Biology and Biochemistry Department
BIO C331
biochemistry Lab

Experiment #9
(A) Extraction and activity of polyphenol oxidase from a banana.
(B)The Isolation of hemoglobin using salt precipitation

Student name: Melak Ottallah
Student number: 1190389

Partners: Ali Milhem, Abdalrahman Sroor, AbdelFattah Tanneneh.

Instructor: Dr. Amanie Abed
Teacher's assistant: Aseel Mhani

Date of experiment: 10/11/2021
Date of submission:13/2/2022

Objective:

(A)The objective of this experiment is to study the effects of ascorbic acid, cysteine, p-hydroxybenzoine, EDTA, and DTT on polyphenol enzyme activity that was extracted from a banana.

(B) The objective of this experiment is Salting out hemoglobin using saturated ammonium sulfate, checking if the salting out solution works, and determining the quantity of protein that was salted out

Introduction:

The involvement of polyphenol oxidase activity (PPO, monophenol monooxygenase, and o-diphenoloxidase) in enzymatic browning in banana fruit has been widely researched. PPO and phenolic chemicals are abundant in banana vegetative tissue, as evidenced by rapid discoloration of leaf, stem, and root tissue following damage and substantial pigmentation of tissue extracts. They stymie biochemical and molecular research in bananas by necessitating time-consuming adjustments to extraction techniques. PPO and phenolic chemicals, on the other hand, may play a vital role in the plant's defense mechanism against pests and diseases, such as root parasitic nematodes. (7)

Various procedures, such as precipitation techniques, affinity chromatography, liquid–liquid extraction, and solid phase extraction, have all been used to isolate and purify proteins from complicated sample matrices. Among these procedures, immobilized metal ion affinity chro-matography is still one of the most extensively used, allowing proteins to be separated using the solid phase extraction concept, which takes advantage of variations in protein affinity for metalions fixed on a solid substrate. Simple manipulations, a relatively large loading

capacity, gentle operating conditions, and separationselectivity imposed by the metal ions and buffers used define such methods. (8)

Materials:

(A)

- Extraction buffer: 5% w/v polyvinylpyrrolidone, 0.5% Triton X-100 in PBS.
- Assay buffer: 0.5% Triton X-100 in PBS.
- 100 mM Catechol in PBS
- Banana.
- 100 mM EDTA in PBS.
- 50 mM p-hydroxybenzoic acid in PBS.
- 2 mM Cysteine in PBS.
- 2 mM Ascorbie acid in PBS.
- 2 mM DTT.
- Acid-washed sand
- Ice
- Mortar and pestle.
- Spectrophotometers; Centrifuges; Centrifuge tubes and Water bath.

(B)

- Test tubes
- Graduated cylinders
- Pipettes
- Balance
- Centrifuge
- Protein solution (hemoglobin)
- Saturated ammonium sulfate solution.

Methods:

(A)

1. a big beaker was filled with ice, then a pestle was brought in and 4.1 grams of fresh bananas were placed in the mortar, followed by 10 ml of extraction solution and roughly two spoons of sand, and the bananas were crushed until homogenous.
2. The homogenate was then transferred to a centrifuge tube and spun for 10 minutes at 4000 rpm.
3. The supernatant was separated to another test tube and put on the ice flask after centrifugation, and then 0.8 mL catechol and 0.8 mL buffer assay solution were added to a cuvette and centrifuged at 420nm.
4. The cuvette was then filled with 50 l of crude enzyme extract, and the absorption reading was measured every 10 seconds for two minutes.

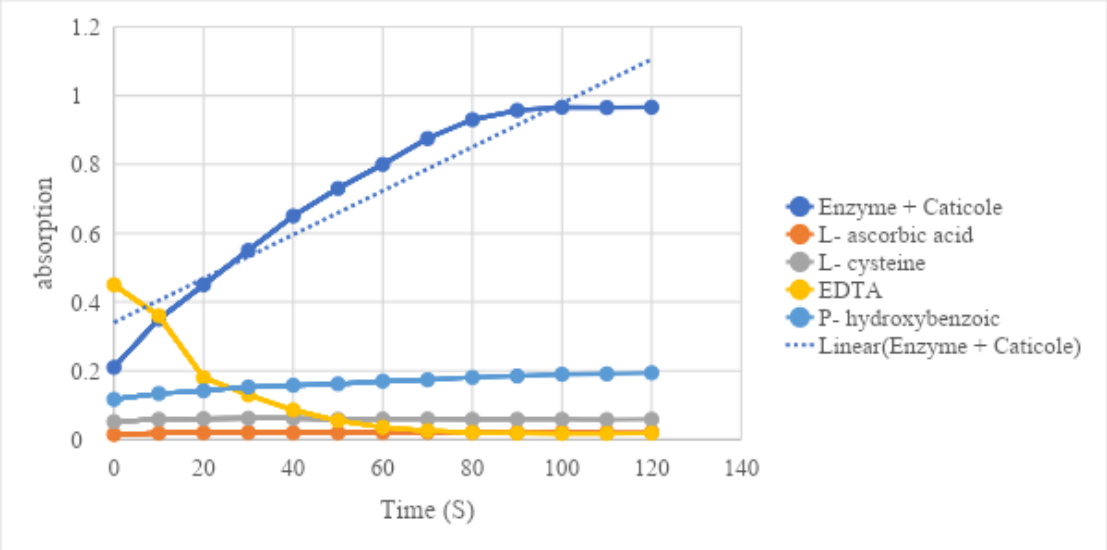
5. In a separate cuvette, 0.8 ml of catechol was added, followed by 0.8 ml of L-ascorbic acid, L-cysteine, p-hydroxybenzoic acid, EDTA, and dithiorthreitol (DTT), in that order, each in a cuvette containing 0.8 ml of catechol, before adding 50 l of enzyme extract and performing the absorbance measurements.

