Purification of polyphenol oxidases from fruit

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# LECTURER VERSION

## Objectives

* Purify a polyphenol oxidase from banana or apple
* Perform an assay for polyphenol oxidase activity
* Perform an assay for protein content
* Analyse data related to enzyme purification and assess reproducibility

## Introduction

Polyphenol oxidase enzyme, also known as tyrosinase, is a copper-containing metalloenzyme which catalyses two distinct chemical reactions using phenolic compounds as the substrates. Polyphenol oxidase is an important enzyme in plants and animals as it responds to cellular damage. In fruit, when they become bruised or damaged, polyphenol oxidases are exposed to various monophenols substrates held in separate cellular compartments. The interaction of the substrate and enzyme leads to the characteristic browning of fruit, for example, cutting an apple or a bruised banana. Expectedly, this enzyme is of great commercial interest, as it affects the post-harvest value and appearance of fruits and vegetables. Various household as well as commercial remedies for the browning of fruit have been investigated and have been a matter of research — these help to maintain an appearance that consumers would find attractive by delaying the browning process. The delaying of browning, in most instances, results from changing the conditions the PPO enzyme finds favourable. Commonly, adding weak acids, food preservatives or chelating agents in small amounts.

In this experiment which will span two weeks, you will use classical biochemical techniques to extract a PPO enzyme from either banana or apple. You will first use protein precipitation to partially purify the protein based on its solubility in a high concentration of ammonium sulfate. A common step in enzyme purification is the removal of unwanted/contaminating proteins or the selective precipitation of proteins of interest. One of the most common methods to achieve this is to utilise the physical properties of proteins in solution- specifically their interaction with solvents. By adjusting the ionic strength of the solution, the water molecules that engage in hydrogen bonding with the protein are more likely to interact with the salt being added. As the water molecules interact less with the proteins, they become more unstable in solution and begin to interact with each other. The proteins quickly precipitate from solution based on the properties of their exposed surface. Salts such as ammonium sulphate are commonly used in purifications such as these, it is highly soluble and readily available and therefore used for this reason.

The salt is usually added as a saturated solution or ground powder. Ground salt ensures that high concentrations of the salt do not develop in pockets or portions of the solution, thus causing incorrect precipitation of proteins. What is paramount to the success of a sound salting out is the slow and careful addition of the salt with constant mixing. Constant mixing will not only ensure that the salt dissolves rapidly but evenly and precipitates the expected proteins. To reduce the high salt concentration from precipitation, another biochemical technique- dialysis, is used. Effectively, this will remove most of the salt used for precipitation which could interfere with further experiments.

After dialysis, the retentate is the partially purified enzyme. This PPE is essential for all the activities of the second week of experiments. To purify an enzyme from a mixture of proteins, an assay for detection of that enzyme is necessary. The assay must be specific for the enzyme of interest, and it should be sensitie since dilution and loss of activity are likely to occur at some points during the purification procedure. In the second week, we will perform assays for PPO as well as assays to determine the total protein content of the various steps. Together you should be able to use this information to comment on the effectiveness of the separation protocol.

## Materials

**A. Extraction of PPO**

1. 1X PBS (pH 7)
2. Powdered ammonium sulfate

* One Eppendorf tube
* One plastic tube with lid (50 mL capacity).
* Dialysis tubing

**C. PPO Assay Solutions**

1. 1X PBS (pH 7)
2. Catechol: 5mM in 1X PBS

**D. Lowry Protein Assay Solutions**

1. Lowry base: 2% Na2CO3 in 0.1N NaOH (LB)
2. Reagent A: 0.02% NaK tartrate / 0.01% CuSO4 in LB
3. Reagent B: Folin Ciocalteu reagent: H2O (1:1)
4. Bovine serum albumin (BSA) standard: 1mg/ml in H2O

## Procedure

**WEEK 1**

PART A: Ammonium Sulfate Precipitation

*This step will take approximately 80 mins.*

1. You will be provided with either bananas or apples, depending on your seating location. Cut the fruit in small pieces with the scalpel provided.
2. Weigh the pieces of cut fruit and add a suitable amount of cold PBS at a ratio of 1 g fruit to 20 mL of buffer. The resultant homogenate is your crude extract (CE).
3. Filter 25 mL of the crude extract per group through Miracloth/cheesecloth into a 50 mL Falcon tube.
4. Save 1 mL of the remaining crude extract in an Eppendorf tube, label the tube appropriately for use the following week.
5. To the crude extract in the Falcon tube, add ammonium sulfate until an 80 % saturation solution is reached. (0.86 g per mL of buffer)
6. Stir the suspension with a glass rod on ice for approximately 30 mins.
7. Centrifuge the sample at 15, 000 rpm for 10 mins at 4 ° C. **Handle the sample with care and be sure not to disturb the pellet, causing it to be resuspended**.
8. Decant the supernatant to a new tube, label appropriately and store on ice. Be careful during this step as the pellet is fragile. (See Appendix 4)
9. Resuspend the pellet with 5 mL 1X phosphate buffer (PBS). Take 500 µl of this sample and store in an Eppendorf tube on ice, this is RP.

PART B: Dialysis

*This step will take approximately 20 mins + overnight incubation.*

1. Load the supernatant into pre-boiled dialysis tubing and seal each end with double knots and universal clips.
2. Place the tubing into a large beaker containing 1X PBS buffer.
3. Allow sample to dialyse overnight at 4 °C.
4. When you retrieve the sample the next week, it will be in an Eppendorf tube labelled with your group number.

