Novel Plant Peroxidase Library Development for Use in Various Biochemical and Practical Applications

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Abstract

Peroxidases are a class of enzymes used by living organisms to catalyze oxidation reactions. Horseradish peroxidase, a Class III heme-containing peroxidase, is almost exclusively used in commercial and research applications for catalyzing oxidation reactions or providing a biochemical marker. Given the tough nature of horseradish root, extracting horseradish peroxidase is labor-intensive resulting in high costs with low yields. As a first step for discovering alternative peroxidases for various applications, this study characterized peroxidase activity for around 50 plant sources using two assays. The guaiacol assay was used to determine general peroxidase activity for the plant source by measuring the conversion of guaiacol to tetraguaiacol by spectrophotometry. The speed and strength of the reaction are directly dependent on the amount of peroxidase present in the sample, quantifying the overall presence of peroxidase present. Second, a fluorophenol assay was used to determine if the plant peroxidase demonstrated defluorination activity especially useful for preventing dye pollution from the textile industry. The results of the assays showed that along with horseradish peroxidase, the skins from pumpkin, zucchini, and butternut squash (all from the Cucurbitaceae family) have maximal activity in the guaiacol assay and the fluorophenol assay with an expected decrease in activity as the concentration of peroxidase was reduced. In addition to these four potent plants, Kudzu, the invasive weed, also demonstrated meaningful activity in both assays. Future studies could include exploring other gourds from the Cucurbitaceae family for peroxidase activity along with the purification and characterization of the already discovered peroxidases with the goal that the new peroxidases will provide more stable, sensitive, and selective oxidizers for various applications.

Introduction

Peroxidases are vital to almost every cell type because they break down reactive oxygen species (ROS) that are made during cellular respiration (1). Since all cells that require oxygen for energy use aerobic cellular respiration, they are vulnerable to ROS which can cause mutations in DNA and damage cellular machinery. Peroxidase from horseradish was the first to be discovered and given its ability to bind to certain molecules it has been extensively used in many applications since then (2). Two major applications that horseradish peroxidase (HRP) is currently used for in the scientific community are Western Blot analysis and the ELISA blood test. These tests are essential for detecting the presence of proteins and antibodies for research purposes as well as medical diagnoses. However, the selectivity of HRP for the desired compounds is not always optimal making the search for better peroxidases important.

The research completed for this project relied on using two general assays that demonstrate a couple of common capabilities of peroxidases. The guaiacol assay (Figure 1) displays peroxidase activity by using a colored compound as a marker. By feeding the reaction with hydrogen peroxide and the colorless compound guaiacol, peroxidase can convert four guaiacol molecules into one tetraguaiacol molecule for every reaction (1). The tetraguaiacol molecule is an oxidized complex of the four guaiacol molecules that is dark orange. Because of this, the change of color as the reaction proceeds is easily recorded by a spectrophotometer which detects the wavelength of light absorbed by tetraguaiacol. The amount of light absorbed allows the amount of tetraguaiacol present to be determined. When this is timed, the amount of peroxidase present can be calculated. Since this oxidation is very simple for a peroxidase to perform, this analysis does not give selectivity information, but it does show the level of peroxidase present in the sample being tested (2). The fluorophenol assay (Figure 2) indicates the capability of the peroxidase to de-fluorinate an aromatic (3). Like the guaiacol assay, 4-fluorophenol is combined with hydrogen peroxide and then treated with peroxidase. Since this reaction causes the fluoride ion to be removed and replaced with a ketone, the fluorescence can be measured (3). With each reaction, one fluoride ion is released, and the intensity of the fluorescence indicates how much peroxidase is present in the sample. This reaction requires more selective and stronger peroxidases because 4-fluorophenol is more stable than guaiacol.

In addition to the above assays, a third analysis was conducted on a few of the most active peroxidases for determining the concentration of heme present in the peroxidases (Figure 3). The process of detection is very similar to the fluorophenol assay and allows the concentration of heme to be determined.

