University of Nevada, Reno

Resin Detoxification in *Dendroctonus ponderosae*: Functional characterizations of Cytochrome P450 Proteins

A thesis submitted in partial fulfillment
of the requirements for the degree of

Bachelor of Science in Biochemistry and Molecular Biology and the Honors Program

by

Joseph Bernardo

Dr. Claus Tittiger, Thesis Advisor

August, 2015

UNIVERSITY OF NEVADA RENO

THE HONORS PROGRAM

We recommend that the thesis
Prepared under our supervision by

JOSEPH M. BERNARDO

entitled

Resin Detoxification in *Dendroctonus ponderosae*:
Functional characterizations of Cytochrome P450 Proteins

Be accepted in partial fulfillment of the requirements for the degree of

Bachelor of Science in Biochemistry and Molecular Biology

_____

Dr. Claus Tittiger, Ph.D., Thesis Advisor

_____

Tamara Valentine, Ph.D., Director, Honors Program

August, 2015

Abstract:

A growing epidemic of pine tree deforestation in North America can largely be attributed to a particular pest, the Mountain Pine Beetle (MPB, *Dendroctonus ponderosae*). Coupling pheromone-coordinated mass attacks with an ability to resist the trees' natural defenses allows the beetles to overwhelm their targets and burrow beneath the tree bark to reproduce. MPB use cytochrome P450 enzymes to both detoxify toxic monoterpene compounds present in tree resin as well as synthesize the pheromones used during tree invasion, suggesting an evolutionary link between the P450 proteins involved in the two processes. This study attempts to contribute to the understanding of this evolutionary link by examining CYP6DH3, a P450 closely related to a known MPB resin detoxifier CYP6DH2. To do so, microsomal CYP6DH3 was expressed in Sf9 insect cells before being separated and incubated with various monoterpene substrates. GC screening revealed that no products could consistently be formed, which suggested an issue with the assay protocol. Various adjustments to the procedure have allowed the results of a previous study (Song et al., 2013) to be replicated, though further testing must be done to confirm whether these reaction conditions apply to CYP6DH3.

Table of Contents

List of Figures

Introduction

For over a decade western North American pine forests have been afflicted by severe infestations of the mountain pine beetle (MPB, *Dendroctonus ponderosae* Hopkins; Coleoptera: Scolytidae). Climate change has caused typical beetle populations to grow to more epidemic levels in new areas in the vast forest regions of British Columbia (Sambaraju et al., 2012). Upwards of 13 million hectares of pine trees have been destroyed by these insects, and as of yet there are no straightforward solutions to curb their spread throughout the forests (Coggins et al., 2011).

The beetles are known to coordinate mass attacks on trees by sensing tree volatiles and communicating with pheromones, both of which can involve the metabolism of monoterpene compounds (Keeling et al., 2013). The biochemical pathways used by *D. ponderosae* to produce their pheromones are not fully characterized, but it is confirmed that major components are biosynthesized *de novo* in addition to modifying host tree monoterpene substances (Blomquist et al., 2010). Understanding the manner by which the beetles accomplish either of these processes could prove useful in developing cost-effective methods of guiding the beetles away from their tree targets in the wild. Feeding the beetles host pine tree tissue results in significant up-regulation of some cytochrome P450 mRNAs (Robert et al., 2013). Since sequence information cannot be used to predict P450 substrate and product profiles, functional characterizations of these proteins are necessary to determine their roles. This information also contributes to the general knowledge of P450 enzymology and insect evolution (Song et al. 2013).

Many mountain pine beetle P450s have yet to be functionally characterized in terms of which monoterpene substrates they are capable of modifying and whether they

are involved in pheromone synthesis or simply used for detoxification purposes. Previous studies have shown that CYP6DH2 has a broad substrate range for monoterpene hydroxylation reactions and functions as a resin detoxifier in MPB (Song 2012). Another uncharacterized P450, CYP6DH3, shares 68% amino acid sequence identity with CYP6DH2 which suggests similar functionality. This study surveys the substrate profile of recombinant CYP6DH3, which was expressed in Sf9 insect cells via a baculovirus vector, isolated using differential centrifugation, and incubated with various monoterpenes. Products of the reactions were to be evaluated using gas chromatography and mass spectrometry to help determine what role this particular P450 protein plays in mountain pine beetle metabolism.

Materials and Methods

Protein Expression

Jeff Nadeau provided all P450 and Housefly (*Musca domestica*) cytochrome P450 reductase (HF-CPR) samples for use in the enzyme assays. Proteins were produced in Sf9 insect cells for 72 h using a baculoviral expression system before harvesting cells and isolating the microsomal proteins via differential centrifugation. Samples were centrifuged for 10 min at 3000 x g (4ºC) to form a pellet, which was then resuspended in 10 mL of cold 100 mM sodium phosphate (pH 7.6) and centrifuged for another 10 min at 3000 x g (4ºC) two times. The pellet was then resuspended in 3 mL of cold cell lysis buffer containing 0.1 M DTT, 0.1 M PMSF, and 1/1000 PIC. Cells were then sonicated on ice and vortexed to form a lysate, which was centrifuged at 10,000 x g for 10 min at 4ºC. Finally, the supernatant was centrifuged at 120,000 x g for 60 min at 4ºC to form a microsomal pellet which was resuspended in 1.5 mL of cold CLB before storing at -20ºC.

