BIOCHEMISTRY I LAB (BCH 3033L)

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BIOCHEMISTRY I LAB SCHEDULE

SPRING 2005

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BIOCHEIMISTRY LABORATORY

EQUIPMENT LIST

GLASSWARE ISSUED	QUANTITY
Beakers	
50 ml	2 ea
100	2
250	2
400	1
1000	1
Bottles, wash	2
Erlenmeyer Flasks	
25 ml	1 ea
50	2
125	2
250	1
500	1
1000	1
Funnels, Glass	2 ea
Cylinders, Graduated	
10 ml	1
50	1
100	1
Separatory Funnel	2
Spatula	2
Test Tube Rack	1
Test Tube Rack, Drying	1
Watch Glass	2

THE TITRATION OF AN AMINO ACID

Equilibrium: When a solute, A, dissolves in water and a chemical reaction occurs to form B, the reaction can be described by the equation A ↔ B. The concentration of B will initially increase very rapidly, then more slowly until its concentration becomes constant. Conversely, the concentration of A will decrease very rapidly at first but also will arrive at a constant value. When the concentration of both components becomes constant, the system is said to be at **equilibrium**.

In the above situation, water does not enter into the reaction but only serves as the solvent. However, even in cases where water does participate directly in a reaction, its concentration does not usually change very much because its concentration is so much greater than the solute molecules'.

It must be kept in mind that, although the concentrations of reactant and product in the above reactions remain constant at equilibrium, the reactions are still proceeding. However, the rates of the forward and backward reactions are identical. This state is referred to as **dynamic equilibrium**.

The concentration of reactants and products at equilibrium for a given reaction will not vary if temperature, pressure, and solvent system remain constant.
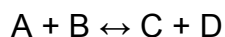
Equilibrium Constant: At equilibrium, the concentrations of all reactants bear a relation to one another. In the simplest case, A ↔ B, the relation can be expressed as:

$$K_{eq} = [B] / [A]$$

The brackets refer to concentration, and **K_{eq}** is simply a number which is constant at a given temperature and pressure. The number is called the **equilibrium constant**.   By convention, the concentration of the reacting species on the right side of the equation is always placed in the numerator and that on the left side is placed in the denominator. If the reaction had been written B ↔ A, then the equilibrium constant would be written as:

$$K_{eq} = [A] / [B]$$

If one considers the reaction:

$$A + B \leftrightarrow C + D$$

The equilibrium constant is obtained by multiplying the concentration of all components on the right side of the expression and dividing this result by the product of the concentrations of components on the left side of the expression. Thus, the equilibrium constant would be written as:

$$K_{eq} = ([C] \times [D]) / ([A] \times [B]) \quad OR \quad K_{eq} = [C][D] / [A][B]$$
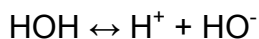
Dissociation: The term dissociation refers to reactions in which a compound or ion breaks up in a solvent into two or more units, at least one of which is an ion.

Consider: $HA \leftrightarrow H^+ + A^-$

The equilibrium constant (also referred to as the **ionization constant**) would be:

$$K_{eq} = \mathbf{K_i} = [H^+] [A^-] / [HA]$$

Water is itself a weak acid which dissociates very slightly according to:

$$HOH \leftrightarrow H^+ + HO^-$$

Thus, the ionization (or '**dissociation**') constant for water should be:

$$\mathbf{K_i} = [H^+] [HO^-] / [HOH]$$

Because the degree of dissociation is so slight relative to the large concentration of water (approximately 55.5M), virtually no change occurs in the water concentration. The product, $K_i[HOH]$, is also constant within the accuracy of most measurements. The value of this product is designated '$\mathbf{K_w}$' and is approximately equal to 1×10^{-14}.

Thus, $\mathbf{K_w} = [H^+] [HO^-] = 1 \times 10^{-14}$

In pure water, the number of H^+ (protons) produced in ionization will equal the number of HO^-. Therefore, the concentration of each of these ions will be about 1×10^{-7} M. If the concentration of one of these ions is increased by the addition of an acid or base, the concentration of the other ion decreases correspondingly so that the product of the ion concentrations still equals 1×10^{-14}.

pH: The degree of acidity, that is the hydrogen ion concentration, [H$^+$], in moles per liter usually involves cumbersome negative exponents. For this reason, the pH scale was devised. The pH is defined as the negative logarithm of the [H$^+$].

$$pH = -\log [H^+] = \log \frac{1}{[H^+]}$$

Thus pure water, where [H$^+$] = 1 x 10^{-7} M, would have a pH of 7 since
-log 1 x 10^{-7} = - (-7) = 7.

It is important to note that when dealing with pH, an increase of 1 pH unit involves a ten-fold difference in [H$^+$] from the next whole number. Thus, one must add ten times more [H$^+$] than already present in a solution at pH 7 in order to bring it to pH 6; at pH 5, there must be 100 times more hydrogen ions present per unit volume than at pH 7.

Dissociation of Weak Acids: An acid is a substance which will liberate protons to a medium. A base is a substance which will combine with a proton. In the general reaction:

$$HA \leftrightarrow H^+ + A^-$$
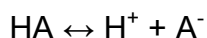
HA is the acid and A$^-$ is the base. Strong acids are almost completely ionized in water. The H$^+$ concentration will be the molar concentration of the added acid. On the other hand, weak acids ionize to a very small extent. Consider the ionization of acetic acid:

$$H_3C\text{-}COOH \leftrightarrow H_3C\text{-}COO^- + H^+$$
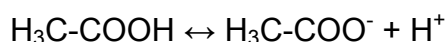
The K$_i$ (ionization or dissociation constant) is about 1.8 x 10^{-5} M. Suppose one was to prepare a O.1 M solution of acetic acid, according to the K$_i$ value, the dissociation of the acid is so slight that the concentration of undissociated acetic acid is still O.1 M. From the given dissociation of acetic acid, it is clear that both the H$^+$ and H$_3$C-COO$^-$ ion concentrations are equal since one of each is formed by the dissociation of one acid molecule.

Recalling that K$_i$= [H$^+$] [H$_3$C-COO$^-$] / [H$_3$C-COOH]

we may now substitute to: 1.8 x 10^{-5} = [H$^+$]^2/0.1 , <u>or</u>

[H$^+$]^2 = 1.8 X 10^{-6}

Accordingly, [H$^+$] = 1.34 x 10^{-3} M

In dealing with weak acids, the most useful characteristic is the K_i value. However, these are small numbers and cumbersome to use. It is convenient to employ the pK notation like that of the pH notation.

$$pK_i = -\log K_i$$

Buffers: A buffered solution contains substances which confer the ability to resist changes in pH. Buffering is of extreme importance in cells and tissues because even small changes in pH may impair, or be fatal to, the organism. Acetic acid and its conjugate base, acetate ion, form an effective buffer in the pH region of the pK value. If both the acid and the acetate ion are present in substantial concentrations when alkali enters the solution, a rise in pH will be resisted according to the reaction:

$$HO^- + H_3C\text{-}COOH \leftrightarrow HOH + H_3C\text{-}COO^-$$
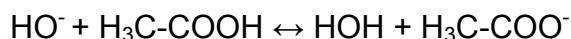
If both the acid and base forms are present in equimolar amounts, the capacity to resist a change in pH in either direction is the same. Consider again the ionization of:
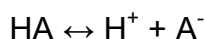
$$HA \leftrightarrow H^+ + A^-$$

$$K_i = [H^+]\,[A^-]\,/\,[HA],\ \underline{or}$$

$$[H^+] = K_i\,[HA]\,/\,[A^-]$$

Taking the negative logarithm of both sides of the equation

$$-\log [H^+] = -\log K_i\,[HA]\,/\,[A^-],\ \underline{or}$$

$$-\log [H^+] = -\log K_i - \log [HA]\,/\,[A^-],\ \underline{or}$$

pH = pK_i + log [A⁻] / [HA]

This expression is known as the **Henderson-Hasselbalch equation (pages 66 - 67 in your textbook)**. From an inspection of this expression it can be seen that pH = pK_i when the [A⁻] (base) is equal to the [HA] (acid).

