family includes a group of alcohol dehydrogenase enzymes (ADHs) found in a diverse range of organisms, which are responsible for interconversion between alcohols and aldehydes or ketones. Since the discovery of ADH in 1937 in *Saccharomyces cerevisiae* (brewer's yeast), ADHs from many organisms have been studied in great detail.[1] In many organisms, the primary function is to eliminate toxic alcohols while generating useful chemicals such as ketones or interconversion to other useful alcohols. However, ADH is likely most prominently known for its ability to run in the opposite direction in yeast to produce alcohol in the process of fermentation for the production of beer and wine. The family of enzymes is also studied clinically for its relationship to alcoholism, drug dependence, and poisoning as well as for industrial applications such as a catalysis in fuel cell or production of enantiomerically pure stereoisomers of chiral alcohols.[2,3,4]

Assay principle

The enzymatic catalysis of alcohol requires the reduction of the cofactor nicotinamide adenine dinucleotide (NAD^+) to the reduced form NADH or the reverse chemical process for the formation of alcohol (Figure 1).

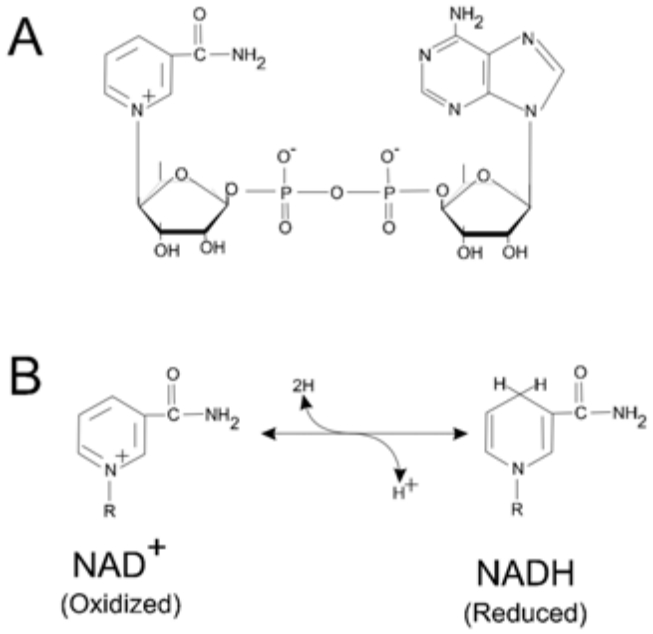

Figure 1. The cofactor NAD^+. (A) Chemical structure of NAD^+ (B) reduction of NAD^+ to NADH during enzymatic catalysis.

Figure 2 illustrates the typical absorbance changes upon reduction of NAD^+ to the reduced form NADH. The reduction of the nicotinamide moiety produces an additional absorbance peak centered at 340 nm. This allows the progression of reactions using the cofactor to be monitored either kinetically or as an endpoint measurement at this wavelength.

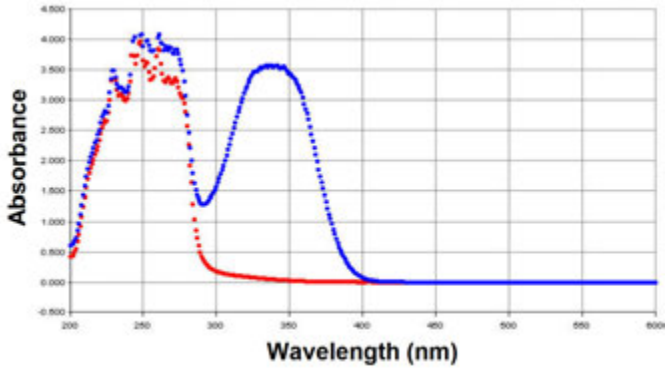

Figure 2. Spectral scans of NADH and NAD^+ solutions. Aliquots (100 µL) of NADH (blue dots) and NAD^+ (red dots) solutions (1 mg/mL) were aliquoted into half area-UV transparent plates and a spectral scan from 200 nm to 600 nm in 1 nm increments performed. Data was plotted using the Agilent BioTek Gen5 microplate reader and imager software.

