Protocol:

Measuring Specific Activity of Malate Dehydrogenase

Synopsis

The purification, characterization and quantitation of proteins depends upon the accurate determination of both the enzyme activity and the protein concentration. These are related in the term specific activity. Protein activity ranges from enzymatic activity to binding to structural roles in cells and can be assayed in a variety of ways including continuous or discontinuous (stop time) enzyme assays.

If we are using an enzyme, like Malate Dehydrogenase, that either consumes or creates NADH, we can use absorbance measurements at 340 nm to quantitate the reaction. The absorbance change will be +ve if we are creating NADH and –ve if we are using NADH. Note, in either case the rate ($\Delta A_{340\ nm}$/minute) is positive!

Each type of assay presents particular problems that must be considered and taken into account to obtain accurate activity measurements. Similarly, there are a number of ways to determine the concentration of the protein in a given solution, and again each type of assay has its own particular limitations and assumptions. The various types of assays and some examples of using such data to characterize a pure protein and to quantitate active protein are discussed below.

Introduction

What is Specific Activity?

In conjunction with the determination of the activity of an enzyme solution, you can use the protein concentration and activity measurement to determine a parameter known as the "specific activity" of an enzyme containing solution:

Specific Activity = Enzymatic Activity/Protein Concentration

As discussed above, the specific activity of a pure protein is a characteristic of that protein just as is its molecular weight or amino acid sequence and can be utilized to follow the purification of the protein.

What are the units of specific activity? The answer is complicated. If enzyme activity is measured in change of absorbance of the substrate as it is converted to product (often the case with Malate Dehydrogenase), the units of enzyme activity could be ΔAbsorbance/minute. If the protein concentration in the cuvette is in mg/mL, then the units of specific activity would be:

(ΔAbsorbance/minute)/(mg/mL)

What is the Turnover Number?

For example, if an activity of 63nmoles/min/ml is determined when one adds 7 pmoles/mL of enzyme to a standard assay, the specific activity and the turnover number of the enzyme would be:

$63{,}000/7 = 9000\ \text{minute}^{-1}$

In such calculations the units of the activity and the units of the protein concentration are arranged to cancel out and the final unit of the turnover number is time^{-1}. ***A turnover number is a specific activity, but one requiring the concentration units to cancel out.*** The units of turnover number are always time^{-1}

Typical calculation for wild type watermelon glyoxysomal Malate Dehydrogenase (wgMDH)

Starting Material:
1mg/mL wgMDH
Dilute 200 fold: Add 5 μL to 1mL of 0.05M Phosphate Buffer, pH 8.

Assay:
In a cuvette, 10 μL of diluted enzyme was added to 3mL assay mix containing 100μM NADH and 200μM Oxaloacetate in 0.05M Phosphate pH 8. Absorbance at 340 nm was observed.

Measurement:
ΔA340 nm = 0.483/minute

Calculations:
Determine rate of reaction in cuvette. First, convert ΔA340 nm/min to ΔNADH concentration/min. Use Beer's law ($A = \varepsilon * c * \ell$; where A = ΔA340 nm and ℓ is length of light path through cuvette) and the extinction coefficient (ε) for NADH at 340 nm (6.22 $mM^{-1}cm^{-1}$):

$$0.483/6.22 = 0.0776 \text{ mM/minute} = 77.6 \text{ μM/minute}$$

That is 77.6 μM NADH is converted to NAD+ per minute.

Determine the protein concentration in cuvette:

$$((1\text{mg/mL}/200 \text{ dilution}) \times 10 \text{ μL})/3010 \text{ μL} = 0.0000166 \text{ mg/mL}$$

Convert to mg/mL protein concentration to μM to have the same units as the reaction rate. The approximate molecular weight of a wgMDH subunit is 34,400 daltons. Recall that MW = g/mol and that M = mol/L:

Convert to μM, multiply by 29: 0.0000166 x 29 = 0.000481mM

Where does the number 29 come from?

The Molecular Weight of wt-WgMDH is 34,400
Thus 34.4mg/mL = 1mM = 1000μM
Thus 1mg/mL = 29μM
Hence if you have a concentration in mg/mL, multiply by 29 to convert to μM.

Alternatively you can set up an equation and solve it

$$\frac{0.0000166\ \text{mg}}{\text{mL}} * \frac{1\ \text{g}}{1000\ \text{mg}} * \frac{1000\ \text{mL}}{1\ \text{L}} * \frac{1}{34{,}400\ \text{MW}}$$

$$= 0.000481\mu\text{M}$$

Calculate the specific activity:

(Rate in cuvette) / (protein concentration in cuvette)

In this case, the rate and protein concentration have the same concentration units. Therefore, the specific activity is also the turnover number:

$$77.6\ \mu\text{M min}^{-1} / 0.000481\ \mu\text{M} = 161{,}330\ \text{min}^{-1}$$

Making Measurements of the Initial Rate of an Enzyme Catalyzed Reaction

Overview:

The measurement of the rate of a reaction depends upon being able to estimate either the amount of substrate [A] present or the amount of product [P] present as a function of time. The rate [often referred to as the velocity, v] of the reaction is simply:

$$\mathbf{v = -}\mathit{d}\mathbf{[A]}/\mathit{d}\mathbf{t = }\mathit{d}\mathbf{[P]}/\mathit{d}\mathbf{t}$$

The rate of the reaction being proportional to the concentration of the reactant, A, where $v = k[A]$, where k is the rate constant of the reaction. Measurement of the velocity as a function of the concentration of A allows the rate constant, *k*, to be determined.

