The Discovery and Analysis of Peroxidase Enzyme in *Pueraria montana*

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Abstract

Peroxidase enzymes are used for a variety of industrial and biotechnological applications because of their ease of purification, broad range of chemical activities, and low cost of use. Identification of quality peroxidase sources that are convenient for enzymatic isolation and give way to high yields of product is a desirable pursuit in biochemical research. Kudzu is an excellent candidate for this pursuit as it displays high catalytic activity in screening assays and is found in abundance. This project seeks to determine the enzyme stability, optimal pH conditions, and possible novel chemical activities of peroxidase isolated from kudzu leaves. The methods for this project include standard protein purification protocols, analytical techniques for evaluation of chemical activities, and purity of the resulting preparations.

Keywords: peroxidase, kudzu, enzyme, biochemical research, chemical activities

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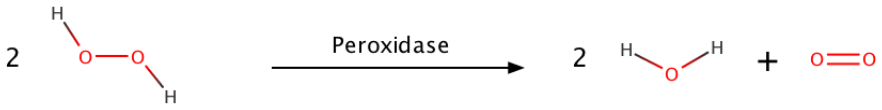
Background

Peroxidases are a group of enzymes belonging to the catalase family that are able to oxidize organic compounds using hydrogen peroxide in order to generate chemical reactions. To complete the oxidation, peroxidases catalyze the detachment of one or two electrons from an organic substrate through single-electron transfer. Hydrogen peroxide is commonly used as the electron acceptor, therefore creating water molecules through the addition of hydrogen atoms (Meunier, 2003). Figure 1 demonstrates, in one of the simplest ways, the ability of peroxidase to utilize hydrogen peroxide to oxidize another compound. In this case the substrate is another hydrogen peroxide, which is oxidized to molecular oxygen. This specific reaction is referred to as a disproportionation reaction.

Figure 1

Schematic Representation of Hydrogen Peroxide Oxidation



As a group of catalases, peroxidases belong to a large family of enzymes that are pervasive in fungi, plants, and vertebrates (Mika & Lüthje, 2003). Schonbein discovered these enzymes in 1855 by treating guaiacol with hydrogen peroxide and plant material (Meunier, 2003). These enzymes are essential for living systems in biology because of their ability to cleave and form oxygen-oxygen bonds. The applications of peroxidases expand beyond biological purposes into the industrial world. These enzymes can be used in the remediation of

commercial dyes as well as the treatment and decolorization of a variety of aromatic dyes in polluted water (Husain, 2009). Additionally, peroxidases can be used in the removal of phenolic contaminants from polluted water sources (Akhtar & Husain, 2006). Medically, peroxidases are used in enzyme immunoassays and diagnostic assays (Yoshida et al., 2003). A diverse range of applications for peroxidases results in a high level of demand for the enzyme and thus a promising market to pursue.

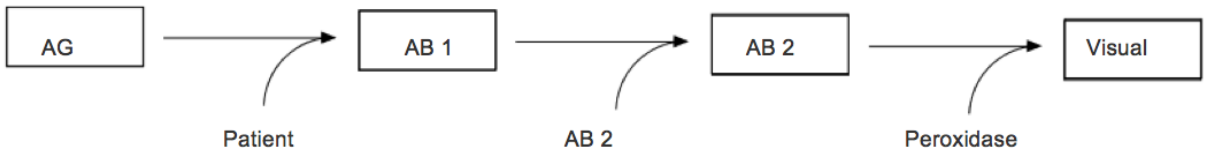
While not all peroxidases require the presence of a heme, it is very common to find peroxidases that contain a heme as the prosthetic group that functions to catalyze the abstraction of electrons via a hydrogen peroxide-dependent mechanism (Meunier, 2003). Research has shown that the active site structures of heme-peroxidases share a strong resemblance in arrangement and the mechanisms for oxidation are nearly parallel (Gumiero et al., 2010). Tetrameric heme-catalases are highly methodical catalysts for the reaction of converting hydrogen peroxide to dioxygen and water.

A commonly researched and well-known peroxidase is the horseradish peroxidase. This enzyme is a heme-peroxidase that catalyzes the one-electron oxidation of many substrates (Harris et al., 1993). Studies have shown that the enzyme reacts with hydrogen peroxide to give a two-electron oxidized species (Harris et al., 1993). Peroxidase from horseradish root is prominently studied because of its wide accessibility and stability. These enzymes have saturated the industrial market with applications in many of the previously mentioned areas, especially nonradioactive immunology assays (Meunier, 2003). For a nonradioactive immunology assay, antigens are applied to the wall of a plate, a patient’s blood sample is added to the wells, secondary antibodies attached to substrate are added, and horseradish peroxidase enzymes are

utilized to provide a visual analysis. In short, the secondary antibodies only stick to the assay if the patient has had or currently has the disease being screened. When peroxidase is applied, the substrate attached to the secondary antibody is reduced providing a color change in the assay (Mondal et al., 2020). This process of using the oxidation properties of peroxidase in immunology assays is displayed in Figure 2.

Figure 2

Nonradioactive Immunology Assays

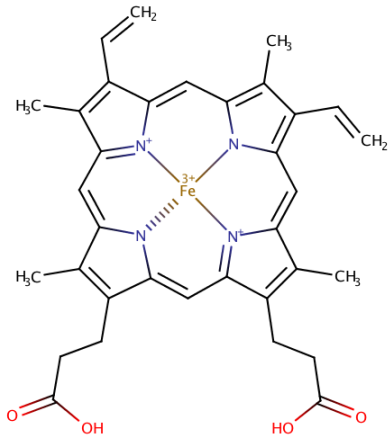


Note. AG represents antigen, AB 1 the antibody from the patient's blood, and AB 2 the secondary antibody bound to a substrate. Luminescence allows results to be visualized.

Horseradish peroxidase is the prototype for peroxidase enzymes, however limitations in certain applications has prompted researchers to explore alternate peroxidase sources that are able to out perform horseradish peroxidase in stability, ease of purification, and ideal optimal conditions. The horseradish peroxidase enzyme contains a heme prosthetic group that is essential for the enzyme's function. Figure 3 shows the chemical structure of the heme prosthetic group found in these enzymes. In the current study, it is understood that, for reasons to be discussed later, the peroxidase discovered is a heme-peroxidase.

