Structural Comparison of Fluoro-phenolic Compound Degradation with Peroxidase Enzymes

Ashton Ware[#], Carson Farmer[*], Gregory M. Raner, PhD[#], Hector E. Medina, PhD[*]

[#]Department of Biology & Chemistry, Liberty University, Lynchburg, VA

[*]School of Engineering, Liberty University, Lynchburg, VA

Abstract

Increased production of fluorinated drugs and agrochemicals over the last few decades has resulted in many concerns over the disposition of fluorinated waste products in the environment. The functional attraction to these compounds arises due to the difficulty in breaking C-F bonds, enhancing stability, and therefore the utility, of these compounds for their desired use. It is this stability that has prompted the concern over the persistent nature of these compounds in the environment. Effective strategies for degrading fluorinated aromatic waste have been developed, however excessive cost of reagents and impractical logistics for use justify the search for novel and inexpensive approaches to the degradation of these environmental waste products. The work presented in this study describes readily available and low-cost plant-based peroxidase catalysts, which were identified using a library screening method and possess the ability to degrade mono and poly fluorinated phenolic compounds. Several of the samples identified have activities that equal or exceed that of horseradish peroxidase, which is the prototypic peroxidase enzyme used in nearly all biotechnological applications involving peroxidases. The much higher abundance of peroxidase enzyme in skin of PKS, butternut squash, yellow squash, spaghetti squash and zucchini, relative to horseradish root, suggests that these novel peroxidase enzymes may be a more cost effective and readily accessible catalyst for this type of fluorophenol metabolism. Ultimately, an understanding of the kinetic properties of the enzymes identified in this study may direct future efforts aimed at developing more effective treatment strategies for these and other classes of environmental toxins, which is a long-range goal of this research.

Purpose of the Project

There are two main objectives of the current study:

Objective 1: Develop analytical methods for quantifying degradation of fluorinated aromatic compounds with multiple fluorine substituents

Objective 2: Evaluate a variety of squash skin peroxidase enzymes and compare their activities toward the degradation of these substrates

Methods

Peroxidase sample production: To extract the peroxidase, the thin-layered vegetable skin was peeled and mixed with 20 mM pH 6.5 Potassium Phosphate Extraction Buffer. For the amount (g) of vegetable skin to the amount (mL) of buffer, a 1:2 ratio was used. The mixture was grinded via mortar and pestle to extract the peroxidase. The crude sample was collected and centrifuged for 5 minutes at 10,000 rpm to pellet any remaining tissue in the sample. The transparent supernatant, containing the desired peroxidase, was transferred to a new vial for experimental use.

Testing for peroxidase with guaiacol: A guaiacol reaction mixture containing 5 mM guaiacol, 5 mM H_2O_2, 50 mM pH 6.5 Potassium Phosphate buffer, and 18.98 mL diH_2O to fill what remained to 20 mL total, was prepared to detect the presence and quantity of peroxidase enzyme for a variety of vegetables. When guaiacol is bound to peroxidase, an orange color is produced. The darker the orange, the more abundant in quantity the peroxidase. Spectrophotometry was used to gather quantitative data on the kinetic rate of activity compared to a blank sample. A spectrophotometer cuvette containing 980 µL of the prepared guaiacol mixture was reacted with 20 µL of each peroxidase sample and measured over 1 minute at 470 nm. Any sample with a measured absorbance rate of 1.2 or higher was diluted due to the possibility of the peroxidase sample being oversaturated with enzyme.

Heme analysis: Using known concentrations of horse radish peroxidase (hrp) and HPLC, a standard curve was generated to determine a vegetable's peroxidase concentration by applying its peak area produced in HPLC. For HPLC, samples were prepared by adding 50 µL of peroxidase sample to a 500 µL volume of 50/50 acetonitrile and diH_2O in 1% TFA. The samples were put on ice for 10 minutes and then centrifuged for 5 minutes at 10,000 rpm to precipitate out any remaining proteins to ensure a purer heme analysis. Heme method was run on HPLC. The peak was detected after 6 minutes at 403 nm for each sample. The peak produced by HPLC was integrated to give a peak area which was used in the standard curve equation to determine the heme concentration.

