A Library of 39 Plant Peroxidases:

Plants from The Families Cucurbitaceae and Brassicaceae

Show Promising Peroxidase and Peroxygenase Activity

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Abstract

Peroxidases are enzymes that reduce reactive oxygen species (ROS) and oxidize organic substances in living cells. The textile industry, wastewater treatment plants, immunological laboratories, and diagnostic laboratories have extensively used horseradish peroxidase for their oxidating needs. This research aims to provide a tool for identifying potentially useful sources of peroxidases in addition to specifically pinpointing a novel peroxygenase in jalapeño seeds. By using two widely accepted peroxidase assays, the guaiacol assay and the fluorophenol assay, fruits of the genus Cucurbita and roots or leaves of the family Brassicaceae were seen to have high peroxidase activity. Additionally, novel peroxygenase activity was discovered in jalapeño seeds. As a result, an informative library of peroxidases was created.

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Introduction

The Peroxidase Reaction Mechanism

Peroxidases, a class of oxidizing enzymes, are used by living cells to react with toxic waste products formed during the necessary energy production common to most organisms. Toxic waste can also be produced during the formation of various molecules necessary for cell structures and maintenance (Abdel-Aty, A., et al., 2021). The toxic wastes products include many free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS are both toxic to the cell in which they are produced because of two reactive electrons positioned on either oxygen or nitrogen. ROS and RNS can travel through various parts of the cell causing unwanted and destructive reactions. Especially dangerous are the reactions with DNA, typically causing mutations that are usually fatal to the cell, but sometimes the reactions can cause cancer by damaging DNA that is necessary to prevent cancer (Cameron, A.M., et al., 2019). This is a potentially even more dangerous outcome since the cancerous cell could multiply and metastasize, overtaking the entire organism. Peroxidases prevent the damage to the cell's DNA by reacting with the free electrons on the ROS or RNS, stopping the molecule from reacting with any other vulnerable portions of the cell. Plants contain three classes of peroxidases, with Class III heme-containing peroxidases being the most widely used commercially (Aghelan, Z., & Shariat, S. Z. S., 2015). Heme-peroxidase contains an iron atom at the center of a porphyrin lattice. The iron-containing heme creates the active site for the entire enzyme because of the ability of iron to change valency to maximize stability. The change in

valency causes the reaction to occur, breaking down the reactive molecule usually into water and a by-product that is either harmless, or must be reacted with again to make it stable.

Current Uses for Horseradish Peroxidase

Significant research has been completed on horseradish peroxidase (HRP), a prominent and small heme-containing peroxidase, because it was likely the first peroxidase discovered (Veitch, N.C., 2004). In its earliest history, horseradish was recorded as making guaiac resin change in color to a brilliant blue. Since then, researchers discovered the cause behind the special properties of horseradish, eventually identifying peroxidase as the enzyme responsible. Even now, research into HRP continues to better understand peroxidases since it is widely used in several commercial, medical, and research applications (Veitch, N.C., 2004).

Commercially, HRP is used to clean wastewater from textile plants and other wastewater processing facilities (Chaojie, Z., et al., 2007; Achar, R.R., et al., 2017). The textile industry contributes large volumes of contaminated wastewater containing residual concentrations of dyes known to be carcinogenic. Once a dye has been used to color fabrics and threads, it is frequently released into rivers or wastewater systems without additional treatment (Al-Tohamy, R., et al., 2022). The dyes commonly used by textile plants, before being cured, are very reactive with DNA since the chemical structure necessary to create color for many dyes contains multiple reactive oxygens (Achar, R.R., et al., 2017). Eco-conscious textile plants use HRP in their wastewater treatment to decolorize the dyes, ensuring that the color of the dye does not inhibit photosynthetic organisms from surviving or that the carcinogens do not harm aquatic life (Al-Tohamy, R., et al., 2022). Wastewater facilities for municipalities and other industries use HRP in activated sludges that contain many other enzymes and organisms that thoroughly clean the water of many toxic contaminates (Chaojie, Z., et al., 2007).

In addition to HRP being used commercially, it is also extensively used by the health care industry for conducting various diagnostic tests. Specifically, the enzyme-linked immunosorbent assay (ELISA) is widely used in diagnostic laboratories to test if a patient has had various infections, either viral or bacterial. For example, during the COVID-19 pandemic, some laboratories ran ELISAs as a cheaper alternative to reverse transcriptase polymerase chain reaction (RT-PCR) to check for self-made antibodies against the virus present in the patient's blood serum after a suspected infection or preventative vaccination (Villafañe, L., et al., 2022). The ELISA test works by binding targeted antibodies or antigens to the components of the assay including HRP. When binding occurs, HRP is activated and breaks down chemicals that give off color. The intensity of color can be measured by a spectrophotometer and relative concentrations of the target can be measured. Although ELISA is one of the main assays used in the medical field that relies on peroxidases, there are three histological staining methods that also use HRP. Immunohistochemistry (IHC), immunocytochemistry (ICC), and immunofluorescence (IF) are techniques used by laboratories to study biopsies of possibly infected or cancerous tissues. Although each differs slightly from the other as to what form the tissues are in when they are tested or how the image of the tissue appears, they all use HRP very much like it is used in the ELISA test.

Interestingly, just as HRP is used for ELISA, IHC, ICC, and IF in medical laboratories, it is also used by research laboratories for the same techniques and a few other heavily utilized imaging methods. Western blots, which reveal the presence or absence of targeted proteins, require the use of peroxidase. As with the previous methods, a sample is separated by protein size or charge using gel electrophoresis and transferred to a synthetic membrane. After treatment with a primary antibody targeting the specific protein, a secondary antibody conjugated to HRP

is added, binding to any primary antibody that found target proteins. Lastly, various reporting molecules are added according to the need of the researcher, which are then modified by peroxidase to appear under various light sources. This single technique is essential to the broad scientific community since proteins are integral to most clinical and informational research projects. Given the broad range of uses in many different fields of research and community life, the importance of peroxidase is evident.

Alternative Sources of Peroxidase

In view of the many applications for peroxidase, extensive research has been devoted to discovering alternatives to HRP that might provide better target binding, cost effectiveness, or a new usage. Various approaches to this goal have been conducted including cloning the HRP gene into bacteria and using selective purification. Many research projects have endeavored to characterize peroxidases from many plant species. Although successful, these studies have not provided a standardized and comparable metric for developing a suitable alternative to using HRP.

For example, a study completed by A. Abel-Aty and colleagues investigated the peroxidases present in garden cress sprouts. It was discovered that there were at least two distinct peroxidases present in the sprouts that were only active at specific pH ranges. Additionally, the two peroxidases had different ionic properties and activities. Ultimately, their purpose in the research was to offer an alternative to HRP specifically for use in industry (Abel-Aty, A., et al., 2021). In a similar manner, research associates R. Achar, and others investigated a peroxidase from the species *Caralluma umbellata*, testing it against HRP in decolorizing common phenolic dyes. As with the garden cress, *C. umbellate* peroxidase (CUP) was capable of decolorizing all but one of the dyes. Additionally, CUP was not as efficient as HRP in oxidizing p-nitrophenol,

an important compound to remove in the wastewater industry (Achar, R.R., et al., 2017). These two studies demonstrate how current research for alternative peroxidases is hampered by the fact that the plants being tested are not tested uniformly. While both appear to be at least as active as HRP, there is no simple way of comparing the two against each other. Since these two studies are merely a small window into the broad field of plant peroxidase research, this issue of non-uniformity is much more widespread.

One assay has been used by many researchers that has helped to provide some uniformity for the search for alternative peroxidases. Specifically, some studies have focused on using a guaiacol assay to characterize various plant peroxidases. A study that heavily utilized the guaiacol assay investigated the peroxidase activity of ginger root (El-Khonezy, M. I., et al., 2020). After purifying their enzyme, they concluded that ginger root could be used for some of the applications listed in the previous section, offering a possible alternative to HRP. Unfortunately, the researchers did not directly compare their findings to HRP, nor is their data easily compared to other plants being tested.

