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Methods of stabilizing a key player in the inflammatory response: co-expression of 5-lipoxygenase and coactosin-like protein

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Methods of stabilizing a key player in the inflammatory response: co-expression of 5-lipoxygenase and coactosin-like protein

by

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Undergraduate honors thesis under the direction of

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Abstract

The enzyme 5-lipoxygenase catalyzes the first reaction in leukotriene biosynthesis, which leads to pathogenic inflammation and associated diseases. In order to study 5-lipoxygenase effectively, the protein should be able to be easily expressed and purified with high yield. However, wild type (or naturally occurring) 5-lipoxygenase is intrinsically unstable, and therefore is hard to work with experimentally. 5-lipoxygenase interacts with a variety of proteins during its activation and catalysis, including coactosin-like protein. Coactosin-like protein causes increased product formation and active 5-lipoxygenase in the cytosol. 5-lipoxygenase and coactosin-like protein were co-expressed and purified on a small scale. Coactosin-like protein improved wild-type 5-lipoxygenase expression, which would be important in obtaining wild-type 5-lipoxygenase for other experiments.

Introduction

Enzymes are proteins that speed up the rate of a biological reaction. They bind their substrates, catalyze the reaction, and release product. The free enzyme is available for another round of catalysis (2). In identifying drug targets to treat disease, enzymes are favorable candidates, as a drug that binds the enzymes can stop a disease-causing reaction to occur. Lipoxygenases are a family of enzymes that catalyze a regio- and stereo-specific dioxygenation of polyunsaturated fatty acids (3). The protein 5-lipoxygenase (5LOX) is an enzyme with a molecular weight of 78 kD that catalyzes the first two steps in leukotriene biosynthesis. Leukotrienes mediate inflammatory responses, which can lead to diseases such as atherosclerosis, asthma, and arthritis (4).

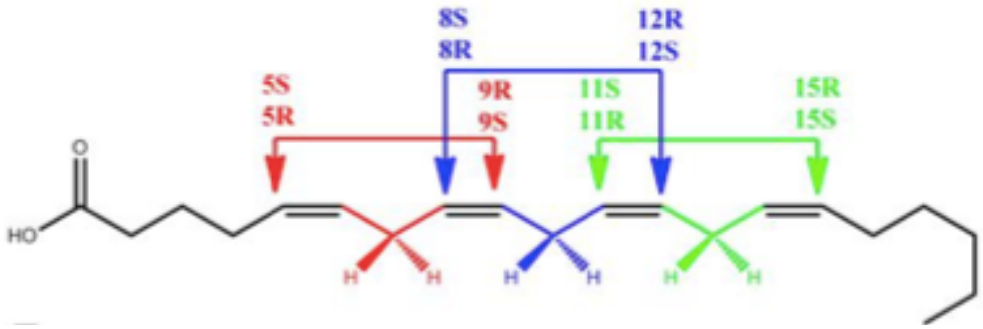

Figure 1. Lipoxygenase specificity for arachidonic acid. 5LOX attacks at the seventh carbon, creating a new double bond at the fifth carbon, in the same way 15LOX attacks the thirteenth carbon to create a double bond at the fifteenth carbon (5).

5LOX works on the omega-6 polyunsaturated fatty acid arachidonic acid found in the cell membrane. Arachidonic acid is an essential fatty acid and is also the substrate for cyclooxygenases that produce prostaglandins, mediators in the inflammatory response (6). 5LOX exhibits specificity for reaction at the seventh carbon; compared to 15-lipoxygenase, which acts on the thirteenth carbon (3). Figure 1 shows the differences in lipoxygenase specificity. 5LOX oxidizes arachidonic acid to 5(S)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE) and immediately dehydrates 5-HPETE to 5(S)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (also known as leukotriene A4 or LTA_4) (7). LTA_4 is then modified by other proteins to form either LTB_4 or LTC_4 (8,9).