Titrations: Titration consists of the stepwise addition of a standardized base or acid to a solution of acid or base. A titration curve is obtained by plotting the pH readings on the ordinate against the milliequivalents of standard acid or base added on the abscissa. A smooth line is then drawn through these points.

EXPERIMENTAL

Each student pair will carry out the titration of an unknown amino acid versus HCl and NaOH using the pH meter. Standardize the pH meter according to the instructions given for the instrument. Obtain linear graph paper from the instructor.

Transfer exactly 50 ml of a O.1 M amino acid solution to a clean, 100ml beaker. Place a thoroughly rinsed teflon-coated stirring bar into the beaker and place the beaker on a magnetic stirrer. Insert the rinsed electrode of the pH meter into the solution and **record the starting pH**. Obtain ~50 ml of 1 N HCl. With the stirrer turned on, add O.2 ml HCl using an appropriate pipettor, wait for the pH value to stabilize on the pH meter and record the pH. Again add O.2 ml HCl while stirring, record the pH and so on. A graph of pH versus milliliters HCl added should be made during the experiment as well as recording the data in tabular form. Continue the titration until the solution reaches a pH of 1.5. Remove the electrode from the solution, wash, and insert it into a beaker of distilled water. Rinse the electrode.

Transfer exactly 50 ml of the same amino acid solution into a clean, 100 ml beaker. Place a teflon coated stirring bar into the beaker and place the beaker on the magnetic stirrer. Insert the rinsed electrode of the pH meter into the solution and **record the starting pH**. Obtain ~50 ml of 1N NaOH. With the stirrer turned on, add O.2 ml NaOH using an appropriate pipettor, wait for the pH value to stabilize on the pH meter and record the pH. Again add O.2 ml NaOH while stirring, record the pH and so on. A graph of pH versus milliliters NaOH added should be made during the experiment as well as recording the data in tabular form. Continue the titration until the solution reaches a pH of 12.0. Remove the electrode from the solution, wash and insert it into the standard pH = 7.0 buffering solution (yellow solution).

Directly from the amino acid titration *curves derive:*

(a) The pK values of the amino acid

(b) The isolelectric point, pI, of the amino acid

What possible amino acid could be contained in the solution? The pK values of selected amino acids are shown in the following table:

pK and pI Values of Common Amino Acids

Amino Acid	pK1	pK2	pK3	pI
Alanine	2.34	9.60	n/a	6.01
Aspartic Acid	1.88	3.65	9.60	2.77
Lysine	2.18	8.95	10.53	9.74
Histidine	1.82	6.00	9.17	7.59

118   Part II   Structure and Catalysis

table 5-1

Properties and Conventions Associated with the Standard Amino Acids

Amino acid	Abbreviated names		M_r	pK_a values			pI	Hydropathy index*	Occurrence in proteins (%)[†]
				pK_1 (—COOH)	pK_2 (—NH$_3^+$)	pK_R (R group)			
Nonpolar, aliphatic R groups									
Glycine	Gly	G	75	2.34	9.60		5.97	−0.4	7.2
Alanine	Ala	A	89	2.34	9.69		6.01	1.8	7.8
Proline	Pro	P	115	1.99	10.96		6.48	1.6	5.2
Valine	Val	V	117	2.32	9.62		5.97	4.2	6.6
Leucine	Leu	L	131	2.36	9.60		5.98	3.8	9.1
Isoleucine	Ile	I	131	2.36	9.68		6.02	4.5	5.3
Methionine	Met	M	149	2.28	9.21		5.74	1.9	2.3
Aromatic R groups									
Phenylalanine	Phe	F	165	1.83	9.13		5.48	2.8	3.9
Tyrosine	Tyr	Y	181	2.20	9.11	10.07	5.66	−1.3	3.2
Tryptophan	Trp	W	204	2.38	9.39		5.89	−0.9	1.4
Polar, uncharged R groups									
Serine	Ser	S	105	2.21	9.15		5.68	−0.8	6.8
Threonine	Thr	T	119	2.11	9.62		5.87	−0.7	5.9
Cysteine	Cys	C	121	1.96	10.28	8.18	5.07	2.5	1.9
Asparagine	Asn	N	132	2.02	8.80		5.41	−3.5	4.3
Glutamine	Gln	Q	146	2.17	9.13		5.65	−3.5	4.2
Positively charged R groups									
Lysine	Lys	K	146	2.18	8.95	10.53	9.74	−3.9	5.9
Histidine	His	H	155	1.82	9.17	6.00	7.59	−3.2	2.3
Arginine	Arg	R	174	2.17	9.04	12.48	10.76	−4.5	5.1
Negatively charged R groups									
Aspartate	Asp	D	133	1.88	9.60	3.65	2.77	−3.5	5.3
Glutamate	Glu	E	147	2.19	9.67	4.25	3.22	−3.5	6.3

*A scale combining hydrophobicity and hydrophilicity of R groups; it can be used to measure the tendency of an amino acid to seek an aqueous environment (− values) or a hydrophobic environment (+ values). See Chapter 12. From Kyte, J. & Doolittle, R.F. (1982) *J. Mol. Biol.* **157**, 105–132.

[†]Average occurrence in over 1150 proteins. From Doolittle, R.F. (1989) Redundancies in protein sequences. In *Prediction of Protein Structure and the Principles of Protein Conformation* (Fasman, G.D., ed) Plenum Press, NY, pp. 599–623.

Adapted from 'Experimental Biochemistry' by Dr. John P. Riehm and Mr. Sherman L. Bonomelli,
The University of West Florida

PAPER CHROMATOGRAPHY OF AMINO ACIDS

The predominant factor in separating solutes by paper chromatography is believed to be partition between two immiscible phases. Other factors such as adsorption and ion exchange also exert an effect. Theoretically, the paper functions as an inert support for the solvent. Partition takes place between the **stationary (paper) phase** and the **mobile (solvent) phase**. _The solute moves in the direction of solvent flow at a rate which is governed by its attraction for either the stationary phase or the mobile phase_. A large number of variables (temperature, solvent composition, type of paper, etc.) exert a marked influence on the rate at which a solvent moves on a chromatogram. Therefore, in making comparisons between known and unknown substances, or chromatogram runs at other times, care must be taken to insure complete reproduction of conditions.

The migration rate of the solute in the direction of solvent flow is defined as the **Retardation Factor (R_f)** value for a given solute in a particular system:

$$R_f = \frac{\text{distance moved by solute}}{\text{distance moved by solvent}}$$

Thus, R_f values vary from 0 to 1.0 and are calculated by measuring from the line of origin. R_f values for particular components can be compared only with other values obtained under precisely similar experimental conditions.

