Effect of Turmeric on the Promoter Activity of the Cyp6a8 Gene of *Drosophila melanogaster*

Honors Senior Thesis

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Abstract:

Cytochrome P450 (CYPs) are a subset of enzymes found in all living organisms. They are vital to many biosynthetic pathways by ridding the body of toxins as well as synthesizing hormones. These enzymes are important because their detoxification property is useful in metabolizing potential pathogens or fatal chemicals such as drugs, food additives, caffeine, etc. One of the foreign compounds that CYP enzymes break down is insecticides. In the fruit fly *Drosophila melanogaster*, certain strains with an over-expression of these P450 genes have been found to be resistant to pesticides. This increased level of expression is found specifically in the Cyp6a8 and Cyp6a2 genes of *Drosophila*. Previous studies have shown caffeine as an inducer of the promoter of these genes. I wanted to study the effects of turmeric on gene expression levels of the Cyp6a8 gene in genetically modified Drosophila of the *0.8 luc 110 (H-ry)* strain. This strain of flies contains a luciferase reporter gene under the control of the Cyp6a8 promoter. Both sexes of flies were used in this experiment. They were allowed to feed for 48 hours, after which fly extracts were prepared and luciferase activity was measured. The results showed that the use of turmeric did significantly increase the expression of the Cyp6a8 promoter. The most significant increase in expression occurred with the use of a turmeric solution concentration of 5mg/mL. These results suggest that the 0.8-kb upstream DNA of the *Cyp6a8* gene has *cis*-acting sequences for turmeric induction. Future studies will determine whether induction by turmeric and caffeine are mediated via the same pathway.

Introduction

Cytochromes P450 (CYPs) are enzymatic proteins that are found in all cells. CYP enzymes are found in all kingdoms of life, even viruses, in varying numbers (Lamb 2009; Feyereisen, 2012; Liu et al, 2015). These CYPs provide detoxification properties that cleanse the cell of harmful substances. They are responsible for recognizing xenobiotics and metabolizing these toxins in chemical systems (Maitra 2002). Xenobiotics are any chemical compounds such as drugs, pollutants, food additives, and insecticides that are foreign to a living system. The removal of these contaminants is an important process, as otherwise such toxins would build up in the body and potentially alter regular cell functions. Although best known for their extremely beneficial detoxification properties, CYPs in rare instances have been proven to harm the body through their natural processes. In addition to metabolizing harmful chemicals, CYPs are also responsible for synthesizing hormones. Over-expression of hormones like estrogen has been correlated to diseases and increased instances of breast cancer (Labas 2017).

Through the detoxification of cells, Cytochromes P450 are able to help organisms cope with the environment. Insects use these CYP enzymes to metabolize environmental xenobiotics like pesticides. Some strains of insects confer resistance to the lethal effects of insecticides (Balabanidou 2016). Scientists have looked at the biological control behind this and discovered an increased expression of Cytochrome P450 genes in insects that are resistant to these chemicals (Maitra 2002). Such studies are often done with *Drosophila melanogaster* fruit flies. There are multiple P450 genes in every species, making it hard to determine specifically which are responsible for this regulation; however, researchers have been able to model which of these genes are up-regulated in resistant strains of *Drosophila*. Out of the eighty three P450 genes present in *Drosophila*, there are six P450 genes that show higher levels of expression in resistant strains as opposed to susceptible ones. Cyp6a8 and Cyp6a2 are two of these CYP genes that are

up-regulated (Bhaskara 2008).

For a gene to become over-expressed there must be an increased transcription of its DNA. RNA polymerase is an enzyme that transcribes the sequence of DNA into mRNA. The mRNA is then transported to a ribosome where it is translated into the protein product of whatever that gene is expressing. Multiple factors can play a role in the increased transcription of a gene such as the amount of RNA polymerase, promoters on the gene that increase the rate of transcription by recruiting RNA polymerase, and other chemical modifications to DNA. To determine the mode of over-expression, it is helpful to determine how expression of the gene is regulated. The presence of certain xenobiotics has been shown to induce the gene promoters of their over-expressed P450 genes (Bhaskara 2008). Since the promoter promotes gene expression, these genes are transcribed at a higher rate. Experiments involving flies exposed to caffeine, a common xenobiotic in many diets, have produced a 4-5 fold induction of promoter activity in treatment groups (Bhaskara 2006).