In order to become active, 5LOX must translocate from the cytoplasm to the nuclear membrane. Once associated with the membrane, 5LOX receives the substrate arachidonic acid from 5-lipoxygenase activating protein (FLAP), though the mechanism for the transfer is not known. The location of 5LOX within the cell may determine what leukotriene is ultimately produced. 5LOX inside the nucleus moves to the inner nuclear membrane and leads to LTB_4, while 5LOX present in the cytoplasm moves to the outer nuclear membrane and leads to LTC_4. Figure 2 shows this pathway. For further review, see Newcomer and Gilbert, 2010.

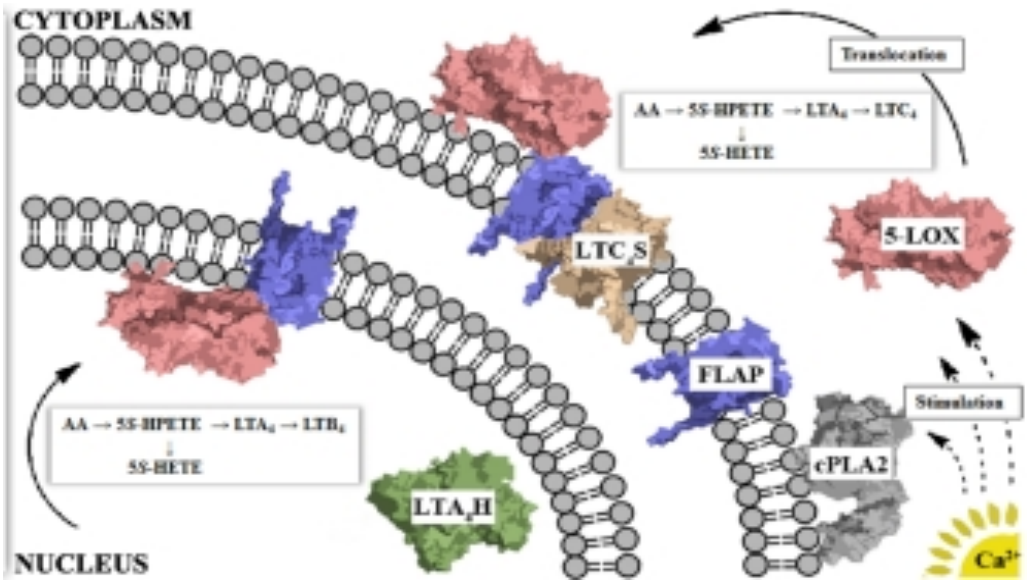

Figure 2. Ca^{2+} elevation causes localization of 5LOX to the nuclear membrane, where it receives arachidonic acid from FLAP. Final product of the 5LOX initiated reaction depends on location of 5LOX: inside the nucleus, 5S-HPETE becomes LTB_4 and outside the nucleus, 5S-HPETE becomes LTC_4 (10).

Because compartmentalization of 5LOX has a large impact in the final products the leukotriene synthesis pathway, finding other 5LOX helper proteins could help in elucidation of the entire activating pathway for 5LOX. Provost *et al.* found an interaction partner in human coactosin-like protein (CLP) (4). CLP is named for its homology to coactosin, an actin-binding protein that slows down actin polymerization (11). CLP, molecular weight 17 kD, can bind F-actin.

Provost *et al*. also looked into the possible interactions between 5LOX, CLP, and actin. 5LOX alone prevented actin polymerization, and they also concluded that 5LOX bound to CLP would prevent actin polymerization. They found no evidence of a tertiary complex containing all three components.

Rakonjac *et al*. demonstrated that CLP could act in the place of phosphatidylcholine (PC, a membrane fatty acid) as a scaffold for 5LOX activity, introducing the possibility of an active 5LOX in the cytosol, though highest production of 5-HPETE and LTA_4 occurred in the presence of both CLP and PC (12).