The R_f is commonly reported at the **hR_f** value, calculated by multiplying the R_f value by 100 to obtain a whole number:

$$hR_f = R_f \times 100$$

In the case of amino acids, the most common means of detection is the ninhydrin reaction, which gives amino groups a red-purple color and is extremely sensitive.

<div align="center">Materials</div>

1. 95% ethanol-NH₄OH (90:10 v/v: base solvent)

2. Whatman No.1 paper (18 x 34 cm)

3. Reference amino acids (10.0 mg/ml)

4. Unknown amino acid mixture (10.0 mg/ml)

5. Chromatographic jars

6. 0.2% ninhydrin solution containing 10% acetic acid in 95% ethanol

Experimental: The origin or starting line of 1 piece of 18 x 34 cm Whatman No. 1 paper is marked 4.0 cm from the bottom and then dotted with 16 spots evenly distributed 2.0 cm apart. Approximately 5.0 µl (0.005 ml) of each standard amino acid solution is placed on nine of the spots of each paper, leaving seven spots for unknown amino acid mixtures (i.e., ~5 – 7 groups can work with the same chromatogram). Completely dry the spots.

The papers are then stapled to form cylinders. The cylinders are **carefully** dropped (origin lines down) into two previously equilibrated chromatographic jars containing the solvent systems listed in the materials section. After a 3 - 6 hour run, papers are removed and allowed to dry <u>overnight</u>. The chromatograms are then sprayed with the ninhydrin spray reagent and dried in an oven at 100°C for 3 - 5 minutes.

After location of the amino acids, the R_f values of the spots are calculated and a tentative identification of the unknown is made by comparison with the R_f values of knowns.

Notes: The spots should be applied so that the liquid does not spread much more than 5 mm and may require more than one application (that is, spot a small portion, let dry and then respot).

The spray reagent is very sensitive and will detect finger prints. Accordingly, wear disposable gloves when handling the paper chromatograms.

THIN-LAYER CHROMATOGRAPHY (TLC) OF AMINO ACIDS

The principle of thin-layer chromatography (TLC) is that a suitable adsorbent is spread in a thin layer on a glass plate, or other suitable support plate. The drop of solution to be analyzed is applied at a known starting point. The plate is placed in a sealed chromatographic chamber with a suitable solvent system. By capillary action the solvent creeps up through the stationary layer and separates the components of the sample into a number of spots. After separation, the plates are dried and the spots identified by the addition of reagents.

TLC has several advantages over paper chromatography:

1) it is faster.

2) it is 10 to 100 times more sensitive.

3) it can utilize a great variety of absorbent materials and is particularly superior for lipophilic substances.

4) it can use more drastic chemical detection methods.

<div align="center">Materials</div>

20 x 20cm prefabricated **cellulose** TLC sheets

Developing chambers

Developing Solvent: Isopropanol: formic acid: water (40:20:10 v/v);

<div align="center">pH 2.5</div>

Pipettes

Known amino acids (10.0 mg/ml)

Unknown samples (10.0 mg/ml)

Spray reagent: ninhydrin (O.1 g), absolute ethanol (70 ml),

glacial acetic acid (21 ml), 2,4,6-collidine (2.9 ml).

Experimental: The solutions of the individual amino acids (5.0 µl) are applied onto a prefabricated cellulose sheet using a spotting guide. The spots should be no more than 0.5 cm in diameter. Before starting the chromatogram, the spots should be allowed to evaporate completely.

The developing solvent is poured into the developing chamber to a depth of 0.7 cm. The TLC plates are loaded in pairs into the chamber and then the cover plate is added. The distance from the solvent to the starting line the TLC plate should be 1 – 2 cm. The chromatogram is allowed to develop until the mobile phase reaches 2 – 3 cm from the top. Remove the TLC plate, dry overnight (hood) and then spray with ninhydrin reagent. After location of the amino acids, the R_f and hR_f values of the spots are calculated and unknowns identified.

Wear disposable gloves when handling the TLC plates.

PROTEIN DETERMINATIONS USING THE BRADFORD AND BICINCHONINIC ACID METHODS

There is no perfect assay method for determining protein concentration. Each of the many methods has numerous advantages and disadvantages. We will consider two of the most widely employed assays to determine protein concentration.
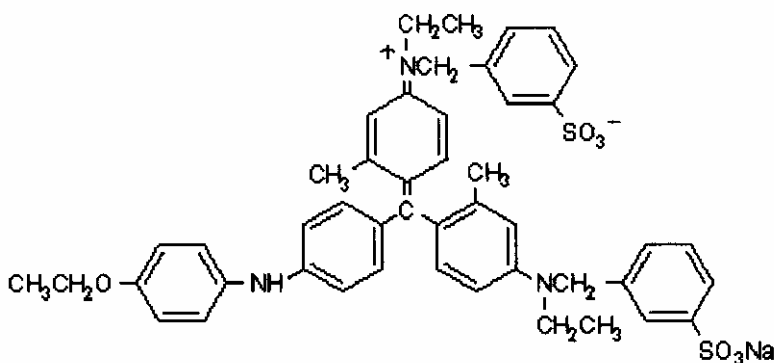
Introduction to the Bradford Assay

Bradford, M.M (1976). Anal. *Biochem.*, **72**, 248.

The Bradford assay is perhaps the most widely employed method for determining protein concentration. It is simple, rapid, relatively inexpensive and quite sensitive. The assay can be performed in the presence of reducing agents such as dithiothreitol and 2-mercaptoethanol which are often added to buffers in which proteins are solubilized. The assay does not yield satisfactory results in the presence of detergents, which are added to buffers in which membrane proteins are solubilized; the detergent interferes with the binding of the Bradford reagent to the protein. The dye reagent is composed of dye solubilized in phosphoric acid and methanol.

The assay is based on the specific binding of Coomassie Brilliant Blue G-250 dye to arginine, tryptophan, tyrosine, histidine and phenylalanine residues in proteins. The dye binds approximately 8 times as well to arginine residues as the other listed aromatic residues. Therefore, the overall reliability of the assay is highly dependent on the primary structure of the proteins being assayed, as well as on the protein chosen to prepare the standard curve. Bovine serum albumin (BSA) is by far the most frequently employed protein to prepare standards. BSA, however, is a poor choice for the Bradford assay. Ovalbumin is preferred. If BSA is used, the concentration obtained is multiplied by 2.1 to obtain a closer approximation of the protein concentration.

The dye in solution is in the cationic form and has an absorption maximum at 470 nm (red). The bound (anionic) form has an absorption maximum of 595 nm, thus the assay is monitored in the spectrophotometer at 595 nm.

Structure of Coomassie Brilliant Blue G-250

Disadvantages of the Bradford assay in addition to those listed above are that the absorption spectra of the cationic and anionic species of the dye partially overlap, the assay is non-linear over wide ranges, the dye binds strongly to quartz cuvettes and some proteins precipitate in the dye reagent. Typically the overlap in spectra are not problematic when using a proper standard curve. The assay should be used over narrow concentration ranges and plastic (or glass) cuvettes should be used. If precipitation of the protein sample is observed, then a small amount of 1 M NaOH can be added to the protein sample prior to assay to reduce precipitation.

Introduction to the Bicinchoninic Acid Assay

Smith, P.K *et al.* (1985) *Anal. Biochem.*, **150**, 76.