Like the ginger root study, peroxidase activity in corn roots was investigated. Researchers A. Mika and S. Lüthje greatly relied on the data collected from several guaiacol assays for their corn root study. Their purpose of researching peroxidases from corn roots was to understand the activity and characteristics of plasma membrane-bound peroxidases, since this specific type of peroxidase had not been researched before (Mika, A., and Lüthje, S., 2003). Although they utilized a broadly accepted method, their purpose for researching was not related to finding an alternative, making their data more difficult to compare against HRP activity.

Another example of a study devoted to characterizing a new peroxidase, but not specifically to provide an alternative to HRP, is one conducted by T. Köktep and his colleagues.

They tested haricot bean peroxidase using the guaiacol assay to find the K_m for the enzyme (Köktepe, T., et al., 2017). Their result showed a relatively high value, indicating that it seems likely that it would not be a suitable substitute for HRP. However, as with most of the other previous articles cited, there is not a direct comparison to HRP, making it difficult to confidently say the plant source is or is not worthwhile as a substitute for HRP.

Along the same lines as the previous studies, several other studies have characterized peroxidases from various plants, such as runner beans, plums, sweet gourds, avocado, and zucchini using the guaiacol assay as the primary test for determining peroxidase characteristics (Oztekin, A., and Tasbasi, S., 2020; Enachi, E., et al., 2018; Koksal, E., et al., 2012; Rojas-Reyes, J., et al., 2014; Wang, X., et al., 2019). However, none of these studies directly compared their results against HRP and many of them do not include any information regarding the capabilities of the peroxidase to complete similar duties normally assigned to HRP.

The Varied Uses of a Broad Library of Peroxidases

Given the evidence presented in the previous paragraphs, it can be discerned that although in-depth study of specific peroxidases is useful, it can turn out to be fruitless if the plant does not contain a high concentration of peroxidase. Because of this, a study superficially analyzing the activity of peroxidases in many different plants is key to directing future peroxidase research. Although the information provided is limited, it reveals enough characteristics of the plant to indicate if further testing is recommended. Having such a resource could prevent hours of research and thousands of dollars from being poured into plant sources that do not have useful peroxidase activity. However, such extensive effort would be warranted once a peroxidase source is identified to fully characterize the protein and its expression. This has already been done for peroxidases confirmed to be active, such as HRP.

As an example, one research project strove to develop a cost-effective method for producing HRP. Genes of 19 peroxidases from horseradish were cloned into the bacteria *Pichia pastoris* with the hope that the highly proliferative bacteria would produce the enzyme at a lower cost, and with higher activity (Krainer, F.W., et al., 2014). Unfortunately, important properties of the enzymes were altered by post-translational glycosylation, decreasing the oxidative activity of the peroxidases, and limiting their usefulness. Although the research was not fully successful, Krainer's study highlights the need for a more cost-effective peroxidase source.

Stemming from this example is another aspect to be considered as a next step after a peroxidase source is characterized. Krainer's study revealed that individual plants can contain many different peroxidases that might be used in different ways. This was also emphasized by a study involving pumpkin peroxidases where alternative gene-splicing was demonstrated to produce distinct peroxidases (Mano, S., et al., 1997). From these examples, it is clear that once a plant can be shown to have valuable peroxidase activity, further investigation is warranted.

Once a plant source is established as useful for peroxidases, it is also important to consider the anatomical and histological location of peroxidase production in highly active plants. An example of such a study is the research completed by S. Carpin and her colleagues into the tissue specific expression of an anionic peroxidase in zucchini seedlings. This study focused on one particular peroxidase, rather than all four of the known peroxidases in the zucchini seedlings (Carpin, S., et al., 1999). Their findings showed that the root walls of the seedlings had the highest presence of anionic peroxidase.

Because this fact pinpoints where the peroxidase is most present, it can be used to ensure that when a peroxidase is being extracted, the part that has the most peroxidase is used. Additionally, it could help elucidate the specific role of the peroxidase. Another characteristic of

peroxidase that is implied in the article by S. Carpin, is the manner of expression of the peroxidase based on the maturity of the plant. Given the highly complex nature of cellular biology, it cannot be assumed that the expression of a peroxidase is steady throughout the lifetime of a plant, or a specific part of a plant.

Considering the studies presented before, in-depth investigation into peroxidases and their activities is warranted and needed. Before this detailed research should be completed, plants with high peroxidase activity first must be identified. Once these have been found, purification and thorough characterization should follow.

The Purpose of the Research Presented

With the foundation laid by the previous information, the necessity of having a uniformly produced library of peroxidases is clear. There is a gap in knowledge surrounding the issue of what plant sources are favorable for comprehensive peroxidase research, or even what categories of plants might be more likely to have activity. This is particularly important with respect to finding a suitable alternative to HRP in its various applications. This research endeavors to provide a library of peroxidases that will fulfill the need of identifying favorable peroxidases in general, but specifically identifying which peroxidases might act as substitutes for HRP, or present new enzymes by identifying novel activities.

The guaiacol assay was used as a primary test to standardize the activities of various plant peroxidases. The use of this assay as a standard in peroxidase research is clearly demonstrated by the multitude of articles cited earlier. The guaiacol assay is particularly useful because it takes advantage of a colorimetric change that is caused by a reaction performed by peroxidase. In the reaction, guaiacol, a phenolic ring with a methoxy group in the ortho position, reacts with hydrogen peroxide. Through multiple secondary reactions involving variations in the

shape of the peroxidase, four guaiacol molecules merge to form tetraguaiacol (Figure 1). Ultimately, the final molecule features two guaiacol pairs bound at the ortho position, having undergone oxidation of the methoxy group. The pairs are also bound to each other by a single carbon-carbon bond in the para position (de Oliveira, F.K., et al., 2021). Tetraguaiacol is a relatively stable molecule since it is non-polar. Considering that the side product is water, the reaction transforms highly reactive molecules into relatively, or completely, harmless chemicals. In addition to tetraguaiacol being a harmless relative of guaiacol, it also has a deep amber color that is easily detected by a spectrophotometer. Guaiacol as the substrate tends to allow for higher V_{max} values for peroxidases, indicating that the enzymes have a fast turnaround time and are quickly able to perform another reaction (de Oliveira, F.K., et al., 2021).

Since the goal of this research was to act as the preliminary step before in-depth research, in addition to cutting costs and time, the peroxidases were tested in their unpurified (crude) form. Although the concentration of peroxidase might vary from plant to plant based on size, standardization was preserved by maintaining the same ratio of plant mass to extraction buffer volume (m/v ratio). One benefit to this method is that the absolute concentrations of the peroxidases were revealed through the lack of purification. High levels of peroxidase activity indicate that the plant is rich in peroxidase. Moderate activity could indicate that purification would yield a substantial amount of enzyme. Low and very low activity might indicate that the tissue is either devoid of peroxidase or has extremely low quantities of the enzyme. Although it might still be the most beneficial to have all 39 peroxidases purified, this is not reasonable given the amount of time and resources this would take. Additionally, the purpose of the research justifies the modified method. The approach of not fully purifying peroxidases prior to assaying is also supported by the research of F. Krainer and colleagues who used a modified purification

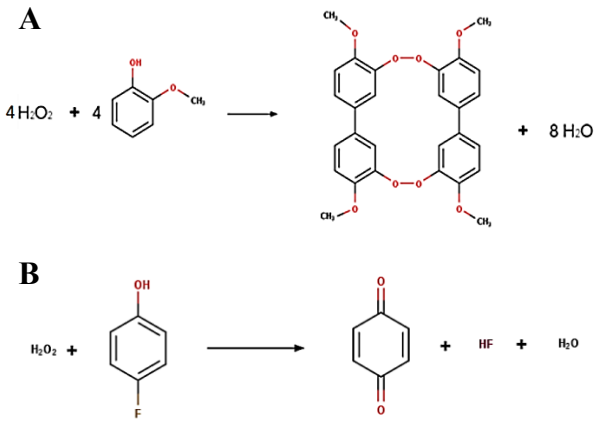

Figure 1. Reaction mechanisms of peroxidase in two assays. (A) The reaction mechanism for the guaiacol assay, with guaiacol as the substrate and tetraguaiacol as the primary product. (B) The reaction mechanism for the fluorophenol assay, with 4-fluorophenol as the substrate and quinone as the primary product.

method to investigate 19 different peroxidases from horseradish (Krainer, F.W., et al., 2014). As another safeguard against skewed data from unpurified samples, unpurified HRP (prepared identically) was used as a standard. Lastly, the linearity of activity of the peroxidases was confirmed by performing progressive dilutions of each enzyme.