Several studies have been done on the amino acids that may aid the two proteins in their interaction. The proteins were shown to bind with 1:1 stoichiometry (13). In CLP, lysine-131 was shown to be important in 5LOX binding, by substitution with alanine (14). Lysine-131 was also substituted with arginine (another basic amino acid) without loss of CLP binding activity, indicating that the basicity of the amino acid important in the 5LOX interaction (4). In 5LOX, tryptophan-102 was absolutely essential in CLP binding. Esser *et al.* proposed a model of interaction between 5LOX and CLP based on these findings, shown in figure 3 (15).

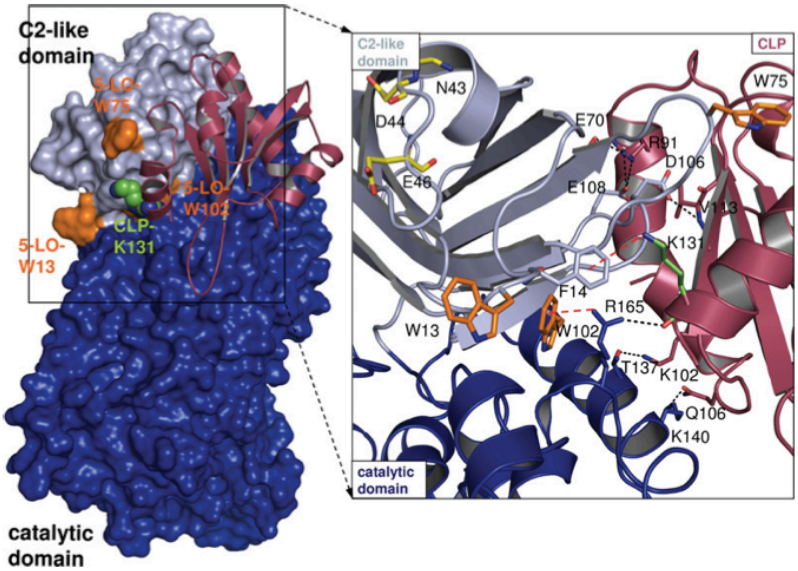

Figure 3. Proposed binding model of 5LOX and CLP, showing the roles of lysine 131 in CLP and tryptophan 102 in 5LOX. Also shown is the interaction between lysine 131 and phenylalanine 14 (15). 5-LOX is space-filled render, the C2-like domain (membrane binding domains) and catalyic domain in blue. CLP is show in cartoon rendering. In the box, both proteins are represented in cartoon rendering with important amino acids in stick,

Provost, Rakonjac and Esser all expressed CLP and 5LOX separately, purified each, and carried out experiments with separate, purified proteins. However, in the cell, proteins have the possibility to be translated simultaneously, with association occurring as the proteins fold or immediately after folding finishes (16). Expressing each protein in separate cell lines from different plasmids does not allow the proteins to interact immediately after folding. Co-expressing 5LOX and CLP, with the idea of increasing the yield of 5LOX due to the stabilizing effect of CLP, was a large part of our experimental design.

There are two ways to force *Escherichia coli* to over-express two proteins. One can transform *E. coli* with two plasmids, each containing one gene of interest. However, many things must be taken into account. First, the chances of both plasmids entering the same cell are very low, and the amount of colonies on the plate would be low. Second, in order to use antibiotics to select for transformed cells, three antibiotics would be used: one for each vector and one for the resistance of the competent cells being transformed. Expression of 5LOX requires Rosetta cells, which carry a plasmid coding for tRNAs that recognize rare codons. Maintenance of this plasmid requires chloramphenicol, which would have to be the third antibiotic. Again, this decreases the chances of successful

transformation. Lastly, the plasmids would also have to have unique origins of replication, to make sure that both plasmids are replicated and passed down to daughter cells. The plasmids may also have two different promoters, meaning two different molecules would be used for induction of protein expression.