To explore genetic regulation of Cytochrome P450 genes, many xenobiotics such as caffeine, curcumin, phenobarbital, and insecticides have been used. The objective of my research was to study the genetic regulation of the Cyp6a8 gene in *Drosophila melanogaster* exposed to turmeric treatments. Turmeric is a spice that is known for its many medicinal benefits including the reduction of inflammation, arthritis, and anxiety (Hewlings 2017). My focus was on the Cyp6a8 gene because other studies including Bhaskara et al. 2006 and 2008 demonstrated that the promoter of that specific P450 gene was induced significantly in the presence of the xenobiotic caffeine. A transgenic Drosophila line was created, using a reporter gene called *luciferase* from a firefly gene that was attached to the promoter of Cyp6a8. Thus, the *luciferase* gene will only be expressed if this promoter is induced. This was the same strain of Drosophila (*0.8 luc 110 (H-ry*)) Bhaskara used to measure gene expression.

Materials and Methods

Fly Strains and Culture Conditions:

The modified strain of *Drosophila melanogaster* used in this experiment was *0.8 luc 110 (H-ry)*. These flies are genetically modified to contain a reporter gene, *luciferase (luc),* which is placed between the promoter and the Cyp6a8 gene. Since the *luc* gene is placed under the presence of the Cyp6a8 promoter, when the CYP gene is transcribed, so is the *luc* gene. Thus, luciferase activity can be measured to determine the activity of the Cyp6a8 gene promoter. The flies were cultured at room temperature on a culture containing agar, cornmeal, yeast, dextrose, sucrose, corn syrup, and water.

Preparation of Turmeric Solution and Treatment of Flies:

A turmeric solution was prepared by mixing 153.8mg of pure turmeric with 15.3mL of sterilized water to create a turmeric suspension that was 10mg/mL. Similar methods were utilized to create suspensions of 5mg/mL and 1mg/mL. Instant fly food was prepared using water and placed into vials. To the treatment vials, 1mL of turmeric suspension was added on top of the food that had already been saturated with water. Since the turmeric solution was a suspension, the turmeric remained on top of the food where it would be consumed by the flies. To the control vials, just water was added to prepare the instant fly food. The flies were etherized and sorted by sex. Upon waking, 20 female or male flies were placed in each vial, depending on the experiment conditions. The flies were placed in the dark for approximately 48 hours before being removed for extract preparation.

Preparation of Fly Extracts:

Following the 48 hours of treatment conditions, the flies were removed from the vials and

etherized. They were then sorted into groups of 5 and placed in 1.5mL Eppendorf tubes. To each tube, 200µL of 1X Cell Culture Lysis Reagent (CCLR) buffer was added. The flies were then homogenized thoroughly using a hand-held homogenizer. The homogenized mixture was centrifuged at 13,000 RPM for approximately eight minutes at room temperature. Once this time had passed, the tubes were carefully removed and placed on ice. From each tube, 100µL of clear supernatant was removed, leaving behind a protein pellet and any unwanted lipids. The 100µL was placed in a new 1.5mL Eppendorf tube and centrifuged again, in the same way as stated previously. After this second centrifugation, 50µL of supernatant was removed from the homogenate and placed in a 1.0mL Eppendorf tube. The tubes were stored at -20°C until they were ready to be used for luciferase and protein assays.

Luciferase Assay:

The Eppendorf tubes containing the fly extracts were removed from the freezer and placed in a rack at room temperature, allowing them to thaw. The luminometer, a machine that measures RLU (relative light reading) was turned on and prepared. After these were set up, LAR (luciferase assay reagent) was removed from the freezer, where it was stored at -80°C. This reagent was set out to thaw, but it was important that it was thawed in the dark to keep it stable, as it becomes unstable when exposed to light. The entire luciferase assay was performed in diffused light as to keep the values as accurate as possible. To begin, 5µL of fly extract and 20µL of LAR were mixed in a fresh 1.0mL Eppendorf tube. The tube was quickly placed in the luminometer and the RLU was measured by the machine after 15 seconds and recorded. The *luc* gene is responsible for luciferase enzyme activity. Luciferase is an enzyme that produces bioluminescence, thus the RLU was able to measure the amount of light produced from the enzyme. Since the transcription of luciferase was dependent on the transcription of Cyp6a8, the

amount of light produced correlated to the amount of Cyp6a8 transcribed. This procedure was performed for all extract samples.