Enzyme catalyzed reactions are a little more complex and derivation of the Michaelis-Menten equation is based on the ability to measure the initial velocity, v_o, defined as the velocity of the reaction immediately after the enzyme steady state has been achieved. Since the determination of the properties of an enzyme depend upon various applications of the Michaelis-Menten equation, it is critically important that the initial velocity of an enzyme catalyzed reaction is accurately measured. The following experiment illustrates this point and will familiarize you with the types of calculations that are involved in measuring the rates of enzyme catalyzed reactions.

Protocol:

Measuring Specific Activity of Malate Dehydrogenase

General Considerations:

The use of saturating substrate concentrations in reaction mixtures to minimize experimental errors has been emphasized. It is also important that reaction rates be measured under conditions where a sufficiently small amount of substrate is utilized so that the rate does not change during the assay as a result of substrate depletion. Similarly, product buildup, which may lead to product inhibition, is to be avoided. In general, a convenient way to test that these factors do not become a problem is to measure activity at a series of protein concentrations: The rate should be directly proportional to the protein concentration, as in Figure 1. Deviations below the line indicate that substrate depletion or product accumulation may be occurring. Deviations from linearity can also result from protein aggregation or subunit dissociation affecting the rate of the catalyzed reaction.

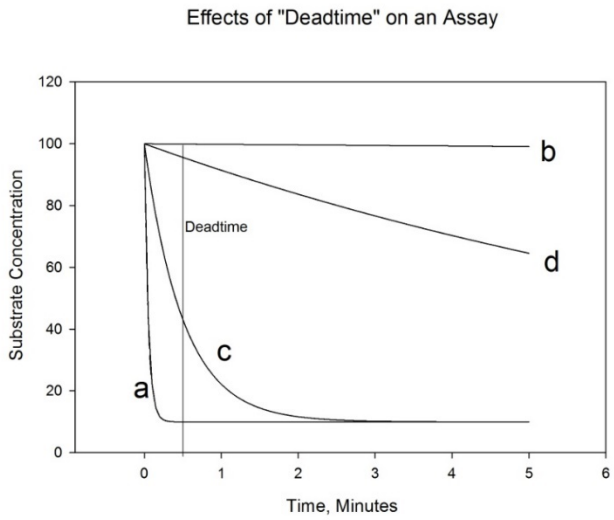


Figure 2: Effects of "Deadtime" on Accurate Initial Rate Determination

For the study of enzyme kinetics, it is important that the rate that is measured is the "Initial" rate of the reaction. In addition to being linearly dependent of the amount of enzyme added, an important criterion of the initial rate is that whatever change is being measured to follow the activity extrapolate to zero change at the start of the reaction- this ensures that the measured rate is indeed the initial rate of the reaction and that some change in the rate of the reaction did not occur in whatever "deadtime" the physical measurement of the rate involves- for example in the direct assays described below for dehydrogenases the deadtime is the time interval between introducing the enzyme, mixing and starting the actual absorbance measurements. This problem is illustrated in figure 2. With an enzyme, such as Malate Dehydrogenase, which catalyzes a reaction that proceeds quickly to equilibrium, a small "deadtime" can lead to a large error in the estimated "initial" rate- however, such a situation is easily detected by the "must extrapolate to zero change at the start of the reaction" rule.

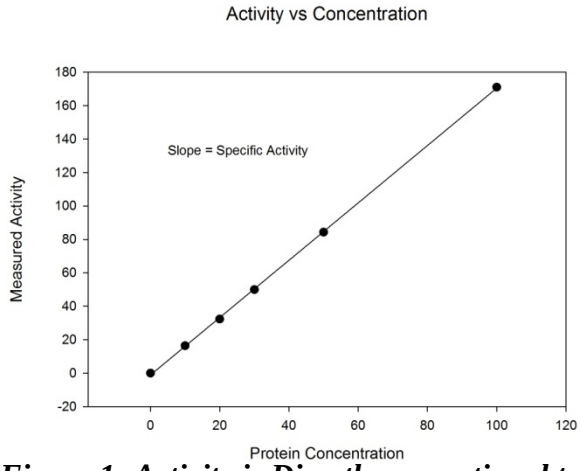


Figure 1: Activity is Directly proportional to Concentration

How do you decide how much enzyme to use? When dealing with an enzyme where you do not know the specific activity, it is important to establish the correct amount of enzyme to use in assays. The trial and error approach is the only option you have. Try some amount (say 10µL of the solution you have) and measure the "rate"- there are three possible outcomes of this experiment- too much was added, too little was added, or approximately the right amount was added, as shown in figure 2- curve d. If too much was added you can make a best guess as to how much too much from the shape of the resultant curve- if by the time you initiated the measurement the reaction was already at, or close to equilibrium you added much too much and probably need

to dilute the enzyme 50-100 fold (curve a). If you added too little of the enzyme to get a reasonably measurable rate (curve b) you need to concentrate the enzyme or simply add more volume of the enzyme until you get a reasonably measurable rate. If you added approximately the right amount the issue is whether or not it extrapolates back to the starting absorbance (usually about 0.6 in an MDH assay) at t = zero, in which case it is fine to continue with your experiment (curve d), or whether the enzyme needs some dilution- curve c- (by either adding a smaller volume- this depends upon how small a volume you are comfortable being able to add accurately- or by diluting maybe 5-10 fold).
You then calculate the initial rate (dA/dt) from the linear region that extrapolates back to the correct absorbance at t = 0. Usually in an MDH assay you can establish conditions where the plot is linear for about 30 seconds or more. Do not include any data from the curved region of the plot as this will distort the initial rate estimate.