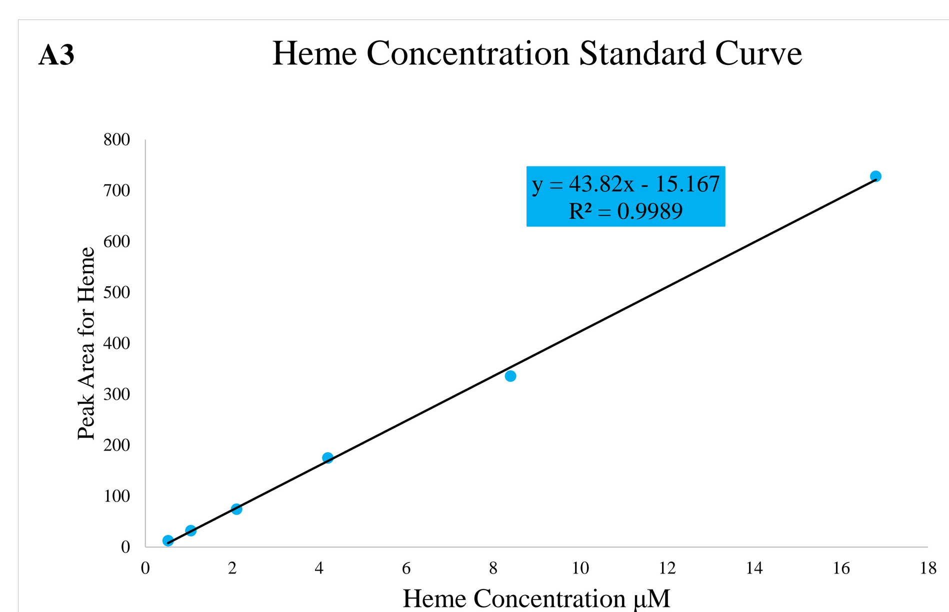
Methods

As a first step before performing any of the assays, crude extracts of each plant sample were made by crushing the material with a mortar and pestle and combined with an extraction buffer. Centrifugation removed all large particles, leaving the peroxidases and some other cellular material. For each dilution, the crude extract was combined with more extraction buffer.

The guaiacol assay was performed by making a stock solution of 5 mM guaiacol, 5 mM H_2O_2, and 50 mM phosphate buffer (pH 6.5). Using 980 µL of this solution and 20 µL of the extract, each sample was measured in triplicate using a spectrophotometer at a wavelength of 470nm and scanned for 1 min. at 10 sec. intervals.

The fluorophenol assay was performed by making a stock solution of 5 mM 4-fluorophenol, 5 mM H2O2, and 50 mM phosphate buffer (pH 6.5). Reactions with each sample were conducted in triplicate with 490 µL of the stock solution and 10 µL of the extract at 30 °C for 5 min. After this, 200 µL of hydrochloric acid was added to stop the reaction at the same time for all the samples. After being centrifuged to remove any leftover proteins, the reaction products were run through a high-performance liquid chromatography column with an injection volume of 25 µL in a 50/50 solvent mixture of acetonitrile and water for 4 min.