Figure 3

Heme Prosthetic Group of Horseradish Peroxidase

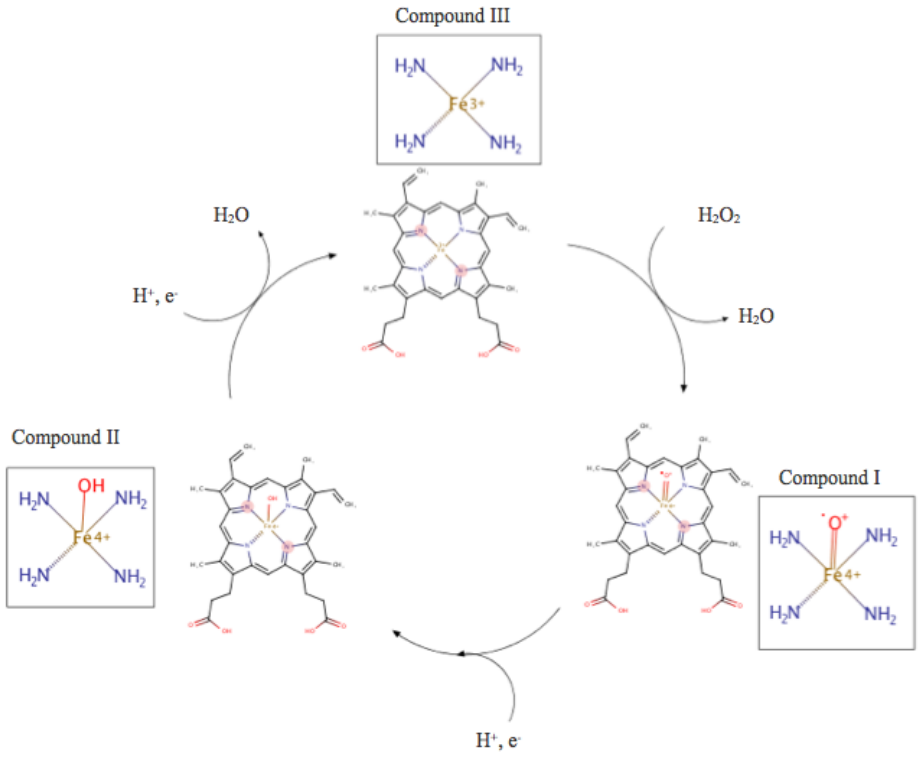


The heme prosthetic group of horseradish peroxidase serves to reduce peroxides so that organic compounds can be oxidized. During the oxidation process, the heme facilitates the transfer electrons from the organic substrate to the peroxide. One defect of the horseradish peroxidase is the radical, depicted in Compound I of Figure 4, generated in the mechanism. The free radical functions as a substrate to oxidize natural substances by removing a hydrogen atom (André et al., 2013). In the horseradish peroxidase mechanism, peroxide binds to the ferric state of Compound III, the oxygen-oxygen bonds of the peroxidase breaks, and the heme is now highly oxidized as shown in Compound I. The highly oxidized heme in Compound I is readily available to generate reactions for peroxidase chemistry. When the heme is at Compound II state, electron transfer occurs to reestablish Compound III by removing the hydroxyl (André et al., 2013). The potential for free radical chemistry occurring poses the risk of creating undesired dimers and other unwanted products (Hobisch et al., 2020). The present study seeks to define the

optimal conditions of peroxidase isolated from kudzu and to understand the underlying mechanism of substrate oxidation, opening up potential opportunities for its use in biotechnological applications, perhaps even some for which horseradish peroxidase is the current enzyme of choice.

Figure 4

Horseradish Peroxidase Mechanism



Note. This scheme represents the heme-iron cofactor oxidation-reduction mechanism in reducing hydrogen peroxide.

Kudzu, officially known as Pueraria Montana, is an invasive weed particularly prevalent among the highways of the southeastern states of the United States. This plant is a climbing vine

plant that forms large fleshy roots and leaflets along the vines (Wong et al., 2011). It was introduced into the United States in 1876 to be planted with the intentions of reducing soil erosion. By 1953 kudzu had been removed from the list of plants approved to reduce erosion, in 1970 it was formally categorized as a weed, and by 1997 it had been placed on the Federal Obnoxious Weed List (Forseth & Innis, 2010). Due to the rapid elongation rates and high photosynthetic rates, the weed poses a threat to the ecosystem of the southeastern states. The weed is prone to shade forest trees and produce biological byproducts that harm the plants surrounding it. The plant thrives in high temperature climates with high levels of carbon dioxide (Forseth & Innis, 2010). Removal of this pernicious species is costly, and currently the removed biomass does not provide any economic benefit.

Kudzu has benefitted other cultures in the past, as it has commonly been used in traditional Chinese medicine to treat fever, acute infectious diarrhea, thirst, diabetes, and cardiovascular diseases (Wong et al., 2011). Specifically, the chemical isoflavone puerarin within the plant aids in treating hypertension of the cardiovascular system. One of the cautions with using kudzu as a medicinal treatment is quality control. Kudzu harvested from different locations allows for slight variances in the chemical composition of the plant, which is a factor prescribers must be aware of and monitor (Wong et al., 2011). Chemical inconsistency as a result of varying harvest locations would likewise affect the research of peroxidase levels in kudzu, which ought to be considered and explored in future kudzu peroxidase studies.

Purpose of the Present Study

The overall goal of this research is to identify if kudzu would be a potentially beneficial source of peroxidase activity. The specific goal of this research is to purify a single peroxidase

sample from one of the plant species, Pueraria montana, known as kudzu, and to evaluate the catalytic properties of the plant in order to determine the potential for industrial applications. Kudzu is an excellent candidate for this pursuit as it shows very high catalytic activity in screening assays and its abundance in the southern states of the east coast. Successful identification of a stable peroxidase with industrial potential would transform an ignominious invasive species into a functional market product. This research seeks to determine the enzyme stability, optimal pH conditions, and possible novel chemical activities. Methods such as standard protein purification, analytical techniques for evaluation of chemical activities, and determination of enzyme purity will be discussed.