CO-Difference Spectrum Analysis

To assess proper folding of the P450 proteins studied, CO-difference spectrum analysis was utilized. Samples were placed in microplates and measured using a SpectraMax M5 Microplate Reader. 50 μL volumes of microsomal P450 protein and Milli-Q purified water were pipetted into plate wells in duplicate to provide a baseline absorbance spectrum against which to compare the CO-treated proteins. These control sample wells were completely sealed away from CO exposure with a sheet of aluminum sealing foil before placing the plate into a plastic container, which was then flooded with CO gas at 0.5 L/min for 3 minutes. The control and CO-exposed samples were then treated with 0.5M sodium hydrosulfite to a concentration of 25 mM before taking absorbance spectra from 400 nm to 500 nm for each well 5, 10, and 15 min afterward.

Substrates and standards

Standard solutions for the following substrates were prepared by a 1:100 fold dilution in pentane: R-(+)-limonene, (±)-α-pinene, 3-carene, myrcene, para-cymene, γ-terpinene, and terpinolene. Standard retention times were identified on the Shimadzu GC-2010 Plus with an HP-INNOWax column using the same GC protocol as described below.

Enzyme Assays

0.40 μM CYP6DH3 1.0 μM CYP9T3 samples were each assayed *in vitro* using 800 μL mixtures in pyrolyzed 4 mL glass vials. The incubation mixtures consisted of 250 μL of microsomal P450 protein, 250 μL of microsomal HF-CPR, 5 μL of 60 mM substrate suspended in pentane, and finally 300 μL of 2.3 mM NADPH dissolved in 200 mM sodium phosphate buffer (pH 7.6) to initiate the reaction. Control mixtures used an

additional 250 μL of 200 mM sodium phosphate buffer (pH 7.6) to substitute for the volume of a P450 protein sample. Reaction mixtures were then immediately placed in a 30°C shaking incubator for 1 hour before extracting the upper organic layer twice with 750 μL 1:1 pentane:ether. The extracted samples were concentrated under N_2 gas stream to approximately 50-100 μL before being stored at -20ºC. GC analysis was conducted on a Shimadzu GC-2010 Plus using an HP-INNOWax column (polyethylene glycol, 60 m, 250 μm i.d., 0.25 μm film). The oven temperature program consisted of: 40ºC hold for 1 min, increase 10ºC/min to 240ºC (20 min), hold at 240ºC for 10 min. 2 uL of sample were injected onto the column per run.

Results

CO-Difference Spectrum Analysis

After isolating microsomal P450 proteins from Sf9 cells, the functionality of these proteins must be confirmed by binding CO to the proteins and observing an absorption at 450 nm. Figure 1 demonstrates properly folded CYP6DH3 proteins with strong absorbance peaks at 450 nm for the 0.43 μM and 0.36 μM samples. Likewise, Figure 2 indicates that CYP9T3 samples were also being produced effectively as shown by the absorbances at 450 nm for the 1μM and 0.96 μM samples. As expected, HF-CPR samples did not exhibit peaks at 450 nm. All P450 protein samples were verified in this way before use in the enzyme assays.

Enzyme Assays

0.40 μM CYP6DH3 was incubated with various monoterpene substrates to determine whether or not the protein functions as a detoxification enzyme as expected. The formation of a new peak in the experimental GC chromatogram that is not present in

the control sample would be taken as indicating a P450-catalyzed reaction product. No detectable products were observed when (R)-(+)-limonene, (±)-α-pinene, 3-carene, myrcene, para-cymene, γ-terpinene, or terpinolene were incubated with CYP6DH3 (Figure 3 and not shown). A previously unpublished study using Oxoplates, microplates containing an oxygen sensor to measure oxygen depletion resulting from P450 enzymatic activity, strongly suggested that CYP6DH3 catalyzed reactions with R-(+)-limonene and, to a lesser degree, 3-carene (J. Nadeau, personal communication).

To verify the assay protocol, 1.0 μM CYP9T3 was incubated with its known substrates, (+)-α-pinene and myrcene at various concentrations (Song et al., 2013). Initial reactions using CYP9T3 and (+)-α-pinene also did not form a product as is shown in Figure 4. Several experiments were conducted to determine the source of the problem such as the addition of Triton X-100 to improve solubility, or sonicating the reaction mixture before incubating to temporarily disrupt the microsomes and allow more substrate molecules to enter the active site. One reaction using CYP9T3 with 60 mM myrcene as substrate finally yielded expected results. Figure 5 depicts the formation of a significant product with a retention time of 17.5 min and other additional peaks nearby. However, these exact same reaction conditions with CYP6DH3 and R-(+)-limonene did not yield an observable product (shown in Figure 6). This assay has yet to be repeated with CYP9T3 and (+)-α-pinene.

