Enzyme Assay

Enzyme assays are based on the measurement of how fast a given (unknown) amount of enzyme will convert substrate to product (the act of measuring a velocity).

From: Animal Biotechnology (Second Edition), 2020

Related terms:

Peptide, Nested Gene, pH, Fluorescence, Mutation, Enzyme Activity, IC50, Escherichia coli

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Enzyme Assays

Robert Roskoski, in xPharm: The Comprehensive Pharmacology Reference, 2007

Spectroscopic Methods

Absorbance Spectroscopy. For some enzyme assays, it is possible to measure the reactant or product directly based on its absorbance properties Fersht (1999). In the reaction catalyzed by glucose-6-phosphate dehydrogenase (EC 1.1.1.49), one product (NADH) absorbs light at 340 nm, making it possible to monitor the reaction by following the increase in absorbance at this wavelength.

Glucose 6-phosphate + NAD_+ → 6-phosphogluconate + NADH + H_+

In other cases, a reactant or product is measured indirectly. With acetylcholinesterase (EC 3.1.1.7), for example, acetylthiocholine is used as a substrate that liberates thiocholine on exposure to the enzyme. The thiocholine reacts with Ellman's reagent to produce a colored product, making it possible to monitor the reaction by following the increase in absorbance.

Acetylthiocholine + H_2O → acetate + H_+ + thiocholine

Thiocholine + Ellman's reagent → colored derivative

Coupled assays are sometimes used to monitor enzyme activity. In this case, the enzymatic reaction of interest is paired with a second reaction that is coupled for

convenient measurement. An example of this is in the assay for hexokinase (EC 2.7.1.1). In this case, an excess of glucose-6-phosphate dehydrogenase and NAD_+ is included in the assay mixture and absorbance at 340 nm is monitored.

Reaction I: Glucose + ATP → glucose 6-phosphate + ADP

Reaction II: Glucose 6-phosphate + NAD_+ → 6-phosphogluconate + NADH + H_+

In this example, reaction I should be rate limiting and the concentration of glucose 6-phosphate should reach a steady state following a lag period. Cleland Cleland (1979) formulated a procedure for ensuring that the coupled assay is providing an accurate measurement of the reaction velocity of the enzyme. If possible, it is preferable to monitor a reaction continuously. With the assays described above, the spectrophotometer can be programmed to give a continuous readout as a function of time. In separation techniques, which may include the use of radioactivity, electrophoresis, or chromatography, discontinuous or end-point assays are employed. Having established a linear time period for an assay, a parameter is measured at a single time point within the linear time period (most preferably, a time point near the middle of the linear phase). The velocity is then determined from the difference in signal at that time point and at the initiation of the reaction. Care must be exercised with end-point assays, and time courses should be checked to confirm linearity. Changes in the incubation conditions, such as temperature, pH, or substrate concentration, can alter the linearity of an assay. For routine enzyme assays, the reaction vessel contains all but one of the components, and the reaction is initiated by adding the missing component (the enzyme or one of the substrates). The other components should be equilibrated in terms of pH, temperature, and ionic strength. The reaction is usually initiated by the addition of a small volume of a concentrated stock solution of the missing component. A small volume ensures that this addition does not perturb the equilibrium conditions already established. When it is not possible to add a small component, the separated components should be at the same temperature, ionic strength, and p H. Although there must be a complete mixing of the two components, vigorous shaking should be avoided as it may denature the enzyme protein. Mixing may be accomplished by inversion of a tube with an attached stopper, or a cuvette using a Parafilm seal. Dilute proteins are often less stable than more concentrated ones, and assays are often performed in the presence of an inert protein, such as bovine serum albumin (0.25 mg/ml), to stabilize the purified enzyme. Experiments should be performed to ensure that the protein is inert. Because albumin, for example, may bind to hydrophobic substrates, it may not always be appropriate for this purpose. For discontinuous assays, the timer can be set and samples aspirated at specific time intervals after mixing. For most spectrophotometers, detection is initiated manually using an instrument panel or a computer keyboard. To add a component to a cuvette, it requires about 20 seconds to place the cuvette in a spectrophotometer, and to begin detection by pressing

a computer key. This time is usually not of great consequence because for most reactions, the assay is run from 10 to 30 minutes. Control measurements include a non-enzyme-containing blank and a non-substrate-containing blank. Such controls ensure that adventitious reactions are not occurring. The control (non-enzyme blank) rate is subtracted from the date generated by the experimental to provide the actual velocity of the enzymatic reaction. For many assays, it is necessary to quench or stop the reaction at a specific time to prevent further production of product. For example, samples may be taken at 5-minute intervals for a predetermined period of time and the product measured by HPLC.