Method

Purification

Kudzu samples were collected along public highways in Campbell County, Virginia, and frozen at -20 °C to prevent degradation. The samples were crushed with a mortar and pestle or blender with a 1:2 ratio of grams of kudzu to milliliters of 20 mM pH 6.5 phosphate buffer. When the sample had been sufficiently broken up, the mixture was compressed so that the liquid was collected as the crude sample. The objective of blending the plant in the buffer and then collecting the liquid was to lyse the cells in order to collect the peroxidase enzymes. However, disrupting the cells cause a wide variety of biochemical molecules to be collected, ranging from enzymes to phospholipids to genetic material. Thus, the next initiative taken was a separation process to focus on isolating the peroxidase enzyme. The crude sample was first centrifuged to separate out large leaf particles. Ion exchange chromatography followed in the purification process. DEAE-Separose® and 10mM pH 6.5 phosphate buffer were added to the column,

treated, and the sample passed through the resin bed. The exchanged sample was collected then underwent precipitation with ammonium sulfate. Increments of ammonium sulfate were added to the solution in order to extract the peroxidase protein. The amounts of ammonium sulfate added to one liter of sample to achieve these concentrations are listed in Table 1. The sample was brought from 0-40% saturation at 0^{o}C, centrifuged, and the supernatant was collected. The supernatant was then brought from 40-70% saturation of ammonium sulfate on ice, centrifuged, and the pellet was separated from the supernatant and suspended in 20 mM pH 6.5 phosphate buffer.

Table 1

Ammonium Sulfate Precipitation

Grams of Ammonium Sulfate Added	Initial Saturation Percent	Desired Saturation Percent		
		40	70	100
	0	226g	436g	697g
	40	0g	187g	418g
	70		0g	209g

Note. The table represents the grams of ammonium sulfate added to 1.0 L of solution to reach desired saturation percent at 0°C. The table is modified from Englard and Seifter (1990).

Following 70% saturation of ammonium sulfate, both the pellet and supernatant were tested for peroxidase activity in order to determine if isolation was successful. Both the pellet and the supernatant revealed peroxidase activity when tested via guaiacol screening. The supernatant of the 70% saturation was then brought to 100% saturation, centrifuged, and the

pellet was separated from the supernatant and suspended in 20 mM pH 6.5 phosphate buffer. The supernatant demonstrated minimal levels of peroxidase and the pellet suspended in buffer contained high levels of peroxidase activity. The solution containing the pellet was suspended in buffer and then was dialyzed to remove any undesired salts remaining in the solution. Dialysis was carried out for approximately 24 hours in 10 mM pH 7.0 phosphate buffer. The purified sample was stored at -20°C in fractions of approximately 2, 5, 15, and 25 mL. Freezing varying fractions of the purified kudzu peroxidase served the purpose of preventing one sample from repeatedly being frozen and thawed, which would inevitably lead to undesired protein denaturation and potentially diminish enzymatic activity.

Guaiacol Screening

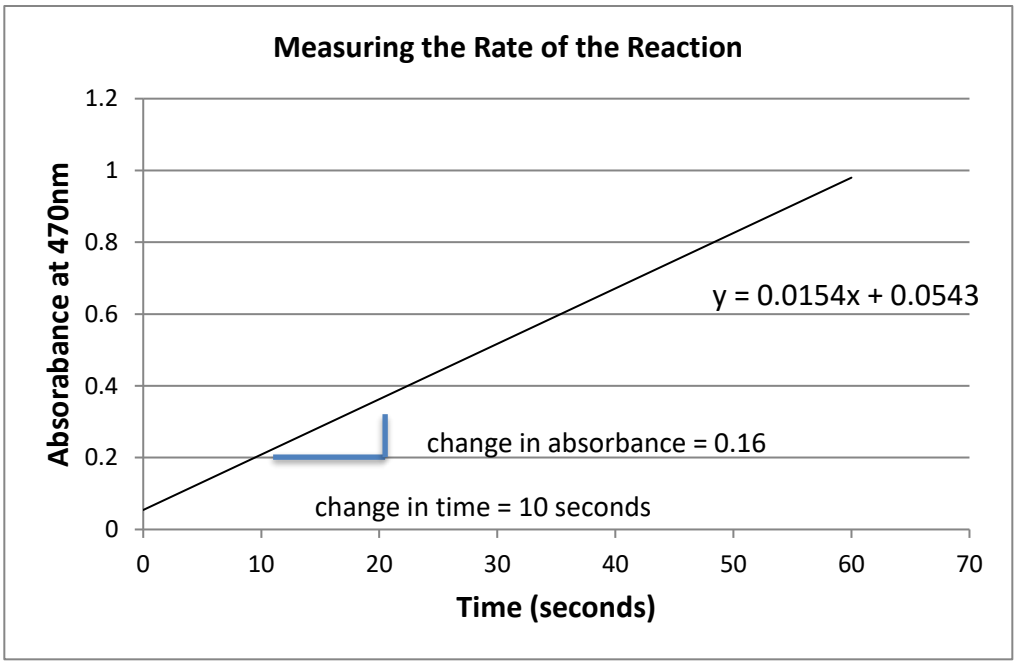
Enzyme activity per microliter of sample was measured within a library of samples in order to identify how kudzu compared to other plant sources in levels of enzymatic activity. Once a purified sample of the enzyme had been isolated, guaiacol screening was performed. The reaction mixture comprised of 4 mM guaiacol, 4 mM hydrogen peroxide, 50 mM phosphate buffer pH 7.5, and deionized water. A 10 μLmL aliquot of the crude sample was mixed with 1.0 mL of the reaction mixture. The value recorded was the average absorbance per second. Any absorbance values larger than 1 were disregarded and the procedure was followed with a diluted enzyme sample.