The bicinchoninic acid assay (or BCA assay) is a popular choice for the assay of protein concentration due to its high sensitivity, linearity over a broad range of protein concentration, compatibility with detergents and compatibility with a wide range of common buffer components and its overall simplicity. The assay requires somewhat more time than the Bradford, though when the reaction is heated in a microwave oven the time difference is negligible. The BCA assay is NOT compatible with the presence of reducing agents in the protein solubilization buffer. The BCA assay is NOT a true end-point method. In other words, the final color continues to develop over time. However, the rate of continued color development at the end of the sample incubation step is slow and does not influence the accuracy of the results.

The assay is based on the reduction of Cu^{2+} to Cu^{1+} by protein in alkaline conditions (the biuret reaction). The cuprous ion formed during the reduction reaction is chelated by two molecules of BCA and the result is a purple-colored product having an absorption maximum of 562 nm. The macromolecular structure of the protein, the number of peptide bonds, and the presence of four specific amino acids (cysteine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA.
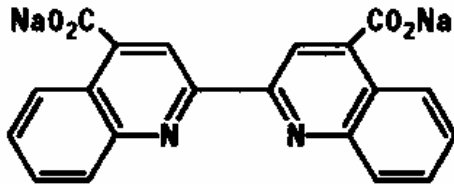
Structure of Bicinchoninic Acid

STEP 1.

Protein + Cu^{+2} $\xrightarrow{\text{OH-}}$ Cu^{+1}

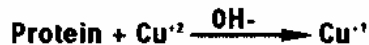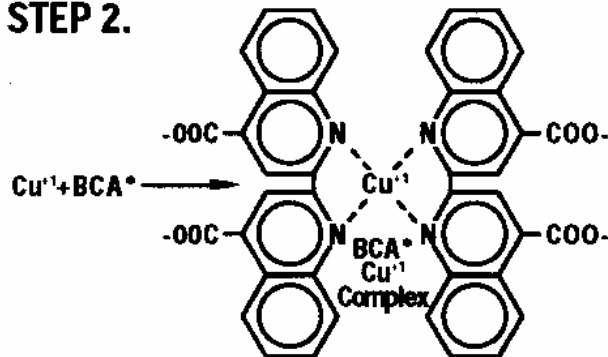**STEP 2.**

Cu^{+1}+BCA$^{\bullet}$ $\longrightarrow$

Figure 4: Proteins react with alkaline copper II to produce copper I. BCA then reacts with copper I to form an intense purple color at 562 nm.

Performing the assays

TO MAKE THE MOST EFFICIENT USE OF TIME, SET UP THE BCA ASSAY FIRST. WHILE THE ASSAY IS INCUBATING AT 60°C FOR AN HOUR, CARRY OUT THE BRADFORD ASSAY.

Protocol for Bradford Assay

The protein standard stock solution contains 1 mg bovine serum albumin (BSA)/ml of sterile, distilled water.

Obtain 8 ml of Bradford reagent from the lab instructor.

Place 5 small test tubes in the test tube rack and label them B, 10, 25, 50, and S.

Make the following additions to each tube as indicated.

	Blank	10 µg	25 µg	50 µg	Sample
BSA Protein Std. (µl)	0	10	25	50	0
Sample Protein (µl)	0	0	0	0	5
Distilled Water (µl)	50	40	25	0	45
Bradford Reagent (µl)	1,500	1,500	1,500	1,500	1,500

Mix the contents of each tube by vortexing.

Wait at least 5 min, and then read the absorbance at 595 nm of the standards and sample, beginning with the blank, using the spectrophotometer. Record each absorbance. Include these data in a table in your lab report. The absorbance should be read within one hour of mixing the standards and samples.

Use the computer in Dr. Ryal's laboratory to calculate the regression coefficient for your standard curve. Include the regression coefficient in your lab report. Use the same software to calculate the protein concentration of your sample. Include this in your lab report. Make any necessary corrections (dilution, etc) to determine the final concentration of protein in your sample **in the appropriate units (mg/ml)**. Include a plot of the standard curve data in your lab report.

Protocol for BCA Assay

Preparation of BCA Working Reagent

Prepare only enough working reagent for the assay. The reagent is stable only for a few hours once it is prepared.

Working reagent is prepared by adding 1 part Cu (II) solution to 50 parts bicinchoninic acid solution.

You will have 5 standards and 1 sample. Each will require 1.0 ml of working reagent for a total of 6.0 ml.

Add 150 µl of Cu (II) solution to 7.5 ml of BCA solution and mix well. The color should be green.

Preparation of Standards and Sample

The protein standard stock solution contains 0.1 mg bovine serum albumin (BSA)/ml of sterile, distilled-deionized water.

Place 6 small test tubes in a rack and label them B, 5, 10, 20, 40, and S.

Make the following additions to each tube as indicated.

	Blank	5 µg	10 µg	20 µg	40 µg	Sample
BSA Protein Std. (µl)	0	50	100	200	400	0
Sample Protein (µl)	0	0	0	0	0	5
Distilled water (µl)	1000	950	900	800	600	995
BCA Working Reagent (µl)	1000	1000	1000	1000	1000	1000

Quickly vortex each tube to mix the contents and place the tubes at 60 to 65°C for 1 h.

Remove the tubes and allow them to cool to room temperature.

Read the absorbance at 562 nm of the standards and sample, beginning with the blank, using the spectrophotometer. Record each absorbance. Include these data in a table in your lab report. The absorbance should be read within one hour of cooling the standards and samples.

Use the computer in Dr. Ryal's laboratory to calculate the regression coefficient for your standard curve. Include the regression coefficient in your lab report. Use the same software to calculate the protein concentration of your sample **in the appropriate units (mg/ml)**. Include this in your lab report. Include a plot of the standard curve data in your lab report.

PURIFICAITON OF PHOSPHOLIPASE D BY HYDROPHOBIC AFFINITY CHROMATOGRAPHY AND INVESTIGATION OF PLD HYDROLYTIC AND TRANSFERASE ACTIVITIES

Introduction

Phospholipase D (PLD) is one of four major types of phospholipases. Each of the major phospholipases is responsible for cleaving phospholipid molecules at specific locations.
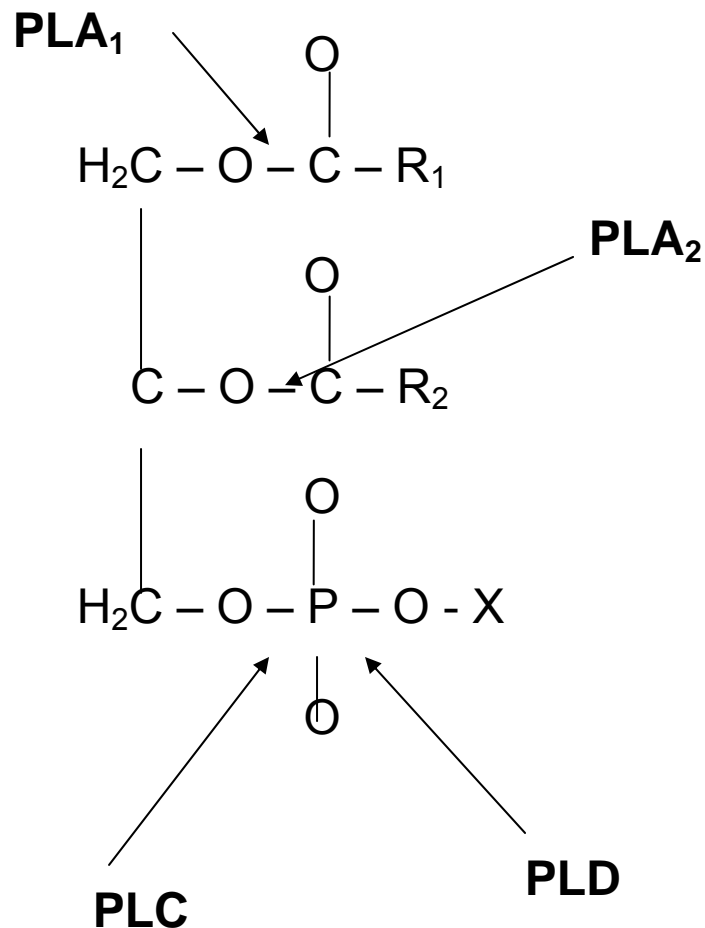
R1 = fatty acid at the sn-1 position
R2 = fatty acid at the sn-2 position
X = phospholipid headgroup (e.g. choline)

The hydrolytic activity of PLD is important in certain lipid-dependent signaling cascades.