As a secondary measure to detect peroxidase activity, a fluorophenol assay was performed. This is another assay for characterizing peroxidases (Buttner, E., et al., 2015). This assay works through a reaction of peroxidase with the substrate fluorophenol in the presence of hydrogen peroxide (Figure 1). In this reaction, the fluoro group on the phenol ring is removed by peroxidase, producing a quinone ring. Since the fluorophenol assay is more specific to the roles filled by HRP, the results from this assay are particularly valuable to finding an alternative to HRP. As with the guaiacol assay, standardization of sample preparation, including for horseradish, allowed activities to be compared against each other.

Materials and Methods

Plant Peroxidase Library: Extraction of Peroxidases

Plants purchased from the local grocery store or gathered from the natural surroundings were washed with water to remove any apparent dirt. Specific parts of some of the plants were isolated. For each plant part or kind, a 1:2 ratio of plant mass (g) to extraction buffer (1.0 M phosphate buffer, pH 7.0) volume (mL), was made. Using a ceramic mortar and pestle, each sample was ground into a smooth liquid or a fine pulp. Absorbent plant material was given more buffer, up to a 1:10 ratio of plant mass to extraction buffer volume. Each sample was then centrifuged at 4000 x g for 15 min and 1000 µL of the supernatant was stored in a microcentrifuge tube at <0° C.

Plant Peroxidase Library: Guaiacol Assay

Triplicates of individual crude plant extracts were run by adding 10 µL of the extract to 490 µL of guaiacol reaction buffer (5 mM guaiacol, 5 mM H_2O_2, 50 mM phosphate buffer, pH 6.5) in a GENESYS 10S UV-VIS spectrophotometer using the kinetic program. The absorbance was measured at 490 nm over 1 minute at 10 second intervals. The same was repeated for crude plant extracts diluted in extraction buffer at 1:10, 1:100, 1:1000 dilutions or up to losing all activity. Samples were run against a blank of pure reaction buffer.

Plant Peroxidase Library: Fluorophenol Assay

As with the guaiacol assay, triplicates of the individual crude samples were run by adding 10 µL of the extract to 490 µL fluorophenol reaction buffer (5 mM 4-fluorophenol, 5 mM H_2O_2, 50 mM phosphate buffer, pH 6.5) for 5 minutes at 30° C. To quench the reaction, 200 µL of 1 M HCl was added to each sample with vortexing. The samples were centrifuged at 14,000 x g for 5 minutes.

Plant Peroxidase Library: High Performance Liquid Chromatography

All samples were read using an Agilent Technologies 1260 Infinity II HPLC programmed with a C_{18} reverse phase HPLC column, a liquid phase of 60% H_2O with 0.1% trifluoroacetic acid and 40% acetonitrile with 0.1% trifluoroacetic acid, a flow rate of 1.200 mL/s, an injection volume of 25 µL, a run time of 4 minutes, and an absorbance read at 254 nm, 275 nm, and 290 nm.

Jalapeño Peroxygenase: Extraction of Peroxygenase

Jalapeño seeds were taken from fresh jalapeños and crushed using a mortar and pestle with a 1:5 ratio of jalapeño seed mass (g) to extraction buffer (20 mM phosphate buffer, pH 7.0) volume (mL). As with the other plant samples, the pulverized seeds with buffer were centrifuged at 4000 x g for 15 minutes and stored at 4-8° C. One 900 µL aliquot, one 990 µL aliquot, and one 980 µL aliquot of the crude extract was separated and to the first aliquot, 100 µL of pure glycerol was added and vortexed, the second aliquot received 10 µL of Tween 20, and the third received 20 µL of Tween 20. All aliquots were stored at 4-8° C.

Jalapeño Peroxygenase: Generic Fluorophenol Assay

Jalapeño seed and pumpkin skin (from the library stock) samples were tested in duplicate using 20 µL of sample with 490 µL of fluorophenol reaction buffer for 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, 60 min, 120 min, 180 min, 240 min, 300 min, 330 min, 2880 min, and 10,080 min using the same method as the one for the library, except that the reaction time was as written above and the samples were quenched with 200 µL of 100% methanol. Another set of samples run alongside these reactions had the methanol added at the beginning instead of at the end to demonstrate that the new enzyme was not quenched by methanol.

Jalapeño Peroxygenase: Glutathione Testing

Crude jalapeño seed samples were processed by the fluorophenol assay as outlined by the Generic Fluorophenol Assay, but instead the samples were run for 2 hours. Two samples were run normally, quenching with methanol at the end and centrifuging for 5 min at 14000 x g. Two samples were also run normally, but before adding the methanol, the solution was given a 1 mM concentration of glutathione, originally intended to quench the reaction. These samples were tested using the same HPLC program outlined for the method "Jalapeño Peroxygenase: Generic Fluorophenol Assay."

Jalapeño Peroxygenase: Glutathione Complex

The same assay as outlined in the previous method section was completed again for two jalapeño seed samples not treated with glutathione, two with glutathione, and two more samples without crude jalapeño seed extraction, but with the 1 mM glutathione. The injection volume was 25 µL, and the flow rate was 1.200 mL/s. For the first three minutes of the run, the liquid phase was 98% H_2O with 0.1% trifluoroacetic acid and 2% acetonitrile with 0.1% trifluoroacetic acid. The next 4 minutes of the run, the liquid phase was changed to 60% H_2O with 0.1% trifluoroacetic acid and 40% acetonitrile with 0.1% trifluoroacetic acid. The last 3 minutes of the run, the liquid phase returned to the first composition of 98%/2% to flush out the column. Results were read at 210 nm, 230 nm, 250 nm, 254 nm, 270 nm, and 280 nm.

Results

Peroxidase Library: Guaiacol Assay

The measure of the activity for the peroxidase is the ΔAbsorbance/min (ΔA/min) with a larger value indicating greater activity since more product is being formed. All numerical data is the triplicate average ΔA/min values at the 1:10 dilution. Ranking was determined based on the

1:10 dilution to ensure that any possible variation in the true activity of the source did not originate from random error. For example, a gradient of concentration in the original sample or interference from a large quantity of other proteins could make the activity appear to be either higher or lower than normal. An overview of the numerical data can be seen in Table 1 and the data is visualized in Figure 2. It should be noted that in the guaiacol assay, butternut squash and pumpkin skin had comparable or higher activity than horseradish peroxidase. Specifically, butternut squash had a ΔA/min of 2.5 with no dilution (1:1), 2.5 at a 1:10 dilution, 0.75 at a 1:100 dilution, and 0.069 at a 1:1000 dilution. Horseradish had slightly lower activity in this assay with a ΔA/min of 2.5 with no dilution (1:1), 1.5 at a 1:10 dilution, 0.15 at a 1:100 dilution, and 0.003 at a 1:1000 dilution. From the top three peroxidases, pumpkin skin had the lowest peroxidase activity with an ΔA/min of 1.3 with no dilution (1:1), 0.68 at a 1:10 dilution, and 0.081 at a 1:100 dilution. The 1:1000 dilution was not tested since the 1:100 dilution was already below an activity of 0.1 ΔA/min. Other notable peroxidases were from Brussel sprouts, kudzu leaf, catnip leaf, and zucchini skin. The following peroxidases had an activity of less than 0.30 but greater than 0.10 at the 1:10 dilution, including yellow squash, radish stems and skin, sweet potato, carrot skin, russet potatoes, broccoli stalks and florets, zucchini seeds, green peas, and green bean seeds. There were twenty-seven other plants or plant parts tested for peroxidase with an activity of less than 0.10 ΔA/min at a dilution of 1:10, indicating that they are likely not suitable for further peroxidase research.