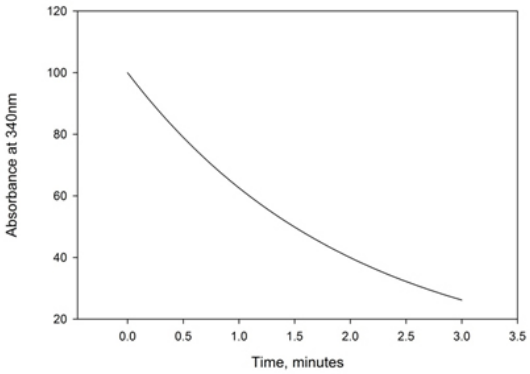


Figure 3 Time course of the reduction of oxaloacetate e catalyzed by malate dehydrogenase using NADH as coenzyme

Once you have established how much enzyme you need to add to give an accurately measurable initial rate it is probably a good idea to test the highest and lowest combinations of substrates that you will use to ensure that you can make good measurements throughout the range you will use during a given experiment.

Continuous Assays: The activity of an enzyme can often be conveniently measured by following either the production of a product or the removal of a substrate. With certain classes of enzymes (e.g., dehydrogenases) the natural substrates are chromophoric and exhibit spectral changes that can be followed directly. For example, malate dehydrogenase catalyzes the reduction of oxaloacetate by the coenzyme NADH:

Oxaloacetate + NADH ←→ Malate + NAD^+

NADH has an absorption band centered at 340 nm with an extinction coefficient of 6.22×10^3 $cm^{-1}M^{-1}$, while NAD^+ has no absorbance at this wavelength. When malate dehydrogenase is added to a mixture of oxaloacetate and NADH, there is a time-dependent loss of absorbance at 340 nm.

If the reaction is allowed to proceed to equilibrium, the "rate" progressively slows until equilibrium is reached. Clearly, the reaction "rate" changes during the time course of the reaction as a consequence of both utilization of substrate and approach to equilibrium. To enable reproducible rate determinations, two aspects of the reaction are determined: (1) the initial rate, as shown in Figure 3, and (2) the rate at saturating substrate concentrations. This rate (the "maximum rate") is calculated using concentrations of, in the case of alcohol dehydrogenase, for example, acetaldehyde and NADH that give an experimentally determined maximum rate (Figure 4a&b)

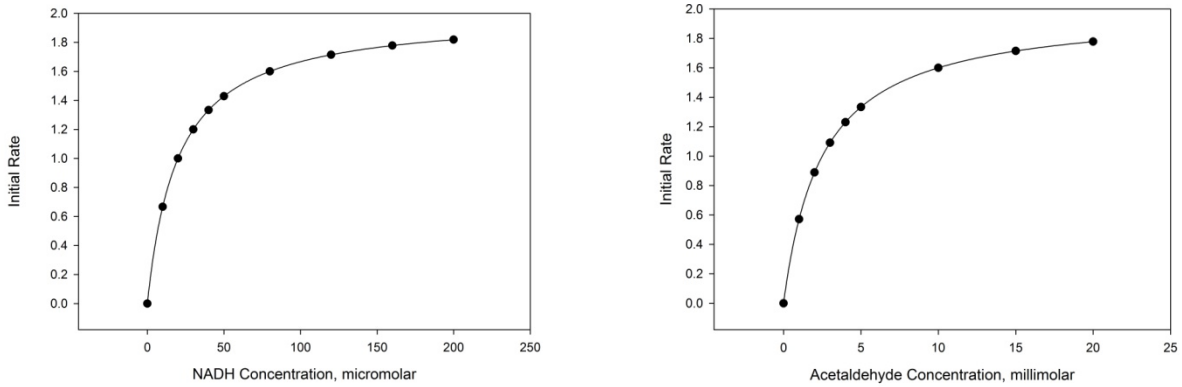


Figure 4. Dependence of the rate of the reaction catalyzed by alcohol dehydrogenase on the concentrations of NADH and acetaldehyde.

These substrate concentrations are used for two reasons, one pragmatic and the other theoretical. If substrate concentrations sufficient to give this maximum rate are used, any experimental error in making up the assay is minimized. From the theoretical standpoint, under these conditions the measured rate of the reaction is dependent only on the concentration of the enzyme, a situation necessary if the enzyme assay is used to determine the activity of the enzyme. The case of malate dehydrogenase is complicated by the fact that the enzyme is known to exhibit substrate inhibition at high concentrations of Oxaloacetate and hence the appropriate Vmax concentrations of oxaloacetate cannot be used. Similarly it is not practical to use "saturating" concentrations of NADH because the Km of the wild type for NADH is in the range of 150-250μM. Since 200mM NADH would have a concentration of 1.24 (usually spectrophotometers have their most accurate measurements between 0.05 and 1 absorbance units) it is unwise to use any NADH concentration above about 200μM. Established "Standard" Assay concentrations for NADH are usually 100μM, in the most accurate range of the spectrophotometer. With Malate Dehydrogenase it is essential to record the concentrations of both NADH and Oxaloacetate that are used in a "Standard" assay

Protocol For Initial Rate Measurements with Malate Dehydrogenase: Continuous Assay

Equipment: Recording spectrophotometer capable of measuring absorbance at 340nm.

Disposable supplies:
1.5 or 4.5 mL plastic cuvettes that pass light at 340 nm
Disposable plastic Pasteur pipettes with about 3 mL volume

Reagents:
Make substrates up fresh and keep on ice (do not premix NADH and Oxaloacetate)
0.05 M Sodium (or Potassium) Phosphate Buffer, pH 8.0
6 mM NADH in H_2O. Make this reagent up more concentrated, check absorbance at 340nm to calculate actual concentration, and dilute as appropriate to 6mM.
12 mM Oxaloacetate in H_2O

3mL Reaction Volumes
If you choose to use 1.5mL cuvettes use 1mL buffer and add 16.5µL amounts of NADH and Oxaloacetate.
2.9 mL Buffer, Blank cuvette in spectrophotometer
Add 50µL NADH stock solution, 50µL Oxaloacetate stock solution, mix and record starting absorbance at 340nm

Measurement:
Add appropriate amount of enzyme, mix, and record A_{340nm} versus time for 30 seconds

Note on Mixing and initiating data collection
Mixing: Pipetting the enzyme up and down a few times with your micropipet DOES NOT EFFECTIVELY MIX THE REACTION. You will get irreproducible data. With the cuvette in the spectrophotometer, hold the Pasteur pipette, air expelled in one hand and the micropipettor with the enzyme in the other. As soon as you pipet the enzyme into the reaction mix, gently suck up the reaction mix into the Pasteur pipet and expel back into the cuvette without introducing bubbles. Start recording A_{340nm} versus time. This effectively mixes the enzyme and reaction mix. You should get reproducible data with this method. Because of the "deadtime" phenomenon discussed early, you should develop a consistent pattern of these steps before starting the recording at A_{340nm}.

