Enzyme and Protein Assays in *Neurospora crassa*

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Overview

In this lab, three samples of crude extract from *Neurospora crassa* will be provided. This extract has been prepared by growing *N. crassa,* collecting it and breaking it open to prepare a crude extract. The extract has been divided into three portions. One portion is untreated, while the second portion has been heated to 90 °C for 10 min and the third portion has had urea added to a final concentration of 5 mM. The goal of this project is to identify which treatment each extract received by measuring both the specific activity for the arginase enzyme as well as the level of urea .

Introduction to colorimetric tests

Colorimetric tests are commonly used in both biology and chemistry. The idea behind using a colorimetric test is that the amount of compound X can be measured if compound X absorbs light. The higher the concentration of compound X in a solution, the higher the absorbance. In many cases, compound X itself is not a chromophore, but it can be reacted with some other compound and the product is a chromophore. Colorimetric tests are based on the proportional relationship between concentration of a chromophore and the amount of light absorbed by that chromophore. This relationship is described by Beer's law which states

$A=\varepsilon \times l \times c$

where ε is a constant called the molar absorptivity(in units of liters/mole cm), l is path length of the spectrophotometer (usually 1 cm) and c is concentration which is in terms of molarity (moles/1). Using this relationship, the concentration of a compound can be determined directly from the absorbance provided molar absorptivity and path length are known. This linear relationship between absorbance and concentration is true for the useful absorbance range; this is generally considered from 0.2 to 1.2. At absorbances greater than 1.2, the linear relationship shows a negative deviation and thus any absorbance value above 1.2 will underestimate the concentration. Absorbances below 0.2 are difficult to read with great accuracy because of the faint color. The higher level of error produces values that are less useful. Molar absorptivity is a constant under a given set of conditions; if pH, temperature or other conditions differ, this value will change. It is also possible to relate the absorbance of a compound to its concentration by use of the standard curve.

The idea of a standard curve is that the relationship between absorbance and concentration is linear provided that the absorbance value is in the useful range. The amount of X in an unknown sample can be measured by comparing the absorbance of the unknown with a series of standards. This is done by plotting the absorbance values vs amount of X and solving for the best straight line which is given in terms of $y=mx + b$ where y is absorbance, x is amount of X, b is the y intercept and m is the slope. The best straight line will give values for the slope and y intercept; it is possible to mathematically solve for amount of X in an unknown sample. Since the standard curve is so important for figuring out the unknown concentration, it is imperative that it be accurate. One way of increasing the accuracy is to use a large number of standards; in this course typically six standards, including a blank, will be used to construct the standard curve.

Introduction to enzyme assays

An enzyme assay is used as a relative measure of the amount of a particular enzyme in a solution. It is based on a measurement of either the amount of product formed or amount of substrate used by an enzyme. This is a relative measure: if a lot of enzyme is present, a large amount of substrate will be used and a large amount of product will be formed.

There are several types of enzyme assays. In one assay, either the product formed or the substrate produced absorbs light at a wavelength in either the visible or ultraviolet part of the spectrum. An example of this type of enzyme assay is that for malate dehydrogenase. This is one of the enzymes of the tricarboxylic acid cycle and it catalyzes the following reaction:

NAD + malate ----------▶ oxaloacetate + NADH.

NADH absorbs light at 340 nm while NAD doesn't. The enzyme can be assayed by measuring the absorbance at 340 nm. When the enzyme is mixed with NAD and malate, the initial absorbance is near 0 and increases over time as NADH is produced.

In many reactions, neither the substrates nor the products absorb light and it is not possible to directly measure the appearance of product or the disappearance of substrate. In this case, another compound or compounds is added which reacts with the products and forms a chromophore. There are many examples of this type of enzyme assays. An enzyme that will be assayed later in the semester is ornithine transcarbamylase (OTC) and is one of those from the arginine biosynthetic pathway in *N. crassa*. This enzyme catalyzes the following reaction:

ornithine + carbamyl phosphate ----------▶ citrulline

Citrulline reacts with a mixture of compounds to produce a purple color.