Peroxidase Library: Fluorophenol Assay HPLC

The measure of the activity for the fluorophenol assay was the peak area of the reaction product found at approximately 1.95 minutes retention time and at a wavelength of 254 nm. A

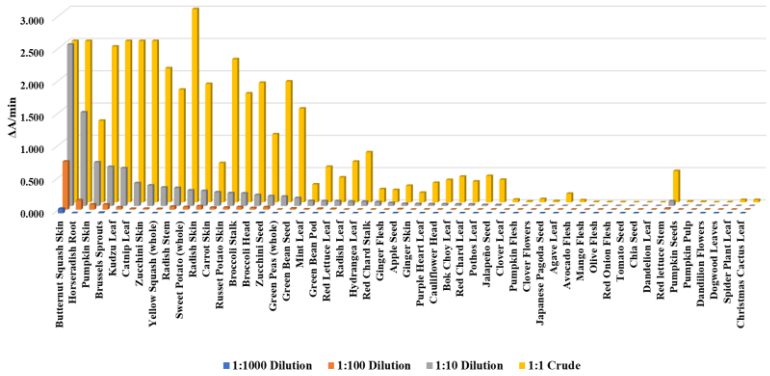

Figure 2. Peroxidase activity of 53 plant sources from a guaiacol assay. The average ΔAbsorbance/min for duplicates of each sample at three dilutions and the original concentration are represented to emphasize the high activity of butternut squash and pumpkin skin. The importance of ranking based on the 1:10 dilution is visible particularly for radish skin, which had an extremely high ΔA/min in the crude sample but had a significantly decreased activity for the 1:10 dilution. Samples with no activity were included to emphasize the relationship between the anatomical and taxonomical factors that might contribute to peroxidase activity.

Table 1. Peroxidase activity of 53 plant sources in a guaiacol assay measured in ΔA/min. Sources were tested until the activity was below 0.025 ΔA/min. Sources are ranked based on activity at the 1:10 dilution.

Rank	Plant part	1:1000 Dilution	1:100 Dilution	1:10 Dilution	1:1 Crude
1	Butternut Squash Skin	0.07	0.75	2.5	2.5
2	Horseradish Root	0.00	0.15	1.5	2.5
3	Pumpkin Skin	0.00	0.08	0.68	1.3
4	Brussels Sprouts	0.01	0.08	0.61	2.4
5	Kudzu Leaf	0.00	0.04	0.59	2.5
6	Catnip Leaf	0.00	0.01	0.35	2.5
7	Zucchini Skin	0.00	0.01	0.32	2.5
8	Yellow Squash (whole)	0.00	0.01	0.29	2.1
9	Radish Stem	0.00	0.05	0.28	1.7
10	Sweet Potato (whole)	0.00	0.04	0.24	3.0
11	Radish Skin	0.00	0.05	0.23	1.8
12	Carrot Skin	0.00	0.03	0.21	0.6
13	Russet Potato Skin	0.00	0.03	0.20	2.2
14	Broccoli Stalk	0.00	0.04	0.20	1.7
15	Broccoli Head	0.00	0.03	0.17	1.9
16	Zucchini Seed	0.00	0.04	0.15	1.1
17	Green Peas (whole)	0.00	0.00	0.15	1.9
18	Green Bean Seed	0.00	0.02	0.12	1.5
19	Mint Leaf	0.00	0.00	0.08	0.28
20	Green Bean Pod	0.00	0.01	0.08	0.55
21	Red Lettuce Leaf	0.00	0.01	0.08	0.39
22	Radish Leaf	0.00	0.00	0.07	0.63
23	Hydrangea Leaf	0.00	0.00	0.07	0.78
24	Red Chard Stalk	0.00	0.00	0.06	0.21
25	Ginger Flesh	0.00	0.00	0.05	0.19
26	Apple Seed	0.00	0.01	0.03	0.26
27	Ginger Skin	0.00	0.00	0.03	0.15
28	Purple Heart Leaf	0.00	0.00	0.03	0.30
29	Cauliflower Head	0.00	0.00	0.03	0.35
30	Bok Choy Leaf	0.00	0.00	0.03	0.40
31	Red Chard Leaf	0.00	0.00	0.03	0.32
32	Pothos Leaf	0.00	0.00	0.02	0.41
33	Jalapeño Seed	0.00	0.00	0.02	0.35
34	Clover Leaf	0.00	0.00	0.00	0.04
35	Pumpkin Flesh	0.00	0.00	0.00	0.01
37	Clover Flowers	0.00	0.00	0.00	0.05
38	Japanese Pagoda Seed	0.00	0.00	0.00	0.02
39	Agave Leaf	0.00	0.00	0.00	0.14
40	Avocado Flesh	0.00	0.00	0.00	0.03
41	Mango Flesh	0.00	0.00	0.00	0.01
42	Olive Flesh	0.00	0.00	0.00	0.00
43	Red Onion Flesh	0.00	0.00	0.00	0.00
44	Tomato Seed	0.00	0.00	0.00	0.00
45	Chia Seed	0.00	0.00	0.00	0.00
46	Dandelion Leaf	0.00	0.00	0.00	0.00
47	Red lettuce Stem	0.00	0.02	0.07	0.49
48	Pumpkin Seeds	0.00	0.00	0.00	0.01
49	Pumpkin Pulp	0.00	0.00	0.00	0.01
50	Dandilion Flowers	0.00	0.00	0.00	0.00
51	Dogwood Leaves	0.00	0.00	0.00	0.00
52	Spider Plant Leaf	0.00	0.00	0.00	0.04
53	Christmas Cactus Leaf	0.00	0.00	0.00	0.04

larger peak area indicates more reaction product was formed in the same amount of time. As with the guaiacol assay, all data was reported as the average of the triplicate data for the sample.

Once the activity disappeared or was greatly reduced, no further dilutions were tested. The raw data and the averages are fully reported in Table 2. A visual representation of the data is in Figure 3. As before, the top three activities were found in pumpkin skin, butternut squash, and horseradish, in a different order than in the guaiacol assay. Pumpkin skin had an average peak area (APA) of 10,000 without dilution (1:1); 10,000 at a 1:10 dilution; 3,100 at a 1:100 dilution;

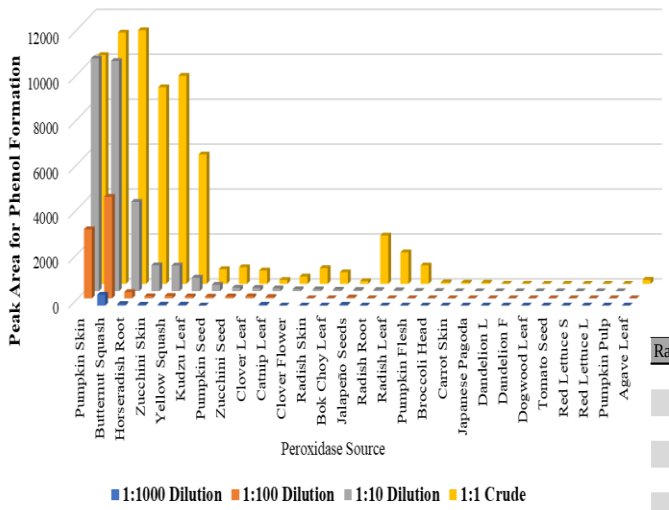

Figure 3. Peroxidase activity of 28 plant sources in a fluorophenol assay. The average ΔAbsorbance/min for duplicates of each sample at three dilutions and the original concentration are represented to emphasize the high activity of butternut squash and pumpkin skin. The importance of ranking based on the 1:10 dilution is visible particularly for radish skin, which had an extremely high ΔA/min. in the crude sample, but had a significantly decreased activity for the 1:10 dilution. Samples with no activity were included to emphasize the relationship between the anatomical and taxonomical factors that might contribute to peroxidase activity.

