

Enzyme Characterization:  
Analysis Of Red Potato Extract For Monophenol Monooxygenase Activity

**References:**

Boyer, R.F. *J. Chem. Educ.*, **1977**, *54(9)*, pp 585-586.

Friedman, M.E.; Daron, H.H. *J. Chem. Educ.*, **1977**, *54(4)*, pp 256-257.

This project involves analyzing a simple extract from red potatoes for activity of the enzyme tyrosinase (monophenol monooxygenase EC 1.14.18.1). Tyrosinases present in both animal and plant cells catalyze the hydroxylation of various monophenols, and the aerobic oxidation of diphenols leading to the production of melanin pigments. The **specific activity** of the extract and determination of kinetic **parameters** based on the simple **Michaelis-Menten** enzyme model will be determined. The reaction, which will be studied spectrophotometrically, is the conversion of the substrate 3,4-dihydroxyphenylalanine (DOPA) to the product dopachrome which is an orange colored o-quinone. **Enzyme units** are obtained by recording the increase in absorbance of a buffered solution of DOPA and potato extract at 475 nm for three minutes. The initial rate of reaction in  $\mu\text{moles dopachrome formed per minute}$  is equivalent to the slope of the linear portion of the absorbance vs. time plot early in the reaction (times less than 3 minutes). Multiplying this slope by the total solution volume (0.00300 L), and divided by the molar absorptivity of dopachrome in a 1 cm cuvet at 475 nm,  $3.60 \times 10^{-3} \text{ M}^{-1}$ , gives enzyme units in  $\mu\text{moles dopachrome formed per minute}$ .

**Solutions:**

- 1) Prepare 250 mL of a 0.1 M sodium phosphate buffer, pH 6.8.  
(pKa = 6.82) Use handbook instructions given on last page.
- 2) Prepare 15.0 mL of a 20.0 mM DOPA(197.19 g/mol) solution in buffer 1.
- 3) Bradford reagent.

**Laboratory:**

A) Preparation of Potato Extract

- 1) Peel and chop a potato and put about 50 g of potato and 50 mL of buffer 1 in a blender cup. Blend for about 1 minute.
- 2) Strain the mixture through gauze and fill two 15 mL centrifuge tubes.
- 3) Centrifuge using the benchtop centrifuge for 10 minutes.
- 4) Transfer supernatant with Pasteur pipet to a **clean** test tube and **keep on ice** until ready to use.
- 5) Before reaction, dilute extract 1:5 with buffer 1.

B) Total Protein Determination by Bradford Method

Prepare a blank and two tubes from **undiluted** extract as below:

Tube	Bradford Rgnt.	Extract	Water
1	4.00 mL	0.00 mL	0.08 mL
2	4.00	0.02	0.06
3	4.00	0.02	0.06

Mix well and read the absorbance of each solution in a cuvet after 15 minutes at 595 nm. Calculate the average protein concentration of the extract using the slope and intercept from the bovine serum albumin standard Bradford assay.

C) Determination of Specific Activity

Run the following reactions in a glass cuvet in a spectrophotometer set at 475 nm.

For each experiment:

- 1) Pipet the buffer into the **clean** cuvet.
- 2) Pipet the DOPA solution into a **clean** test tube.
- 3) When ready to begin, pipet the diluted extract into the cuvet and mix well.
- 4) Rapidly **mix the two solutions and begin timing** the reaction.
- 5) Put the cuvet in the spectrophotometer. Record the absorbance of the solution every 20 seconds for 3 minutes.

Tube	Buffer	Diluted Extract	DOPA(20 mM)
1	1.90 mL	0.100 mL	1.00 mL
2	1.80	0.200	1.00
3	1.70	0.300	1.00
4	1.60	0.400	1.00

D) Determination of the Michaelis-Menten Constants

Run the following reactions in a glass cuvet in a spectrophotometer set at 475 nm.

For each experiment:

- 1) Pipet the buffer into the **clean** cuvet.
- 2) Pipet the DOPA solution into a **clean** test tube.
- 3) When ready to begin, pipet the diluted extract into the cuvet and mix well.
- 4) Rapidly **mix the two solutions and begin timing** the reaction.
- 5) Put the cuvet in the spectrophotometer. Record the absorbance of the solution every 20 seconds for 3 minutes.