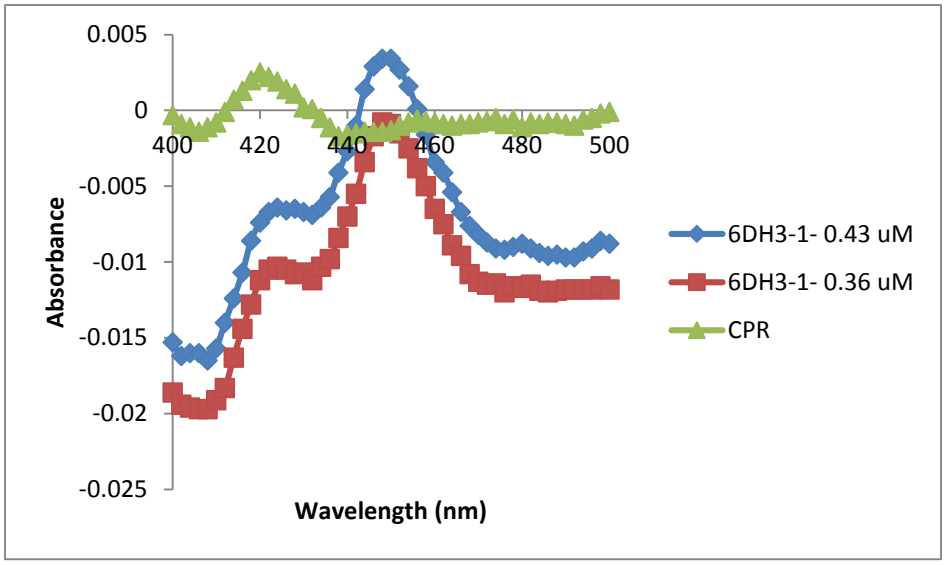


Figure 1. CO-difference spectrum analysis of recombinant CYP6DH3.

Sf9 microsomes containing CYP6DH3 were exposed to CO and reduced with sodium hydrosulfite to induce an absorbance peak at 450 nm as shown with 0.43 and 0.36 µM concentrations.

