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Increasing Lipoxygenase Solubility through Fusion of Highly Acidic Peptides

by

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Undergraduate honors thesis under the direction of

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Introduction

Lipoxygenases (LOXs) are highly stereo- and regio-specific enzymes which catalyze the oxygenation of polyunsaturated fatty acids. The main substrates of LOXs are arachidonic acid (AA) in animals and linolelate in plants. In addition to being expressed in plants and animals, LOXs have also been found in ascomycetous fungi and prokaryotes. Much remains unknown of lipoxygenase structure and function, but the majority of research has demonstrated that many LOX metabolites are incredibly harmful. The hydroperoxy-eicosatetrataenoic acids (HpETEs) generated by LOXs have been shown to influence tumor generation and metastasis, anaphylaxis, and organelle degradation.

One major impediment facing the lipoxygenase field is the inability to express a soluble LOX at high concentrations as a recombinant protein. Lipoxygenases are prone to aggregation and improper folding when expressed in *E. coli,* therefore prohibiting crystallization and activity assays. Currently, solubility enhancing fusion tags have been shown to have great success in solubilizing proteins that are difficult to express. Recent studies by Su et al. (2007) and Zou et al. (2008) focused on highly acidic peptides derived from the DH5α *E.coli* DNA as solubility enhancing fusion tags. Based on their results, we fused two highly acidic *E. coli* proteins, yjgD and msyB to several human lipoxygenases.

Biological Significance of Lipoxygenases and their Metabolites

5LOX

5LOX oxygenates the C-5 of arachidonic acid to make 5-HpETE (5-hydroperoxy-eicosatetraenoic acid), which can be reduced to 5-Hydroxyeicosatetraenoic acid (5-HETE) after it leaves the active site. While 5-HETE has no biological role, it can be later turned into 5-oxo-eicosatetraenoic acid (5-oxoETE) by the enzyme 5-hydroxy-eicosatetraenoic acid dehydrogenase (5-HEDH). 5-HEDH is fully activated after neutrophils activate NADPH oxidase for respiratory burst and release $NADP^+$, a necessary cofactor for 5-oxoHETE production (Buczynski, et al., 2009). 5-oxoETE is a chemoattractant for neutrophils, eosinophils and monocytes and is responsible for increases in intracellular Ca^{2+} levels, as well as actin polymerization (O'Flaherty, et al., 2000). An alternative pathway exists for 5-HpETE if it remains in the active site. In this position, it can undergo catalytic rearrangement into leukotriene A_4 (LTA_4), which can then be converted to leukotriene C_4 (LTC_4) and a variety of leukotriene B_4 (LTB_4) isoforms through enzymatic catabolism.

Leukotrienes are known to have several dangerous effects on the body, most notably causing endothelial contraction. Dahlen et al. (1980) found the effects of small amounts of LTC_4 to be up to 1,000 times more potent than the same dose of histamine. Also, in trials, histamine immediately bound to sample epithelial cells and remained for one minute while LTC_4 took two minutes to get maximum result, but continued stimulation for over twenty minutes (Smedgard, et al., 1982). Administered amounts of LTD_4 are equally effective, however it remains uncertain whether the LTD_4 causes the endothelial contraction, or if the reaction is due to LTD_4 being converted into LTC_4. Moreover, the leukotrienes bind tightly to their target tissue, and inhibitors such as isoprenaline have strong but short lived effects, as the inhibitor can be easily washed away from sample cells, and the leukotrienes remain bound (Dahlen, et al., 1980). LTC_4 is the primary component in the slow reacting substances of anaphylaxis (SRS-A), the main component in the anaphylaxis of asthma (Walch, et al., 2000).

Healthy pulmonary artery endothelial cells produce a constitutively low amount of 5-LOX, therefore generating a small amount of 5-HPETE. Under normal conditions, this amount is too small to be generated into a significant amount of leukotrienes. However, in stressed conditions, 5-LOX expression is increased, providing the possibility for harmful doses of leukotrienes to be generated. It is very possible these stressful conditions include pulmonary hypertension and allergic response (Zhang, et al., 2002). LTC_4 and LTB_4 cause a drop in leukocytes but not platelets; possibly inferring leukocyte trapping in one area. LTC_4 affects peripheral airways and causes increased transpulmonary pressure. Therefore, leukotriene production can lead to hypertension, especially when working in conjunction with other causative agents (Smedgard, et al., 1980). However, some evidence does exist that leukotrienes may serve a beneficial purpose in the body. Leukotrienes may be necessary to regulate vascular tone. LTD_4 actions on receptors in epithelial tissue indirectly cause a relaxation through the induction of the nitric oxide pathway. Nitric oxide is a potent vasodilator, among other things, that is released by epithelial cells, neurons and macrophages. In fact, many endothelial tissue grafts lacking sufficient 5-LOX activity have been ineffective (Allen, et al., 1992).

Aside from asthma and hypertension, 5 LOX metabolites have been implicated in cancer as well. 5 LOX mRNA can be detected in most lung, colon and prostate cancer. Moreover, we know 5 LOX is expressed in pancreatic cells only when cancer is present. When different pancreatic cells were tested, the highest number of 5-LOX and LTB_4 receptors occurred in the cancerous pancreatic ductal cells (Hennig, et al., 2002). This discovery supports a common theory that pancreatic cancer is derived from the ductal cells, and that 5-LOX does have a carcinogenic agenda.

12*S*-LOX

While all tissues contain a normal amount of 12*S*-LOX, copious amounts of the enzyme are found in tumors developing from the colon, prostate, stomach and skin. It is known that 12LOX induces cell adhesion to vascular endothelial tissue through increased integrin expression (Piao, et al., 2008). 12*S*-HETE can interact with integrins on the cell surface and the actual cytoskeleton to enhance the adhesion and movement of cells through the endothelium. 12*S* HETE interaction is comparable to autocrine motility factor and believed vital for metastasis (Tang, Honn, 1994). The TNFα/NFκβ pathway induces 12*S*-LOX expression as part of the host inflammatory response. 12*S*-LOX has also been linked to neoplastic formation in the epidermis, and is thought to act as a proliferation promoter among these cells. In mouse epidermal tissue, Baicalein, a lipoxygenase inhibitor, had little effect on large tumors, however it did suppress proliferation among small masses of tumor cells. Therefore, we can deduce 12*S*-LOX is important in the beginning stages of tumorigenesis (Piao, et al., 2008).

Interestingly, after prolonged ultraviolet B exposure, an increase in lipoxygenase catalysis was documented. Sunburn is a relatively common occurrence after increased unprotected exposure to UV light. The UV rays cause hydrolysis of the membrane and thus release fatty acids to initiate an inflammatory response. During sunburn, initially, many prostaglandins are released in the first 24 hours and may account for the erythema and inflammation. However, later after exposure, 12LOX and 15 LOX are being expressed at significantly higher rates. At 72 hours after exposure, 12*S*-HETE and 15-HETE are present at 10 times their normal concentration. 12*S*-HETE is believed to be a chemoattractant for

leukocytes, thus increasing the inflammatory response, but 15-HETE involvement is most likely a counter to the inflammation (Rhodes, et al., 2009).