When you have collected data, calculate the rate over the linear region that extrapolates back to the starting absorbance. You can check that you have the initial rate by calculating the rate over two time periods within this range. If it is the initial rate, you will get essentially the same rate from each segment.

Typically, if you have a reasonable concentration of enzyme, you will see an absorbance change of 0.05- 0.2 during the course of the assay. If you let the reaction run for a longer time period, it will eventually reach equilibrium and the absorbance will be about 0.02.

Calculating the Rate of the Reaction

Since the mM extinction coefficient of NADH at 340nm is 6.22 mM^{-1} cm^{-1}, you simply divide the ΔA_{340nm}/min by 6.22 to obtain the rate in mM NADH consumed per minute (remember mM is a concentration, not an amount).

Stopped Time Assay

Reagents
Stop Solution: 1 M Na_2CO_3 dissolved in water, pH should be around 12

Stop Time Assay: For a single reaction, combine the following components *except enzyme* into a microfuge tube, cap and **vortex**. Incubate all solutions at 25°C for 5-10 min prior to starting reaction.

- 700 µl assay buffer

- 100 µl 2 mM NADH
- 100 µl 10 mM OAA
- 100 µl Enzyme solution **Do not add enzyme until ready to start the reaction!

- Once the reaction is started, mix well by vortexing and incubate in 25°C water bath.
- Stop the reaction by adding 100 µl of stop reagent at the determined time and vortex immediately.
- Transfer 300 µl of each sample into a 96 well plate. Read absorbance at 340 nm.
- Include samples without NADH (negative control) and another sample with NADH but without MDH (no reaction control).
- Ensure reaction is linear over the time of the assay by performing a series of different times and dilution of enzyme.
- Calculate ΔA = starting absorbance (ie. the no reaction control) – the absorbance of the sample
- ΔA / min = ΔA divided by the total time of reaction. Calculations of Enzyme Activity are then as described above.

General Comments on Enzyme Assays

Assay method: As discussed above, here are two common methods of determining the activity of an enzyme: stop time assays and a real-time or continuous assays.A stop time assay is just that; start the reaction and stop or read the results at a given time. This is the easiest way to do many assays at one time, **BUT** there are two things that need to be considered before doing this form of the assay. First, is the assay linear? In other words, in the time that I am running the assay,, is the product being produced (or substrate converted) at a linear rate? If the conditions of the assay tube are such that the reactants (substrate) are depleted or the products are inhibiting the enzyme, then you **CANNOT** use this assay. Second, is the compound you are measuring stable enough to wait to read and are the conditions used to stop the enzyme, i.e. acid or base too harsh to maintain the structure of the readout? .

Absorbance: Most specs can only read between 0.01 and 3.0 abs units. At either end of this range there will be too much noise.

Always run a control assay – This is an assay that does not contain enzyme. It will tell you any drift in the baseline absorbance. If you get an appreciable amount of drift, you will have to subtract this ΔOD/min from your enzyme assay tube. If it is about zero, then baseline corrections are not needed. The control assay will also tell you what the starting absorbance is.

Proper Rates: This depends on each enzyme. For MDH, a rate of 0.05 to 0.4 ΔOD/min is good enough. If the rate is over too fast (*see above*,) then dilute the enzyme. If you are not certain how much to dilute the enzyme, do a 1:2 or 1:5. I have included notes in the MDH assay for our favorite expressed enzyme.

Run a positive and negative control: Always include a sample that has every component except NADH. The absorbance from these samples represent the absorbance when no/little NADH is left and the reaction is exausted substrate. ALSO include a sample with NADH but NO enzyme. This is the starting

concentration/absorabance. Samples that have the same absorbance did not have an active enyme or enough enzyme to accuratly be measured (below threashold of detection). ALWAYS keep the total volume the same; thus replace your NADH or MDH with an equivilant volume of enzyme assay buffer.

Run a positive enzyme assay control: Use a sample that you know has the enzyme. Often this can be from an extract or some purified protein already prepared.

Temperature: Bring all solutions to room temp before starting assays. The easiest way to do this is mix the next set of tubes while assaying one set. Enzyme should always be on ice before adding to the enzyme cocktail or it will denature. 10^{o}C can bring about a 2 fold change in kinetics. Be consistent.

Measuring and Pipetting: This is another problem area. Day to day variations or even batch to batch changes in how you make up your enzyme or substrate solutions will cause a lot of error. For the MDH assay, there is more than enough solution to conduct many assays.

Calculating enzyme units: You will sometimes see amounts or concentrations of enzymes expressed as units or units/mL. By definition, 1 Unit of enzyme catalyzes the conversion of 1 µmole of substrate to product per minute. To calculate the units in any spectrophotometric based assay, Beer's law is used: $A = \varepsilon\, l\, C$
Where A = absorbance ($M^{-1}\ cm^{-1}$), b = pathlength of the cell (1 cm), c = concentration of the absorbing species (M) and ε = the molar extinction coefficient.

Determining the Concentration of Malate Dehydrogenase (or total Protein in a solution if not pure).