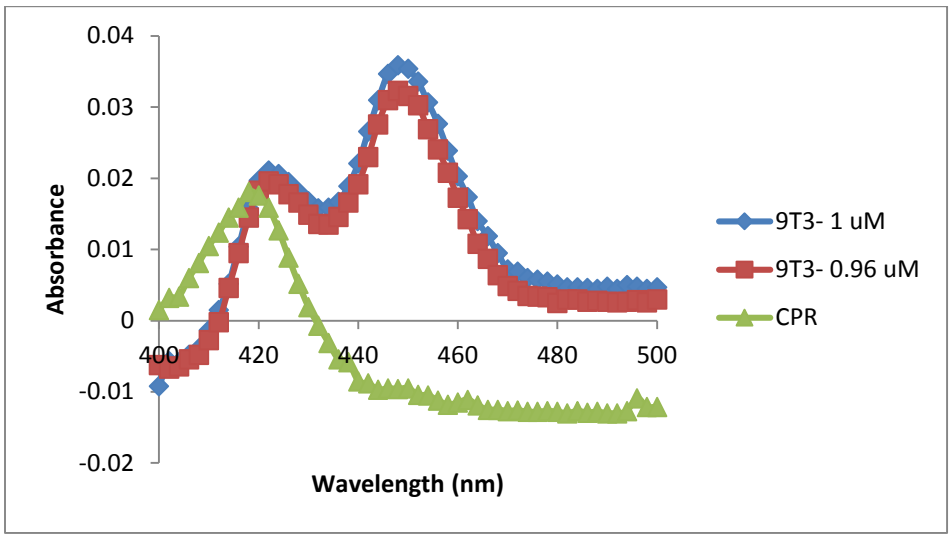
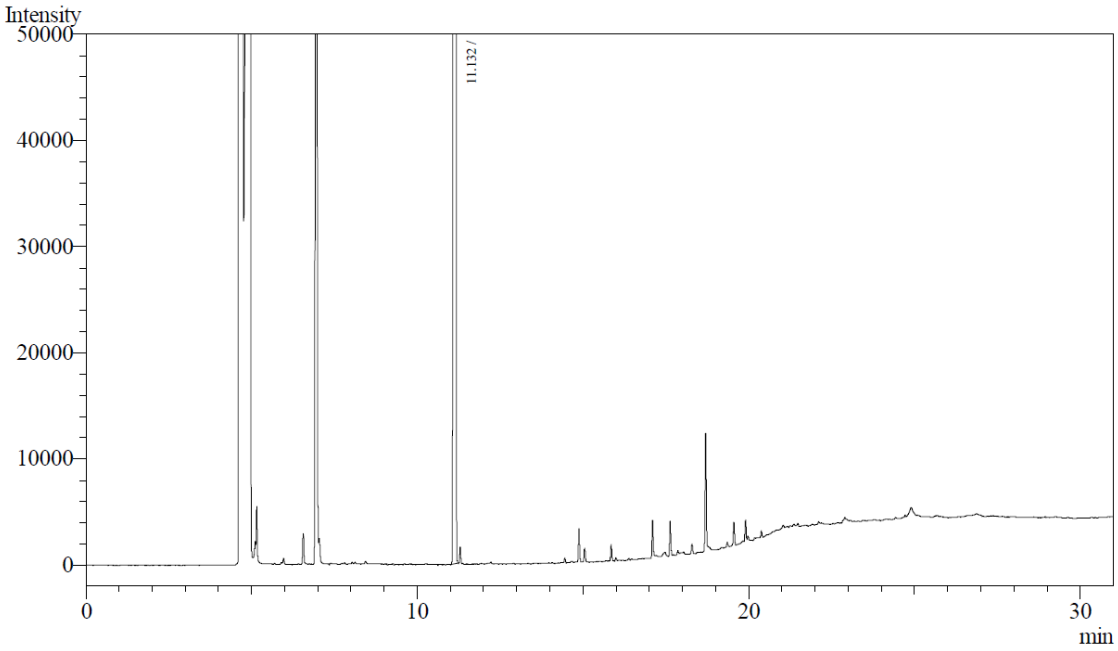
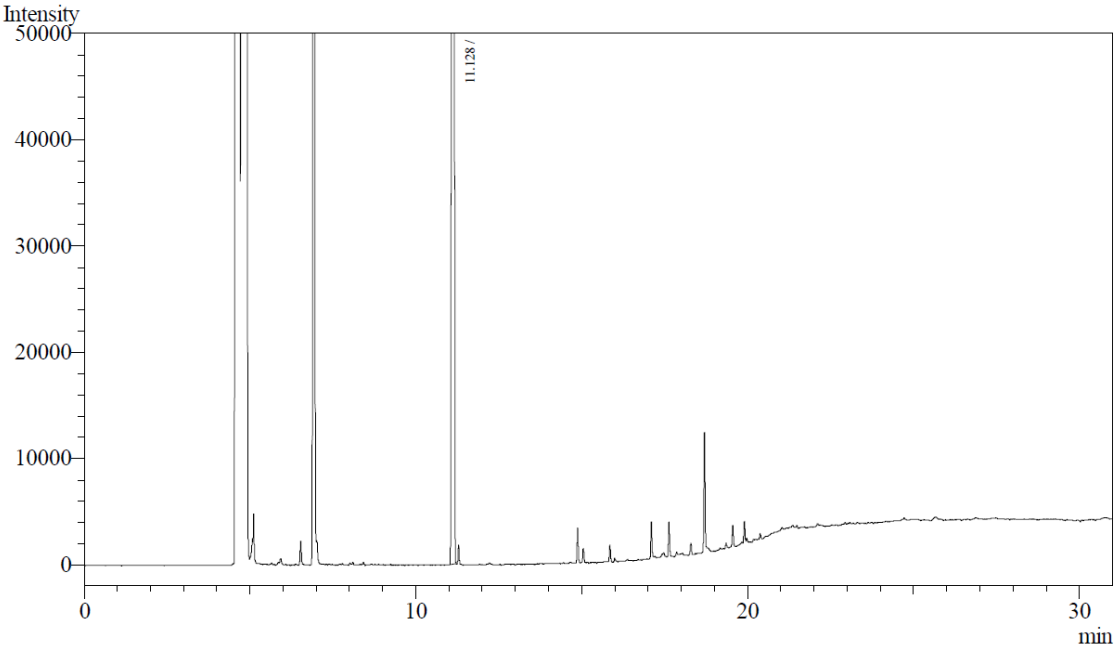


Figure 2. CO-difference spectrum analysis of recombinant CYP9T3.

Sf9 microsomes containing CYP6DH3 were exposed to CO and reduced with sodium hydrosulfite to induce an absorbance peak at 450 nm as shown with 1.0 and 0.96 µM concentrations.



