Structural and Functional Characterization of Human Lipoxygenases: The Key Players in Inflammation and Cancer

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

by

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February, 2017

University of Hyderabad
(A Central University established in 1974 by an act of parliament)
HYDERABAD – 500 046, INDIA

CERTIFICATE

This is to certify that the thesis entitled "**Structural and Functional Characterization of Human Lipoxygenases: The Key Players in Inflammation and Cancer**" submitted by **Mr. Kumar Reddy Kakularam** bearing registration number 10LAPH04 in partial fulfillment of the requirements for award of Doctor of philosophy in the School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

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1. Reddy KK, Vidya Rajan VK, Gupta A, Aparoy P, Reddanna P (2015) *BMC Res Notes*. 16;8:152. (Chapter 4, Objective 1)

B. Presented in the following conferences

1. SBCIC (University of Hyderabad), 2013 **(International)**
2. Host-pathogen interactions (NIAB), 2014 **(International)**
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Further the student has passed the following courses towards fulfillment of coursework requirement for Ph.D

Course Code	Name	Credits	Pass/Fail
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2. AS 802	Research Ethics & Management	2	Pass
3. AS 803	Biostatistics	2	Pass
4. AS 804	Analytical Techniques	3	Pass
5. AS 805	Lab Work	4	Pass

Supervisor **Head of the Department** **Dean of School**

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DECLARATION

I hereby declare that the results of the study incorporated in the thesis entitled "**Structural and Functional Characterization of Human Lipoxygenases: The Key Players in Inflammation and Cancer**" has been carried out by me under the supervision of **Prof. Pallu Reddanna** at Department of Animal Biology, School of Life Sciences. The work presented in this thesis is a bonafide research work and has not been submitted for any degree or diploma in any other University or Institute.

Date:

Kumar Reddy Kakularam
(10LAPH04)

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Dedicated
To My Family
&
My Mentor
Prof. P. Reddanna

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Abstract

Lipoxygenases (LOXs) are one of the enzyme classes of arachidonic acid pathway involved in the biosynthesis of eicosanoids responsible for various cellular functions and pathological implications. There are six different functional LOXs in humans and are classified based on their stereo/regio specific insertion of oxygen on polyunsaturated fatty acids. These enzymes share a common mode of action and will metabolize same substrate with only difference being the position of oxygen insertion. This leads to the production of different classes of lipid mediators that work differently based on milieu. LOX-derived metabolites are associated with auto-immune diseases, cancers, allergic diseases, and asthma. In addition, genetic variations in LOXs have been attributed for increased risk in many diseases such as coronary artery disease, breast cancer, osteoporosis, bronchial asthma, schizophrenia, and autosomal recessive congenital ichthyosis. Hence, it is essential to characterize different LOXs at the structural level to examine the important amino acids that are responsible for their distinct activities. In order to achieve this, in the present study, we employed molecular modelling approaches to distinguish structural and functional similarities/diversities and identified the key amino acid determinants at the active sites of human 15-Lipoxygenase, human 12S-Lipoxygenase, human 5-Lipoxygenase, and human 12R-Lipoxygenase. Later, the identified amino acids were functionally characterized for their effects on enzyme catalysis and reaction specificity *in vitro* through site-directed mutagenesis. Further studies were also done to address the molecular mechanisms involved for the observed effects in the mutants by monitoring induced structural and conformational changes by performing molecular dynamic simulation analysis. This study led to identification of important amino acids in human LOXs that are essential for enzyme functionality and reaction specificity.

Abbreviations

°C	:	Degree centigrade/ Degree Celsius
HODE	:	hydroxyoctadecadienoic acid
HOTrE	:	hydroxyoctadecatrienoic acid
HETE	:	hydroxyeicosatetraenoic acid
HPETE	:	hydroperoxyeicosatetraenoic acid
HETE-met	:	hydroxyeicosatetraenoic acid methyl ester
AA	:	Arachidonic acid
ALA	:	α-Linolenic Acid
COX	:	Cyclooxygenase
DHA	:	Docosahexaenoic acid
EPA	:	Eicosapentaenoic acid
GC-MS	:	Gas chromatography-mass spectrometry
kDa	:	Kilo Dalton
LA	:	Linoleic acid
LOX	:	lipoxygenase
HRP	:	Horseradish peroxidase
LT	:	Leukotriene
LX	:	Lipoxin
mg	:	milligram
min	:	Minute(s)
mL	:	Milliliter
mM	:	millimolar
nm	:	nanometer(s)
ns	:	nano seconds
NSAIDs	:	Non-steroidal anti-inflammatory drugs

PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PG	:	Prostaglandin
PUFAs	:	Polyunsaturated fatty acids
RP-HPLC	:	Reverse phase- High pressure liquid chromatography
SDS	:	Sodium dodecyl sulfate
Tris	:	Tris-(Hydroxymethyl) aminomethane
UV	:	Ultraviolet
µM	:	Micro molar
RMSD	:	Root mean square deviation
RMSF	:	Root mean square fluctuations
A/Ala	:	Alanine
T/Thr	:	Threonine
G/Gly	:	Glycine
F/Phe	:	Phenylalanine
S/Ser	:	Serine
E/Glu	:	Glutamic acid
Q/Gin	:	Glutamine
His	:	Histidine
Leu	:	Leucine
Arg	:	Arginine
Val	:	Valine
Ile	:	isoleucine
Met	:	Methionine
Lys	:	Lysine
ALI	:	aliphatic interactions

PII	:	aromatic interactions
ACC	:	hydrogen bond acceptors
DAC	:	mixed donor/acceptors
Fe	:	Iron
h	:	Human
r	:	Rabbit
m	:	Mouse
p	:	Pig
Rv	:	Resolvin
PMN	:	Polymorphonuclear leukocytes
HX	:	Hepoxilin
MaR	:	Maresin
Å	:	Angstrom
LB	:	Luria-Bertani
NCBI	:	National centre for biological information
SDM	:	Site-directed Mutagenesis
PDB	:	Protein data bank
H-bonds	:	Hydrogen bonds

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CHAPTER 1

INTRODUCTION

1.1. Inflammation

Inflammation is body's defense mechanism against external stimuli such as tissue damage, microbial invasion, toxins, etc. Five major features can characterize inflammation; Swelling, Heat, Redness, Pain, and Loss of function (Figure 1.1) [Lawrence et al., 2002]. Inflammation helps in removal of harmful agents from the body, for tissue repair, and for tissue healing. It can be classified as either acute or chronic inflammation. Acute inflammation is the primary response of the body, whereas chronic inflammation is a more complex and prolonged process due to a persistent inflammatory stimulus [Lawrence and Gilroy, 2007].

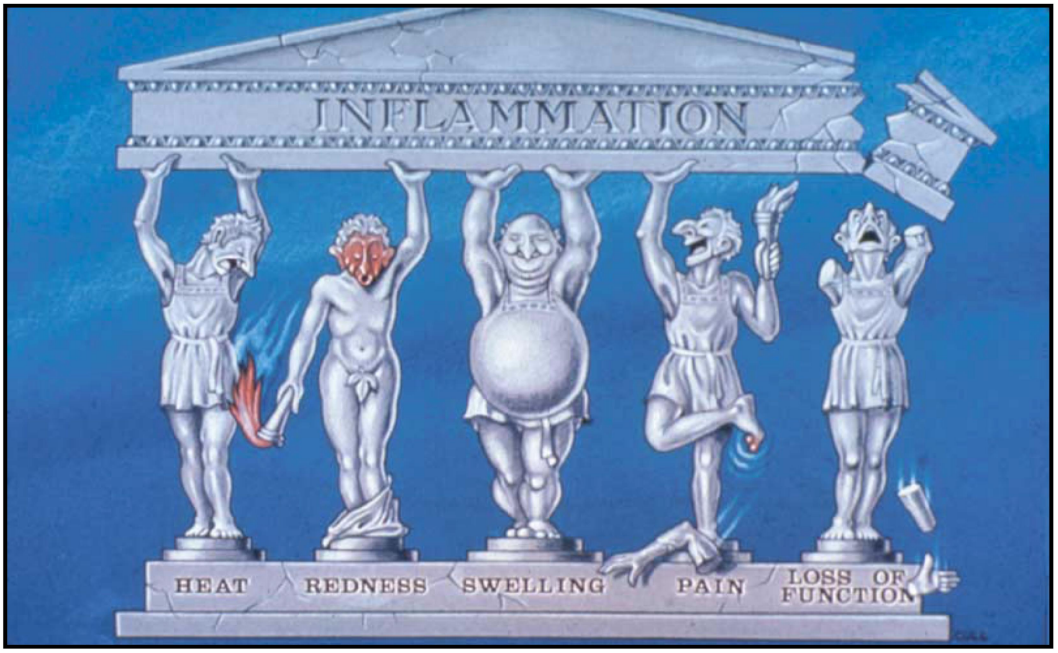

Figure 1.1: Five cardinal signs of inflammation; (Source: Lawerence T et.al, *Nat Rev Immunol*, 2002.)

Inflammation is a beneficial host response; however, prolonged inflammation contributes to many disease states such as rheumatoid arthritis, cancer, neurological, autoimmune, and cardiovascular diseases [Lawrence and Gilroy, 2007; Serhan et al., 2015a]. So, resolution of inflammation is an important mechanism that is regulated by various endogenous mediators. Pro-inflammatory mediators such as cytokines, eicosanoids, chemokines, and vasoactive amines coordinate the initial events of acute inflammation [Headland and Norling, 2015]. Lipoxins, resolvins, protectins and cyclopentenone prostaglandins, on other hand, act as anti-inflammatory mediators in regulating chronic inflammation by suppressing pro-inflammatory gene expression,

leukocyte migration and activation, followed by phagocytosis and apoptosis [Lawrence et al., 2002].

1.2. Cancer

Cancer is one of the most common causes of deaths worldwide. It can be characterized as an uncontrolled growth of cells with a metastatic potential to invade any tissue in the body. Cancer can occur in any tissue of the body, and the causes include physical, chemical and biological factors that induce genomic instability [Pavlova and Thompson, 2016]. Initiation, promotion, and progression are the three different sequential stages of cancer. Genomic changes such as point mutations, base deletions, and chromosomal rearrangements lead to irreversible cellular changes and contribute to the initiation of cancer cells. The development of cancer cell into a tumor is promoted by survival, clonal expansion, and progression which involve ample growth in tumor size and activating invasion and metastasis. Ten hallmarks of cancer that govern normal cells into a cancerous cell are illustrated in Figure 1.2. They represent the fundamental properties that are essential for cancer development.

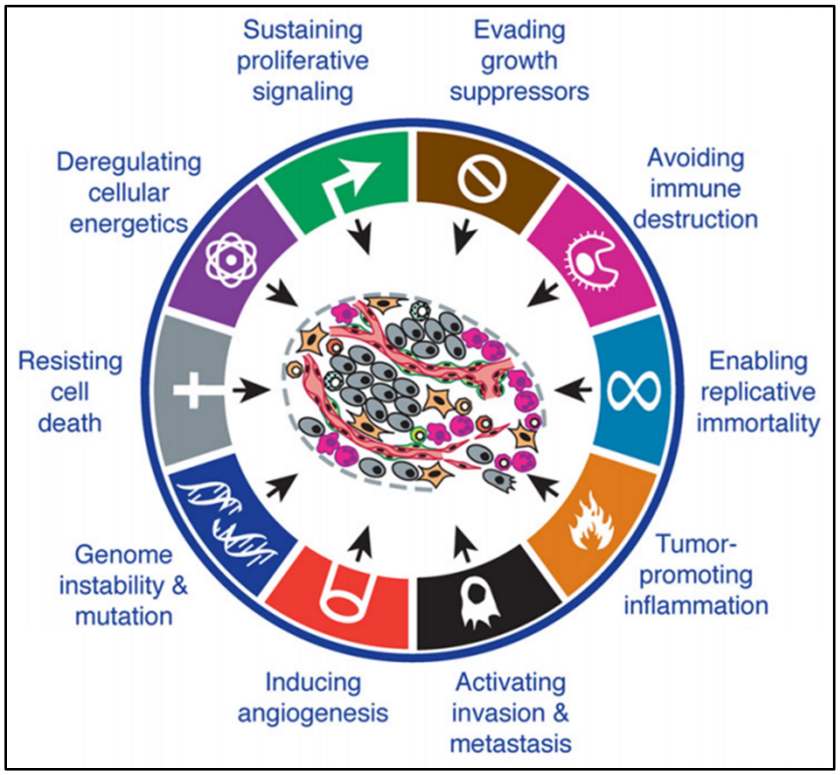

Figure 1.2: Acquired capabilities of cancer during the multistep development of human tumors;

Source: Douglas H et.al, *cell,* 2011.

Sustaining proliferative signaling involves deregulation in growth promoting signals that govern the cell growth and cell division cycle in maintaining tissue homeostasis by cancer cells for their advantage. Cancer cells negatively regulate cell proliferation (evading growth suppressors), hijack apoptosis and autophagy (resisting cell death) and acquire reproductive immortality that serves as natural barriers for cancer development [Pavlova and Thompson, 2016]. In order to grow tumors, the cancer cell needs adequate amounts of nutrients and oxygen for which blood supply is necessary. Cancer cells can induce angiogenesis, a process for formation of new blood vessels. Metastasis is the spread of cancer cells from the site of the primary tumor to distant metastases. Invasion and metastasis involve a sequence of discrete steps: local invasion, intravasation, extravasation, micrometastases, and colonization. Inflammation is considered as the seventh hallmark of cancer as it plays a critical role in cancer development [Lu et al., 2006; Pavlova and Thompson, 2016]. Eicosanoids being the key players of inflammation, they have a critical role in the initiation, progression, and maintenance of cancers. It promotes cell proliferation, cell survival, angiogenesis and free radical production that affect DNA [Wang and DuBois, 2010]. Cancer cells can reprogram energy metabolism in order to fuel cell growth and division and are also capable of evading destruction by the body's immune defenses. Based on the cellular origin, cancers can be classified as carcinomas (epithelial in origin), sarcomas (mesenchymal in origin), leukemia's and lymphomas (blood cell in origin). According to world health organization, an estimated 14 million new cases and 8.2 million cancer-related deaths occurred worldwide in 2012, and the numbers are expected to rise by about 70% over the next 2 decades. The treatment options available include surgery, radiation therapy, targeted therapy, chemotherapy, hormone therapy and precise medicine and are based on the stage and type of cancer. However, the complexity of the disease varies with the kind of cancer and most importantly between individuals suffering from a similar kind of cancer making it difficult to develop universal therapies. Hence, an enormous line of research is being focused on understanding and identifying molecular mechanisms responsible for developing novel therapeutic approaches.

1.3. Eicosanoids

Eicosanoids are a family of lipid mediators that are generated majorly from arachidonic acid (AA) metabolism. AA is a 20 carbon ω-6 polyunsaturated fatty acid with four cis double

bonds and is produced by the action of phospholipase A2 and other phospholipases from membrane phospholipids. Eicosanoids generated via AA pathway are associated with both physiological and pathological functions [Kuhn et al., 2015]. Vasoconstriction, vasodilatation, anti-aggregation, and pro-aggregation of platelets, bronchoconstriction, bronchodilation in bronchi, inhibition of gastric acid secretion in the stomach, filtration and renal blood flow in the kidney are some of the physiological functions associated with eicosanoids [Harizi et al., 2008]. During inflammation, eicosanoids production will be increased at the site of inflammation resulting in processes ranging from inflammatory responses to chronic tissue remodeling, cancer, asthma, rheumatoid arthritis and autoimmune disorders [Pidgeon et al., 2007]. Typically, the share of pro- and anti- inflammators in the milieu governs the progression or resolution of inflammatory diseases. Inflammation is a complex process which is regulated by many metabolic pathways and eicosanoids are not the only one that defines the disease condition.

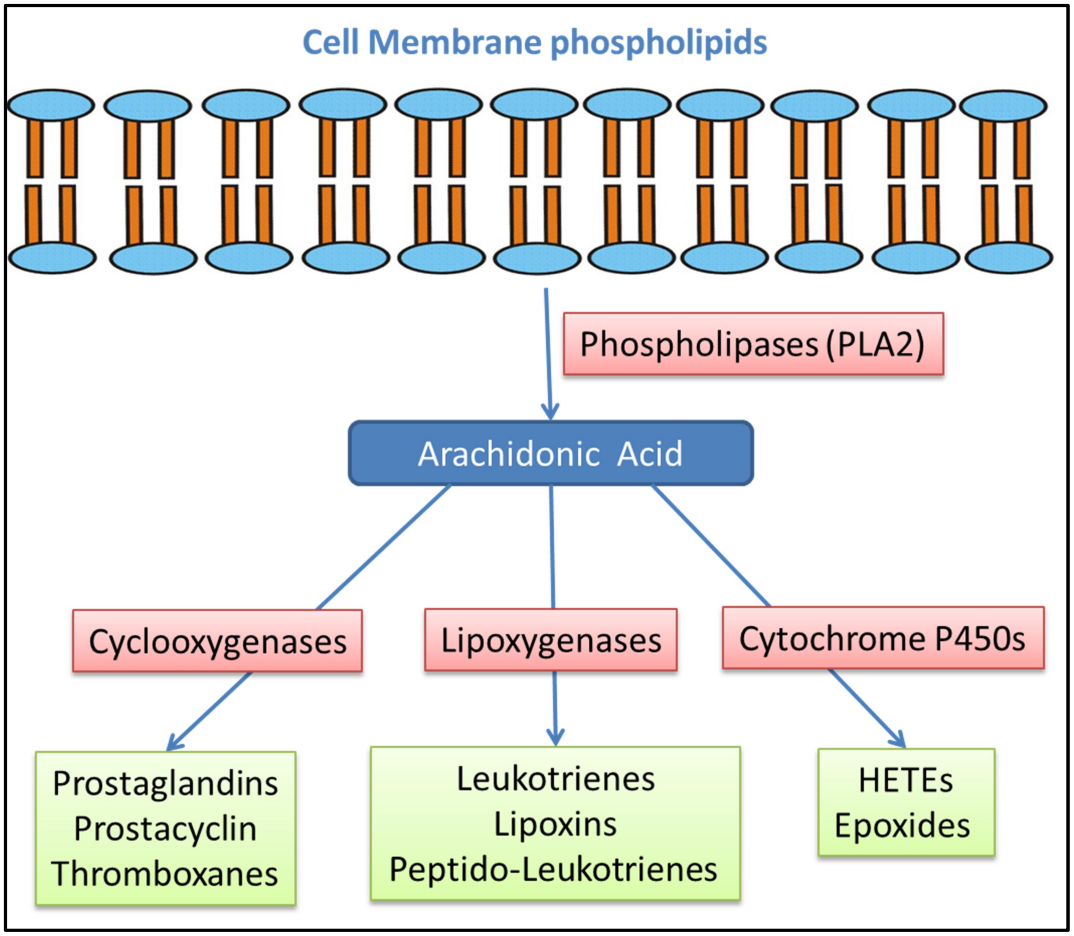

Figure 1.3: Arachidonic acid metabolism: Arachidonic acid released from membrane phospholipids will be metabolized by lipoxygenase, cyclooxygenase, and cytochrome P450 pathways

Three different enzymatic pathways can metabolize AA; lipoxygenase (LOX), cyclooxygenase (COX), and cytochrome P450 (Figure 1.3). Prostaglandins (PGs) and thromboxanes (TXs) are generated from COX enzyme pathway that consists of mainly two enzymes, COX-1, and COX-2. COX-1 is constitutively expressed in kidney, liver, and stomach whereas COX-2 is an inducible form expressed mainly at the site of inflammation [Rocca and FitzGerald, 2002]. COX-1 generated PGs help in maintaining normal body functions such as regulation of gastric acid, stomach mucous production, and kidney water excretion. On the other hand, PGs generated by COX-2 are a result of stimulation of inflammatory cytokines and growth factors and are associated with various inflammatory diseases [Harris et al., 2002]. Similarly, LOX enzymatic pathway generated leukotrienes (LTs), and lipoxins (LXs) are also shown to play a major role in various physiological and pathological processes, including cancers [Mashima and Okuyama, 2015].

1.4. Lipoxygenases

1.4.1 General Introduction of LOXs

Lipoxygenases are non-heme iron-containing dioxygenases that metabolize polyunsaturated fatty acids by adding one mole of oxygen to produce hydroperoxy derivatives. LOXs have been identified and characterized in various plants and mammals, and recently they have also been identified in bacteria [Vance et al., 2004], coral [Brash et al., 1996], moss [Senger et al., 2005] and fungi [Heshof et al., 2014]. AA is the major substrate for LOXs in mammals. LOX-derived AA-pathway produces various lipid mediators that have diverse roles in various pathological and physiological conditions [Harizi et al., 2008]. In addition to AA, some of these enzymes also metabolize linoleic acid, alpha-linolenic acid, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), ester lipids, biomembranes, and lipoproteins. In humans, till date, six different LOXs have been identified. These enzymes have been classified based on their regio- and stereo-specific incorporation of molecular oxygen on AA into 5-, 12S-, 12R-, and 15-LOXs [Ivanov et al., 2010]. They can also be classified based on their localization as leucocyte type (5-LOX, 12S-LOX, and 15-LOX-1) and epidermis type (12R-LOX, 15-LOX-2, and

ELOX3) LOXs. The metabolites generated by different types of LOXs with AA as the substrate are summarized in Figure 1.4.

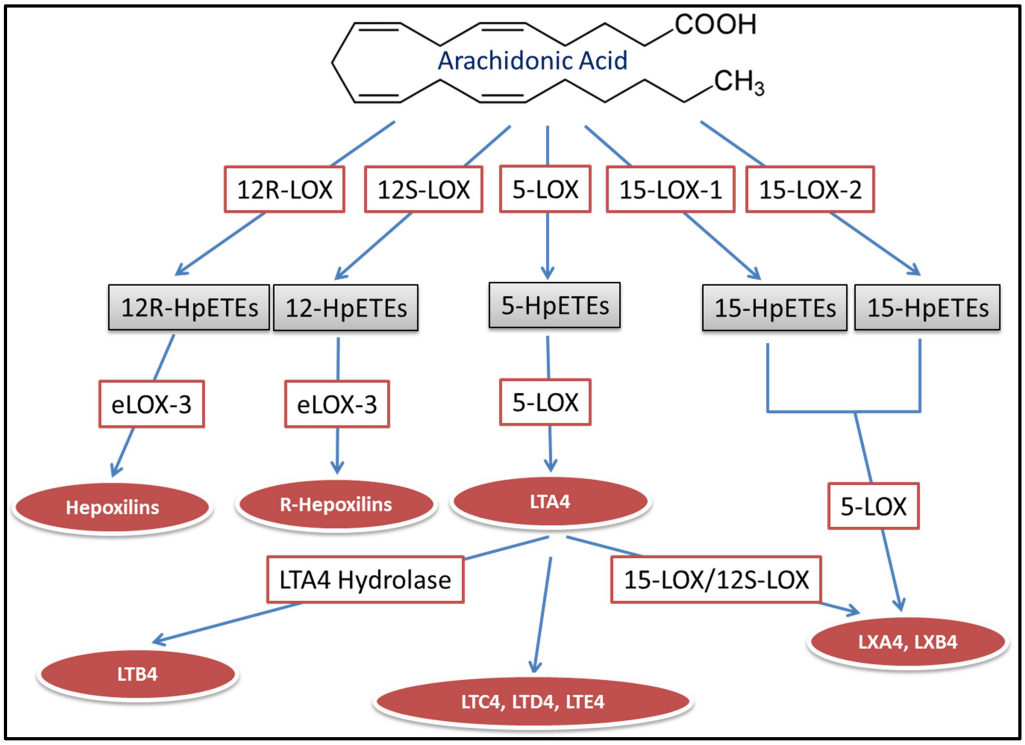

Figure 1.4: Lipoxygenase pathway of arachidonic acid: Lipoxygenases can metabolize arachidonic acid into different classes of lipid mediators comprising of HETEs, leukotrienes, lipoxins, and hepoxilins

The molecular mass of LOXs ranges from 75 to 80 kDa and sequence length from 662 to 711 amino acids. They share a similar structural architecture with two conserved domains; N-terminal beta barrel domain and catalytically active C-terminal domain (Figure 1.5). The amino-terminal or N-terminal domain is of 25 to 30 kDa and consists of only β barrels. C-terminal domain is a catalytically active domain of 55-60 kDa composed of mainly alpha helices. LOXs consist of one molecule of iron (ferrous form) per one mole of enzyme and iron is octahedrally coordinated to 5 amino acids and a water molecule or a ligand (Figure 1.6). This octahedral geometry is conserved in all the human LOXs involving three conserved histidines, one isoleucine, and one more histidine that is replaced with glutamine in the case of 5-LOX. The iron is usually in ferrous form (FE(II)) and will be converted into catalytically active ferric form (FE(III)) after the activation by trace lipid hydroperoxides [Haining and Axelrod, 1958].

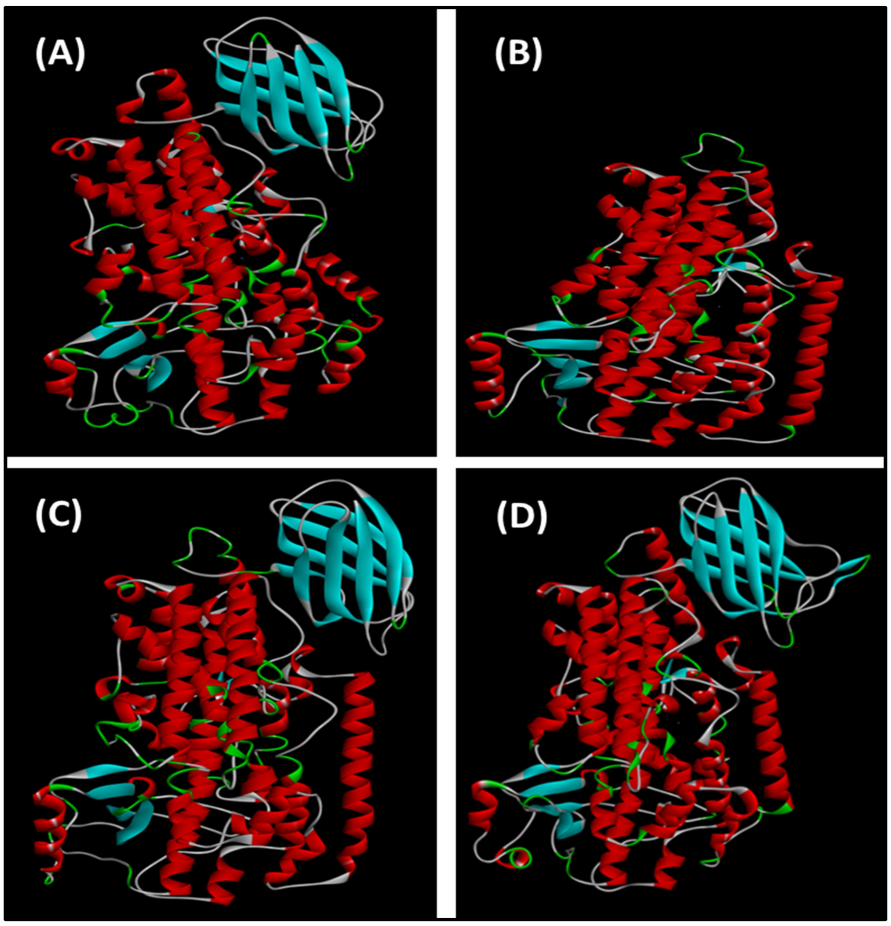

Figure 1.5: Crystal structures of different human lipoxygenases exposing the structural domains (A) 5-LOX, (B) truncated version of 12S-LOX, (C) 15-LOX and (D) 15-LOX-2

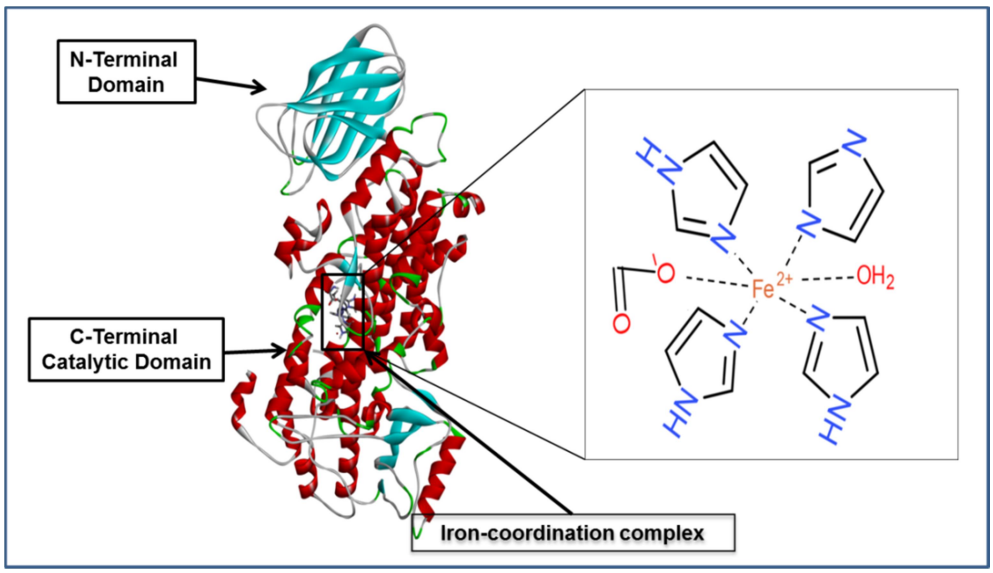

Figure 1.6: Crystal structure of human 15-lipoxygenase revealing N-terminal, C-terminal catalytic domains, and its non-heme iron coordination geometry

1.4.2 Human 5-Lipoxygenase

The h5LOX is the most widely studied and characterized LOX among all the six human LOXs. Unlike all other LOXs, h5LOX gene, ALOX5 is localized separately on chromosome 10 and contains 14 exons. This enzyme catalyzes AA to 5(S)-hydroperoxy-6-trans-8, 11, 14-cis-eicosatetraenoic acid (5-HPETE) that will be further converted into 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE). In addition to dioxygenase activity h5LOX also possess leukotriene synthesizing activity that results in conversion of 5-HPETE to 4-{(2S, 3S)-3-[(1E, 3E, 5Z, 8Z)-1, 3, 5, 8-Tetradecatetraen-1-yl]-2-oxiranyl} butanoic acid (LTA_4) [Rådmark et al., 2007]. LTA_4 is further converted into LTB_4, LTC_4, LTD_4, and LTE_4 by the action of different enzymes (Figure 1.7). The 5-LOX primarily expresses in various leukocytes that include neutrophils, eosinophils, monocytes, macrophages, dendritic cells, mast cells, and B-lymphocytes. Different from other LOXs, h5LOX requires co-factors like calcium, ATP, and FLAP (five lipoxygenase activating protein) for its catalysis [Rådmark and Samuelsson, 2005]. The enzyme translocates to the nuclear membrane upon activation by increased Ca2+ concentration and forms complex with FLAP to metabolize AA. The activity of 5-LOX is short lived, and enzymes undergo auto-inactivation which could be one of the mechanisms for controlling the synthesis of pro- and anti-inflammatory mediators.

The h5LOX contains 663 amino acids with two distinct structural domains: N-terminal domain containing only beta-sheets and C-terminal catalytic domain containing mainly alpha-helices. The N-terminal domain is of approximately 120 amino acids and confers Ca2+-dependent membrane binding, and is necessary for nuclear envelope translocation. The larger catalytic domain is of ~550 amino acids and harbors the non-heme catalytic iron. The iron is octahedrally coordinated with 3 histidines (367, 372, and 550), one asparagine (554), and C-terminal isoleucine (673) and the sixth coordinate will be either water or ligand. The X-ray crystal structure for the apo-form of h5LOX is available at a resolution of 2.4 Å [Gilbert et al., 2011]. To achieve this, the authors had replaced some of the amino acids which they claimed to be a 5-LOX specific destabilizing sequence with *plexaurahomomalla* 8RLOX sequence. There are no co-crystalized substrates available not only for h5LOX but also for all other solved mammalian LOX structures making it difficult to understand the binding mode of the substrate. Leukotrienes are the biologically active h5LOX metabolites which are involved in various

physiological and pathological processes [Sharma and Mohammed, 2006]. Physiological functions of leukotrienes include vasoconstriction, vasodilation in blood vessels, and bronchoconstriction in bronchi. Due to their roles in inflammation, leukotrienes are associated in the pathogenesis of inflammatory and immune diseases. Elevated levels of h5LOX both in genomic and proteomic levels were observed in different types of cancers such as colorectal [Tuncer and Banerjee, 2015], prostate [Gupta et al., 2001], breast [Jiang et al., 2003], lung [Poczobutt et al., 2016] and bladder [Hayashi et al., 2006] cancers. For example, mRNA and protein levels of 5LOX were up-regulated in more than 65% of the 91 colorectal tumors in comparison with adjacent normal mucosa samples [Soumaoro et al., 2006]. *In vitro* and *in vivo* studies with 5-LOX inhibitors in various cancers significantly reduced the cancer proliferation, migration, and invasion [Hayashi et al., 2006; Chen et al., 2009]. As a result, many inhibitors were in the clinical trials. For example, zileuton which is already on the market for treating asthma is in phase-II clinical trials for the treatment of lung, head, and neck cancer [Bishayee and Khuda-Bukhsh, 2013]. Elevated levels of 5LOX were also observed in other diseases such as allergy [Joshi and Praticò, 2014], asthma, rheumatoid arthritis, sepsis, and atherosclerosis [Liu and Yokomizo, 2015]. Consequently, many studies are focused on developing potential drug candidates towards h5LOX and in elucidating the molecular mechanisms underlying its effects.

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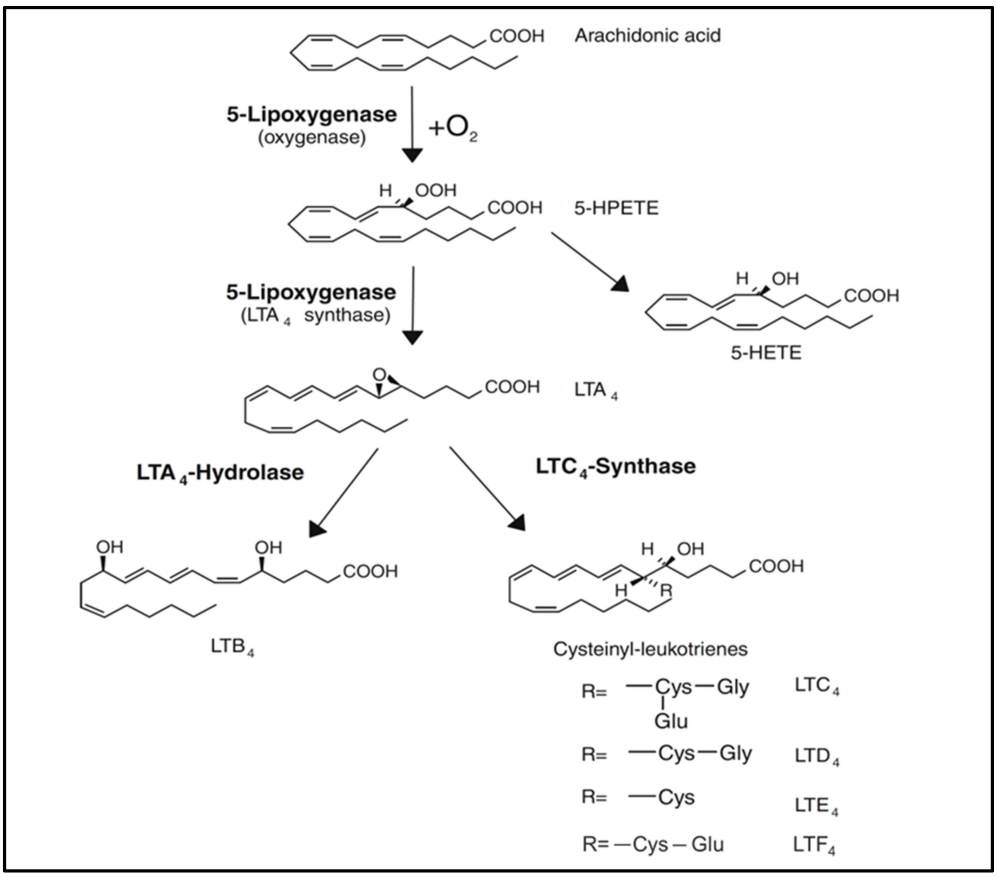

Figure 1.7: Human 5-lipoxygenase pathway of leukotrienes biosynthesis from arachidonic acid; Source (O. Rådmark et al. *Biochem Biophy Res Commun* 2010) with modifications

functions of leukotrienes include vasoconstriction, vasodilation in blood vessels, and bronchoconstriction in bronchi. Due to their roles in inflammation, leukotrienes are associated in the pathogenesis of inflammatory and immune diseases. Elevated levels of h5LOX both in genomic and proteomic levels were observed in different types of cancers such as colorectal [Tuncer and Banerjee, 2015], prostate [Gupta et al., 2001], breast [Jiang et al., 2003], lung [Poczobutt et al., 2016] and bladder [Hayashi et al., 2006] cancers. For example, mRNA and protein levels of 5LOX were up-regulated in more than 65% of the 91 colorectal tumors in comparison with adjacent normal mucosa samples [Soumaoro et al., 2006]. *In vitro* and *in vivo* studies with 5-LOX inhibitors in various cancers significantly reduced the cancer proliferation, migration, and invasion [Hayashi et al., 2006; Chen et al., 2009]. As a result, many inhibitors were in the clinical trials. For example, zileuton which is already on the market for treating asthma is in phase-II clinical trials for the treatment of lung, head, and neck cancer [Bishayee and Khuda-Bukhsh, 2013]. Elevated levels of 5LOX were also observed in other diseases such as

allergy [Joshi and Praticò, 2014], asthma, rheumatoid arthritis, sepsis, and atherosclerosis [Liu and Yokomizo, 2015]. Consequently, many studies are focused on developing potential drug candidates towards h5LOX and in elucidating the molecular mechanisms underlying its effects.