In addition to the hydrolytic activity of PLD, the enzyme also possesses a phosphatidyl transferase activity. The phosphatidyl group of the phospholipid is transferred to an alcohol, such as glycerol or ethanol to produce a new phospholipid. Depending on the alcohol, the new phospholipid can be one not found in nature.

Until relatively recently phospholipase D was a difficult enzyme to purify. In the early 1990s, a method for purifying PLD in a single chromatographic step was developed (Biol. Chem. Hoppe-Seyler **373**, 81 (1992)). The method employs hydrophobic affinity chromatography using the hydrophobic resin octyl-Sepharose. The crude protein extract is applied to a column containing octyl-Sepharose. Phospholipase D binds to the matrix in the presence of calcium ions and is eluted from the column with buffer containing EDTA (ethylenediamine tetraacetic acid, a cation chelator).

PROTOCOL

I.   Preparation of Crude Cabbage Extract (this step will be done for you).

Obtain approximately 450 g of the inner leaves of Savoy cabbage and tear them into small pieces.

Add 150 g of leaf material to approximately 230 ml of distilled-deionized water contained in a blender.

Homogenize the leaf material using several pulses in the blender. Pour the homogenate into a large beaker.

Repeat the homogenization using another 150 g of leaf material and another 230 ml aliquot of water. Combine the homogenate with the first. Repeat the homogenization with the remaining leaf material and combine with the first two homogenates.

Using 4 layers of cheesecloth, filter the homogenate and collect the green liquid.

Centrifuge the liquid at 10,000 rpm for 10 min in the Thermo-IEC centrifuge.

Heat the supernatant to 55 °C and hold at that temperature for 3 minutes.

Cool the supernatant in an ice water bath.

Centrifuge the supernatant at 10,000 rpm for 25 min. Collect the supernatant from this centrifugation.

Determine the volume of the supernatant and add ½ volume of cold ethanol and mix.

Store the sample at -20°C overnight.

Centrifuge the sample at 10,000 rpm for 10 min and discard the supernatant.

Resuspend the pellet 3x, using 6 ml of water each time, and centrifuging after each resuspension. Pool the supernatants following each centrifugation.

The pooled supernatants represent the **crude extract**.

Store refrigerated until the chromatography step.

II.   Hydrophobic Chromatography

Obtain a small chromatography column containing 2 ml of octyl-Sepharose resin.

Open the plug at the bottom of the column and let the storage buffer drain. Wash the resin with 5 ml of **Buffer A**.

Add 2 ml of crude extract to the column and allow it to fully enter the resin.

Elute the column 4x with 3 ml of 30 mM PIPES buffer pH 6.2 containing 50 mM $CaCl_2$ **(Buffer A).**

Collect the run through in tubes on ice and keep on ice. Collect each 3 ml elution separately.

Elute the column 2x with 3 ml of 10 mM PIPES pH 6.2 containing 30 mM $CaCl_2$ **(Buffer B).**

Collect the run through in tubes on ice and keep on ice. Collect each 3 ml elution separately.

Elute the column 4x with 3 ml of 10 mM PIPES buffer pH 6.2 containing 0.1 mM EDTA **(Buffer C).**

Collect the run through in tubes on ice and keep on ice. Collect each 3 ml elution separately.

III.   Qualitative Assay for PLD Activity

In the wells of a porcelain spot plate, place the following:

50 µl of sample
300 µl of reaction mixture (see below)
Mix by gently pipeting up and down several times.

Incubate at room temperature for 10 to 15 min.

Hydrolysis of phosphatidylcholine lowers the pH due to ionization of the phosphate group. This causes a color change in the pH indicator from yellow to red.

Composition of reaction mixture:

0.5 mg/ml phosphatidylcholine
0.3 mM sodium dodecyl sulfate
0.1 mM methyl red
40 mM calcium chloride
Adjusted to pH 6.2

Pool all of the active fractions (except the crude extract) and store refrigerated.

IV.   Demonstration of Phosphatidyl Transferase Activity

In two separate microfuge tubes add:

100 µl of PLD (purified)
50 µl of 50% alcohol solution or water (as control)
750 µl of 2.5 mg/ml phosphatidylcholine in diethyl ether.
Mix for 30 min. (Vortex every 5 minutes)

Add 100 µl of 1 M HCl.
Invert to mix.

Briefly microfuge to separate the phases. The top phase is the ether phase, and the bottom phase contains the "lipids".

Spot 20 µl of each ether layer on a **silica gel TLC plate** along with PC standard and develop in a solvent of chloroform/methanol/acetic acid (65:25:10; v/v).

Place the plate in a TLC tank containing iodine crystals to visualize the separated phospholipids.

Note: The octyl-sepharose can be regenerated by washing in SDS-NaOH-EDTA solution, followed by extensive washing in water. It can then be stored refrigerated in 33% ethanol.

PLD is most stable in buffers lacking calcium.

GEL ELECTROPHORESIS OF PROTEIN AND MOLECULAR WEIGHT ESTIMATION

This lab will demonstrate the estimation of protein Molecular Weight (MW) by denaturing polyacrylamide electrophoresis. As the concentration of polyacrylamide that makes up the gel is increased, the pore size of the gel decreases. Smaller MW polypeptides migrate the greatest distance in the gel, and higher MW proteins migrate the least distance. When the gel has run to completion, been stained and destained (to visualize the polypeptides), a graph will be plotted to determine the MW of the "unknown" proteins present in the sample. The graph will be constructed (by hand) on semi-log graph paper. The migration distance (millimeters) of protein markers with known MW's will be plotted on the x-axis and their corresponding MW (converted to scientific notation, i.e., 1.65×10^5 Da = 165,000 Da) on the y-axis. A non-linear line will be hand-fit to the points, and migration distance of the "unknown" polypeptides will be used to estimate their respective MW. *See the example graph on page 26.*

I. Preparation of Crude Samples

Concentrate the protein by precipitating it with 5 volumes of -20°C Acetone O/N

Centrifuge small aliquots for 10min at 10,000 g

Discard supernatant, air dry the protein

Add 100 µl sample buffer to the dry pellet
Sample Buffer: Tris-HCl, pH = 8.0

SDS, glycerol (to increase density of samples)

10% Beta-mercaptoethanol (BME) (**reducing agent**)

Heat 100°C, 5 min (**solubilize the proteins, reduce disulfide bonds, allows SDS to bind to all proteins**)

10µl Tracking dye added to each sample
Bromophenol-Blue (BPB) – similar to methyl-red, a pH indicator; BPB is initially a red/rust color, but turns blue if pH is appropriate (~8.0); BPB's molecular weight is less than all peptides, thus the blue band runs with the ion front of the buffer and indicates ("**tracks**") when the gel is done running

II. Preparing a Denaturing or Reducing Gel

A 'denaturing' or 'reducing' gel separates proteins based only on their MW because all proteins carry a net negative charge from the SDS added. Commercial gels are available; however, if you find the need to augment your protocol by changing the polyacrylamide concentration, you have to buy more, expensive gels! Unpolymerized acrylamide in its monomeric form is a neurotoxin that is absorbed through the skin and can be airborne when dry; its effects are cumulative, so **WEAR GLOVES!!!!!**   **A denaturing gel has two different types of gels.** The **Separating/Resolving** gel is added first, found on the bottom of the gel, consists of 12.5% polyacrylamide (for our experiment) and separates proteins by MW. The **Stacking** gel is added after the Separating Gel has polymerized, is found on the top of the gel, consists of 4.0% polyacrylamide (for our experiment) and separates proteins by a gross association of "large" versus "small" proteins. Both the separating and stacking gels consist of the same components.