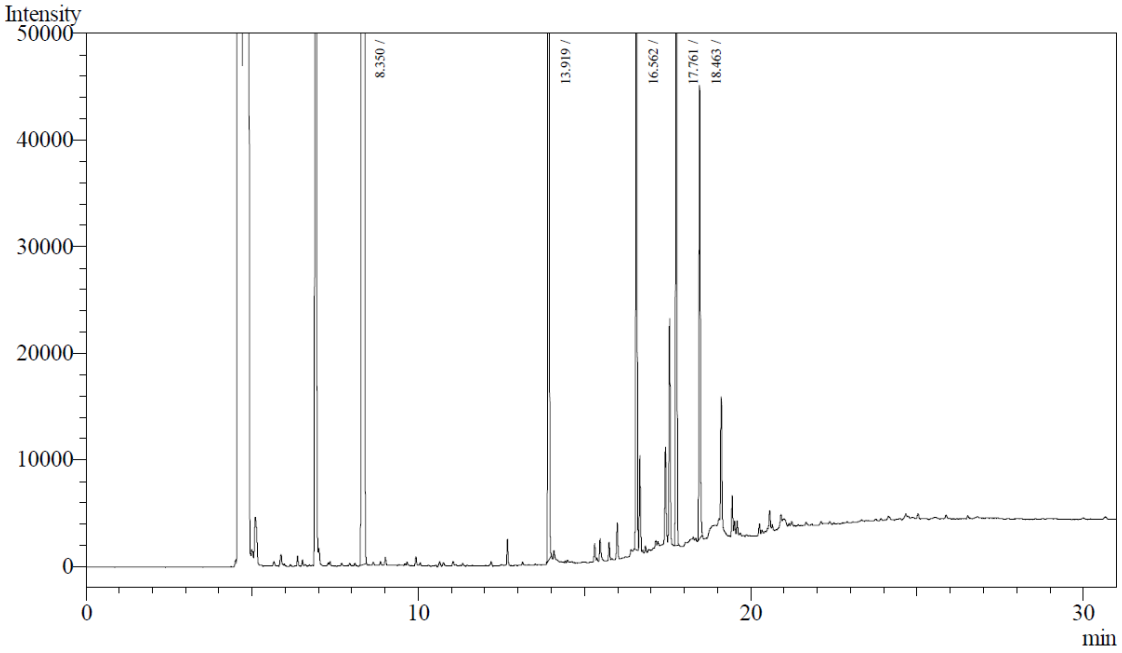
Experimental sample containing CYP6DH3 and HF-CPR with R-(+)-limonene



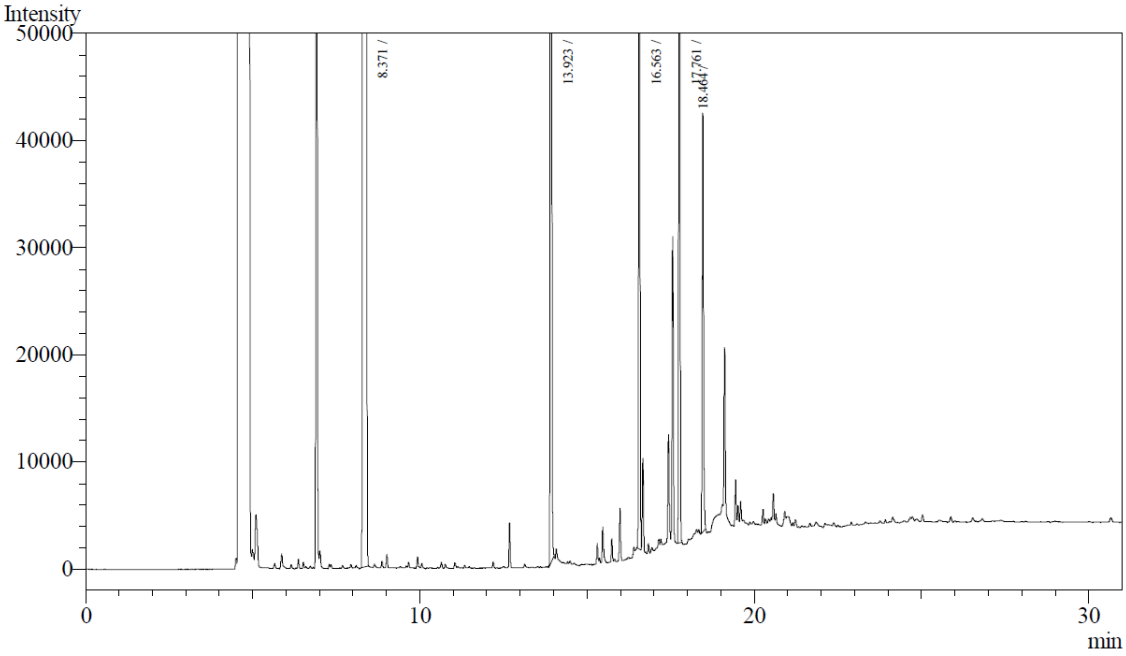
Control sample containing HF-CPR only with R-(+)-limonene

Figure 3. Gas chromatogram of pentane-ether extracts of CYP6DH3 incubated with R-(+)-limonene.

Despite Oxoplate readings (not shown), no products were extracted from the incubations. The retention time of the substrate R-(+)-limonene is 11.13 min.