Photo by Sally Hess



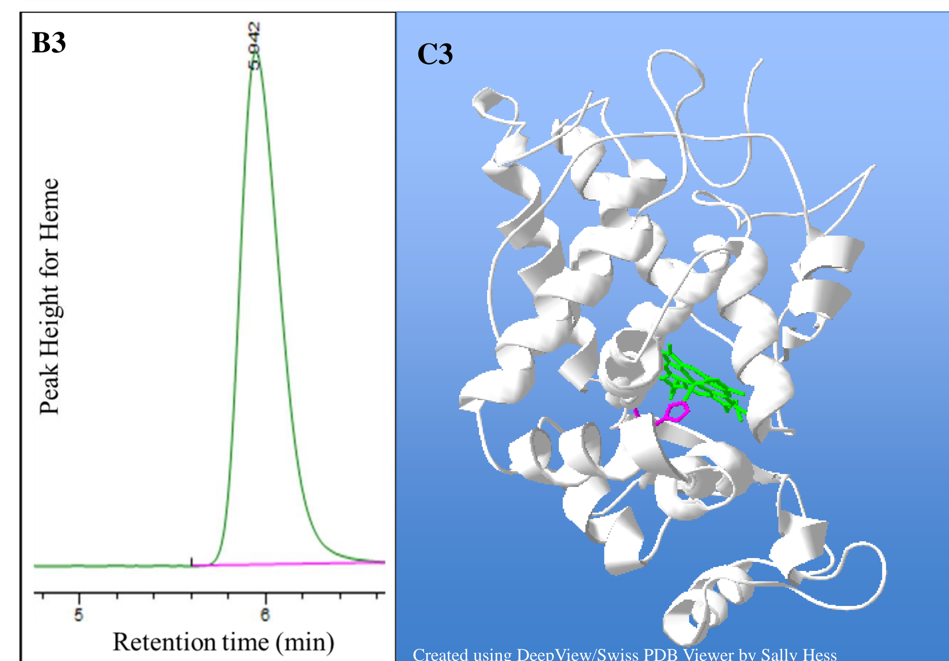
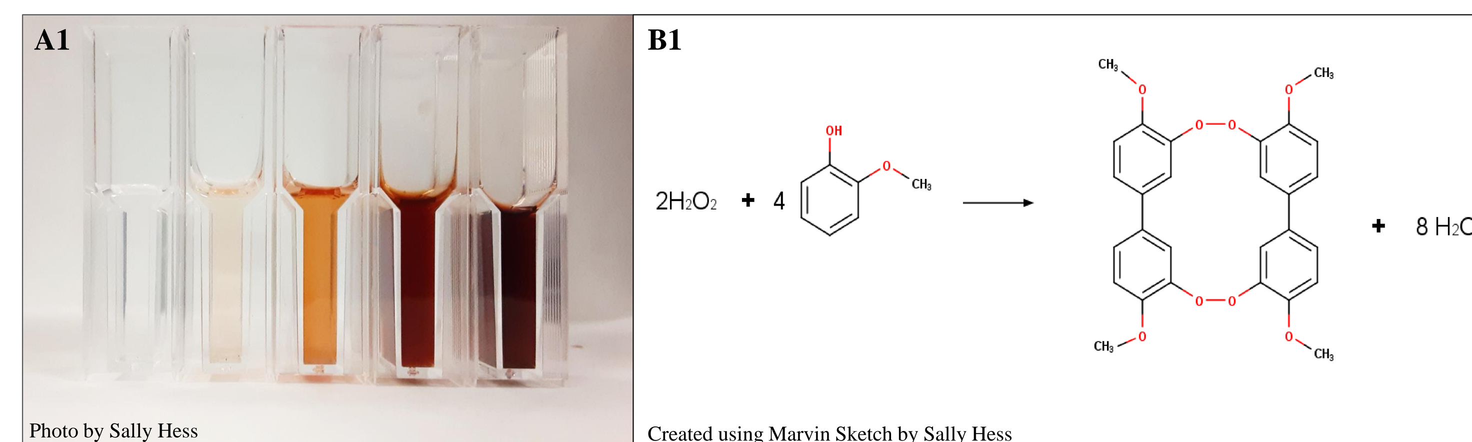


Figure 3. Heme analysis was used to determine heme concentration in novel peroxidases. (A) A standard curve of heme concentration assembled using known concentrations of heme tested using high-performance liquid chromatography (HPLC) to give a measurable area under the curve. (B) An HPLC chromatogram showing an example heme curve. Heme appears at a retention time of 5.942 min from the injection into the chromatography column. The computer program calculates the area under the curve which can be converted to heme concentration using the standard curve. (C) A computer-generated protein model of horseradish peroxidase (HRP) showing the heme group (green) with Histadine 171 (pink) bound to it. A heme group is a porphyrin ring that makes up the active site of many peroxidases including HRP. Heme concentration indicates how efficient the molecule could be.