Components of Polyacrylamide Gels

1. Acrylamide
2. Bis-acrylamide
3. Tris Buffer
4. Water
5. SDS
6. Ammonium Persulfate (APS) – **catalyst** to drive polymerization; if it is wet, no polymerization occurs; if added to water, you should hear a snap, crackle, pop noise as this indicates the APS is GOOD!
7. TEMED (N-N-N'-N'-Tetramethylethylenediamine) – accelerant

III. Pouring and Running the Gel

Acquire 2 different-sized glass plates and 2 spacers (1.0 mm thick)

Pour polyacrylamide solution (Separating Gel) and take care that no bubbles are introduced

Add water-saturated butanol to top of separating gel, giving the gel a flat surface

Wait one hour for the separating gel to polymerize, now acrylamide is non-toxic!!

Pour stacking gel and add comb, wait for polymerization

Remove slips and bottom spacer

Add Running Buffer (Tris-Glycine with SDS, pH 8.8) to fill top and run over lip

Watch out for current killing bubbles, remove them with a 10cc syringe

Use thin, gel-loading tips to load samples with micropipettor

 Load Volume Series (10µl, 20µl, 30µl, 40µl)

Load the gel smartly, don't dawdle

Don't load two outermost lanes, which often become "stretched" and resolve poorly

80V until tracking dye <u>completely in stacking gel</u>, then turn up to 160V

Remove gel, rinse with water

Add Destain solution (with Coomassie Blue) – 30% MeOH, 20% Acetic Acid, 50% Water for 1 hour

Shake O/N in Destain (without Coomassie Blue)

Repeat Destain (without Coomassie Blue) until background is light enough, but bands are still dark enough for accurate interpretation of gel
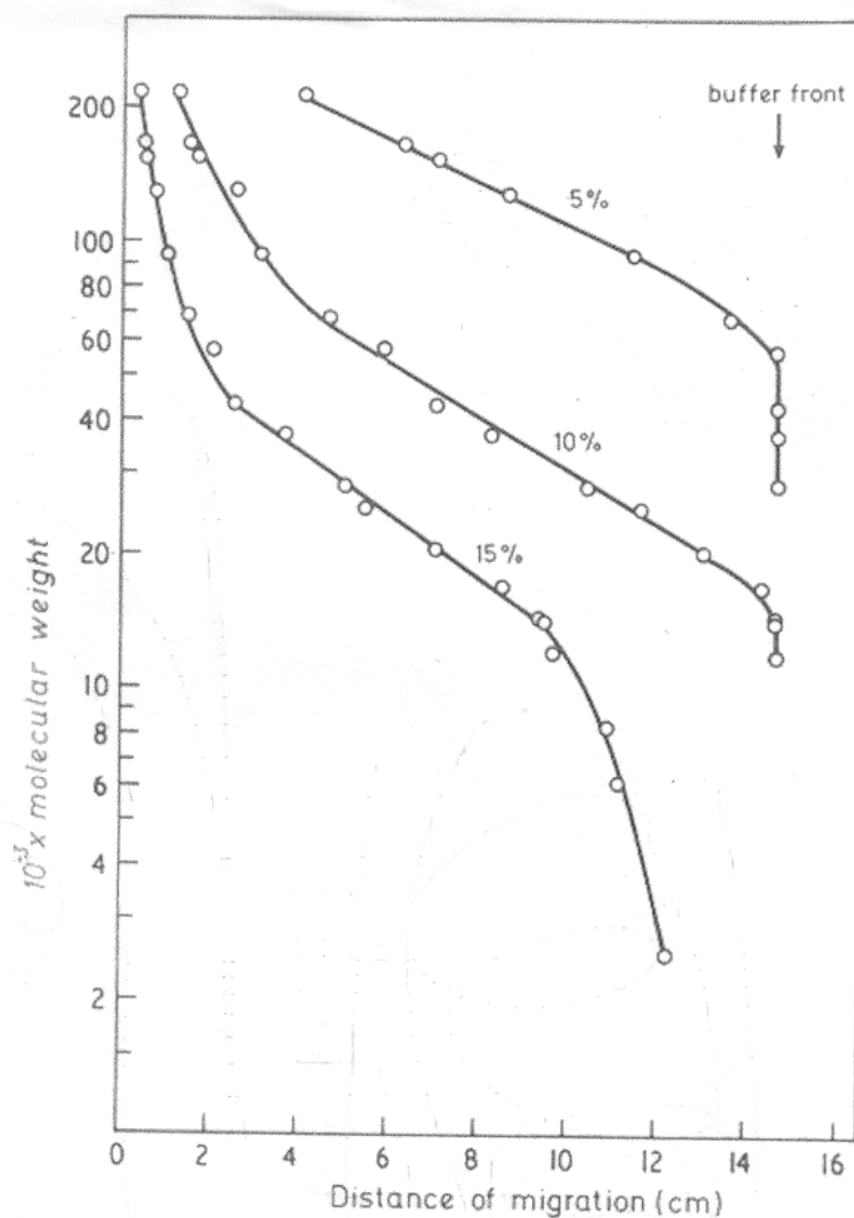
Figure 6. Calibration curves of $\log_{10}$ polypeptide molecular weight versus distance of migration during SDS-PAGE in slab gels using the SDS-discontinuous buffer system. The polyacrylamide gels used were uniform concentration 5%, 10%, or 15%. The polypeptide markers, in order of decreasing molecular weight, were myosin (mol. wt. 212,000), RNA polymerase β' (165,000) and β (155,000) subunits, β-galactosidase (130,000), phosphorylase a (92,500), bovine serum albumin (68,000), catalase (57,500), ovalbumin (43,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), chymotrypsinogen A (25,700), soybean trypsin inhibitor (20,100), horse heart myoglobin (16,950), horse heart myoglobin cyanogen bromide cleavage fragment I + II (14,404), lysozyme (14,300), cytochrome *c* (11,700), horse heart myoglobin cyanogen bromide cleavage fragments I (8,159), II (6,214), and III (2,512). The molecular weights of horse heart myoglobin and its cyanogen bromide cleavage fragments are calculated from the primary sequence given in ref. 129. References to the molecular weights of the other polypeptides are given in *Table 6*.
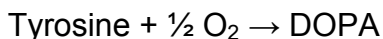
ENZYME KINETICS – PART ONE

# DETERMINING K_m AND V_{max} FOR TYROSINASE

Background

Tyrosinase is found in nearly all cells. It is present in many plants, fungi (in particular, mushrooms), and mammalian cells. The enzyme catalyzes reactions using a variety of substrates. It can oxidize a number of phenolic residues. In animal systems, it was named for its activity on the amino acid tyrosine. When tyrosine is the substrate, tyrosinase has the ability to form dopaquinone, an intermediate in the production of the pigment melanin. Dopaquinone spontaneously forms an orange-red pigment called dopachrome (the pigment found in the hair of red-heads). Dopachrome must undergo a final reaction to form the black/brown pigment melanin (which is found in virtually all human skin cells).