12*R* LOX and eLOX

12*R* LOX and eLOX share 50% sequence homology and are both found in the epidermis. Despite this similarity, eLOX is strangely unique (Buczynski, et al., 2009). eLOX is in fact a hydroperoxide isomerase (hepoxilin synthase) that uses the product of 12*R*-LOX, 12*R*-HpETE to synthesize an epoxyalcohol (Yu, et al., 2004). Mutations in either 12R LOX or eLOX are related to psoriasis and ichthyosis. Ichthyosis is a rare skin disorder, which gives the skin a dry, scaly look, similar to fish skin. There are several variations of the disease, all treated similarly with topical creams or keratanoids to rehydrate the skin. 10% of patients with Autosomal Recessive Congenital Ichthyosis (ARCI) were found to have mutations in 12*R*LOX or eLOX. It has been suggested that ARCI patients with normal 12*R*-LOX and eLOX have an abnormality elsewhere in the same pathway. The end product that must be incorporated into the membrane is a hydroxyepoxyalcohol, specifically, 12(*R*) -hepoxilin A3 and 12(*R*)-hydroperoxy-eicosatetraenoic acid (12*R*-HpETE) (Elias, et al., 2008).

The generation of 12*R*-LOX knockout mice yielded incredibly abnormal results. Knockout mice born without the 12*R*-LOX gene did not survive longer than five hours and showed substantial transepidermal water loss. Prenatal mice begin expressing 12*R*-LOX at embryonic day 15, and the epidermal barrier is formed by E17. In the womb, injected dye showed the epidermis of the knockout mice was penetrable from embryonic day 16.5-17.5. During this time, normal mice make an impenetrable barrier. Closer analysis revealed the stratum corneum was abnormally organized and lacked a fillagrin monomer. Therefore, 12*R*-LOX is essential to epidermal barrier formation (Epp, et al., 2007). The linkage to icthyosis may be a compensation of hyperkeratosis to account for a 12*R*-LOX defect.

Hepoxilins and Lipoxins

Certain rat 12 LOXs are known to make hepoxilins. Hepoxilin A_3 (HXA_3) is connected with intracellular calcium release, neutrophil migration, insulin secretion, and modulation of neuronal signaling. (Buczynski, et al., 2009) We have not discovered the ability for hepoxilin synthase activity in human LOXs (Nigam, 2009). However, Yu et al. (2003) showed production of a HXA_3 stereoisomer after eLOX was given 12*R*-HpETE as a substrate. In the same experiment, 12*R*-HpETE was found to be the best substrate for eLOX activity, which further suggests these two enzymes work in unison in human epithelial tissue (Yu, et al., 2003).

Lipoxins are generally produced during an inflammatory response by transcellular interaction and biosynthesis. Biosynthesis always involves two lipoxygenases; the two corresponding proteins determine whether Lipoxin A_4 (LXA_4) or Lipoxin B_4 (LXB_4) is produced. LTA_4 from 5LOX can be made into LXA_4 by 15-LOX and LXB_4 by 12 LOX. Also, 15-HETE from 15 LOX can be taken up by leukocytes and turned into LXA_4 by 5 LOX (Buczynski, et al., 2009). Lipoxins have numerous anti-inflammatory responses and promote a return to homeostasis. They first stimulate the epithelial cells to release nitric oxide and prevent leukocyte adhesion. Lipoxin can arrest movement of the cytoskeleton, thus stopping neutrophil

diapedesis. It also stops leukocyte migration into the tissue by partially inhibiting organ fibrosis of the vascular and smooth muscle tissue (Serhan, et al., 2008). Finally, lipoxins also reduce pain in a manner similar to NSAIDS, but are over 100 times more potent (Bannenberg, et al., 2004).

15-LOX

15-LOX 1 is found predominately in reticulocytes, and it is the only mammalian LOX for which a crystal structure is available (Gillmor et al., 1997) When found in the reticulocyte, lipoxygenase can inactivate mitochondria. The lipoxygenase targets the sulfhydryl groups in the mitochondrial matrix by invading through connection with the fatty acids in the membrane. Once these sulfhydryl groups are inactivated, instability and certain disappearance of the mitochondria occur. This behavior was previously only found to occur in the reticulocyte, forcing the belief that erythrocyte cytosol has some nulling effect. (Rapoport, et al., 1978) However, this same organelle destroying behavior has linked 15-LOX to several neurodegenerative diseases, such as stroke, Alzheimer's disease and Parkinson's disease. 15-LOX can be activated by radical oxidative species during heightened immune responses, and can serve to amplify this signal for oxidative stress. These conditions, along with neuron degeneration, which may be caused by 15-LOX metabolites as well, call for further study of the enzyme’s role in neurodegenerative disease (Leyen, et al., 2009).

Unlike the other mammalian LOXs, 15 LOX1 has been shown to utilize linoleic acid or arachidonate as a substrate, and is able to acquire these substrates from lipid complexes without additional enzymatic help. 15 LOX can be activated during association with the biomembrane and has the unique ability to acquire LDL for oxidation. All research limits this ability to 15-LOX found within monocyte-macrophages (Cathcart, Folcik, 2000). The problem with LDL oxidation is that linoleic acid is the largest component of LDL. 15-LOX oxidation of linoleic acid can result in 9-Hydroxy octadecadienoic acid (9-HODE) or 13-Hydroxy octadecadienoic acid (13-HODE) formation. 9-HODE and 13-HODE are found to be highly concentrated in atherosclerotic lesions, leading to the belief that oxidized LDLs are primarily responsible for the cytotoxic effects of the disease (Jira, et al., 1998).When arachidonic acid is the substrate, the product of 15-LOX1 catalysis is 15-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HpETE). It is relatively unstable and may be subject to reduction or enzymatic catalysis. One secondary product of particular importance is LXA_4, as noted earlier (Kuhn, 1997).

In pancreatic cancer cells, the expression of 15-LOX1 is downregulated, while 5-LOX expression is increased. Pancreatic cancer has one of the worst prognoses of any cancer, due to late diagnoses and lack of treatment options. When 15-1LOX activity was restored to these cells, proliferation decreased and apoptosis was induced. Therefore, 15- LOX1 must have some ability in regulating pancreatic cancer. 15 LOX 1 is shown to indirectly inactivate 5-HpETE production as 15-HETE competes with arachidonic acid for the active site of 5-LOX. Therefore, in addition to promoting apoptosis, it may silence proliferation signals from within the lipoxygenase family (Petrich, et al., 1996). However, 15-HETE has been linked to a pro-tumorigenic effect in prostate cancer (Hennig, et al., 2007).