The activity per microliter was calculated by measuring the average change in absorption over a given time and then dividing by the tetraguaiacol extinction coefficient. The values of absorption measured by the spectrometer are directly correlated with the concentration using the extinction coefficient as proportionality constant. The longer the reaction took place, the more

products were formed, the darker the solution became, and the larger the absorption value was. However, the rate of the reaction was not always linear, so a line of best-fit was applied to the most linear portion in order to represent the average speed of the reaction for a given time. In this study, most reactions were measured for one minute. The rate of the reaction was determined by the slope of the best-fit line. Dividing the change in absorbance by the change in time allowed for the rate of the reaction to be calculated. For instance, the representative data shown in Figure 5 allows the method of determining the rate of the reaction to be understood. For this specific example, an approximate change in absorbance of 0.16 divided by an approximate change in time of 10 seconds yields a value of 0.016 absorbance units (AU) per second as the rate of the reaction. Given that these values were visually estimated from the best-fit line, they are not exact when compared to the best-fit line equation generated. The line of best-fit provides a more specific average rate of the reaction being 0.0154 AU per second. Using the extinction coefficient for the product tetraguaiacol (26.6 mM-1cm-1) allows the conversion of AU into concentration of the solution and identifies the amount of product formed over a given time.

Figure 5

Rate of Reaction



Note. An example of using absorbance values to understand the average rate of a reaction.

The rate of a reaction can give some indication to enzyme activity, but it does not yield information regarding how much activity occurs per mL of each peroxidase sample. The following equation was utilized for calculating the activity of peroxidase per milliliter:

$$\text{Activity}\left(\frac{\text{Units}}{\text{mL}}\right) =$$

$$\left(\frac{\text{change in absorbance}}{\text{time}}\right)(2)\left(\frac{1}{0.0266\ \text{micromolar}}\right)\left(\frac{1}{\text{microliters of enzyme}}\right)\left(\frac{1\ \text{liter}}{1000\ \text{microliters}}\right)(1\ \text{milliliter})$$

Once the value had been derived from the equation, the quantity of activity per microliter of each crude peroxidase sample was determined, and could be directly compared. Comparison of these values was used to assess the potential of each plant as a source peroxidase activity.

pH Selectivity

Guaiacol analysis was performed with varying pH buffers in order to determine the ideal pH for maximized kudzu peroxidase activity. The pH values selected to test in the experiment were 5.5 to 8.0 in increments of 0.5. The reaction mixture comprised of 8.8 μL guaiacol from a 20 mM stock and 8.0 μL of hydrogen peroxide from a 20 mM stock brought up to 20 mL with deionized water. The reaction mixtures were made by mixing 950 μL of the reaction mix, 50 μL of phosphate buffer at 20 mM, and 5 μL of the kudzu peroxidase. The kudzu peroxidase was always added last, the solution was quickly mixed to evenly distribute the reagents, and the absorbance was measured in a spectrometer. The cuvettes used in the spectrometer had a path length of 1.00 cm. The value recorded was the average absorbance per second. Any absorbance values larger than 1 AU per min were disregarded and the reaction was repeated with a diluted enzyme sample. An identical method for calculating the activity per microliter of enzyme was performed as previously mentioned in the guaiacol screening of the plant library.

Peroxidase Quantification via Heme Analysis

The well-understood nature of horseradish peroxidase can be employed as a reference point for measuring the heme content in other peroxidase sources. Powdered commercial horseradish peroxidase enzyme was brought into solution with deionized water. The concentration was determined by measuring the heme absorbance in the spectrometer and using the known extinction coefficient for horseradish peroxidase. A 1:1 dilution was performed by mixing 1 mL of the horseradish peroxidase solution with 1 mL of deionized water. This was repeated several times with each 1 mL horseradish sample being taken from the previously diluted sample. Absorbance was measured using absorbance at 403 nanometers (e=100,000 M-

1cm1). The equation M1V1=M2V2, with M and V representing molarity and volume, could then be applied to determine the concentration for each dilution in the series. High performance liquid chromatography, otherwise referred to as HPLC, was used to measure the amount of heme activity in the samples. 100 μL of each horseradish dilution were mixed with 500 μL of the heme analysis reaction mixture. The heme analysis reaction mixture was 50% acetonitrile and 50% deionized water, with an added 0.1% trifluoroacetic acid. These results were graphed to assemble a standard curve, which could then be used with other peroxidase enzymes to quantify their heme content. The same HPLC procedure was also followed with a purified sample of peroxidase isolated from kudzu in order to determine heme concentration.

Michaelis-Menten Kinetics and Lineweaver-Burke Plot

The solution for the Michaelis-Menten and Lineweaver-Burke plot was composed of 5 mM hydrogen peroxide, 50 mM pH 6.5 phosphate buffer, 5-40 mM guaiacol, and 1 μL of purified kudzu. Originally 10 μL of purified kudzu peroxidase was added to the reaction mix, but the sample was too high in enzymatic activity to produce reliable results. Thus the enzyme was diluted with a 1:10 ratio of enzyme to deionized water, which was then added to the reaction mix. The total volume of the reaction mix was brought to 1 mL by adding the necessary amount of deionized water. The hydrogen peroxide was added to the reaction mix last so that the solution could be quickly shaken to distribute the reagents evenly and then placed in the spectrophotometer, which was set to 470 nanometers. The rate of the reaction was measured in ten second intervals for one minute to produce a numeric value for the slope of the reaction. This value was recorded as the absorbance for each individual solution with a unique guaiacol

concentration. This information was then analyzed using Michaelis-Menten plot and Lineweaver-Burke plots.

Gel Electrophoresis via SDS-PAGE

Gel electrophoresis was performed to verifying the purity of the isolated kudzu peroxidase. Fractions from the crude sample and the isolated enzyme were run on the gel alongside a protein ladder. A pre-cast mini-protean gel produced by Bio-Rad Laboratories that was compatible with tris/glycine buffers was used with tris/glycine buffer. The protein ladder used was a 30,000-200,000 molecular weight marker set sold by Sigma Aldrich. The gel apparatus was constructed, buffer was added, and the prepared samples were loaded in a volume of 15 μL. To prepare the samples for electrophoresis a loading buffer had been added to both the samples and the protein ladder, heated at 100°C for 5 minutes, and then these materials were loaded into the gel. Electrophoresis was done for 45 minutes at 100 volts. The gel was removed from the plastic framework, washed in a destaining solution for 30-45 minutes, and then washed in deionized water for approximately 12 hours.

