Systematic Purification Scheme for Peroxidase Library Samples

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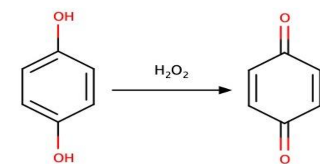
Purpose of Project

This project sought the development of a systematic purification method for peroxidase enzymes from crude plant sources. Peroxidases represent valuable biotechnological tools for a wide range of applications, however, the majority of these applications have been developed utilizing a single member of the peroxidase family, that being the enzyme isolated from horseradish root. Development of a universal purification strategy that is effective for the isolation of peroxidases from a vast number of plant sources may open the door for development of novel applications, or identify more effective peroxidase enzymes for many of the current applications. This prepared method is a simple step-by-step process, starting from the freshly harvested plant, and produces a partially purified sample of selected plant peroxidases that is are higher in specific activity. The method involves grinding crude plant material in 20mM buffer, followed by the addition of 40% ammonium sulfate, which causes precipitation of high molecular weight contaminating protein. The high salt soluble fraction is subjected to liquid chromatography involving a phenyl-Sepharose resin and produces highly enriched peroxidase enzyme. Two assays were used in this process, a guaiacol assay to determine enzyme activity and a Bradford assay to determine the amount of protein in the samples. These tests were conducted throughout each step of the purification process to confirm that the highest specific activity was found in the partially purified fraction of each sample.

Background Information

Peroxidases are a class of enzymes that are found in all plants. These heme-containing enzymes use hydrogen peroxide to catalyze a wide range of chemical oxidations, many with important biotechnological implication. An example of a chemical oxidation carried out by peroxidases is shown below:



Horse radish peroxidase (HRP) has emerged as the the prototypical peroxidase due to the relatively high amount of easily extracted enzyme in its roots. It is currently used in a variety of immunohistochemical applications, such as, ELISA and Western blotting, as well as bioremediation, biosensor technology and waste-water treatment, to name a few. Despite its widespread use, HRP is merely 1 of thousands of peroxidases found in nature, thus the isolation and characterization of novel peroxidases has great potential for expanding and/or improving the use of these enzymes as biotechnological tools. The current project is intended to provide a simple protocol for the purification of peroxidases, which may facilitate discovery of novel applications or the identification of more effective catalysts for those applications currently in use.

Methods

Peroxidase sample production: In this study, various vegetables were used to collect the peroxidase enzyme. This was done by peeling 2 grams of each vegetable's thin layer of skin and mixing it and mashing it with 4 grams .02 M pH 6.5 Potassium Phosphate sample buffer in a mortar and pestle. For further experiments, the ratio of grams of vegetable skin to mL of sample buffer was set to 1:2. These samples were then centrifuged at 10000 rpm for 5 minutes to remove plant residue.

In this study, a much larger sample of each vegetable's thin layer of skin was peeled off and mixed/crushed using a mortar and pestle with a .02 M pH 6.5 Potassium Phosphate sample buffer. This was done with a ratio of 1:2 (grams of vegetable skin: mL of sample buffer). The samples were about 30-50 mL each. These samples were then placed in the big centrifuge and centrifuged at 18000 rpm for 15 minutes at 4°C. This made all the plant residue collect at the bottom, to isolate the peroxidase more. The supernatant of each sample was taken out and put in a beaker in an ice bath. Then ammonium sulfate was added to the sample to make the solution 40% of ammonium sulfate's saturation in water at 0°C. Once the salt fully dissolved it was brought back to the big centrifuge where it was once again centrifuged at 18000 rpm for 15 minutes at 4°C. The supernatant of this sample was collected. The sample that was collected was run through a column with resin that trapped the product as the solution ran through it. Once the sample was collected at the top, water was run through, and the sample was eluted into fractions. Once the fractions were collected the column was cleaned with 10 mL of a 20% methanol solution and then 20 mL of the .02 M sample buffer before adding the next sample to ensure no contamination. The fractions that were collected had their activities measured using a guaiacol assay and Bradford assay to measure protein content. The data was recorded to measure the specific activity of each sample by dividing the guaiacol activity by the protein content. The protein content of each sample was measured using a standard curve that was made with a 2 mg/mL BSA solution to measure protein concentration, with 1 mL of Bradford reagent and 33 µL of the sample being used. This was tested in a spectrophotometer at 595 nm and the absorbance was measured. The guaiacol activity was measured by the absorbance rate per minute. A guaiacol mixture was prepared using 22 µL pure guaiacol, 20 µL H2O2, 2 mL 1M ph 6.5 Potassium Phosphate buffer, and 38 mL H2O to test the different samples. Guaiacol binding to peroxidases creates an orange color and the darker the orange, the more peroxidase present in the sample. A spectrophotometer was used to quantify the data gathered from each sample by measuring the kinetic rate compared to a "blank" sample. 990 µL of the guaiacol mixture was mixed with 10 µL of each peroxidase sample and the absorbance rate at 470 nm for 1 minute was measured. Samples that recorded above a 1.2 absorbance rate, which had a very dark orange color, were diluted due to those samples possibly being oversaturated with peroxidase.