Table 2. Peroxidase activity of 28 plant sources in a fluorophenol assay measured by reaction product peak area. Some sources are missing data for the 1:1000 dilution due to a lack of enzyme stability or low activity in the 1:100 dilution. Sources with peak areas below 51 units were not tested further. All sources were ranked by the activity in the 1:10 dilution.

Rank	Plant part	1:1000 Dilution	1:100 Dilution	1:10 Dilution	1:1 Crude
1	Pumpkin Skin		3100	10400	10000
2	Butternut Squash	480	4500	10200	11000
3	Horseradish Root	72	280	3970	11000
4	Zucchini Skin	40	92	1200	8800
5	Yellow Squash	41	110	1100	9300
6	Kudzu Leaf	42	86	600	5800
7	Pumpkin Seed	0	76	280	660
8	Zucchini Seed		85	150	740
9	Clover Leaf		89	150	600
10	Catnip Leaf	41	63	130	180
11	Clover Flower	0		84	330
12	Radish Skin	0	0.0	84	700
13	Bok Choy Leaf	0	0.0	66	520
14	Jalapeño Seeds	39	40	54	120
15	Radish Root	0	0.0	50	2200
16	Radish Leaf	0	0.0	42	1400
17	Pumpkin Flesh	0	0.0	14	820
18	Broccoli Head	0	0.0	8	68
19	Carrot Skin	0	0.0	0.0	54
20	Japanese Pagoda	0	0.0	0.0	54
21	Dandelion L	0	0.0	0.0	11
22	Dandelion F	0	0.0	0.0	9.5
23	Dogwood Leaf	0	0.0	0.0	5.4
24	Tomato Seed	0	0.0	0.0	4.3
25	Red Lettuce S	0	0.0	0.0	2.0
26	Red Lettuce L	0	0.0	0.0	2.0
27	Pumpkin Pulp	0	0.0	0.0	1.7
28	Agave Leaf	0	0.0		190

and a 1:1000 dilution was not tested because the original crude extract became inactive. The next peroxidase, in order of activity, was butternut squash which had 11,000 without a dilution (1:1); 10,000 at a 1:10 dilution; 4,500 at a 1:100 dilution; and 480 at a 1:1000 dilution. In comparison, horseradish root had 11,000 without a dilution (1:1); 4,000 at a 1:10 dilution; 280 at a 1:100 dilution; and 72 at a 1:1000 dilution. With a similar order of activity as the last assay, zucchini skin, yellow squash, kudzu leaf, pumpkin seeds, zucchini seeds, clover leaf, and catnip leaf all

had activities higher than an APA of 100 for the 1:10 dilution. This could indicate that these sources could be investigated further with the possibility of useful results. There were many plant sources and parts that had activity lower than an APA of 100, most of which correspond to ones which were not active in the guaiacol assay. In order of decreasing APA, there was clover flower, radish skin, bok choy leaf, jalapeño seeds, radish root, radish leaf, pumpkin flesh, broccoli florets, carrot skin, Japanese pagoda seeds, dandelion leaves and flowers, dogwood leaf, tomato seeds, red lettuce stalk and leaves, and pumpkin pulp.

Jalapeño Peroxygenase: Optimal Reaction Time for Unique Product

When jalapeño seeds were tested for peroxidases using the fluorophenol assay, it was observed that there was a distinct product peak not seen in any of the other plants tested. Since this new product might indicate a novel peroxygenase, effort was made to characterize it. The primary experiment for initial characterization of the jalapeño seed peroxygenase (JSP) was the fluorophenol assay run at progressively longer periods of time to determine the optimal reaction time. The area of the unique peak at a retention time of 2.47-2.52 minutes was used as a measure of activity and only signals from an absorbance of 270 nm were used for the data. The absorbance wavelength of 270 nm was seen to be the optimal absorbance. Averages of duplicate trials are reported for each reaction time length and raw data can be found in Table 3 with a visual representation in Figure 4. All samples were run in methanol for the duration of the reaction to demonstrate the capability of the enzyme to function in a hostile environment. It was determined that the optimal reaction time was 60 min since the APA for the product peak reached its maximum at this time with a value of 360. Reactions that were run longer than this time also produced peaks with a relatively stable APA ranging from values of 290-340. However, after 300 minutes, the stability of the product appeared to decline as the APA

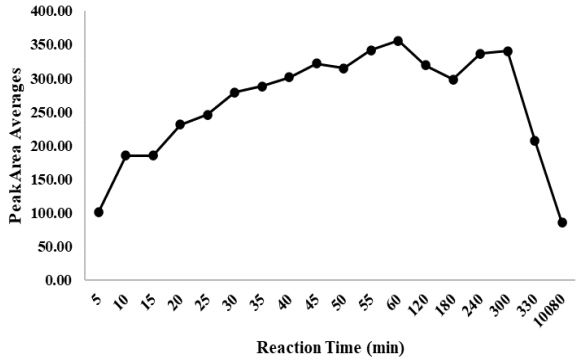

Figure 4. Optimal reaction time for jalapeño peroxygenase. The reaction time that yielded the most product (based on peak area) was at 60 min. Relatively the same levels of product were made and remained stable through 300 min. After 300 min. the stability of the product decreased significantly.

Table 3. Raw data for optimal reaction time for jalapeño peroxygenase. For 50 min. and 55 min. only one sample was tested.

Reaction Time (min.)	Peak Area 1	Peak Area 2	Peak Area Averages
5	89	114	100
10	190	180	190
15	195	175	190
20	234	229	230
25	237	255	250
30	278	280	280
35	301	275	290
40	302	302	300
45	324	320	320
50	315	--	310
55	342	--	340
60	395	316	360
120	349	291	320
180	322	275	300
240	347	327	340
300	339	342	340
330	241	174	210
10080	13	13	13

decreased significantly. The pumpkin skin extraction run alongside the JSP showed no peaks at the retention time span given, until the reaction time reached 240 min. Even at that time, the average peak area (APA) was only 11, and at 300 min, it was only 10.

Jalapeño Peroxygenase: Stability Over Time

Storage stability of JSP was determined using the fluorophenol assay, using the APA for the unique product peak found at either 0.48-0.59 minutes of retention time for the C_{18} reverse phase HPLC column or 2.46-2.49 minutes. All samples were measured in duplicate at an absorbance of 270 nm. All data for the stability testing can be seen in Table 4 with a visual representation in Figure 5. The initial activity of the standard crude jalapeño seed extract (SC-JSE) and the glycerol-treated jalapeño seed extract (GT-JSE) was 330 units, the 1% Tween 20 treated jalapeño seed extract (1TT-JSE) had initial activity of 370, and the 2% Tween 20 treated jalapeño seed extract (2TT-JSE) had initial activity of 380. These values show that the initial

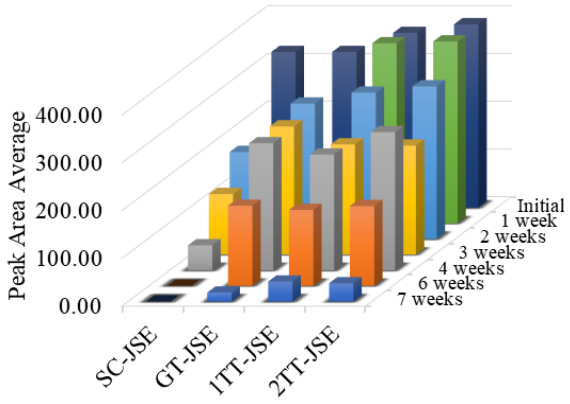

Figure 5. Peroxygenase storage stability in hydrophobic solutions. Untreated jalapeño seed extraction (SC-JSE) lost activity within two weeks, but all three peroxygenase samples treated with a hydrophobic solution maintained high activity through the fourth week. Although the glycerol treated sample (GT-JSE) and the 1% Tween 20 treated sample (1TT-JSE) maintained activity, the 2% Tween 20 treated sample (2TT-JSE) had the highest activity overall.