Materials and methods

Standard 96-well UV transparent microplates (part number 3636), were purchased from Corning (Tewksbury, MA). A calibration standard of NADH was purchased from Sigma-Aldrich (St. Louis, MO) as preweighed vials (part number 8129). A stock solution was prepared in TE pH 8.0 aqueous buffer as the diluent. The concentrations of NADH solutions were validated by absorbance at 340 nm in a 1 cm pathlength cuvette using a Shimadzu UV-1700 spectrophotometer (Columbia, MD). Further dilutions were then made in TE buffer and 160 µL aliquots of each dilution were dispensed into microplate wells as triplicates. Absorbance measurements were made using an Agilent BioTek 800 TS absorbance reader at 340 nm. Alcohol dehydrogenase from Baker's yeast (part number A3263-30KU) and NAD^+ (part number 10127965001) were purchased from Sigma-Aldrich.

All enzymatic reaction measurements were made in enzyme diluent buffer (10 mM sodium phosphate buffer pH 7.5 (10 mM Tris, 0.1% (w/v) bovine serum albumin). Briefly, the enzyme activity of ADH was estimated using a 1 cm cuvette following reconstitution in enzyme diluent buffer following the manufacturers' recommendations. Microplate-based reactions were performed in a total volume of 160 µL consisting of 32 µL of 50 mM phosphate buffer (f.c. = 10 mM), 10 µL of 15 mM NAD^+, 10 µL of ~ 20 units/mL ADH in enzyme buffer, and initiated with the addition of 50 µL of alcohol substrate.

Instrumentation

An Agilent BioTek 800 TS absorbance reader was used for reading absorbance either in kinetic mode or as an endpoint measurement with a 340/40 nm bandpass filter. Measurements were typically performed by reading every 5 seconds for 6 minutes.

Data reduction

The absorbance change calculated for one-minute intervals during the linear kinetic interval were calculated. The calculated values represent the maximum velocity of the reaction (V_{max}) in absorbance units per minute. V_{max} values were then converted to µmoles NADH per minute per mg ADH protein (µmoles [NADH] min^{-1} mg^{-1}) by interpolation from the NADH standard curve. Reaction rate data were determined for various alcohol concentrations. Reaction rate data were plotted versus alcohol concentrations for generation of Michaelis-Menten plots by exporting data to GraphPad Prism (La Jolla, CA). Lineweaver-Burk plots were generated as double-reciprocal plots of the above data for determination of Michaelis-Menten constants (K_m and V_{max}) for each substrate.

Results and discussion

NADH standard curve

The NADH standard curve was prepared using a 1:2 serial dilution in TE starting from a stock concentration of 325 µM as determined from a 1 cm pathlength cuvette. Measurements were made by pipetting 160 µL of each concentration into a microplate in triplicate. The total amount of NADH present in each well was plotted versus the absorbance measurements at 340 nM (Figure 3). The standard curve was then generated in Gen5 using a least squares linear regression fit and used for the subsequent determination of the amount of cofactor reduced to NADH during enzymatic reactions with various alcohol substrates.

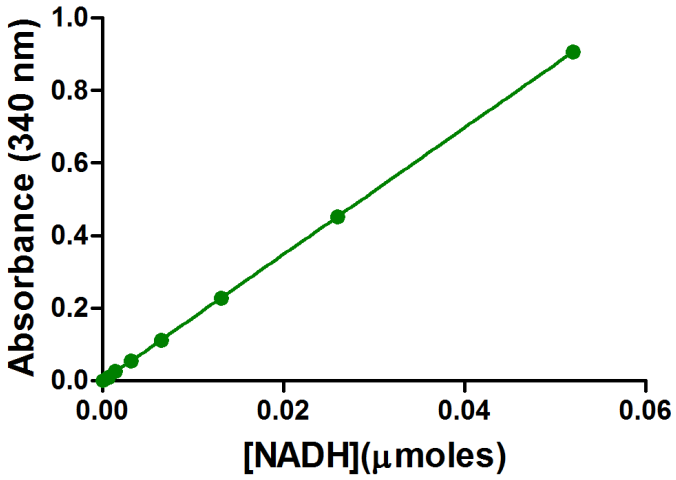

Figure 3. NADH concentration curve measured using absorbance at 340 nm. Serial dilutions of NADH ranging from 0 to 0.05 µmoles per well were made in TE (pH 8.0) aqueous buffer. The absorbance was determined using an Agilent BioTek 800 TS absorbance reader. Agilent BioTek Gen5 microplate reader and imager software was used for reader control and data capture, as well as linear regression analysis of the data.