Each chromatographic analysis may take 30 minutes to complete. Methods for terminating the reaction usually involve denaturation of the enzyme by adding acid, or immersion in a boiling water bath. The activity of some metalloenzymes can be quenched with EDTA or other metal ion chelator. Most enzyme assays are based on spectroscopic techniques, with the two most commonly used being absorption and fluorescence Fersht (1999). The wavelength used for following the reaction rate should be one that yields the greatest difference in absorption between the substrate and the product. Absorption measurements are performed with a standard spectrophotometer with the samples contained in specialized cells, or cuvettes. Disposable plastic cuvettes that hold 1 or 3 ml samples are commercially available. Their use is restricted to the visible wavelength range (350-800 nm). Quartz cuvettes must be used (glass and plastic absorb light in the UV range) for measurements at wavelengths less than 350 nm. The path lengths for cuvettes is provided by the manufacturer. Popular path lengths are 1.00 cm, 0.40 cm, and 0.20 cm. An increasing number of assays are performed with 96 well microtiter plates with plastic or quartz bottoms. The concentration of a substance that absorbs light at specific wavelengths can be determined from Beer's law:

$A = \varepsilon cl,$

where A is the absorbance of the sample at a specified wavelength, c is the concentration of the sample, l is the path length, and ε is the extinction coefficient, or molar absorptivity. ε has the units of $M^{-1}\ cm^{-1}$ or $mM^{-1}\ cm^{-1}$. If the value of ε is known for a particular substance, it is possible to calculate the concentration of that substance in a solution by measuring its absorption in that solution. Using Beer's law, it is possible to calculate the rate of change of the concentration during the course of a reaction. A potential error using absorption measurements results from a deviation from Beer's law because it holds only for a finite range of absorption values. Thus, because it may not be applicable with absorbencies greater than 1, assays should be designed to keep experimental values less than this. It may be possible to circumvent some of these problems by using cuvettes with shorter path lengths. A sample with an absorbance of 1.00 in a 1.00-cm path length cell will have an absorbance of 0.20 in a 0.20-cm path length cell. In general, working with an ab-

sorbance of around 0.5 represents a compromise between minimizing optical noise inherent in a spectrophotometer and having a reasonable signal to measure. The turbidity in a solution can scatter light and produce apparent absorbance. Filtration or centrifugation may be used to remove these particles. Although alterations in path length circumvent some deviations from linearity (i.e., when the absorbance is so high that the spectrophotometer cannot make an accurate measurement), frequently the deviations are due to intermolecular interactions among the absorbing species. Dimerization (or formation of higher order eximers) occurs at higher concentrations of the absorbing species. In these cases, Beer's law will be obeyed if the concentration of product is decreased. This can be accomplished by either slowing the reaction (by decreasing the enzyme concentration) or by taking measurements over a shorter period of time (before deviations from Beer's law are encountered).

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Drug Discovery Technologies

G.A. Holdgate, in Comprehensive Medicinal Chemistry III, 2017

2.07.2.16 Enzyme Kinetics

Enzyme assays, which often can be followed using biophysical readouts, for example, quantification of the amount of a fluorescent product, can also be used to measure binding kinetics. However, knowledge of the inhibitor mechanism of action (MoA) is required in order to access the relevant parameters. An example is the derivation of the kinetic parameters for inhibitors of the enoyl-acyl carrier protein reductase, FabI, from forward reaction progress curves (enzyme assay time courses) through a detailed understanding of the MoA of the inhibitors coupled with computational methods.[3]

Jump dilution methods offer a more direct way to estimate inhibitor dissociation rate constants. In a similar way to the large dilution experiments described earlier, the enzyme is incubated with a saturating concentration of test compound, and this mixture is rapidly diluted into a large excess of substrate. Product accumulation is then again measured as a function of time, and the observed rate of enzyme activity recovery fits to a single exponential in order to derive the inhibitor dissociation rate constant. Two important factors in conducting this type of experiment are that the inhibitor concentration falls well below its apparent inhibition constant, K_i, upon dilution and that the final enzyme concentration, following the dilution, is sufficient to generate enough product for reliable detection.