**WEEK 2**

PART C: Enzyme Kinetics of Polyphenol Oxidase

*This step will take approximately 50 mins.*

1. Label 3 tubes for each sample from the last week (CE, RP, PPE), they can be labelled *‘diluted.’*
2. In each tube prepare a 1: 10 dilution of the samples provided. *e.g*. (0.2 mL sample in 1.8 mL buffer).
3. Label 10 assay tubes, 3 tubes for each sample from the last week (CE, RP, PPE). *E.g*. CE1, CE2, CE3, RP1…, and a tube containing 2.95 mL of buffer for the blank.
4. Each assay for the samples will have a total volume of 3 mL, to the tubes add 0.1 mL of the appropriate diluted fraction (CE, RP, PPE) and 2.85 mL buffer.
5. Add 0.05 mL catechol to al the assay mixtures above, mix briefly. **Only do this when ready to measure the absorbance**.
6. Record the absorbance of the assay tube immediately after at wavelength 352 nm every 30 seconds for 2 minutes.
7. Record your results in a table you have constructed.

PART D: Lowry Protein Assay

*You will determine the total amount of protein (polyphenol oxidase and other "contaminating" proteins) present in the crude extract (CE), ammonium sulfate precipitation supernatant (SUP) and the resuspended pellet (RP), using a standard colourimetric assay.*

1. Set up 13 assay tubes. Dilute 0.7ml of BSA standard with 6.3ml of Reagent A. Add 0 (blank), 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 ml of the diluted standard to tubes 1-7.
2. Dilute 0.1ml of CE with 0.9ml of Reagent A. Then dilute 0.5ml of the diluted CE with 4.5ml of Reagent A to give a final dilution of 1:100. Dilute 0.6 ml of RP with 5.4ml of Reagent A.
3. Add 0.25 and 0.5 ml of diluted CE to tubes 8 & 9, add 0.5 and 1 ml undiluted sup to tubes 10 & 11, and 0.25 ml of diluted and 0.5 ml undiluted to tubes 12 & 13.
4. Add Reagent A to each tube to give a final volume of 3ml. Mix well (Vortex mixer) and let stand at least 10 min.
5. Add 0.3ml of Reagent B to each tube, mixing each sample with the Vortex mixer immediately after adding Reagent B. Let stand for at least 30 min.
6. Read and record the absorbance at 640nm. (Blue colour intensity is proportional to protein concentration).

## Calculations

From the data collected in C, determine the relative concentrations of PPO in CE, RP and PPE, in Δabs352 min-1 ml-1. (Assume abs352 at zero time is zero). For each, average the 3 values, unless one is obviously “off”, or not in congruence with the other two.

The total enzyme activity started with, that is the activity of the freshly homogenized fruit, in Δabs min-1, is the enzyme concentration of the CE multiplied by the total volume of CE used in the purification. Similarly, determine the total enzyme activity left in the RP and recovered PPE. Account for dilutions, you should carefully review the procedure above to determine areas where you performed dilutions. The ratio of activity recovered to activity loaded (X100%) is the yield for the purification procedure.

From the data collected in D, construct a standard curve for the Lowry protein assay (abs640 vs μgBSA = protein). Plot the best straight line (or smooth bow), forcing it through the origin. From the standard curve, determine the μg protein in each of the tubes containing CE, RP or PPE. Use these values to calculate the protein concentrations of CE, RP and PPE in either μg ml-1 or mg ml-1. Again, average the 3 values for each, unless one is “off”. Determine the total μg or mg of protein in CE and in PPE by multiplying the concentration by volume. Account for dilutions similar to before.

The enzyme activity per mg (or μg) protein for CE and for PPE is the specific activity. The ratio of specific activities (PPE/CE) is the -fold purification (eg 3-fold, 10-fold) for the purification procedure.

## References

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Price, Nicolas., Narin, Jacqueline. et al. Exploring Proteins, Oxford University Press (2009).

# STUDENT VERSION (RESULTS)

Table 1: Activity of Banana PPO in the crude at various volumes

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Time/ s | A352nm | | | | | |
| 0.5 mL CE | 0.2 mL CE | 0.1 mL CE | 0.01 mL CE | 0.002 mL CE | 0.001 mL CE |
| 0 | 0.237 | 0.139 | 0.096 | 0.059 | 0.035 | 0.033 |
| 30 | 0.551 | 0.452 | 0.291 | 0.128 | 0.061 | 0.051 |
| 60 | 0.539 | 0.601 | 0.451 | 0.163 | 0.078 | 0.061 |
| 90 | 0.525 | 0.587 | 0.573 | 0.188 | 0.090 | 0.068 |
| 120 | 0.521 | 0.565 | 0.629 | 0.216 | 0.098 | 0.072 |

Table 2: Determination of banana PPO content in crude extract, supernatant, re-suspended pellet and retentate using Bio-Rad Bradford Assay.

|  |  |  |  |
| --- | --- | --- | --- |
| BSA Standard/ mg/ mL | A595nm | Protein Sample | A595nm |
| 0.01 | -0.012 | Crude Extract | 0.119 |
| 0.025 | 0.01 |
| 0.05 | 0.046 | Supernatant | 0.001 |
| 0.075 | 0.066 |
| 0.125 | 0.098 | Re-suspended Pellet | 0.286 |
| 0.400 | 0.318 |
| 0.800 | 0.624 | Retentate | 0.165 |