The 15-LOX2 isozyme was discovered in 1997, and has been found predominately in mammalian hair, prostate, lung and corneal cells. It shares 80% structural homology with murine 8-LOX, and both have been found to produce 8,15-diHETE *in vivo* (Buczynski, et al., 2009).

Normal human prostate (NHP) cells show the highest expression of 15-LOX2 at a constitutive level. 15-LOX expression is also constantly mediated by the presence of SP1 and SP3, growth factors which increase and decrease expression, respectively. At the end of a NHP cell's life span, the expression of 15-LOX2 is upregulated. These preliminary studies lead us to believe 15-LOX2 metabolites have some regulatory ability over prostate cell proliferation. Coincidentally, in many prostate cancer cells, the expression of 15-LOX2 has been completely silenced (Tang, et al., 2007).

Lipoxygenase Structure

Mammalian 5, 12, and 15 lipoxygenases share 35-80% amino acid homology (662-676 aa) and are about the same size (75-80kDa) (Gillmor, et al., 1997). One common feature shared by all lipoxygenases is the production of tightly controlled, regio-specific and stereospecific hydroperoxy fatty acids.

Catalytic C-terminal

The catalytic domain is the most conserved sequence between lipoxygenases in all phyla. The mammalian catalytic domain contains eighteen α-helices, and shares an interface of 1600 $Å^2$ with the N-terminal β-barrel domain (Brash, 1999). The active site contains a non-heme iron that is bound by four surrounding histidines and the carboxyl terminal isoleucine. Interestingly, the histidine- isoleucine support structure is conserved in all known mammalian lipoxygenases, and plants only vary the structure by replacing a histidine with an aspargine residue (Andreou, Feussner, 2009). All LOXs also share an identical sequence of oxygenation of the polyunsaturated fatty acid substrate: hydrogen removal, radical rearrangement, and oxygen addition.

Step 1: Proton Extraction

It is currently believed that lipoxygenases employ a proton coupled electron transfer in order to form the fatty acid radical. A bisallylic methylene abstracts the hydrogen, and the iron atom readily accepts the electron while the proton removed is transferred to a bound hydroxide (Kuhn, et al., 2005). Therefore the ferric Fe^{3+}-OH complex is effectively reduced to Fe^{2+}-OH_2. Though skepticism remains on the exact method of radical formation, the conserved non-heme iron's involvement is deemed crucial.

Step 2: Radical rearrangement

The hydrogen abstraction occurs at C-7, C-10, or C-13 of the arachidonic acid, and each lipoxygenase uses its highly specific cavity to direct the radical to a specific face of one particular carbon (Buczynski, et al., 2009). While a common mechanism for radical formation involves oxygen, there is no evidence of Fe-O_2 or Fe-OOH, merely Fe-OH. There are several theories that oxygen may be involved as a peroxyl binding the opposite (anatarafacial) side of the cation. Peroxide radicals have various effects on cabonyl radicals, including β-Fragmentation, double bond isomerization from *cis* to *trans* bond,

and cyclization. Most notably, peroxide radicals can also remove hydrogens from the fatty acid. While the initial hydrogen abstraction does not involve the use of a peroxyl, the radical rearrangement could utilize such a common factor. However, if the radical did exist and did accept the hydrogen from the fatty acid, it could be very quickly transferred, leaving no evidence of a hydroperoxide end product. The speed of a peroxyl reaction does not match that of a lipoxygenation, therefore radical involvement is highly disputed (Schneider, et al., 2007). Instead, it has been suggested the fatty acid may adapt to a dioxygen bridged allyl radical at the active site (Kuhn, Thiele, 1999).

Step 3: Oxygenation

There is no evidence of oxygen binding to the ferric iron before radical formation. The participation of O_2 in generating specificity of hydrogen removal is not supported by data in the literature, warranting the suggestion that O_2 does not bind until needed for oxygenation (Schneider, et al., 2009). However, there is little proof of how and when the atmospheric oxygen actually enters the active site.

The possibility of oxygen diffusion into the active site is highly unlikely. The ferric ion is located deep in the interior of the catalytic domain, in a cavity so hydrophobic it lacks solvent. Therefore, it is highly unlikely the atmospheric oxygen could even permeate to the correct area (Jankun, et al., 2008). Secondly, as lipoxygenase activity is highly regio- and stereo-specific, it is highly unlikely the oxygen could be added in the same manner if it randomly diffused to the active site (Kuhn, et al., 2005).

A possible channel for atmospheric oxygen entry has been analyzed in the soybean LOX 1 that has direct access to the exact spot of oxygenation on the linoleate. A similar conserved channel has not been found in the crystallized 15-LOX 1 structure, but in highly oxidized solvent, the oxygen has been noted to travel in the same area (Kuhn, et al., 2005). It is important to remember that the protein has never been crystallized with substrate in the active site; therefore, conformational changes could open such a passage.

Active Site Specificity

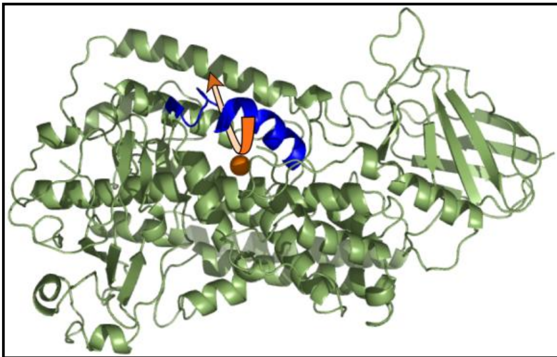
The active site among all lipoxygenases is thought to be conserved; in fact, several studies using molecular models of the various animal LOXs and crystal structures of plant LOXs have found all sites to be almost perfectly imposable onto each other (Kuhn, et al., 2005). Therefore, the study of substrate entry for plant lipoxygenation is largely relevant to mammalian catalysis.

In the soybean LOX structure proposed by Youn et al. (2006), there are three branches that make up the active site. Two of the branches make up a hydrophobic cavity which houses the substrate. The first branch is the entrance for linoleic or linolenic acid, and has conserved basic residues that hold the carboxyl of the substrate in place. The second branch is an extra pocket found to contain several water molecules. The substrate is held between these two branches, and resembles a boot. The third branch is believed to be an oxygen channel, that deposits oxygen at C-9 or C-13, depending on the specific LOX. The third branch is not conserved within all plant LOXs, and can be blocked in some cases (Youn, et al., 2006) The space related model, based on studies of plant LOXs, dictates the methyl end of

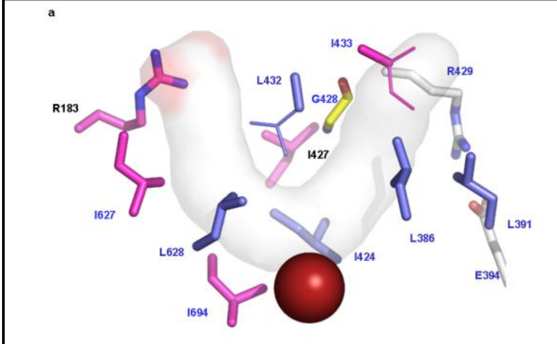
the fatty acid will always penetrate first, and the different residues present in the active site account for the regio-specificity. Increasing the depth of the cavity by mutating even a single residue has shown to have dramatic influences on regio-specificity (Borngraber,et al., 1999). In 13-LOX, a histidine or phenylalanine residue takes up space near the end of the pocket, prohibiting fatty acid entrance into the deepest section of the cavity. This blockage forces oxygenation at C-13 because of proximity. Consequently, in 9 LOX, a valine residue is present at the same site, giving more space than the bulky aromatic residue of 13 LOX. This allows the linoleate to penetrate deeper into the cavity and oxygenation at C9 may occur (Andreou, Feussner, 2009).