The two methods of choice include measuring the absorption properties of the protein, measuring the change in absorbance properties of a dye [coomassie brilliant blue: often referred to as Bradford Dye] as it binds to a protein.

Before we start lets examine the assumptions that are made in each of these methods. With the absorbance measurements at 280nm we are assuming that Beer's Law is obeyed, that is that the absorbance at a fixed wavelength of a given protein is directly proportional to its concentration:

Absorbance = concentration x extinction coefficient (x pathlength, usually 1cm)

Why might this not be true? Some proteins show concentration dependent aggregation affects which could cause anomalies. It is important to establish that over the range of concentrations that you might use that Beer's Law is strictly obeyed. The second problem is of course that you need to know the extinction coefficient for the given protein- more on that assumption later.

With the Dye Binding method for example, or various chemical methods that result in a color change, you are assuming that all proteins behave essentially the same way, either binding the same amount of dye ***and*** causing the same extent of color change, or that all proteins react equally on a per weight basis with the chemical reagent

that triggers the color change. The experiments that are described here reveal the extent that such an assumption is valid..

Standard Curves: Construction, Use & Limitations

Introduction

The molecular life sciences are a field that in many ways depends upon accurate quantitation of macromolecules such as proteins, carbohydrates or nucleic acids, or of small molecules such as metabolites. Standard Curves are used in many different ways in biology and chemistry and usually provide for relatively rapid and fairly accurate estimates of the amount of a particular molecule. A standard curve is simply an assay in which an experimentally measured response of some sort that depends upon the amount of "stuff" that is put into the assay. One of the most common ways this type of analysis is used is to determine the amount of protein (either specific or generic) that is present in a solution. Typically a plot of measured response (Y axis) versus the Amount of the Standard (X axis) is plotted and used to determine the amount present in some sample of the unknown. The process is outlined in the figure below. Usually once the standard curve has been constructed, some volume of the unknown is used in an identical assay and the response measured. The value of the measured response is then located on the standard curve and the amount of "standard" that gave that measured response identified.

While such an approach seems straight forward there are a number of potential pitfalls that you must be aware of when using standard curves.

First, one must consider the type of Standard "Curve" that is being constructed. This depends upon one of two types of measured response that one might use. The first is a response that depends upon a molecular property of the compound being measured and obeys fundamental laws of either chemistry or physics while the second depends upon some type of binding or equilibrium phenomenon. In the first case the standard curve is actually a straight line, while in the second the curve is actually a curve that results from a saturation phenomenon. These are represented below for the types of data one might get in the experiments discussed here that demonstrate the construction and use of standard curves in the molecular life sciences. In figure 1, panel A is shown the type of data one might get when measuring the absorbance (a defined molecular property of a specific protein) of a protein (the experimentally measured response) as a function of the amount of protein put in the cuvette. The second panel, B, shows the results of a "Bradford" assay (where the color of a dye changes upon binding to a protein)for the determination of protein concentration.

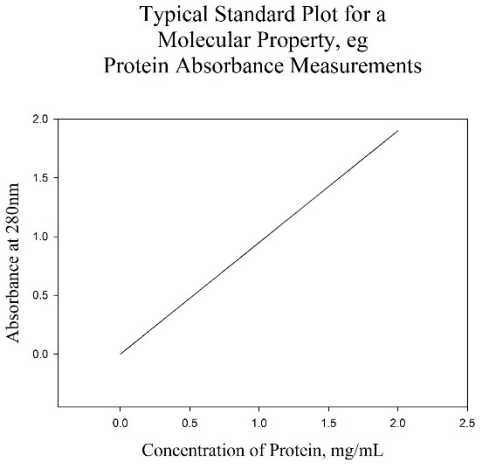


Figure 1A: Determination of the extinction coefficient of a protein: Absorbance is plotted as a function of the concentration of protein and the slope of the line is the mg/mL extinction coefficient of the protein which allows a determination of protein concentration from a single absorbance measurement at 280nm.

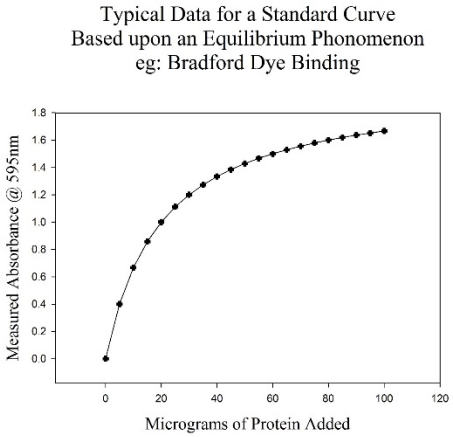


Figure 1B: Results of an experiment where increasing concentrations of a protein are incubated with a fixed concentration of "Bradford" Dye- Coomassie Brilliant Blue and the resulting absorbance at 595nm measured. A "blank" with dye but no protein is subtracted from the measurements.

Using a Standard Curve

Once the standard curve has been constructed, a sample of the "unknown" is used under the same assay conditions. For example in the Bradford Assay above a given volume of the unknown would be subjected to exactly the same assay conditions and the resultant absorbance measured, for example 30µL of unknown gives an absorbance of 0.78 at 595nm.Locating 0.78 on the Y axis and going to the curve before dropping a perpendicular to the X axis indicates that 16 micrograms of protein would produce that color in the standard assay (figure 2). Hence 30µL of the unknown solution contains 16 micrograms of protein and the concentration of protein in the unknown is 16/30 = 0.53 micrograms per microliter or 0.53mg/mL.