1.4.3 Human 12S-Lipoxygenase

Human 12SLOX also called as arachidonate 12-lipoxygenase, and platelet-type 12-LOX is encoded by ALOX12 gene that is localized on chromosome 17 in a joint gene cluster along with all other LOXs (except 5-LOX) [Dobrian et al., 2011]. It has a molecular mass of approximately 75 kDa. In humans, it expresses in blood platelets and in skin. The h12SLOX metabolizes AA at C12 carbon position to produce 12-HETE. It is also involved in the biosynthesis of lipoxins, hepoxilins, and maresins from other fatty acids and their reaction intermediates. Human 12SLOX consists of 663 amino acids that shape into two domains consisting of approximately 115 and 550 amino acids. The smaller domain (N-terminal domain) consists of parallel and antiparallel beta sheets, and the bigger domain (catalytic domain) mainly consists of alpha-helices. The non-heme iron is positioned in the catalytic domain and forms coordination covalent complex with four histidines (360, 365, 540, and 544) and a c-terminal isoleucine (663) [Tresaugues et al.,]. The iron is usually in inactive ferrous form, and the oxidation to ferric form is required for catalysis. The crystal structure of the catalytic domain of h12SLOX at a resolution of 2.6 Å was reported with few missing amino acids at the active site [Tresaugues et al.,]. For a better understanding of the structural properties, the crystal structure of porcine 12SLOX (catalytic domain) [Xu et al., 2012], the ortholog of h12SLOX can serve as a suitable model. The metabolites formed via 12SLOX pathway have been implicated in various biological processes such as platelet activation, atherogenesis, regulation of angiogenesis, endothelial cell proliferation and migration, cancer cell growth, and neuronal apoptosis [Dobrian et al., 2011]. The AA derived 12HETE has pro-inflammatory effects whereas lipoxins, hepoxilins, and maresins show anti-inflammatory and pro-resolving effects [Serhan et al., 2015b].

1.4.4 Human 15-Lipoxygenase

Human 15LOX is encoded by ALOX15 gene which is located on chromosome 17 [Dobrian et al., 2011]. It is a two-domain structure of 663 amino acids with a molecular weight

of 75 kDa. The enzyme contains one non-heme iron that is coordinated with His361, His366, His541, His545, and Ile663 per mole of enzyme [Gillmor et al., 1997]. The enzyme is of S-enantiomeric chirality and metabolizes fatty acids to hydro (per)oxyeicosatetraenoic acids which will be further reduced to their hydroxy derivatives. The h15LOX inserts molecular oxygen at carbon 15 as the name suggests producing 15HpETE from AA. In addition to 15-lipoxygenation, this enzyme also possesses lower 12-lipoxygenation specificity to produce 12-HpETE. The h15LOX possesses broad substrate specificity, and the substrates include linoleic acid, alpha-linolenic acid, phospholipids, triacylglycerols, lipoproteins, cholesterol esters, and complex biomembranes [Kuhn et al., 2002]. The h15LOX constitutively expresses at high levels in immature red blood cells, airway epithelial cells, and eosinophils. Low expression levels of h15LOX in polymorphonuclear leukocytes, vascular cells, uterus, and atherosclerotic lesions [Wittwer and Hersberger, 2007] were also reported. The h15LOX is responsible for the synthesis of lipoxins, hepoxilins, protectins, and resolvins which exhibit various cellular functions. The enzyme can be catalytically active even after removal of the N-terminal domain consisting of beta-sheets [Walther et al., 2002].

The first mammalian lipoxygenase crystal structure solved was for rabbit 15LOX which is an ortholog of h15LOX [Gillmor et al., 1997]. This structure illuminated great detail on the structural features of mammalian LOXs, provided insights on substrate alignment and led to the identification of sequence determinants responsible for positional specificity. The substrate binding cavity of 15LOXs is boot-shaped pocket comprising of mostly hydrophobic amino acids. The substrate enters from the top of the binding cavity with its methyl end first. The bottom of the cavity consists of hydrophobic amino acids Phe353, Ile418, and Ile593 that may interact with the hydrophobic end of the fatty acid. In fact, these amino acids play a crucial role in the localization of C13 carbon in close proximity of iron for hydrogen abstraction. Alterations in the volume of the cavity changed the preference of hydrogen abstraction on bisallylic carbon resulting in different product profile of the enzyme [Borngräber et al., 1999]. Another critical assumption drawn from the structure was that carboxylic group of fatty acid forms a salt bridge with positively charged Arg403 located at the entrance of the binding pocket [Schwarz et al., 1998]. The exchange of arginine with neutral lysine strongly affected the substrate affinity and the reaction rate supporting the significance of the interaction. However, the similar effects were not seen in h5LOX suggesting that it may be specific only for h15LOX [Schwarz et al., 2001].

1.4.5 Epidermis-type Lipoxygenases

Human 12RLOX is the only LOX that generates R-specific hydroperoxy derivatives from fatty acids. It is encoded by ALOX12B gene located on chromosome 17 [Krieg et al., 2001]. The enzyme functions in sequence with heLOX-3, an epidermis type LOX that possesses hydroperoxide isomerase activity to generate hydroxyepoxyeicosatrienoic acids. The gene for eLOX3 is ALOXE3 located on chromosome 17 [Krieg et al., 2001]. Different from other LOXs, h12RLOX and eLOX3 contains proline-rich extra domain of 31 and 41 amino acid residues respectively located near the substrate entrance of the binding pocket. The surface exposed other domain may attribute to the enzymatic activity of the two isoforms and may have a function in protein–protein interactions. There are no crystal structures available for both the enzymes. The expression of h12RLOX and heLOX3 is restricted to the differentiated cell compartments suggesting a function of these enzymes in terminal keratinocyte differentiation. The h12RLOX along with eLOX3 have been incriminated in late epidermal differentiation. In particular, they are involved in the maintenance of one of the three principal layers (epidermis, dermis, and subcutis) of skin, i.e. the stratum corneum. The skin expresses different LOX-isoforms such as h12RLOX, heLOX3, h15LOX-2, and h12SLOX. Targeted inactivation of these LOXs, particularly h12RLOX and heLOX3 resulted in impaired water barrier function. The knockout mice of h12RLOX and heLOX3 die shortly after birth due to rapid dehydration [De Juanes et al., 2009]. Whereas the knockout mice of h12SLOX are viable and reproduce normally this could be due to the compensatory effect of other enzymes [Johnson et al., 1998]. The transgenic mice overexpressing 12RLOX are viable, breed normally but develop scaly dermatitis symptoms, similar to psoriasis in the adult stage (Naireen, thesis, 2014). However, the molecular mechanisms of how they induce impaired water-barrier function are still unknown. Naturally occurring in h12RLOX and heLOX3 are associated with skin disease known as autosomal recessive congenital ichthyosis (ARCI). Analysis of the genetic polymorphisms in h12RLOX and heLOX3 in a group of 250 patients suffering from ARCI showed these enzymes as the second most common cause after transglutaminase-1. Functional analysis of identified mutations *in vitro* showed impaired catalytic activity suggesting a causal relationship with pathogenesis [Eckl et al., 2009].

Human 15-LOX have 2 designated types; reticulocyte or leukocyte type 15-LOX-1 (15-LOX) and epidermis type 15-LOX-2. ALOX15B is the gene responsible for human 15-LOX-2 that is located on chromosome 17 and consists of 14 exons and 13 introns [Krieg et al., 2001]. Human 15-LOX-2 exclusively prefers AA and entirely metabolizes it to 15-HETE while 15-LOX-1 prefers LA and processes dual specificity with AA (15 and 12 oxygenations). The enzyme was discovered by Brash et al. in human skin [Brash et al., 1997]. In contrast to 15-LOX-1, the expression of 15-LOX-2 is limited to only a few tissues such as prostate, lung, and cornea suggesting its possible role in the regulation of organ-specific functions or differentiation or possible alterations in disease states. The likely role of h15-LOX-2 in prostate cancer and atherogenesis has been suggested. The h15LOX-2 expression is present in normal human adult prostate and in the case of prostate cancer impaired expression is observed. As this enzyme acts as a negative cell cycle regulator in the prostate, the enzymes were suggested as an anti-tumor suppressor in prostate carcinoma. The higher expression of h15-LOX-2 has been observed in advanced atherosclerotic lesions suggesting its role in the pathogenesis of atherosclerosis. Recently, polymorphisms in h15-LOX-2 have been associated with coronary artery diseases [Ye et al., 2013]. Functional characterization of the mutant enzymes showed no major differences indicating that the h15-LOX-2 metabolites are not involved and the underlying mechanisms yet to be addressed. Higher expressions of the enzyme were also reported in the major atherosclerotic plaques, and its expression has been suggested as a tissue marker for the atherosclerotic carotid artery [Moller et al., 2012].

1.5 Pro-inflammatory, Anti-Inflammatory, and Pro-resolving Properties of Lipid Mediators generated via Lipoxygenase Pathways

LOXs produce a variety of lipid mediators that play an essential role in inflammation by oxygenation of polyunsaturated fatty acids [Harizi et al., 2008]. The lipid mediators biosynthesis can occur in a single cell or through transcellular activities by different multiple cell types cooperation, and they may exhibit their actions by paracrine and/or autocrine mechanisms. Leukotrienes, lipoxins, HETEs, HpETEs, hepoxilins, resolvins, maresins and protectins are the different classes of lipid mediators produced by LOX-pathway. Among these, leukotrienes, lipoxins, HETEs, and HpETEs are generated from AA, a 20 carbon ω6 fatty acid. AA is released into the cell from the membrane phospholipids by the action of cytosolic phospholipase A2,

which is then rapidly oxygenated by the enzymes: COXs, LOXs, and cytochrome p450. The other enzymes, COXs, and cytochrome P450 produce prostaglandins, thromboxanes, prostacyclins and epoxyeicosatrienoic acids (EETs) which mostly exhibit pro-inflammatory functions [Bogatcheva et al., 2005]. Linoleic acid, another ω6 fatty acid consisting of 18 carbons can also be metabolized by LOXs into 9-, and 13-hydroxyoctadecadienoic acids (HODEs). The other group of fatty acids that are metabolized by LOX-pathway is eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) of ω-3 PUFAs. They act as precursors for the synthesis of resolvins, protectins, and maresins which act as effective pro-resolving mediators. In general, lipid mediators generated through h5LOX and h12SLOX are pro-inflammatory while h15LOX-2 metabolites are anti-inflammatory and the role of h15LOX metabolites is controversial.

Acute inflammation is initiated by prostaglandins and leukotrienes which regulate the early events of inflammatory responses. Excessive activation of these mediators and perturbations of counter-regulatory mechanisms account for chronic inflammation associated with many diseases [Lawrence and Gilroy, 2007]. After the acute inflammatory response, switching of lipid mediators from pro-inflammatory eicosanoids to anti-inflammatory/pro-resolving lipoxins, resolvins, and protectins lipid mediators begins (Figure 1.8). Pro-resolving mediators stimulate the recruitment of non-phlogistic monocytes, phagocytosis of apoptotic neutrophils, removal of inflammatory debris, clearance of neutrophils from epithelial surfaces, and sequestration of pro-inflammatory mediators [Serhan et al., 2015a].

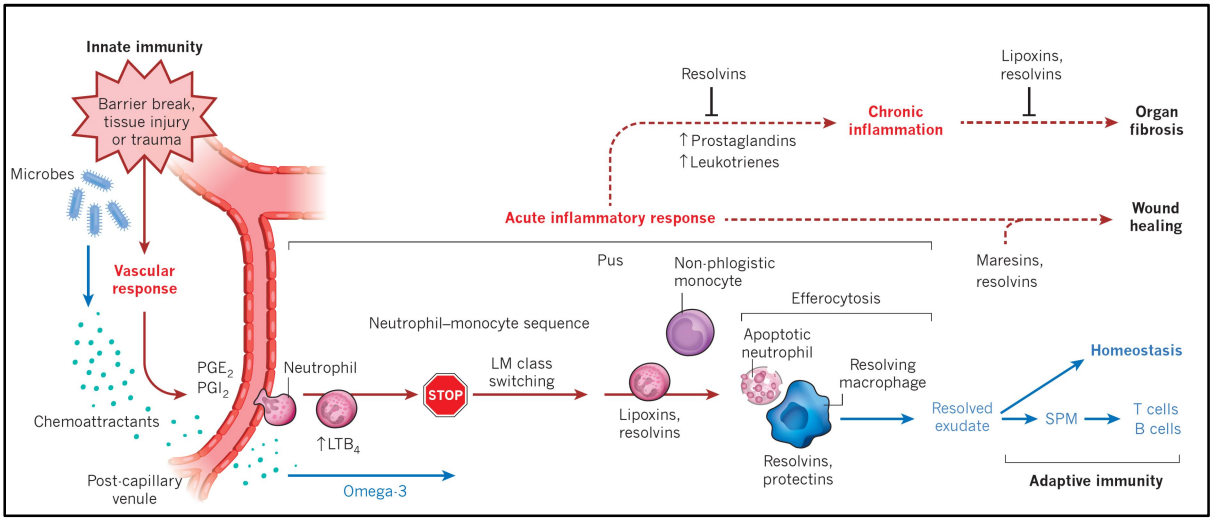

Figure 1.8: Lipid mediators in the acute inflammatory response, resolution and other outcomes; Source: Charles N. Serhan, Immunity, 2014.

1.5.1 Pro-inflammatory Mediators

As mentioned earlier, inflammation is a beneficial process adopted by organisms to challenge infection, injury, and external stimuli. This involves recruitment of different cell types to the site of inflammation and production of various mediators at the site of inflammation to regulate the inflammatory profile and promote the return of affected tissues to homeostasis and function. An enormous body of research has been done in identifying the pro-inflammatory mediators responsible for the progression of inflammation and the mechanisms involved. Leukotrienes are the most widely studied among the entire pro-inflammatory lipid mediators generated via LOX-pathway. Hepoxilins are another class of pro-inflammatory mediators from LOX-pathway that exhibit pro-inflammatory properties.

1.5.1.1 Leukotrienes

Leukotrienes, as the name suggests contain three conjugated double bonds in their structure and are predominantly produced in leukocytes. The h5LOX catalyzes the first step in leukotriene production i.e. conversion of AA into 5-HpETE that is further converted into LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4 by the action of a different class of enzymes [Haeggström and Funk, 2011]. In addition oxygenase activity, h5LOX also possesses leukotriene biosynthesis activity that results in the production of LTA_4 from 5HpETE [Samuelsson et al., 1987]. LTA_4 is the first metabolite among leukotrienes and is highly unstable at physiological pH 7.4. LTA_4 can be further metabolized either to LTB_4 which is a non-cysteinyl leukotriene by the action of LTA_4 hydrolase or to cysteinyl leukotrienes LTC_4, LTD_4, LTE_4, and LTF_4 by the action of glutathione-S-transferase, γ-glutamyl transpeptidase (GGTP), and dipeptidase, and GGTP respectively (Figure 1.9). A direct conversion of LTC_4 to LTF_4 by the action of carboxypeptidase has also been demonstrated [Reddanna et al., 2003]. Substantial evidences suggest a key role for leukotrienes as pro-inflammatory mediators in the progression of acute inflammation. The critical balance between leukotrienes along with other lipid mediators is essential for human health.

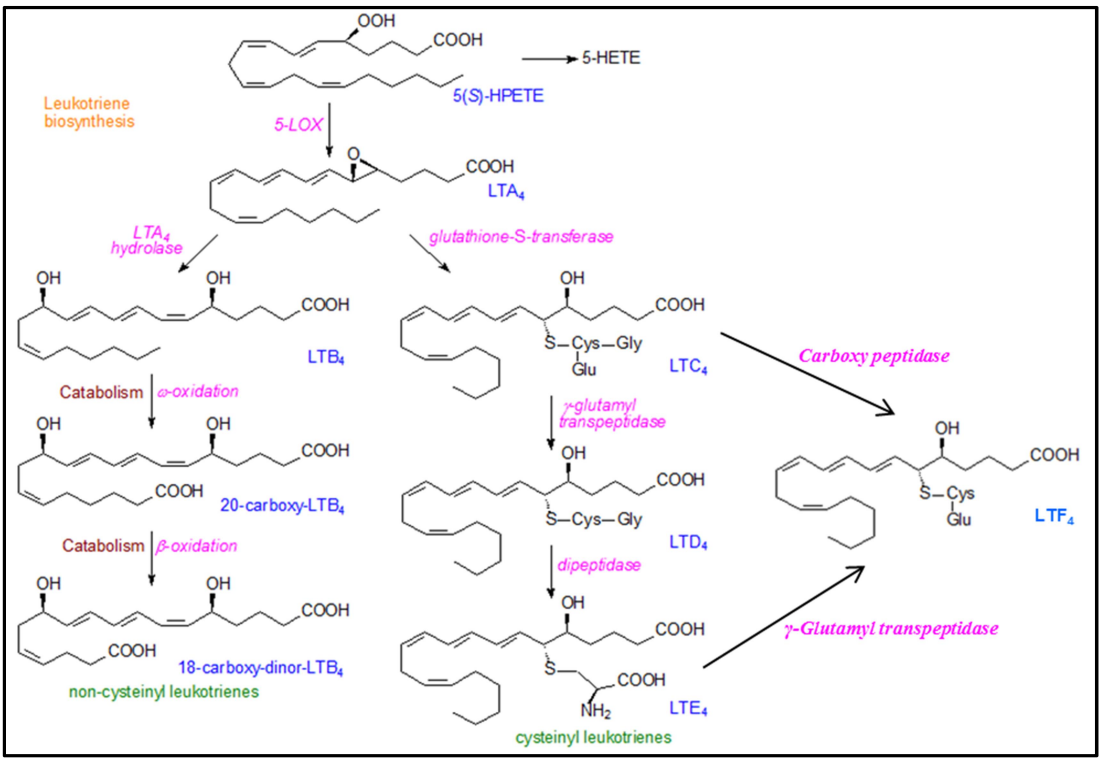

Figure 1.9: Leukotriene biosynthesis displaying their structures and different enzymes involved; Source: http://www.lipidhome.co.uk/ with modifications

Leukotrienes are synthesized by transcellular mechanisms. For instance, LTA_4 is synthesized in the neutrophils, transported to erythrocytes or platelets that possess LTA_4 hydrolase for LTB_4 synthesis and lacks 5-LOX. These potent chemotactic agents stimulate cellular responses via G-protein coupled receptors BLT1, BLT2, $CysLT_1R$, and $CysLT_2R$ [Bäck et al., 2014]. The functions such as increased vascular permeability [Dudek and Garcia, 2001], secretion of lysosomal enzymes, nitric oxide formation, phagocyte chemotaxis, intracellular signaling, and smooth muscle contraction are mediated by leukotrienes during inflammation [Lawrence et al., 2002]. In addition to their role in inflammation, they are also associated with many auto-immune diseases, cardiovascular diseases, Alzheimer's disease, Parkinson's disease, atherosclerosis, sepsis, cancer, and asthma [Haeggström and Funk, 2011]

1.5.1.2 Hepoxilins

Hepoxilins are another class of pro-inflammatory lipid mediators containing hydroxyl and epoxy functional groups and are derived from polyunsaturated fatty acids such as AA, and linoleic acid. 12SLOX, eLOX3, 12RLOX and hepoxilin synthase are the enzymes involved in

the biosynthesis of hepoxilins. Hepoxilins can be classified into A-type or B-type based the position where the functional groups are located on the fatty acid. AA will be metabolized by 12SLOX to produce 12SHpETE which can be either reduced to 12HETE or to HXA3 by the action of hepoxilin synthase. The other route for the synthesis of hepoxilins is in the epidermis of humans where linoleic acid will be metabolized by 12RLOX to 9R-HPODE and further to A-type and/or B-Type hepoxilins by eLOX3. Among all identified hepoxilins, mainly, HAX_3 has been elucidated for its role in inflammation. HXA_3 can recruit neutrophils to infected and inflamed sites and is associated with mobilization of intracellular calcium into the cytosol in neutrophils [Dho et al., 1990]. Hepoxilins may possess both pro-inflammatory and anti-inflammatory properties. In skin, they have pro-inflammatory properties whereas in neutrophils they exhibit anti-inflammatory properties [Pace-Asciak, 2015]. Hepoxilins biosynthesis was also observed in the pineal gland of the brain. There, they contribute to increased sensitivity towards pain during inflammation due to their probable involvement in melatonin production.

1.5.2 Anti-inflammatory and/or pro-resolving mediators

The dietary supplementation of the omega-3 fatty acids is well-established since a long time due to their proven beneficial effects in many disease states. However, the mechanisms behind their effects were not known until recently. Essential fatty acids, DHA and EPA are the substrates for a class of lipid mediators derived by LOX isozymes that exhibit constructive functions in different cellular events (Figure 1.10). In inflammation, they act as a switch in lipid mediator class to trigger the self-limited response of acute inflammation. These lipid mediators are called as specialized pro-resolving mediators and comprise lipoxins, resolvins, protectins, and maresins [Serhan et al., 2015a]. Except for lipoxins, all other metabolites are generated from fatty acids DHA and EPA and execute their functions via different inflammatory cell types. SPM restore the acute inflammatory milieu to homeostasis by stopping PMN transmigration and chemotaxis, blocking the production of prostaglandins and leukotrienes, recruitment of non-phlogistic monocytes, uptake and removal of apoptotic PMN, and enhancing antimicrobial defense mechanisms [Serhan et al., 2015b] [Buckley et al., 2014].

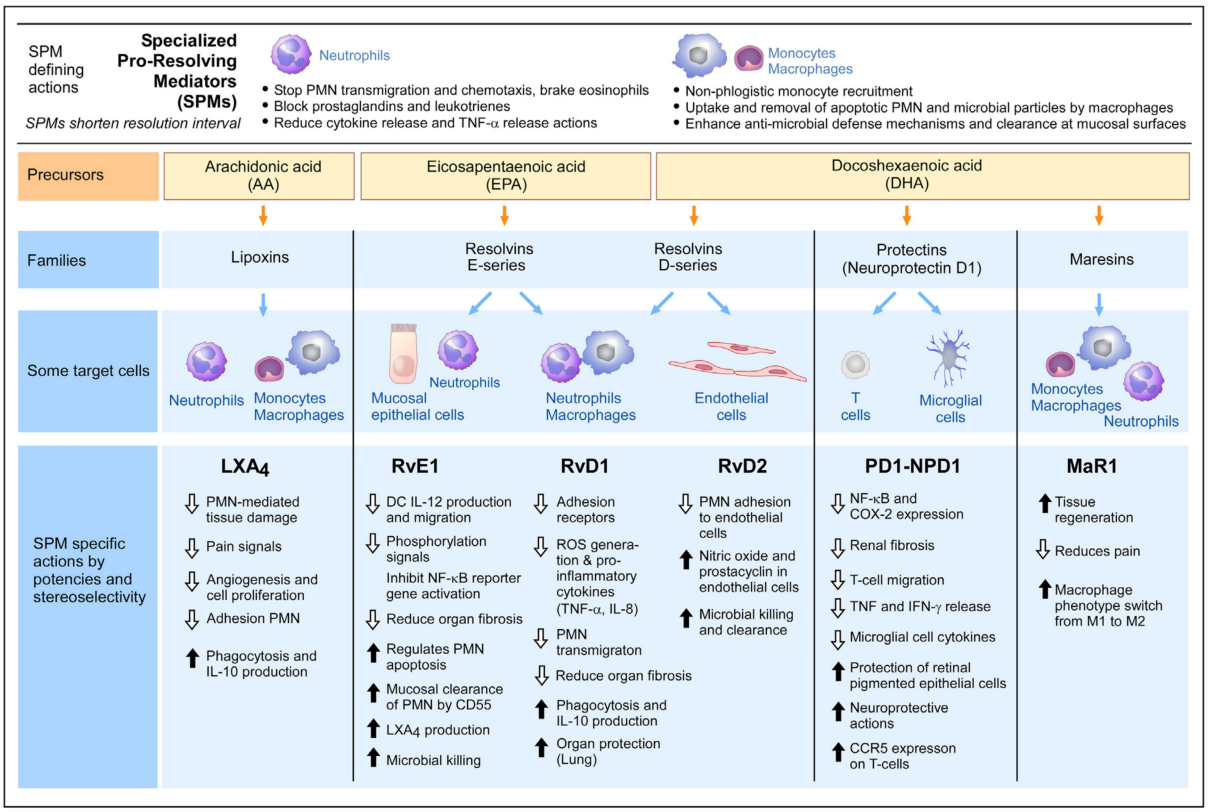

Figure 1.10: Special pro-resolving mediators, target cells, parent substrates, and their representative potent actions; Source: Christopher D. Buckley et. al, immunity, 2014.

1.5.2.1 Lipoxins

Lipoxins are the very well characterized mediators among the LOX-derived metabolites after leukotrienes. They are the first discovered LOX-derived anti-inflammatory mediators that share high structural similarity with leukotrienes but exhibit complementary and/or counter-regulatory mechanisms during the inflammation. Among the LOX-derived lipid mediators, lipoxins are the only anti-inflammatory mediators that are generated from an omega-6 fatty acid, AA. Their biosynthesis can take place via two independent pathways and involves transcellular mechanisms (Figure 1.11). AA will be converted into 15HpETE by the action of 15-LOX in epithelial cells and translocated into neutrophils whereby the action of 5-LOX, LXA_4 hydrolase, and LXB_4 hydrolase, 15HpETE will be further converted into LXA_4 and LXB_4 [Samuelsson et al., 1987]. In the later pathway, the AA will be converted into LTA_4 in the leukocytes, and the same will be metabolized into lipoxins by the action of 12-LOX in the platelets. An important third pathway is also identified that involves aspirin-triggered COX-2 generating R-specific

15(R)-HpETE intermediate that will be transformed into a different class of epi-lipoxins by subsequent actions of LOXs [Spite and Serhan, 2010].

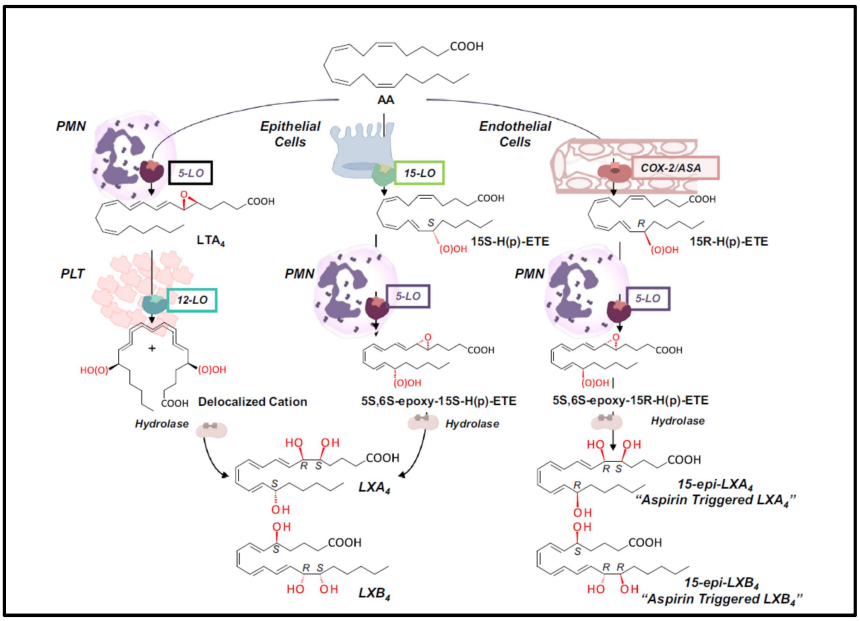

Figure 1.11: Cellular biosynthetic pathways of lipoxins and epi-lipoxins; Source: Romano M et. al, J Inflamm Res. 2015.

Lipoxins play a regulatory role in both innate and adaptive immune systems [Chandrasekharan and Sharma-Walia, 2015]. They modulate anti-inflammatory and pro-resolution properties via modulating the levels of pro-inflammatory genes, inhibiting PMN and eosinophil chemotaxis, superoxide anion generation, PMN vascular adhesion, transendothelial and transepithelial migration, stimulating monocyte chemotaxis, enhancing bacterial phagocytosis and reducing the release of inflammatory cytokines [Romano et al., 2015]. A large body of research in *in vitro* and *in vivo* models demonstrates their role in physiological and pathological conditions. For instance, the functions of lipoxinA_4, 15-epi-LipoxinA_4, and their related intermediates in different clinical and preclinical studies are summarized in Figure 1.12.

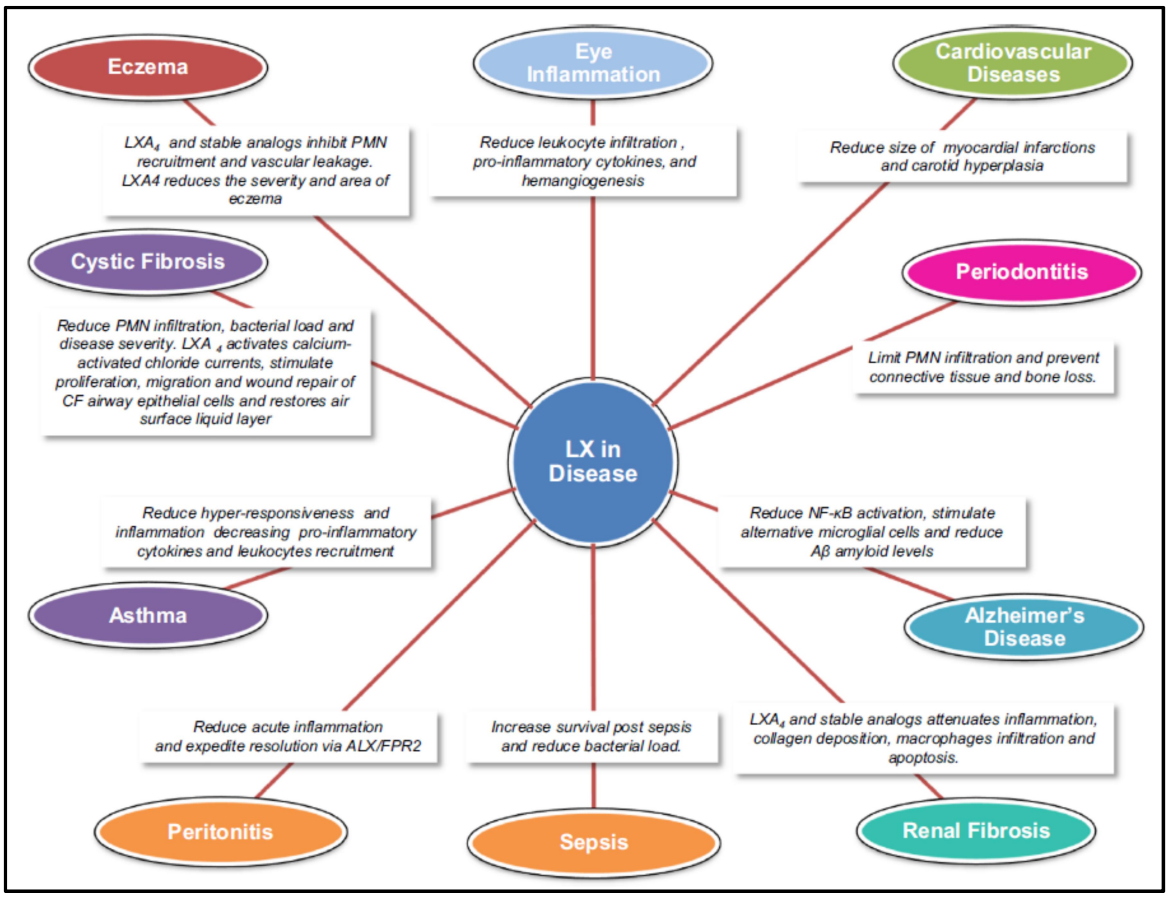

Figure 1.12: Main actions of LXA_4, 15-epi-LXA_4 and related analogs in preclinical and clinical models of the diseases; Source: Mario Romano et. al, J Inflamm Res. 2015.

1.5.2.1 Resolvins

Resolvins are generated from omega-3 fatty acids EPA and DHA. They are classified into E-series and D-series based on the parent substrates they use: i.e., D-series resolvins are metabolites of DHA, and E-series resolvins are metabolites of EPA [Buckley et al., 2014] (Figure 1.13). They were initially identified by spectrometry-based lipidomic analysis of the exudates harvested *in vivo* during the resolution phase of inflammation [Serhan et al., 2009]. The enzymes associated with the biosynthesis of resolvins are 15-LOX, 5-LOX, aspirin-treated COX-2, and cytochrome p450 [Serhan et al., 2002]. The synthesis of resolvins can happen via two different enzymatic pathways leading to the production of R-specific and S-specific lipid mediators. Human 15LOX catalyzes the first step in the synthesis of S-specific resolvins. Hydroperoxy moiety (with S-specificity) will be introduced at carbon positions 17 and 18 in DHA and EPA respectively [Serhan et al., 2002]. The intermediates generated then will be

oxidized by the action of 5-LOX to produce different S-type resolvins. Whereas, in the latter pathway, aspirin-treated COX-2 or cytochrome P450 catalyzes the initial step of introducing hydroperoxy moiety (with R-specificity) followed by 5-LOX catalysis to produce R-specific resolvins. A series of D-series (RvD1-5) and E-series (RvE1-4) have been identified and were elucidated for their role in different inflammatory and disease models [Chen et al., 2008].