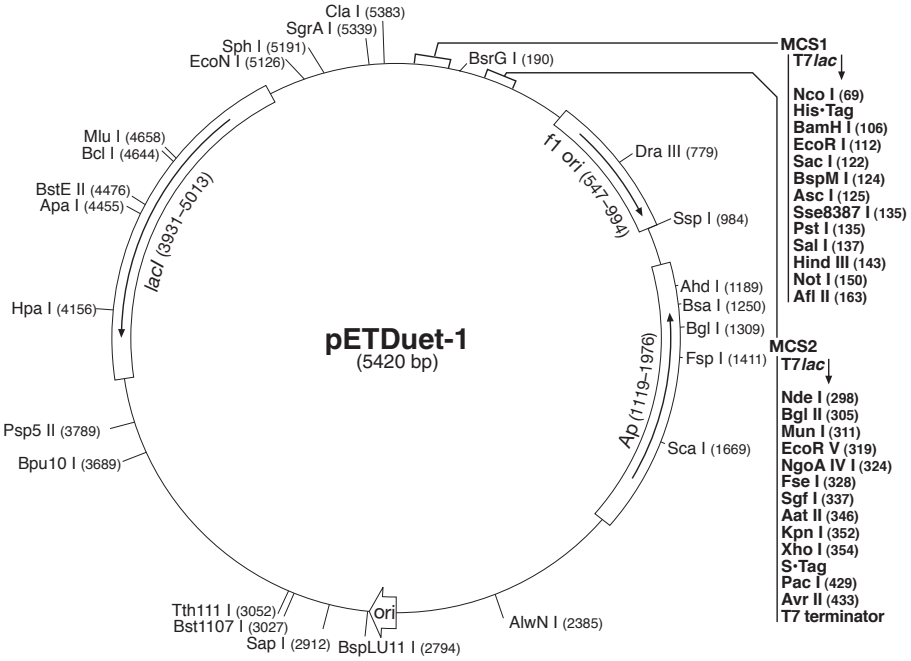

Figure 4. Vector map of pETDuet-1, showing multiple cloning sites in which CLP and 5LOX were inserted (17).

We decided to use the pETDuet-1 plasmid, a vector that contains two sites into which one can clone genes of interest. Figure 4 shows the pETDuet-1 vector map. The genes for 5LOX and CLP are present on one plasmid with one antibiotic resistance gene and one origin of replication. By transforming just one plasmid into competent cells, (1) chances of success are intrinsically higher, (2) only one antibiotic needs to be used to select for one plasmid, and (3) the

plasmid will be replicated and passed to daughter cells without competing with any other plasmid origins of replication. One inducer (IPTG) can be used for both proteins, because they use the same *lac* promoter sequence.

Wild-type 5LOX (5LOX-wt) is inherently unstable and difficult to express, purify, and crystallize (5,18). In order to obtain enough purified protein for experiments, large cultures of transformed *E. coli* had to be purified at once and yielding low amounts of purified protein. Then, because the protein is inherently unstable, it denatured easily at room temperature, making experiments difficult and causing more expression/purification rounds. Dr. Nathaniel Gilbert was able to make mutations to the domains believed to contribute to this instability and obtained a crystal structure of his mutant, known as 5LOX-octa (19). The stable-5LOX structure has allowed for insight into the 5LOX reaction mechanism as well as comparison with other lipoxygenase structures and mechanisms. By expressing CLP and 5LOX-wt simultaneously, we will be able to see if CLP contributes to improving expression of 5LOX-wt. We also want to see if the 5LOX-wt and CLP bind tightly to form their complex in solution and during affinity chromatography purification.

Materials & Methods

Cloning

In order to create a protein expression system, the genes of choice must be inserted into plasmids, which are circular double stranded DNA that can be transformed into bacteria. Restriction enzymes are used to cut certain DNA sequences, leaving “sticky” ends that are complementary to each other. The same restriction enzymes are used on the gene of interest and the plasmid so that they have complementary sticky ends, and two restriction enzymes are used to ensure that the genes of interest is inserted into the plasmid in the correct orientation. The sticky ends of each DNA strand hydrogen bond to the other due to their complementarity. DNA ligase can then covalently link the two pieces of DNA, creating a circular plasmid now containing an insert of the gene of interest. Essentially, genes are “cut” out of one plasmid and “pasted” into another (Figure 5).