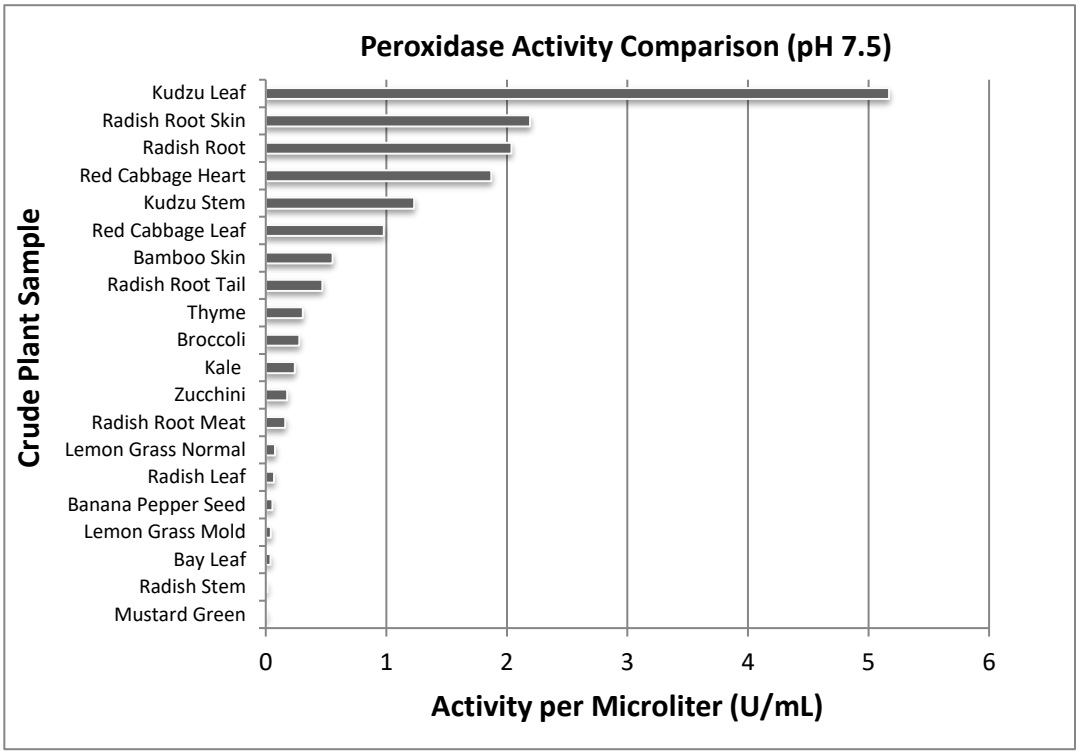
Results

Guaiacol Screening

An identical guaiacol analysis procedure was applied to a wide variety of plant samples prepared using a standardized protocol in which 2 mL of buffer and 1 g of plant material were ground using a mortar and pestle, and the supernatant was collected by centrifugation. In this way, one may obtain an estimate of the extractable peroxidase activity per gram of plant material. As depicted in Figure 6 and Table 2, the results revealed abundant peroxidase levels in the kudzu leaf sample.

Figure 6

Peroxidase Activity Comparisons



Note. The figure provides a visual representation of the relative peroxidase activity in U/mL for varying crude plant sources as identified by guaiacol kinetics.

Within the initial screening for peroxidase activity, no other plant was remotely as enzymatically active as the kudzu sample. Of the selected plant sources, the greatest rival was the radish root skin, which did not display 50% of the enzymatic activity that kudzu did. Only three of the explored crude samples displayed 25% of the activity that kudzu contained. These results suggest, therefore, that the invasive weed kudzu may be a rich source of peroxidase activity, and warrants further study with regard to its potential application for industrial biocatalysts or remedial applications. As more specific details of the enzyme selectivity and the

mechanism of the enzymatic activity are explored, they would either further solidify the value of kudzu peroxidase activity or dampen the initial enthusiasm associated with this finding. It is also important to note that these values represent the peroxidase activity prior to purification.

Table 2 lists that quantified amount of peroxidase activity per microliter upon guaiacol analysis. Some plants, such as mustard greens and banana pepper seeds, contained less than 0.1 peroxidase activity per microliter of crude sample. The average peroxidase activity per microliter of crude sample was 0.80. In comparison, the crude kudzu peroxidase activity was more than six times greater than the average between all of the samples. These initial screening results are indicative of promising peroxidase activity that could serve industrial purposes.

Table 2

Numerical Values for Guaiacol Screenings

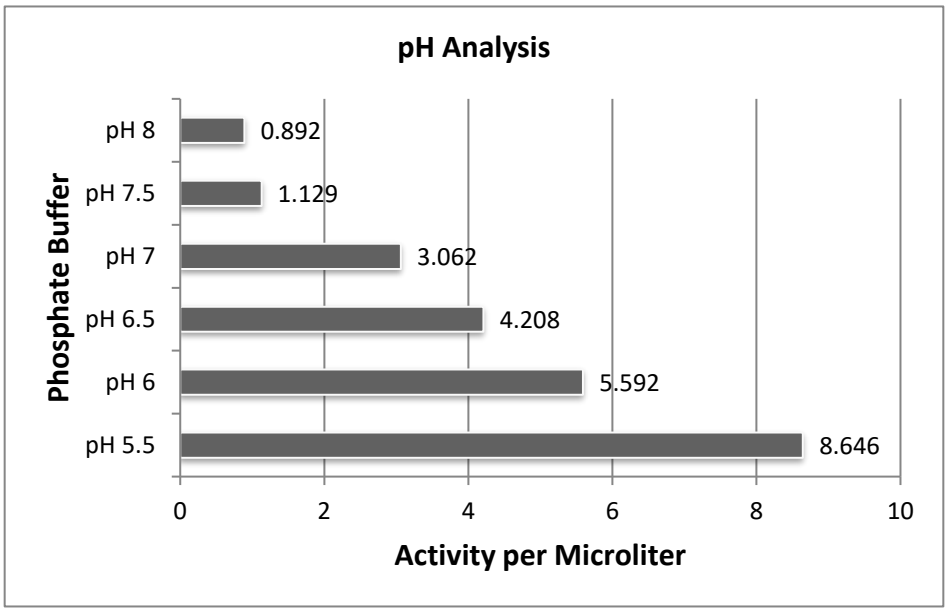
Plant	Units per Microliter
Mustard Green	0.0077
Radish Stem	0.015
Bay Leaf	0.0385
Lemon Grass Mold	0.042
Banana Pepper Seed	0.054
Radish Leaf	0.069
Lemon Grass Normal	0.077
Radish Root Meat	0.162
Zucchini	0.178
Kale	0.24
Broccoli	0.277
Thyme	0.308
Radish Root Tail	0.469
Bamboo Skin	0.554
Red Cabbage Leaf	0.98
Kudzu Stem	1.23
Red Cabbage Heart	1.87
Radish Root	2.038
Radish Root Skin	2.192
Kudzu Leaf	5.17

pH Selectivity

In order to determine the optimal enzyme conditions and pH selectivity purified kudzu peroxidase underwent a series of guaiacol assays with varying pH levels. Figure 7 provides a visual of the results.

