Potato Polyphenol Oxidase (PPO) Assay and Data Analysis

Introduction

Potato polyphenol oxidase (PPO) is an enzyme that is activated upon injury to the potato, e.g., sliced with a knife, cut with a spade, poked with a pitch-fork **[1]**. It is this enzyme that causes the potato to turn brown where it shows damage **[2,3]**. The same enzyme (polyphenol oxidase -- PO) is also found in apples and bananas and grapes **[4]**.

PPO catalyzes the following reaction (note that **Figure 1** shows the benzoquinone as the product of catechol oxidase catalysis and that **Figure 2** illustrates the o-quinone as the product of polyphenoloxidase catalysis – same things, different names):

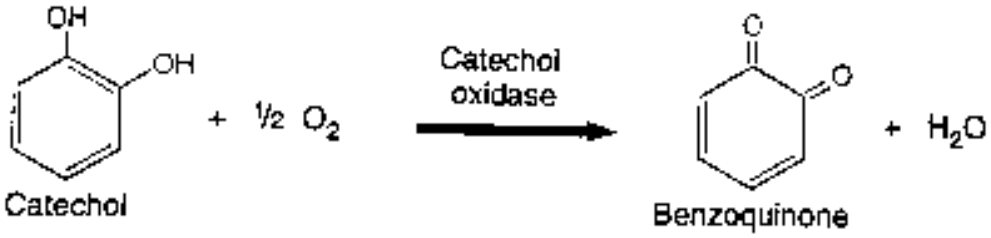

Figure 1. Catechol oxidase (PPO) catalyzes the oxidation of catechol to benzoquinone. The oxidation product is used to synthesize melanins.

Figure 2. Polyphenoloxidase (PPO) catalyzes the oxidation of catechol to o-quinone. The oxidation product is used to synthesize melanins.

The benzoquinone/o-quinone product is then utilized to synthesize melanins – pigments that cause the damaged tissues to turn reddish-brown. It is these pigments that will be measured in the laboratory to determine the K_M and V_{max} of this enzyme. In addition, the effects of three proposed inhibitors of PPO will be studied, as well: Tyrosine, *trans*-Cinnamic Acid and Phenylthiourea (PTU).

Classic Michaelis-Menten enzyme kinetics have been discussed in lecture and will not be discussed as part of this lab write-up. The applications of Lineweaver-Burke analysis discussed in lecture will be applied and discussed in the "Results" and "Discussion" sections.

Materials

The materials required for this experiment are tabulated, below:

Blender (Food Processor)	Distilled water	A potato
24 -- Disposable test tubes	Test tube rack	Pencil
Spectrophotometer at 480 nm	24 -- Disposable cuvets	Parafilm
Disposable pipets	Scissors	Waste container
Clock and Parafilm™	Ice bath	Catechol (0.03M)
Tyrosine (0.01M)	*trans*-Cinnamic Acid (0.01M)	Phenylthiourea (0.01M)
Glass stirring rods	Beakers (for dissolving substrate and inhibitors)	Pan balances
Buchner funnel	Filter paper	Filter tubing
Large disposable pipets	Pipet bulb	Lab cart

Methods

The method for this experiment is a conglomeration of several methods, optimized for this experiment **[5-7]**.

Set up 4 rows of test tubes – 6 test tubes per row (refer to **Table 1**, below). Label the tubes in Row 1 as 1 through 6. Label the tubes in Row 2 as 1-tyr through 6-tyr. Label the tubes in Row 3 as 1-t-CA through 6-t-CA. Label the tubes in Row 4 as 1-PTU through 6-PTU.

Into each test tube in each row, pipet 3 mL distilled water. Into the first tube (#1) of each row add 5 drops of distilled water. Into the second tube (#2) of each row add 4 drops of distilled water. Into the third tube (#3) of each row add 3 drops of distilled water. Into the fourth tube (#4) of each row add 2 drops of distilled water. Into the fifth tube (#5) of each row add 1 drop of distilled water. Into the sixth tube (#6) of each row add no additional water.