On the other hand, the orientation dependent model suggests regio-specificity varies dependent upon how the fatty acid enters the active site. In plants, only in recent years has the fatty acid penetration been researched in a backwards motif; it was previously assumed the linoleate entered the active site with its methyl group penetrating deepest. This tail first model always explained the hydrogen removal at C-11 and subsequent oxygenation at C13. However, in recent years, researchers have begun to explain the presence of linoleate 9-lipoxygenation by suggesting the linoleate enters backwards, with the carboxyl end penetrating deepest. There may be a positively charged group on an arginine residue near the innermost part of the cavity (Kuhn, et al., 2005). In theory, the positive charge could draw the linoleate into the enzyme in a head first manner, in order to react with the carboxyl. If this bridge connection was to form, the linoleate would be in a different position and could more readily oxygenate at C9, instead of C-13, which is the product of methyl-terminal entry (Andreou, Feussner, 2009). Interestingly, Hornung et al. (2008) made a discovery that a LOX isoform from *Mormordica charantia* seeds that carries a glutamine at the position where other plant LOXs harbour either histidine/phenylalanine (linoleate 13-LOX) or valine (linoleate 9-LOX) residues. This discovery decreases the validity of the space oriented model, as glutamine is not bulky enough to restrict linoleate access, but not small enough to allow deepest permeation. Instead, it provides additional proof that regiospecifity may depend on the linoleate's direction of entry into the cavity. In this case, if the methyl end entered first, a C13 oxygenation would occur, while if the carboxyl end entered, a C9 oxygenation would ensue (Hornung, et al., 2008).

Browner and colleagues (1996) suggested a boot shaped cavity formed the active site after observing the crystal structure of rabbit reticulocyte 15-LOX1 with an inhibitor bound. The cavity is 20Å deep and 6Å wide, allowing enough room for AA residence after penetration through a side entrance (Gan, et al. 1996). The cavity is lined by the residues found in helices 9, 11, 12, and 21 and makes a sharp 90° turn after approaching the catalytic iron, giving the cavity a boot-like shape (Gillmor, et al., 1997). Gillmor et al. proposed the AA could enter with the carboxylic head first, and would interact with Arg 403.However, there are challenges to the boot-like shape. Choi et al. (2007) compared the crystal structures of 15-LOX1 with and without inhibitor. When inhibitor was not bound, the cavity was short and shallow, but had an entrance connecting the solvent to the cavity. The crystal structure for 15-LOX1 with inhibitor was deeper, but the entrance to the protein exterior was blocked by helix α2. The flexibility of mammalian lipoxygenases could allow for this conformational change, but the discovery does raise questions on the credibility of a boot shaped cavity model for substrate binding.



The U shaped cavity proposed by Neau et al. (2009): (Above) The U shaped cavity in relation to the lipoxygenase structure. (Below) AA in the proposed LOX U shaped active site, with conserved L and I residues (blue), Coffa site (yellow) and catalytic iron (red).



There is also a possibility that the active site of animal LOXs is a U-shaped channel rather than a boot-shaped cavity. Neau et al. (2009) examined this possible structure through crystallization of 8R-LOX, a coral lipoxygenase that shares 40 % identity with human 5-LOX. In the bottom arch of the U shape lies the catalytic iron atom, across from leucine 432. This particular leucine is postulated to position the arachidonic acid for hydrogen abstraction at C-10. In the crystallized lox structure, the absence of an electron density for the L432 side chain is consistent with the theory that L432 functions in securing the arachidonic acid for pentadiene attack. The rest of the channel contains four leucines and one isoleucine that are identical in all animal LOXs. The hydrophobicity of these residues also helps slide the arachidonate into place. The advantage of the U-shaped channel is that it can be open on both ends or closed on one end. Thus, at least in theory, AA could enter the U-shaped channel of all LOXs tail-first. The side of entry would depend on the stereo-specificity of a given LOX. In the case of the human LOXs, AA would enter one side of the channel of 15*S* and 12*S* LOXs, but it would enter the 5- and 12*R*-LOXs from the other side (Neau, et al., 2009).

The LOX regiospecificity is far from understood. In addition to understanding the shape of the active site, more research must be conducted on the actual configuration of the fatty acid substrate at the active site. Fatty acids take on different flexibility at different temperatures and pHs, meaning we must consider the actual orientation of the fatty acid in order to align it into the site and study its interaction with the enzyme. Luckily, mammalian enzymes are prone to more flexibility, and unlike the rigid plant lipoxygenases, the mammalian LOXs may be able to oxygenate several conformations of one fatty acid (Andreou, Feussner 2009).

Even if the oxygen channel theory proves true, devices need to be in place to control product specificity. The most valid theory is steric shielding (Coffa, et al., 2005; Schneider, et al., 2007; Andreou, Feussner, 2009). In an effort to control specificity during oxygenation, several residues are shielded by devices such as methylation in order to allow O_2 to move where needed. This mechanism restricts oxygen access to parts of the active site, and possibly even the fatty acid substrate (Schneider, 2007). One residue in specific may dictate the actual specifics of steric shielding.

12*R*-LOX is the only mammalian lipoxygenase known to produce a hydroperoxy fatty acid with *R*-chirality (Buczynski, et al., 2009). There is a substantial degree of sequence identity between R and S lipoxygenase active sites, prompting the search for very small factors capable of impacting stereospecificity. Coffa et al. (2005) evaluated residues conserved in *R* LOXs against those conserved in *S* LOXs. The results led to the discovery of the Coffa site. At this particular site, glycine is always present in

R lipoxygenases, while alanine is conserved in all but one *S* lipoxygenase. During experimentation, the alanine was mutated to glycine in *S* lipoxygenases and the glycine was mutated into alanine in *R* lipoxygenases. The resulting products were overwhelming changed not only in stereospecifity but regiospecificity as well. For instance, when the glycine from 8*R*- lipoxygenase from the coral *Plexaura homomalla* was mutated into alanine, the major product (98%) was 12-*S* HETE (Coffa, Brash 2004). This residue may be a prime example of shielding. Regardless of the carbon to which the oxygen was added, the presence of alanine/glycine switch does in fact result in conserved stereo-specificity (Andreou, Feussner, 2009). While this sheds light on the specificities of lipoxygenase, other aspects need to be considered additionally to truly understand stereospecificity (Coffa, et al., 2005). For example, the mutant enzymes produced by Coffa and Brash (2004) have severely compromised catalytic activity.