Most Sensitive Regions of a Standard Curve

When a standard curve is based upon a molecular property and is linear as in figure 1A, all regions of the curve are equally sensitive, upto any physical limitations of the measurements. When however the standard curve is based on an equilibrium phenomenon as in figure 1B, the curve can be divided into 3 regions, as shown in figure 3. The first region, at low amounts/concentrations of the standard is close to linear and is the most sensitive (biggest differences between small increments of the standard). The second region can still give meaningful estimates of the concentrations of unknowns but the third region (close to the "equilibrium" position) should be avoided as small changes in the measured parameter would indicate large changes in the estimated concentration. At the limit when the parameter no longer changes with changing amounts of the standard, no useful information can be obtained other that an estimate of the minimum amount that could give the response.

Constructing a "Standard Curve"

Protocol:

Measuring Specific Activity of Malate Dehydrogenase

To illustrate the ways in which Standard Curves are constructed and used we will consider the types of experiments that one might perform to determine the concentration of an unknown amount of protein in a solution. In addition we will discuss the experiments that reveal the types of problems (or limitations) that you might encounter in such experiments For such a laboratory experiment you would be provided with several "known" protein solutions at indicated concentrations and "unknown" protein solutions whose concentration you wished to determine. We will discuss two or three different methods to determine the protein concentrations of these solutions which illustrate both the quantitative aspects of the approaches and the limitations and assumptions that you must make to interprete the data.

In addition to familiarizing you with common methods of protein concentration determination, it should be emphasized that when some sort of assumption is made or some type of "standard" is used to calibrate a method, the results are only as good as the "assumption" or "standard" is representative of the protein whose properties you are trying to determine.

Methods to Determine Protein Concentration

These methods include measuring the absorption properties of the protein, measuring the change in absorbance properties of a dye [coomassie brilliant blue] as it binds to a protein, and using a chemical reaction which results in creation of a color.

Before we start lets examine the assumptions that are made in each of these methods. With the absorbance measurements at 280nm we are assuming that Beer's Law is obeyed, that is that the absorbance at a fixed wavelength of a given protein is directly proportional to its concentration:

Absorbance = concentration x extinction coefficient (x pathlength, usually 1cm)

Why might this not be true? Some proteins show concentration dependent aggregation affects which could cause anomalies. It is important to establish that over the range of concentrations that you might use that Beer's Law is strictly obeyed. The second problem is of course that you need to know the extinction coefficient for the given protein- more on that assumption later.

With the Dye Binding method for example, (or various chemical methods that result in a color change), you are assuming that all proteins behave essentially the same way, either binding the same amount of dye ***and*** causing the same extent of color change, or that all proteins react equally on a per weight basis with the chemical reagent that triggers the color change.

Utilization of uv Absorbance Measurements

The absorbance properties of a protein are contributed primarily by three residues, phenylalanine, tyrosine and tryptophan but since proteins have individual percentage compositions that may vary from each other it is hard to generalize about the absorbance properties of a given protein. Some proteins contain few if any tryptophan residues while other proteins may contain many tryptophan residues. Most proteins contain tyrosine and

phenylalanine but again there are variable % compositions of these amino acids too depending upon the protein. Further complications arise from the fact that local environment [as illustrated in some of the more advanced experiments in this section] affect the absorbance properties of the individual amino acids as well.

Measurements of the absorbance of a protein solution at 280nm offers a convenient way to estimate the concentration of a given protein [when it is the only species in solution absorbing at 280nm] if the mg/mL extinction coefficient is known. The problem of course is that quite often one wants to determine the total protein concentration of a solution without any knowledge of the different types and amounts of actual proteins that may be present. Can uv measurements at 280nm still be of use? The answer of course is yes, but certain assumptions need to be made. One of these assumptions: that the average mg/mL extinction coefficient for protein is 1.0 you have no control over, the other, that protein is the only thing contributing to the absorbance of the solution at 280nm you have some control over. The most likely 'contaminants" are nucleic acid or nucleotide containing compounds which have a much stronger absorbance at 260nm than at 280nm, where as proteins absorb more strongly at 280nm than 260nm. Frequently by measuring the ratio of the absorbances at 280nm and 260nm you can estimate the "purity" of the protein contributing absorbance at 280nm. Typically a pure protein has a 280nm:260nm ratio around 1.75 whereas a nucleic acid has a ratio around 0.5 (RNA)-0.55 (DNA)(Wilfinger et al 1997, Switzer & Garrity, 1989) Nucleic acids also have absorbance at 235nm and measuring both 280nm and 235nm absorbance allows the protein concentration in mg/mL to be estimated from the equation:

$$\text{mg protein/mL} = (A_{235} - A_{280})/2.51$$

(Whitaker & Granum, 1980)

A major source of absorbance in the 26-280nm region is often buffer salts, and in particular when purifying His-Tagged proteins, imidazole. Fortunately buffer salts can usually be removed effectively by equilibrium dialysis. Malate Dehydrogenase eluted from a NiNTA resin usually contains high (250-500mM) Imidazole which must be removed by equilibrium dialysis prior to determining concentration by uv measurements.

For example 1mL of elution fraction dialyzed versus 1 Liter of for example 5mM Phosphate buffer for 8-12 hours to reach equilibrium will result in a concentration of imidazole in the sample of 0.25-0.5mM imidazole. A second dialysis against another 1 liter of buffer would reduce the imidazole to 0.25-0.5µM concentrations

To obtain the 280nm: 260nm ratio for each protein you can simply scan the absorbance spectrum from 220 to 400nm and calculate the ratio. To scan, first blank a quartz cuvette with buffer, then add, for example, 200µL of protein and scan. The resultant spectrum allows the calculation of the absorbances at 260nm and 280nm.

To determine the concentration of an unknown concentration of a known protein using absorbance measurements one simply determines the absorbance at 280nm of the solution using an appropriate dilution if necessary and divides the absorbance by the extinction coefficient of that protein.