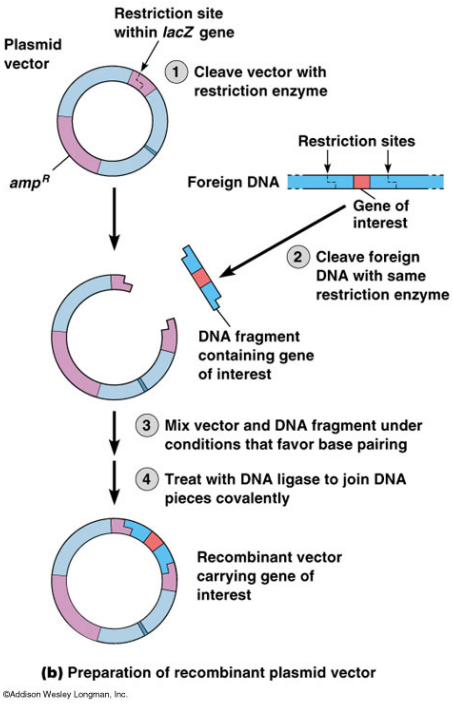

Figure 5. “Cut and Paste” cloning method (1)

The plasmid used was pETDuet-1. First, CLP and pETDuet were digested with NdeI and AvrII, ligated and transformed into alpha-select cells. A miniprep of plasmids was performed, resulting in stock of pETDuet-CLP plasmid. 5LOX-wt

(containing sequences for a His-tag) was amplified by PCR from pET14b-5LOX-wt. pETDuet-CLP was digested with XbaI and BamHI, and 5LOX-wt was digested with XbaI and BglII. BamHI and BglII leave complementary sticky ends, allowing for ligation of the two strands. pETDuet-CLP and 5LOX-wt were ligated and transformed into alpha-select cells. A miniprep of plasmids was performed, providing a stock of 5LOX-wt/CLP in pETDuet.

Expression

We transform our newly constructed plasmid into special *Escherichia coli* cell lines. Specifically, we used Rosetta 2(DE3) cells. The Rosetta cell line expresses tRNAs that recognize rare codons that *E. coli* do not naturally express, but that occur in mammalian genes. This kind of competent cell is useful when trying to express mammalian proteins in the bacteria *E. coli*. “DE3” indicates that the cells contain the gene for lambda phage T7 RNA polymerase, which is activated by addition of the inducer IPTG (20). Therefore, the Rosetta cells (prokaryotic cells) are able to make our protein of interest (a eukaryotic protein) in excess, allowing for purification and further study.

5LOX-wt/CLP in pETDuet was transformed into Rosetta 2(DE3) cells on a small scale (3mL media) to ensure proteins were being expressed. 5LOX-wt/CLP in pETDuet and 5LOX-wt in pET14b were transformed into Rosetta for large-scale expression. Colonies were picked and inoculated into 25mL of LB and grown at 37°C and 220 rpm. 5mL of overnight culture was inoculated into 500 mL of TB and grown for 4 hours at 37°C and 220 rpm. The temperature was

changed to 20°C for 20 hours. Select flasks were induced with 500 mM IPTG 24 hours after inoculation. Seven hours after induction, 1mL aliquots of cells were pelleted and stored at -80°C.

Pellet Lysis & Gels

In order to compare the expression protocols, aliquots are lysed and run on an SDS-PAGE. SDS-PAGE stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis, an analytical technique. Sodium dodecyl sulfate coats the proteins, denaturing them and conferring a uniform negative charge. When subjected to an electric field, proteins migrate toward the positive node based solely on their polypeptide chain size. Coomassie blue is a compound that forms a complex with proteins at positive amine groups and can be seen in visible light, and it can detect as little as 0.5 $\mu g/cm^2$ of protein present in the gel matrix (21).

Pellet mass was measured, cells were lysed with 300 uL BugBuster®, and the suspension was mixed in cold room for 10 minutes. Cells were centrifuged and 200uL of supernatant was collected, 10 uL of which was run on SDS-Page at 150 V for 60 minutes. Gels are stained with Coomassie blue and destained for analysis.