Regulatory N-terminal domain

The N-terminal domain is much smaller than the C-terminal domain, ranging from 115-125 amino acids compared to its counterpart with over 500 residues. The function of the N-terminal domain of all LOXs is presumed to be to target the enzyme to the membrane. The membrane targeting is best understood in 5-LOX and coral 8*R*-LOX. The N-terminal domains of both of these LOXs are similar in size, structure and/or amino acid sequence to mammalian lipases. These two LOXs, like the lipase, also bind calcium. On the other hand, 15-LOX and lipoprotein lipase share only 23% amino acid identity, even though the structures of their N-terminal domains are similar. The most intriguing structural similarity is a hydrophobic β barrel domain. The lipoxygenases and human lipases are cytosolic, but must extract fatty acid substrates from the membrane, and due to the β barrel's exterior location on these proteins, membrane binding occurs in this region. In the LOX's case, the loop creates an interface between the membrane and active site (Gillmor,et al., 1997). Like the C2 domain in lipases, the N-terminal domains of 5-LOX and 8R-LOX have high affinity Ca^{2+} binding loops which require Ca $^{2+}$ for membrane binding. (Kulkarni, et al., 2002; Oldham, et al., 2005) Once the N-terminal domain of 5-LOX has bound calcium, it requires the presence of FLAP (5-LOX activating protein) for substrate aquisition. The structure of FLAP allows a large enough space for arachidonic acid to laterally diffuse from the nuclear membrane into the active site of 5 LOX (Buczynski, et al., 2009). While other lipoxygenases respond to calcium changes, 5 LOX is the only human lipoxygenase that is able to bind calcium. Binding of calcium presumably evokes a conformational change that displays amino acids with hydrophobic side chains such as trp, phe, and tyr, on the surface of the C-2 like domain. These residues, in turn, could mediate membrane binding. Rabbit reticulocyte 15-LOX does not have a Ca^{2+} binding loop, but small increases of calcium have been noted to enhance membrane binding. This activity could be the result of calcium salt bridges between negative membrane phospholipids and acidic LOX residues (Walther, et al., 2004) The lipoxygenase has been proven to use esterified polyenoic fatty acids from the membrane as well, therefore prompting the search for membrane binding mechanisms. Three hydrophobic residues in the 15-LOX1 N-terminal β-barrel domain, Tyr 15, Phe 70 and Leu 71 are specifically linked to enzyme interaction with membrane lipids (Kuhn, et al., 2005).

The N-terminal β barrel shares a 1600 $Å^2$ interface with the catalytic domain, and is connected to it by a short peptide sequence (Kuhn, et al., 2005). The two major domains of all lipoxygenases are connected by a short sequence that extends from approximately amino acid 220 to amino acid 330.

Upon deletion of these residues, a lipoxygenase is unavailable for purification, indicating the sequence's significance in protein folding. It has been proposed this region is a PDZ regulatory domain. These domains are typically 100 amino acids long and largely responsible for connecting homo and heterocomplexes. Also, they are usually found in proteins associated with signal proliferation, such as our lipoxygenases. Although remarkable structural similarities are apparent, little amino acid homology is present between the β-barrels of LOXs and known PDZ domains (Jankun et al., 2008).

Obviously, our knowledge of catalysis by and structure of lipooxygenases is far from complete. Yet, it is vital to continue studying them due to all of the implications of LOX intermediates and metabolites in diseases. These molecules influence numerous biological functions from bronchial constriction, neuronal death, cancer, to overall inflammation. The major impediment to mammalian LOX research is the inability to express any of them at high levels as recombinant proteins.

Solubility Enhancing Fusion Tags

Translation and protein folding are significantly faster in *E. coli* than eukaryotes. Therefore expression of an active eukaryotic protein in *E.coli* requires overcoming several problems. Over the years, vector and media enhancements have created more success in actually translating the protein, but problems remain in solubility and purification (Esposito, Chatterjee, 2006). Expression of a soluble protein requires prohibition of aggregation. Aggregation occurs in instances of high protein concentration, when the proteins can readily interact with each other and impede native folding. Due to this additional binding and buildup of improperly folded target protein, most of the expressed protein product is aggregated and cannot be used for enzymatic or structural studies (Zhang, et al., 2004).The tendency of eukaryotic proteins to aggregate when expressed in *E. coli* probably results from the combination of a fast rate of translation, coupled with a slow rate of folding (Widmann, Christen, 2000). Lipoxygenases are subject to aggregation when expressed in *E. coli*. This, coupled with the inherent instability and autoinactivation of some of them, has made expressing lipoxygenases extremely difficult.

Over time, new fusion tags have been generated, each with better solubility, efficacy or additional features. Two commonly used fusion tags, maltose binding protein (MBP) and glutathione-S-transferase, have risen to the top of the most frequently used list due to their additional capability as an affinity tag. During purification, any protein fused with MBP will bind to an amylose resin, while any protein fused with GST will bind glutathione resin. (Chatterjee, Esposito, 2006) The dual impact of these two fusion peptides promotes success in the two most difficult steps in protein generation, solubility and purification. However, there have been several occasions where the addition of fusion peptides MBP and GST have disrupted the native folding of the enzyme or even caused aggregation through self-association (Kapust, Waugh, 1999). MBP and GST are also quite large (40kDa and 26kDa, respectively) and significantly changes the molecular weight and characteristics of the target protein once fused. Thus, the search continues for a more effective solubility enhancing fusion protein.

Zhang et al. (2004) found huge increases in solubility of a number of difficult to express proteins by adding a fusion tag with a large negative net charge. In fact, solubility increased as more negative charges were added, regardless of size of the peptide fusion. When additional N terminal domains were

added, the size and net charge were doubled, the target protein was found to be even more soluble. Adding a duplicate maintained the average charge per residue, but overall covered a greater surface area. Therefore, the effects were more universal across the protein surface (Kim, 2007).

Two highly acidic fusion peptides: msyB and yjgD

The research and results of Zhurong Zou and associates gave us incentive for fusing two particularly acidic peptides to the human lipoxygenases 12*R*-LOX, 15-LOX1, 15-LOX2, and eLOX. Human 5-LOX was excluded from the experiment because a crystal of 3Å resolution has been obtained by our group.