Enzyme assay

Enzyme assays were performed in triplicate and initiated sequentially, for each substrate concentration, by the addition of 50 µL of the alcohol substrate. Triplicate measurements were captured kinetically to ensure capture of the linear portion of substrate and cofactor conversion. The sequential initiation for each substrate concentration was required given the rapid rate of substrate and cofactor depletion. Reagents, including enzyme, cofactor, and substrate were optimized to insure reaction rates were ~0.1 OD/min. A 1:2 serial dilution of ethanol was prepared to give a final concentration in the assay ranging from 0.8 to 100 mM and of 2-propanol from 3.9 to 500 mM.

Initial reaction rates were calculated for the first 60 seconds for each reaction by subtracting the initial OD from t = 60 seconds. The ΔOD340 nm was converted to µmoles [NADH] min^{-1} mg^{-1} for each substrate concentration as described above and plotted versus substrate concentration. Michealis-Menten plots were generated in Graph Prism using standard methods and subsequently the double-reciprocal Lineweaer-Burk plots using the Michaelis-Menten constants (Figure 4 and Table 1).

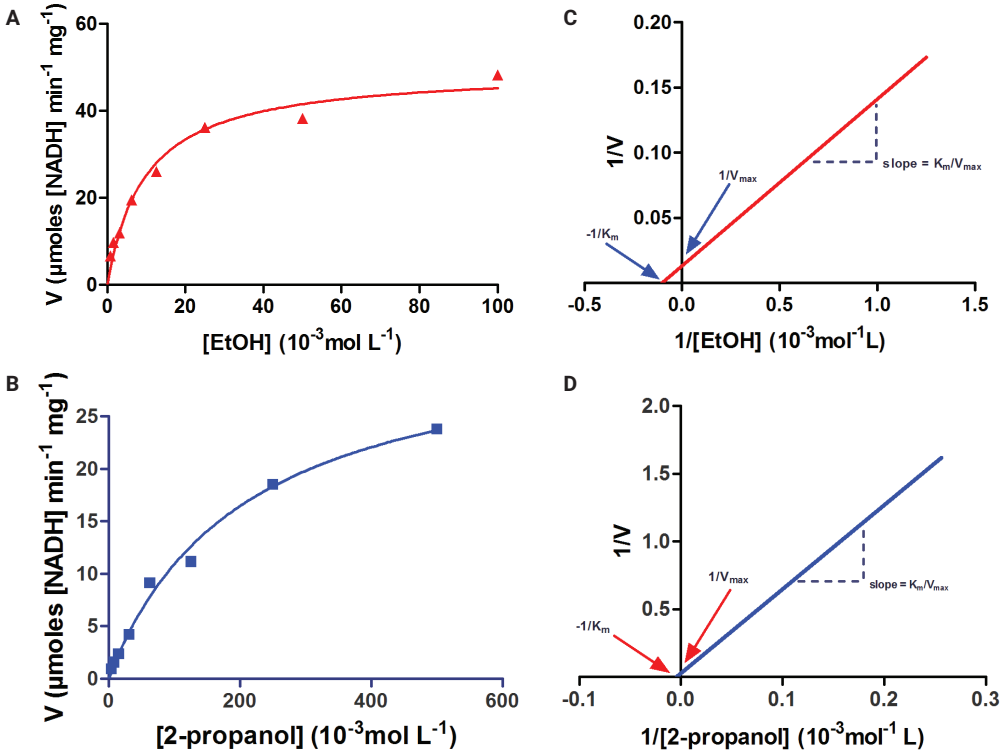

Figure 4. Enzyme activity for ADH from *S. Cerevisiae*. Michaelis-Menten (A, B) and Lineweaver-Burk plots (C, D) for the substrates ethanol and 2-propanol, respectively, were generated for determination of K_m and V_{max}.

Table 1. Michaelis-Menten constants.

	Ethanol	2-Propanol
K_m (mM)	9.7	207.5
V_{max} (µmoles•min^{-1}•mg^{-1})	49.6	33.48

Conclusion

Microplate-based assay formats are the workhorse of many labs performing absorbance spectroscopy that require a higher throughput or multiple experimental conditions. Instrumentation that uses filter-based optical systems for sample measurement can provide simple, affordable solutions for ease of workflow and increased throughput. The analysis of alcohol dehydrogenase from Baker's yeast using the Agilent BioTek 800 TS absorbance reader illustrates a typical workflow solution for the kinetic analysis of various alcohol substrates by monitoring the absorbance of NADH following enzymatic reduction of NAD^+.

References

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