Pellet Lysis & Batch Purification

Chromatography is a technique that involves a mobile phase with a certain physical property (eg. hydrophillicity) run past a stationary phase with a different or opposing physical property (eg. hydrophobicity). Affinity chromatography uses

small beads (also known as resin; made of agarose, silicone or some other inert material) on which a chelating agent (cobalt or nickel) is attached as the stationary phase. Your protein of interest in solution is the mobile phase. When cloning the protein of interest, sequence for a purification tag can be included so that the protein contains a sequence of six or more histidine residues. The tag specifically interacts with the chelating agent, allowing the protein of interest to stick to the resin. An increasing gradient of a molecule that mimics the tag is washed over the resin, displacing or eluting the protein of interest at a specific concentration. Fractions collected during elution allow you to collect only your protein of interest for further purification or study. After running the fractions on an SDS-PAGE gel, the gel is stained with SYPRO Ruby gel stain. SYPRO Ruby is a fluorescent gel stain that is more sensitive than Coomassie Blue, detecting as little as 0.25-1 ng of protein (22)

5LOX-wt/CLP pellet was lysed with BugBuster®, pepstatin, DNAse, DDM and PMSF. Lysate was sonicated for 5 minutes at 50% and centrifuged at 17500rpm in an SS34 rotor for 30 minutes. Lysate was brought to 10% imidazole concentration by adding Buffer B (20 mM Tris, 500 mM NaCl, 200 mM Imidazole). 150 µL of lysate was applied to 100 µL cobalt resin and washed with 500 µL of Buffer A (20 mM Tris, 500 mM NaCl, 20 mM imidazole). A stepwise gradient of 0%, 10%, 20%, 30%, 40%, and 50% of buffer B was run over the resin, with each flowthrough collected separately. Aliquots from each flowthrough (0%, 10%, 20%, 30%, 40%, and 50%) and the cobalt beads were run on 10%

SDS-PAGE gel at 150 V for 60 minutes. Gels were stained with SYPRO Ruby overnight and imaged on Typhoon imager.

Western Blot

A Western blot is a technique that uses antibodies to detect small amounts of a protein of interest. From an SDS-PAGE gel, the proteins are transferred onto a hydrophobic (PVDF) membrane and soaked in a primary antibody that recognizes an epitope or portion of the protein of interest. Then the membrane is soaked in a secondary antibody that recognizes an epitope of the primary antibody. The secondary antibody also contains some sort of marker for visualization, either a fluorescent molecule or an enzyme that catalyzes a reaction that forms a visual or chemiluminescent product. Western blotting is very sensitive, detecting as pico- to femtograms of protein, and it is specific to the protein of interest by virtue of the antibodies (23). For example, a protein may be present in small amounts that do not stain with Coomassie blue. Anther example of when to use a Western blot is if a different protein of a similar molecular weight of your protein of interest blocks you from seeing the protein of interest.

Cell aliquots were lysed and run on 10% SDS-PAGE gel as previously described. Filter paper was soaked in transfer buffer, and the PVDF membrane was soaked in methanol. Components were placed on the Turbo western blot tray in this order: three filter papers, PVDF membrane, SDS-PAGE gel, three filter papers. A turbo transfer machine was run at 1 mini gel setting for 7 minutes. SDS-PAGE gel was stained with Coomassie blue and destained for analysis of

transfer to make sure the transfer was successful. PVDF membrane was soaked in ~7 mL blocking solution (5% BSA in TBS) for 1 hour. The membrane was incubated in 6 mL of primary antibody (rabbit anti-5LOX; 5% BSA in TBS, 0.1% Tween-20, 1:1000 dilution) for 1 hour. Membrane was washed three times in ~10 mL of wash solution (TBS, 0.1% Tween-20) for 10 minutes. The membrane was incubated in 6 mL of secondary antibody (goat anti-rabbit, 637nm; 5% BSA in TBS, 0.1% Tween-20, 1:10,000 dilution). The membrane was washed again as previously described. The membrane was washed in ~10 mL of 1x PBS for 10 minutes and placed between two pieces of filter paper wrapped in foil to dry. The membrane was imaged on Typhoon under 610 Cys 5 settings.