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VITAMINS | Overview

V. Spitzer, U. Höller, in Encyclopedia of Analytical Science (Second Edition), 2005

Enzyme Assays

Enzyme assays are mainly used for determination of body status of vitamins. As vitamins usually function either as coenzymes or building blocks of coenzymes, the activity of the vitamin-dependent enzymes is a measure of vitamin status. Usually, the assay is carried out by determining the enzyme activity with and without activation by added coenzyme. The activity can be monitored by measuring changes in concentration of substrates or products during the reaction. An activation coefficient can be deduced, which reflects the status of the enzyme investigated, and thus the vitamin status. Most assays are conducted with whole blood or the separated erythrocyte fraction. They can be automated with clinical analyzers. Disadvantages include difficulties in assay standardization, instability of the enzymes during storage, and misleading results, e.g., due to conditions other than vitamin deficiency leading to low apoenzyme concentrations.

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Immobilized Cells

Susan Linko, ... Yi-Hong Zhu, in Progress in Biotechnology, 1996

2.4 Analytical methods

Enzyme assays. *Endo-1,4-β-glucanase activity* [1,4-(1,3;1,4)-β-D-glucan-4-glucanohydrolase; EC 3.2.1.4] xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) *activity* and *filter paper* (*FP*) *activity* were determined spectrophotometrically at 540 nm as described in [13], Lignin *peroxidase activity* was determined spectrophotometrically at 310 nm at 23 °C room temperature as described by Linko and Haapala [20]. The activities were reported either as nkat ml^{-1} or for lignin peroxidase as units (U) per liter (1 U = 16.67 nkat).

Soluble protein. Soluble protein was precipitated with 10% trichloroacetic acid and determined spectrophotometrically at 750 nm according to Lowry [21], using Bovine serum albumin as standard.

Total reducing sugars. Total reducing sugars were determined colorimetrically at 540 nm by the dinitrosalicylic acid (DNS) method [22], and *glucose* by the method of Nelson [23].

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Clinical Enzymology

JOHN W. KRAMER, in Clinical Biochemistry of Domestic Animals (Third Edition), 1980

F Kinetics

Enzyme assays are performed under conditions for optimal enzyme activity. Concentrations of substrate, cofactors, activators, pH, and temperature are maintained constant so that the only variable is the enzyme. These are the conditions of zero-order kinetics, conditions at which the reaction rate is independent of initial substrate concentration. Thus, an enzyme as assayed in the laboratory may not have the same natural functional activity in the cell, where different conditions may exist.

The approximation of zero-order kinetics becomes limited when the rate of enzyme activity is very high. Reagents for an enzyme generally have only sufficient substrate concentration to maintain zero-order kinetics for the time period of the assay or for magnitudes of enzyme activity two to three times greater than the activity normally occurring in the patient's serum. When one is performing an enzyme assay, it is imperative that the limitations of zero-order kinetics be understood. If the results of the assay exceed the limits of zero-order kinetics, the enzyme activity is in error and should be reported only as “greater than” that value established as the upper limit of zero-order kinetics. Alternatively, the assay can be repeated on a diluted sample, or the size of the sample can be reduced and appropriate dilution corrections made.

Determination of the maximal amount of enzyme activity that a procedure can measure requires knowledge of the equilibrium of the reaction and the concentration of substrate. An equilibrium constant (K_{eq}) of 100 indicates that there will be 100 times more product than substrate when the reaction has gone to completion. In this case, 99% of the substrate is converted to product. However, if $K_{eq} = 1$, as in the GPT reaction, only 50% of the substrate is converted to product at equilibrium and the reaction appears to stop. In some cases, if the reaction is reversible, the product may be reconverted to substrate as quickly as product is formed.

The K_{eq} may be altered by “trapping” the product or converting it to another form. In the reverse LDH reaction, lactate is converted to pyruvate by LDH, and, in order

to drive the reaction to pyruvate, hydrazine is used to "trap" pyruvate. This form of "trapping" permits a greater amount of activity to be determined with the same amount of substrate than would be possible if no "trapping" reagent were used.