Another enzyme that has an indirect assay is arginase. This enzyme will be assayed in this project as well as future projects. Arginase catalyzes the following reaction:

arginine ----------▶ ornithine + urea

The enzyme is assayed by measuring the amount of urea produced. Enzyme activity is measured in terms of units which are the μmole product formed/ min. All enzyme assays are done for a fixed period of time. This assay is done in two steps. The enzyme is mixed with substrate and incubated at a fixed temperature for a fixed period of time. At the end of the time period, the enzyme must be stopped from catalyzing the reaction and this requires denaturation of the enzyme. Usually, either a strong acid or base is added to the reaction mixture. In the second step, the amount of urea produced is measured. A portion of the reaction mixture is removed and mixed with a solution of isonitrosopropiophenone in sulfuric acid/phosphoric acid and boiled. This solution reacts with urea to produce a pink product whose absorbance can be measured.

The enzyme assay measures the total amount of urea in the extract. Any urea that was already present in the extract would also be measured. To take into account an indigenous urea, the procedure is modified slightly to do what is known as a 0 minute control. In this control, the order of adding reagents is modified. Substrate is mixed with acid and then the enzyme is added. Since the enzyme is denatured by the acid, it cannot form urea from arginine. Any urea that is measured using the colorimetric test must have been present in the extract. To subtract for the endogenous urea, the amount of urea in the 0 min control is subtracted from the amount of urea produced in the enzyme assay.

Instructions

1. You will receive portions of samples A, Band C. Each of these samples came from the crude extract. One sample has been heated to 90 °C for 10 min, one has had urea added to a 5 mM concentration and one has not been treated. The goal is to identify the treatment that the samples received.

2. Each sample should be assayed for both protein by the Lowry assay and arginase activity. These values can be combined to determine both the arginase specific acitivity and the indigenous level of urea. Each of these assays are described in the following pages.

3. Calculate the specific activity for samples A, Band C.

Notes

1.) During the class period, the extracts should be stored on ice to prevent denaturation and degradation. Between class periods, the extracts will be frozen at -20 C.
2.) In any enzyme assay, the timing is critical since the time of incubation is included in the calculation. Before you begin the assay, devise a schedule so that the assay is timed correctly.
3.) The assay calls for temperatures of 37°C and this requires either a water bath or heating block.
4.) 0 min controls must also be done for the enzyme assay.
5.) The colorimetric procedures require the addition of an appropriate volume. What is an appropriate volume? It is a volume that gives an absorbance value in the range of the standard curve. It is only possible to make guesses as to what this volume is before starting the assay. If the volume produces an absorbance of less than 0.2, the assay should be repeated with a larger volume. If the absorbance is greater than 1.5, the assay should be repeated with a smaller volume.
6.) Absorbance can be measured using one of several spectrophotometers. , There will be several Spec 20 spectrophotometers available in the lab and these require special glass cuvettes. There are also two UV-visible spectrophotometers available in F-009. These require special plastic cuvettes that will be provided.

Arginase Assay

Reference: Davis, R.H. and Mora, J. J *Bacteriology(1968)* 96, 383-388.

Introduction: Arginase is one of the arginine catabolic enzymes and like many enzymes, it requires a metal co factor. In the case of arginase, the co factor is manganese. The reaction catalyzed by arginase is:

arginine ⟶ ornithine + urea

The urea can be measured colorimetrically. This assay consists of two parts. In the first part, the enzyme assay is done: the enzyme is mixed with manganese and then arginine is added. After an incubation period the assay is stopped by adding perchloric acid. The strong acid solution denatures the enzyme and prevents and further activity. The product is then assayed in a second step. An aliquot from the first assay is mixed with reagents that produce a pink color in the presence of urea. The amount of urea can be measured by extrapolation using a standard curve.