Now, into the first tube (#1) of each row add no (0) drops of catechol. Into the second tube (#2) of each row add 1 drop of catechol. Into the third tube (#3) of each row add 2 drops of catechol. Into the fourth tube (#4) of each row add 3 drops of catechol. Into the fifth tube (#5) of each row add 4 drops of catechol. Into the sixth tube (#6) of each row add 5 drops of catechol.

Table 1, below, summarizes the above text:

Tube #	**1**	**2**	**3**	**4**	**5**	**6**
mL Distilled Water	3	3	3	3	3	3
gtts Distilled Water	5	4	3	2	1	0
gtts catechol	0	1	2	3	4	5
Tube #	**1-tyr**	**2-tyr**	**3-tyr**	**4-tyr**	**5-tyr**	**6-tyr**
mL Distilled Water	3	3	3	3	3	3
gtts Distilled Water	5	4	3	2	1	0
gtts catechol	0	1	2	3	4	5
Tube #	**1-t-CA**	**2-t-CA**	**3-t-CA**	**4-t-CA**	**5-t-CA**	**6-t-CA**
mL Distilled Water	3	3	3	3	3	3
gtts Distilled Water	5	4	3	2	1	0
gtts catechol	0	1	2	3	4	5
Tube #	**1-PTU**	**2-PTU**	**3-PTU**	**4-PTU**	**5-PTU**	**6-PTU**
mL Distilled Water	3	3	3	3	3	3
gtts Distilled Water	5	4	3	2	1	0
gtts catechol	0	1	2	3	4	5

Table 1. Tabular summary of test tube organization for the experiment from initial study through the three potential inhibitors.

By row, each #1 tube is now 0M catechol; each #2 tube is 0.006M catechol; each #3 tube is 0.012M catechol; each #4 tube is 0.018M catechol; each #5 tube is 0.024M catechol; each #6 tube is 0.03M catechol – this is the concentration of substrate ([S]) for your kinetic studies.

Next, into each of the "#-tyr" tubes, add 5 gtts of 0.01M tyrosine. Into each of the "#-t-CA" tubes, add 5 gtts of 0.01M *trans*-cinnamic acid. Into each of the "#-PTU" tubes, add 5 gtts of 0.01M PTU. Each row of tubes now contains its potential PPO inhibitor.

NOTE: PTU and catechol are poisonous and are to be handled with care. Wash your hands after using these chemicals and do not mouth pipet them. In addition, with the exception of catechol, these chemicals don't "like" to dissolve in water. Ask your instructor for assistance with this.

Chop the potato into small cubes and place in the blender. Add 300 mL distilled water and pulse the blender until the potato is blended in the water. Set up the Buchner funnel with filter paper and attach to the aspirator on the sink. Turn the water on as high as it will go and filter the potato extract. You want to save the filtrate (the liquid in the filter flask) for your assay. The potato pulp and filter paper may be discarded in the trash.

The PPO extract and all tubes are now ready to be taken to the spectrophotometer for the assay.

Make sure the visible light source is on and that the spectrophotometer is set on absorbance. Set the spectrophotometer at 480 nm. Place a cuvet with water in it in the cuvet holder. Close the lid. Press the "Calb" button and wait until the LED reads "0.000".

Use the following protocol for Tube #1 for every tube – one at a time.

Using a disposable pipet, place 8 drops of your PPO extract in the tube. Fairly quickly, cover the tube with a piece of Parafilm ™ and invert to mix without shaking (shaking allows air to mix with the protein and denature it). Pour enough out of the test tube into a cuvet to fill the cuvet about two-thirds full. Place in the spectrophotometer and read as soon as the LED stabilizes the time zero value of the absorbance at 480 nm. Time the sample for 30 more seconds and read the Absorbance at 480 nm. Record both readings in the following data table. Remove the sample, place in an appropriate container, and repeat with each tube until done.