Tyrosinase catalyzes the first two reactions in the following sequence: the oxidation of tyrosine and the subsequent oxidation of DOPA.

Tyrosine + ½ O_2 → DOPA

2 DOPA + O_2 → 2 Dopaquinone + 2H_2O

Dopaquinone → Leukodopachrome

Leukodopachrome + Dopaquinone → Dopachrome + DOPA

Enzyme (tyrosinase) activity can be measured by monitoring the appearance of dopachrome pigment at a wavelength of 475 nm. The absorbance allows for the standard spectrophotometric analysis of tyrosinase activity by determining the rate of dopachrome formation from the substrate L-DOPA. Tyrosinase exhibits typical Michaelis-Menten kinetics.

The summary reaction for the laboratory experiments is:

L-DOPA + ½ O_2 → Dopachrome
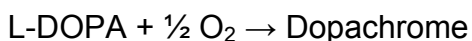
Tyrosinase catalyzes the oxidative decarboxylation of L-DOPA (sometimes used in the treatment of Parkinson's disease) to dopachrome.
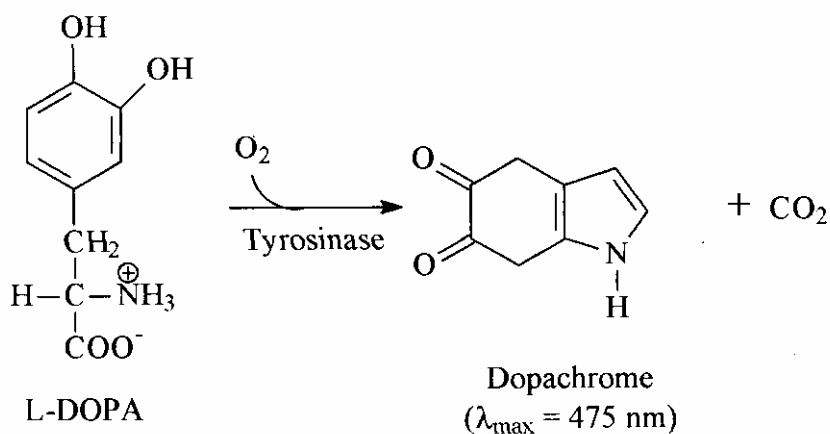
L-DOPA → Dopachrome ($\lambda_{max} = 475$ nm) + CO_2, catalyzed by Tyrosinase with O_2

EXPERIMENTAL

Materials

0.1 M sodium phosphate buffer, pH 7.0
Enzyme – tyrosinase (100 units/ml in buffer) – keep on ice AT ALL TIMES.
Substrate – 15 mM L-DOPA in buffer

Spectrophotometer
Countdown timer
Cuvettes
Ice bucket

I. Determining the optimum amount of enzyme for use in the standard assays.

It is necessary when determining enzyme kinetic parameters to establish what amount of enzyme will provide a convenient catalytic rate for the assays. The "convenient" rate is one that will be neither too fast nor too slow. By performing the experiment below, determine the amount of enzyme to use in subsequent experiments that will give a reaction rate of approximately 0.2 A_{475}/min.

Procedural Details

For each reaction, combine the appropriate volumes of **buffer and substrate** in a cuvette. Cover the cuvette with a small piece of Parafilm and mix by inversion. Zero the spectrophotometer with this solution. Remove the cuvette, add the appropriate volume of enzyme, mix by inversion, and return the cuvette to the spectrophotometer. **Record the absorbance every 30 sec for 3 min.**

	Volume in ml				
Buffer	1.0	0.8	0.6	0.4	0.2
Substrate	0.5	0.5	0.5	0.5	0.5
Enzyme	0.0	0.2	0.4	0.6	0.8
Final Rxn Vol.	1.5	1.5	1.5	1.5	1.5

Plot rate (A_{475}/min) versus volume of enzyme solution. Absorbance should ALWAYS be plotted on the y-axis.

II. Determination of K_m and V_{max}

To determine K_m and V_{max} for the tyrosinase reaction, perform the following experiment using a constant amount of enzyme with varying amounts of substrate. The concentrations that result from the dilutions given in the table below are 0.25, 0.5, 1.0, 2.5, and 5 mM. The final reaction volume is 1.5 ml in every case. Follow the same guidelines as given above for obtaining and recording the absorbencies.

	Volume in ml				
Buffer	____	____	____	____	____
Substrate	0.025	0.05	0.1	0.25	0.5
Enzyme	____	____	____	____	____ (amt you determined experimentally).

1. *Plot A_{475} versus time for each run.*
2. *Convert the absorbance-based rates (A_{475}/min) to standard concentration-based rates (µmoles/min) using the Lambert-Beer Law and the volume of the reaction mixture. The molar extinction coefficient of dopachrome is 3600 M^{-1} cm^{-1}.*

Additional information.

The concentration units used are millimolar (mM).
A 1.0 mM solution contains 1 mmole per liter = 1000 µmoles per liter = 1 µmole per ml.

The Lambert-Beer Law is A = ecl, where A = absorbance, e = molar extinction coefficient, c = concentration, l = light path (1 cm).

3. *Prepare a Lineweaver-Burke plot (1/Vo versus 1/[S]) and determine the K_m and V_{max} for the tyrosinase reaction from this plot.*

ENZYME KINETICS – PART TWO
INHIBITION OF TYROSINASE

EXPERIMENTAL

Materials

0.1 M sodium phosphate buffer, pH 7.0
Enzyme – tyrosinase (100 units/ml in buffer) – keep on ice AT ALL TIMES
Substrate – 15 mM L-DOPA in buffer
Inhibitor I – 10 mM sodium azide in buffer – **wear gloves, sodium azide is hazardous!!**
Inhibitor II – 5 mM sodium benzoate in buffer
Inhibitor III – 5 mM cinnaminic acid in buffer

Spectrophotometer
Countdown timer
Cuvettes
Ice bucket
Microliter pipettes

Methods

Reaction I – Perform an assay WITHOUT inhibitor 0.25, 0.5, 1.0, 2.5, and 5 mM substrate and the optimal amount of enzyme determined in the previous lab. Take absorbance readings every 30 sec over a 3 min reaction time. (**you just did this in Part One!!**)

Reaction II – Perform the assay described in Reaction I above, but include 0.1 mM of ONE of the inhibitors.

Reaction III – Perform the assay described in Reaction II above, but include 0.2 mM of the SAME inhibitor.