Table 4. Jalapeño peroxygenase stability over time. The activity of the enzyme over 7 weeks was determined by HPLC product peak area. Data for the untreated sample (SC-JSE) and the glycerol treated sample (GT-JSE) was not recorded at the first week. Overall, the samples treated with 1% Tween 20 (1TT-JSE) and 2% Tween 20 (2TT_JSE) maintained activity the best, but 2TT-JSE ultimately maintained the best activity.

Time	SC-JSE	GT-JSE	1TT-JSE	2TT-JSE
Initial	330	330	370	380
1 week			380	380
2 weeks	180	280	300	320
3 weeks	130	270	230	230
4 weeks	52	270	240	290
6 weeks	0	170	160	170
7 weeks	0	19	42	38

activity of the four samples was relatively stable, although it might appear that Tween 20 gives the enzyme more stability. After one week, the samples treated with hydrophobic solutions did not experience any relative deactivation. By week two, SC-JSE activity was declining rapidly, but the other three samples still had relatively stable activity. Even though the activity of all the samples continued to decrease, the three hydrophobic samples maintained relatively high activity through the fourth week. Data for the fourth week showed SC-JSE activity at an APA of 53, while GT-JSE activity was at 270. 1TT-JSE activity had an APA of 240, and 2TT-JSE activity was 290. The fifth week, the samples were not tested. By the sixth week, all SC-JSE activity was lost, but the activity of GT-JSE, 1TT-JSE, and 2TT-JSE was still showing an APA over 100 for all three. In the final week of testing, all samples showed negligible activity with APA for each being less than 15 units.

Jalapeño Peroxygenase: Glutathione Quenching and Complexing

As the next step in characterizing JSP, a new quenching method was employed using glutathione. Instead of glutathione only reacting with products actively being made, glutathione also appeared to react with the already made primary product of the reaction (Figure 6). This was evidenced through the fact that when treated with glutathione, the product peak disappeared completely. Since the secondary reaction might have produced a more stable product suitable for identification as an aid to characterization, a new method was conducted as outlined in the "Jalapeño Peroxygenase: Glutathione Complex" section of the methods. As expected, no primary product peak was observed, but evidence for a unique product peak was seen at 0.406 minutes. Only the sample containing both the enzyme and glutathione gave rise to this peak, and the controls each showed no peak at that time.

Specifically, both controls, one containing only the enzyme, no glutathione, and the other only containing glutathione, no enzyme, produced no peaks at a retention time of 0.395-0.410. The experimental sample gave consistent results with relatively equal heights of the peak between duplicates. Since this was the very beginning of the investigation, no qualitative values of peak area were found or recorded.

Discussion

The Relationship Between Peroxidase Activity and Plant Taxonomy

The driving purpose in developing a peroxidase library was to discover novel peroxidases that could be further characterized for use in industrial applications as an alternative to HRP. However, another motivation was to provide the biochemical research community with a resource that identified plants that potentially contain useful peroxidases. To better clarify what

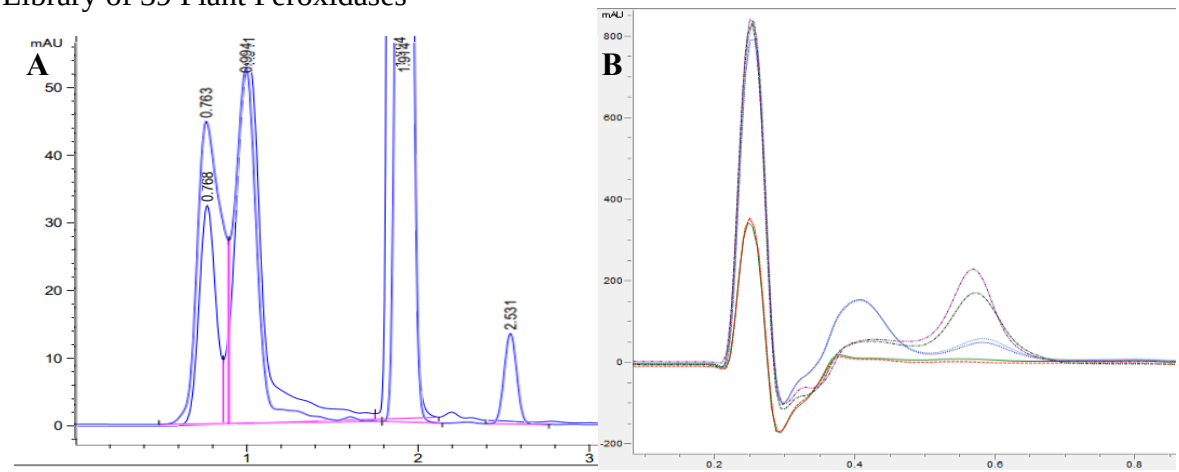

Figure 6. The destruction of the primary product by glutathione to form a more stable secondary product as seen for jalapeño peroxygenase. (A) Two chromatographs of jalapeño seed extract were overlayed to show the loss of the reaction peak in the sample treated with glutathione. The original sample, reacted without glutathione, shows a peak at 2.531 minutes. Alternatively, the sample reacted with glutathione displays no peak at ~ 2.5 minutes. This result demonstrates the secondary reaction that is hypothesized to occur between the primary product and glutathione. (B) This overlayed chromatograph with an original sample containing no glutathione (green and red lines), a sample reacted with glutathione (dark blue and teal lines), and reaction buffer with glutathione (dark green and burgundy lines) shows a unique peak in the reacted sample at 0.406 minutes. Since a peak at that time is not present in either of the two controls, it is reasonable to hypothesize that the novel peak is the secondary product of the reaction between the primary product and glutathione.

plants might be better suited for peroxidase research, Table 5 organizes the plants tested into their taxonomic categories, starting with the *Clade*. In plant taxonomy naming, there have been several iterations of classifications, with the most recent being that of the angiosperm phylogeny group's (APG) fourth system published in 2016 (APG IV. Byng, J.W., et al., 2016). Subclades separate plants that all qualify under the very broad classification of angiosperms and generally either as eudicots or monocots. Under eudicots, there are two large clades, smaller than the previous distinction, but containing a very broad variety of plants. These are then divided into orders. A simplified taxonomic naming scheme was utilized, that disregards subfamilies, tribes, and any other narrow classifications that add confusion for the purpose of this paper, namely, to generally identify more promising plant orders, families, or genera that might contain plants with

Table 5. Fifty-two plant parts organized by scientific classification and peroxidase activity. The primary activity used for ranking was the guaiacol assay. The fluorophenol assay was secondary activity.