Resolvins showed promising benefits in many diseases and infections such as *E. coli* peritonitis infection [Serhan et al., 2015b], colitis [Arita et al., 2005], acid-induced lung injury [Seki et al., 2010], acute lung injury, cecal ligation and puncture sepsis [Spite et al., 2009], and burn/wound injury [Bohr et al., 2013] by increasing survival and by shortened resolution . For example, administration of RvE1 in aspiration pneumonia model is associated with a reduction of pro-inflammatory cytokines, improved survival and increase bacterial clearance [Seki et al., 2010]. Similarly, administration of RvD2 is involved in decreased expression of pro-inflammatory mediators (PGE2, LTB_4, IL-17, and IL-10), intraphagosomal vacuolar ROS production, and enhanced bacterial phagocytosis in murine polymicrobial sepsis [Spite et al., 2009]. When applied to the wound sites, RvE1, RvD1, and RvD2 each reduce neutrophilic infiltration and stimulate reepithelialisation thereby decreasing the time required for wound closure [Greene et al., 2015]. The common mechanisms of resolvins include suppression of NF-κB gene activation, reduced pro-inflammatory mediator's expression, increased phagocytosis and enhanced efferocytosis.

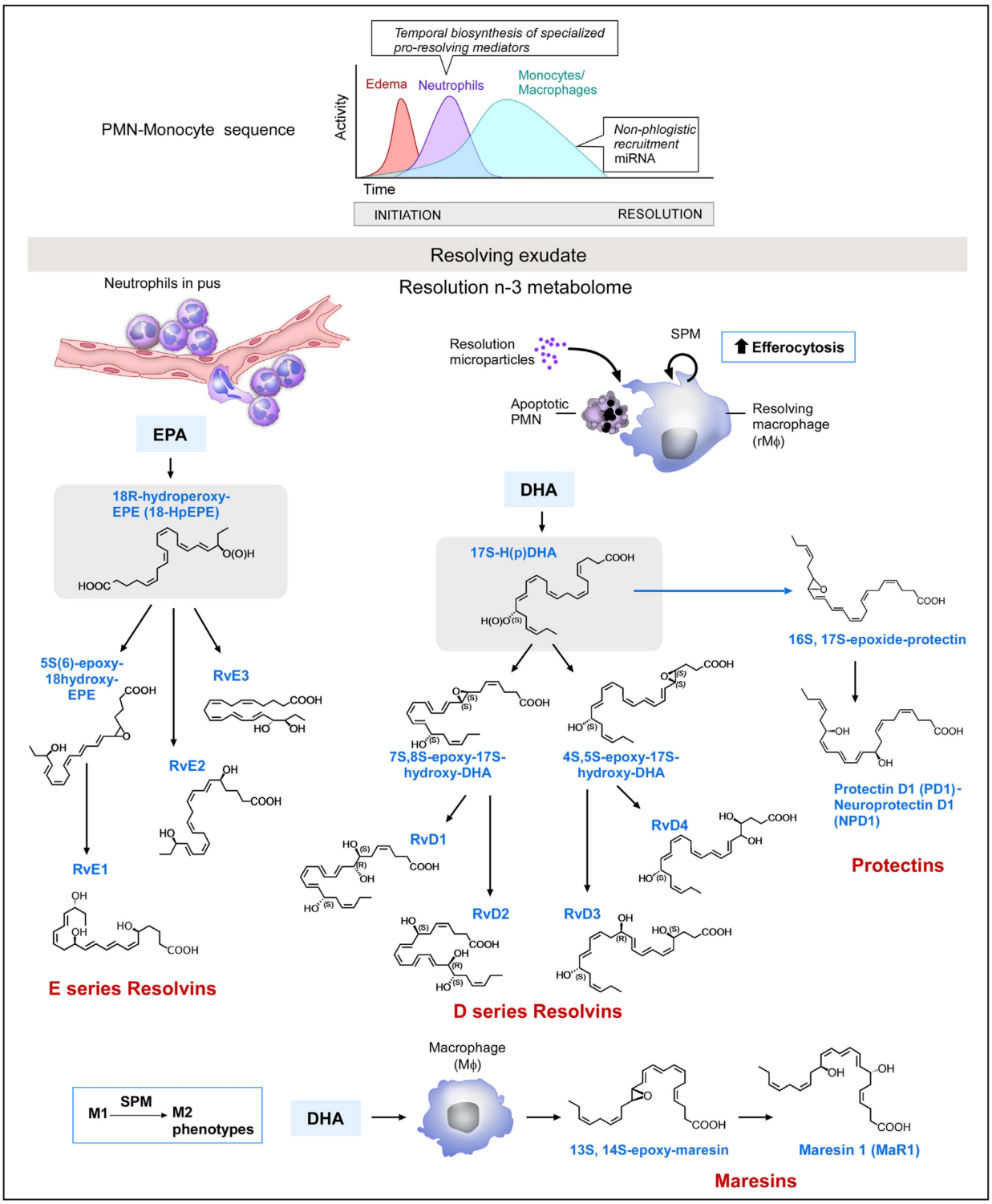

Figure 1.13: Outcome of an acute challenge (upper panel) and the resolution: omega-3 metabolome;
Source: C.N. Serhan et. al, Immunity 2014.

1.5.2.3 Protectins

DHA serves as a precursor for the synthesis of protectins. Similar to resolvins, 15LOX catalyzes the oxygenation at carbon 17 to produce 17S-hydroperoxy DHA intermediate that will be later converted into protectin D1 by enzymatic epoxidation and hydrolysis (Figure 1.13). Protectin D1 contains two hydroxyl groups at 10^{th} and 17^{th} carbons of DHA and synthesized by human mononuclear cells and Th2 $CD4^{+}$ T-cells [Hong et al., 2003]. Administration of PD1 in different experimental disease models increased survival and shortened resolution of inflammation. For instance, in an experimental model of asthma, mice prior to aerosol allergen challenge, administration of PD1 intravenously protected animal from the development of airway hyperresponsiveness and T-cell mediated inflammation. It also dampened the levels of pro-inflammatory mediators, Th2 inflammatory cytokines, and airway mucus [Levy et al., 2007]. The target cells for protectins are T cells and endothelial cells. PD1 mediated responses include decreased NF-κB and COX-2 expression, renal fibrosis, T-cell migration, TNF and IFN-γ release, and microglial cell cytokines and increased protection of retinal pigmented cells, neuroprotective actions, and the CCR5 expression on T-cells (Figure 1.10) [Buckley et al., 2014].

1.5.2.4 Maresins

Maresins are the lipid mediators generated in macrophages from the endogenous DHA. Human 12SLOX catalyzes the initial step of maresins biosynthesis. It oxygenates DHA at carbon-14 position to produce 14S-hydroperoxy DHA which will later be converted by an enzymatic catalysis to MaR1 (7R, 14S-dihydroxy-docosahexaenoic acid) and non-enzymatically to MaR2 (13R, 14S-dihydroxy-docosahexaenoic acid) (Figure 1.13) [Serhan et al., 2015a]. Enzymatic catalysis of MaR1 involves epoxidation and hydrolysis of 14S-hydroperoxy DHA for which the responsible enzymes are not yet characterized. Maresins appear in the resolution phase of the inflammation and show strong actions in regulating inflammation resolution, for tissue regeneration, and in resolving pain. Biosynthesis of MaR1 by macrophage involves an active 13S, 14S-epoxide-maresin intermediate that stimulates the phenotype switch from M1 macrophage to M2 macrophage that is associated with anti-inflammatory functions.

1.6 Reaction Mechanism of LOXs

The reaction mechanism of LOXs includes four consecutive steps; hydrogen abstraction, radical rearrangement, oxygen insertion, and radical rearrangement (Figure 1.14) [Coffa et al., 2005]. Hydrogen abstraction will be done at double allelic methylene group leading to the formation of a free radical. If we consider AA as a substrate, hydrogen abstraction can be done at three different diene positions, which are C7, C10, and C13 (Figure 1.14). In addition to position, as these diene carbon atoms also have pro-S and pro-R hydrogen, hydrogen abstraction can be done with six different possibilities (Figure 1.14). However, these enzymes are highly specific in the abstraction of particular hydrogen thereby controls the specificity of the reaction. It is believed that the position of hydrogen abstraction depends on the orientation of substrate in the active site of these enzymes. The free radical thus formed can be rearranged in either +2 or -2 directions leading to the possibility of generating six different intermediates for AA [Andreou and Feussner, 2009]. If hydrogen abstraction is done at the C7 position in AA, penta dienyl radical can take either C5 or C9 position; similarly, for C10 and C13, rearrangement can be at C12 & C8 and C15 & C11 positions respectively (Figure 1.14). In a non-enzymatic AA oxidation, considering the stereospecific selection of hydrogen and free radical rearrangement can result in 12 different HPETEs. However, most of the mammalian LOXs produce higher amounts of specific HPETEs and share of different HPETEs formed varies with localization of the enzyme, temperature, and pH [Walther et al., 2009]. In the final step, molecular dioxygen is inserted antarafacially, and the peroxy radical formed will be reduced by an electron from ferrous iron. In addition to dioxygenase reaction, LOXs also catalyze hydroperxidase and leukotriene synthase reactions.

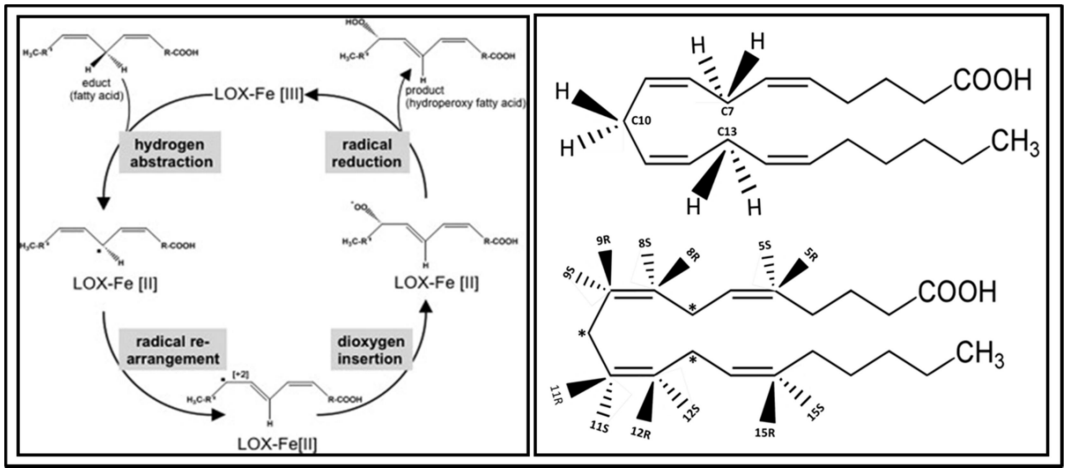

Figure 1.14: The lipoxygenases reaction mechanism explaining the four steps, available diene centers and hydrogens that can be abstracted during hydrogen abstraction step from arachidonic acid and all the possible metabolites that can be formed by non-enzymatic reaction of arachidonic acid oxygenation.

1.7 Reaction specificity of Lipoxygenases

1.7.1 Regiospecificity of LOXs

LOXs are extremely specific for their reaction mechanism and are capable of producing both regio and stereospecific derivatives from the fatty acids. Two existing hypotheses can explain the regiospecificity of LOXs; Space-based or volume based hypothesis and orientation based hypothesis (Figure 1.15) [Gardner, 1989] [Prigge et al., 1998] [Andreou and Feussner, 2009]. According to the volume based hypothesis, the volume of the binding site is the crucial determinant of the specificity of the enzyme [Borngräber et al., 1999]. Whereas in orientation based hypothesis, the way substrate enters into the binding site i.e. whether the methyl end or carboxylic end of the fatty acid enters first decides the specificity of the products [Prigge et al., 1998]. The logic behind both the hypotheses is that the steric conformation of the substrate in the binding site should favor hydrogen abstraction at a specific diene center in different LOXs. For example, in the case of h5LOX, the orientation of AA should be in such a way that C7 carbon should be close to catalytic iron compared to C10 and C13 carbons. Therefore, the amino acids that line the binding cavity of h5LOX play a decisive role in the movement and orientation of AA favoring the specific activity of the enzyme.

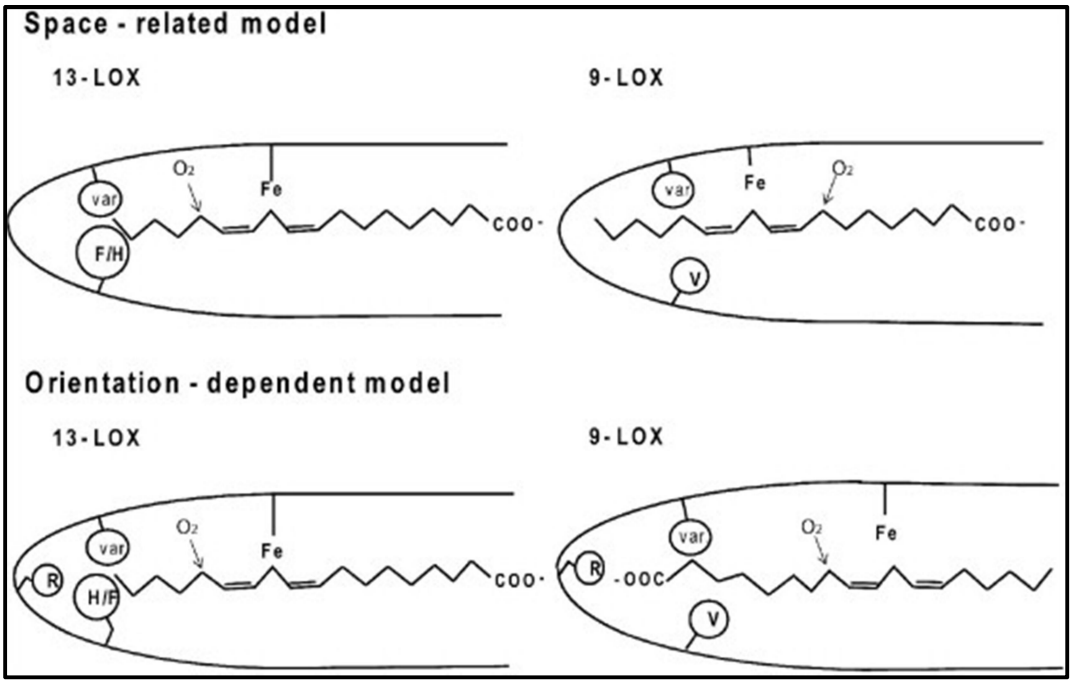

Figure 1.15: Comparison of existing space-related and orientation related models that explain the substrate alignment at the active site of lipoxygenases; Source: A. Andreou and I. Feussner, *Phytochemistry*, 2009.

1.7.1.1 Space-based Hypothesis

Molecular modeling studies and sequence comparison studies even before the crystal structures are solved led to the identification of crucial amino acids that are responsible for differences in the volume. The volume of the binding site varies in different LOXs; h5LOX has a ~20%, and h12SLOX has ~6% bigger binding site cavities compared to h15LOX-1 [Schwarz et al., 2001]. Sloane et al. and his colleagues had performed site-directed mutagenesis in h15LOX-1 and identified the amino acids Ile & Met at positions 418 & 419 as sequence determinants for the regiospecificity [Sloane et al., 1995]. Space filling amino acid exchanges of theses residues in h15LOX-1 resulted in mutants that exclusively metabolized AA to 12-HETE instead of 15-HETE. Later mutagenesis studies on the same/counter amino acids in r15LOX-1, p12SLOX, h12SLOX reconfirmed that the volume is very critical in defining the regiospecificity of LOXs. The explanation of this phenomenon is that, if the size of the volume pocket is more, the substrate can slide deeper into the pocket to choose an optimum favorable conformation. For example, the volume is more for h5LOX which results in AA to penetrate more making C7 carbon close to the iron. This hypothesis is based on the assumption that always methyl end of

the fatty acid slides first in the active site. Phe353, Ile418, Met419, and Ile593 are the positional determinants for h15LOX-1, and all these residues alter specificity due to alterations in the volume [Sloane et al., 1995, Borngräber et al., 1999]. Similarly, Phe359, Ala424, Asn425, and Ala603 are the sequence determinants for the h5LOX (Figure 1.16) [Schwarz et al., 2001] and replacing them with bigger amino acids reduced space at the bottom of substrate binding cavity (Figure 1.16) thus the product specificity [Hofheinz et al., 2013].

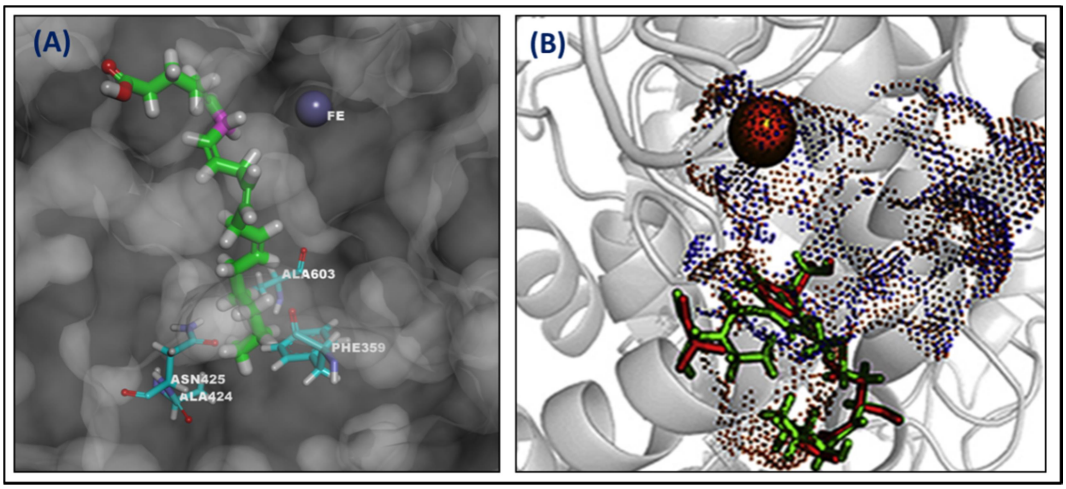

Figure 1.16: Active site volume of human 5-LOX correlated with 15-lipoxygenase activity. (A) Active site of human 5-LOX with substrate arachidonic acid showing sequence determinants and (B) overlay of active site volumes of human 5-LOX (orange dots) and its 15-lipoxygenating mutant (blue dots); Source: Hofheinz et. al, *Arch Biochem Biophy*, 2013.

1.7.1.2 Orientation-based Hypothesis

Orientation based hypothesis suggests that in h15LOX-1 the substrate AA enters the active site with its methyl end ahead leading to orientation favoring oxygenation at C15 carbon. In contrast, in h5LOX, the reverse head to the tail orientation of substrate i.e. carboxyl end enters first into the active site leading oxygenation at the C5 carbon of AA. This hypothesis was first explained in corn and soybean LOXs [Egmond et al., 1973]. However, the major concern of this hypothesis is the energy penalty associated with placing the polar carboxylate at the highly hydrophobic environment of the binding site [Prigge et al., 1998].

The unavailability of substrate bound mammalian LOX crystal structure is the reason behind the lack of unifying conclusion in understanding the rationale behind the regiospecificity

of the isozymes. Taken together, it can be concluded that both the hypotheses are appropriate in explaining the positional specificity. Targeted alterations of fatty acid may force the inverse head to tail orientation for oxygenation at C5 carbon by h15LOX isozyme, but it may not be the case in h5LOX. The h5LOX may prefer the head to the tail orientation of the fatty acid, and the volume may play a crucial role in determining the positional specificity. Conversion of the hydrophobic environment at the bottom of the substrate binding cavity to hydrophilic may alter the orientation of fatty acid. For instance, when V603 of h15LOX-2 is mutated to histidine, the V603H mutant metabolized AA to 8-HETE and the alterations in pH impaired with the specificity [Walther et al., 2009]. The reason behind this could be decrease in energy penalty associated with the reverse orientation of AA in the pocket. Together, the positional specificity of LOXs mainly depends on the volume of the pocket and the substrate structure rather than on the enzyme itself. Other physiological factors like pH [Gardner, 1989], temperature, and concentration of substrate [Kühn et al., 1990] are also shown to affect the specificity in LOXs. Different from the above studies, Gilbert and his associates demonstrated that phosphorylation mimicking mutant (Ser663Asp) of h5LOX changed oxygenation position of AA from five to fifteen [Gilbert et al., 2012]. However, the same effect was not seen when the corresponding serine in human, mice, and zebrafish were mutated to aspartic acid [Adel et al., 2014].

1.7.2 Stereo-Specificity of Lipoxygenases

In addition to positional specificity, LOXs also exhibit R and S stereochemistry. Epidermis-type h12RLOX is the enzyme that produces R specific products, unlike all other human LOXs where S chirality is preserved. There are very few studies that explain the mechanism by which mammalian LOXs control stereo-specificity. Coffa and his group showed that the single amino acids alanine and glycine which are highly conserved in all S and R LOXs respectively are the sequence determinants for stereo-specificity [Coffa and Brash, 2004]. They further validated them by converting alanine to glycine, and vice versa in mouse 8SLOX, h15LOX-2, h12RLOX and coral 8RLOX and all the mutants not only altered their stereospecificity but also showed a shift in regio-specificity [Coffa et al., 2005]. Interestingly, the carbon that is specific for hydrogen abstraction in all the wild types remained unaltered even in the mutants. The change in positional specificity can be reasoned to the movement of radical formed on specific carbon after the hydrogen abstraction. For example, in h12RLOX, hydrogen

abstraction takes at the C10 position leading to the formation of radical that moves in +2 direction resulting 12R-HETE. Whereas in h12RLOX Gly441Ala mutant, the radical displaced in both +2 and -2 directions resulting in 8- and 12-HETEs (1.4:1 ratio). The 8HETE methyl ester mainly contained S-enantiomer (91%) and 12HETE methyl ester contained R-enantiomer (92%) [Coffa and Brash, 2004].Based on their results, the authors hypothesized a model (Figure 1.17) to explain the mechanistic reasons behind the effects. According to their hypothesis, AA takes tail-first orientation, and L-hydrogen will be abstracted in 8RLOX & 12SLOX. Ala/Gly residue is in opposite direction of iron, and the substrate will be positioned in between catalytic iron and the residue. Due to the difference in the volumes, antarafacial oxygenation will be at the 8R position in case of glycine, and larger alanine prevents this resulting oxygenation deep in the binding pocket at 12S position (Figure 1.17). Similarly, in the case of 8SLOX and 12RLOX, antarafacial oxygenation occurs at a 12R position in the case of Gly and deeper in the active site at the 8S position for Ala with the difference being the D-hydrogen abstraction and head to the tail orientation of the substrate (Figure 1.17) [Coffa et al., 2005].

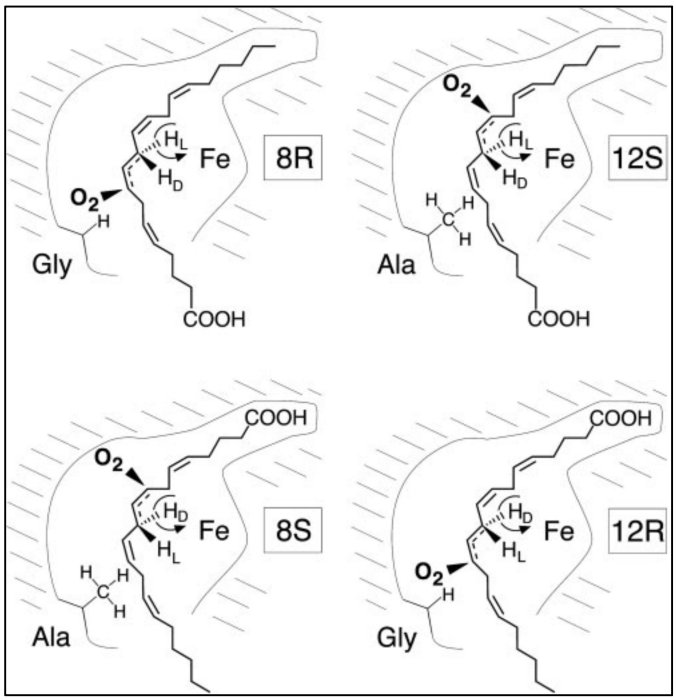

Figure 1.17: Schematic representation explaining the effect of alanine and glycine in 8S-, 8R-, 12S-, and 12R-Lipoxygenases for their stereospecificity; Source: G. Coffa et al, *Biochem Biophys Res Commun*, 2005.

1.8 Site-directed Mutagenesis studies on Lipoxygenases

Even before the crystal structures were available, scientists have worked towards the identification of necessary amino acids that are crucial for LOXs specificity. The mutational studies performed for understanding their functional importance can be broadly summarized as follows; Identification of iron-ligating residues, sequence determinants responsible for regio/stereo specificities, understand the function of N-terminal domain, to stabilize the structure for crystallization, for defining the active site residues, to elucidate the diffusion channel by which oxygen enters the active site, characterization of functional importance of SNPs identified in various diseases and etc.

Initial SDM experiments were performed to elucidate the significance of amino acids that are highly conserved in different LOXs. Zhang and his group have mutated six conserved histidines of h5LOX and showed that histidines at positions 367, 372, and 551 are very critical for enzyme activity as their mutations showed no enzyme activities [Zhang et al., 1992]. In a follow-up study, they quantified the iron content in wild type and mutants and concluded that they are involved in the binding of non-heme catalytic iron [Zhang et al., 1993]. Later SDM studies on LOXs were mainly focused on identifying the sequence determinants that are responsible for the specificity. First sequence determinant reported in mammalian LOXs was Met418 from h15LOX-1 which on exchange with valine resulted in the conversion of AA to both 12 and 15 HETEs in almost equal volumes In later experiments, the authors were successful in the conversion of h15LOX-1 specificity from 15-oxygenation to 12-oxygenation. The double mutant having Ile417Val and Met418Val mutations converted AA into 12 and 15HETE in the ratio of 20:1[Sloane et al., 1995]. The volume was shown to be critical for the reaction specificity and was further validated in other LOXs such as r15LOX, p12SLOX, and h12SLOX. As the binding site of h5LOX is bigger, additional mutations at Phe359 and A603 are performed in order to achieve 15-oxygenation [Schwarz et al., 2001]. However, epidermis type h15LOX-2 and h12RLOX didn't follow similar strategy indicating that volume concept is not applicable for all LOXs. Alanine that is conserved in S-type LOXs which is replaced with glycine in R-type LOXs was shown to be a critical determinant for stereospecificity [Coffa and Brash, 2004].

Qing-Fen and his group employed molecular modeling approach to generate enzyme-substrate complexes based on soybean lipoxygenase-1 structure to identify Arg402 as important substrate binding determinant [Gan et al., 1996]. They mutated Arg403 to leucine/lysine and then suggested that the carboxylic group of fatty acid forms ionic interaction with positively charged Arg403. After X-ray crystal structure of the rabbit reticulocyte was solved; additional mutagenesis studies were carried out in different mammalian LOXs. For instance, in the crystal structure, it has been observed that Arg403 forms an electrostatic interaction with Gln399 and Glu176 [Choi et al., 2008]. So, studies were done to check the importance of that interaction in terms of enzyme catalysis, secondary structure and tertiary structure [Di Venere et al., 2013]. In another study, a molecular dynamic simulation approach on X-ray coordinates was used to identify the diffusion channels by which oxygen enters the catalytic center [Saam et al., 2007]. The study was further validated my blocking one of the identified oxygen channels by introducing space filling amino acid (Leu367Phe) mutation.

Naturally occurring genetic polymorphisms in human LOXs have been associated with the frequency of various diseases by many epidemiological correlation studies. The functional consequences of these SNPs at the enzymatic level were evaluated for their importance by generating site-directed mutations. For instance, Thomas and his group in collaboration with us evaluated the non-synonymous genetic variations in h5LOX, h12SLOX, h15LOX, and h15LOX-2 reported in the human 1000 genome project for their functionality [Horn et al., 2013]. Most of these genetic variations are located on the enzyme surface and unaltered the enzyme functionality [Horn et al., 2013]. They suggested that accumulation of loss-of-function variations are prevented in the coding regions of these enzymes due to evolutionary pressure in the human population. In another study, genetic polymorphisms in h12RLOX and heLOX3 associated with autosomal recessive congenital ichthyosis (ARCI) are evaluated for their role in enzyme functionality by generating mutants. All the identified mutations *in vitro* showed impaired catalytic activity suggesting a causal relationship with the pathogenesis of the disease.

1.9 Molecular dynamics

Molecular dynamic simulation studies give valuable insights on the structural flexibility, conformational flexibility accessible and the interaction profile of the binding partners of the biological molecules [Hospital et al., 2015]. The existence of life is nothing but the interactions

between various biological molecules like protein-protein, protein-ligand/substrate, protein-solvent, nucleic acid-protein, and nucleic acid- ligand/substrate at the atomic level. Hence, understanding the mechanisms responsible for their action at an atomic level is essential. In recent years, molecular dynamic simulations combined with experimental validation have led in interpreting and pursuing innumerable biological aspects. This method calculates the time-dependent behavior of a molecular system. Most of the biological reactions happen in the range of nanoseconds (ns) to seconds. Unfortunately, with the available computational power, and developed methodologies it may be tough to address the complex phenomena that happen in second's time, such as protein folding, but it's not impossible. The molecular dynamic programs, algorithms, and computational expertise are evolving, and in near future simulation, calculations may increase from ns to seconds. At present, the molecular dynamic simulation calculations are being widely used to study the structural re-arrangements [Gebremichael et al., 2008], conformational changes, modifications in the chemical environment of catalytic domain upon ligand binding [Young et al., 2007], allosteric regulation [Vettoretti et al., 2016], and mutation-induced structural changes [Linder et al., 2015] of the proteins and nucleic acids. The X-ray crystal coordinates available in PDB are the primary requirement for performing molecular dynamic simulations. These structures provide immense understanding about the structural features of the biological molecules, but they only provide coordinates for a single conformation. In biological scenarios, flexibility and dynamics in proteins and nucleic acids are essential for their functionality. Molecular dynamics is an excellent tool to study those mechanisms. The basic rationale behind molecular dynamic simulation is, allowing the molecules of the system to interact with each other for a defined period of time and calculating potential functions integrating Newton's equations of motion (Figure 1.18). The trajectories and inter-atomic potentials of the atoms at each step are computed using molecular mechanics force fields. Force field consists parameters for both bonded terms (bond vibrations, angle vibrations, and torsion potentials) and non-bonded terms (van der Walls and electrostatic interactions) which will be used in calculating the total potential energy of the system [González, 2011]. In the present study, we exploited the advantages of molecular dynamic simulation methodologies to decode the mutation-induced structural and conformational changes in the protein structure and their effects on substrate binding.

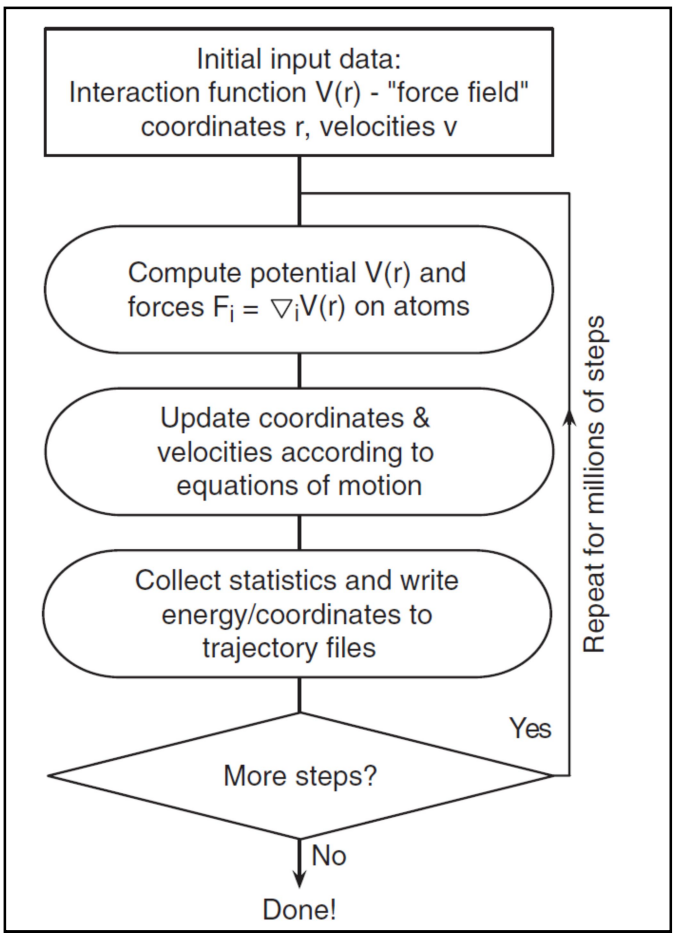

Figure 1.18: Simplified flowchart of a conventional molecular dynamics simulation; Source: Lindhah ER et al., *Methods Mol Biol.* 2008)

CHAPTER 2

RATIONALE OF THE STUDY

LOXs are one of the enzyme classes of AA pathway involved in the biosynthesis of eicosanoids responsible for various cellular functions and pathological implications. There are six different functional LOXs in humans and are classified based on their stereo/regio specific insertion of oxygen on polyunsaturated fatty acids. These enzymes share a common mode of action and will metabolize same substrate with only difference being the position of oxygen insertion. This leads to the production of different classes of lipid mediators that work differently based on milieu. Leukotrienes, the classical pro-inflammatory mediators along with prostaglandins, are generated via h5LOX pathway. The enzyme catalyzes the first two steps of leukotriene biosynthesis i.e. conversion of AA to 5S-HpETE and later to LTA_4 [Peters-Golden M et al., 2003]. LTA_4 is later converted into either into LTB_4 or LTC_4 LTD_4, and LTE_4 by enzymes LTA_4 hydrolase, LTC_4 synthase, gamma-glutamyl transpeptidase and dipeptidases [Joshi and Praticò, 2014]. Lipoxins and resolvins which show anti-inflammatory properties are synthesized by h15LOX pathway. In cancer, h5LOX and h12SLOX are proved to stimulate angiogenesis and tumor growth, whereas h15LOX-2 has an anti-tumorigenic role. Unlike other LOXs, h15LOX-1 can have both pro-tumorigenic and anti- tumorigenic activity [Hyde and Missailidis, 2009]. In addition to inflammation and cancer, LOX-derived metabolites also play a role in allergic diseases, GERD (gastroesophageal reflux disease) and asthma [Hay et al., 1995]. Furthermore, genetic variations in human LOXs have been attributed for increased risk in many diseases by different epidemiological correlation studies. For instance, heterozygous allele carriers of Thr560Met SNP in ALOX15 gene have significantly higher risk for coronary artery disease [Assimes et al., 2008]. Another SNP (Gln261Arg) in ALOX12 was correlated for the pathogenesis of breast cancer [Prasad et al., 2011], osteoporosis [Harsløf et al., 2011], and schizophrenia [Kim et al., 2010]. Similarly, ALOX5 SNP (Glu254Lys) was associated with higher risk in bronchial asthma [Bai et al., 2008]. Genetic polymorphisms in h12RLOX and heLOX3 are associated with rare skin dysfunction known as autosomal recessive congenital ichthyosis [Eckl et al., 2009]

Hence, understanding the mechanisms by which LOXs metabolize AA has been an active area of research from past 70 years from their discovery. As reviewed LOXs have divergent roles in oxygenation of PUFAs, in terms of positional and stereospecificity of oxygen insertion. These divergent metabolites have diametrically opposite functions, from pro-inflammatory to anti-inflammatory. Several of site-directed mutagenesis studies have identified critical amino acids regulating the positional/stereo specificity of LOXs and thus their functions. It would be interesting to identify various naturally occurring mutations in the

populations and how they affect the catalytic efficiencies in terms of substrate and regiospecificities. In this direction the present study was taken up on various LOXs to identify key amino acids involved in the alterations in the structure and functions.

In different LOXs, the active sites have modifications to effect specific reactions; hence, it is imperative to identify critical conserved/non-conserved amino acids at the active sites of these LOXs. The share of different eicosanoids exist in the milieu will define the progression or resolution of a disease. As LOXs metabolize the common substrate AA, it will be interesting to see how these enzymes control their reaction specificity. In order to understand this, in the present study, we employed molecular modeling approaches to distinguish structural and functional similarities/diversities and to identify the key amino acid determinants at the active sites of LOXs. Further, we evaluated the effects of the identified amino acids on the LOX reaction by performing mutational exchanges and by analyzing the product profile of the mutants by chromatography. The mutants that showed significant differences with wild types were further subjected to molecular dynamic simulation analysis to check the structural and conformational changes induced due to mutational exchanges.