Figure 1. Preparation of peroxidase Samples of vegetable skin and sample buffer crushed with mortar and pestle to form final sample that will be used for purification.

Figure 2. Purification process Purification was done by means of adding ammonium sulfate to a beaker of the peroxidase sample in an ice bath. This was followed by use of giant centrifuge to remove any precipitate or plant residue left over.

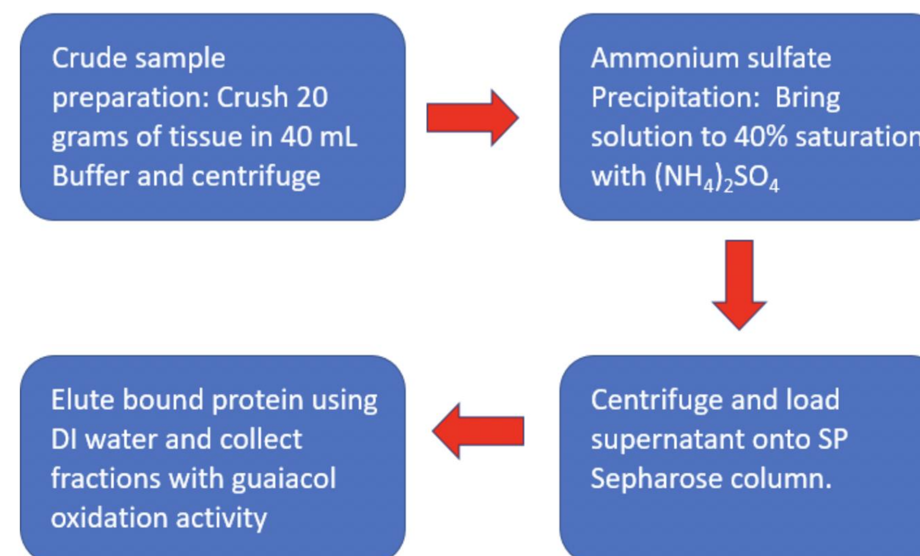


Figure 3. Purification scheme A scheme that illustrates the various steps needed for sample purification within this study.