Enzyme assays in clinical biochemistry are generally carried out in one of two ways. The first is an "end point" procedure analogous to a colorimetric assay. The sample is added to the reaction mixture, and, after a suitable incubation period, the reaction is stopped by the addition of a reagent which destroys or inhibits the enzyme activity. The amount of substrate used or the amount of product produced during the incubation period is measured. This end point method is subject to a variety of errors as when the activity exceeds the limits of the substrate concentration, or if product inhibition occurs.

The second, "kinetic" assay procedure requires sequential reading either manually or with a constant recording device. A kinetic assay procedure is more sensitive, more accurate, and more easily controlled than end point assays, because the reaction rate can be visualized. The constant recording device is extremely useful for this purpose because the linearity of the reaction rate can be easily seen. The primary advantages of the kinetic method in clinical enzymology are as follows: (1) When high enzyme activities are encountered, the reaction rate can be determined before substrate is exhausted whereas, in an end point assay, it has to be repeated when the substrate is exhausted; and (2) if an activator is present, it can be detected on the graph (Fig. 1).

Fig. 1. Illustration of potential hazards of using a end-point enzyme assay. Line *ACE* is a zero-order reaction that permits accurate determination of enzyme activity for the entire reaction time. Curve *ABE* initially is a zero-order reaction of high rate followed by a reduction in rate, possibly due to exhaustion of substrate. Assay at point *E* would be in error. Curve *ADE* has an initial lag phase which also would be erroneous.(Reproduced with permission from J. B. Henry, 1963.)

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Marine Enzymes and Specialized Metabolism - Part B

Christopher R. Reisch, in Methods in Enzymology, 2018

6 Summary and Conclusions

The DmdA enzyme assay described here is relatively simple to perform and requires no special equipment with the exception of an HPLC for separation and quantification of the reaction products. The methods provided earlier can be used as a framework for designing assays to suit a specific purpose. Several aspects of the method, including cell lysis, reaction volumes and timing, and details of the HPLC separation, can be adapted to work with materials on-hand. Though the enzyme substrate THF is irreversibly oxidized in air, the assay can be performed under aerobic conditions with the protocol supplied here. Lastly, the enzyme was stable with addition of EDTA to the buffers, which should enable easy workflows for cell lysis and enzyme assay.

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Cellulases

James E. McDonald, ... Alan J. McCarthy, in Methods in Enzymology, 2012

4.6 Cellulase activity assays

Several enzyme assays are available for the detection and quantification of cellulolytic activity in isolated strains. Three main approaches are used:

(1) *Measurement of* hydrolysis *products such as reducing sugars and total sugars*: Reducing sugar assays include the dinitrosalicyclic acid (DNS) method (Miller, 1959) and the Nelson–Somogyi method (Somogyi, 1952). The anthrone–H_2SO_4 (Viles and Silverman, 1949) and phenol–H_2SO_4 (Dubois *et al.*, 1956) methods can be used to measure total soluble sugars released by extracellular enzymes in culture supernatants.

(2) *Measurement of reductions in substrate quantity via total carbohydrate assays*: The anthrone–H_2SO_4 (Viles and Silverman, 1949) and phenol–H_2SO_4 (Dubois *et al.*, 1956) methods are the most widely used. However, these techniques are

limited to the study of pure celluloses, as derivatives of other carbohydrates (3) may interfere with the quantification of cellodextrins (glucose equivalents). *Measuring changes in the physical properties of substrates*: Historically, physical characteristics including turbidity, viscosity, swollen factor, disruption of cellulose structure, and strength of cellulose fibers have also been used to assess cellulase activity.

For a comprehensive description of these methods, the reader is referred to the review of Zhang *et al*. (2006).