Objectives of the study:

- *In Silico* identification of critical amino acids at the binding sites of lipoxygenases that are responsible for either functionality or specificity

- *In vitro* evaluation of identified amino acids by site-directed mutagenesis and by analyzing the alterations in enzyme catalysis due to mutational exchanges

- To understand the molecular mechanisms involved for observed transformations by the mutants compared to wild-type enzymes: Molecular dynamic simulation analysis

CHAPTER 3

MATERIALS & METHODS

3.1 Materials

3.1.1 Chemicals and Commercial Kits

All the chemicals and kits used in the present study with their manufacturers/providers details are summarized in Table 3.1.

Chemicals	Manufacturer/Provider
Acetic Acid	SDFCL Fine-chem Limited, India
Acrylamide	Sigma-Aldrich, Co. St. Louis, USA
Adenosine 5′-triphosphate (ATP)	Sigma-Aldrich, Co. St. Louis, USA
Ammonium Persulfate	Sigma-Aldrich, Co. St. Louis, USA
Ampicillin	Sigma-Aldrich, Co. St. Louis, USA
Arachidonic Acid	Nu-Chek Prep, Inc. MN, USA
Bisacrylamide	Sigma-Aldrich, Co. St. Louis, USA
BL21-(DE3)plyss Cells	Invitrogen Life Technologies, USA
Calcium chloride ($CaCl_2$)	Sigma-Aldrich, Co. St. Louis, USA
Coomassie Brilliant Blue	Sigma-Aldrich, Co. St. Louis, USA
Dipalmitoyl Phosphatidylcholine	Sigma-Aldrich, Co. St. Louis, USA
Ethanol	Changshu Yangyuan Chemical, China
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Co. St. Louis, USA
Glycine	Sigma-Aldrich, Co. St. Louis, USA
HRP conjugated anti-goat IgG secondary antibody	Santa Cruz Biotechnology, USA

HRP conjugated anti-HIS antibody	Santa Cruz Biotechnology, USA
HRP conjugated anti-rabbit IgG secondary antibody	Santa Cruz Biotechnology, USA
Human 12R-Lipoxygenase Antibody	Santa Cruz Biotechnology, USA
Human 15-Lipoxygenase Antibody	Santa Cruz Biotechnology, USA.
Human 5-Lipoxygenase Antibody	Santa Cruz Biotechnology, USA.
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich, Co. St. Louis, USA
Kanamycin	Sigma-Aldrich, Co. St. Louis, USA
Linoleic Acid	Nu-Chek Prep, Inc. MN, USA
Luria Bertani Agar	Hi-Media Laboratories Pvt Ltd., India
Luria Bertani Broth	Hi-Media Laboratories Pvt Ltd., India
Lysozyme	Sigma-Aldrich, Co. St. Louis, USA
Methanol	Standard Reagents Pvt. Ltd., India
Arachidonic Acid Methyl Ester	Nu-Chek Prep, Inc. MN, USA
Na_2HPO_4	Hi-Media Laboratories Pvt Ltd., India
NaH_2PO_4	Hi-Media Laboratories Pvt Ltd., India
Ni Sepharose 6 Fast Flow	GE Healthcare Life Sciences, USA
Nitrocellulose Membrane	Merck Millipore, India
Phosphate-buffered Saline (PBS)	Sigma-Aldrich, Co. St. Louis, USA
Qiagen Miniprep Plasmid Isolation Kit	QIAGEN India Pvt. Ltd., - India

Quik Change Site-directed Mutagenesis Kit	Agilent Technologies, India
Sodium Chloride (NaCl)	Hi-Media Laboratories Pvt Ltd., India
Sodium Dodecyl Sulfate (SDS)	Roche Diagnostics, Germany
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Co. St. Louis, USA
Tris-Base	Merck Specialities Pvt. Ltd., India
Tween 20	Sigma-Aldrich, Co. St. Louis, USA
West Pico Chemiluminescent Substrate Kit	Thermo Scientific, RockFord, USA.
XL-1 Blue Cells	Invitrogen Life Technologies, USA
α-Linolenic Acid	Nu-Chek Prep, Inc. MN, USA

Table 3.1: The chemicals and kits used for the present study and their manufacturers/providers

3.1.2 Primers

The forward and reverse primers used for the generation of mutants in h15-, h12S-, h12R- and h15-LOXs are listed in Table 3.2.

S. No	Mutation	Primer Sequence
1	Human 15-Lipoxygenase Glu356Gln	**Forward Primer:** 5'- TTCCAGCTCCATCAGCTGCAGTCTCAT-3' **Reverse Primer:** 5'-ATGAGACTGCAGCTGATGGAGCTGGAA-3'
2	Human 12S-Lipoxygenase Glu356Gln	**Forward Primer:** 5'-TTCCAACTGCACCAGATCCAGTATCAC-3' **Reverse Primer:** 5'-GTGATACTGGATCTGGTGCAGTTGGAA-3'
3	Human 15-Lipoxygenase Gly364Thr	**Forward Primer:** 5'-CATCTTCTGAGGACACACTTGATGGCT-3' **Reverse Primer:** 5'-AGCCATCAAGTGTGTCCTCAGAAGATG-3'
4	Human 12S-Lipoxygenase	**Forward Primer:**

	Thr364Gly	5’-CACTTGCTGAACGGTCACCTGGTGGCT-3’ **Reverse Primer:** 5’-AGCCACCAGGTGACCGTTCAGCAAGTG-3’
5	Human 15-Lipoxygenase Phe414Ser	**Forward Primer:** 5’- GACATGGGAATTTCCGACCAGATAATG-3’ **Reverse Primer:** 5’- CATTATCTGGTCGGAAATTCCCATGTC-3’
6	Human 12S-Lipoxygenase Phe414Ser	**Forward Primer:** 5’-GATGGAGGAATTTCTGATAAGGCAGTG-3’ **Reverse Primer:** 5’-CACTGCCTTATCAGAAATTCCTCCATC-3’
7	Human 5-Lipoxygenase Phe421Ser	**Forward Primer:** 5’-GAGTGTGGCCTCTCTGACAAGGCCAAC-3’ **Reverse Primer:** 5’-GTTGGCCTTGTCAGAGAGGCCACACTC-3’
8	Human 12R-Lipoxygenase Ser451Phe	**Forward Primer:** 5’-GAGGGGGGGCTCTTTGCCAAGGGCATG-3’ **Reverse Primer:** 5’-CATGCCCTTGGCAAAGAGCCCCCCCTC-3’
9	Human 15-Lipoxygenase Ala557Phe	**Forward Primer:** 5’-TGGGTGCCTAATTTTCCCTGCACGATG-3’ **Reverse Primer:** 5’-CATCGTGCAGGGAAAATTAGGCACCCA-3’
10	Human 12S-Lipoxygenase Ala557Phe	**Forward Primer:** 5’-TGGGTCCCTAATTTTCCATGCACAATG-3’ **Reverse Primer:** 5’-CATTGTGCATGGAAAATTAGGGACCCA-3’
11	Human 5-Lipoxygenase Ala567Phe	**Forward Primer:** 5’-TGGATCCCCAATTTTCCCCCAACCATG-3’ **Reverse Primer:** 5’-CATGGTTGGGGGAAAATTGGGGATCCA-3’
12	Human 12R-Lipoxygenase Phe595Ala	**Forward Primer:** 5’-TGGATGCCCAACGCCCCAGCGTCCATG-3’ **Reverse Primer:** 5’-CATGGACGCTGGGGCGTTGGGCATCCA-3’
13	Human 15-Lipoxygenase Gly597Ser	**Forward Primer:** 5’-ACTTGGCAGCTGAGCAGACGCCAGCCC-3’ **Reverse Primer:**

		5’-GGGCTGGCGTCTGCTCAGCTGCCAAGT-3’
14	Human 12S-Lipoxygenase Ser598Gly	**Forward Primer:** 5’-TCATGGCATCTGGGTCGCCGCCAGCCA-3’ **Reverse Primer:** 5’-TGGCTGGCGGCGACCCAGATGCCATGA-3’
15	Human 15-Lipoxygenase Val661Ile	**Forward Primer:** 5’- GTGGAAAACAGTATTGCCATCTAAAAG-3’ **Reverse Primer:** 5’-CTTTTAGATGGCAATACTGTTTTCCAC-3’
16	Human 12S-Lipoxygenase Val661Ile	**Forward Primer:** 5’-ATAGAGAACAGTATTACCATCTGAAAG-3’ **Reverse Primer:** 5’-CTTTCAGATGGTAATACTGTTCTCTAT-3’

Table 3.2: Forward and reverse primers used for the generation of mutants in h15-, h12S-, h12R- and h15-LOXs

3.2 Methods

3.2.1 Molecular Modeling Approach for Identification of Crucial Amino Acids

3.2.1.1 Identification of Active Site Amino Acids

None of the solved crystal structures of human LOXs have been co-crystalized with the substrate, AA. Hence, in this study, we have developed a 15-LOX-1/AA co-crystal structure by employing constraint docking methodology exploiting known structural information. Site-directed mutagenesis studies and activity assays on 15-LOX-1 showed that charged side chain of arginine 403 forms an ionic interaction with ionized carboxylate [Schwarz et al., 1998]. Similarly, activity assays with different fatty acids having diene centers at different positions showed that hydrogen abstraction by the iron would be done at different positions in different LOX reactions [Andreou and Feussner, 2009]. In 15-LOX-1, the hydrogen abstraction will be done at the C13 position, which means C13 carbon atom should be in close proximity to iron. Hence, keeping this as two distance constraints, we performed molecular docking using GOLD (Genetic Optimization of Ligand Docking) software, a docking program based on genetic algorithm [Verdonk et al., 2003]. Before docking, AA was drawn using chemsketch software, converted to mol2 file and later to GJF file. The Gaussian software was used to optimize the AA structure by DFT-based method

using 6/31G basis set. The optimized structure of AA was then used for further docking calculations. Crystal coordinates of rabbit reticulocyte 15-LOX-1 (PDB Id: 1LOX) were taken from protein data bank (http://www.rcsb.org/pdb/home/home.do). During docking, the default algorithm speed was selected. The number of poses for each inhibitor was set to 100, and early termination was allowed if the top three bound conformations of a ligand were within 1.5 Å RMSD. Input parameters of the GOLD were set to allow octahedral coordination geometry to iron. The best and the most energetically favorable conformation of AA was selected, and the amino acids that are in 10 Å radius around AA were taken as active site residues.

3.2.1.2 Sequence Analysis

Protein sequences of human 5-LOX (UniProtKB/Swiss-Prot: P09917.2), human 12S-LOX (UniProtKB/Swiss-Prot: P18054.4), human 12R-LOX (UniProtKB/Swiss-Prot: O75342.1), and human 15-LOX-1 (UniProtKB/Swiss-Prot: P16050.3) were taken from NCBI protein database in FASTA format. Multiple sequence alignment was done using the ClustalW2 program by employing default parameters. Later, the critical amino acids are identified by manually observing the differences and similarities in the alignment file for the active site residues.

3.2.1.3 Comparative Analysis of Binding Sites Using MultiBind Software

MultiBind reveals the common physicochemical patterns (receptor-based pharmacophore) that may be responsible for the binding of a small molecule in a set of binding sites [Shulman-Peleg et al., 2008]. Molecular surface of the binding site is calculated and determined by the solvent accessible surface points that are located on the surface of the binding partner. The program performs multiple alignments of binding sites and recognizes the structurally conserved physicochemical and geometrical patterns that may be responsible for the binding. MultiBind develops receptor-based pharmacophore model for the protein of interest. Each amino acid in a binding site is represented by points in 3D space termed pseudo centers as described by Schmitt *et al.*, 2002. Each pseudo center represents one of the following physicochemical properties essential for protein–ligand interactions: hydrophobic, aliphatic (ALI), aromatic interactions (PII), hydrogen bond donors (DON), hydrogen bond acceptors (ACC), and mixed donor/acceptors (DAC). The algorithm finds a set of transformations which will superimpose the binding sites in a manner that will maximize the physicochemical score of the matched properties. Each pattern that was recognized on the

surface of other proteins was scored using the physicochemical scoring function of MultiBind. MultiBind score explains the similarities in the binding sites of the proteins compared. A high score implies that the physicochemical properties of the proteins are quite similar. Before submitting the enzyme-AA complexes generated by docking methodologies, the four-stage protocol was set up for energy minimizations of the complexes. Minimization at each stage was performed using 100 steps of steepest descent and 1500 steps of conjugate gradient algorithms for minimization. In the first stage, water molecules were minimized keeping the substrate and protein atoms fixed. This will relieve any bad contacts involving water molecules in the initially solvated system. In the second stage, the hydrogen atoms of the whole system were allowed to relax. This step relaxes the hydrogen atoms prior to relaxing heavy atoms. In the third stage, all the atoms of the inhibitor and the solvent are allowed to move during optimization. This stage establishes the preferred interactions. In the fourth and final stage, all the protein atoms within 15 Å from the center of the inhibitor (water molecules, protein atoms, and the ligand) were allowed to relax. In this study, binding site comparison was made by taking two enzymes at a time (5-LOX & 12S-LOX, 5-LOX & 15-LOX-1, and 15-LOX-1 & 12S-LOX) and also by taking all enzymes (5-LOX, 12S-LOX, and 15-LOX-1) together.

3.2.2 Generation of Mutants by Site-Directed Mutagenesis

Site-directed mutagenesis (SDM) was performed for the identified amino acids in human 5-, 12S-, 12R-, and 15-LOXs using the Quik Change Site-Directed Mutagenesis Kit following manufacturer's protocol. The standard PCR was performed by adding primers specific for mutation, PfuTurbo DNA polymerase, dNTPs, and reaction buffer (Figure 3.1) followed by Dpn1 digestion for cleaving parent plasmid. The cycling parameters used for performing PCR are shown in Figure 3.2. The plasmids were then transformed into XL-1 blue competent cells and cultured on LB agar plates overnight after transformation. Three colonies were picked, cultured in LB broth overnight at 37 ^{o}C. The plasmid DNA was isolated from the isolated clones, and LOX insert was completely sequenced for confirmation of the mutation. The plasmid with the desired mutation was then used for expression and purification of the enzyme. The typical reaction mixture and PCR conditions used for generating mutation are summarized below.

5 µl of 10× reaction buffer
1 µl (10 ng) of LOX plasmid
1.25 µl (125 ng) of oligonucleotide forward primer
1.25 µl (125 ng) of oligonucleotide reverse primer
1 µl of dNTP mix
Double distilled water to a final volume of 50 µl
Then add
1 µl of PfuTurbo DNA polymerase (2.5 U/µl)

Figure 3.1: The volumes and concentration of ingredients used in the PCR reaction mixture

Cycling Parameters for the QuikChange Site-Directed Mutagenesis Method

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12–18	95°C	30 seconds
		55°C	1 minute
		68°C	2 minutes/kb of plasmid length

Figure 3.2: The cycling parameters used for PCR

3.2.3 Lipoxygenase Enzymes Expression and Purification

3.2.3.1 Expression of Lipoxygenases in *E. coli* Bacterial System

5-LOX gene cloned in pRSETA (generously given by Prof. Kuhn), 12S-LOX and 15-LOX-1 genes cloned in pQE9 (generously given by Prof. Kuhn) and 12R-LOX cloned in Pet28b (generated in house) [Nairen Thesis] were taken and checked for inserts by running agarose gel electrophoresis after digesting the plasmids at 37 ^{o}C for 1 hour with insert specific restriction enzymes. The plasmids were then transformed into BL21 (DE3) Plyss cells. Approximately 10 ng of plasmid DNA was taken and incubated with BL21 (DE3) Plyss cells on ice for 30 min followed by heat shock at 42 ^{o}C for 45 sec and incubation in ice for 2 minutes. 1 ml of LB medium was added to the cells and cultured in orbital shaker for 1 hour at 37 ^{o}C and 180 RPM. The cells were then centrifuged at 5000 rpm for 5 min, resuspended in 100 ul of LB medium and cultured on plasmid specific (Ampicillin resistance for 5-, 12S- and 15-LOX; Kanamycin resistance for 12R-LOX) LB agar plate for overnight in 37 ^{o}C incubator. A single colony was taken and inoculated in 10 ml LB media and cultured for

overnight at 37 ^{o}C at 180 RPM. 100 ml of fresh LB media was inoculated with 1ml of overnight culture and allowed the cells to grow till OD reaches 0.6. Induction was done using IPTG (1 mM final concentration). The expression level of protein was checked initially by performing SDS page and later with Western blotting by using enzyme-specific antibodies. For SDS page, an equal concentration of protein samples from uninduced and induced cultures were taken and mixed with appropriate amount of loading buffer. The protein samples were then heated for 10 min at 99 ^{o}C before running on 10% SDS page. The gel was then stained either by coomassie or silver staining by following standard procedures for detection of protein bands. The same procedures were followed for mutant enzymes of 5-, 12S-, 12R-, and 15-LOXs as well.

3.2.3.2 Enzyme purification by affinity chromatography

For enzyme purification, 1 liter liquid culture (LB medium containing appropriate antibiotic) was inoculated with BL21 expression cells transformed with an LOX-containing plasmid that are grown overnight at 37 °C. The culture was allowed to grow till the OD reaches 0.5 to 0.6, and the expression of the recombinant protein was induced by addition of 1 mM IPTG (final concentration) and the culture was kept either at 30 ^{o}C for 4 hrs or at 18 ^{o}C overnight at 200 RPM. Then, bacteria were spun down, washed and resuspended in PBS. Later, cells were lysed by incubating cells with lysozyme for 30 min in ice and sonication for 10 min. Cell debris was removed by centrifugation, and the lysis supernatant was used for further purification. As all the LOXs are cloned as His-tag proteins at the N-terminal, we used metal ion immobilized affinity chromatography using imidazole as eluting agent. Lysis buffer was added to a 10 ml column containing 0.4 ml Ni-NTA beads. The column was then washed with 10 ml of washing buffer (50 mM phosphate buffer with 300 mM sodium chloride) containing 20 mM imidazole to remove loosely bound proteins. Then tightly bound proteins were eluted from the column with elution buffer (50 mM phosphate buffer with 300 mM sodium chloride) containing 300 mM imidazole. Five fractions were collected, and the majority of LOX-containing fraction 2 was stored at -80 ^{o}C till used for activity assays.

3.2.4 Activity Assays for Lipoxygenases

LOX enzyme fractions were incubated for 10 min at room temperature with 0.1 mM AA except for 12R-LOX where arachidonic acid methyl ester was used. In case of 5-LOX, the reaction was carried out in the presence of 0.4 mM $CaCl_2$, 1.4 µg/ml dipalmitoyl phosphatidylcholine, 0.1 mM EDTA and 0.1 mM ATP (final reaction volume of 0.5 ml). The

hydroperoxy compounds then formed were reduced with sodium borohydride to the corresponding hydroxy derivatives, the mixture was acidified to pH 3 by adding acetic acid, and 0.5 ml of ice-cold methanol was added for precipitating the protein. Aliquots of the clear supernatant after centrifugation for 10 min at 14000 rpm and at 8 ^{o}C were then injected to RP-HPLC.

3.2.5 Quantification Lipoxygenases Generated Products by RP-HPLC

HPLC analysis – HPLC of the LOX products was performed on a Shimadzu HPLC equipped with a Hewlett-Packard diode array detector 1040 A by recording the absorbance at 235 nm. Reverse phase-HPLC was carried out on a C18 Gravity column with a solvent system of methanol/water/acetic acid (85/15/0.05, by volume) at a flow rate of 1 ml/min. The 15-HETE standard was also injected during each evaluation of mutant to identify the products based on their retention time. Absorbance was checked at 235 nm as the hydroxy derivatives have highest absorbance spectra at this wavelength. Individual peak areas in each chromatogram were taken for further comparison analysis between wild-type and mutant enzymes. The Individual peaks collected were later subjected to gas chromatography-mass spectrometry (GC-MS) for the confirmation of the HETE structures (data not shown).

3.2.6 Western Blotting

To normalize the LOX content, immunoblotting was carried out. 10µl of protein samples of both wild-type and mutants were mixed with SDS sample buffer and ran on SDS-PAGE. After electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane, and the blot was developed by enzyme-specific antibodies except for 12S-LOX where we used conjugated anti-His tag antibody. For this purpose, the membrane was blocked by using 5 % milk solution followed by three washes with PBS containing tween20 for 15min each. Later, the membrane was incubated with primary antibody for 1 hour followed by three washes 15 min each. Respective secondary bodies were used to quantify the intensity of the immunoreactive band at 75 kDa densitometrically, and expression of the wild-type enzyme was set to 100%.

3.2.7 Molecular Dynamics Simulations of Lipoxygenases

Molecular dynamics is a widely used computational approach for studying the conformational changes and fluctuations in the proteins over a period of simulation. In this

study, Desmond software integrated into Maestro for graphical user interface advantage was used for performing molecular dynamics simulations. Each simulation was performed for a period of 10 ns. Crystal coordinates of rabbit 15-LOX (PDB ID 2P0M), human 5-LOX (PDB ID 3O8Y) and pig 12S-LOX (PDB ID 3RDE) were taken from protein data bank. OPLS_2005 force field parameters were used, and mutants were generated by *in silico* mutations. Before performing simulation, the protein was solvated in TIP3P water model in the cubic box to mimic the biological environment. As iron has no proper force field parameters, we restrained the movement of iron and protein atoms within 5 A^{o} of the iron. Default parameters available in Desmond were used, and the relaxation protocol available was selected for the simulation. Conformational changes and fluctuations due to mutation were analyzed in wild-type and mutants by comparing RMSF, Hydrogen bonds, and RMSD during the calculated 10 ns simulation. In the case of r15LOX/AA complex, the same methodology was followed except that the simulation calculation was only for 5 ns. The analysis included the comparison analysis of the binding patterns of AA throughout the calculation in both wild-type and mutants.

CHAPTER 4

RESULTS & DISCUSSION

4.1 Identification of Binding Site Amino Acids by Sequence Analysis and Constraint Docking

4.1.1 Sequence Analysis

Sequences of human LOXs considered in the present study were taken from the UniProt database. Sequence analysis was performed using the clustalW2 program to check the sequence similarity between h5-LOX, h12S-LOX, h15-LOX-1, and h12R-LOX. As we can see in **Table 4.1**, h12S-LOX and h15-LOX-1 share highest sequence similarity with an alignment score of 65.51 indicating that these two enzymes are very similar compared to other LOXs. Except for the above mentioned LOXs, all LOXs have a sequence alignment score close to 40 with each other indicating less sequence similarity. Sequence analysis helped us to identify the amino acids that are conserved in all LOXs and also amino acids that differ in different LOXs compared to other LOXs. However, it is difficult based on these results to conclude important amino acids that are critical for the enzyme catalysis. This is because, even though various studies based on site-directed mutagenesis and analyzing the metabolites generated using different fatty acids having diene centers at different positions [Newcomer and Brash, 2015a] suggested the importance of some of the amino acids, yet critical active site amino acids are unknown [Newcomer and Brash, 2015b]. As these enzymes contain iron at the catalytic center, it's straight forward to define the cavity where the substrate binds. The iron-containing cavity shares atoms with more than 40 amino acids making it difficult to pick amino acids for evaluation for their importance. In addition to this, none of the available LOXs crystal structures are co-crystalized with fatty acid substrates making it more difficult to identify active site amino acids. Hence, in the present study, we exploited the advantage of constraint docking methodology to identify the active site amino acids.

	Human 5-LOX	Human 12S-LOX	Human 12R-LOX	Human 15-LOX-1
Human-5LOX	100	41.99	41.31	40.09
Human 12SLOX		100	37.92	65.51
Human 12RLOX			100	35.7
Human 15SLOX				100

Table 4.1: Pairwise alignment score between human LOXs

4.1.2 Molecular Docking

Molecular docking has become a very important tool for understanding the binding behavior of substrates and inhibitors in the active site of the enzymes. It fits the ligand in a proper orientation that is more favorable for efficient binding and deciphers the crucial interactions that are responsible for the binding. Constraint docking is a module available in this tool where the user can restrain a particular interaction such as distance or hydrogen bond between ligand and protein atoms. It is known that hydrogen abstraction will happen at different diene centers in different lipoxygenases [Kuhn et al., 2005]. Gan QF and his colleagues showed that side chain of Arg403 forms an ionic interaction in h15LOX-1 with the carboxyl group of fatty acid [Gan et al., 1996]. In h5LOX, the hydrogen abstraction will be done from C7 carbon which means C7 carbon atom should be in close proximity to iron [Kuhn et al., 2005]. The respective amino acid of Arg403 of h15LOX-1 is Arg411 in h5LOX. By considering the above two important interactions, we performed constraint docking in h5LOX taking arachidonic acid as the substrate. The docked complex (Figure 4.1) was then taken for further analysis. In order to evaluate important amino acids, we have considered all the amino acids that are within 10 A^{o} range from the docked AA in the h5LOX as active site amino acids. These identified amino acids are further analyzed for their differences and similarities in comparison with different LOXs. The partial sequence alignment file can be seen in Figure 4.2. Even though LOXs share sequence similarity close to 40 percent in most of the cases, highest sequence similarity was observed for identified binding site amino acids. In addition to this sequence alignment method, we also used structural alignment methods before selecting amino acids for further evaluation for catalytic efficiency and reaction specificities by in vitro experiments.

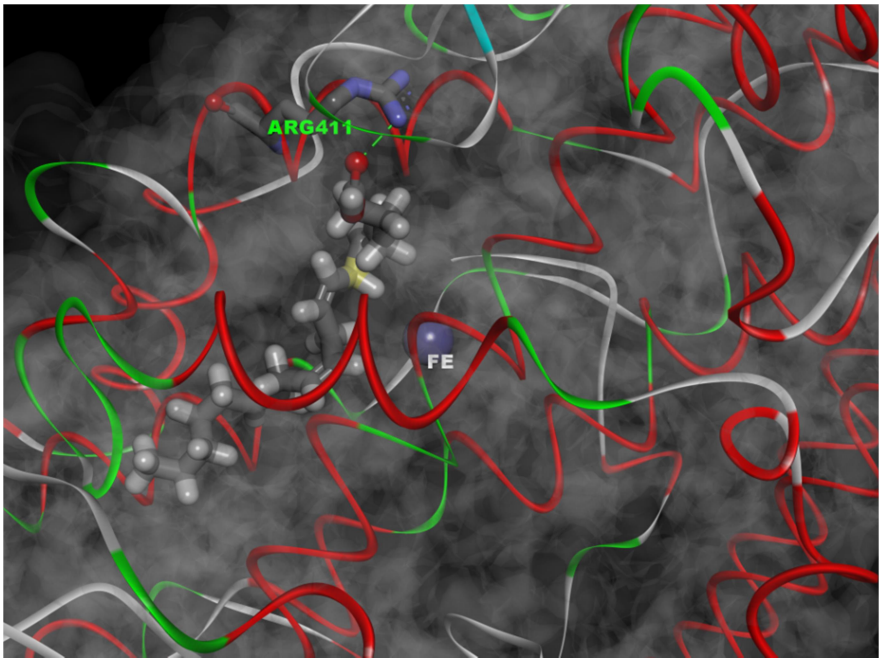

Figure 4.1: Docked complex of 5-LOX showing arachidonic acid, Arg411 and FE in the binding site

```
12R-LOX       MMPIAIQLS--QTPGPDCPIFLPSDSEWDWLLAKTWVRYAEFYSHEAIAHLLETHLIAEA 408
5-LOX         IVPIAIQLN--QIPGDENPIFLPSDAKYDWLLAKIWVRSSDFHVHQTITHLLRTHLVSEV 377
12S-LOX       LQPMVIQIQPPSPSSPTPTLFLPSDPPLAWLLAKSWVRNSDFQLHEIQYHLLNTHLVAEV 369
15-LOX        LLPMVIQLQLPRTGSPPPPLFLPTDPPMAWLLAKCWVRSSDFQLHELQSHLLRGHLMAEV 369

12R-LOX       FCLALLRNLPMCHPLYKLLIPHTRYTVQINSIGRAVLLNEGGLSAKGMSLGVEGFAGVMV 468
5-LOX         FGIAMYRQLPAVHPIFKLLVAHVRFTIAINTKAREQLICECGLFDKANATGGGGHVQMVQ 437
12S-LOX       IAVATMRCLPGLHPIFKFLIPHIRYTMEINTRARTQLISDGGIFDKAVSTGGGGHVQLLR 429
15-LOX        IVVATMRCLPSIHPIFKLIIPHLRYTLEINVRARTGLVSDMGIFDQIMSTGGGGHVQLLK 429

12R-LOX       AVEGDPELQSWVQEIFKECLLGRESSGFPRCLRTVPELIRYVTIVIYTCSAKHAAVNTGQ 585
5-LOX         VVEEDPELQDFVNDVYVYGMRGRKSSGFPKSVKSREQLSEYLTVVIFTASAQHAAVNFGQ 557
12S-LOX       IVKGDPELQAWCREITEVGLCQAQDRGFPVSFQSQSQLCHFLTMCVFTCTAQHAAINQGQ 546
15-LOX        AVKDDPELQTWCREITEIGLQGAQDRGFPVSLQARDQVCHFVTMCIFTCTGQHASVHLGQ 546

12R-LOX       MEFTAWMPNFPASMRNPPIQTKGLTTLETFMDTLPDVKTTCITLLVLWTLSREPDDRRPL 645
5-LOX         YDWCSWIPNAPPTMRAPPPTAKGVVTIEQIVDTLPDRGRSCWHLGAVWALSQFQENELFL 617
12S-LOX       LDWYAWVPNAPCTMRMPPPTTKEDVTMATVMGSLPDVRQACLQMAISWHLSRRQPDMVPL 606
15-LOX        LDWYSWVPNAPCTMRLPPPTTK-DATLETVMATLPNFHQASLQMSITWQLGRRQPVMVAV 605

12R-LOX       GHFPDIHFVEEAPRRSIEAFRQRLNQISHDIRQRNKCLPIPYYYLDPVLIENSISI 701
5-LOX         GMYPEEHFIEKPVKEAMARFRKNLEAIVSVIAERNKKKQLPYYYLSPDRIPNSVAI 673
12S-LOX       GHHKEKYFSGPKPKAVLNQFRTDLEKLEKEITARNEQLDWPYEYLKPSCIENSVTI 662
15-LOX        GQHEEEYFSGPEPKAVLKKFREELAALDKEIEIRNAKLDMPYEYLRPSVVENSVAI 661
```

Figure 4.2: Partial Sequence alignment of h5, h12S, h12R, and h15LOXs; Amino acids highlighted in cyan indicate the identified active-site amino acids

4.1.3 Exploration of Binding Site Patterns in Lipoxygenases

4.1.3.1 Pairwise comparison of LOXs

Receptor-based pharmacophore models were generated for h5LOX, p12SLOX, and r15LOX-1 to elucidate important amino acids in the binding sites. We couldn't include h12RLOX in this study as this enzyme doesn't have a crystal structure. Initially, pairwise comparison was done between h5LOX & p12SLOX, h5LOX & r15LOX-1, and p12SLOX & r15LOX-1. Pairwise alignment helps in the elucidation of important amino acid similarities between each enzyme with the other enzymes.

4.1.3.1.1 Human 5-Lipoxygenase and Rabbit 15-Lipoxygenase

Comparison of h5LOX and h15LOX-1 resulted in the identification of 14 common physicochemical properties (Table 4.2) with an alignment score of 41.92. Higher alignment score implies higher similarity between the binding sites of compared proteins. Observed common physicochemical properties are seven ALI, four PII, one DAC, and two ACC. Hydrophobic nature of fatty acid could be reasoned for the hydrophobic microenvironment in the binding sites of LOXs. List of all the common interactions identified is summarized in Table 4.2. Out of 14 properties identified 11 represent properties that form hydrophobic interactions. The amino acid Phe present at the bottom of the binding site at position 359 and 353 in h5LOX and h15LOX-1 respectively showed common PII interaction. This Phe has been shown to be a positional determinant in both of these LOXs [Borngräber et al., 1996]. Mutation of Phe 359 with space filling Trp in h5LOX resulted in the production of more 15-HETE compared to the wild-type [Schwarz et al., 2001]. Glu357 of h15LOX-1 and Glu363 of h5LOX showed common hydrogen bond acceptor property. These amino acids are close to catalytic iron center with differed charged side chains i.e. negative charge for Glu and neutral charge for Gln; hence, we have selected this amino acid for further evaluation by *in vitro* experiments. Interestingly, only h5LOX contains Glu at this position where as h15LOX-1, h12SLOX, and h12RLOX contains Gln.

r15LOX		h5LOX		Property Type
Res.ID	Res. Name	Res.ID	Res. Name	
353	Phe	359	Phe	PII
357	Glu	363	Gln	ACC
357	Glu	363	Gln	ACC
361	His	367	His	DAC
361	His	367	His	PII
362	Leu	368	Leu	ALI
366	His	372	His	PII
404	Ala	410	Ala	ALI
405	Arg	411	Arg	ALI
408	Leu	414	Leu	ALI
409	Val	415	Ile	ALI
415	Phe	421	Phe	PII
593	Ile	603	Ala	ALI
594	Val	604	Val	ALI

Table 4.2: Common physico/chemical features observed by MultiBind in r15LOX, and h5LOX; aliphatic interactions (ALI), aromatic interactions (PII), hydrogen bond acceptors (ACC), and mixed donor/acceptors (DAC)

Iron coordinating residues His361 of h15LOX-1 and His367 of h5LOX showed two common properties that include DAC and PII interactions. His372 and His366 which are also one of the iron co-coordinating residues in h5LOX and h15LOX-1 showed one common PII interaction. These histidines are highly conserved in all LOXs that are involved in iron binding, and any mutation completely inactivates the enzyme stability and function [Zhang et al., 1992]. Leu at positions 362 and 368 in h15LOX-1 and h5-LOX respectively showed common ALI interaction. As this Leu is conserved in all LOXs, we didn't consider this amino acid for further evaluation. Ala at positions 410 and 404 in h5LOX and h15LOX-1 that showed common ALI interaction is the stereo determinant of LOXs that is highly conserved in all S-type LOXs that is replaced by Gly in R-type LOXs [Coffa and Brash, 2004]. Conversion of Ala to Gly at this position in four different LOXs resulted in a change of stereo-specificity of the products. Side chains of Arg404 and Arg411 of r15LOX-1 and h5LOX contributed for common ALI interaction. In h15LOX-1, this Arginine i.e. Arg403 was shown to form ionic interactions with a carboxylic group of AA [Schwarz et al., 1998]. Other common ALI interactions were contributed

by Leu408 & Leu414, Val409 & Ile415, Ile593 & Ala603 and Val594 & Val604 of r15LOX-1 & h5LOX respectively. Leu at positions 408 and 414 in r15LOX-1 and h5LOX is conserved in all LOXs. Even though corresponding amino acids of Val 409 and Val594 of r15LOX-1 are different in other LOXs, due to the limitation of this study we did not perform any mutational exchanges of these amino acids to check their importance. Ala603 is at the bottom of the active site in h5LOX which is replaced by space filling amino acids in other LOXs. The exchange Ala603Ile along with other positional determinant exchanges Phe359Trp, Alsa424Ile, and Asn425Met resulted in complete conversion of m5LOX to anti-inflammatory m15LOX [Hofheinz et al., 2013]. Similarly in h12RLOX where the corresponding amino acid of Ala603 i.e. Val 631 when replaced with less space filling amino acids Gly and Ala resulted a shift in the positional specificity [Meruvu et al., 2005]. The mutants produced more of 11-HETE instead of 12-HETE. Common PII interaction was observed due to the alignment of Phe at positions 415 & 421 of r15LOX-1 and h5LOX. Phe is conserved in all human S-type LOXs whereas h12RLOX contains Ser at this position. So, we have selected this amino acid for further evaluation as this exchange results in a change of polarity and size of the binding site.