Table 1. Heme Concentrations

Sample	Heme Concentration (µM)
Jalapeno Seeds	0.382
Zucchini Skin	0.706
Butternut Squash	0.859
Kudzu Leaf	0.529



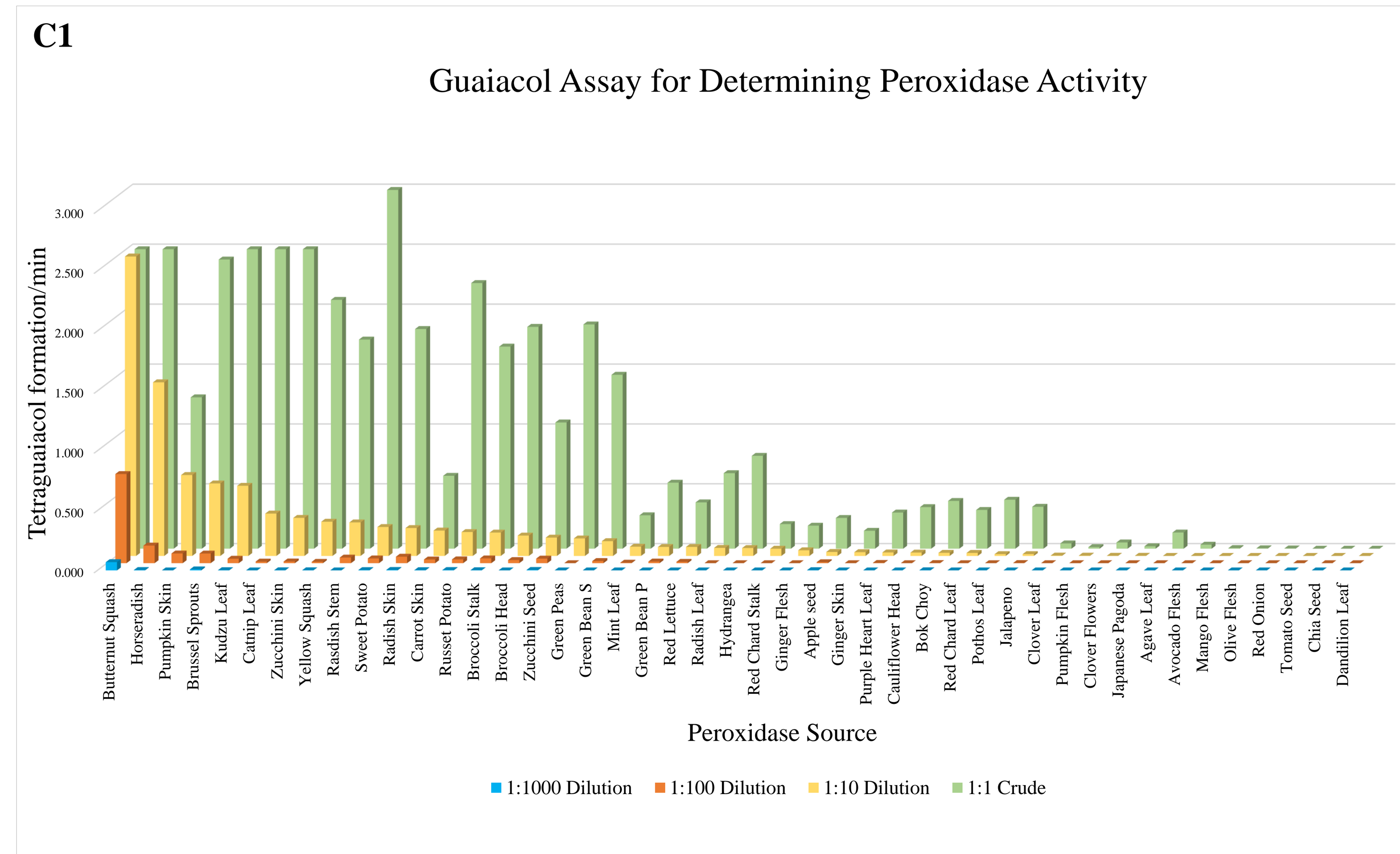


Figure 1. Peroxidase activity was measured by quantifying the guaiacol reaction. (A) A gradient of guaiacol/tetraguaiacol mixtures in cuvettes was used to measure light's absorbance at a wavelength of 470nm, the optimal absorbance for tetraguaiacol. The far-right cuvette has no tetraguaiacol and the far-left cuvette has the highest concentration of tetraguaiacol. (B) The reaction between hydrogen peroxide (H2O2) and guaiacol (1) to form water and tetraguaiacol (2). Guaiacol is colorless while tetraguaiacol has a deep orange-to-brown color allowing the spectrophotometer to detect a difference. (C) Activity of peroxidases from novel sources in the guaiacol assay, measured with an undiluted sample, a 1:10 dilution, a 1:100 dilution, and a 1:1000 dilution. Butternut squash demonstrates the highest activity with horseradish and pumpkin skin following closely behind. Peroxidase activity was ranked based on activity in the 1:10 dilution. Samples with very low activity (less than 0.050 tetraguaiacol/min were not tested beyond the undiluted sample. Some inactive samples were excluded from the graph.