1. *Obtain data for the other two inhibitors from lab groups who used a different inhibitor.*

2. *Plot the data with and without inhibitor using a Lineweaver-Burke Plot (1/Vo vs 1/[S]. Determine K_m, V_{max}, $K_{m(app)}$, and $V_{max(app)}$.*

3. *Determine if the inhibition is competitive or noncompetitive for each inhibitor by inspection of the plot and calculate K_i for each inhibitor.*

The uninhibited assay will provide values for K_m and V_{max}. The inhibited assays will provide **apparent** values for K_m and V_{max} ($K_{m(app)}$ and $V_{max(app)}$). K_i is calculated for competitive and noncompetitive inhibition by the following equations:

Competitive: $K_i = K_m \times [I] / Km_{(app)} - K_m$
Noncompetitive: $K_i = V_{max(app)} \times [I] / V_{max}$

Example Calculation

NADPH was found to competitively inhibit "examplase", an enzyme that normally uses NADP$^+$ as substrate.

The following results were obtained:

Conc. of Inhibitor (NADPH)	K_m	$K_{m(app)}$
0.00 mM NADPH	0.0197	-------
0.06 mM NADPH	--------	0.0392
0.12 mM NADPH	--------	0.0529

$K_i = K_m$ x [I] / $Km_{(app)}$ - K_m

For 0.06 mM NADPH:

K_i = (0.0197) x (0.06) / (0.0392) – (0.0197)

K_i = 0.00118 / 0.0195

K_i = 0.0605 mM

A similar calculation is done for 0.12 mM inhibitor and **the mean K_i is calculated**.

EXTRACTION AND CHROMATOGRAPHIS ANALYSIS OF

Tetrahymena thermophila LIPIDS

Various lipids play important roles in a variety of cellular functions. Phospholipids are a major structural component of cell membranes and form semi-permeable barriers that allow only certain molecules and ions to pass in and out of the cell and membrane-bound organelles. Other lipids serve as storage molecules/energy reserves. Still other lipids function in transmembrane signaling cascades, act as hormones, vitamins, and cofactors.

Given the diversity of lipid structure and lipid function, it is often necessary to extract the total compliment of lipids from cells or tissues for purification and further study. The following lipid extraction protocol is widely used to obtain a sample of total lipids present in cells or tissues.

LIPID EXTRACTION

1. Add 10 ml chloroform and 20 ml methanol to a 125 ml separatory funnel and mix by inversion. Cap with ground glass stopper.

2. Rapidly chill a cell culture to $5^{o}C$ using the Cryo-Cool.

3. Sediment cells by centrifuging in the cold at approximately 2000 x g for 5 min.

4. Decant the supernatant, retain the pellet.

5. Add 9 ml of cold dd-water to the pellet and resuspend the cells by inversion.

6. Pipet the cell suspension into a 125 ml separatory funnel that contains 10 ml chloroform and 20 ml methanol (the one you prepared in step 1).

7. Mix by inversion and vent the funnel to release trapped gases.

8. Wait 10 min, and then add 10 ml chloroform. Mix. Vent.

9. Wait 10 min, and then add 10 ml dd-water. Mix. Vent.

10. Allow phases to separate fully so that both the upper aqueous phase and the lower chloroform phase are clear. This takes several hours to overnight.

11. Remove the ground glass stopper and carefully drain the lower phase containing lipids into a 50 ml round bottom flask. Remove as much of the lower phase as possible without allowing any of the precipitated material at the interface to enter the round bottom flask.

12.   Add 2 to 3 drops of ethanol to disperse any water present. Rotary evaporate the sample to near dryness at 40°C.

13.   Add a Pasteur pipet full of chloroform/methanol (6:1, v/v) to the round bottom flask. Swirl a few times to solubilize any lipid stuck to the wall of the flask. Transfer the solution to a small, glass screw-cap tube. Repeat and add the second wash to the first.

14.   Dry the sample completely under a gentle stream of N_2.

15.   Add 200µl chloroform/methanol (6:1, v/v) to the dry sample. Flush tube with a little N_2, immediately cap tightly and store at –20 ^{o}C for no longer than 1 month.

THIN LAYER CHROMATOGRAPHY OF LIPIDS

1. Obtain a silica gel coated thin layer plate.

2. Spot 5, 10, 20, and 40 μl of the total lipid extract in separate lanes. Take care to keep the spot size as small as possible. Cap the sample tube between applications to prevent excessive evaporation of the solvent.

3. Allow the sample spots to dry completely, then place the TLC plate in the appropriate TLC tank containing the mobile phase.

4. Allow the solvent front to migrate to approximately 1 inch of the top of the plate.

5. Remove the plate and allow it to dry completely. If the mobile phase is not allowed to completely evaporate before detection in iodine, the background will be too dark!

6. Place the plate in a TLC tank that contains iodine crystals. Iodine will bind to organic molecules, especially unsaturated ones. Yellow-brown spots will begin to appear.

7. Remove the plate, circle the spots using a pencil, and place the plate silica-gel side DOWN on a piece of plastic wrap. Fold the edges of the plastic wrap over the back of the plate. This will impede the dissipation of the iodine so that the spots will remain visible for a longer period.

8. Scan the plate for a permanent record.

9. Identify the separated lipids and calculate Rf values.

Shown at the right is a typical separation pattern resulting when *Tetrahymena* phospholipids are resolved in the solvent system used in the laboratory exercise. The NEUTRAL LIPIDS migrate WITH THE SOLVENT FRONT (not shown in the Figure). SL = sphingolipid; AEPL = aminoethylphosphonolipid; di-PG = cardiolipin.

You may see TWO small spots in the SL region of your plate. Both are sphingolipids. The Figure shown at the right was obtained by chromatographing *Tetrahymena* lipids that had been radiolabeled with ^{32}P. Following TLC, the plate was placed against X-ray film to localize the radioactivity rather than staining the lipids with iodine.
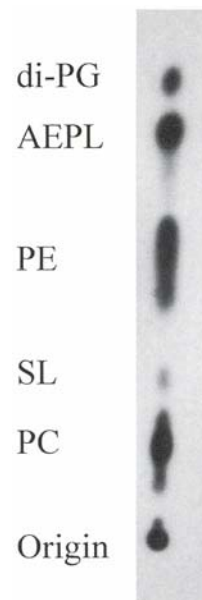
SOLVENT SYSTEMS FOR THIN LAYER CHROMATOGRAPHY

Neutral Lipid Solvent Systems

These solvent systems separate the neutral lipids (free fatty acids, triacylglycerols, glyceryl ethers, diacylglyerols, tetrahymenol, sterols, fatty alcohols, fatty aldehydes, etc) leaving the phospholipids at the origin of the plate.

Hexane/diethyl ether/acetic acid (70:30:1, v/v)

Or

Petroleum ether/diethyl ether/acetic acid (70:30:1, v/v)

Mix the solvents in an Erlenmeyer flask and pour into a filter paper lined TLC tank. Place the tank lid on the tank and a weight (a reagent bottle for example) on top of the lid. ***Allow the tank to equilibrate for 1 h before use.*** The solvent system remains useable for 2 to 3 days.

To prevent oxidation of fatty acids add 0.005 % butylated hydroxytoluene to the solvent mixture.

Polar Lipid Solvent Systems

These solvent systems separate the phospholipids (PA, PE, PI, PS, PC, diPG, AEPL) from each other with the total neutral lipids migrating with the solvent front.

Chloroform/acetic acid/methanol/dd-water (75:25:5:2.2, v/v)

Or

Chloroform/acetic acid/methanol/dd-water (65:30:5:2, v/v)

Mix the solvents in an Erlenmeyer flask and pour into a filter paper lined TLC tank. Cover as for neutral lipid system. ***Allow the tank to equilibrate overnight before use.*** The solvent system remains useable for 1 week.