The above discussion uses the 0.1% (1mg/mL) extinction coefficient but for a pure protein this is easily converted to a molar extinction coefficient using the molecular weight of the protein. If the extinction coefficient for the protein has not been experimentally determined, Pace and Coworkers, (1995, 1997) have developed an equation based upon the amino acid composition for estimating the molar extinction coefficient of a protein:

$$\varepsilon_{280} = (5500 \times n_{Trp}) + (1490 \times n_{Tyr}) + 125 \times n_{S\text{-}S})$$

Which takes into account the molar absorbances of Tryptophan, Tyrosine and Disulphide bonds and the number (n) of tryptophans, Tyrosines and Cystines in the protein.

How Do Absorbance Measurements work with Mixtures of Proteins?

The problem with a mixture of proteins is that they can all have quite different mg/mL extinction coefficients, as illustrated by some sample proteins in table 1

Table 1
Extinction Coefficients for Sample Proteins

Protein	mg/mL Extinction Coefficient	MilliMolar Extinction Coefficient
Immunoglobulin	1.4	
Bovine Serum Albumin	0.66	
αCasein	1.01	
Watermelon glyoxysomal Malate Dehydrogenase	0.475	
Glutamate Dehydrogenase	0.93	

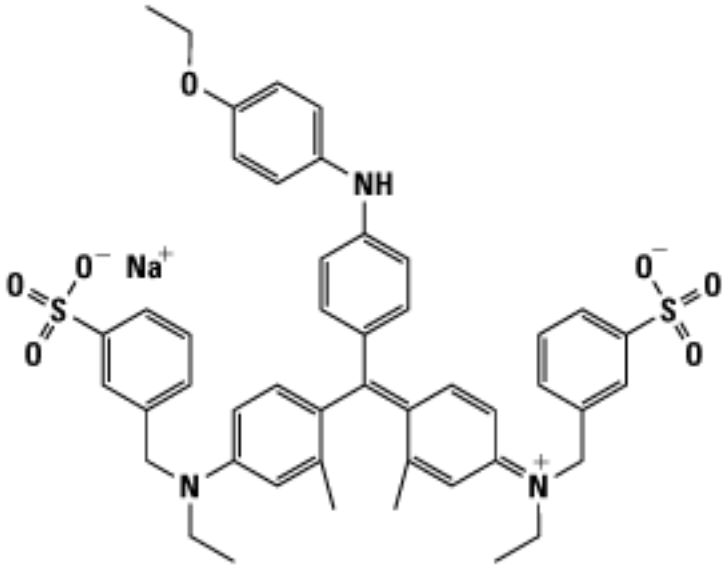
You will notice that Watermelon glyoxysomal Malate Dehydrogenase has a quite low extinction coefficient (0.475) which results from the fact that it contains no tryptophan sidechains. Other forms of MDH often have 1 or more tryptophans and as a result have higher extinction coefficients.

For a mixture of proteins it is often assumed that the average mg/mL extinction coefficient is 1.0 and this is not an unreasonable assumption. If a particular protein is being purified from a mixture this assumption could create inaccuracies as the protein is purified if the actual extinction coefficient of the protein in question is significantly different from 1.

Bradford Dye Binding Assay

***a]* Spectrum of Free and Bound Bradford Dye**

The Bradford dye binding method for the determination of the concentration of a protein solution depends on the observation the the dye Coomassie Brilliant Blue G-250



undergoes a color transition, as shown in figure 2, on going from a cationic species [with a wavelength of 470nm] to a neutral species [maximum at 650nm] to an anionic species, with a maximum at 595nm. The equilibria between these species are shifted when the dye binds to a protein due to the effects of the local environment on the protonation of the ionizable groups on the dye.

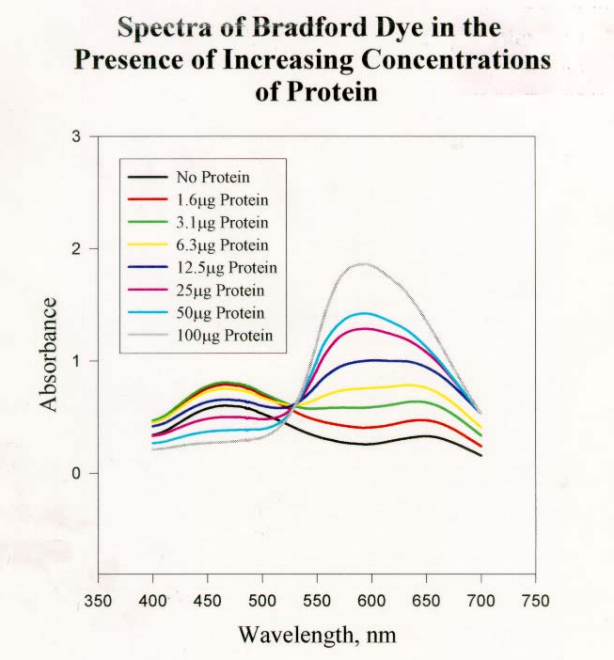
Figure 2

***b]* Construction of a Standard Curve for the Bradford Assay**

Protocol:

Measuring Specific Activity of Malate Dehydrogenase

For each of the three protein solutions that you used in part 1 you will construct a standard curve using the Bradford Dye assay.



To construct a standard curve for this assay, you will set up a series of incubations, containing identical buffer components and amount of dye, but differing in the amount of protein. In this curve you will utilize protein amounts from 0 to 100µg, in a total sample volume of 100µL.