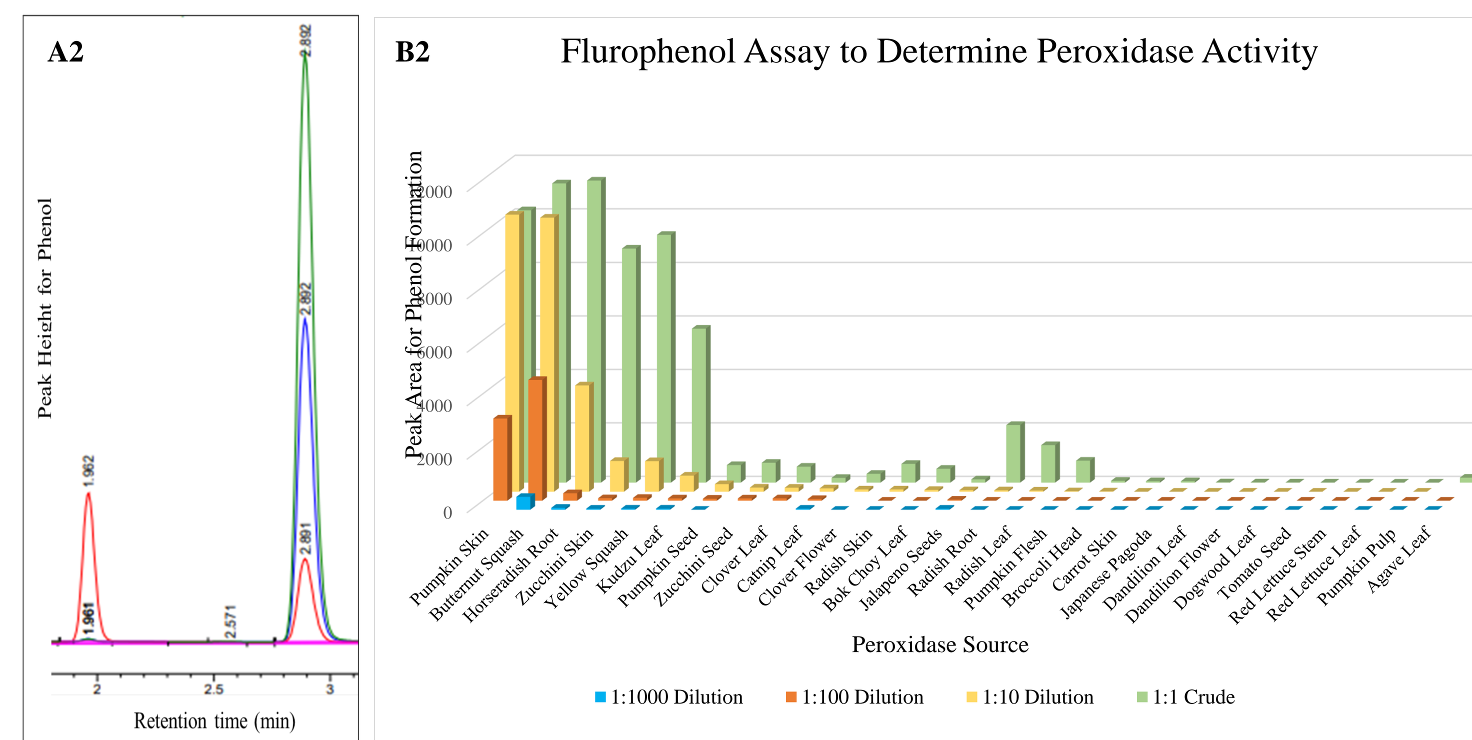


Figure 2. Detection of defluorination to determine peroxidase activity. (A) An HPLC chromatogram was obtained from testing a novel peroxidase. The peak at 1.962 minutes (red) is the peak for phenol, the defluorinated compound while the peak at 2.891 minutes is the peak for 4-fluorophenol (red), the initial substrate. The blue and green peaks represent compounds of the solvent. As with the heme chromatogram, the peak area is calculated by the computer program. (B) Defluorination activity of different peroxidases ranked by highest activity at a 1:10 dilution. Undiluted crude samples, 1:10 dilutions, 1:100 dilutions, and 1:1000 dilutions were tested as needed. Pumpkin skin showed the highest activity with butternut squash also having high activity. Horseradish had significantly less activity in this assay. Any samples with a peak area of less than 50 were not tested beyond the crude sample.

Results and Discussion

Butternut squash skin and pumpkin skin both expressed high levels of peroxidase activity in the guaiacol and fluorophenol assay when compared to horseradish root. Butternut squash had activity outside of the range of detection for both the crude and 1:10 dilution in guaiacol while pumpkin had an activity of 1.265 ΔA/min for the crude and 0.676 ΔA/min. for the 1:10 dilution. The slightly diminished activity in the pumpkin skin could be attributed to the extracted concentration of the pumpkin skin being about 100 mg/mL lower than that of both the horseradish and the butternut squash. However, the fluorophenol assay showed that pumpkin skin had an activity of 10,183 U^2 for the crude and 10,356 U^2 for the 1:10 dilution. In comparison, butternut squash had an activity of 11,186 U^2 for the crude and 10,244 U^2 for the 1:10 dilution. Aside