4.1.3.1.2 Rabbit 15-Lipoxygenase and Human 12S-Lipoxygenase

Pairwise comparison of r15LOX-1 and p12SLOX resulted in the identification of 23 common physico/chemical properties. The properties included11 ALI, 2 ACC, 5 PII,1 DAC, and 1 DON interactions (Table 4.3). Similar to higher sequence similarity score, these two enzymes showed highest structural alignment score of 58.3 compared to other pairwise alignments. Common amino acids of r15LOX-1 and p12SLOX that contributed for physico/chemical interactions are Phe353, GlU357, His361, Leu362, His366, Ile400, Arg403, Leu 408, Phe415, Ile593, Gln 596, Leu59, and Ile673. Amino acids Ile418, Met419, and Val594 of r15LOX-1 and Val418, Val419, and Thr594 of p12SLOX which are different in both the enzymes also shared common ALI interactions. Ile418 and Met419 of r15LOX-1 and the respective amino acids Ala418 and Val419 of p12SLOX are the sequence determinants for the positional specificity [Borngräber et al., 1996]. Conversion of these amino acids with space altering amino acids resulted in the shift of product specificity not only in r15LOX-1 and p12SLOX but also in r15LOX-1 and h12SLOX. The other different amino acid, Val594 of r15LOX-1, corresponds to Thr593 in h15LOX-1 is different in all human LOXs (Val, Ser, and Leu in 5-, 12S- and 12R-

LOXs). Due to their dissimilarity, it is a good idea to evaluate the effect of this amino acid exchanges in all LOXs, however, due to the limitation of the present study, we couldn't perform this mutation. As most of the amino acids are similar in both the LOXs and the amino acids that are different have been already evaluated, we haven't considered any amino acids for *in vitro* studies based on this comparison.

r15LOX-1		**p12SLOX**		**Property Type**
Res.ID	Res. Name	Res.ID	Res. Name	
353	Phe	353	Phe	PII
357	Glu	357	Glu	ACC
357	Glu	357	Glu	ACC
357	Glu	357	Glu	ACC
361	His	361	His	DAC
361	His	361	His	PII
362	Leu	362	Leu	ALI
366	His	366	His	PII
400	Ile	400	Ile	ALI
403	Arg	403	Arg	PII
403	Arg	403	Arg	ACC
403	Arg	403	Arg	ALI
404	Ala	404	Ala	ALI
405	Arg	405	Arg	ALI
408	Leu	408	Leu	ALI
415	Phe	415	Phe	PII
418	Ile	418	Val	ALI
419	Met	419	Val	ALI
593	Ile	593	Ile	ALI
594	Val	594	Thr	ALI
596	Gln	596	Gln	DON
597	Leu	597	Leu	ALI
663	Ile	663	Ile	ACC

Table 4.3: Common physico/chemical features observed by MultiBind in r15LOX, and p12SLOX; aliphatic interactions (ALI), aromatic interactions (PII), hydrogen bond acceptors (ACC), and mixed donor/acceptors (DAC)

4.1.3.1.3 Human 5-Lipoxygenase and Pig 12S-Lipoxygenase

A pairwise comparison study of p12SLOX and h5LOX led to the identification of 13 common physicochemical properties. The properties observed included six ALI, four PII, two ACC, and one DAC (Table 4.4). The amino acids that contributed for properties in p12SLOX include Phe353, Glu357, His 361, Leu362, His366, Ala404, Arg405, Leu408, Phe415, Ile 593, and Thr594. The corresponding amino acids of h5LOX that shared properties with p12SLOX are Phe359, Gln363, His367, Leu368, His372, Ala410, Arg411, Leu414, Phe421, Ala603, and Val604. The amino acids Phe359 and Ala603 of h5LOX have been previously evaluated for their importance and are shown to be sequence determinants that control the specific orientation of AA for 5-oxygenation [Vogel et al., 2010]. Amino acid Gln363 of h5LOX shared two hydrogen

p12SLOX		**h5LOX**		**Property Type**
Res. ID	Amino Acid	Res. ID	Amino Acid	
353	Phe	359	Phe	PII
357	Glu	363	Gln	ACC
357	Glu	363	Gln	ACC
361	His	367	His	DAC
361	His	367	His	PII
362	Leu	368	Leu	ALI
366	His	372	His	PII
404	Ala	410	Ala	ALI
405	Arg	411	Arg	ALI
408	Leu	414	Leu	ALI
415	Phe	421	Phe	PII
593	Ile	603	Ala	ALI
594	Thr	604	Val	ALI

Table 4.4: Common physico/chemical features observed by MultiBind in p12SLOX, and h5LOX; aliphatic interactions (ALI), aromatic interactions (PII), hydrogen bond acceptors (ACC), and mixed donor/acceptors (DAC)

bond acceptor properties withGlu357 of p12SLOX similar to r15LOX. As glutamine and glutamic acid differ in charge and this amino acid is at the core of the substrate binding cavity, we have considered this amino acid for *In Vitro* evaluation. Rest of the amino acids that showed

common properties except Thr594 of p12SLOX and Val 604 of h5LOX are similar in both the LOXs. Based on this comparison, we have only selected one amino acid (Gln363 of h5LOX) for further studies.

4.1.3.1 Multiple Alignment of all three Lipoxygenases

Multiple alignments of all the three LOXs binding sites indicated a common pattern of eight physiochemical properties, namely one DAC, four ALI interactions, two ACC and one PII contacts. Table 4.5 lists all the identified common physicochemical properties that are similar for each of the enzymes. Among the amino acids responsible for the common property, two leucines and one histidine (iron-coordinating ligand) are conserved in all LOXs, and amino acids that are different at positions 593, 593 and 603 of r15-, p12S-, and h5S-LOXs respectively are the sequence determinants. The amino acids that are different and yet shared two common hydrogen acceptors are Glu357, Glu357, and Gln363 of r15-, p12S-, and h5S-LOXs respectively. These amino acids have already been considered for the present *in vitro* evaluation based on previous pairwise alignments of LOXs. Other different amino acids Val409 (r15LOX), Val409 (p12SLOX), and Ile415 (h5LOX) were not included in the present study.

r15LOX		**p12SLOX**		**h5LOX**		**Property**
Res. ID	Amino Acid	Res. ID	Amino Acid	Res. ID	Amino Acid	**Type**
357	Glu	357	Glu	363	Gln	ACC
357	Glu	357	Glu	363	Gln	ACC
361	His	361	His	367	His	DAC
361	His	361	His	367	His	PII
362	Leu	362	Leu	368	Leu	ALI
408	Leu	408	Leu	414	Leu	ALI
409	Val	409	Val	415	Ile	ALI
593	Ile	593	Ile	603	Ala	ALI

Table 4.5: Common physico/chemical features observed by MultiBind in r15LOX, p12SLOX, and h5LOX; aliphatic interactions (ALI), aromatic interactions (PII), hydrogen bond acceptors (ACC), and mixed donor/acceptors (DAC)

The pairwise comparison indicated that 15- & 12S-LOX share high structural similarity followed by 15- & 5-LOX and 5- &12S-LOX. The majority of amino acids that line the substrate binding cavity are hydrophobic in nature with only a few exceptions. As there is a non-heme iron bound octahedrally, the histidines that are involved in this binding contributed for common hydrogen bond properties. Other amino acids that shared common hydrogen bonds properties are glutamine/glutamic acid and arginine which is believed to be involved in ionic interaction with a carboxylic group of fatty acid. Rest of the common features observed at the active sites are ALI and PII interactions which are hydrophobic interactions. This can be reasoned to the hydrophobic nature of the substrates and can be said that the most important interactions that the substrate form with protein is hydrophobic.

The receptor-based pharmacophore models generated for the binding sites and sequence analysis of LOXs led to the identification of amino acids that may play an important role either in specificity or in enzyme catalysis. Figure 4.3 summarizes the amino acids that were evaluated for their effects in different LOXs in this study. The rationale followed for selection of amino acids for evaluation was the difference in terms of properties of the amino acids identified and the location of those amino acids at the binding site.

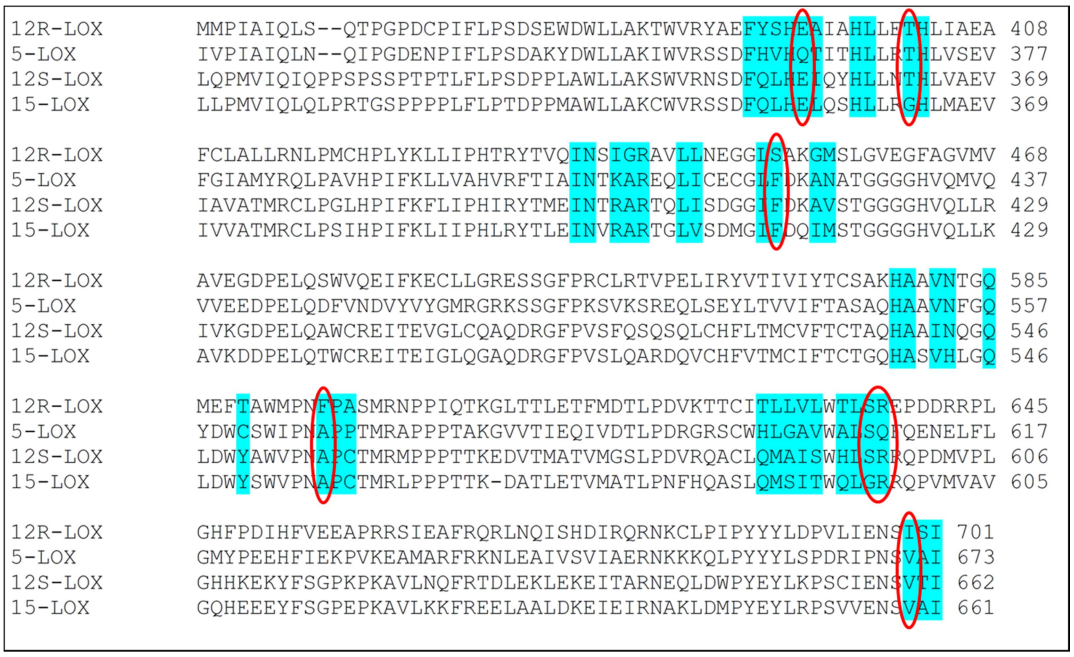

Figure 4.3: Partial Sequence alignment of 5-, 12S-, 12R- and 15-LOXs; Amino acids highlighted in cyan indicate the identified active-site amino acids and the regions circled in red are selected for *in vitro* experiments

A total of 16 mutants were generated, and their effects on the functionality of the enzymes were evaluated by investigating the reaction products from enzyme reactions. The enzymes h5LOX, h12SLOX, h15LOX-1, and h12RLOX were considered for amino acid exchanges, and the modifications include a change in volume, polarity, hydrophobicity, and charge.

4.2 Functional Characterization of Crucial Amino Acids in Human 5-, 12S-, 12R-, and 15-Lipoxygenases

Site-directed mutagenesis experiments were performed on highly conserved amino acids at the binding sites of different LOXs to elucidate their importance in terms of specificity of the reaction. Most of these studies are mainly focused on identifying the sequence determinants that are responsible for the positional and stereo specificity [Schwarz et al., 2001; Sloane et al., 1995; Coffa and Brash, 2004]. For instance, the first sequence determinant was reported for h15LOX-1 where conversion of methionine at position 418 to smaller valine resulted in dual positional specificity [Sloane et al., 1995]. Later experiments by site-directed mutagenesis led to identification of a series of sequence determinants that can change one type of LOX to another type LOX upon volume based modifications. However, most of these studies are focused on the bottom of the active site but not on the amino acids located at the catalytic center. Hence, in the present study, we tried to characterize the important amino acids at the catalytic center of the LOXs for their probable role in enzyme catalysis and positional specificity.

The important amino acids identified by molecular modeling approaches were further evaluated for their importance by generating mutants. Site-directed mutagenesis approach was used to insert amino acid exchanges mutations, and mutation induced effects were evaluated by analyzing the product profile of the mutants and by comparing them with wild-type enzymes. The *E. coli* bacterial system was used for the expression and purification of all the LOX wild-types and their mutants. The mutations inserted were confirmed by sequencing before proceeding to enzyme purification and activity assays. For comparison purpose, we calculated the relative enzyme activity of the mutants in comparison with the wild-type enzymes. To calculate this, equal volumes of wild-type and mutant protein containing elutions were incubated with substrate arachidonic acid and allowed the reaction to proceed for 10 mins. The generated HETEs were then quantified based on the peak areas of the HPLC chromatogram, and the normalized LOX protein expression was estimated basing on the intensity of immunoreactive band on western blot. The specific activity of wild-type is taken as 100 %, and the ratio of a number of HETEs formed to normalized protein expression of the mutant in reference to wild-type is considered as a relative specific activity of the mutant. The shares of different HETEs formed were estimated by taking the ratio of individual peak areas to the total peak areas contributing for HETEs.

4.2.1 Purification and Activity Assays of Human 5-, 12S-, 12R- and 15-Lipoxygenases

4.2.1.1 Human 15-Lipoxygenase Wild-Type

The ALOX15 gene cloned in the pQE9 vector was used for purification, expression, and site-directed mutagenesis. Before inserting mutations in the ALOX15 gene, we standardized the h15LOX protein expression and purification conditions. To achieve this, initially, we transformed the ALOX15 containing pQE9 plasmid into BL21 (DE3) plyss expression cells and induced the protein expression by adding IPTG. However, leaky expression observed in overnight grown XL-1 cells was higher than regular BL21 (DE3) plyss cells based protein expression system. So, throughout this study, we used XL-1 cells for expressing h15LOX wild-type and its mutants. The h15LOX present in the cell lysate was purified as a His-tag fusion protein on an Ni-NTA column using imidazole as a competitive binder (Figure 4.4). Further, western blotting was used to confirm the h15LOX expression in the lysate by using h15LOX antibody (Figure 4.5).

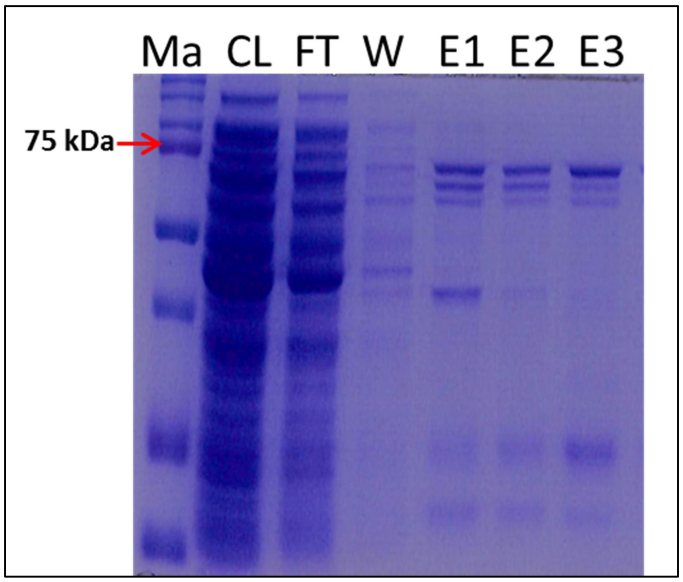

Figure 4.4: SDS-PAGE analysis of various fractions during purification of human 15-lipoxygenase wild-type; MA (Marker), CL (Cell lysate), FT (Flow through), W (Wash), E1 (Elution 1), E2 (Elution 2), and E3 (Elution 3).

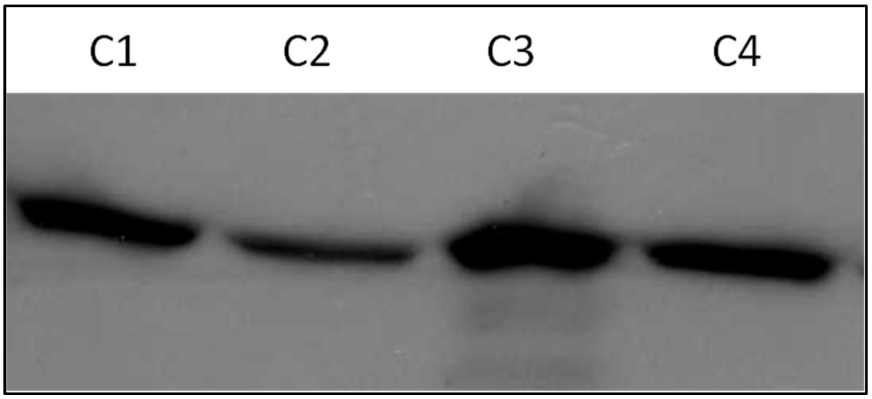

Figure 4.5: Western blot analysis of human 15-lipoxygenase wild-type; Equal volumes of the cell lysates from four cultures grown by taking different colonies were separated through SDS-PAGE and subjected to western blot analysis using 15LOX specific antibody; C1 (Clone-1), C2 (Clone-2), C3 (Clone-3), and C4 (Clone-4).

The same protocol was employed for expression and purification of all the h15LOX mutants generated in this study. The enzyme activity of the purified h15LOX was then checked by using AA as the substrate and injecting generated metabolites to the C18 column on an HPLC. As shown in Figure 4.6 the majority of the metabolite formed was 15-HETE with trace amounts of 12-HETE with retention times of approximately 9.5 min and 10.5 minutes respectively.

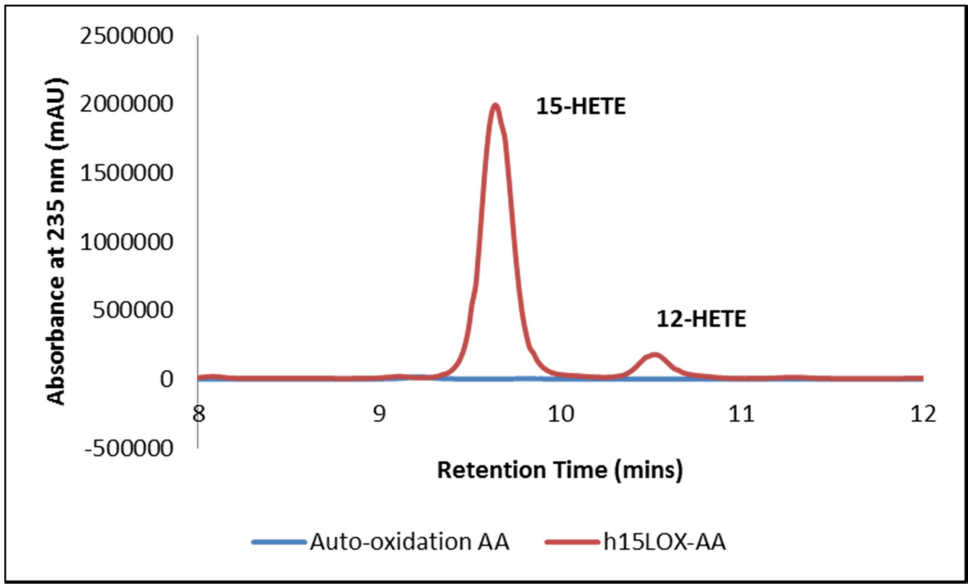

Figure 4.6: RP-HPLC analysis of the products formed by h15LOX wild-type and auto-oxidized products of arachidonic acid.

4.2.1.2 Human 12S-Lipoxygenase Wild-Type

The h12SLOX gene (ALOX12) cloned in the pQE9 vector was used for the expression and purification of the enzyme. Similar to h15LOX, h12SLOX also showed higher expression in overnight cultured XL-1 blue cells than regular BL21 (DE3) plyss cells based protein expression system. Hence, we used XL-1 cells for expression of h12SLOX as recombinant His-tag protein (Figure 4.7). Western blotting was done with HRP-conjugated anti-his antibody to confirm the expression of the enzyme in expressed cells (Figure 4.8). The enzyme was then purified by affinity chromatography by eluting the protein using different concentrations of imidazole. The HETEs generated after incubating the purified enzyme with arachidonic acid were extracted and analyzed by HPLC. The major product formed was 12HETE with a retention time of approximately 9 minutes (Figure 4.9).

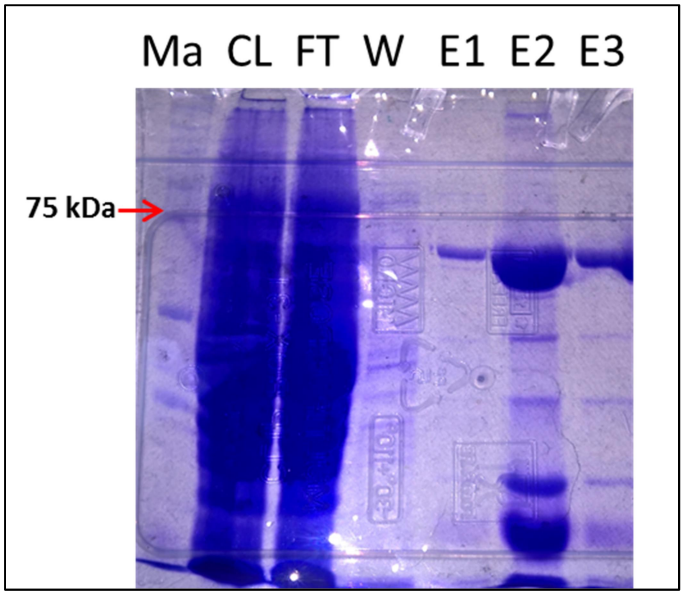

Figure 4.7: SDS-PAGE analysis of various fractions during purification of human 12S-lipoxygenase wild-type; MA (Marker), CL (Cell lysate), FT (Flow through), W (Wash), E1 (Elution 1), E2 (Elution 2), and E3 (Elution 3).

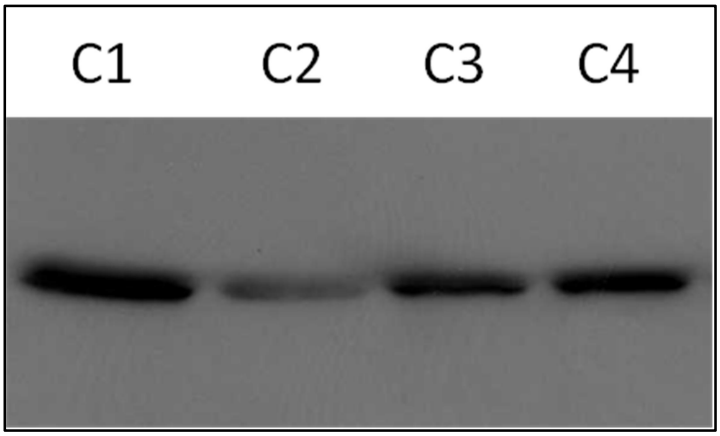

Figure 4.8: Western blot analysis of human 12S-lipoxygenase wild-type; Equal volumes of the cell lysates from four cultures grown by taking different colonies were separated through SDS-PAGE and subjected to western blot analysis using HRP-conjugated anti-His antibody; C1 (Clone-1), C2 (Clone-2), C3 (Clone-3), and C4 (Clone-4).

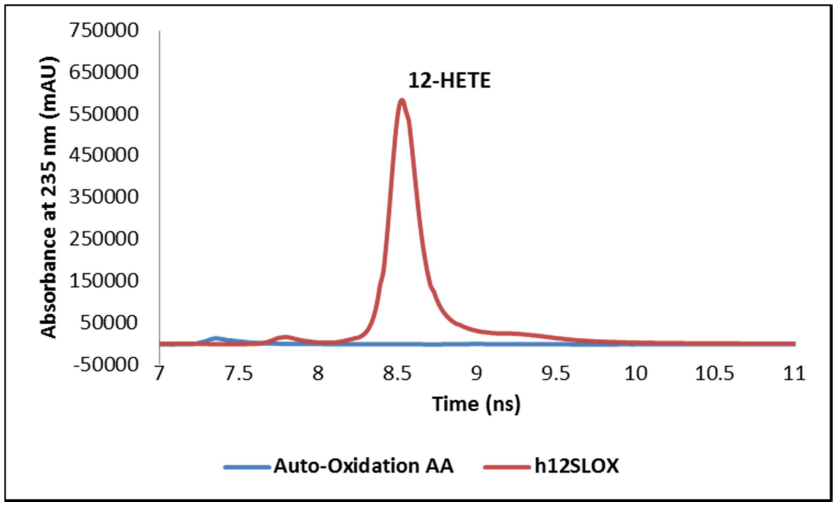

Figure 4.9: RP-HPLC analysis of the products formed by h12S-Lipoxygenase wild-type and auto-oxidized products of arachidonic acid.

4.2.1.3 Human 5-Lipoxygenase Wild-Type

The ALOX5 gene cloned in pRSET-A vector was used for expression, purification of h5LOX as a His-tag fusion protein. Compared to h15LOX and h12SLOX the expression level of h5LOX was very limited. BL21 (DE3) plyss cells transformed with h5LOX containing plasmid were cultured, and the expression of recombinant protein was induced by addition of 1mM IPTG (final concentration). The cell lysate was then subjected to purification by affinity

chromatography (Figure 4.10). The purification profile of h5LOX in the elutions collected can be seen on the western blot in Figure 4.11. The elution 2 and 3 which have most of the enzyme were pooled and used for activity assays. The arachidonic acid oxygenation products of h5LOX consisted mainly 5-HETE with very few traces of 8HETE and 12HETE with retention times close to 9 and 12 minutes respectively (Figure 4.12). Out of all the reaction products produced by h5LOX, 80% of them were 5-HETE while the rest 20% are 8-and12-HETEs.

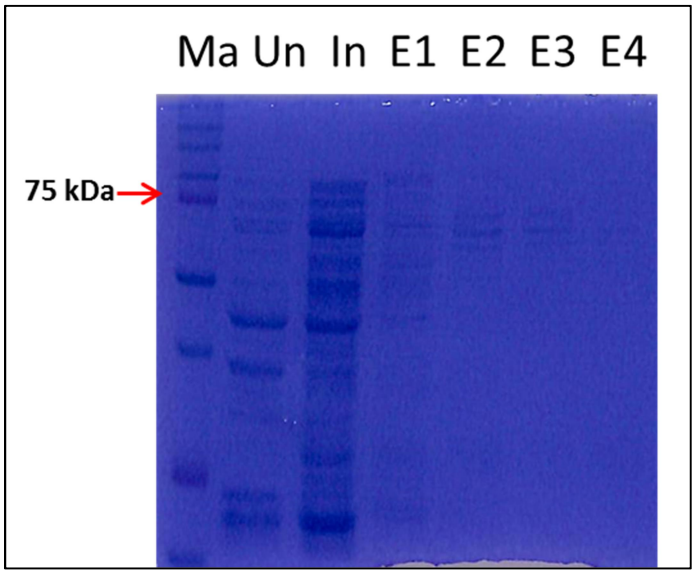

Figure 4.10: SDS-PAGE analysis of various fractions during purification of human 5-lipoxygenase wild-type; MA (Marker), Un (Un-induced), In (Induced), E1 (Elution 1), E2 (Elution 2), E3 (Elution 3), and E4 (Elution 4).

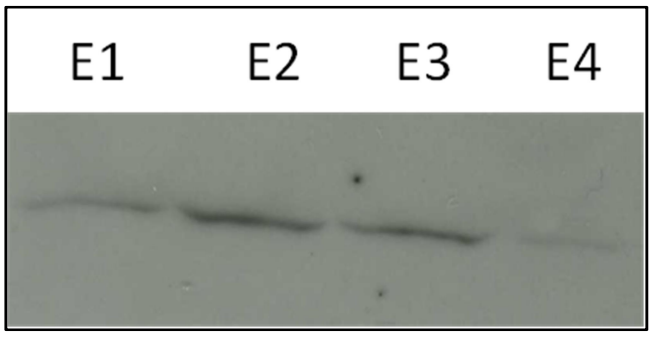

Figure 4.11: Western blot analysis of human 5-lipoxygenase wild-type; Equal volumes of the elutions collected after affinity chromatography were separated on SDS-PAGE and subjected to western blot analysis using h5LOX specific antibody; E1 (Elution-1), E2 (Elution -2), E3 (Elution -3), and E4 (Elution -4).

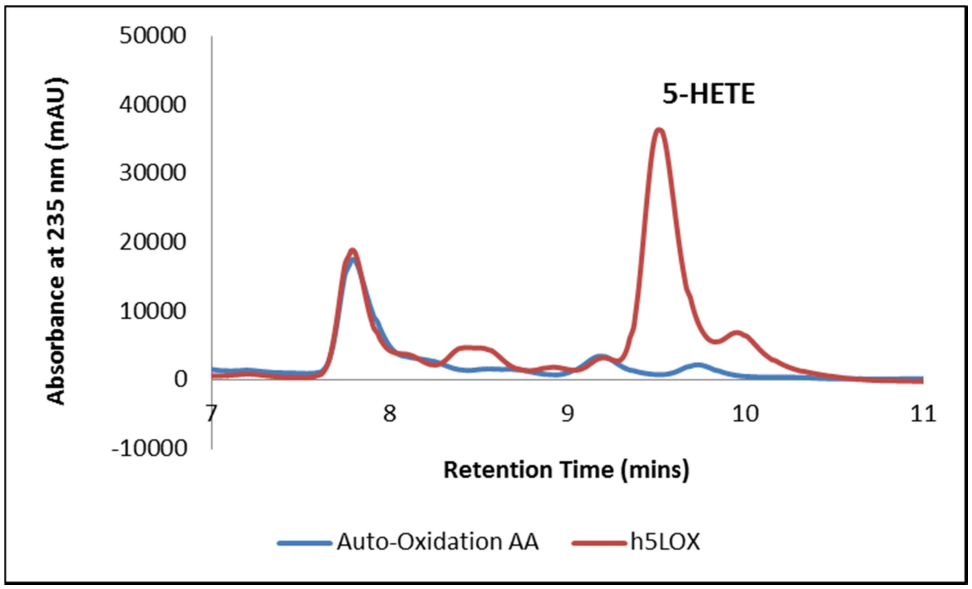

Figure 4.12: RP-HPLC analysis of the products formed by h5-Lipoxygenase wild-type and auto-oxidized products of arachidonic acid

4.2.1.4 Human 12R-Lipoxygenase Wild-Type

Human 12RLOX gene was cloned into a pET28b vector. For expression, BL21 (DE3) plyss cells were used. Most of the expressed protein was coming in inclusion bodies with only very limited soluble protein available (Figure 4.13). This could be due to hydrophobic nature of the protein as this enzyme expresses mainly in the epidermis. The purified enzyme didn't exhibit any enzymatic action against arachidonic acid, so, we used arachidonic acid methyl ester for the activity assays. The products formed are 12R-HETEmet and 8R-HETEmet with HPLC retention times of 15 and 15.5 minutes respectively (Figure 4.14). This is comparable to the results reported earlier for murine 12RLOX where the authors used insect cell system for the expression of the enzyme [Meruvu et al., 2005]. When arachidonic acid methyl ester was used as the substrate for murine 12RLOX, the products formed comprised of 12-, 11-, 9-, and 8-HETEmet's in the ratio of 63, 18, 7, and 12 respectively.

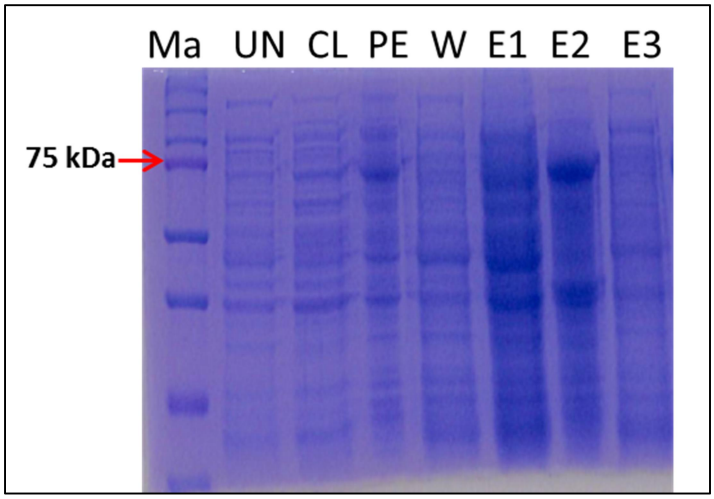

Figure 4.13: SDS-PAGE analysis of various fractions during purification of human 12R-lipoxygenase wild-type; MA (Marker), Un (Un-induced), CL (Supernatant of cell lysate), PE (pellet of cell lysate), E1 (Elution 1), E2 (Elution 2), and E3 (Elution 3).

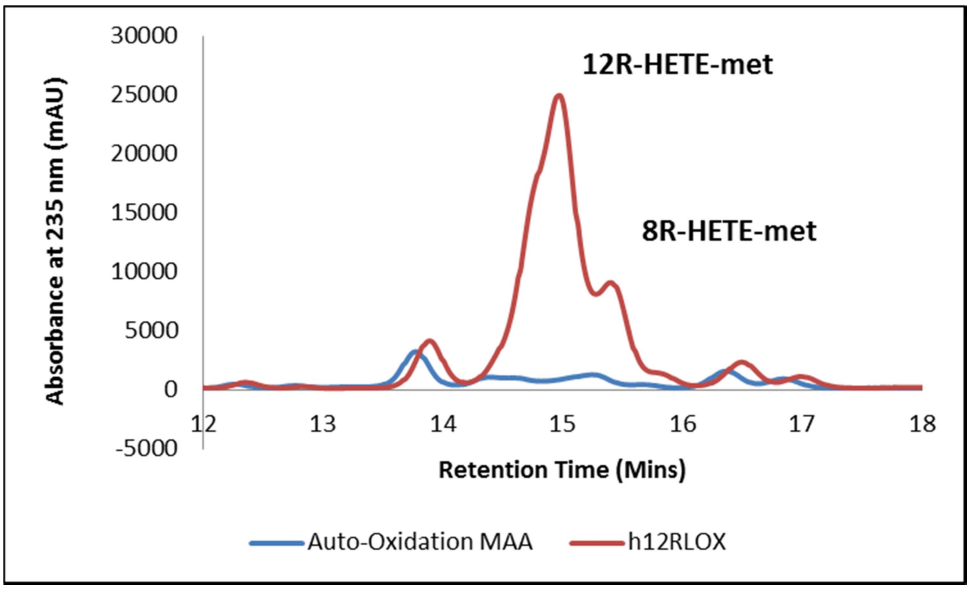

Figure 4.14: RP-HPLC analysis of the products formed by h12R-Lipoxygenase wild-type and auto-oxidized products of arachidonic acid methyl ester.

4.2.2 Functional Characterization of Human 15-Lipoxygenase Glu356Gln and Human 12S-Lipoxygease Glu356Gln Mutants

The amino acid glutamic acid present in h15-, h12S-, and h12R-LOXs is replaced with glutamine in h5LOX. These amino acids are located on the opposite side of the non-heme iron in line with the substrate and contribute for common hydrogen bond acceptor in structure-based

pharmacophore models. Even though they shared same physiochemical property, they have different charge groups on their side chains; glutamic acid has a positively charged side chain whereas glutamine carries neutral charged side chain at physiological pH. Hence, we have investigated the role of glutamic acid in h15LOX and h12SLOX by mutating it with glutamine by employing site-directed mutagenesis.

4.2.2.1 Human 15-Lipoxygenase Glu356Gln Mutant

The h15LOX Glu356Gln mutant was expressed, purified, and analyzed for its outcomes. Activity assays were performed for both the h15LOXGlu356Gln mutant and h15LOX wild-type after expressing them on similar conditions followed by purification. Similar to wild-type, the major product formed was 15-HETE with a little share of 12-HETE (in the ratio 86:14) indicating positively charged side chain of glutamic acid might not contribute to the regiospecificity of the h15LOX (Figure 4.15). The h15LOX Glu356Gln mutant exhibited 93% of the relative specific activity compared to wild type (Table 4.6).