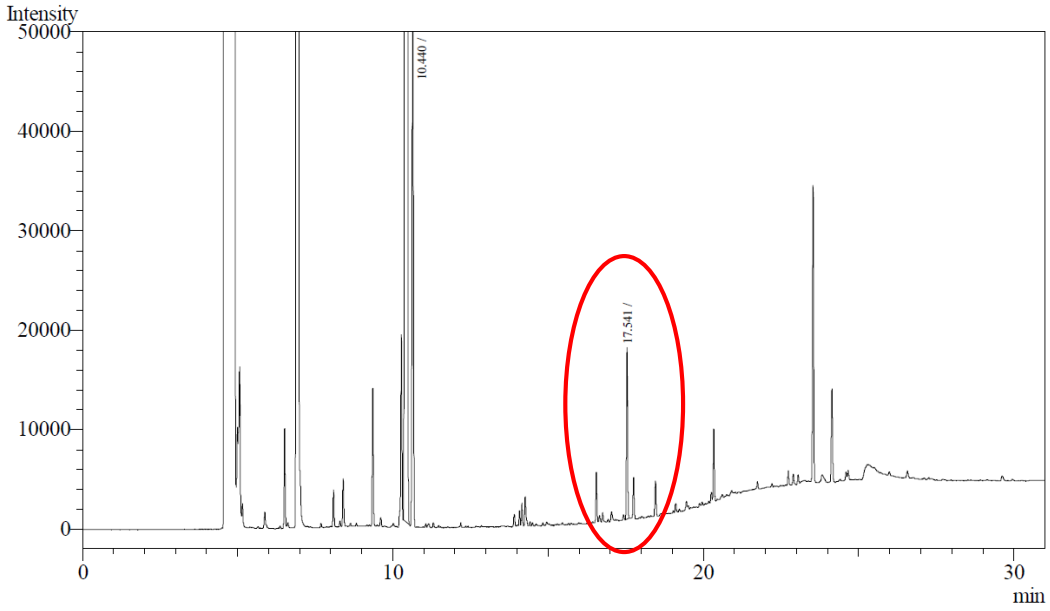
Experimental sample containing CYP9T3 and HF-CPR with (+)-α-pinene



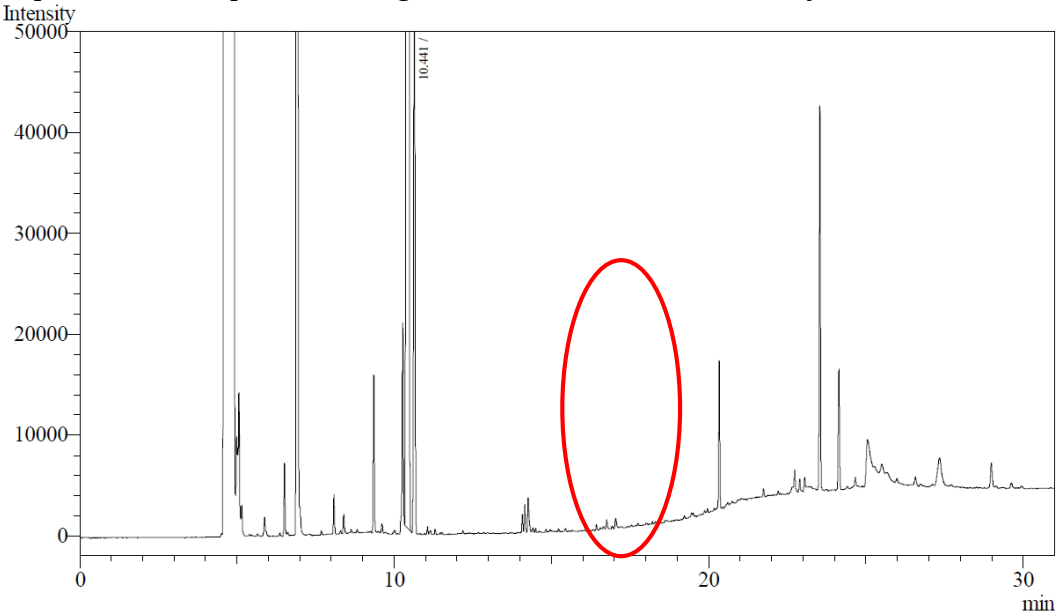
Control sample containing HF-CPR only with (+)-α-pinene

Figure 4. Gas chromatogram of pentane-ether extracts of CYP9T3 incubated with (+)-α-pinene.

Incubating CYP9T3 with (+)-α-pinene still did not form a product, confirming an issue with the assay protocol. The retention time of substrate (+)-α-pinene is 8.37 min.