Fusion tags show great promise in increasing solubility among difficult to express proteins. Much work has been done to generate new fusion tags, including the modified version of the Wilkinson-Harrison solubility model, which predicts the solubility of a peptide based on NGPS% and DEKR %. NGPS% accounts for the number of turn causing amino acids (Asn, Gly, Pro and Ser) in the peptide, while DEKR% calculates the acidity of the peptide by analyzing the Asp and Glu vs. Lys and Arg residues. This formula has been used to predict the solubility of over 4000 *E. coli* proteins in the SwissProt protein database (Harrison, 2000). Su et al. (2007) used the database as a resource to find four fusion tags of similar molecular weight, but different pIs. Of the fusion peptides sampled, MsyB was found to have the largest DEKR%, lowest NGPS% and a very large net negative charge at pH 7.0. It was also found to produce the most soluble target protein, prompting the notion that the acidity of a fusion peptide can take priority over other parameters when enhancing solubility. (Su, et al., 2007)

Zou et al. (2008) worked with Su to determine the most probable solubility enhancing fusion peptides. However, in addition to the newly found msyB and several other popular fusion tags, the team added yjgD and a fragment of a RNA polymerase subunit to their target proteins. YjgD and msyB are highly acidic and their pI values have been estimated at 3.5. The target proteins were EK (bovine enterokinase), TEV (tobacco etch virus protease), and rbcL (the large subunit of Rubisco). Each of these target proteins share common protein isolation problems. EK is normally highly expressed, however rarely soluble. TEV is prone to forming large aggregates, making it insoluble as well. Finally, rbcL is stable and highly soluble when associated with rbcS in the chloroplasts of plants, but is relatively insoluble once expressed in *E. coli*. YjgD increased EK solubility by 63%, TEV by 61% and rbcL by 50-60%. MsyB had similar results. Other fusion tags such as SUMO, Trx, and MBP were less effective in solubilizing EK and TEV. Furthermore, msyB and yjgD were the only proteins with a profound effect on the solubility of rbcL. Based on these results, we decided to fuse msyB or yjgD to the N-terminus of each human LOX.

There is much uncertainty concerning the exact reason why these fusion proteins relay such enhanced solubility in their target proteins. However, the small size of each (14-15kDa) and highly acidic nature play a vital role. Zhang et al. (2004) proposed the negative charge could repel other polypeptides that are simultaneously translated but not yet folded, therefore prohibiting aggregation. Also, because these enzymes are derived from *E. coli* cells, there is a higher success rate in translation and rapid folding of msyB and yjgD. Each of the original target proteins share similarity to our lipoxygenases; the size of rbcL, the insolubility of EK, and aggregation of TEV. Therefore, the msyB and yjgD fusion tags

were separately cloned onto a pET28Δthr plasmid already encoding one of the human lipooxygenases 12*R*-LOX, 15-LOX1, 15-LOX2, or eLOX.

Materials and Methods

Vector Construction

Lipoxygenase genes were sequenced and purchased from ATCC (American type Culture center) as such: 15Lox 1(image number 4778890 cloned out of vector pCMV-SPORT6.ccdb), 15Lox 2 (image number 5179700 cloned out of vector pCMV-SPORT 6), 12*R*Lox (image number 5441563 cloned out of vector pOTB7), and Elox (image number 40011849 cloned out of vector pCR-BluntII-TOPO).

All lipoxygenase-fusions were constructed onto the backbone plasmid pet 28bΔ thr. The thrombin cutting site was excised from the plasmid after causing interference in past experiments. Thrombin excision was performed prior to experimentation, as well as lipoxygenase ligation into the pET 28bΔthr.

The yijd and msyB fragment were amplified from DH5α *E. coli* template DNA by polymerase chain reaction with Pfu Ultra II Hot start DNA polymerase (Stratagene). Briefly, the *E.coli* cells were boiled in water for 3 minutes, centrifuged for 5 minutes and 1 µl of the supernatant was used as template in the PCR. The primers were synthesized by Eurofins MWG Operon and included an Nde I site at the 5' and 3' termini, therefore ensuring ligation into the ORF of pET28bΔ thr. The sequences are listed in the table below.

Primer	Sequence
5’ msyB	5'-CTTCTTCATATGGCAAACCCGGAACAACTGGA-3'
3’msyB	5'-CTTCTTCATATGGTGGCGAACTCCGTCATCGT-3'
5’ yjgD	5'-CTTCTTCATATGGAAGAAGCCCATTGACG-3'
3’ yjgD	5'-CTTCTTCATATGACGTTCATCCCACTCATCAGC-3'

Vectors were digested with NdeI and treated with calf intestine alkaline phosphatase (Promega) to prevent self-ligation of the vector. Each lipoxygenase-containing plasmid was ligated to yijD or msyB, using T4 DNA ligase (New England Biolabs). All DNA was purified using Promega Wizard ® SV gel and PCR clean up system.

α-select competent cells Gold efficiency *E. coli* (Bioline) were grown on LB agar plates containing kanamycin following transformation with pET 28b Δthr plasmid with lipoxygenase and fusion peptide and grown overnight. Picked colonies were grown overnight at 37°C in TB in the presence of kanamycin. The DNA was extracted from cells using the ZR Plasmid Mini Prep Kit (Zymogen), and stored in 10mM Tris-HCl, pH 8.5, .1mM EDTA. The prepared DNA underwent a PCR cycle using the 5' primer from the

corresponding fusion protein (yijd or msyB) and 3' primer from the corresponding lipoxygenase (15lox1, 15lox2, 12*R*lox, elox). The purpose of this PCR was to detect the orientation of the fusion peptide in the plasmid. Due to the double Nde I cut, a fusion peptide could have been inserted incorrectly, and transcription would not be favorable. This PCR test ensures only those plasmids with the correct sequence orientation are used. Samples of the prepared DNA are run on DNA 1.2% agarose gel to check for accuracy.

Protein Expression and SDS-PAGE analysis

Each lipoxygenase-fusion plasmid was transformed into *E. coli* BL21 (DE-3) Rosetta 2 cells (Calbiochem/Novagen) and plated on MDAG agar (Studier, 2005) containg kanamycin and chloramphenicol. The resulting colonies were then picked and grown overnight in Luria broth supplemented with 5052, trace metals, and amino acids (Studier, 2005). The cells were centrifuged then resuspended in 50mM Tris/200mM NaCl, pH 8 and sonicated to release protein. The cells were then centrifuged again, and dithiothreitol was added to the soluble supernatant to a final concentration of 0.1M. After removal of the soluble supernatant, .1M DTT/Na2CO3 was added to the insoluble pellet and it was resonicated. A solution of SDS/loading dye was added to each sample before insertion in 100°C water bath. 15 µL of each soluble and insoluble sample were subjected to electrophoresis on a 10% acrylamide gel at 5 watts then stained with Coomassie brilliant blue R250. Images of protein gels were digitally stored.