from butternut squash and pumpkin skin, other sources that performed well were plants from the family Cucurbitaceae. Yellow squash and zucchini skin were in the top ten most active samples. Surprisingly, kudzu was relatively active along with catnip. The activity of the sweet potato skin in the guaiacol assay did not behave in a normal manner since the crude measurement was extremely high, while the 1:10 dilution was significantly lower than several other samples. This could have arisen from improper mixing before making the dilution. When specifically looking at the different parts of the pumpkin tested, it is clear that the vegetable as a whole does not have an even distribution of peroxidase throughout since the pumpkin flesh in the guaiacol and fluorophenol assays had very low activity and pumpkin pulp was even less active. The heme analysis showed that, unsurprisingly, butternut squash had the highest heme concentration of the samples tested. This confirms the hypothesis that heme concentration is related to peroxidase activity. More samples will be tested using heme analysis in the future to make a more complete view of the relationship between heme content and peroxidase activity. In conclusion, butternut squash and pumpkin skin both demonstrate activity similar enough to horseradish root that a more detailed analysis of these sources of peroxidase should be considered soon.

Summary

1. Pumpkin Skin and Butternut Squash skin possessed classical peroxidase activity comparable to Horseradish root.
2. The total peroxidase concentration found in Pumpkin skin was the highest among 20 samples tested.
3. Both Butternut Squash skin and Pumpkin skin showed very high activity with fluorinated aromatic substrates.

References

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