Figure 7

pH Analysis



Note. The effect of pH variance on the activity of peroxidase.

Figure 7 shows that acidic conditions were optimal for kudzu peroxidase in comparison to neutral or basic conditions. Enzymatic activity levels were almost ten times as great when using a phosphate buffer with a pH of 5.5 rather than a pH of 8.0. Due to the phosphate buffer only spanning a pH range from 8.0 to 5.5, another chemical class of buffer would need to be used to gather a more comprehensive set of data values. With the current range of pH exploration, it can be seen that there is a general trend of increased activity with an increase in hydrogen ion concentration. It cannot, however, be determined if the pH of 5.5 is the pH at which peak activity occurs or if a more extreme acidity might produce even greater enzymatic activity. This is an important area of interest in future kudzu peroxidase research projects. It is

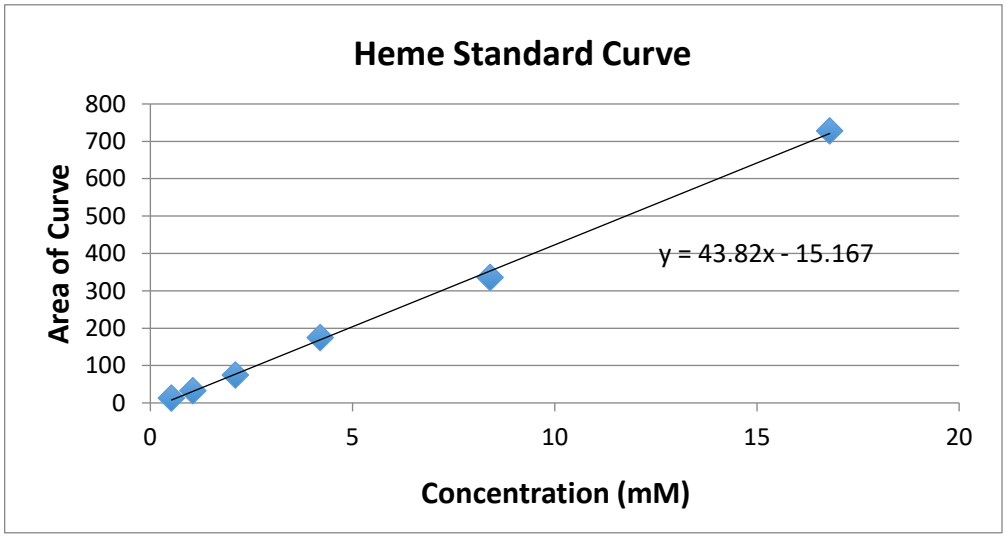
likely that if the peak for enzymatic activity is not a pH of 5.5, it will be close to a pH of 5.5. This estimate is based on studies that indicate a pH of or below 4.5 caused deterioration in the secondary structure of horseradish peroxidase (Chattopadhyay & Mazumdar, 199). Discovering the optimal pH for peroxidase isolated from kudu will give insight into specific industrial applications.

Peroxidase Quantification via Heme Analysis

The established heme standard curve was used to evaluate the heme concentration in kudzu peroxidase. Each peroxidase molecule contains one heme prosthetic group, thus giving a 1:1 ratio of heme to peroxidase. Given that horseradish peroxidase is widely studied and well understood, a standard curve was generated using horseradish peroxidase at varying concentrations. The results are given in Figure 8.

Figure 8

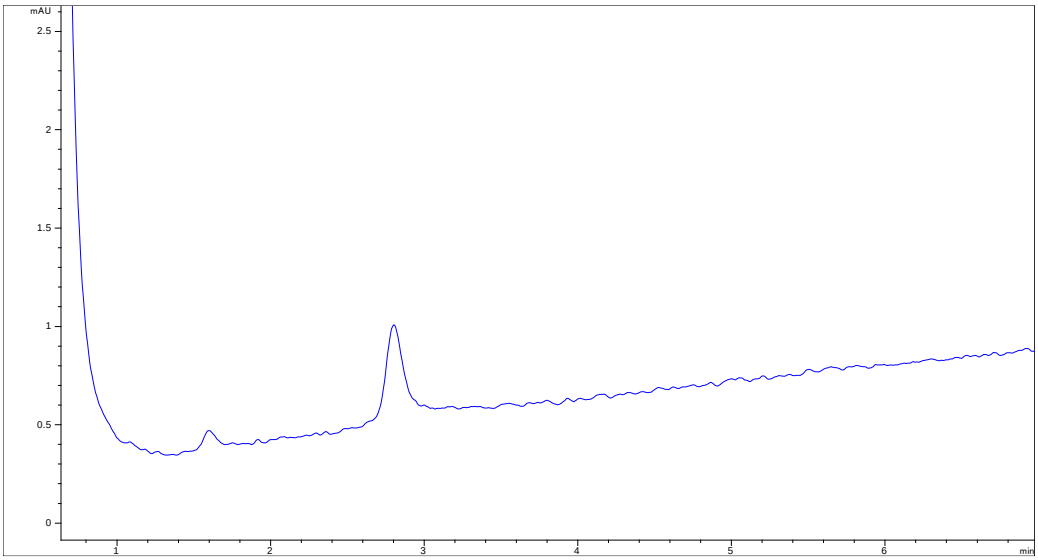
Quantifying Heme Content via Horseradish Peroxidase



Using the same method for the standard curve and the kudzu analysis, a value was generated for kudzu peroxidase. The presence of a kudzu peroxidase HPLC peak at the same retention time as a horseradish peroxidase peak confirms that the peroxidase contains a heme as the prosthetic group. Since it is known that the horseradish peroxidase contains a heme as the prosthetic group, identical peaks equate to identical prosthetic groups. A sample of purified peroxidase from kudzu underwent HPLC heme analysis and the results are shown in Figure 9. The value under the integrated HPLC curve was 4 mAU× sec and this value replaced the y value in the standard curve equation y = 43.82x - 15.167. The value x was calculated to determine the concentration of heme. The calculated concentration was 0.44 μM. Since it is known that there is a 1:1 ratio of heme prosthetic groups to peroxidase molecules, the concentration of heme also yields the concentration of peroxidase.