	Share of 15:12-HETE	Relative Activity (%)
h15LOX-WT	90:10	100
h15LOX-E356Q	86:14	93

Table 4.6: The relative specific activity and share of 15-HETE and 12-HETE formed for h15LOX wild-type and h15LOX Glu356Gln mutant taking arachidonic acid as the substrate

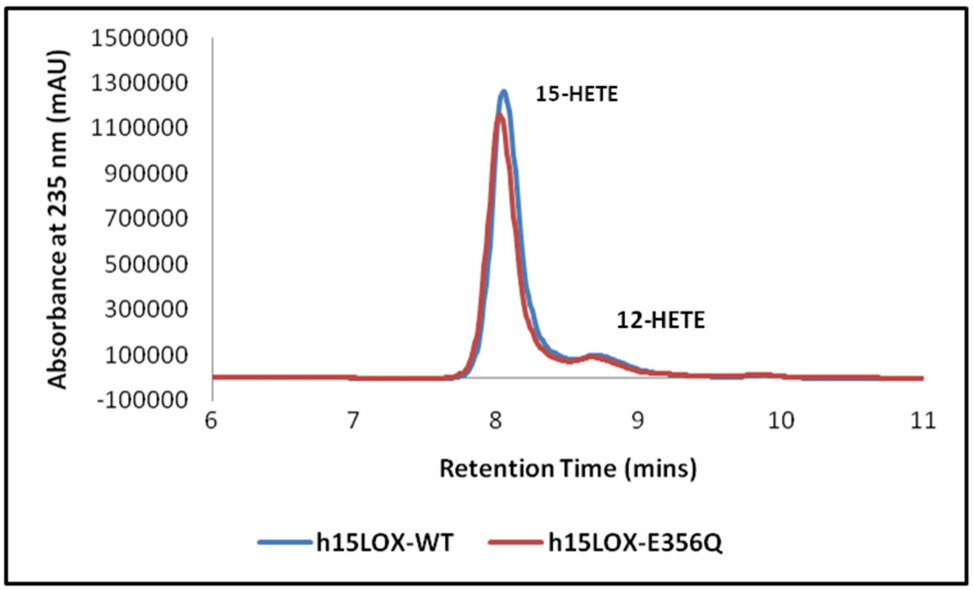

Figure 4.15: RP-HPLC analysis of the products formed by h15-Lipoxygenase wild-type and its Glu356Gln mutant with arachidonic acid as the substrate

4.2.2.2 Human 12S-Lipoxygenase Glu356Gln Mutant

Conversion of glutamic acid to glutamine in h12SLOX also didn't show much variation in terms enzyme catalysis and positional specificity compared to wild-type. The h12SLOX Glu356Gln mutant showed 117% relative enzyme activity (Table 4.7) and majority of the product formed was 12-HETE with a retention time of 8.5 mins (Figure 4.16)

	Share of 12-HETE	Relative Activity (%)
h12SLOX-WT	>99	100
h12SLOX- E356Q	>99	117

Table 4.7: The relative specific activity and share of 12-HETE formed for h12SLOX wild-type and h12SLOX Glu356Gln mutant taking arachidonic acid as the substrate

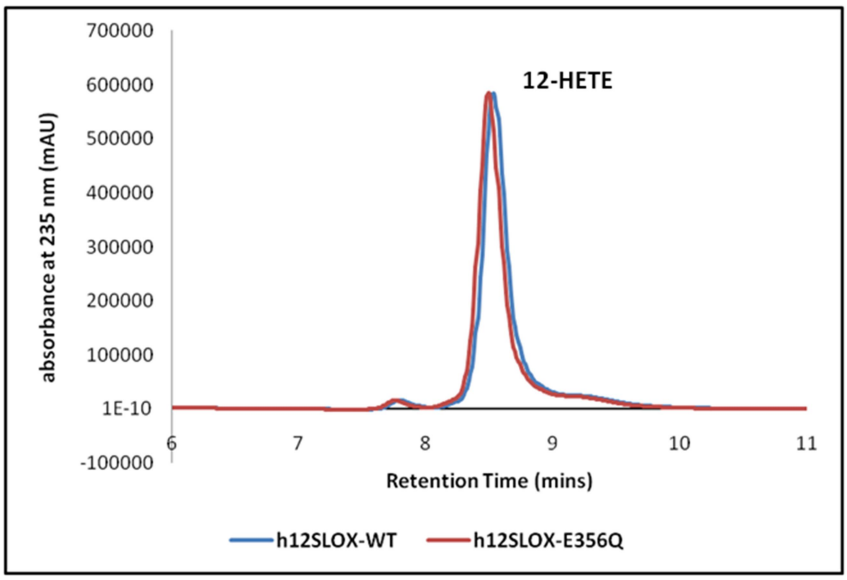

Figure 4.16: RP-HPLC analysis of the products formed by h12S-Lipoxygenase wild-type and its Glu356Gln mutant with arachidonic acid as the substrate

In case of h15-LOX and h12SLOX the radicals generated at C13 and C10 carbons respectively during enzyme catalysis moves in +2 directions while in h5LOX the radical generated at C7 carbon moves in -2 direction [Andreou and Feussner, 2009]. The amino acid glutamic acid and glutamine are located at the core of active site in h15LOX and h12SLOX. Hence we hypothesized that the difference in charge may play a role in deciding the movement of the radical. However, the conversion of glutamic acid to glutamine didn't induce significant changes in the relative specific activity or the positional specificity in h12SLOX and h15LOX. Based on these results, it can be suggested that the amino acid glutamine don't interfere with the enzyme catalysis and regio-specificity or both the amino acids glutamine and glutamic acid are capable of contributing the necessary requirements for the enzyme action.

4.2.3 Functional Characterization of Human 15-Lipoxygenase Gly364Thr and Human 12S-Lipoxygease Thr364Gly Mutants

The arachidonic acid enters the active site of lipoxygenases from the top the binding site where the N-terminal domain is located [Gan et al., 1996]. A large body of research has been conducted to find out which end i.e. carboxyl or methyl end of the substrate enters first into the active site [Coffa et al., 2005]. There are studies which support both the possibilities depending upon the type of LOX [Mitra et al., 2015]. Amino acids threonine and glycine of h12S- and h15-LOXs respectively are located at the entrance of the binding sites, and mutational exchange

modifies the polarity of the binding site entrance. So, we evaluated the importance of these amino acids by generating h12SLOX Thr364Gly and h15LOXGly364Thr.

4.2.3.1 Human 15-Lipoxygenase Gly364Thr Mutant

The conversion of glycine to threonine showed no difference in the positional specificity of h15LOX Gly364Thr mutant. The mutant converted arachidonic acid to 15- and 12-HETE in the ratio of 90:10 that is similar to the wild-type h15LOX (Table 4.8). The relative specific activity of h15LOX Gly364Thr was 1.3 fold higher than the wild-type (Table 4.8). The chromatograms showing the pattern of reaction products formed after AA oxygenation were shown in Figure 4.17.

	Share of 15:12-HETE	Relative Activity (%)
h15LOX-WT	88:12	100
h15LOX-G364T	90:10	129

Table 4.8: The relative specific activity and share of 15-HETE and 12-HETE formed for h15LOX wild-type and h15LOX Gly364Thr mutant taking arachidonic acid as the substrate

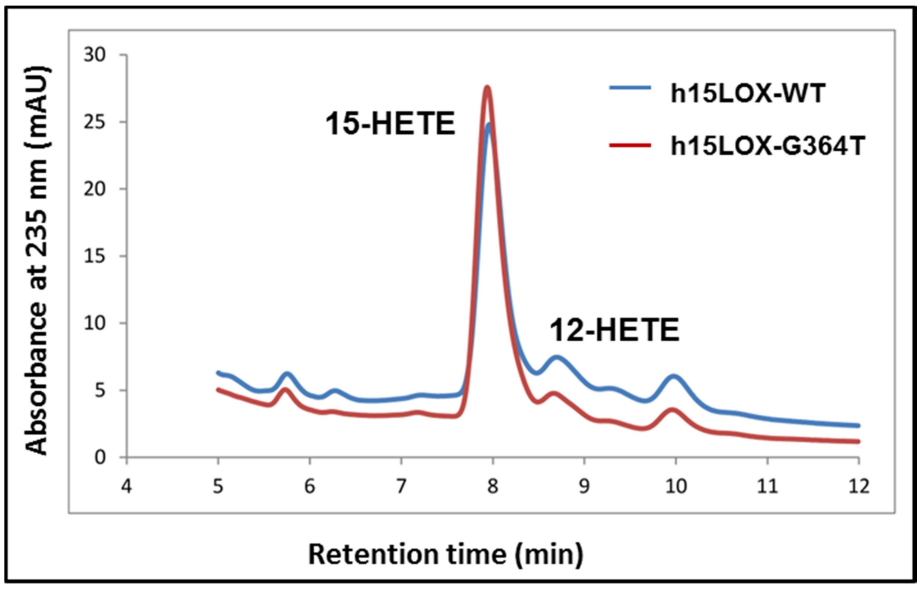

Figure 4.17: RP-HPLC analysis of the products formed by h15-Lipoxygenase wild-type and its Gly364Thr mutant with arachidonic acid as the substrate

4.2.3.2 Human 12S-Lipoxygenase Thr364Gly Mutant

The h12SLOX Thr364Gly mutant showed 2.7 fold higher relative specific activity compared to the wild-type h12SLOX (Table 4.9). Positional specificity of the enzyme was not affected due to the exchange of threonine to glycine. The only product formed on oxygenation of substrate arachidonic acid was 12-HETE with a retention time of 8.5 mins (Figure 4.18).

	Share of 12:15-HETE	Relative Activity (%)
h12SLOX-WT	95:5	100
h12SLOX-T364G	99:1	273

Table 4.9: The relative specific activity and share of 12-HETE formed for h12SLOX wild-type and h12SLOX Thr364Gly mutant taking arachidonic acid as the substrate

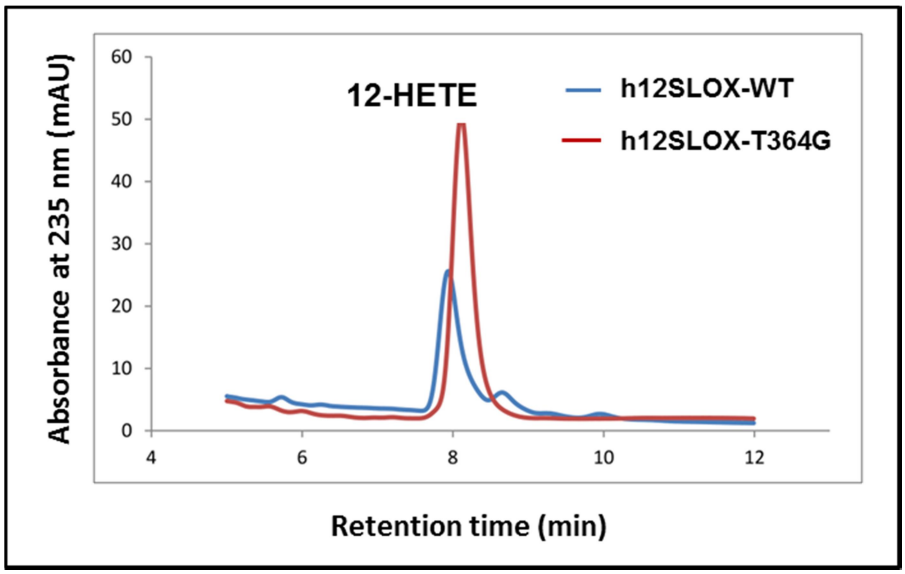

Figure 4.18: RP-HPLC analysis of the products formed by h12S-Lipoxygenase wild-type and its Thr364Gly mutant with arachidonic acid as the substrate

The change in polarity at the active site of the h15LOX and h12SLOX altered the efficiency of the enzyme catalysis. Both the mutants showed higher relative enzyme activities compared to their respective wild-types.

4.2.4 Functional Characterization of Human 15-Lipoxygenase Phe414Ser, Human 12S-Lipoxygenase Phe414Ser, Human 5-Lipoxygenase Phe421Ser, and Human 12R-Lipoxygease Ser451Phe Mutants

The amino acid phenylalanine is conserved in all S-product specific human LOXs whereas the same is replaced with serine in R-product specific human 12RLOX. This amino acid is located on the opposite side of non-heme iron in line with substrate binding cavity. The fatty acid substrate will be aligned differently in this area for the proximal advantage of hydrogen-abstraction in different lipoxygenases. Most of the amino acids that line the catalytic center of LOXs are hydrophobic amino acids which can be reasoned to the hydrophobic nature of the fatty acid chains. Phenylalanine is the closest fatty acid on the opposite side of the hydrogen abstraction locality. In order to characterize the importance of phenylalanine, initial experiments were done on h15LOX and h12SLOX by mutating it to serine. As the amino acid exchanges interfered with the enzyme function in both h12S- and h15-LOXs, we further extended our analysis to h5LOX and h12RLOX. The exchange alters the size and polarity of the binding environment at the catalytic centers of LOXs.

4.2.4.1 Human 15-Lipoxygenase Phe414Ser Mutant

Conversion of phenylalanine to serine strongly interfered with the enzyme catalytic activity. The h15LOX Phe414Ser mutant exhibited only 3% relative enzyme activity with AA as the substrate (Table 4.10). It also altered the positional specificity of the reaction when arachidonic acid was used as a substrate. Mutant exhibited higher 12-lipoxygenation compared to the wild-type; however, the amounts of the HETEs formed are very less to label it as a sequence determinant. The ratio of 15-HETE to 12HETE has changed to 70:30 in the mutant enzyme while wild-type exhibited 94:6 ratio (Table 4.10). We further checked whether this trend would change with LA and ALA as the substrates; but, the effects were same with h15LOX Phe414Ser still being inactive with relative specific activities of 6% (LA) and 5% (ALA) (Table 4.10). The chromatogram of RP-HPLC showing the peaks of products generated from the AA, LA, and ALA by mutant and the wild-type enzyme are shown in Figure 4.19.

	Share of 15:12-HETE	Relative Activity (%) (AA)	Share of 13:9-HODE	Relative Activity (%) (LA)	Share of 13:9-HPOTrE	Relative Activity (%) (ALA)
h15LOX-WT	94:6	100	90:10	100	>99	100
h15LOX-F414S	70:30	3	78:22	6	60:40	5

Table 4.10: The relative specific activity and share of HETEs, HODEs, and HPOTrEs formed for h15LOX wild-type and h15LOX Glu356Gln mutant taking arachidonic acid, linoleic acid and alpha-linolenic acid as the substrates

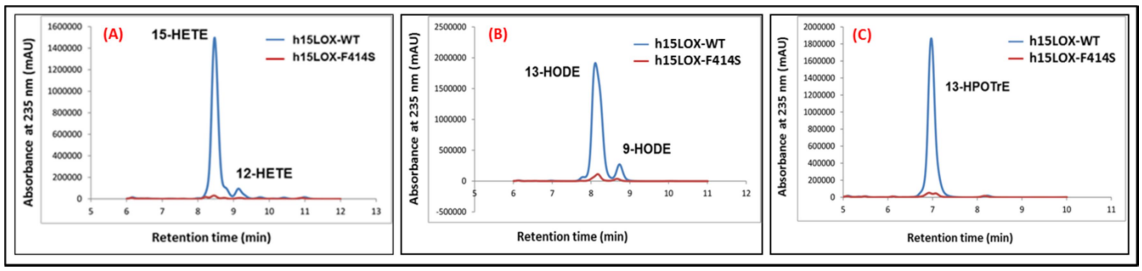

Figure 4.19: RP-HPLC analysis of the products formed by h15-Lipoxygenase wild-type and its Phe414Ser mutant with (A) arachidonic acid, (B) linoleic acid, and (C) alpha-linolenic acid as the substrates

4.2.4.2 Human 12S-Lipoxygenase Phe414Ser Mutant

Phenylalanine mutated to serine at position 414 in h12SLOX showed reduced relative enzyme activity. In contrast to catalytically inactive h15LOX Phe414Ser mutant, the h12SLOX Phe414Ser mutant was catalytically functional with 12% relative enzyme activity compared to its wild-type (Table 4.11). Specificity of the reaction was slightly altered due to the mutation; mutant showed higher 15-lipoxygenation ability (Table 4.11). HPLC chromatograms of reaction products generated from AA were shown in Figure 4.20.

	Share of 12:15-HETE	Relative Activity (%) (AA)
h12SLOX-WT	94:6	100
h12SLOX-F414S	57:43	12

Table 4.11: The relative specific activity and share of 12-HETE and 15-HETE formed for h12SLOX wild-type and h12SLOX Phe414Ser mutant taking arachidonic acid as the substrate

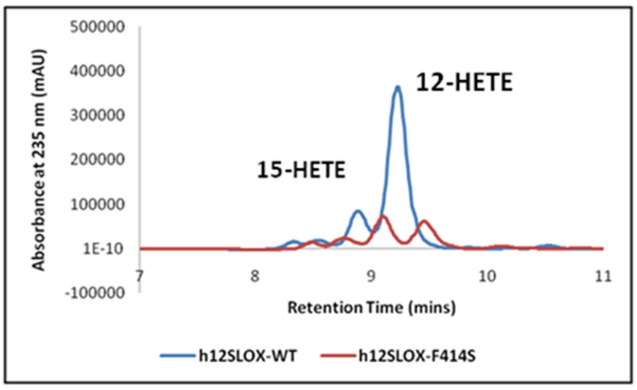

Figure 4.20: RP-HPLC analysis of the products formed by h12S-Lipoxygenase wild-type and its Phe414Ser mutant with arachidonic acid as the substrate

4.2.4.3 Human 5-Lipoxygenase Phe421Ser Mutant

The h5LOX Phe421Ser mutant was catalytically inactive. It exhibited only 5 % relative enzyme activity with a negligible change in positional specificity of the reaction (Table 4.12). When AA was used as a substrate, it produced HETEs (5- to 8-/12- HETEs) in the ratio of 99:1 while the wild-type showed the ratio of 80:20. But then again, 5% relative enzyme activity implies an almost catalytically inactive version of the enzyme. Therefore, phenylalanine can't be concluded as a sequence determinant responsible for the positional specificity of h5LOX. HPThe wild-type h5-LOX as well as h5LOX Phe421Ser mutant didn't exhibit any catalytic activity against LA and ALA. HPLC chromatogram of reaction products generated from AA is shown in Figure 4.21.

	Share of 5:8/12-HETE	**Relative Activity(%)**
h5LOX-WT	80:20	100
h5LOX-F421S	99:1	5

Table 4.12: The relative specific activity and share of 5-HETE and 8-/12-HETE formed for h5LOX wild-type and h5LOX Phe421Ser mutant taking arachidonic acid as the substrate

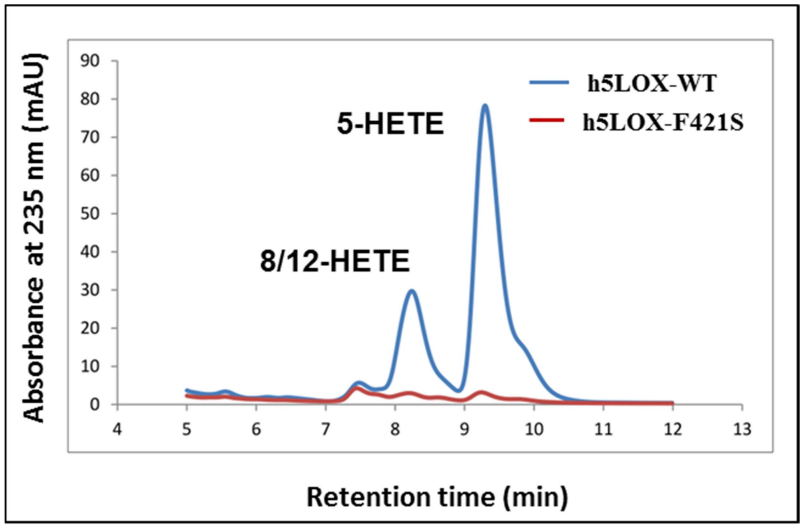

Figure 4.21: RP-HPLC analysis of the products formed by h5-Lipoxygenase wild-type and its Phe421Ser mutant with arachidonic acid as the substrate

4.2.4.4 Human 12R-Lipoxygenase Ser451Phe Mutant

All the S-type human LOXs exhibited diminished catalytic activity when the phenylalanine was mutated to serine. To check whether the reverse mutation i.e. conversion of serine to phenylalanine also have similar effects or not, we generated h12RLOX Ser451Phe mutant. Quantification of the catalytic assay products generated by taking arachidonic acid methyl ester as a substrate on RP-HPLC showed a complete loss of catalytic activity for the mutant. The h12RLOX Ser451Phe mutant only had 9% relative enzyme activity with little change in the product profile compared to wild-type (Table 4.13). The mutant enzyme generated 12R-metHETE and 8R-metHETE in the ratio of 62:37 whereas wild-type produced in the ratio of 78:21 (Table 4.13). As you can see in the RP-HPLC chromatogram (Figure 4.22), there are almost no detectable metabolites for the h12RLOX Ser451Phe mutant.

	Share of 12R:8R-HETE-met	Relative Activity (%)
h12RLOX-WT	78:21	100
h12RLOX- S451F	63:37	9

Table 4.13: The relative specific activity and share of 12R-HETE-met and 8R-HETE-met formed for h12RLOX wild-type and h12RLOX Ser451Phe mutant taking arachidonic acid methyl ester as the substrate

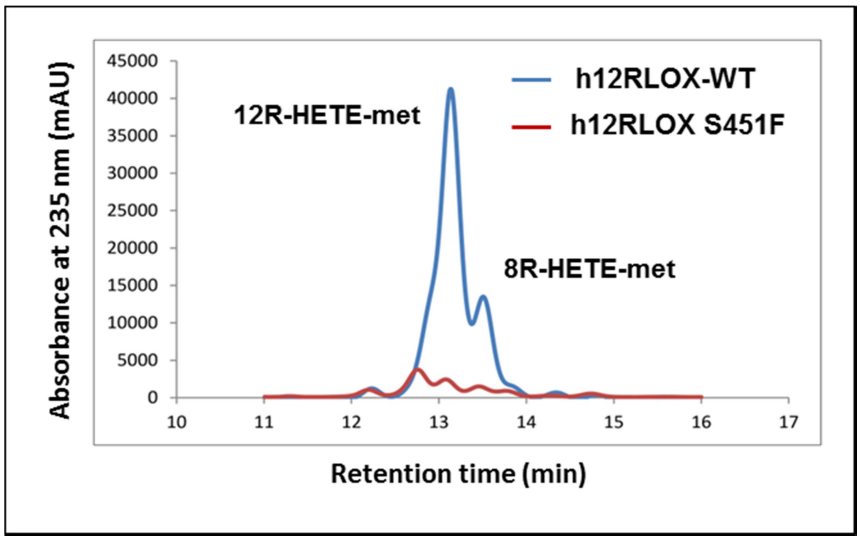

Figure 4.22: RP-HPLC analysis of the products formed by h12R-Lipoxygenase wild-type and its Ser451Phe mutant with arachidonic acid methyl ester as the substrate

The amino acid exchange from phenylalanine to serine and vice versa in all the four human LOXs resulted in the loss of activity. The h15LOX mutant didn't exhibit any change in their catalytic efficiency even when LA and ALA were used as the substrates. All the mutants showed variations in product profile indicating the probable role of phenylalanine/serine in the positional specificity. This can be correlated with earlier experiments performed by Qing-Fen Gan and his group [Gan et al., 1996]. In their study, in order to define the binding site of h15-LOX they have mutated phenylalanine 414 to isoleucine & tryptophan and showed that this amino acid is critical for the binding of the substrate. The Phe414Ile and Phe414Trp mutants exhibited change in positional specificity with AA as the substrate. Phe414Ile mutant showed dual positional specificity (61:39) while Phe414Trp exhibited more 15-lipoxygenation ability (96:4) compared to h15LOX wild-type (90:10). The affinity towards AA was decreased with the decrease in size of the amino acid located at this position [Gan et al., 1996]. Taken together with the present study, it can be concluded that the phenylalanine and serine are very critical in enzyme catalysis and also play an important role in positional specificity for all the LOXs. In addition, we also postulate that this phenylalanine may play a critical role in the stereo specificity as this is conserved in all human S-type LOXs and studies to validate this hypothesis are in progress.

4.2.5 Functional Characterization of Human 15-Lipoxygenase Ala557Phe, Human 12S-Lipoxygenase Ala557Phe, Human 5-Lipoxygenase Ala567Phe, and Human 12R-Lipoxygease Phe595Ala Mutants

The size of the binding site has been attributed for the positional specificity of the LOXs. Modifications in the amino acids located at the bottom of the active site resulted in the conversion of h5-, h12S-, and h15-LOXs to LOXs with different positional specificity. For example, m5LOX has been converted as 8-, 12-, and 15-lipoxinating enzyme with variations in the volume [Hofheinz et al., 2013]. The amino acid alanine in all the three LOXs is located at the bottom of the active site, and replacement with phenylalanine may prompt modifications in the binding site. Hence, the amino acid alanine at positions 557, 557, and 567 in h15-, h12S-, and h5-LOXs respectively is replaced with phenylalanine. Similarly, reverse mutation i.e. phenylalanine to alanine at position 595 was also generated for evaluation in h12R-LOX. Mutants generated in all the four LOXs were later characterized for their importance in enzyme catalysis and regiospecificity.

4.2.5.1 Human 15-Lipoxygenase Ala557Phe Mutant

The amino acid alanine at position 557 in h15LOX was mutated to generate h15LOX Ala557Phe mutant. The enzyme characterization by enzyme catalysis with AA showed complete loss of catalytic efficiency in the mutant. No products were formed by the mutant, and it exhibited only 8% relative enzyme activity with reference to the wild-type enzyme (Table 4.14). Analysis of reaction products by RP-HPLC showed trace amounts of 15-HETE, with almost null amounts of 12-HETE (Figure 4.23). Further, we checked whether the mutant behaves differently with other substrates LA and ALA. The mutant was still inactive and showed relative enzyme activities of 3 % and 4% with LA and ALA respectively (Table 4.14). The positional specificity appeared to be altered in the case LA and ALA oxygenation, but as the enzyme activity is almost null (Figure 4.23) this could be an insignificant change.

	Share of 15:12-HETE	Relative Activity (%) (AA)	Share of 13:9-HODE	Relative Activity (%) (LA)	Share of 13:9-HPOTrE	Relative Activity (%) (ALA)
h15LOX-WT	94:6	100	90:10	100	>99	100
h15LOX-A558F	94:6	8	73:27	3	52:48	4

Table 4.14: The relative specific activity and share of HETEs, HODEs, and HPOTrEs formed for h15LOX wild-type and h15LOX Ala557Phe mutant taking arachidonic acid, linoleic acid and alpha-linolenic acid as the substrates

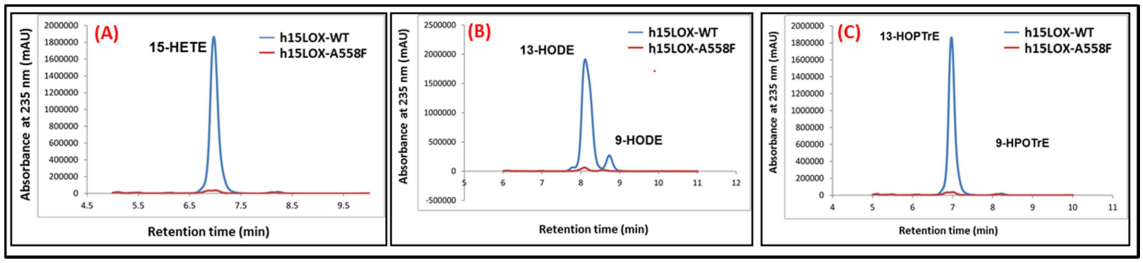

Figure 4.23: RP-HPLC analysis of the products formed by h15-Lipoxygenase wild-type and its Ala 557Phe mutant with (A) arachidonic acid, (B) linoleic acid, and (C) alpha-linolenic acid as the substrates

4.2.5.2 Human 12S-Lipoxygenase Ala557Phe Mutant

The amino acid conversion of alanine to phenylalanine in h12SLOX at position 557 significantly altered the enzyme function. The h12SLOX Ala557Phe mutant was catalytically inactive when AA used as a substrate. The relative enzyme activity compared to wild-type was only 8% (Table 4.15). In the case of regio-specificity, the mutant showed higher 15-lipoxygenation (71:29) compared to the wild-type. The RP-HPLC chromatograms showing the peaks corresponding to the products formed by the mutant and the wild-type can be seen in Figure 4.24.

	Share of 12:15-HETE	Relative Activity (%) (AA)
h12SLOX-WT	94:6	100
h12SLOX-A557F	71:29	8

Table 4.11: The relative specific activity and share of 12-HETE and 15-HETE formed for h12SLOX wild-type and h12SLOX Phe414Ser mutant taking arachidonic acid as the substrate

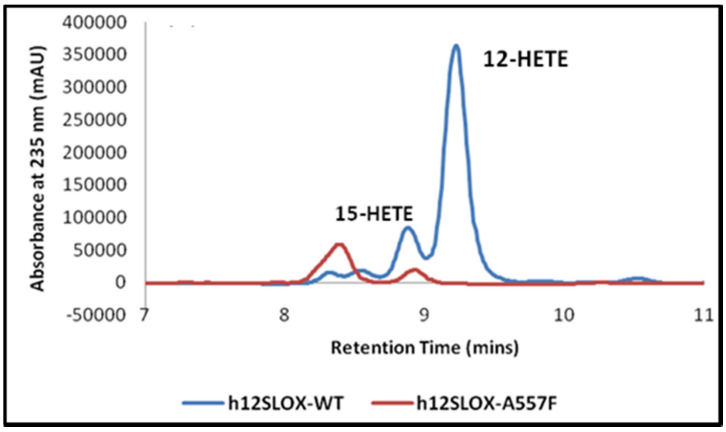

Figure 4.24: RP-HPLC analysis of the products formed by h12S-Lipoxygenase wild-type and its Ala557Phe mutant with arachidonic acid as the substrate

4.2.5.3 Human 5-Lipoxygenase Ala567Phe Mutant

Conversion of alanine to phenylalanine in h5LOX also resulted in complete loss of enzyme activity similar to h15- and h12S-LOXs. The h5LOX Ala567Phe mutant exhibited 5% relative enzyme activity compared to wild-type h5LOX whereas reaction specificity of the reaction was moderately altered. When AA was used as the substrate, the h5LOX mutant produced only 5-HETE while h5LOX produced 5-HETE and 8-/12-HETEs in the ratio of 80:20 (Table 4.16). The RP-HPLC chromatogram displaying the peaks corresponding to 5-, and 8-/12-HETEs can be seen in Figure 4.25.

	Share of 5:8/12-HETE	Relative Activity (%)
h5LOX-WT	80:20	100
h5LOX-A567F	99:1	5

Table 4.16: The relative specific activity and share of 5-HETE and 8/12-HETE formed for h5LOX wild-type and h15LOX Ala567Phe mutant taking arachidonic acid as the substrate

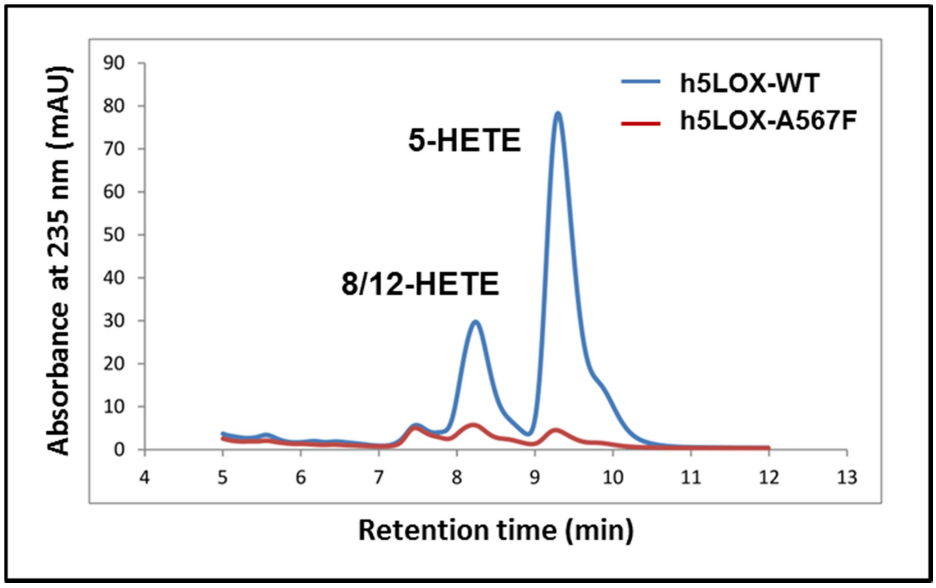

Figure 4.25: RP-HPLC analysis of the products formed by h5-Lipoxygenase wild-type and its Ala567Phe mutant with arachidonic acid as the substrate

4.2.5.4 Human 12R-Lipoxygenase Phe595Ala Mutant

The reverse mutation of alanine to phenylalanine in h12RLOX exhibited relative enzyme activity of 106% compared to wild-type. The analysis of reactive products formed after incubation of h12RLOX Phe595Ala with arachidonic acid methyl ester on RP-HPLC showed the presence of 12R-HETE-met and 8R-HETE-met (Figure 4.26). The ratio of both the hydroxyl products was 75:24 which is very much similar to the h12RLOX wild-type (Table 4.17). This implies that phenylalanine in h12RLOX won't contribute to enzyme catalysis or positional specificity of the enzyme. Also Phe at 595 in h12RLOX can be effectively replaced with Ala for catalysis though positional specificity is not affected.

	Share of 12R:8R-Met-HETE	Relative Activity(%)
h12RLOX-WT	78:21	100
h12RLOX-F595A	75:24	106

Table 4.17: The relative specific activity and share of 12R-HETE-met and 8R-HETE-met formed for h12RLOX wild-type and h12RLOX Phe595Ala mutant taking arachidonic acid as the substrate

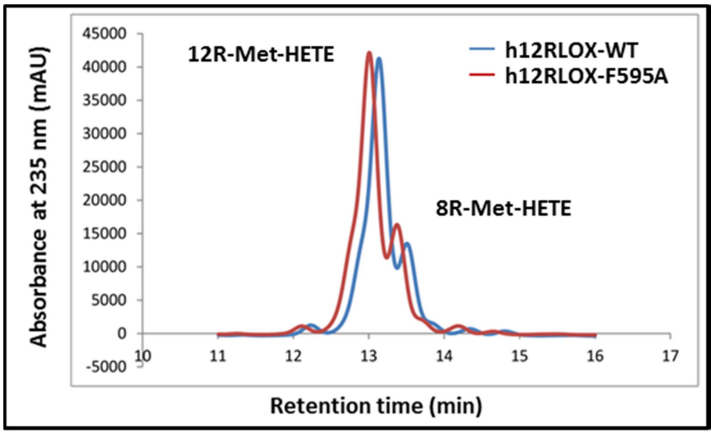

Figure 4.26: RP-HPLC analysis of the products formed by h12R-Lipoxygenase wild-type and its Phe595Ala mutant with arachidonic acid methyl ester as the substrate

Amino acid exchange from smaller alanine to bigger phenylalanine in all the human S-specific LOXs resulted in null variants of the enzyme. Conversely, exchange of space filling phenylalanine with alanine in h12RLOX did not affect the efficiency of the enzyme. The regiospecificity of the enzyme was unaltered due to insertion of the mutations in all the four LOXs. This implies a change in volume at the site of mutation won't affect the regiospecificity of the LOXs. As the difference of amino acid is amongst S-type and R-type LOXs, there is a possibility of altered stereospecificity of the products. The experiment for identification of the chirality of the generated products is still in progress. There is another possibility for the exhibited effect, which is the size of the phenylalanine. As this amino acid is comparatively very big, it may induce steric inference that may cause a change in the binding site geometry which may be essential for the catalysis.

4.2.6 Functional Characterization of Human 15-Lipoxygenase Gly597Ser and Human 12S-Lipoxygease Ser598Gly Mutants

Amino acid glycine at position 598 in h15LOX is different from other LOXs that have serine at this position. These amino acids are located in the core of the active site where the enzyme catalysis occurs. As these amino acids differ in the polarity they carry on their side chains, we evaluated the importance of the functional group they behold by generating mutations in h12SLOX and h15LOX.