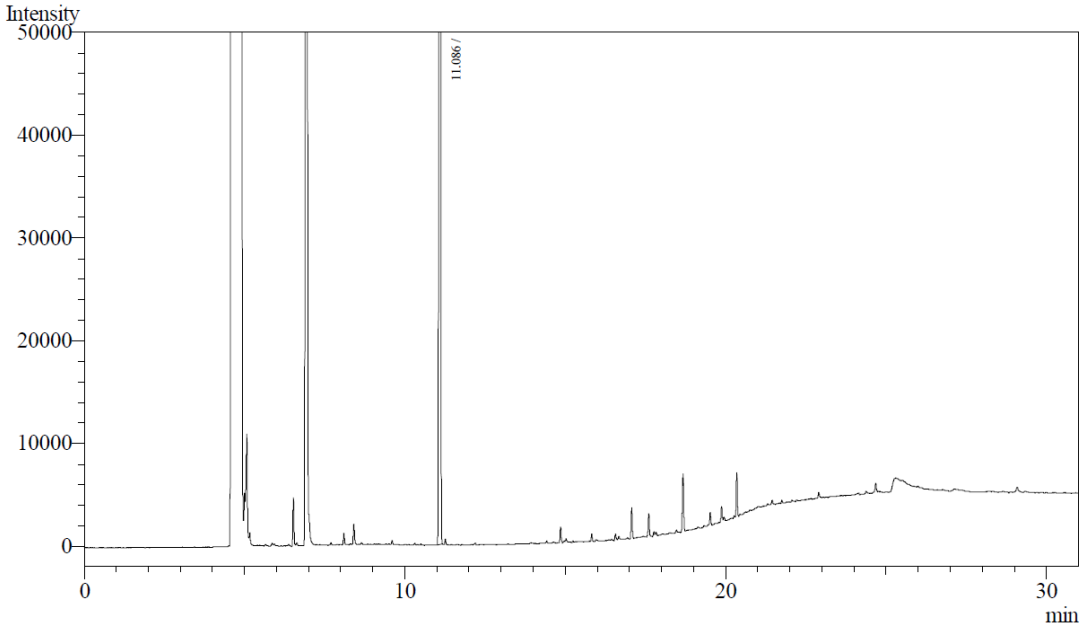
Experimental sample containing CYP9T3 and HF-CPR with myrcene



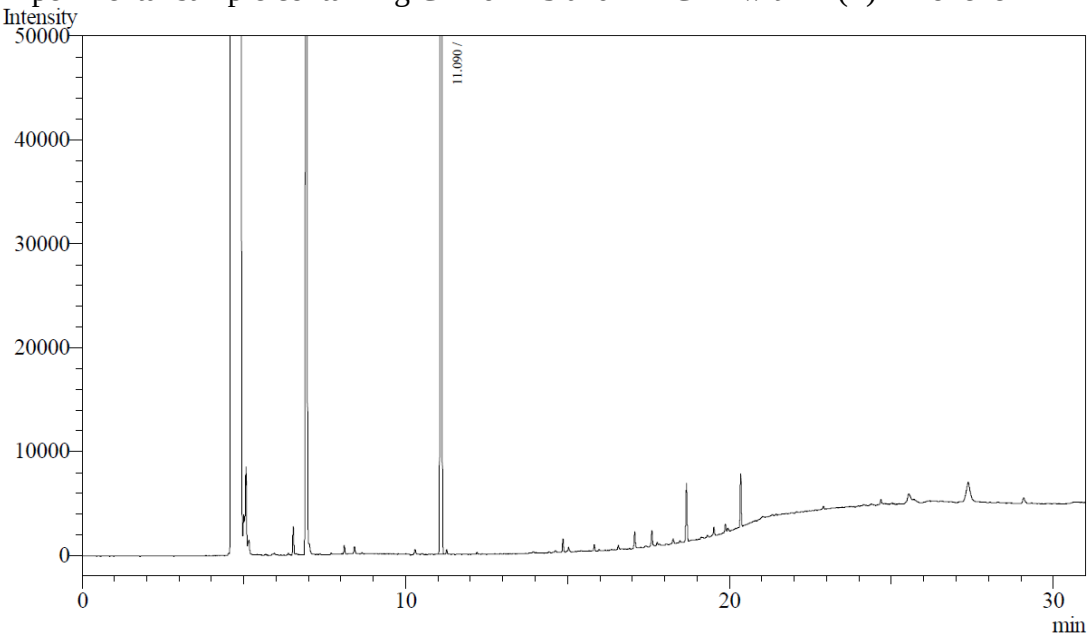
Control sample containing HF-CPR only with myrcene

Figure 5. Gas chromatogram of pentane-ether extracts of CYP9T3 incubated with myrcene.

Switching the substrate to myrcene and diluting it to 60mM before adding it to the reaction mixture resulted in the formation of a product at 17.54 minutes. The retention time of substrate myrcene is 10.41 min.



Experimental sample containing CYP6DH3 and HF-CPR with R-(+)-limonene



Control sample containing HF-CPR only with R-(+)-limonene

Figure 6. Second gas chromatogram of pentane-ether extracts of CYP6DH3 incubated with R-(+)-limonene.

Using a lower concentration of substrate did not result in an observable product being formed. CYP6DH3 must require different reaction conditions from CYP9T3 to work properly. The retention time of R-(+)-limonene is 11.09 min.

Discussion

As an invader that burrows through defensive layers of tree bark and resin, *Dendroctonus ponderosae* must be equipped with an array of detoxification enzymes to resist the variety of host tree monoterpene compounds present. MPB uses hydroxylated forms of some monoterpenes as pheromone components to coordinate their mass attacks on trees, which suggests an evolutionary link between monoterpene detoxification and pheromone production. Sequence homology suggests that CYP6DH3 is involved in detoxification by hydroxylating monoterpenes. This study attempted to confirm this hypothesis by screening for and determining the identity of any product formation catalyzed by CYP6DH3.