Your instructor/professor will assist you in disposal of reagents and mixed cuvets and test tubes.

Data Collection Table

				5 gtts 0.01M inhibitor		5 gtts 0.01M inhibitor		5 gtts 0.01M inhibitor	
		WITHOUT Inhibitor		WITH Tyr		WITH t-Cinnamic Acid		WITH PTU	
		A 480 readings		A 480 readings		A 480 readings		A 480 readings	
	[Catechol] M	30 sec	0 sec	30 sec	0 sec	30 sec	0 sec	30 sec	0 sec
Tube 1	0.000								
Tube 2	0.006								
Tube 3	0.012								
Tube 4	0.018								
Tube 5	0.024								
Tube 6	0.030								

Results and Data Manipulation

The following table summarizes the data collected from the four different samples:

				5 gtts 0.01M inhibitor		5 gtts 0.01M inhibitor		5 gtts 0.01M inhibitor	
		WITHOUT Inhibitor		WITH Tyr		WITH t-Cinnamic Acid		WITH PTU	
		A_{480} readings		A_{480} readings		A_{480} readings		A_{480} readings	
	[Catechol] M	30 sec	0 sec	30 sec	0 sec	30 sec	0 sec	30 sec	0 sec
Tube 1	0.000	0.271	0.271	0.123	0.123	0.218	0.216	0.234	0.225
Tube 2	0.006	0.322	0.264	0.160	0.135	0.317	0.281	0.234	0.232
Tube 3	0.012	0.391	0.303	0.171	0.136	0.291	0.251	0.249	0.242
Tube 4	0.018	0.423	0.313	0.189	0.142	0.365	0.304	0.248	0.236
Tube 5	0.024	0.394	0.289	0.196	0.149	0.404	0.328	0.260	0.244
Tube 6	0.030	0.411	0.293	0.201	0.142	0.338	0.272	0.251	0.238

The following table summarizes velocity determinations along with reciprocal substrate concentrations and reciprocal velocity information:

	1/[Catechol] (M^{-1})	V	1/V	V Tyr	1/V Tyr	V t-CA	1/V t-CA	V PTU	1/V PTU
Tube 1		0.000		0.000		0.004	250.000	0.018	55.556
Tube 2	166.667	0.116	8.621	0.050	20.000	0.072	13.889	0.004	250.000
Tube 3	83.333	0.176	5.682	0.070	14.286	0.080	12.500	0.014	71.429
Tube 4	55.556	0.220	4.545	0.094	10.638	0.122	8.197	0.024	41.667
Tube 5	41.667	0.210	4.762	0.094	10.638	0.152	6.579	0.032	31.250
Tube 6	33.333	0.236	4.237	0.118	8.475	0.132	7.576	0.026	38.462

Remember that the calculation of the velocity of the reaction is as follows:

$$V = \frac{A_{480}^{30} - A_{480}^{0}}{0.5 \text{ min}} = \frac{Change\ in\ absorbance}{\text{min}}$$

Lineweaver-Burke Analysis of PPO without any inhibitor is shown, below, **Figure 3**:

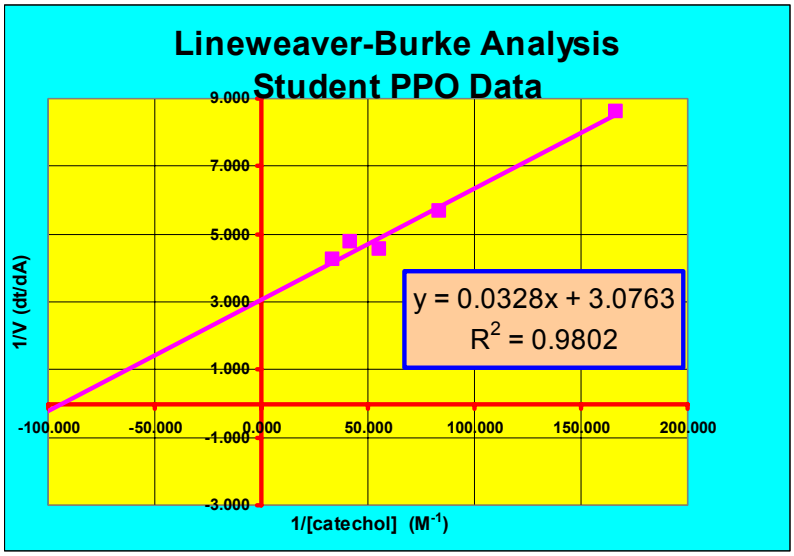

Figure 3. Lineweaver-Burke analysis of PPO. No inhibitor is present. V_{max} is 0.325 absorbance units per minute. K_M is 0.0107 M. R^2 indicates that the data follows the equation for a straight line within Excel's parameters of linear regression.

Lineweaver-Burke analysis of PPO in the presence of 0.01M tyrosine (tyr) is shown in **Figure 4**:

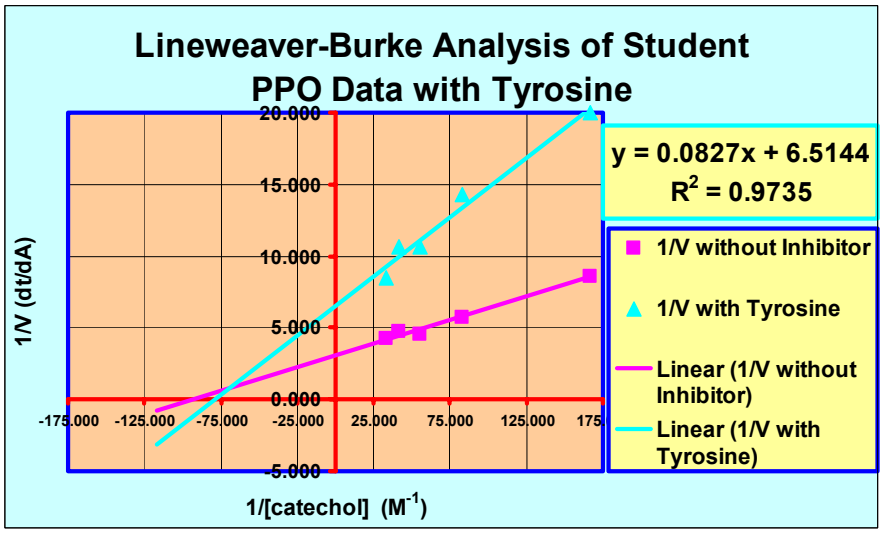

Figure 4. Lineweaver-Burke analysis of PPO in the presence of 0.01M tyrosine. Note that V_{max} has decreased and that, for all intents and purposes, K_M has not been impacted. R^2, again, indicates that the data follows the equation for a straight line within Excel's parameters of linear regression.

Lineweaver-Burke analysis of PPO in the presence of 0.01M trans-cinnamic acid (t-CA) is shown in **Figure 5**:

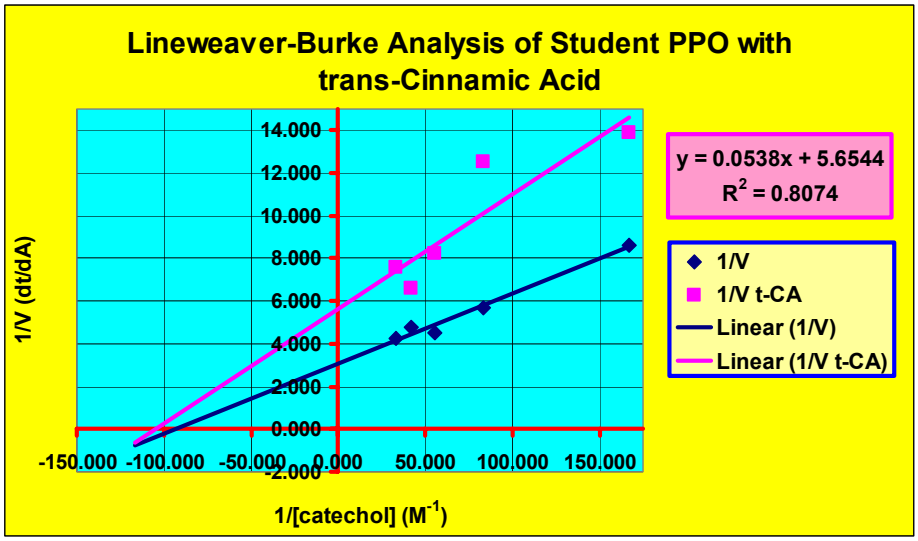

Figure 5. Lineweaver-Burke analysis of PPO in the presence of 0.01M trans-cinnamic acid. Note that V_{max} has decreased and that, for all intents and purposes, K_M has not been impacted. R^2, however, suggests that the data does not quite follow the equation for a straight line within Excel's parameters of linear regression.

Lineweaver-Burke analysis of PPO in the presence of 0.01M phenylthiourea (PTU) is shown in **Figure 6**:

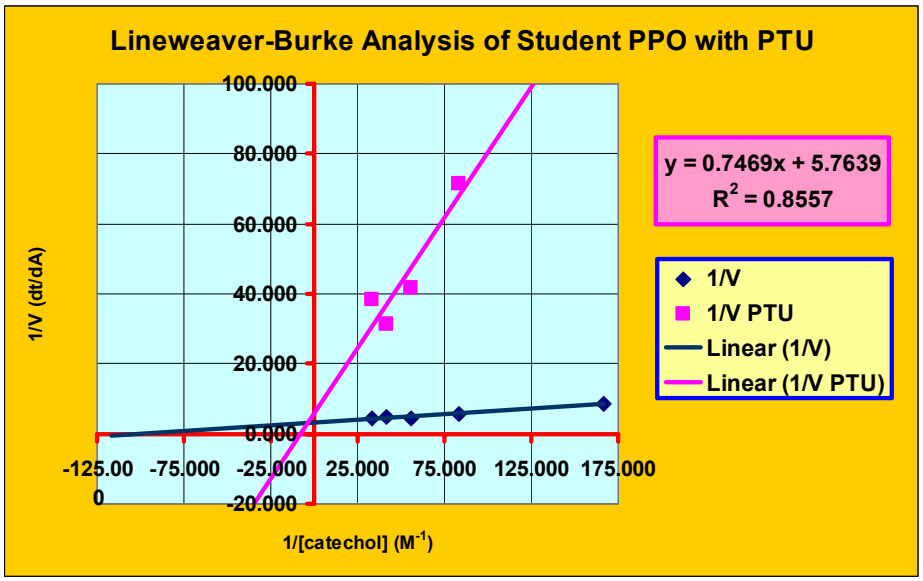

Figure 6. Lineweaver-Burke analysis of PPO in the presence of 0.01M phenylthiourea (PTU). Note that V_{max} , for all intents and purposes, has not been impacted, but that K_M has been impacted. R^2, however, suggests that the data does not quite follow the equation for a straight line within Excel's parameters of linear regression.

Discussion

Potato polyphenol oxidase was chosen for this experiment because of the ease of acquisition of potatoes. The enzyme is also easily studied in the lab (with appropriate safety precautions) without requiring any expensive equipment or sophisticated solutions, e.g., buffers set at specific pH's. PPO can be easily prepared simply in water and stored on ice just before use. One might note that as soon as the potato is cut, PPO is activated and the reaction begins in the blender and continues after filtration. It is this activation that storing on ice slows down.