Fluorophenol metabolism analysis: To assess the peroxidase's ability to metabolize fluorophenol compounds, a reaction mixture was prepared, for each peroxidase species, with 5 mM H_2O_2, 50 mM pH 7.0 Phosphate buffer, and 1 mM fluorophenol. diH_2O was used to reach the desired reaction mixture volume. The species-specific peroxidase was added to the reaction mixture, and the metabolism of the fluorophenol was measured at 2 minutes and 10 minutes by pipetting 2 0.5 mL portions of reaction mix at the respective times into HPLC vials containing 200 mL 100% methanol and 100 mL 1 M HCl to halt further metabolism. All peroxidase concentrations were normalized to hrp concentration. For the PKS peroxidase, a 3.57 µM concentration was added to the reaction mix. For horse radish peroxidase, 3.13 µM, for butternut squash peroxidase (bsp), 0.844 µM, for spaghetti squash peroxidase (ssp), 0.720 µM, for yellow squash peroxidase (ysp), 1.27 µM, for zucchini peroxidase (zhp), 0.971 µM. After all samples were reacted, HPLC vials were loaded into the HPLC and ran under the appropriate fluorophenol method based on the number of fluorines attached to the phenol.

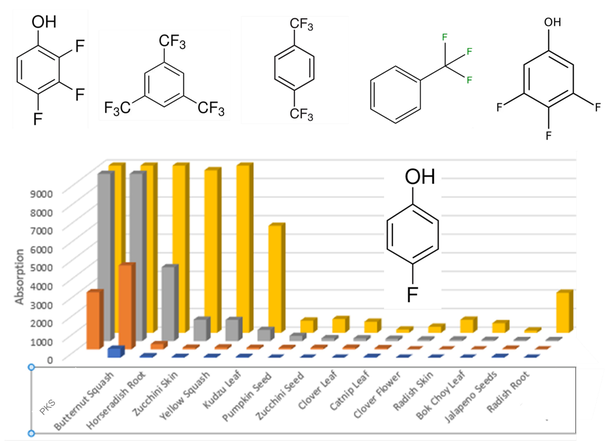

Figure 1. Peroxidase Activity From Guaiacol Assay(A.) A graph measuring absorbance rate at micromoles per minute for a library of vegetables at dilutions 1:1, 1:10, 1:100, and 1:1000.

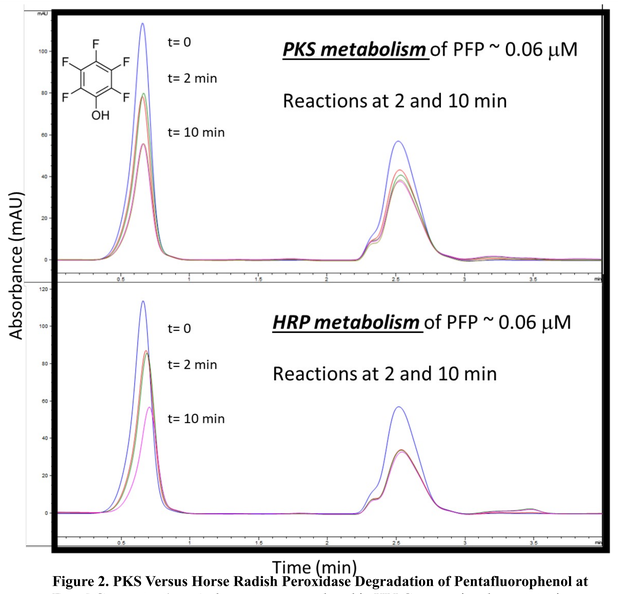

Figure 2. PKS Versus Horse Radish Peroxidase Degradation of Pentafluorophenol at Equal Concentrations A chromatogram produced in HPLC comparing the prototypic peroxidase from horse radish with the novel peroxidase derived from PKS.

Figure 3. Peroxidase Extraction From Crude Samples A flow chart demonstrating the process of extracting peroxidase enzymes from crude samples.

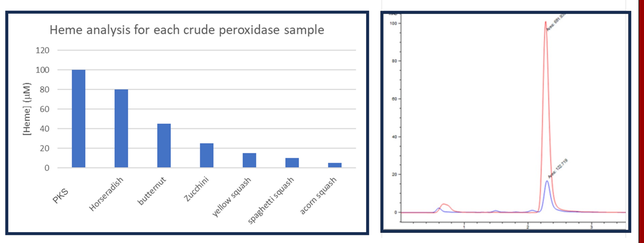

Figure 4. Heme Content in Concentration (µM) for Various Peroxidases A graph shows the heme concentrations recorded from heme analysis in HPLC for the variety of squash and vegetable crude samples (left). The chromatogram (right) shows the heme concentration in the PKS crude sample (red) versus the horse radish crude sample (blue).