Clade	Clade	Order	Family	*Genus*	Guaiacol activity	Fluorophenol activity	Plant part
Eudicots	Rosids	Cucurbitales	Cucurbitaceae	*Cucurbita*	1	2	Butternut Squash Skin
Eudicots	Rosids	Cucurbitales	Cucurbitaceae	*Cucurbita*	3	1	Pumpkin Skin
Eudicots	Rosids	Cucurbitales	Cucurbitaceae	*Cucurbita*	7	4	Zucchini Skin
Eudicots	Rosids	Cucurbitales	Cucurbitaceae	*Cucurbita*	8	5	Yellow Squash (whole)
Eudicots	Rosids	Cucurbitales	Cucurbitaceae	*Cucurbita*	16	8	Zucchini Seed
Eudicots	Rosids	Cucurbitales	Cucurbitaceae	*Cucurbita*	35	17	Pumpkin Flesh
Eudicots	Rosids	Cucurbitales	Cucurbitaceae	*Cucurbita*	48	7	Pumpkin Seed
Eudicots	Rosids	Cucurbitales	Cucurbitaceae	*Cucurbita*	49	34	Pumpkin Pulp
Eudicots	Rosids	Brassicales	Brassicaceae	*Armoracia*	2	3	Horseradish (whole)
Eudicots	Rosids	Brassicales	Brassicaceae	*Brassica*	4		Brussels Sprouts
Eudicots	Rosids	Brassicales	Brassicaceae	*Raphanus*	9		Radish Stem
Eudicots	Rosids	Brassicales	Brassicaceae	*Raphanus*	11	12	Radish Skin
Eudicots	Rosids	Brassicales	Brassicaceae	*Brassica*	14		Broccoli Stalk
Eudicots	Rosids	Brassicales	Brassicaceae	*Brassica*	15	18	Broccoli Head
Eudicots	Rosids	Brassicales	Brassicaceae	*Raphanus*	22	16	Radish Leaf
Eudicots	Rosids	Brassicales	Brassicaceae	*Brassica*	29		Cauliflower Head
Eudicots	Rosids	Brassicales	Brassicaceae	*Raphanus*		15	Radish Root
Eudicots	Rosids	Brassicales	Brassicaceae	*Brassica*	30	13	Bok Choy (whole)
Eudicots	Rosids	Fabales	Fabaceae	*Pueraria*	5	6	Kudzu Leaf
Eudicots	Rosids	Fabales	Fabaceae	*Lathyrus*	17	21	Green Peas
Eudicots	Rosids	Fabales	Fabaceae	*Phaseolus*	18		Green Bean Seed
Eudicots	Rosids	Fabales	Fabaceae	*Phaseolus*	20		Green Bean Pod
Eudicots	Rosids	Fabales	Fabaceae	*Peltogyne*	28		Purple Heart Leaf
Eudicots	Rosids	Fabales	Fabaceae	*Trifolium*	34	9	Clover Leaf
Eudicots	Rosids	Fabales	Fabaceae	*Trifolium*	37	11	Clover Flowers
Eudicots	Rosids	Fabales	Fabaceae	*Styphnolobium*	38	23	Japanese Pagoda Seed
Eudicots	Rosids	Rosales	Rosaceae	*Malus*	26		Apple Seed
Eudicots	Rosids	Sapindales	Anacardiaceae	*Mangifera*	41	25	Mango Flesh
Eudicots	asterids	Lamiales	Lamiaceae	*Nepeta*	6	10	Catnip Leaf
Eudicots	asterids	Lamiales	Lamiaceae	*Mentha*	19		Mint Leaf
Eudicots	asterids	Lamiales	Oleaceae	*Olea*	42	20	Olive Flesh
Eudicots	asterids	Lamiales	Lamiaceae	*Salvia*	45	27	Chia Seed
Eudicots	asterids	Solanales	Convolvulaceae	*Ipomoea*	10		Sweet Potato (whole)
Eudicots	asterids	Solanales	Solanaceae	*Solanum*	13		Russet Potato (whole)
Eudicots	asterids	Solanales	Solanaceae	*Capsicum*	33	14	Jalapeño Seed
Eudicots	asterids	Solanales	Solanaceae	*Solanum*	44	31	Tomato Seed
Eudicots	asterids	Apiales	Apiaceae	*Daucus*	12	22	Carrot Skin
Eudicots	asterids	Cornales	Hydrangeaceae	*Hydrangea*	23	24	Hydrangea Leaf
Eudicots	asterids	Cornales	Cornaceae	*Cornus*	51	30	Dogwood Leaf
Eudicots	asterids	Asterales	Asteraceae	*Lactuca*	21	33	Red Lettuce Leaf
Eudicots	asterids	Asterales	Asteraceae	*Taraxacum*	46	28	Dandelion Leaf
Eudicots	asterids	Asterales	Asteraceae	*Lactuca*	47	32	Red Lettuce Stem
Eudicots	asterids	Asterales	Asteraceae	*Taraxacum*	50	29	Dandelion Flower
Eudicots		Caryophyllales	Amaranthaceae	*Beta*	24		Red Chard Stalk
Eudicots		Caryophyllales	Amaranthaceae	*Beta*	31		Red Chard Leaf
Eudicots		Caryophyllales	Cactaceae	*Schlumbergera*	53		Christmas Cactus Leaf
Monocots	Commelinids	Zingiberales	Zingiberaceae	*Zingiber*	25		Ginger Flesh
Monocots	Commelinids	Zingiberales	Zingiberaceae	*Zingiber*	27		Ginger Skin
Monocots		Alismatales	Araceae	*Pothos*	32		Pothos Leaf
Monocots		Asparagales	Asparagaceae	*Agave*	39	19	Agave Leaf
Monocots		Asparagales	Amaryllidaceae	*Allium*	43	26	Red Onion Flesh
Monocots		Asparagales	Asparagaceae	*Chlorophytum*	52		Spider Plant Leaf
Magnoliids		Laurales	Lauraceae	*Persea*	40		Avocado Flesh

high concentrations of peroxidase. The data collected does not constitute a complete delineation of peroxidase activity in various plant families, but instead provides a general outline of where further research should be focused.

From the data in Table 5, it appeared that plants from the eudicot clade generally contained more relative activity than those from either the magnoliid or monocot clades. Within the eudicot clade, the Rosid subclade also had greater relative activity than the Asterid subclade. We see that the order of Cucurbitales dominates with several organisms with high relative activity. The Brassicales order also presents many organisms with significant levels of peroxidase activity.

Unfortunately, due to limited availability of organisms to choose from, all plants from these two orders also came from the same family respectively, Cucurbitaceae and Brassicaceae. All tested organisms from the Cucurbitaceae family also came from the same genus, *Cucurbita*. Given these limitations, the assumption that plants from these orders will contain high relative peroxidase activity needs more evidence from other families of these orders to confirm the hypothesis.

Peroxidase activity in the genus *Cucurbita* appears to be elevated. Looking specifically at various *Cucurbita* species tested, it should be noted that only the fruits of the species were examined. Additionally, some species of *Cucurbita* are sold only when the fruit is immature. For example, zucchini and summer squash are usually only sold at an immature age because at that point in growth, the rind of the fruit is edible. Other *Cucurbita* fruits are only sold in their fully mature state, when the rind has completely hardened for winter storage such as pumpkins, butternut squash, acorn squash, spaghetti squash, and others. Both the zucchini and yellow squash were only tested in their immature state, possibly affecting their level of

peroxidase activity. Previous research into garden cress sprouts was only completed for six-day-old sprouts (Abel-Aty, A., et al., 2021). It is also reasonable that the activity of peroxidase will change over the life of the plant, according to the needs of the plant. To ensure that the optimal age of the fruit is used for research, zucchini and yellow squash should be tested at full maturity. Additionally, more fruits from the *Cucurbita* genus should be investigated to give a broader sense of potential peroxidase sources.

Plants in the Brassicaceae family offer potential peroxidase sources. As mentioned earlier, the second most important family in this study was the Brassicaceae family because several species displayed elevated peroxidase activity. As expected, horseradish, correctly called *Armoracia rusticana* from the Brassicaceae family, demonstrated exceptional activity, but other organisms from the genus *Brassica*, within the family Brassicaceae, were also very active. Brussels sprout (*Brassica oleracea*) peroxidase was the fourth most active in the guaiacol assay out of the plants tested. Broccoli, a subspecies of *B. oleracea*, was the fourteenth most active in the guaiacol assay. Surprisingly, cauliflower, another subspecies of *B. oleracea* was only the 29th peroxidase in the guaiacol assay. *Raphanus raphanistrum*, commonly known as the radish, was also very active being ninth in guaiacol peroxidase activity. From this evidence, other organisms from this family might also contain relatively high levels of peroxidase activity.

Other important orders to consider for further peroxidase research were found. Beyond the genus *Cucurbita* and the Brassicaceae family, some plants of the family Fabaceae were relatively higher in peroxidase activity. For example, kudzu was significantly active in both the guaiacol and fluorophenol assays ranking fifth and sixth respectively. This discovery could provide state and international governments a beneficial solution to the infestations of kudzu in their territories. Catnip leaves of the family Lamiaceae were sixth and tenth in activity for each

respective assay. Interestingly, chia seeds, also of the Lamiaceae family were very inactive, placing forty-fifth and twenty-seventh respectively. This comparison highlights that although certain orders, families, or genera might appear to be better choices for investigative peroxidase research, there will be some species or portions of plants that do not contain any significant amounts of peroxidase.