Results

We chose to incorporate the yjgD and msyB fusion protein onto the human 12*R* LOX, 15 LOX-1, 15LOX-2, and eLOX in hopes of increasing the amount of recombinant soluble protein. These LOXs had been previously cloned into the pET28bΔthr plasmid using a 5' Nde I site and 3' HindIII or Xho I site. The yjgD or msyB fragment was cloned into the NdeI site at theN terminal of the LOX sequence, leaving the hexa-histidine tag on the outer N-terminus of the fusion protein. Clones containing either yjgD or msyB in the correct orientation were identified by the polymerase chain reaction (PCR) using the yjgD or msyB forward primer and the reverse primer for the appropriate LOX.

In preliminary studies in our laboratory, Dr. Bartlett analyzed the amino acid sequences of all of the human LOXs for rare codons in *E. coli*. She found that each LOX had more than 15 rare codons, and some of them had adjacent rare codons. Rare codons slow down translation, thus stalling the ribosome. If the ribosome has stalled for an extended amount of time, it can altogether quit translation and dislodge the protein prematurely. Maria Waight expressed an yjgD/eLOX fusion in the *E. coli* host cells BL21 (DE3), C41, C43, and BL21 (DE3) Rosetta 2. Expression of

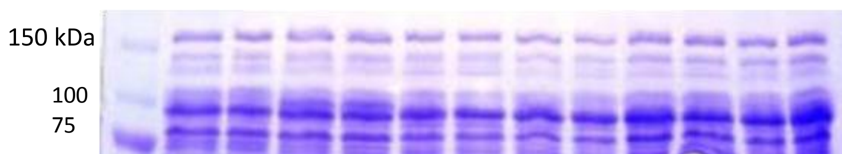


Figure 1 : SDS-PAGE gel of various cells. Lanes 1-4 are Rosetta cells, lanes 5-8 are from C41 cells, and lanes 9-12 are from C43 cells. Lanes 1,2,5,6,9 and 10 contain eLOX (~80kDa) and lanes 3,4,7,8,11 and 12 contain eYjgD/LOX (~100kDa).

eLOX was obtained only when it was fused to yjgD and only in BL21(DE3) Rosetta2 cells (Figure 1).

Once checked for the presence and correct orientation of yjgD and msyB by digestion with restriction enzymes and PCR, each LOX fusion was expressed small scale in *E. coli* BL21(DE-3) Rossetta 2 cells. Samples were subjected to SDS-PAGE.

	M	1	2	3	4	5	6	7	8	9	10	11	12	13
Wild type		+	+							+	+			
MsyB/LOX				+	+	+						+	+	+
YjgD/LOX							+	+	+					

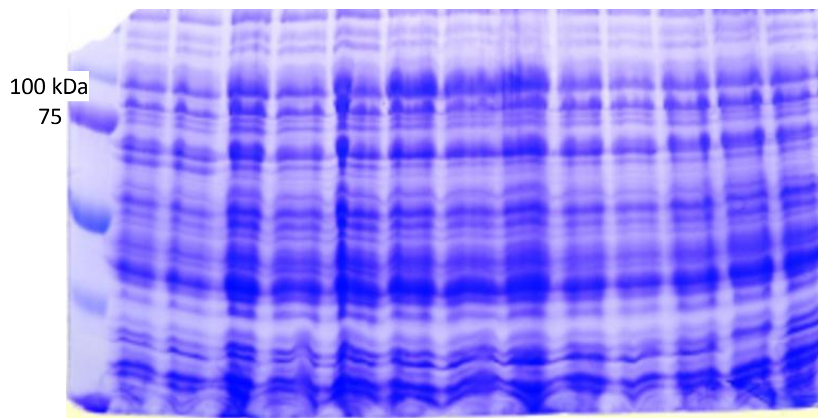


Figure 2: SDS-PAGE of soluble lysates from Rosetta cells. Lanes 1-8 are 15LOX-2 and lanes 9-13 are 15-LOX1.

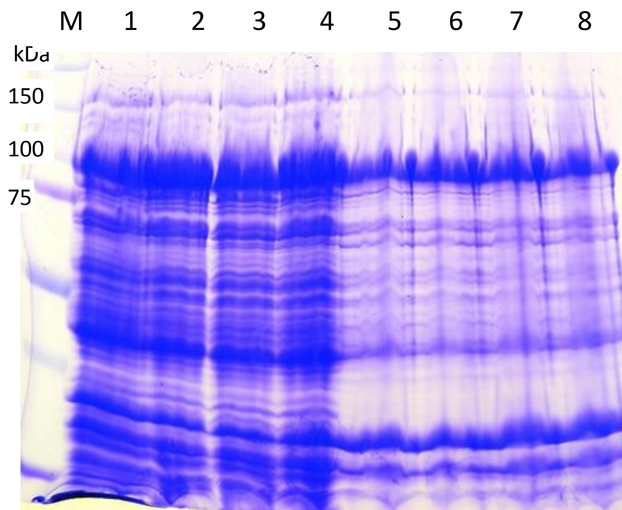


Figure 3: SDS PAGE gel of lysates from Rosetta cells transformed with yjgD/15LOX2. Lanes 1-5 show soluble protein, lanes 5-8 show insoluble protein. yjgD/15LOX2 can be found at ~100 kDa marker.

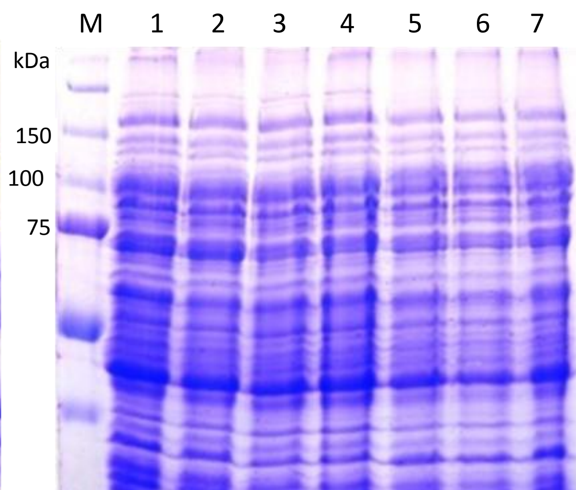


Figure 4: SDS PAGE gel of soluble lysates from Rosetta cells. Lanes 1,2 are 12RLOX(~150kDa); lanes 3,4 are msyB/12R-LOX (~100kDa; lanes 5,6 and 7 are YjgD/12R-LOX.