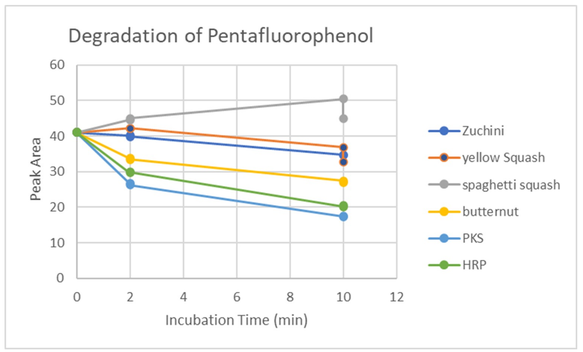

Figure 5. Degredation of Pentafluorophenol Using a Variety of Squash and Vegetable Samples A graph displaying measured degradation at 2 minutes and 10 minutes from HPLC analysis.

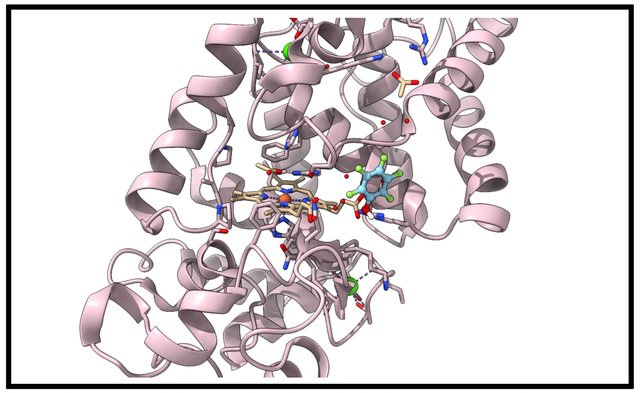

Figure 6. Crystallographic Structure of Peroxidase Binding Site to Fluorophenol Compound A simulated structure shows the binding site for peroxidase to bind to the mono or polyfluorophenol.

Results and Discussion

After initial screening of 52 crude plant peroxidase samples for 4-fluorophenol oxidation activity, it was determined that the skin of several squash samples are excellent candidates for further studies aimed at identifying biocatalysts for degradation of fluorinated aromatic compounds of the type illustrated in Figure 1. Of note, the PKS and butternut squash skin were particularly high in this activity, as indicated in the graph for 4-fluorophenol. As a result, the PKS sample was initially chosen to evaluate pentafluorophenol degradation alongside the classical Horseradish peroxidase enzyme. The two enzyme samples were normalized for their concentration and compared at 2 and 10 min incubation times using an HPLC assay we developed. As shown in Figure 2, when both enzymes were used at a concentration of 0.060 mM, similar degradation profiles were observed, with the PKS having slightly higher activity than HRP. Having established the ability of these plant peroxidases to degrade pentafluorophenol, several other samples from the squash family were isolated according to the scheme shown in Figure 3. This method produced crude peroxidase samples from the skin of butternut squash, spaghetti squash, yellow squash, zucchini squash and acorn squash, to go along with the PKS and horseradish samples already described. Once crude samples were prepared, the total heme content for each was measured using an HPLC-based assay, also developed in this lab, as a means to approximate total peroxidase concentration (Figure 4). Consistent with prior studies, the butternut and zucchini produced higher concentrations than yellow squash, acorn squash and spaghetti squash, but less than PKS. With the known concentrations of the samples, all six were normalized to the same concentration and compared. This allowed us to determine the intrinsic activity of each enzyme toward the degradation of this perfluorinated substrate. As shown in Figure 5, the PKS sample appears to possess the highest overall intrinsic activity in pentafluorophenol degradation. This is consistent with previous data on 4-fluorophenol as well. Kinetic studies are planned in which the binding affinities of the different enzymes toward this substrate will be determined. Figure 6 shows an active site model of the horseradish peroxidase enzyme with the pentafluorophenol substrate docked. This binding mode is very similar to the known binding of phenol to the active site in which the electron transfer reaction associated with this reaction occurs between the two propionate groups along the heme edge shown in the diagram. A similar binding mode is envisioned for the squash peroxidases, though no crystal structure is currently available for any of them. Although the data is preliminary, the observation that PKS peroxidase appears to outperform HRP in this particular reaction is encouraging, given the high abundance of peroxidase in PKS, and the relative ease of purification compared to horseradish root. In addition, other studies from our lab have demonstrated that butternut skin peroxidase has a greater thermal stability than HRP, which may also be a significant consideration in the development of biocatalysts for this type of function.

Summary

- PKS and horseradish peroxidase enzymes showed high intrinsic activity in the degradation of pentafluorophenol, under mild (pH 6.5 @ 23 °C) reaction conditions.
- PKS may be a viable low-cost source for biocatalysts capable of degrading fluorinated aromatic compounds in industrial applications

References and Acknowledgments

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