Reagents

For the enzyme assay:
1. 100 ml of 0.1 M glycine/NaOH pH 9.5
2. 50 ml of 0.25 M arginine, pH 9.5
3. 50. mi of 10 mM $MnCl_2$
4. 10 % perchloric acid-

For the colorimetric assay
1. 10 ml of 4 % isonitrosopropiophenone in 95% ethanol
2. 200 ml of 3:1 mixture of concentrated phosphoric acid: concentrated sulfuric acid
3. 100 ml of 1 mM urea standard

Arginase assay instructions:

1. Label 13 x 100 mm test tubes.

2. Add 0.2 ml of qlycine buffer, 0.5 ml of enzyme solution and 0.1 ml of manqanese chloride. Incubate at 37 °C for 10 min. This pre-incubation step is necessary to allow the enzyme to bind with the manganese which is a co-factor .

3. Initiate the enzyme assay by adding 0.1 ml of arginine and continue to incubate at 37 °C.

4. After 30 min. stop the assay by addinq 1.0 ml of the perchloric acid ($HCL0_4$) solution.

5. An aliquot of this will be used to assay for urea.

Urea colorimetric assay

1. Label 13 x 100 mm test tubes.

2. Add volumes of urea standards that contain the following:
   0, 100, 250, 500, 750 and 1000nmole of urea

3. For the samples remove an appropriate volume from the arginase assay mixture.

4. Bring the total volume of both samples and standards up to 2.0 ml with distilled water .

5. Add 1.0 ml of the phosphoric acid/sulfuric acid mixture. Mix well

6. Add 0.1 ml of 4% isonitrosopriopophenone

7. Cap using the while bacto caps.

8. Carefully place in a boiling water bath for 1 hour.

9. Remove the solutions and let them cool to room temperature.

10. Measure the absorbance at 540 nm.

Notes on the urea colorimetric assay

1. This assay calls for the use of extremely acidic conditions so use caution.

2. The colored compound produced is somewhat light sensitive, so read the absorbances shortly after the tubes have cooled off.

Lowry assay

Introduction: The Lowry assay for protein is a colorimetric assay for the total amount of proteins. Proteins alone are not chromophores but they can be reacted with compounds and produce chromophores. Since this is an assay for all proteins, any, added reagent must react with all. proteins. The original paper that describes this assay is one of the most cited biochemistry papers of all time and this assay is widely used. The Lowry assay is actually a combination of two colorimetric assays. One of the colorimetric reagents reacts specifically with tryosine resides and another colormetric reagent reacts with the peptide bond.

Similar to other colorimetric assays, the absorbance of an unknown solution is related to the concentration by using a standard curve. For Lowry assay, any protein could act as as a standard but the most commonly used protein is BSA(bovine serum albumin). This is a protein from cow's blood. It is cheap and readily available and for this reason has become a standard.

Reference:

Journal of BioloQical Chemistry, 193(1951), page 265.

Reagents

1.) 100 ml of 2.68% potassium sodium tartrate
2.) 100 ml of 1% copper (II) sulfate pentahydrate
3.) 500 ml of 2% sodium carbonate in 0.1 M NaOH
4.) 50 ml of 1 N Folin reagent I **.**5.)1 L of 0.5 M NaOH
6.) 100 ml of 1 *mg/ml* BSA standard

Reaqents that have to be prepared fresh:
1.tartrate/copper(ll) sulfate/sodium carbonate: Mix 1 part of sodium potassium tartrate, 1 part of copper(ll) sulfate pentahydrate. and 100 parts of sodium .carbonate in 0.1 M NaOH.

Instructions

1. Label 13 x 100 mm test tubes. .

2. For the standards. use the BSA solution to prepare the following:
..0, 100' 150. 200. 250 and 300 *LJQ* BSA Do the standard curve in duplicate-

3. For the samples, add an appropriate volume.

4. For all the test tubes bring the total volume up to 1.2 ml with distilled water.

5. Add 3.0 ml of tartrate/copper (II) sulfate/ sodium carbonate solution. Mix well with a vortexer.

6. After 10 min. add 0.3 ml of Folin reaqent. **Vortex each tube immediately I after the addition of Folin reagent**

7. **Let the tubes sit for 30 min and then measure the absorbance at 720 nm.**