Results & Discussion

5LOX-wt and CLP in pETDuet were co-expressed and purified by batch purification. Results are show in Figure 6. CLP was washed off of the cobalt beads at a low concentration in the imidazole gradient, indicating that it was not bound tightly to 5LOX-wt, which was immobilized on the resin by its affinity tag. Lane 2 shows CLP present (at 14 kD) washing off with the non-specific binding, indicating that no stable 5LOX-wt/CLP forms when the proteins are co-expressed.

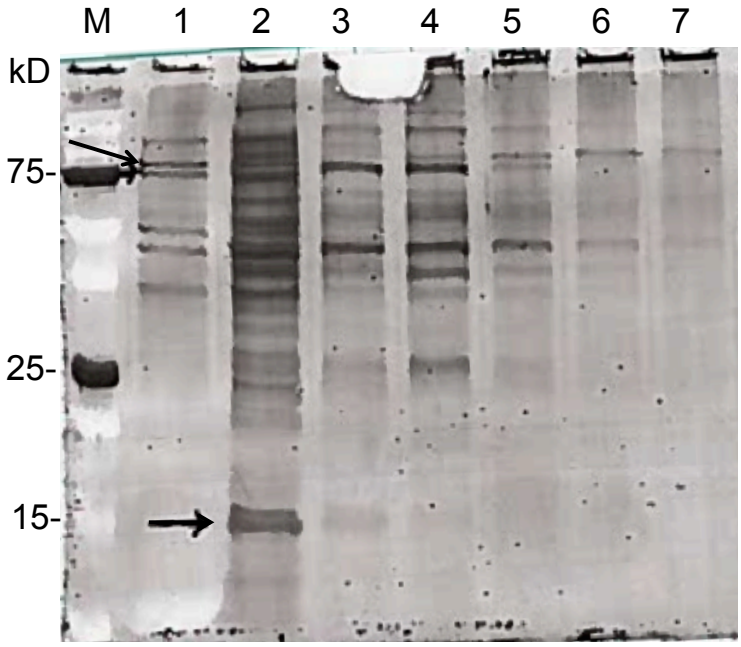

Figure 6. Sypro Ruby stained batch purification gel. From left to right, lanes include marker (M), cobalt beads (1), 0% buffer B (2), 10% (3), 20% (4), 30% (5), 40% (6), 50% (7). Each band represents proteins, with smaller molecular weight proteins running to the bottom of the gel and higher molecular weight proteins at the top of the gel. Intensity of the band corresponds to the amount of protein present: dark, thick bands indicate more protein and light, thin bands indicate less protein.

The plasmids pET14b containing 5LOW-wt and pETDuet containing 5LOX-wt and CLP were expressed separately. One flask of each was induced with IPTG, and the other flask was not induced. After lysis and analysis with Coomassie-stained SDS-PAGE gel (Figure 7), the gels were examined and differences in 5LOX-wt expression were observed. 5LOX-wt in pET14b does not express as well when it is induced as when it is not induced, shown in lanes 1 and 2. 5LOX-wt expression in the presence of CLP is approximately the same as 5LOX-wt alone in the absence of induction. However, when 5LOX-wt is induced in the presence of CLP, both 5LOX-wt and CLP expression improves, shown in lane 4.