The Relationship Between Plant Part and Peroxidase Activity

In the previous section of the discussion, we addressed the idea that there could be a relationship between the taxonomic designation of the plant and its peroxidase activity. Additionally, it should be noted that it appears that peroxidase activity is greatly influenced by what part of the plant was tested. Pumpkin skin, for example, had the highest peroxidase activity in the fluorophenol assay, and the third highest in the guaiacol assay. All other parts of the pumpkin were much less active and some, like pumpkin pulp, were completely inactive. It is interesting to note that most other seeds tested in this study were also very inactive. This could be from a dramatic change in environment for the peroxidase. If the host cells of the peroxidase have a high lipid content, it might be that the peroxidase was inactivated by the hydrophilic environment of the storage buffer. Another possibility is that because the storage buffer was hydrophilic, the peroxidases in the plant cells congregated and remained surrounded in a hydrophobic shield of fatty cytosol. In another way, the activity of the peroxidase might have been dampened from a lack of fatty cofactors.

As mentioned in the last paragraph, pumpkin skin performed extremely well in both assays, as did other fruit skins. It is reasonable that the most peroxidase concentration should be found in the exposed skin of the fruit, specifically since the cells of the rind would be the most abused by the elements such as foreign chemicals, sunshine, insects, and other cell-stressing

factors. These factors would warrant the presence of peroxidases to ameliorate any effects of damaged cells releasing large amounts of ROS.

Another example of how the part of the plant seemed to play a significant role in peroxidase activity was in red lettuce, specifically in the guaiacol assay. Red lettuce leaves ranked twenty-first in peroxidase activity, while the stem of the leaf ranked as forty-seventh. Interestingly, other plants that did not possess very high activity in either assay also did not display many differences between the parts of the plant. For example, clover leaves and flowers both had similar activities in each assay, as did green bean seeds and pods, and red chard stalks and leaves. This might indicate that in general, the entire plant is not a good candidate for peroxidase research and that care should be taken to choose the most efficient portion of the plant to eliminate waste.

Variations in Peroxidase Activity Based on The Assay

While most of the samples behaved in an expected manner, showing similar activity in both assays, some plants defied this expectation by being more active in one or the other of the assays. The top three plants in each assay may have changed order in which was most active for each assay, but ultimately, all three, butternut squash, pumpkin, and horseradish, all placed in the top three for both assays. In contrast, clover leaves and flowers ranked at thirty-fourth and thirty-seventh, respectively, in the guaiacol assay. But in the fluorophenol assay, they ranked ninth and tenth respectively. These two rankings for clover are very different and suggest a unique model of peroxidase activity in the plant. When compared against butternut squash, which had no distinction between the two assays, the difference is clear. Because of this, a discrepancy in activities, like the one seen in clover, should be investigated more deeply. This odd relationship

is also demonstrated beautifully in jalapeño seeds and pumpkin seeds, which both performed better in the fluorophenol assay than in the guaiacol assay.

Deeper Investigation into Novel Activity in Jalapeño Peroxidase/Peroxygenase

In completing the generalized fluorophenol assay of the jalapeño seed peroxidase, the peak mentioned in the results appeared where no other plant showed a peak at that retention time. This new product gave rise to a suspicion that a different enzyme was at work since other plants did not show this activity. Additionally, the enzyme was not quenched by methanol, indicating it might be more robust. However, since the product peak only positively indicates novel activity, the nature of the enzyme had to be investigated further.

In testing the crude extract of the jalapeño seeds it was observed that the activity of the new enzyme was declining rapidly with time, indicating that it might not be stable in the storage buffer. As mentioned in a previous section, it is likely that the normal environment of the enzyme is more hydrophobic than the storage buffer was, since it was a phosphate buffer. As shown in the results, the storage stability of the peroxygenase was increased in the presence of detergents. Since all three additives were detergents, with Tween 20 being more potent, it is hypothesized that the enzyme was more readily available for performing oxidation. If the nature of the enzyme is hydrophobic, it is possible that the enzymes congregated together to avoid the hydrophilic portions of the suspension. Since the detergents would emulsify the hydrophobic with the hydrophilic, the enzymes might not have congregated together, making more enzymes available for activity. Additionally, the structure of the enzyme might have been better preserved.

The reaction time experiment was important in establishing an optimal reaction time length given the specific concentrations of the reaction buffer. Given the results of an hour reaction, it would suggest that all of the limiting reagent had been converted to product. In the

case of the fluorophenol reaction, both hydrogen peroxide and 4-fluorophenol could act as limiting reagents by very small concentration differences in the preparation of the reaction buffer. Since it is not possible to determine which reagent acted as the limiting reagent, given the naturally occurring variation in measurement, it can only be concluded that for the specific amounts of reagents used, the greatest concentration of product was formed after 60 minutes of reaction time.

The stability of the primary product was also observable from the reaction time experiment because the amount of product decreased after 300 minutes as mentioned in the results. Since it should be assumed that approximately the same amount of product was made in each reaction for each time tested beyond the apex of 60 minutes, the decrease in the presence of product when measured can be attributed to the breakdown of the product itself and not the deterioration of the enzyme, although this would be occurring as well.

Understanding the secondary reaction with glutathione to isolate the product. From the results of adding glutathione at the end of the fluorophenol assay and the complete disappearance of the product peak, it was hypothesized that the glutathione had reacted with the product. This could cause the mobile phase solubility of the product-glutathione complex to change, thereby moving the peak to a different place on the chromatogram. Typically, glutathione is used to quench a reaction by reacting with free radicals as they are formed, which was the original purpose of adding it. However, it seemed that glutathione not only quenched the reaction, but also reacted with already formed products. If glutathione only reacted with products being made actively, the new product peak should have been observed even after adding glutathione. But since the peak disappeared, it is reasonable to believe that the product reacted with glutathione.

The disappearance of the peak in the environment of glutathione offered more insight into the nature of the product. A stable product should not have reacted with glutathione, revealing that the product of the new enzyme was unstable. However, the presumed new complex might be stable enough to investigate more thoroughly. The presence of a possible new complex was revealed by the final fluorophenol assay that had the unique peak. Since the peak was only observed in the samples run normally and quenched with glutathione, this might be the product complex that is desired. This is especially encouraging since the other samples, either run without glutathione or with only glutathione, did not have a peak at that retention time. There is a possibility that the new peak is due to some other reaction between compounds found in the jalapeño seeds and glutathione, but it could not be entirely from unreacted jalapeño seed compounds since the peak was absent from the plain jalapeño reaction.

Further study should be done to enhance the signal of the new peak such as altering the column size, changing the mobile phase, or increasing the concentration of the complex by increasing reagent concentrations and lengthening the reaction time. Once the new complex is characterized to the greatest extent it can be by HPLC, liquid chromatography and mass spectrometry (LC-MS) should be used to isolate the complex and provide a map of its structure. Using this data, the original product can be more clearly understood by using retrosynthesis techniques based on the original glutathione, 4-fluorophenol, and the new glutathione complex. This information could start to reveal what kind of enzyme was present in the jalapeño by forming hypotheses about the mechanism of the reaction along with intermediates. Additionally, insight into the shape and method of the active site of the enzyme could be gained and lead to more significant research. The unknown enzyme is particularly attractive given its ability to

function in >30% methanol. Further study of the enzyme should include research into its kinetic properties, crystallographic structure, and a full assessment of its enzymatic capabilities.

Conclusion

It is evident from the library of peroxidases that there are several valuable sources of peroxidase, with many coming from the Cucurbitaceae and Brassicaceae orders. Particularly, the peroxidases in butternut squash and pumpkin skin should be investigated thoroughly as possible alternatives to HRP. Additionally, the novel activity seen in JSP should be delved into since it might present an enzyme capable of unique reactions for industrial purposes given its ability to function in high concentrations of methanol. Through this research, it is evident that the sphere of peroxidase research is expansive and has the possibility to grow. So many plants remain untested and the full characteristics of already known peroxidases have yet to be completely discovered. This library of peroxidases will hopefully act as resource for many other biological and chemical scientists to dive into the topic of peroxidases with more background knowledge about peroxidase activity in a broad array of plant sources.

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