4.2.6.1 Human 15-Lipoxygenase Gly597Ser Mutant

The amino acid exchange from glycine to serine at position 597 resulted in increased relative enzyme activity in the h15LOX Gly597Ser mutant compared to the wild-type (Table 4.18). The mutant enzyme showed 1.5 fold higher catalytic efficiency without altering the positional specificity of the enzyme reaction. The only product formed after incubation with arachidonic acid was 15-HETE which was eluted at a retention time of 10.5 minutes on RP-HPLC (Figure 4.27).

	Share of 15-HETE	Relative Activity (%) (AA)
h15LOX-WT	>99	100
h15LOX-G597S	>99	152

Table 4.18: The relative specific activity and share of 15-HETE formed for h15LOX wild-type and h15LOX Gly597Ser mutant taking arachidonic acid as the substrate

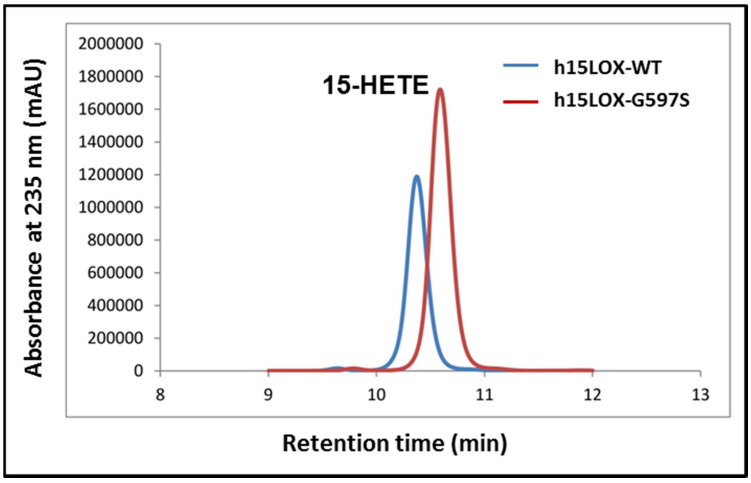

Figure 4.27: RP-HPLC analysis of the products formed by h15-Lipoxygenase wild-type and its Gly597Ser mutant with arachidonic acid as the substrate

4.2.6.2 Human 12S-Lipoxygenase Ser598Gly Mutant

Conversion of serine to glycine in h12SLOX behaved similarly to the wild-type. The h12SLOX Ser598Gly had a relative enzyme activity of 102% with the major substrate being 12-HETE (Table 4.19). This implies that serine at position 598 in glycine doesn't play any role in the enzyme catalysis or the regiospecificity of the h12SLOX enzyme. The overlaid RP-HPLC chromatogram displaying 12-HETE peaks from the h12SLOX wild-type and h12SLOX Ser598Gly mutant can be seen in Figure 4.28.

	Share of 12-HETE	Relative Activity (%)
h12SLOX-WT	>99	100
h12SLOX- S598G	>99	102

Table 4.19: The relative specific activity and share of 12-HETE formed for h12SLOX wild-type and h12SLOX Ser598Gly mutant taking arachidonic acid as the substrate

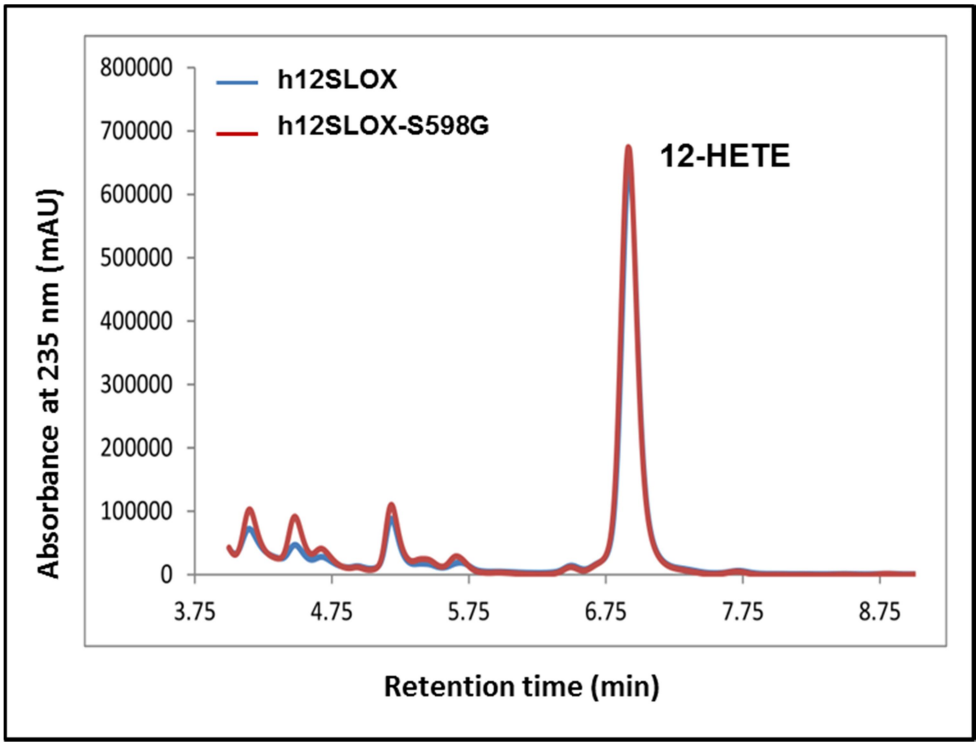

Figure 4.28: RP-HPLC analysis of the products formed by h12S-Lipoxygenase wild-type and its Ser598Gly mutant with arachidonic acid as the substrate

Conversion of serine to glycine in h12SLOX and glycine to serine in h15LOX at positions 598 and 597 respectively didn't alter the regiospecificity of both the LOXs indicating that these amino acids don't play any role in positional specificity. While in the case of relative enzyme activity, only h15LOX Gly598Ser showed 1.5 fold higher relative activity implying the exchange of serine might induce added advantage for the enzyme catalysis in h15LOX.

4.2.7 Functional Characterization of Human 15-Lipoxygenase Val659Ile and Human 12S-Lipoxygease Val660Ile Mutants

Amino acid valine which is sequentially aligned in h15-, h12S-, and h5-LOXs at positions 659, 660 and 671 respectively is conserved in all the S-type LOXs whereas the same is replaced with isoleucine in h12RLOX. These amino acids are located in the core of the catalytic site preceding the C-terminal isoleucine which is a part of iron coordination complex. Hence, we evaluated the importance of valine by mutating it to isoleucine in h15LOX and h12SLOX.

4.2.7.1 Human 15-Lipoxygenase Val659Ile Mutant

The h15LOX Val6659Ile exhibited reduced relative enzyme activity; mutant exhibited 57% relative enzyme activity in comparison to the wild-type (Table 4.20). Incubation of the mutant with arachidonic acid produced 15HETE similar to the wild-type h15LOX. Overall, the conversion of valine to isoleucine in h15LOX impaired the enzyme catalysis with no change in the positional specificity of the enzyme. The 15-HETE formed by the mutant wild-type taking AA as the substrate can be seen in RP-HPLC chromatogram (Figure 4.29)

	Share of 15-HETE	Relative Activity (%) (AA)
h15LOX-WT	>99	100
h15LOX-Val659Ile	>99	57

Table 4.20: The relative specific activity and share of 15-HETE formed for h15LOX wild-type and h15LOX Val659Ile mutant taking arachidonic acid as the substrate

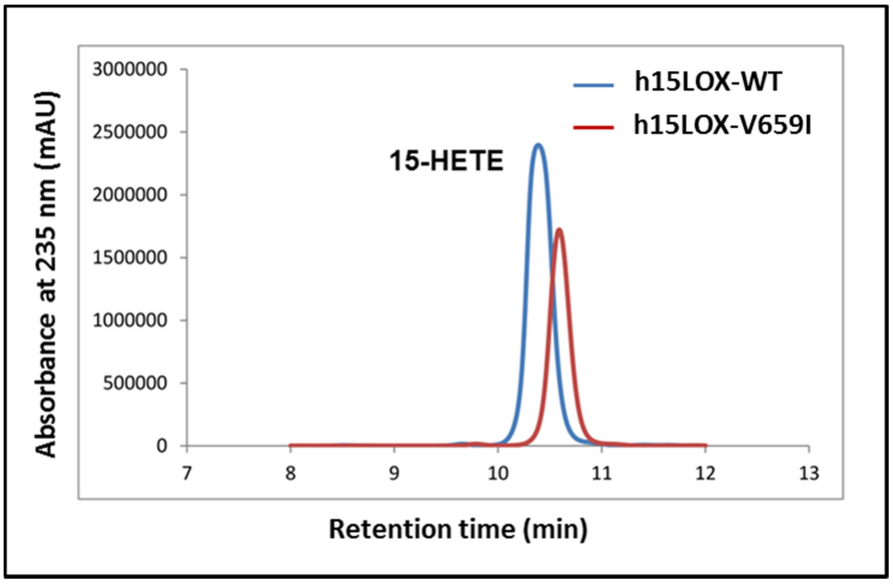

Figure 4.29: RP-HPLC analysis of the products formed by h15-Lipoxygenase wild-type and its Val659Ile mutant with arachidonic acid as the substrate

4.2.7.1 Human 12S-Lipoxygenase Val660Ile Mutant

Mutational exchange of valine to isoleucine in h12SLOX also did not affect the regiospecificity of the h12SLOX. The h1SLOX Val660Ile mutant produced 12HETE upon incubation of the enzyme with arachidonic acid similar to wild-type (Table 4.21). The mutant exhibited relative enzyme activity of 86% compared to the wild-type (Table 4.21). All in all, h12SLOX Val660Ile didn't show any change with respect to regio-specificity or enzyme activity compared to wild-type. The RP-HPLC chromatogram (Figure 4.30) showed only 12-HETE as AA oxygenation metabolites for the wild-type and mutant.

	Share of 12-HETE	Relative Activity (%) (AA)
h12SLOX-WT	>99	100
h12SLOX-Val660Ile	>99	86

Table 4.21: The relative specific activity and share of 12-HETE formed for h12SLOX wild-type and h12SLOX Val660Ile mutant taking arachidonic acid as the substrate

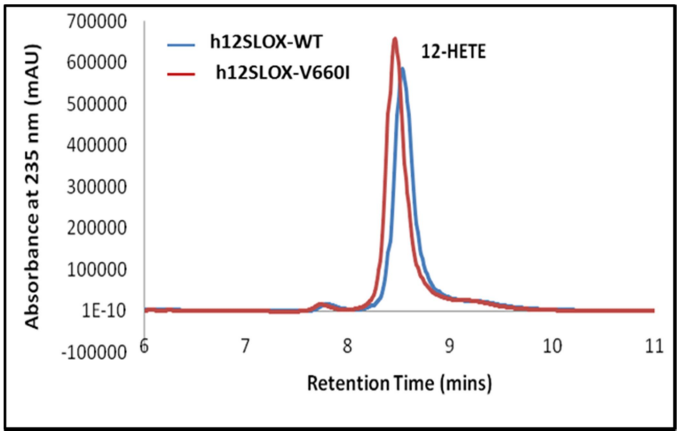

Figure 4.30: RP-HPLC analysis of the products formed by h12S-Lipoxygenase wild-type and its Val660Ile mutant with arachidonic acid as the substrate

The amino acid exchange of valine to isoleucine which is comparatively of bigger size did not induce any changes in the regiospecificity of h12S-, and h15-LOXs. However, the exchange partially affected the enzyme's catalytic efficiency meaning valine may contribute for

the catalysis of the enzyme or isoleucine may induce alterations that may interfere with the enzyme function.

Functional characterization of important amino acids identified by molecular modelling approaches resulted in identification of amino acids that are important/not-important for enzyme catalysis and regio-specificity. Amino acid phenylalanine at positions 414, 414, and 421 in h15LOX, h12SLOX, and h5LOX respectively and serine at position 451 in h12RLOX are important for enzyme catalysis and positional specificity as the mutations of these amino acids resulted in complete loss of activity in all the four LOXs. This conversion alters the binding site volume and polarity at the catalytic center in all the LOXs. Various studies have successfully demonstrated the role of volume on the positional specificity of LOXs [Sloane et al., 1995, Borngräber et al., 1999, Hofheinz et al., 2013]. However, all the amino acids evaluated in the previous studies are at the bottom of the active site while phenylalanine we evaluated is at the catalytic center of the human LOXs opposite to iron in line with substrate. In another study, to identify potential stereo determinant, coffa and his colleagues altered the polarity and volume of the binding site [coffa et al., 2005]. Sequence alignments indicated conserved glycine in all R-type of LOXs which is substituted with alanine in all S-type of LOXs except for mouse platelet type 12S-LOX which had serine at this position. They evaluated the importance of glycine and alanine by mutating them in both S-type and R-type LOXs and concluded them as the stereo-determinants. Similar to phenylalanine we evaluated in the present study, alanine/glycine are also on the opposite side of the non-heme iron in line with substrate and are very close to phenylalanine/serine structurally. In the same study, they also checked the importance of serine by introducing it in the place of alanine and glycine in S-type (mouse 8S-LOX and human 15-LOX-2) and R-type LOXs (Coral 8R-LOX and human 12R-LOX) respectively. The product analysis of mutants showed change in stereo specificity in R-type LOXs with no impact in S-type LOXs. In addition, mutation of alanine/glycine with space filling amino acids valine and threonine resulted in complete loss of activity in mouse 8S-LOX, human 12R-LOX, and human 15-LOX-2 [coffa et.al, 2005]. Conversely, in a different study, exchange of phenylalanine (414) with bigger tryptophan in human 15LOX resulted in increased catalytic efficiency while with smaller isoleucine resulted in reduced enzyme activity with modifications in the positional specificity [Gan et al., 1996]. Taken together, it can be concluded that the change in the volume at catalytic center will affect the catalytic efficiency and specificity of the LOX-reaction.

Alanine at position 557, 557 and 567 in h15LOX-1, h12SLOX, and h5LOX respectively is conserved in all S-type human LOXs. The corresponding amino acid of this alanine is replaced with phenylalanine in h12RLOX. The volume of the binding site in LOXs plays an important role in determining the positional specificity. Differences in the volume of LOXs alter the orientation of arachidonic acid in such a way that the specific diene centre where the initial hydrogen abstraction happens stays in close proximity to iron. As alanine is at the bottom of the active site in h15LOX-1, h12SLOX, and h5LOX, we exchanged it with phenylalanine to check whether the change in volume has any effect on the specificity of the enzymes. Similarly, alanine at positions 557, 557, 567 positions in h15LOX, h12SLOX, and h5LOX respectively interfere with enzyme catalysis leading to complete loss of activity in the mutants. Conversely, conversion of phenylalanine to alanine at corresponding position (595) in h12RLOX had no effect on the enzyme functionality of the mutant implying that only alanine in S-type LOXs is important or exchange of phenylalanine impairs with enzyme catalysis. Glutamic acid at positions 356 and 356 located at the core of the active sites in h15LOX and h12SLOX respectively when mutated to glutamine (present in h5LOX) didn't alter the positional specificity or enzyme catalysis in both the mutants. The corresponding amino acid in h12RLOX, glutamic acid 394, is reported for polymorphism in patients suffering with autosomal recessive congenital ichthyosis [Lesueur et al., 2007]. The amino acid is replaced with lysine and the functional characterization of this exchange is yet to be evaluated. Amino acids glycine 364 & valine 659 of h15LOX and threonine 364 & valine 660 of h12SLOX didn't show significant variations in terms of positional specificity or enzyme catalysis indicating no probable role. When checked for SNPs for all the amino acids considered in this study, none of them except glutamic acid 394 of h12RLOX have polymorphisms [Lesueur et al., 2007]. This can be correlated with our previous collaborative study, where we reported that there is evolutionary pressure on these enzymes preventing the accumulation of loss of function variations in the human population [Horn et al., 2013]. Conversion of phenylalanine (414, 414, and 421 in h15-, h12S-, and h5-LOXs respectively) to serine and alanine (557, 557, and 567 in h15-, h12S-, and h5-LOXs respectively) to phenylalanine has resulted in complete loss of activity in all the three LOXs. In order to understand the molecular mechanisms behind these effects further studies were taken up by performing molecular dynamic simulations.

4.3 Molecular Dynamic Simulations for Understanding the Effects due to Mutation

Molecular dynamic simulation studies will help in understanding the molecular motion of protein atoms at the microscopic level. They provide additional information in terms of structural flexibility, modifications due to binding of the ligand/substrate, and process of protein folding for the solved X-Ray crystallography and NMR structures. Mutations of the protein residues can lead to the alterations in the dynamic or structural behavior thereby impairing the enzyme function. Single amino acid changes in human LOXs have resulted in enzymatically inactive enzymes. For example, exchange of threonine to methionine at position 560 (Thr560Met) in h15LOX-1 resulted in enzymatically inactive variant *in vitro* [Assimes et al., 2008]. Thr560Met is a SNP reported in coronary artery patients and the heterozygous allele carriers of this SNP had significantly increased risk for the disease [Assimes et al., 2008]. When checked for the molecular mechanism behind the loss of activity by molecular modelling, the only difference observed was lack of a single hydrogen bond in mutant. Mutating the hydrogen bonding partner of Thr560 i.e. Gln294 also resulted in loss of activity and conversion of Thr560 with serine that will retain the hydrogen bond reversed the effect [Schurmann et al., 2011]. Based on these data it can be concluded that hydrogen bond was the reason for the loss of activity in supporting the mechanism. Similarly, most of the SNPs reported for h12RLOX in the patient families suffering with ARCI are also enzymatically inactive [Eckl et al., 2009]. However, the exact mechanism by which these SNPs in h12RLOX affect its enzyme activity is yet to be addressed. In the present study, conversion of phenylalanine to serine in h15LOX, h12SLOX, and h5LOX at 414, 414, and 421 respectively resulted in significantly reduced enzyme activity compared to wild types. The reverse mutation i.e. conversion of serine to phenylalanine in h12RLOX also resulted in complete loss of activity. The other amino acid exchange that also impaired with the enzyme activity is conversion of alanine to phenylalanine in h15LOX, h12SLOX, and h5LOX at positions 557, 557, and 567 respectively. So, we have performed molecular dynamics studies on all the above mutants to understand the molecular mechanism behind their effects. Due to the unavailability of h15LOX-1 crystal structure, the crystal co-ordinates of r15LOX which shares 81% sequence similarity with 99% sequence coverage against h15LOX was used to perform calculations. Even though crystal structure of h12SLOX is solved [Tresaugues et al.,], there are missing residues at the binding site, so, the crystal co-ordinates of truncated version of p12SLOX

[Xu et al., 2012] were used for calculations. There are no crystal co-ordinates available for h12RLOX; hence, we have excluded this enzyme in calculations.

The crystal structure coordinates of h5LOX (3O8Y), p12SLOX (3RDE), and r15LOX-1 (2POM) were taken from protein data bank (http://www.rcsb.org/pdb/home/home.do). A 10 ns molecular dynamic simulation was performed for h5LOX, p12SLOX, and r15LOX using Desmond software. The proteins were solvated in TIP3P water model in a cubic geometry to mimic the cellular environment prior to simulation analysis considering default relaxation protocol available in Desmond. The OPLS 2005 force field parameters were used with the periodic boundary conditions in the NPT ensemble. The movement of catalytic iron and atoms within 5 Å from it were restrained as non-heme iron doesn't have proper force field parameters. Later, after simulation we checked if restraints had any effect on the co-ordination geometry of iron and its interacting residues. The distances of interacting atoms from iron during the simulation ranged in acceptable ranges for all the three LOXs (Figure 1). So, we followed the same strategy for all the mutant proteins of the three LOXS. The important parameters of the protein such as Root mean square deviation (RMSD), Root mean square fluctuations (RMSF), energy parameters, and total number of intra molecular hydrogen bonds were analysed with the time dependent function. The RMSDs for the main chain atoms of the protein in comparison with initial structure over 10 ns simulation were calculated to check the deviation of protein from the input structure. Further, we monitored the RMSF fluctuations of each residue to determine dynamic behaviour of protein residues over the simulation. Hydrogen bonds play a major role in the structural stability, ligand/substrate binding and protein flexibility. Therefore, we have also monitored the changes in hydrogen bonding patterns due to insertion of mutations in comparison with wild-type enzymes. The properties evaluated are stability, dynamic movement, and modifications in the number of hydrogen bonds of the protein.

4.3.1 Wild Type LOXs

4.3.1.1 Rabbit 15-Lipoxygenase Wild-Type

As the crystal structure for the human 15-LOX is not solved, we used crystal co-ordinates of rabbit reticulocyte 15-LOX (r15LOX). The h15LOX shares more than 80% sequence similarity with r15LOX. First crystal structure for r15LOX was solved in the year 1997 which

was later revised to 2.4 Å resolution [Gillmor et al., 1997]. The revised structure consists of two monomers of which one of the monomer contains an inhibitor RS7. To maintain uniformity with the h5LOX and p12SLOX, we have used ligand free chain 'A' for all the calculations and for the generation of *in silico* mutants.

The crystal co-ordinates of the r15LOX (2P0M) [Choi et al., 2007] were taken from the PDB and the ligand free chain 'A' was taken for simulation calculations. Simulation run of 10 ns using Desmond molecular dynamics software was performed. The average changes in the displacement of the r15LOX from its initial structure over 10 ns simulation were monitored by calculating RMSD. The RMSD values of the backbone atoms are within 2 Å meaning there are no big conformational changes. Secondary structural elements movement was also monitored by calculating RMSF profile of individual amino acids. As you can see in the Figure 4.31, higher fluctuations can be observed for the loop regions and fewer fluctuations for alpha-helical and beta-strand regions. Five regions of r15LOX corresponding to the high peaks in the Figure 4.31 are the most fluctuated during the simulation. These amino acid regions are; 87-90, 119-124, 255-260, 324-328, and 432-434. All of these regions are on the surface of the protein (Figure 4.32) and there are no studies exposing the importance for any of the observed regions. We believe that the higher fluctuations could be due to their secondary structure element, as all these amino acids contribute for connecting loop regions.

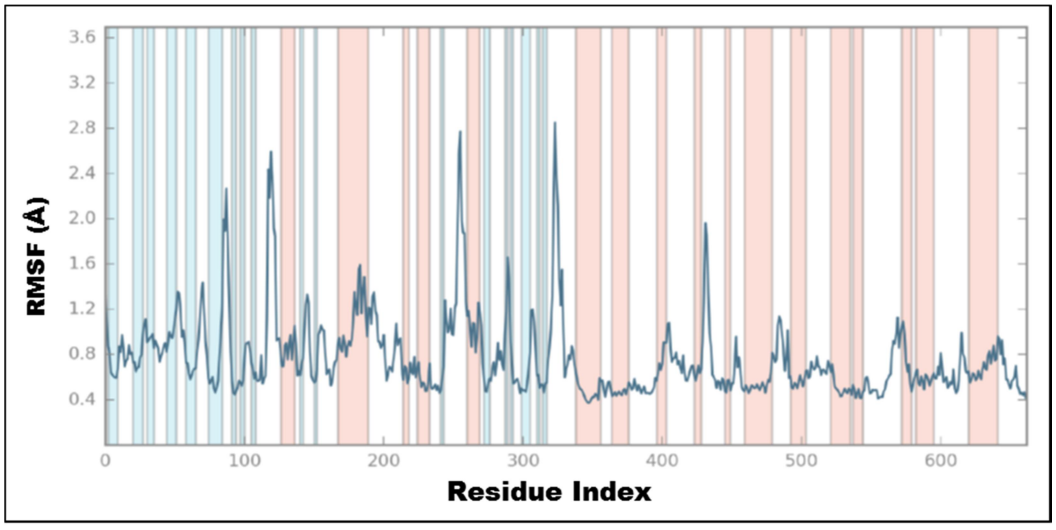

Figure 4.31: RMSF of the amino acids of the r15LOX wild-type displaying their secondary structural elements helices (pink), sheets (blue) and loops (white).

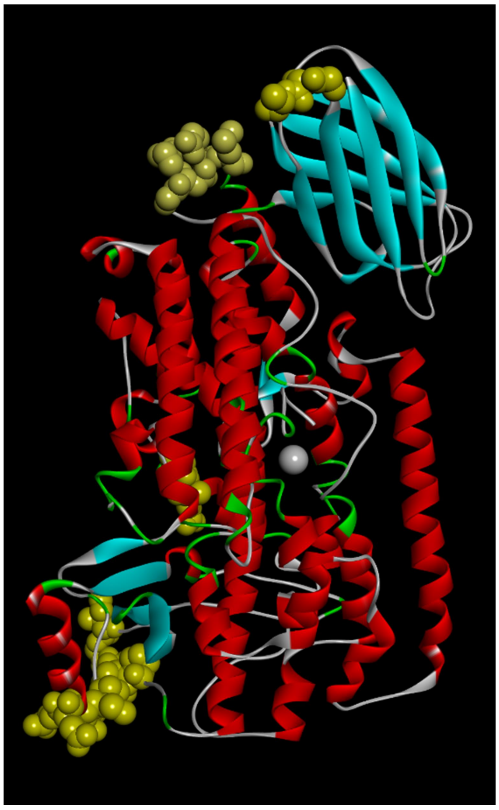

Figure 4.32: Crystal structure of r15LOX displaying the highly fluctuated amino acid regions (yellow) and non-heme iron (white) in sphere representation

4.3.1.2 Pig 12S-Lipoxygenase Wild-Type

The crystal structure for the catalytic domain of human12S-LOX is available at a 2.6 Å resolution [Tresaugues et al.,]. However, it has missing residues at the binding site; so, we took crystal co-ordinates of the pig 12S-LOX for performing molecular dynamic simulation calculations. The p12SLOX has sequence similarity of more than 66% with h12SLOX and lacks n-terminal domain co-ordinates. Hence, we proceeded with the catalytic domain of p12SLOX for all the calculations and *in silico* mutation studies. The crystal structure contains four monomers of p12SLOX and we took chain 'A' for the calculations and for *in silico* mutant's generation

The RMSD values of the back bone atoms of p12SLOX amino acids are less than 2 Å indicating no major change in average displacement from the initial structure. RMSF analysis for the 10 ns simulation showed two regions with most flexibility (Figure 4.33). These amino acid regions are 324-326 & 602-603; which are on the surface of the protein and are part of extended loops and are not part of the binding site cavity (Figure 4.34). Similar to h12SLOX 324-326

region, the corresponding amino acids in h15LOX showed maximum flexibility. Amino acid of the other region, proline 602 forms hydrogen bond with serine 210 of chain 'D'. Therefore, this region may contribute for oligomer formation in p12SLOX and higher flexibility could be due to absence of these interactions.

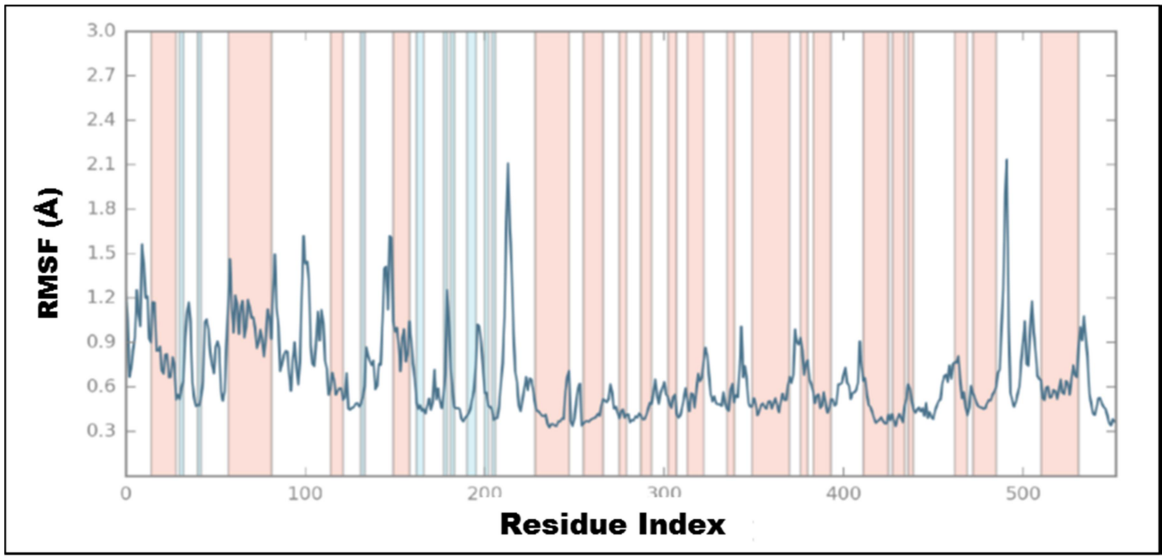

Figure 4.33: RMSF of the amino acids of the p12SLOX wild-type displaying their secondary structural elements helices (pink), sheets (blue) and loops (white).

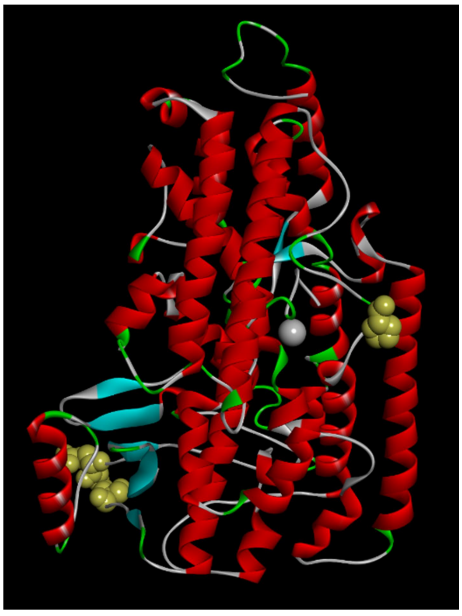

Figure 4.34: Crystal structure of p12SLOX displaying the highly fluctuated amino acid regions (yellow) and non-heme iron (white) in sphere representation

4.3.1.3 Human 5-Lipoxygenase Wild-Type

Human 5-LOX crystal structure was solved by Gilbert et al at a resolution of 2.4 Å in the year 2011 [Gilbert et al., 2011]. This is the first human LOX crystal structure solved that contained both the domains after the h12SLOX which only had catalytic domain. However, authors had to introduce some mutations in the h5LOX to make it more stable for generating crystals. Later, they also solved arachidonic acid bound crystal structures for the stable-5LOX and its mutants [Gilbert et al., 2012]. But then again, the new structures have missing residues at the binding site and when we tried modelling them without removing arachidonic acid from structure; all possible modelled co-ordinates are overlapping with the arachidonic acid. So, we couldn't develop the full structure of the stable 5-LOX with arachidonic acid. Hence, we had to use the substrate-free stable 5-LOX structure for generating the mutants and for performing molecular dynamic simulation calculations.

The RMSD profile of the protein during the 10 ns simulation was within 2 Å indicating no significant deviation due to the calculation. Further, RMSF profiles of the amino acids were examined to identify the region with high/low structural flexibility. RMSF profile of h5LOX can be seen in Figure 4.35. Amino acids contributing for secondary structural elements, helices and β-sheets, showed minimum fluctuations whereas loop regions showed higher fluctuations (Figure 4.35). RMSFs were comparatively higher for the loop regions present in the n-terminal domain than that of catalytic domain. The highest peak was observed for the loop region of n-terminal domain consisting amino acids 73-77. Modelling and mutagenesis studies suggest these loop regions as probable calcium binding ligands as this region structurally similar with that of cPLA2 domain [Hammarberg et al., 2000]. The amino acid regions 652-654 and 328-334 showed higher fluctuations in the catalytic domain. So, we further examined the localization of these loops in the crystal structure to infer the functional importance. Amino acids 328-334 are on the surface of the protein and contribute for the connecting loop of small beta barrel domain and the following helix (Figure 4.36). They are the closest amino acids to other monomer present in the dimer crystal structure of h5LOX. Interaction analysis between both the h5LOX monomers showed existence of a single hydrogen bond between proline 331 of chain A and tyrosine 515 of chain B. As these is the only region in interacting distance with the other monomer, we believe that these loop regions may play very critical role in dimerization of the h5LOX. The higher

fluctuations in this region could be due to use of monomer and molecular dynamics of the dimer may give more insights.

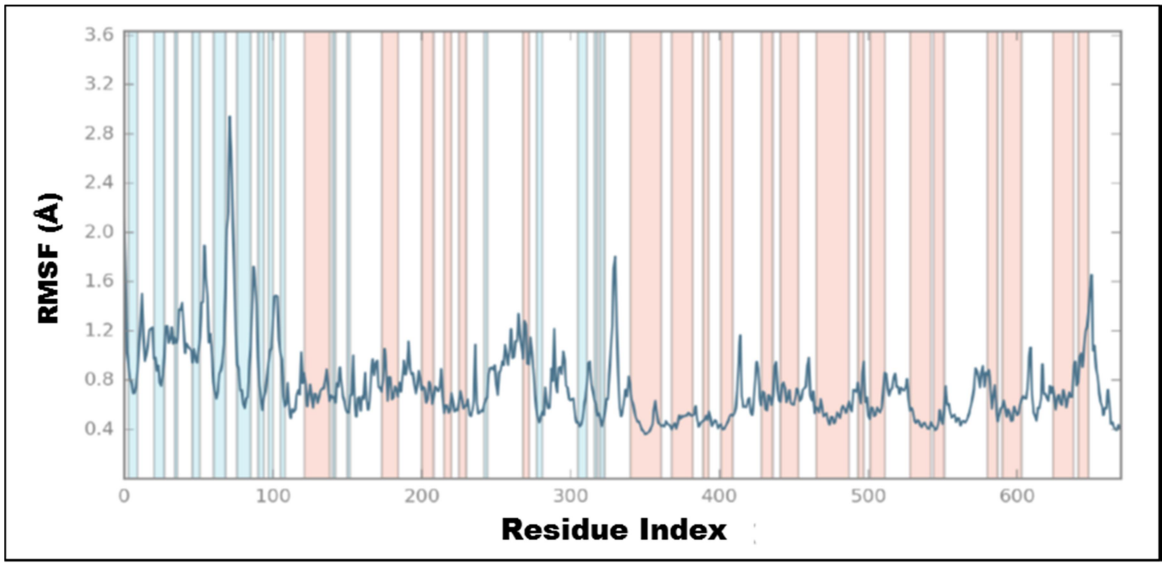

Figure 4.35: Root mean square fluctuations of the backbone atoms of the h5LOX wild-type displaying their secondary structural elements helices (pink), sheets (blue) and loops (white).

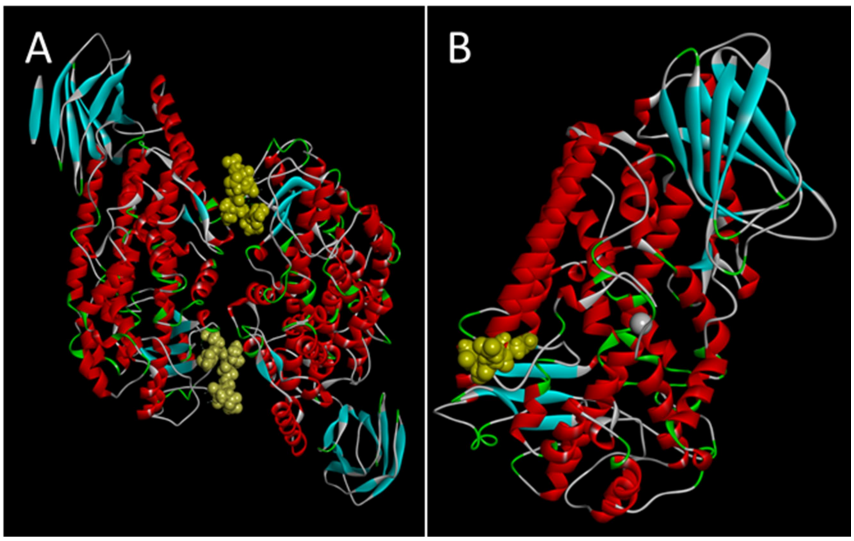

Figure 4.36: Crystal structure of h5LOX displaying (A) amino acids involved in dimer formation (yellow) and (B) highly fluctuated amino acid regions (yellow) and non-heme iron (white) in sphere representation

The other region, i.e. 652-654 is the mutated region. In order to achieve structurally stable structure for crystallization, this lysine rich region of h5LOX was mutated with murine 8RLOX sequence [Gilbert et al., 2011]. This region helps carboxyl terminal to penetrate in to the binding site for c-terminal carboxylate to interact with catalytic iron. Higher fluctuations of this

region could be due to the mutations in this region or it is a highly flexible region of h5LOX that might confer instability [Gilbert et al., 2011].