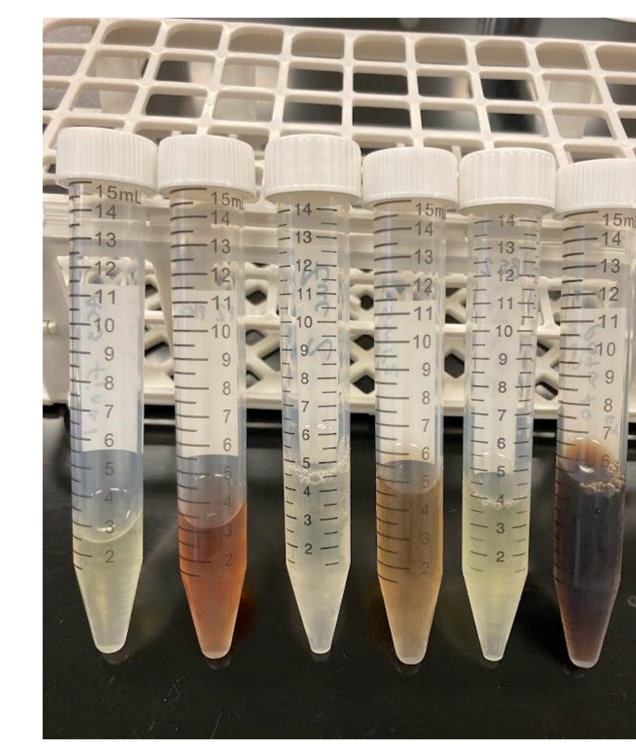


Figure 4. Column collection fractions Different fractions were collected as purified samples of peroxidase with ammonium sulfate were run through a column, collecting the sample from the solution. Then water was run to collect the sample and was eluted in different fractions which held enzymatic activity.

Sample:	Type:	Guaiacol Absorption:	Dilution:	Volume (in mL):	Total activity (in units):	Protein-Bradford Absorption:	Total Protein:	Specific Activity:
Acorn Squash	Crude	0.34	/	33	42	0	33	**1.27**
	40%	0.32	/	36	42	0	10.8	**3.88**
	Column	0.89	2	2.9	20	0	1.16	**17.24**
Pumpkin	Crude	0.318	100	33.1	3957	0.589	37	**106.9**
	40%	0.303	100	30.5	3474	0.59	34	**102.2**
	Column	0.207	1000	3	3823	0.596 (1:5)	21.4	**178.6**
Brussel Sprouts	Crude	0.693	/	13.1	34	0.311	7.48	**4.5**
	40%	0.577	/	12	26	0.246	5.7	**4.6**
	Column	0.91	/	4.5	15.9	0.33	2.7	**5.88**
Sweet Potato	Crude	0.279	10	26	272.7	0.116	4.92	**55.4**
	40%	0.255	10	27	258.8	0.04	1.09	**237.4**
	Column	1	10	5	188	0.091	0.7	**268.6**
Russet Potato	Crude	0.808	/	32	97.2	0.471	28.32	**3.43**
	40%	0.663	/	29	72.3	0.169	8.5	**8.51**
	Column	0.644	5	5.4	65.4	0.501	5.1	**12.82**
Zucchini	Crude	0.786	/	35	103.4	0.265	16.85	**6.14**
	40%	0.694	/	37	96.5	0.177	11.43	**8.44**
	Column	0.367	10	5.1	70.36	584	5.64	**12.48**
Jalapeno Seed	Crude	0.038	/	14.5	2.07	0.457	12.44	**0.166**
	40%	0.02	/	15.5	1.17	0.278	7.86	**0.149**
	Column	0.139	/	3.6	1.88	0.557	3.79	**0.496**

Figure 5. Final specific activity The image above shows the various numbers used to find and quantify data to find specific activity of each sample. Guaiacol absorption was done using the guaiacol assay and protein absorption was found using the Bradford assay. The specific activity was found by dividing the guaiacol absorption by the protein absorption.