Figure 3, above, represents classical Lineweaver-Burke analysis of enzymatic activity; in this case for potato polyphenol oxidase (PPO). V_{max} is 0.325 absorbance units per minute. K_M is 0.0107 M. R^2 indicates that the data follows the equation for a straight line within Excel's parameters of linear regression.

Classically, V_{max} is not measured in "absorbance units per minute". Classical units are discussed in lecture. For the purposes of this experiment, however, those units were chosen to simplify the experiment.

Figure 4, above, illustrates Lineweaver-Burke analysis of PPO in the presence of 0.01M tyrosine. Note that V_{max} has decreased and that, for all intents and purposes, K_M has not been impacted. R^2, again, indicates that the data follows the equation for a straight line within Excel's parameters of linear regression.

The function of catecholase (another name for PPO) is to provide quinone compounds that are used to produce melanins. Tyrosine is used to synthesize melanins in humans. Based upon both its structure and its human functions, one would expect that tyrosine might inhibit PPO. Based upon the results of this experiment, it seems that PPO "sees" tyrosine as a non-competitive inhibitor. Since non-competitive inhibitors inhibit in an allosteric manner, it seems reasonable that tyrosine regulates PPO activity by a negative feedback mechanism, i.e., to shut down the enzyme at a binding site not identical to the active site of the enzyme.

Figure 5 illustrates Lineweaver-Burke analysis of PPO in the presence of 0.01M trans-cinnamic acid. Note that V_{max} has decreased and that, for all intents and purposes, K_M has not been impacted. R^2, however, suggests that the data does not quite follow the equation for a straight line within Excel's parameters of linear regression.

Trans-cinnamic acid tends to not strike one as a metabolite, i.e., tyrosine is something that PPO is continuously exposed to as a result of biological activity and the need for tyrosine in protein synthesis. Trans-cinnamic acid doesn't serve the same purpose, hence, that it is also a non-competitive inhibitor of PPO is somewhat surprising.

That the data doesn't conform as closely to Excel's linear regression as did the analyses of enzyme and enzyme-plus-tyrosine, suggests that technique may be a bit of an issue, i.e., consistency between sample preparation and analysis might could be rendered improved.

Knowing that all samples (short of adding enzyme) were prepared by the same person and that the only variables impacting analysis are enzyme addition, sample inversion and reading the spectrophotometer tend to suggest the latter contributing to the deviation away from the regression equation.

Figure 6, above, illustrates Lineweaver-Burke analysis of PPO in the presence of 0.01M phenylthiourea (PTU). Note that V_{max} , for all intents and purposes, has not been impacted, but that K_M has been impacted. R^2, however, suggests that the data does not quite follow the equation for a straight line within Excel's parameters of linear regression.

That PTU is a competitive inhibitor (based on these results) of PPO comes as a surprise. PPO contains copper(II) ions as an inorganic cofactor. The copper perpetuates a redox cycle between the Cu(II) and Cu(I) ions much as during collagen synthesis and catecholamine synthesis. PTU is well known to react with and bind to the copper ion to inactivate PPO.

Typically, competitive inhibition occurs as a result of the inhibitor interacting with the enzyme's active site, competing with the substrate, blocking it out of binding in the pocket. Competitive inhibitor may be "swamped out" by the addition of substrate and the enzyme reactivated. This effect was not studied in this experiment. It would be helpful to study the effect of PTU on the enzyme in varying concentrations of PTU to more clearly understand this inhibitive effect.

Conclusions

Trans-cinnamic acid and tyrosine are both non-competitive inhibitors of PPO. PTU is a competitive inhibitor of PPO – per this experiment.

In the future, it would be of interest to explore the inhibitive properties of phenol and DOPA given their similar structures to either catechol or catechol derivatives.

This experiment is easily performed in the one semester organic chemistry laboratory setting and consistent results may be obtained with minimal laboratory experience.

Acknowledgements

Without the good natured participation of Erin, Marlo, Travis and Alvin (CHEM 220 Students, Spring 2005), this experiment would not have been completed or accomplished as efficiently or as effectively.

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