The yjgD fusion produced high levels of soluble 15-LOX1 and 15-LOX2. When compared to the wild type, LOXs fused with yjgD showed greater solubility. As can be seen in figure 2, there is ample soluble 15LOX-2 expression in lanes 6, 7, and 8 (yjgD/15-LOX2) when compared to lanes 1 and 2 (wild

type 15-LOX2). Similarly, yjgD/15-LOX1 expression exceeds wild type expression as can be seen in figure 2. Each LOX fusion resulted in approximately two times as much soluble lipoxygenase protein when compared to insoluble; Figure 3 presents these results. Figure 4 displays the amount of the yjgD/ 12*R*-lipoxygenase in the soluble and insoluble fractions of *E. coli* lysates. While the yjgD/12*R*-LOX fusion expressed poorly, we still obtained sufficient protein to yield a stained band after SDS-PAGE. Obviously, yjgD does improve expression of soluble LOXs. The two gels containing 15-LOX2 (figures 2 and 3) show that not only is there an overall improvement in protein expression, but also most of the protein being produced has been made more soluble. However in all studies, msyB either failed to increase expression of soluble protein, or resulted in less soluble protein than yjgD fusions. As can be seen in figure 2, the msyB/15-LOX2 fusions (lanes 3, 4, and 5) expressed better than 15-LOX2, but not as abundantly as the yjgD/15-LOX2 fusions. For this reason, fusions with yjgD were expressed large scale and assayed for catalytic activity.

Two fusions, yjgD/12*R*LOX and yjgD/15-LOX1 were selected for large scale expression by autoinduction (Studier, 2005). YjgD/15-LOX 1 had robust expression.

One special feature of the pET28bΔthr plasmid is the addition of a hexa-histidine tag to the N-terminus of the target protein. We have previously used this tag to purify expressed lipooxygenases. The highly acidic yjgD peptide was inserted immediately behind the tag, so their proximity raised concerns of interference with the His tag's ability to bind the nickel affinity column. The figure to the right shows the purification of yjgD/15-LOX using batch-wise binding to a nickel affinity resin and elution with 250mM imidazole. In the figure at the right, lane 1 contains a sample of total soluble protein, lane 2 contains a sample of unbound protein, lane 3 contains a sample of the last wash, and lane 4 contains a sample of the protein eluted with imidazole. The elutant (lane 4) contains a substantial amount of yjgD/15-LOX1 (at 100 kDa). Lane 2 shows that some of the fusion did not bind the resin. At the point we do not know whether this fusion has lost the His-tag or the capacity of the affinity matrix to bind the His-tag was exceeded. Additional proteins were found in the elutant, but better results are expected with a nickel affinity column and elution with a gradient of imidazole.

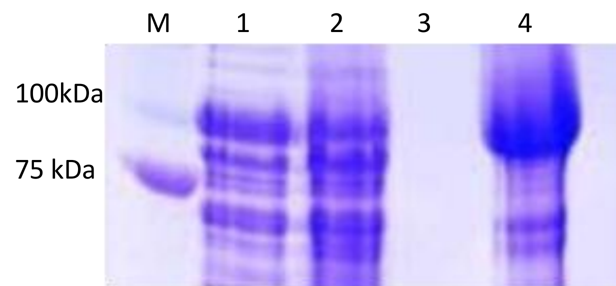


Figure 5: Purification of yjgD/15-LOX 2 after large scale autoinduction. Lane 1:total soluble protein; lane 2: first wash; lane 3: last wash; lane 4: elutant

For the yjgD/15-LOX assay, 50μM arachidonic acid was used as substrate and conversion to 15-H(p)ETE was monitored by absorbance at 235nM. This generated an initial velocity (v_0) of 15-HETE production at 4800μmol/min/μmol protein. Thus, we can conclude that the fusion protein has robust 15-LOX activity. For the 12*R*LOX assay, we did not expect such high activity, as previous determinations of 12*R*-LOX activity were not substantial. In fact, Siebert et al. (2001) found no activity when mouse wild type 12*R*-LOX was given arachidonic acid as a substrate. However, our yjgD/12*R*-LOX catalysis recorded an initial velocity of HETE production at 0.027 μmol/min/μM 12*R*-LOX. This number is low, yet significantly above background, indicating activity. 15-LOX1 and eLOX will be assayed at a later date, but

results of the assays for the 15- and 12*R*-LOX activity give proof that the yjgD fusion peptide does not interfere with activity.

Discussion

Presently, the central problem in the lipoxygenase field is the inability to express these enzymes abundantly and in a cost-effective manner. Without a large amount of protein, purification, crystallization and activity assays cannot be performed. The results of the current study demonstrate that hyperacidic fusion tags are an essential key in obtaining high-level expression of human LOXs, and the yield of our yjgD fusions holds considerable potential for crystal trials and structure-function studies. Most importantly, the yjgD protein does not interfere with LOX activity or affinity purification

The yjgD fusion is successful mainly due to its small size and highly acidic nature. One proposed reason for the high solubilization rate of these acidic proteins is their repulsion to each other. The repulsion of the negative charge prohibits aggregation during the protein folding stage, therefore allowing adequate time to fold properly and spontaneously, without interactions from surrounding target proteins. Several groups concur this is most likely mechanism for success, rather than a protein chaperone (Zhang, et al., 2004). Also, the fact that these peptides are added at the N-terminal plays into the repulsion theory. Producing the highly acidic peptides first enables an early anti-aggregation mechanism (Kim, et al., 2004). Therefore as synthesis of the protein occurs, it is left untouched and able to assemble at its own rate.

Though crystallization of our fusion LOXs has not been attempted, we do anticipate slight structural rearrangements due to the yjgD addition. Even if the additional peptide does not interfere with protein rearrangement, the highly acidic nature of the fusion peptide could cause repulsion between proteins. In order to achieve a useful crystal for x-ray diffraction, all proteins must be tightly packed in an aligned matrix. However, the repulsion of the fusion peptide would most likely interfere with tight packing. There is a possibility of neutralizing the large net negative charge by adding an arginine solvent (Vedadi et al., 2006). However, more than likely, the yjgD peptide will require removal for crystal trials and certainly will have to be removed for studies of membrane binding. Cleaving the fusion peptide from its current position would also remove the histidine tag and eliminate our possibility of purification. Future studies in Dr. Bartlett's lab hope to change the position of the yjgD relative to the histidine tag and insert a TEV protease cleavage site between yjgD and the His tag. This way, the negative net charge would be present to reduce aggregation during protein folding and increase solubility, but could be cleaved without removing the histidine tag.

Conclusion

The search for solubility enhancing materials is far from over. However, the results from this experiment and its predecessors show there is great potential in fusion tags, specifically highly acidic fusion tags. The solubility of human lipoxygenases was greatly increased after the addition of yjgD, and my results show that the fusion proteins can be purified via the His tag and that they are catalytically active. However, further study is required to analyze the full effectiveness of yjgD fusion as well as any negative implications this protein fusion may have. The yjgD/LOX fusion has yet to be crystallized;

therefore we do not know if the yjgD addition will interfere with the formation of a densely packed crystal.

Overall, we hope the fusion of the solubility tag yjgD will enhance study in the lipoxygenase field. Lipoxygenases have been are extremely hard to obtain in soluble form; therefore, our results with the yjgD/LOX fusions hold great importance. As discussed earlier, it is vital to understand these enzymes in order to begin strategies to negate their harmful effects; however, we must be cautious not to interfere with the beneficial effects of 12*R*LOX, 15-LOX2, and lipoxins.

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