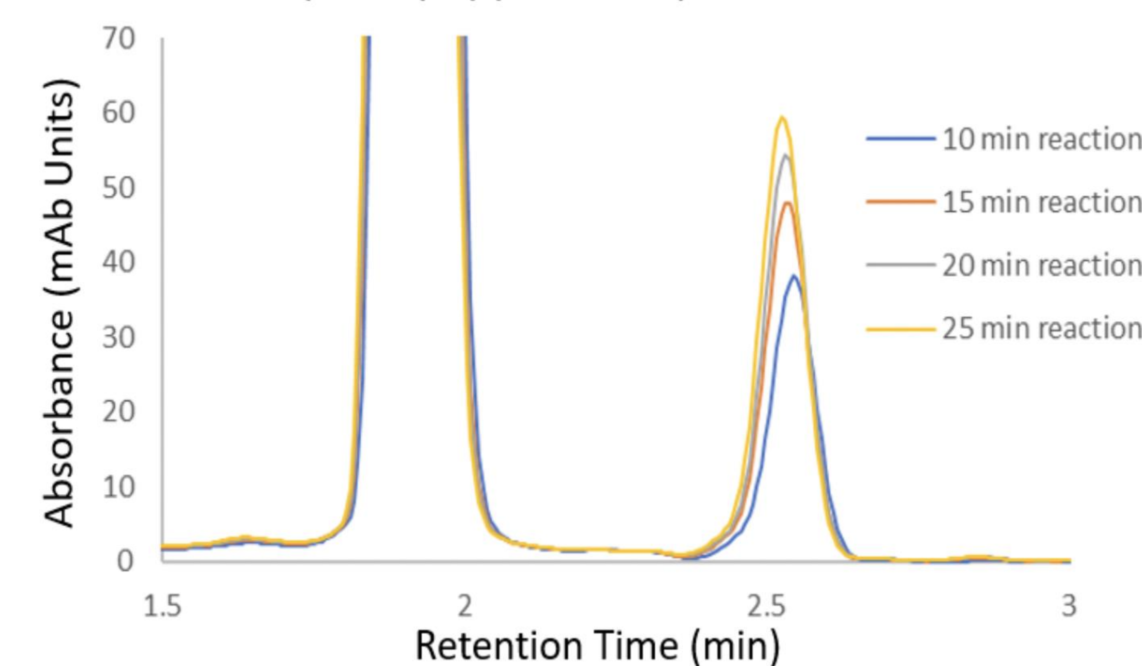


Figure 6. Jalapeño chromatogram A chromatogram is shown with a second for the jalapeño peppers. This indicates a product of an alternative mechanism that can be researched in future studies.

Results and Discussion

The purification strategy used for this project begins with the liberation of the peroxidase enzyme from the crude plant sample through the mechanical disruption of the tissue using a mortar and pestle, as shown in Figure 1. Due to the hydrophilic (water soluble) nature of the peroxidase enzyme, an aqueous buffer system is utilized in this first step. A total of 7 different crude plat samples were utilized in these trials. The samples included: Acorn Squash skin, Pumpkin Skin, Zucchini Skin, Russet Potato Skin, Sweet Potato Skin, Whole Brussel sprouts, and Jalepeno pepper seeds. It should be noted that for each of the samples, it was determined that only certain parts of the plant possessed high peroxidase activity. For example, activities were limited to the skin of the potato and squash samples, so peeling the raw sample first produced a much more enriched peroxidase sample. The Jalepeno seeds also possessed much more activity than the flesh as well. After crushing the samples, centrifugation produced a cleared supernatant high in peroxidase activity. This is referred to as the crude sample identified in Figure 5. Ammonium sulfate precipitation using a 40% saturated solution was then used and shown in Figure 2. This step removed higher molecular weight protein from the sample, but leaving the peroxidase still in solution. The high salt is also important for the subsequent chromatography step, illustrated in Figure 4. This chromatography step utilizes a hydrophobic solid phase consisting of phenyl-sepharose, which takes advantage of the selectivity of the peroxidases for aromatic (phenyl) substrates. The high salt optimizes the hydrophobic interaction between the enzyme and the solid phase, leading to effective binding of the enzyme to the column. Reducing the ionic strength of the mobile phase, then allows for the elution of the peroxidase from the column, and the collection of the sample in a small volume, which results in an overall refinement and concentration of the peroxidase in a single step. Sample collected from the column are shown in Figure 4.

The table shown in Figure 5 was generated by measuring the total peroxidase activity recovered at each step, and dividing this total activity by the total amount of protein in the recovered samples at each stage of purification allows for determination of the Specific activity of each sample (Specific activity is used to measure relative purity of the sample). According to this table, each sample was significantly increased in specific activity using this simple 2-step purification method.

Summary

- For each of the seven samples tested, this method produced an increase in specific activity between 2 and 15-fold, demonstrating the universal utility of this method
- Recovery of activity was very high for each sample, with minimal loss of total activity observed across all sample
- The Jalapeño seed demonstrates the value of this method in identifying novel peroxidase activities, which is a long-term goal of this project.

References and/or Acknowledgments

K.M. McLellan, D.S. Robinson, The heat stability of purified spring cabbage peroxidase isoenzymes, Food Chemistry, Volume 26, Issue 2, 1987, Pages 97-107, ISSN 0308-8146, https://doi.org/10.1016/0308-8146(87)90120-8.