(B)

1. The absorbance of the hemoglobin solution was determined after 10 ml of hemoglobin was centrifuged at 3000 rpm for 5 minutes,
2. the supernatant was collected, and the spectrophotometer was blanked at 577 nm with water.
3. The hemoglobin solution was then pipetted into a centrifuge tube in 4 mL increments.
4. An equivalent amount of saturated ammonium sulfate solution was added dropwise to the protein solution while stirring until a precipitate formed.
5. After centrifuging the mixture for 15 minutes at 10,000 g, the precipitate was obtained by carefully eliminating as much supernatant as feasible.

Data and results:

(A)



Graph 1: time vs absorption for different additions and their affect on ppo.

(B)

Theoretical absorbance of original Hb solution = 0.640 nm

Absorbance after salting out = 0.599 nm

Percentage Yield = 93.6%

Percentage Error = 6.4%

Discussion:

(A) And (B)

The enzyme activity grows with time until it achieves its maximum activity, when the line stops moving up, as seen in the initial absorbance measurement of the enzyme without the

addition of any acid. Ascorbic acid, which is regarded an anti-browning agent, aids in the conversion of benzoquinone to catechol when added, as seen in graph 1.

The addition of ascorbic acid to the reaction mixture inhibited PPO activity in a concentration-dependent manner. According to the graph above, it reduces the amount of benzoquinone for which absorption is measured. To summarize, at high concentrations, ascorbic acid can instantly convert the formed quinone to the original substrate (catechol), whereas at lower concentrations, it acts as a competitive inhibitor. Because cysteine combines with the resulting Quinone to produce colorless molecules, it functions as a competitive inhibitor.) (1)

When EDTA is introduced, the activity begins at a higher value and rapidly falls; this may be explained by the nature of EDTA as a chelating agent, where it combines with the ions on the active site of the enzyme, and therefore the line's nature. (A reaction is occurring) (2) The most efficient PPO inhibitor should be hydroxybenzoic acid (3) Monohydroxybenzoic acids inhibit PPO significantly; in most situations, the inhibition is competitive, with a binding affinity equivalent to that of the substrate. (4), which explains why the line is more effective than other inhibitors since this sort of inhibition is competitive, allowing the PPO reaction to proceed.

The results show that a sufficient amount of saturated ammonium sulfate solution is an excellent salting out reagent. The absorption values are lower after using the solution, indicating that part of the protein has been salted out, causing the absorbance values to rise. Salts impact the solubility of proteins, either salting them in or salting them out. We can test if a solution is as good as salting out/in solution using the salt precipitation technique. Protein precipitation is

frequently utilized in biological product downstream processing, as well as to organize proteins and purify them from different impurities.

Conclusion:

Using the salt precipitation approach, we can see if a solution is as good as salting out/in solution. Protein precipitation is often used in the downstream processing of biological products, as well as to organize and purify proteins from various contaminants. Food browning has proven to be a difficult problem for the food business. Several remedies have been offered, the most common of which is the use of anti-browning chemicals that block the PPO activity that causes browning. Anti-browning chemicals are used to prevent enzymatic browning and maintain the color of fresh-cut fruit. Furthermore, anti-browning agents offer a variety of outstanding features that drive product demand in the end-use sector, namely the food business.

References:

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Appendix:

- Percentage Yield = Experimental Absorbance after salting out/ Theoretical absorbance of original Hb solution*100% = (0.599/0.640)*100 = 93.6%

- Percentage Error = ((Experiment Absorbance after salting out - Theoretical absorbance of original Hb solution) / Theoretical absorbance of original Hb solution) *100 % = ((0.599-0.640) (0.640) * 100% = 6.4%

- What are the effects of each of the molecules above on enzymatic activity?

- Each molecule acts as a PPO inhibitor, lowering the enzyme's activity, as discussed in the discussion.