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Theory of Extraction Techniques

M.W. Ducey, ... S.M. Lunte, in Comprehensive Sampling and Sample Preparation, 2012

2.25.4.2.6 Enzyme Assay

The use of enzyme assays (especially coupled assays) allows for the rapid and, in some cases continuous, analysis of MD perfusate with very low detection limits, and without the need for a separation step (imparted by the high specificity of the enzyme). To date, most MD-coupled enzyme assays employ nicotinamide adenine dinucleotide (NAD)-dependent enzymes and produce fluorescent or electrochemical active products. These assays have been reviewed by Obrenovitch and Zilkha.[113] In general, the use of MD-coupled enzyme assay allows for temporal resolution on the order of 2 min and is amenable to analytes that can serve as substrates for an NAD- or NADP-dependent enzyme.

To provide one example, Obrenovitch and coworkers examined changes in L-glutamate in ischemic rat brains.[114] Using glutamate dehydrogenase and NAD^+, NADH was produced and quantified by fluorescence detection. Glutamate concentrations were monitored continuously over a 30 min perfusion of 100 mM potassium ion. The authors were able to demonstrate the relationship between glutamate concentration and epileptic episodes with much greater temporal resolution in comparison to an off-line HPLC method.

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Bacterial and Protozoan Rhomboid Proteases

Sinisa Urban, in Handbook of Proteolytic Enzymes (Third Edition), 2013

Reaction Optima

The pure enzyme assay established that the rhomboid polypeptide is itself catalytic [13], and thus led to the acceptance of rhomboid into the Enzyme Commission as the only family of intramembrane serine proteases (EC 3.4.21.105). It also showed that rhomboid proteases are not produced as zymogens like many other proteases, nor do they require processing of substrates to prime cleavage, like other intramembrane proteases that rely on a site-1 protease. The *in vitro* assay allowed the effect of environment (ions, pH, membranes), exogenous small molecules (inhibitors, peptides) and engineered rhomboid and substrate mutants to be studied directly on the rate of proteolysis as well as cleavage site preference. Remarkably, rhomboid catalysis is notably robust to changes in salt, pH 5 to 8.5, and divalent ions, and does not require any cofactors or energy source [13]. Current assays use physiological salt of 150 mM and pH of 7.4.

For membrane proteases, the choice and concentration of detergent used in the assays is perhaps the most influential parameter on the reaction. About a dozen detergents have been tested explicitly, and non-ionic detergents with longer alkyl chain lengths support proteolysis to the highest degree [13]. This is probably because longer alkyl chains are more similar to those in natural membranes (16–18 carbons). The minimum detergent tail length that supports rhomboid activity is nine carbons [13]. Detergent must be kept above its critical micelle concentration to avoid membrane protein aggregation, but high concentrations themselves inhibit proteolysis. Currently most assays are conducted in 0.1% dodecyl-□-D-maltoside (DDM), which has a tail length of 12 carbons. However, given the thousands of detergents that are now available, with more being developed nearly each day, it is likely that further improvements are possible (albeit unnecessary).

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Investigation of Enzymes in Biological Preparations

Trevor Palmer BA, PhD, CBiol, FIBiol, FIBMS, FHEA, Philip L. Bonner BSc, PhD, in Enzymes (Second Edition), 2011

15.2.1 Introduction

The purpose of enzyme assay is to determine how much of a given enzyme, of known characteristics, is present in a tissue homogenate, fluid or partially purified preparation. It is important that the specimens be treated carefully prior to assay, if the results are to have any meaning. The maintenance of cellular organization and integrity is a characteristic of life, and cellular destruction by natural processes (autolysis), accompanied by changes in enzymes and breakdown of cofactors, commences on the death of an organism or the isolation of a tissue. Autolysis is minimized if a tissue is kept cold, so specimens for enzyme assay are usually stored at temperatures below 4°C, both before and after the preparation of homogenates. Many enzymes are easily denatured at high or even moderate temperatures, so this is a further reason why all types of specimens or preparations should be kept cold prior to enzyme assay. (Note that these factors apply to the preparation of specimens for the investigation of enzyme characteristics as well as for enzyme assay.)

Since changes due to autolysis and denaturation must increase with time of storage, it will be apparent that the assays should be performed with a minimum of delay. If some delay is unavoidable, it may be necessary to store the specimens at very low temperatures (e.g. – 60°C) to prevent loss of enzyme activity. However, freezing of tissues might increase the disruption of cells, so there can be no general rule about storage. The stability of the enzyme in question at various temperatures, and the nature of the specimen or preparation (see section 17.3), all need to be taken into account.

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