Tube	Buffer	Diluted Extract	DOPA(20 mM)
1	2.40 mL	0.200 mL	0.400 mL
2	2.00	0.200	0.800
3	1.60	0.200	1.20
4	1.20	0.200	1.60

## Report:

- 1) One complete report will be required per team that follows the **directions for team lab reports** in the handout distributed. The objective of this project is to determine the specific activity and Michaelis-Menten constants for tyrosinase in the simple extract. The steps to calculate these are described below.
- 2) For each experiment in C and D above, plot absorbance(475 nm) vs. time(minutes) on Excel, and determine the slope of the linear portion of the graph (omit points if graph starts to curve). Dividing each slope by  $3.60 \times 10^{-3} \text{ M}^{-1}$  and then multiplying by  $0.00300 \text{ L}$  will yield the initial velocity of each reaction in  $\mu\text{moles dopachrome formed per minute}$ . This quantity is defined as the number of enzyme activity units.
- 3) From the total protein concentration determined by the Bradford Method, taking into account dilutions, and the enzyme activity units determined for the experiments in part C, calculate the **Specific Activity** of the potato extract defined as  $\mu\text{moles dopachrome formed per minute per mg of total protein}$ . Report the average specific activity obtained for the four experiments with estimated uncertainty.
- 4) For each experiment in part D, determine the actual substrate concentrations in the reaction mixtures from dilutions. Each experiment will then have a substrate concentration (i.e. [DOPA]) and an initial velocity. Plot the data as Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf plots to determine  $K_M$  and  $V_{\max}$  for the tyrosinase catalyzed reaction.
- 5) Report a single value for  $K_M$  and for  $V_{\max}$ . Estimate an uncertainty for each value based on the different plots.

## PREPARATION OF BUFFERS FOR USE IN ENZYME STUDIES\*

G. Gomori

The buffers described in this section are suitable for use either in enzymatic or histochemical studies. The accuracy of the tables is within  $\pm 0.05$  pH at 23°. In most cases the pH values will not be off by more than  $\pm 0.12$  pH even at 37° and at molarities slightly different from those given (usually 0.05 M).

The methods of preparation described are not necessarily identical with those of the original authors. The titration curves of the majority of the buffers recommended have been redetermined by the writer. The buffers are arranged in the order of ascending pH range. For more complete data on phosphate and acetate buffers over a wide range of concentrations, see Vol. I [10].\*

\*From Gomori, in *Methods in Enzymology*, Vol. 1, Colowick and Kaplan, Eds., Academic Press, New York, 1955, 138.

Table 12  
PHOSPHATE BUFFER\*

x mL of A	y mL of B	pH	x mL of A	y mL of B	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	90.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

\*Stock solutions

A: 0.2 M solution of monobasic sodium phosphate (27.60 g of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  in 1,000 ml)

B: 0.2 M solution of dibasic sodium phosphate (53.61 g of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  in 1,000 ml)

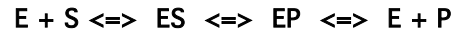
**Recipe** for 200 mL of 0.1 M buffer at desired pH:

**x ml of solution A + y ml of solution B, diluted to a total of 200 ml.**

# Kinetics of Enzymes Following the Michaelis-Menten Model

Determination of Parameters  $K_m$  and  $v_{max}$

Michaelis-Menten Mechanism



Michaelis-Menten Equation

$$v_0/v_{max} = \frac{[S]_0}{(K_M + [S]_0)}$$

Lineweaver-Burk Equation

$$1/v_0 = (K_M/v_{max})(1/[S]_0) + 1/v_{max}$$

Eadie-Hofstee Equation

$$v_0 = -K_M(v_0/[S]_0) + v_{max}$$

Hanes-Woolf Equation

$$[S]_0/v_0 = (1/v_{max})([S]_0) + K_M/v_{max}$$

Table 1. Data from Michaelis-Menten Kinetic Experiments on Enzyme

$[S]_0$ (mM)	$v_0$ ( $\mu$ moles/min)	$1/[S]_0$	$1/v_0$	$v_0/[S]_0$	$[S]_0/v_0$
0.00	0.000				
2.70	0.123	0.370	8.13	0.0456	22.0
5.40	0.179	0.185	5.59	0.0331	30.2
8.09	0.210	0.124	4.76	0.0260	38.5
10.80	0.226	0.093	4.42	0.0209	47.8
36.00	0.280				
100.00	0.300				