Protocol for Using the Bradford Dye Binding Assay

Equipment: Spectrophotometer capable of reading absorbance at 595nm

Disposable Supplies
1.5mL Plastic spectrophotometer cuvettes
Disposable plastic Pasteur pipettes

Reagents
0.05M Phosphate Buffer
1mg/mL BSA in 0.05M Phosphate Buffer pH 8
Bradford Reagent
Unknown Sample

The Basics:
In this assay you will set up the standard curve using varied amounts of the "standard" protein BSA to a total volume of 800 microliters and then add 200 microliters of a Bradford Dye concentrate to give a final total volume of 1000 microliters- you must make sure that everything is thoroughly mixed before quantitating the results. The Unknown that you will use must be handled the same way.
To achieve the protein amounts that you will need for the standard curve for the Bradford Bye Binding Assay, you must first prepare a series of cuvettes containing different amounts of the protein standard, Bovine Serum Albumin (BSA) from a 1mg/mL stock BSA solution, using 0.05M phosphate buffer , pH 8, as the dilutant to give protein amounts from 100μg down to about 1μg per 800μL of added sample. In the protocol below a BSA range of 0-10micrograms is used which effectively keeps the "standard curve" in the linear range.

Set Up for Standard Curve and Unkowns

Tube Number	**Desired Amount of Protein, μg**	**Volume (μL) of 1mg/mL BSA Added**	**Volume (μL) of Buffer Added to give a total of 800μL**
1	0	0	800
2	2	2	798
3	4	4	796
4	6	6	794
5	8	8	792
6	10	10	790
Unknown	??	1-10 as appropriate	790-799 to make a total of 800

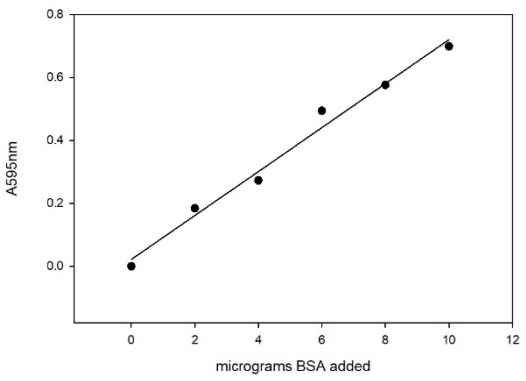
Add 0.2mL of reagent, Mix each tube, incubate for 5 minutes at room temperature, and determine the absorbance at 595nm, using a cuvette with buffer in place of the protein sample as the blank (tube 1 in above table).

For the unknowns, add sufficient volume of the sample to give an absorbance that lies on the standard curve

Typical Data for a standard curve under these conditions would be:

Tube Number	**Desired Amount of Protein, μg**	**Volume (μL) of 1mg/mL BSA Added**	**Absorbance at 595nm obtained by blanking on tube 1**
1	0	0	0
2	2	2	0.184
3	4	4	0.273
4	6	6	0.494
5	8	8	0.576
6	10	10	0.699
Unknown	? to be determined	10μL unknown added	0.317

Data Analysis



Plot the standard curves, using Absorbance at 595nm vs μg of BSA for the Bradford assay.

Using linear regression, the slope of the standard line is 0.0699+/- 0.0043 A595nm/microgram protein and the intercept is 0.022+/- 0.026 (indistinguishable from zero as expected)

The line is described by the equation:

A595nm = 0.0699 x micrograms of protein

Note that the x axis of the graph is micrograms of protein- it didn't matter what volume we added, just the final number of micrograms.

From the standard curve determining the number of micrograms of protein in the unknown sample is quite simple. The unknown gave an absorbance of 0.317 and we simply solve the equation:

0.317 = 0.0699 x micrograms of protein to get the micrograms of protein in the unknown.

0.317/0.0699 = 4.54 micrograms of protein.

To express the results in mg/mL of the original stock solution of the unknown, we know that 10μL of the unknown (the amount we put in the assay) contained 4.54 micrograms and hence the sample contained

4.54/10 = 0.454 micrograms/microliter, which is the same as 0.454mg/mL

Protocol to Determine the Concentration of Watermelon Glyoxysomal Malate Dehydrogenase Using UV measurements

Equipment: uv vis specvtrophotometer capable of scanning from 240-400nm

Quartz Cuvettes, 1mL or 3mL

Disposable Reagents

0.05M Phosphate Buffer, pH 8.0

Disposable plastic Pasteur pipettes

Method

Dialyze unknown sample against 0.05M (or less) Phosphate Buffer- keep a sample of the post dialysis buffer for control

Place 1mL of 3mL buffer in quartz cuvette

Blank spectrophotometer, collect spectrum of buffer from 240nm to 400nm

Add appropriate amount of unknown

Scan spectrum from 240nm to 400nm. Spectrum 1

Repeat with post dialysis control buffer using the same volume as established above. (this will tell you whether any uv absorbing species remain in the dialyzed sample) Spectrum 2

Calculations

Subtract spectrum 2 from spectrum 1 to give sample spectrum

Determine absorbance values in sample spectrum at 260nm, 280nm and 340nm

The concentration of Pure Watermelon Glyoxysomal MDH is then determined from:

Concentration in mg/mL in the cuvette = (A280nm - A340nm)/ 0.475

Where 0.475 is the mg/mL extinction coefficient at 280nm of watermelon Glyoxysomal malate dehydrogenase (remember different forms of malate dehydrogenase will have different mg/mL extinction coefficients due to varying Tryptophan content)

The concentration in the original sample depends upon the total volume in the cuvette and the volume added and you need the "Dilution Factor"

Dilution Factor = Total Volume in Cuvette/Volume added

For example if you added 100mL to 3mL of buffer (to give a total volume of 3.1mL) the dilution factor would be:

Dilution Factor = 3100/100 = 31

The concentration of protein in the original stock solution is then:

Stock Concentration = Concentration in Cuvette x Dilution Factor

For example if A280 is 0.068 obtained when 100mL of sample is added to 1mL buffer and A340 is 0.004

The concentration of wgMDH in the cuvette would be:

(0.068 – 0.004)/0.475 = 0.135mg/mL

The dilution Factor would be 1100/100 = 11

And the concentration in the original stock solution would be

0.135 x 11 = 1.48mg/mL