BCA Protein Assay:

The Pierce™ BCA Protein Assay kit was used to determine the amount of protein present in each fly extract. At first, ten glass test tubes were lined up in a rack, two for each of the varying amounts of BSA (Bovine Serum Albumin) standard: 0, 12.5, 25, 37.5, and 50µL. A 2mg/mL stock BSA solution was diluted using equal amounts of BSA and 1X CCLR. This yielded a solution with a concentration of 1mg/mL BSA in 0.5X CCLR. Increasing amounts of the 1mg/mL BSA in 0.5X CCLR standard were added in duplicate to the ten prepared glass test tubes in intervals: 0, 12.5, 25, 37.5, 50µL. The tubes were then filled to 50µL in volume by adding 0.5X CCLR. For each fly extract, two test tubes were prepared. To all test tubes, 40µL of 0.5X CCLR was added. Then 5µL of fly extract was added to each test tube. To all of the fly extracts and the standard tubes, 1mL of BCA reagent was added and the tubes were mixed gently. After this preparation, all tubes were placed in an incubator at 37°C for thirty minutes. A spectrophotometer was then used to read the absorbance or optical density of the samples at 595nm wavelength after the first ten tubes were run as standards to create a standard protein curve. Final data for the extracts was normalized by combining these measurements with the luminometer RLUs to determine the RLU/µg protein for each fly extract sample.

Results and Discussion:

To investigate the effects of different turmeric treatments on Cyp6a8 promoter activity, adult Drosophila flies of the *0.8 luc 110 (H-ry)* strain were allowed to feed for 48 hours on food prepared with varying amounts of turmeric. First, the flies were treated under simple control conditions. Strains of female flies treated with 10mg/mL turmeric were compared to untreated flies. The effect of 10mg/mL of turmeric on Cyp6a8 promoter activity significantly increased gene transcription compared to the control group. Following the initial experiment, further trials were conducted comparing the effects of varying amounts of turmeric (Figures 2 & 3). These experiments displayed changes in promoter activity based on different amounts of turmeric concentration used in the treatment, with a peak effect at 5mg/mL turmeric. This was further confirmed by the fold induction of female flies. Those treated with 5mg/mL turmeric displayed a fold induction average of 7.05 whereas females treated with 10mg/mL showed a fold induction of only 4.93. Finally, experiments were conducted to compare the effects of 10mg/mL turmeric treatments between male and female flies (Figure 4). This data displayed no significant difference on the effects of turmeric between male and female flies. This data further identifies turmeric as a xenobiotic inducer of the Cyp6a8 gene, with a peak induction level at a concentration of 5mg/mL turmeric.

Turmeric is a spice known around the world for its health benefits. It aids with health problems such as inflammation, arthritis, and anxiety to name a few. These health benefits are due to the curcumin in turmeric, but studies have shown that curcumin on its own does not have the same benefits due to its poor bioavailability (Hewlings 2017). Many studies have been done with pure curcumin, but it is hard to find ones specifically performed with turmeric. Additional studies done with turmeric at a concentration of 5 mg/mL would be ideal for next steps in experimenting, as this was the concentration for peak induction in the experiments of this study.

Figure 1. Induction of Cyp6a8 gene promoter in female flies treated with turmeric. Each bar represents average of triplicate determination.