Figure 9

HPLC Heme Peak for Kudzu



Note. The peak at 2.9 minutes is the desired heme detection that was integrated for quantitative analysis.

Michaelis-Menten Kinetics and Lineweaver-Burke Plot

The Michaelis-Menten and Lineweaver-Burke Plot are two of the simplest ways to illustrate kinetic data for enzymes, and their application allows for effective understanding of various aspects of enzymatic activity. The plots demonstrate an enzyme's affinity for a substrate, reflected in the value Km, and the highest speed at which the reaction occurs, represented by Vmax. When the reagents are first introduced to each other, equilibrium is rapidly established between enzyme bound to the substrate and enzyme free from substrate. A low Km is favorable because it implies that less of the substrate is necessary to reach saturation and thus that there is a high affinity between the molecules. Vmax, demonstrates the quickest the reaction can occur and when the enzyme is entirely saturated. Even if the sample were to have a higher substrate concentration than the concentration at which Vmax occurs, the reaction would not take place any faster due to an absence of free enzyme molecules to bind to the surplus of substrates molecules.

The Km for the peroxidase isolated from kudzu was calculated to be 16 mM (Figure 10). The Vmax for the kudzu peroxidase was 0.31 mM/min (Figure 11). The equation in Figure 11 was used to calculate both the Km and Vmax.

Figure 10

Kudzu Michaelis-Menten Kinetics

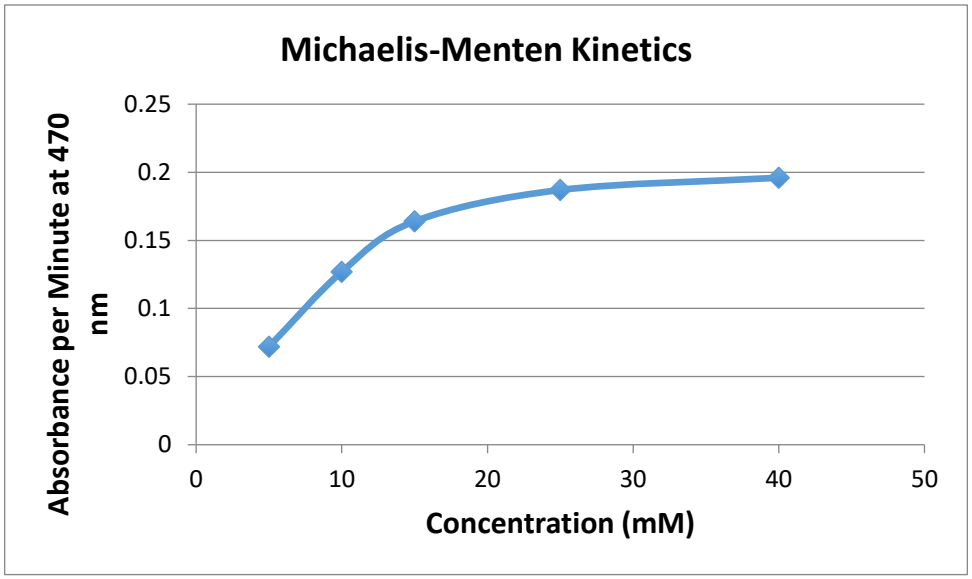
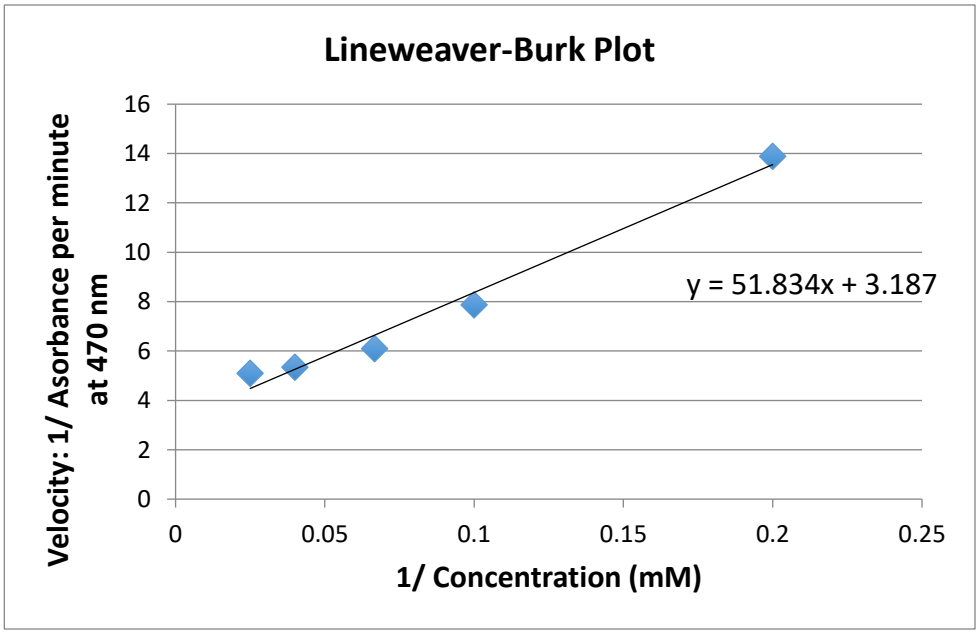


Figure 11

Kudzu Lineweaver-Burk Plot.