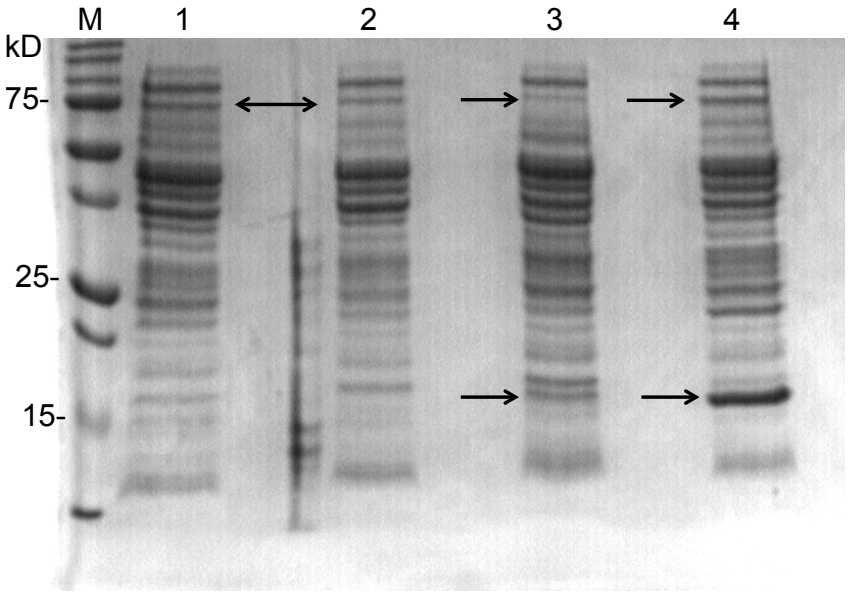

Figure 7. Coomassie blue stained SDS-PAGE gel of lysed aliquots grown at different conditions A) 5LOX-wt, B) 5LOX-wt induced, C) 5LOX-wt/CLP, D) 5LOX-wt/CLP induced. Recall the molecular weights of the proteins of interest: 5LOX is 78 kD and CLP is 14 kD. The positions of the 5-LOX and CLP bands are noted with arrows.

Western blotting of the lysate using an internal antibody confirms that 5LOX-wt is present, shown in Figure 8.

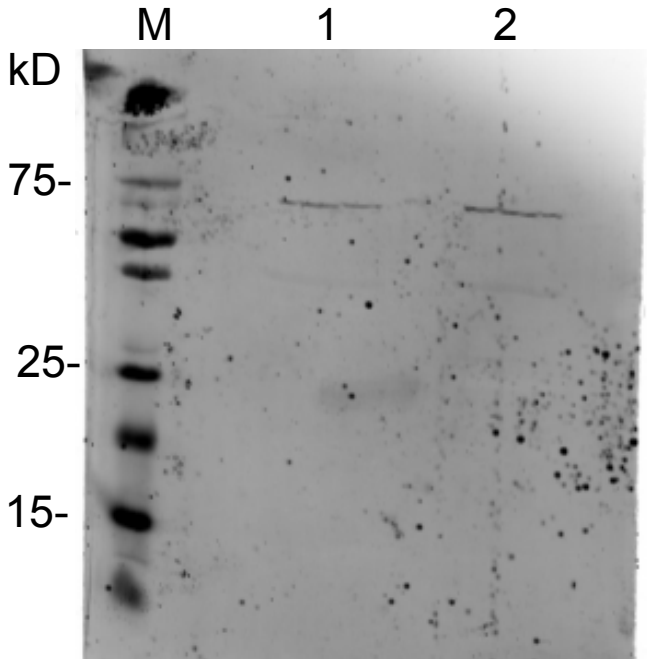

Figure 8. Western Blots of anti-body 5LOX-wt.

Conclusions

Though we were not able to demonstrate the interaction between 5LOX-wt and CLP, the results show that CLP did have a significant stabilizing effect on 5LOX-wt when they were co-expressed. More experiments need to be performed in order to optimize the expression protocol. A Western Blot using the tail antibody for 5LOX-wt should be performed to confirm the presence of full-length 5LOX. Purifying a large expression of 5LOX-wt co-expressed with CLP will allow for direct comparison of purification yields of purification of 5LOX-wt alone. 5LOX co-expressed with CLP also needs to be assayed for activity, as a functional enzyme is crucial in any further data obtained from this expression protocol. However, the data described in this thesis has laid groundwork for future experiments on 5LOX.

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