P450 proteins contain a heme cofactor that allows a properly folded protein to absorb light at 450nm when reduced and complexed with CO. In order to ensure that the CYP proteins used were properly folded, CO-difference spectra were taken after isolating P450 microsomes from Sf9 cells. Figures 1 and 2 demonstrate properly folded samples of CYP6DH3 and CYP9T3 respectively. The concentrations of the enzymes in each sample are also measured using this method.

No products were consistently observed using CYP6DH3 as a catalyst. Generally, the only peaks were from the solvent, the substrate, or contaminants from either the microsomal fraction or an outside source. This observation contradicted evidence from Oxoplate results that strongly suggested the enzymes were significantly active in the presence of R-(+)-limonene and some other monoterpenes, implying an issue in the assay protocol. Protocol diagnostics were conducted using CYP9T3 and its known substrates: (+)-α-pinene and myrcene. The lack of product formation using (+)-α-pinene (shown in

Figure 4) confirmed that reaction conditions in the incubation tubes were not satisfactory. After adjusting many variables in the reaction conditions such as the addition of Triton X-100, a product was finally formed from myrcene using CYP9T3. Experiments prior to this used 2 µL of pure (+)-α-pinene substrate, meaning that it is unclear whether the switch to myrcene or simply making a 100-fold dilution of the substrate beforehand contributed to the formation of the product at 17.54 min as seen in Figure 5. Either case could feasibly be the source of the problem as having too high of a concentration of substrate can result in substrate inhibition limiting the formation of product, and the (+)-α-pinene stock used contained a number of contaminants that could interfere with the reaction or otherwise mask a product peak in the gas chromatogram. As Figure 6 illustrates, the same success could not be replicated using CYP6DH3 and R-(+)-limonene under identical conditions during the incubations. Further experiments must be conducted to make this distinction and see if the same reaction conditions can produce a product using CYP9T3 and (+)-α-pinene.

Since the formation of a product using CYP6DH3 has still not been achieved, more considerations involving enzyme kinetics must be made. For example, it is possible that higher concentrations of CYP6DH3 are needed to produce a measurable amount of product. Another possibility is that the hydrophobic substrate cannot effectively reach the active site of the enzyme. The use of a shaking incubator was meant to account for this possibility. Perhaps the 800 µL total volume of the reaction is too large for the concentration of enzyme being used and the reaction must occur in a tighter space. This theory could account for why there is observable activity in the Oxoplate wells, but no product formation in the enzyme assays. This idea may be tested by using smaller

amounts of the reaction substituents and/or placing them in a smaller container than the 4 mL glass vials used in this study.

Since no new products could be consistently formed with CYP6DH3, mass spectrometry data was not collected for any of the extraction samples. Ultimately, there is still no conclusive data regarding CYP6DH3's substrate profile. Further adjustments must be made to provide proper conditions for the reactions to occur with confidence.

References

Blomquist, G. J., Figueroa-Teran, R., Aw, M., Song, M., Gorzalski, A., Abbott, N. L., . . . Tittiger, C. (2010). Pheromone production in bark beetles. Insect Biochemistry and Molecular Biology, 40(10), 699-712. doi:10.1016/j.ibmb.2010.07.013

Coggins, S. B., Coops, N. C., Wulder, M. A., Bater, C. W., & Ortlepp, S. M. (2011). Comparing the impacts of mitigation and non-mitigation on mountain pine beetle populations. Journal of Environmental Management, 92(1), 112-120. doi:http://0-dx.doi.org.innopac.library.unr.edu/10.1016/j.jenvman.2010.08.016

Keeling, C. I., Henderson, H., Li, M., Dullat, H. K., Ohnishi, T., & Bohlmann, J. (2013). CYP345E2, an antenna-specific cytochrome P450 from the mountain pine beetle, dendroctonus ponderosae hopkins, catalyses the oxidation of pine host monoterpene volatiles. Insect Biochemistry and Molecular Biology, 43(12), 1142.

Robert, J. A., Pitt, C., Bonnett, T. R., Yuen, M. M. S., Keeling, C. I., Bohlmann, J., & Huber, D. P. W. (2013). Disentangling detoxification: Gene expression analysis of feeding mountain pine beetle illuminates molecular-level host chemical defense detoxification mechanisms. PloS One, 8(11), e77777.

Sambaraju, K. R., Carroll, A. L., Zhu, J., Stahl, K., Moore, R. D., & Aukema, B. H. (2012). Climate change could alter the distribution of mountain pine beetle outbreaks in western canada. Ecography, 35(3), 211-223. doi:10.1111/j.1600-0587.2011.06847.x

Song, M. (2012). Mountain pine beetle (MPB; dendroctonus ponderosae) pheromone component biosynthesis, cytochromes P450 and monoterpene metabolism in bark beetles

Song, M., Kim, A. C., Gorzalski, A. J., MacLean, M., Young, S., Ginzel, M. D., Tittiger, C. (2013). Functional characterization of myrcene hydroxylases from two geographically distinct ips pini populations. Insect Biochemistry and Molecular Biology, 43(4), 336.