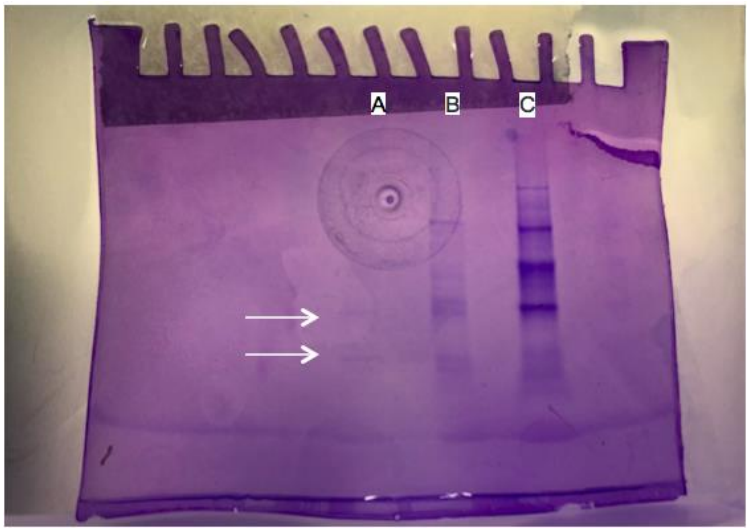


Gel Electrophoresis via SDS-PAGE

Gel Electrophoresis was able to establish that peroxidase isolated from kudzu was successfully isolated and purified (Figure 12). The lanes A, B, and C resemble the purified peroxidase, crude peroxidase, and the protein ladder samples respectively. One important observation from the gel result is that bands within the crude sample do not appear in the purified sample. This leads to the conclusion that non-peroxidase proteins were removed in the purification process

Figure 12

Gel Analysis to Determine Purity of Peroxidase Sample



Note. Lane A was the sample that had undergone the purification procedure, lane B was the crude sample, and lane C was the protein ladder. The two arrows point to the two protein bands detected in the purified sample.

Given the molecular weights provided in the protein ladder, it can be concluded that the kudzu peroxidase either has (1) a molecular weight slightly above 30,000 or (2) a molecular

weight significantly lower than 30,000. Smaller proteins travel further down the gel, thus allowing for these molecular weights to be concluded from the sorted proteins.

Discussion

The present study was initiated to determine whether or not Pueraria montana contained peroxidase enzymes and if so, the chemical parameters of those enzymes. It was successfully determined that kudzu does contain a bountiful amount of peroxidase activity. Furthermore, these peroxidase enzymes are capable of being isolated from the plant source with relatively simple procedures. While the most efficient isolation method has yet to be discovered, gel analysis revealed that the current purification scheme was successful.

The pursuit of discovering the chemical parameters was mostly successful, as some general characteristics are now understood. Many chemical specifics have yet to be discovered about the enzyme, which is expected given the novelty of the source. High initial screenings via guaiacol analysis on crude kudzu indicate that peroxidase have a strong presence in kudzu, especially in comparison to the other potential sources examined. Within the selected plant species selected for the peroxidase library, few samples remotely rivaled the activity in kudzu. As expected, pH selectivity screenings determined that acidic conditions are ideal for kudzu peroxidase and generate the highest levels of activity. However, further analysis would be necessary to determine the specific pH at which activity peaks. If pH-activity profile differs from that of horseradish peroxidase, then it might reveal an additional unique factor to kudzu peroxidase that could increase its industrial value. Heme analysis revealed that there was a peak at the expected retention time of the HPLC analysis, strongly pointing to the kudzu peroxidase

being a member of the heme-peroxidase family. The enzyme also proved to be relatively simple to isolate, which benefits companies seeking profit in a stable source of extraction.

The most prominent rationale for pursuing kudzu industrially is the enormous surplus of the plant plaguing the highways of the southeastern states of the United States. Currently, the plant serves no practical function and is only a nuisance. Rather than enduring the financial burden of removing the plant species as waste, it would be more economic to allow companies to pursue opportunity to profit. Additionally, the abundance of kudzu allows for large chemical screenings on the peroxidase isolated without fear of squandering valuable resources.

Future Research

Initial screenings for peroxidase activity in kudzu have proven to be strongly interesting and therefore open up the opportunity to pursue further chemical assays. The plant source is novel and there remains much to be discovered. Guaiacol screening could be performed with varying temperatures to identify which temperature is optimal for the reaction. It would also be beneficial to discover a purification process that is most efficient for industry financially and for quality of the product. Additionally, there are a wide number of reagents that could be used in assays to determine the mechanism of peroxidase isolated from kudzu. Some reagents identified as promising candidates for chemical screenings are 4-fluorophenol, 3,4-difluorophenol, 3,4,5-trifluorophenol, and pentafluorophenol. These reagents would provide insight into the mechanism that peroxidase isolated from kudzu use by studying the products produced and the chemical mechanisms known within the structural components of each reagent. Specific knowledge of the chemical aspects of kudzu peroxidase brings about the ability to determine distinct industrial applications and areas of potential profit.

An area of focus that currently remains unexplored and would be beneficial information for considering industrial potential is the variance of peroxidase levels in kudzu removed from different harvest locations. Data would be simple to collect via crude guaiacol screenings that do not require the purification process. This would highlight the most remunerative locations for an industry to remove kudzu and isolate peroxidase. Furthermore, the data collected would provide insight into the degree to which peroxidase concentrations vary in separate regions. Additionally, it would be wise to further reproduce chemical examinations described within this thesis in order to strengthen the support for kudzu being a promising source of peroxidase and to more specifically determine the optimal conditions for the enzyme. While the initial evidence was clear in establishing that peroxidase is plentiful in kudzu, there is always benefit in reproducing results to more strongly establish the data and conclusions. There are seemingly endless opportunities for future research with Pueraria montana, as the plant is newly introduced to the peroxidase library.

Conclusion

The purpose of the present study was to identify whether or not kudzu would be a candidate for peroxidase industrial use. Given that the invasive species currently serves no value and would require a financial burden to remove form public facilities, the potential to redirect the plant and gain profit is highly desirable. Given the rapid rate of growth of kudzu, the plant promises a steady source of peroxidase. Results from the study of the chemical attributes of peroxidase isolated from kudzu strongly affirm the plant as a favorable source of peroxidase activity. Further chemical analytics would need to be generated in order to determine specific industrial outlets for peroxidase isolated from kudzu, but there is no doubt that kudzu holds

promise as a source of peroxidase enzyme. There is certainly the potential for profit in resourcing the invasive species for peroxidase production, and there are numerous future areas of research to pursue in this newly emerging peroxidase source.

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