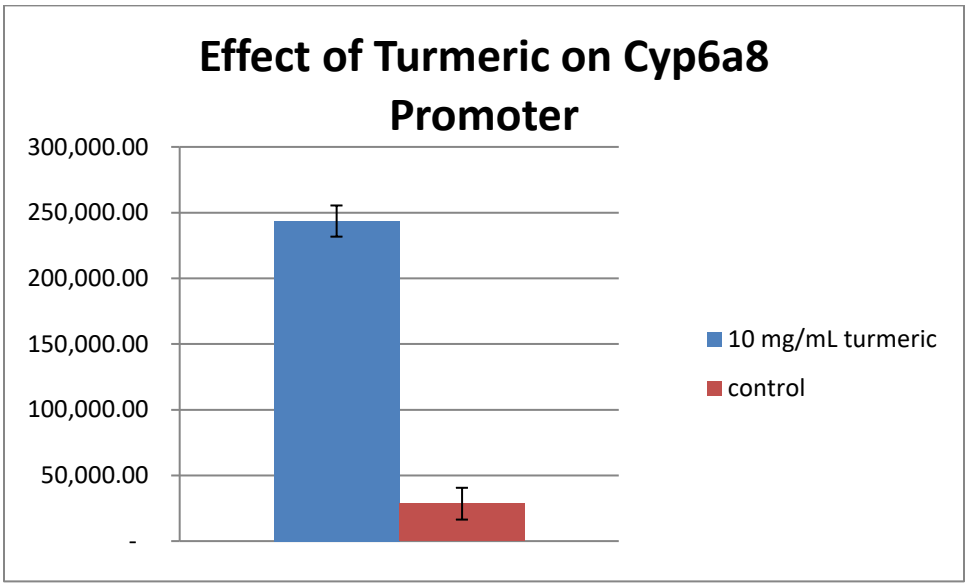


Figure 2. Induction of Cyp6a8 gene promoter in female flies treated with varying turmeric concentrations. Each bar represents average of triplicate determination.

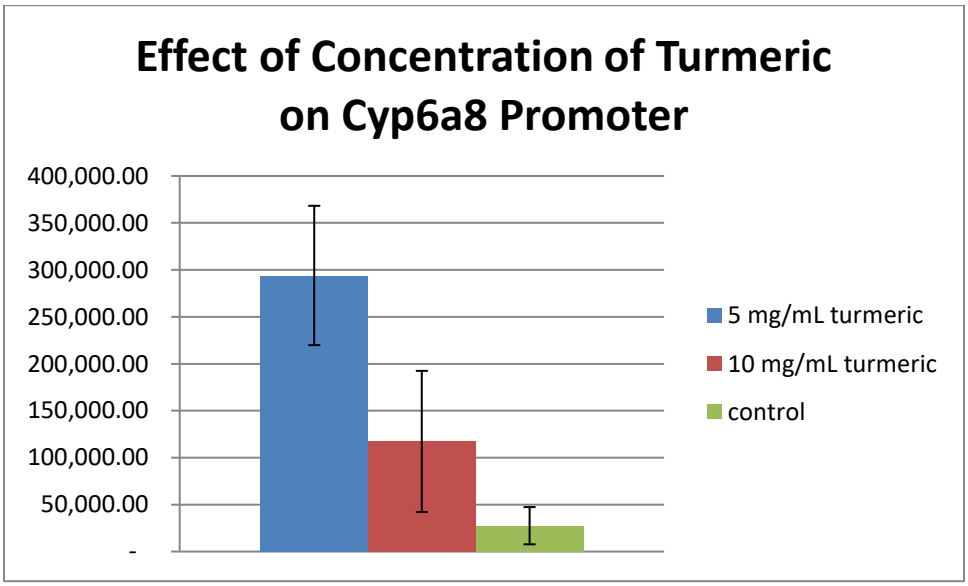


Figure 3. Induction of Cyp6a8 gene promoter in female flies treated with varying turmeric concentrations. Each bar represents average of triplicate determination.

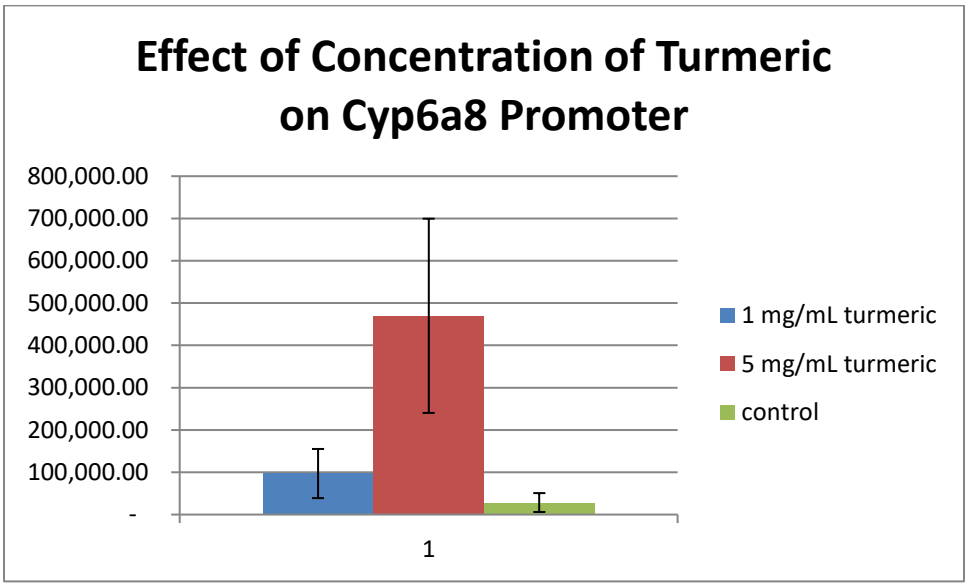


Figure 4. Induction of Cyp6a8 gene promoter in male and female flies treated turmeric. Each bar represents average of triplicate determination.

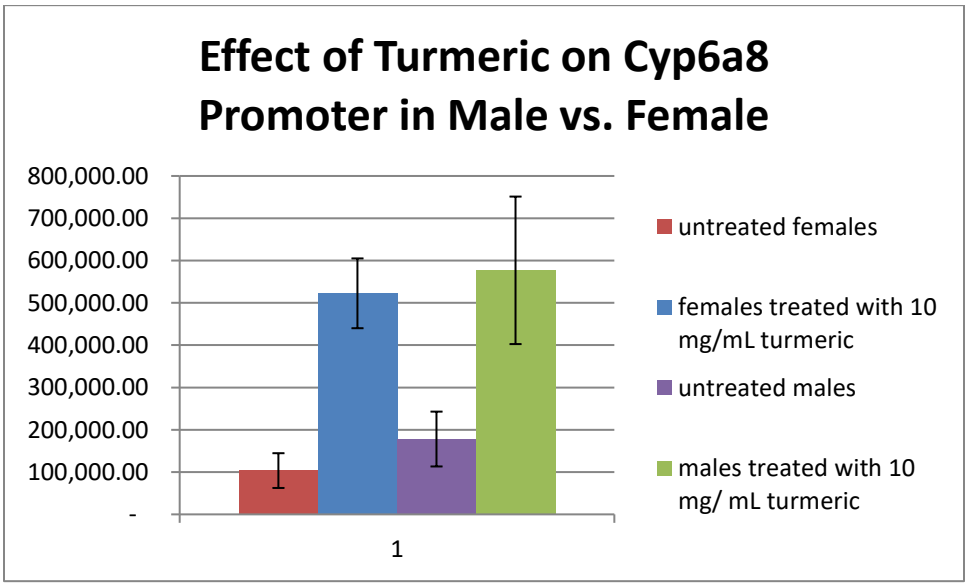


Table 1. Results of Cyp6a8 promoter induction in Drosophila of the 0.8 luc 110 (h-ry) stain exposed to varying turmeric concentrations. Average RLU/µg protein calculated from data collected from luciferase assay and BCA protein assay.

Experiment	Sex	Treatment	RLU/µg protein*	Standard Deviation	Fold Induction
1	female	10 mg/mL turmeric	243,577.36	11,855.43	8.55
	female	5 mg/mL turmeric	294,039.50	74,209.84	10.68
2	female	10 mg/mL turmeric	117,309.77	72,152.17	4.26
	female	1 mg/mL turmeric	97,034.34	58,076.61	16.53
3	female	5 mg/mL turmeric	469,958.59	229,621.50	3.41
	female	10 mg/mL turmeric	103,352.50	54,999.09	1.86
4	male	10 mg/mL turmeric	334,176.29	93,952.16	3.3
	female	10 mg/mL turmeric	522,566.91	82,488.35	5.05
5	male	10 mg/mL turmeric	576,927.29	174,360.50	3.24

*Mean of triplicate determination

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