4.3.2 Conversion of Phenylalanine to Serine

4.3.2.1 Rabbit 15-Lpioxygenase Phe415Serine

The conversion of phenylalanine 414 to serine in h15LOX resulted in complete loss of activity (Chapter 4.2). The corresponding amino acid of phenylalanine in r15LOX is at position 415. *In silico* mutant of r15LOXF415S was generated on Maestro and molecular dynamic simulation for 10 ns was performed. The RMSD, RMSF and total number of hydrogen bonds over the calculation were monitored and compared with wild-type r15LOX. RMSD values of the mutant are around 2 Å and were slightly higher than mutant (Figure 4.37). The deviation was observed after 2 ns and stayed higher for rest of the simulation. Further, differences in the amino acid fluctuations in comparison with wild-type due to insertion of mutation were monitored. The RMSF profile of wild-type and mutant are shown in Figure 4.38. Loop regions of r15LOXF415S in n-terminal domain and one region in catalytic domain showed more and three regions in catalytic domain showed decreased fluctuations in comparison with wild type. Amino acid regions 433-434 and 255-257 showed increased fluctuations whereas amino acid regions 68-70, 103-105, 115-118 and 603-605 showed fewer fluctuations compared to wild type. None of the differentially fluctuated amino acids belong to binding site or shown to have any specific function in catalysis. The total number of hydrogen bonds are decreased in the mutant (Figure 4.39) after 2ns simulation. Overall, there are no induced explainable effects in the mutant in comparison with wild type were observed that may account for loss of activity.

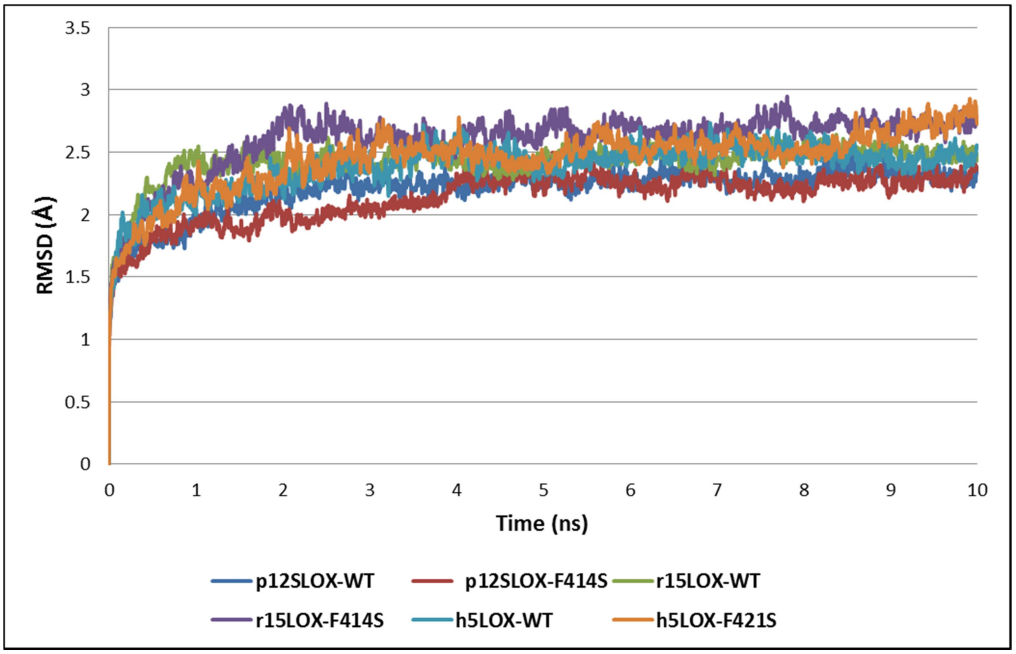

Figure 4.37: Root mean square deviation of the backbone atoms of the r15LOX, p12SLOX, and h5LOX wild-types and r15LOX Phe415Ser, p12SLOX Phe414Ser, and h5LOX Phe421Ser mutants

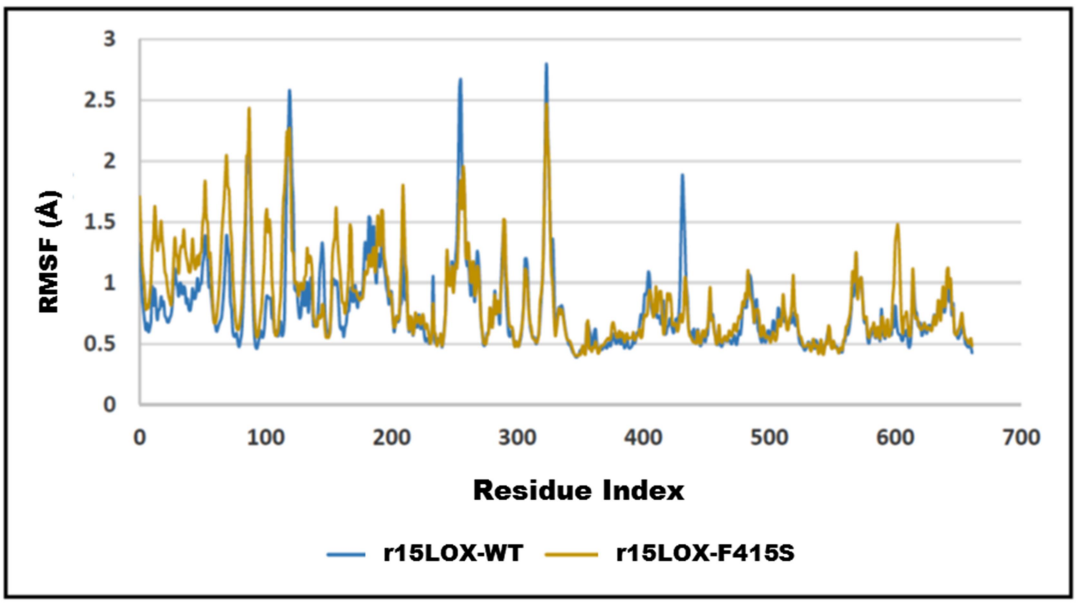

Figure 4.38: Root mean square fluctuations of the backbone atoms of the r15LOX wild-type and r15LOX Phe415Ser mutant

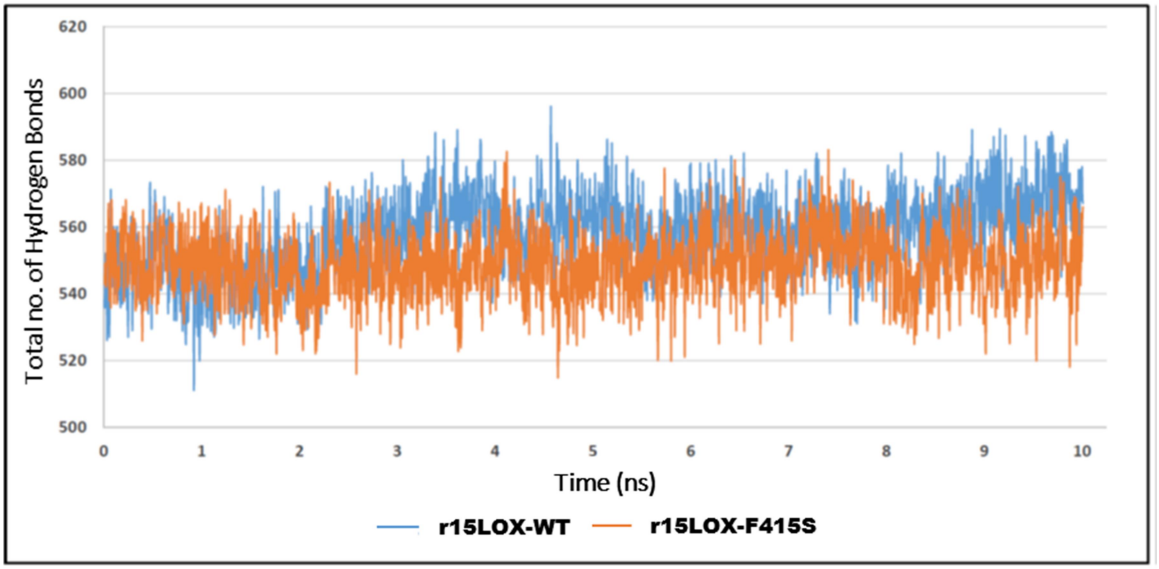

Figure 4.39: Total number of hydrogen bonds of the r15LOX wild-type and r15LOX Phe414Ser mutant over 10ns simulation

4.3.2.2 Pig 12S-Lipoxygenase Phe414Ser

The conversion of phenylalanine to serine in h12SLOX resulted in reduced relative activity in the mutant (Chapter 4.2). The mutation was introduced *in silico* in p12SLOX and effect on structural flexibility and stability was studied by performing a 10 ns simulation. RMSD profile over the simulation showed no significant distortions from the native structure. The RMSD values monitored were less than 2 Å (Figure 4.37). Differences in amino acid fluctuations by measuring RMSF showed only few differences with wild-type. Amino acid regions 602-603 showed decreased whereas 121-126 and 486-487 showed increased fluctuations in the mutant. The amino acids proline 602 and threonine 603 forms interactions with other monomer in the crystal structure of p12SLOX. Probably, insertion of the mutation may alter the ability to form oligomers as these amino acids fluctuations are reduced. But then again, if that is the case, the activity shouldn't be impaired because these enzymes are enzymatically active even as monomers [Shang et al., 2011]. The corresponding amino acids in r15LOX also showed increased fluctuations compared to wild-type r15LOX. However these amino acids are not in binding distance in the r15LOX with counter monomers. But, in ligand bound chain (B) of r15LOX, the isoleucine 603, share a hydrogen bond with lysine 171. This lysine is a part of α-helix that showed maximum displacement in comparison with ligand-free conformer of r15LOX. Therefore, there is a possibility that mutation may alter the movement of this helix leading to

impaired enzyme activity. The other amino acid regions of p12SLOX F414S mutant, 121-126 and 486-487 are on the surface of the protein and there are no studies exposing the importance of these amino acids. The total number of hydrogen bonds are quite similar throughout the 10ns simulation in p12SLOX Phe414Ser mutant compared to p12SLOX wild-type (Figure 4.41).

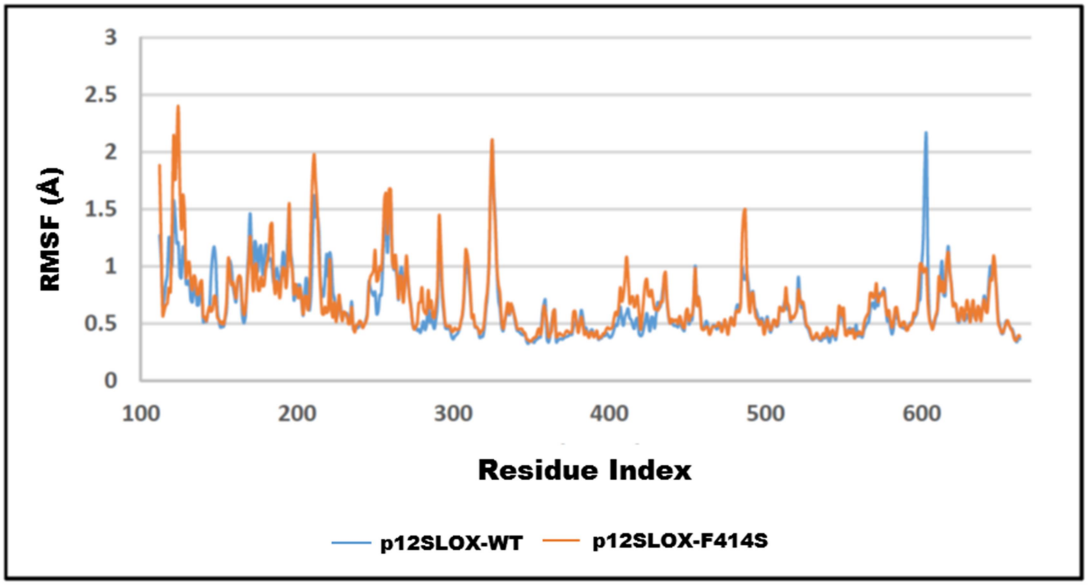

Figure 4.40: Root mean square fluctuations of the backbone atoms of the p12SLOX wild-type and p12SLOX Phe414Ser mutant

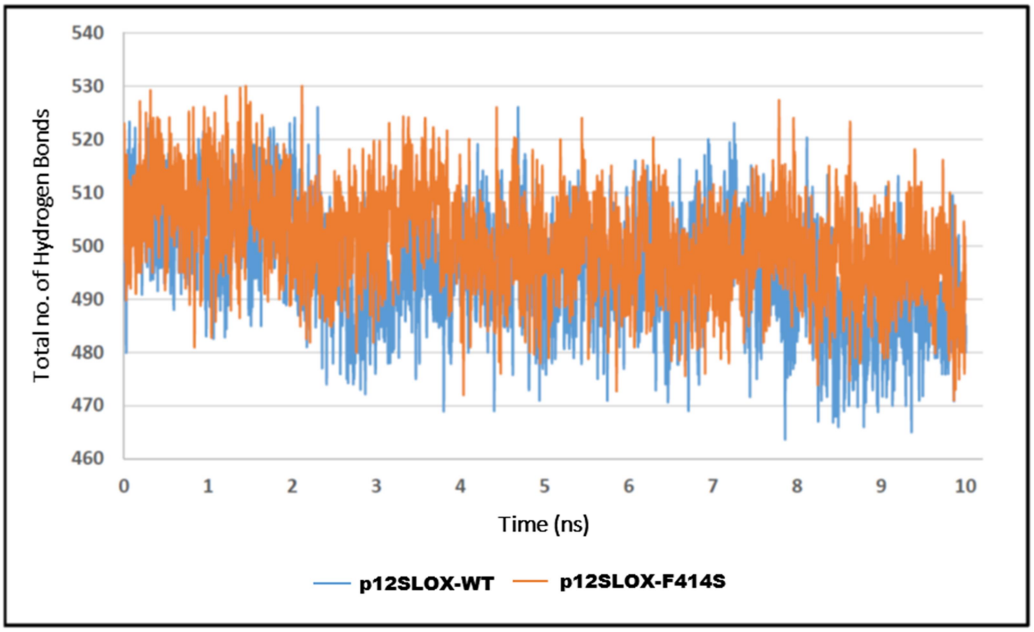

Figure 4.41: Total number of hydrogen bonds of the p12SLOX wild-type and p12SLOX Phe414Ser mutant over 10ns simulation

4.3.2.3 Human 5-Lipoxygenase Phe421Ser

The conversion of phenylalanine 421 to serine resulted in complete loss of activity in h5LOX (Chapter 4.2). Hence, *in silico* mutation was inserted in the h5LOX and simulation of 10 ns was performed to monitor the structural stability and flexibility of the mutant in comparison to the wild-type h5LOX. The RMSD values are less than 2 Å indicating no significant deviation from the native structure (Figure 4.37). However, the enzyme showed increased variation in the RMSD values which means the structure is highly flexible and can have many conformers (Figure 4.37). RMSF profile also showed increased variation compared to the wild type h5LOX (Figure 4.42). Except for 74-78 region in the n-terminal domain which showed lesser fluctuations, other differentially fluctuated regions showed increased fluctuations compared to wild type. The most affected region is from 423 to 434 residues. These residues are part of an adjacent helix that forms interactions with phenylalanine 421 in wild-type. The interactions include hydrogen bond between phenylalanine 421 and asparagine 425, a pi-pi stacked interaction between phenyl ring of phenylalanine 421 and imidazole ring of histidine 432 and many aliphatic interactions. Amino acids alanine 424 and asparagine 425, the positional determinants of the h5LOX [Schwarz et al., 2001], are also present in this region. The possibility for increased fluctuations could be loss of pi-pi interaction by serine in the mutated h5LOX. As these amino acids contribute for the binding site, this would have affected the topology of the binding site leading to impaired enzyme catalysis, observed in the present study.

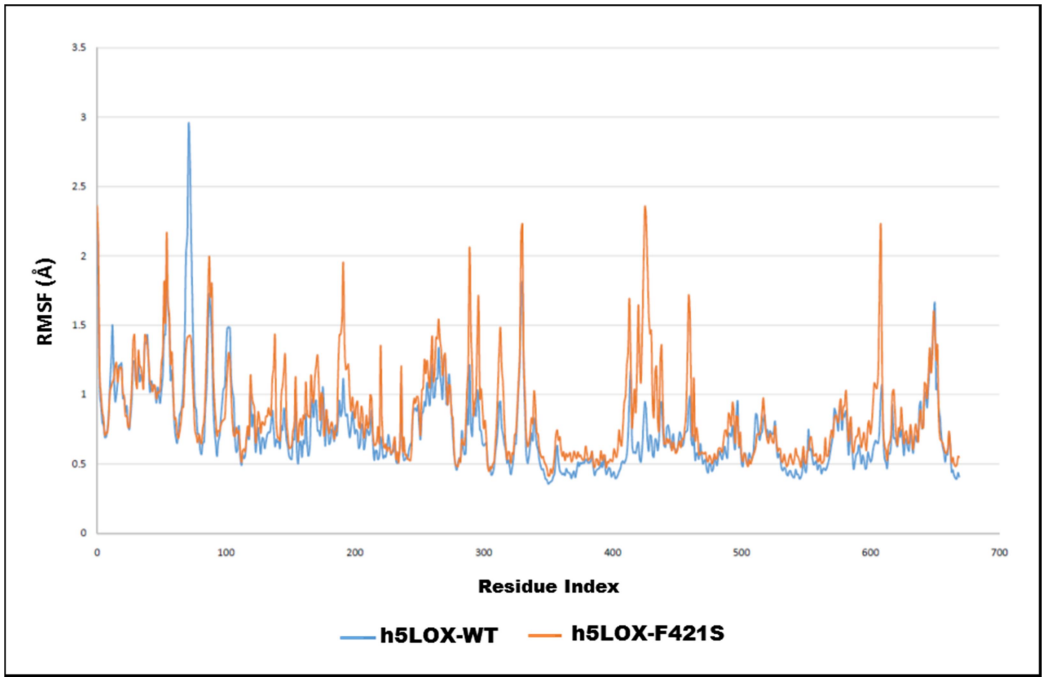

Figure 4.42: Root mean square fluctuations of the backbone atoms of the h5LOX wild-type and h5LOX Phe421Ser mutant

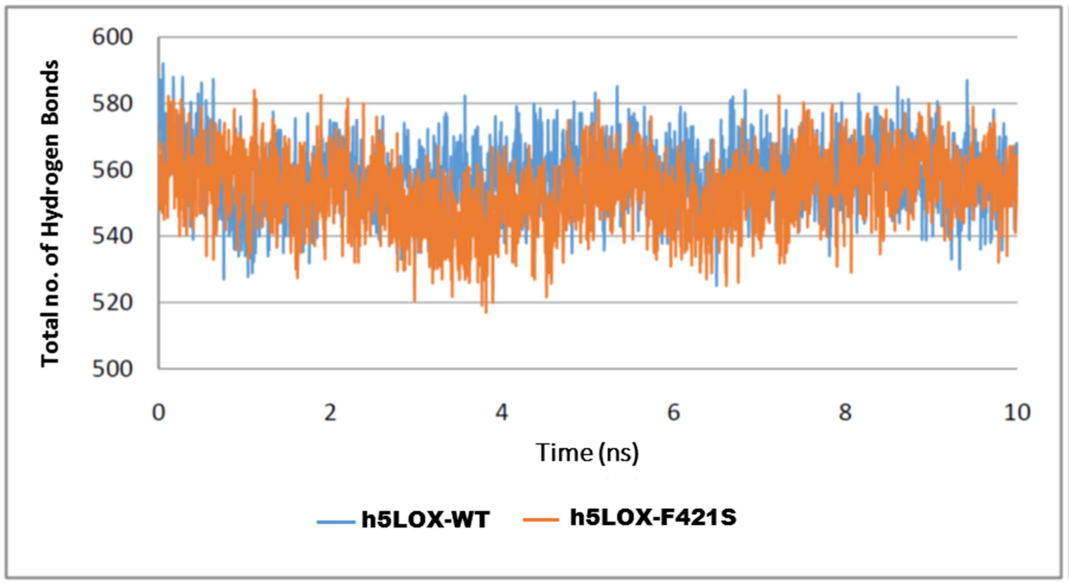

Figure 4.43: Total number of hydrogen bonds of the p12SLOX wild-type and p12SLOX Phe414Ser mutant over 10ns simulation

The other amino acid regions that showed increases in fluctuation are 193-195, 293-294, 463-464, and 611-612. Amino acids from 193-195, 293-394 are part of the neighbouring secondary structural elements of mutation containing small helix, hence, fluctuations could be due to mutation induced structural changes. The amino acids from 463-464 and 611-612 are on

the opposite side of the protein from the site of mutation and are surface exposed. Sequentially corresponding regions of amino acids, 611 and 613 of h5LOX also showed differential fluctuations in p12SLOX and r15LOX. The total numbers of hydrogen bonds were mostly similar throughout the simulation in h5LOXF421S mutant and h5LOX wild-type (Figure 4.43). Overall, due to the mutation, there are no significant structural deviations and change in total number of hydrogen bonds but the conformational flexibility of the enzyme is greatly affected.

Molecular dynamics simulation for the conversion of phenylalanine to serine in h5LOX, p12SLOX, and r15LOX suggested differential variations in the amino acid profiles compared to their wild-types. The only region that showed differential variation in all the three LOXs was 602-603, 602-603 and 611-613 in r15LOX, p12SLOX, and h5LOX respectively. These amino acids are in r15LOX and p12SLOX are involved in the formation of dimer while in h5LOX they are on the opposite side of the monomer interaction territory. Importantly, introduction of serine/exchange of phenylalanine didn't affect the binding site amino acids or its topology. Based on these studies, it can be concluded that the exchange of phenylalanine to serine wont effect the structural integrity of the LOXs that are essential for their catalysis and the loss of function may be associated with substrate interaction with enzyme. To validate this, we further performed molecular dynamic simulation of r15LOX/Arachidonic acid complex.

4.3.3 Conversion of Alanine to Phenylalanine

Amino acid alanine at positions 557, 557, and 567 in h15LOX, h12SLOX, and h5LOX respectively is conserved in all S-type LOXs which is replaced with phenylalanine at position 595 in h12RLOX. In order to evaluate the importance of alanine in S-type LOXs and phenylalanine in R-type LOXs, site-directed mutagenesis studies were undertaken. The amino acid exchange of alanine to phenylalanine resulted in significant reduction in relative enzyme activity in all the S-type LOXs in comparison with their respective wild-types (Chapter 4.2). However, the reverse exchange, i.e. conversion of phenylalanine to alanine in h12RLOX did not alter the enzyme catalysis (Chapter 4.2). Alanine is located at the bottom of the active site and is a part of small helix. In order to understand the molecular mechanism behind the loss of activity in all S-type LOXs, we performed molecular dynamic simulation analysis of the mutant enzymes and compared the differences observed with the wild-type enzymes.

4.3.3.1 Rabbit 15-Lipoxygenase Ala558Phe

In silico mutation was generated at position 558 in r15LOX crystal structure that corresponds to amino acid 557 in h15LOX. The amino acid alanine was mutated to phenylalanine and the mutant was subjected for 10 ns molecular dynamic simulation calculation. The amino acid exchange will decrease the binding site volume and may induce steric hindrance due to larger amino acid leading to structural dis-orientation. However, the RMSD profile over the simulation ranged in acceptable range and is quite similar to the wild-type enzyme. This indicates that there is no significant mutation induced structural deviation from the native structure (Figure 4.44). The RMSF profile of all the amino acids for the r15LOXA558F mutant and r15LOX wild-type is shown in Figure 4.45. Amino acid regions 187-191, 182-183, and amino acid 103 showed higher fluctuations whereas 255-258, 118-124, and 432-434 showed lesser fluctuations in mutant compared to wild-type (Figure 4.45). The amino acids 187-191 and 182-183 are part of a helix that has been shown to undergo structural modifications upon ligand binding [Shang et al., 2011].

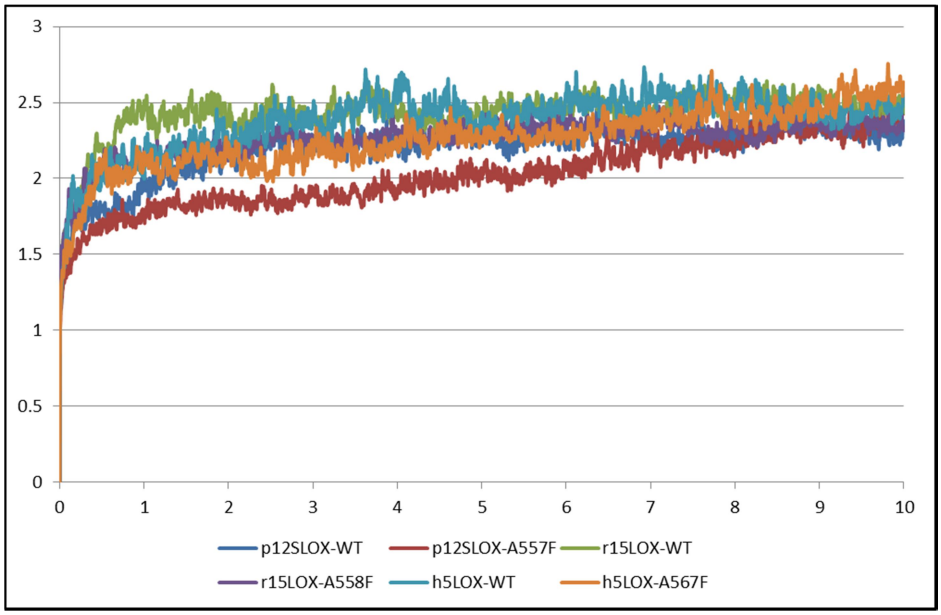

Figure 4.44: Root mean square deviation of the backbone atoms of the r15LOX, p12SLOX, and h5LOX wild-types and r15LOX Ala558Phe, p12SLOX Ala557Phe, and h5LOX Ala567Phe mutants

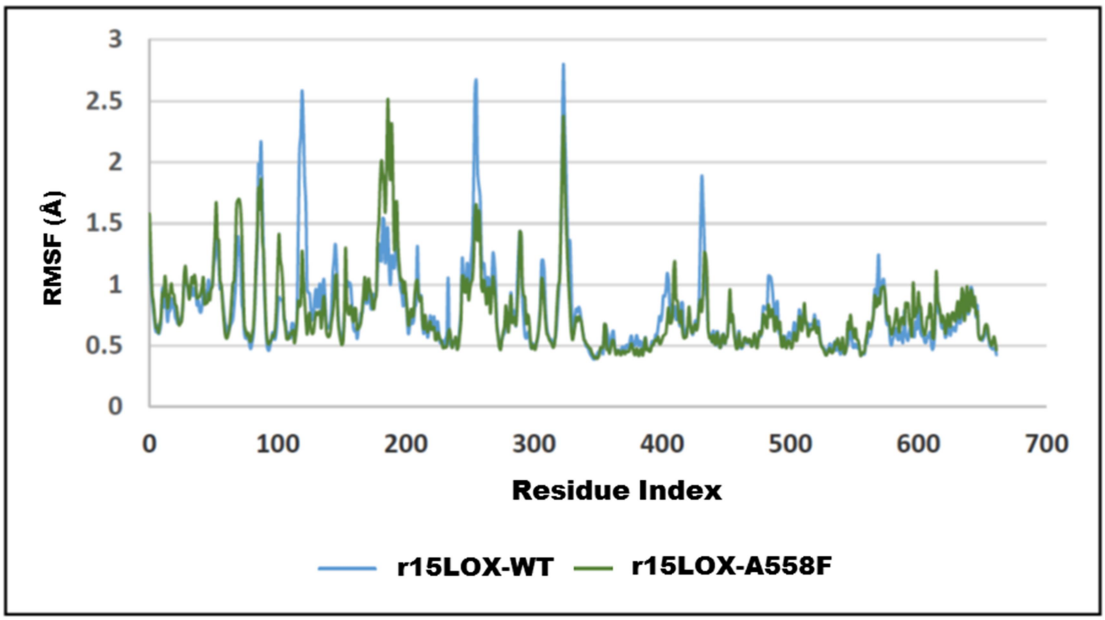

Figure 4.45: Root mean square fluctuations of the backbone atoms of the h5LOX wild-type and h5LOX Ala558Phe mutant

The comparison of ligand bound (closed form) and apo-enzyme (open form) crystal structures showed maximum difference for these helices. The two helices are separated by ~12 Å and the secondary structural elements are different. The closed form has five turns whereas the open form has seven turns. However, how this increased fluctuations can affect enzyme catalysis are unexplainable. Amino acids 255-258 and 432-434 that showed lesser fluctuations in r15LOXA558F also showed differential fluctuations (increased) in r15LOXF415S. All this amino acids are part of extended loops and are located on the surface of the protein. No studies have reported or exploited the importance of these amino acids. Probably the differential fluctuations could be due to their secondary structural elements. Further, the analysis of total number of hydrogen bonds in the mutant were analysed over the simulation. Mutant showed noticeable decrease in the no of hydrogen bonds after 2 ns simulation (Figure 4.46) compared to wild-type. After completion of the10 ns simulation, there is a significant decrease suggesting mutation induced conformational changes in the protein. This means even though there are no significant changes in the native structure, there are loss of many interactions that may have affected the enzyme's structural integrity that is essential for catalysis.

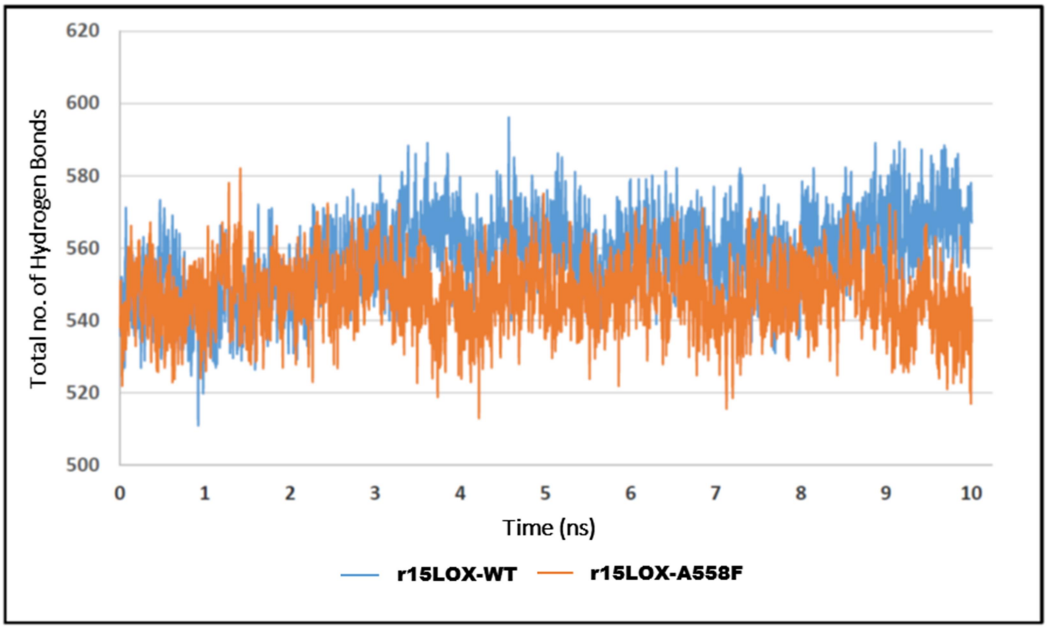

Figure 4.46: Total number of hydrogen bonds of the r15LOX wild-type and r15LOX Ala558Phe mutant over 10ns simulation

4.3.3.2 Pig 12S-Lipoxygenase Ala557Phe

The p12SLOX Ala557Phe mutant was generated *in silico* for analysing the mutation induced structural modifications in the protein. In comparison to h12SLOX-WT, the mutant had different RMSD profile. The maximum deviation from the initial conformation in wild-type appeared immediately after the start of simulation and the updated conformation persisted with minor deviations till the end of the simulation (Figure 4.44). In case of p12SLOX Ala557Phe mutant, even though the maximum deviation happened immediately, the noticeable structural deviations appeared till the end of simulation (Figure 4.44). The RMSF profile showed maximum fluctuations at the N-terminal end of the truncated h12SLOX Ala557Phe mutant in comparison to h12SLOX wild-type (Figure 4.47). The other amino acid region 250-258, which is surface exposed and located far from the catalytic cavity also showed increased fluctuations compared to wild-type. Conversely, corresponding amino acids of 250-258 in r15LOXA558F mutant showed decreased fluctuations. Amino acid region 170-177 showed fewer fluctuations in the mutant. This region is part of the helix that undergoes structural modifications on ligand binding. Conversely, in case of h15LOX A558F mutant, amino acids of the same helix showed higher fluctuations compared to the h15LOX wild-type. The other region that showed decreased fluctuations is 602-603 that is involved in the binding of the other monomer in the crystal

structure. These amino acids in h12SLOX Phe414Ser mutant also showed decreased fluctuations. Comparison of total number of hydrogen bonds in wild-type and mutant showed no significant differences (Figure 4.48). Overall, conversion of alanine to phenylalanine induced structural modifications to the active site amino acids that are essential for catalysis.

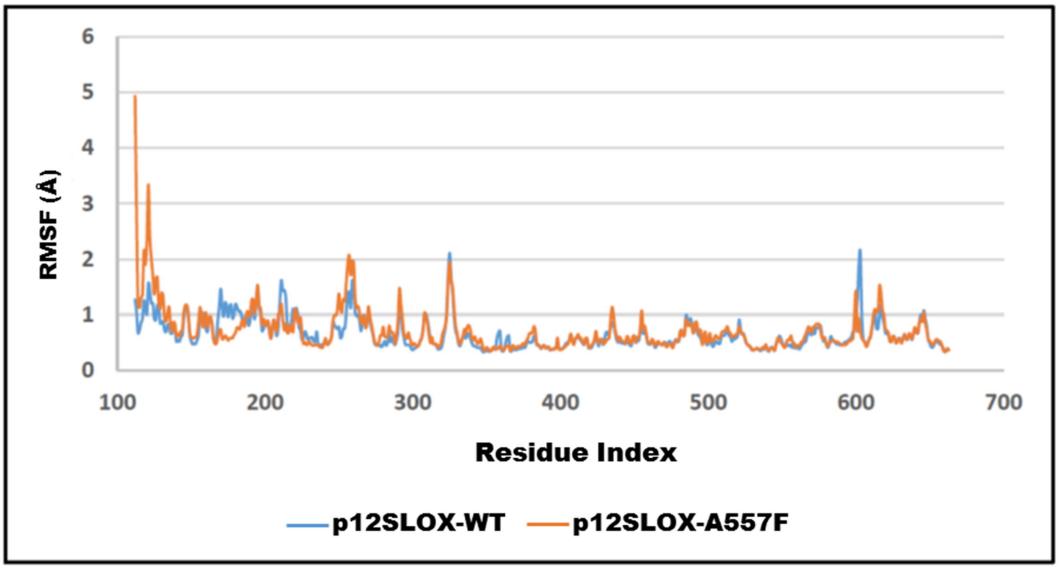

Figure 4.47: Root mean square fluctuations of the backbone atoms of the p12SLOX wild-type and p12SLOX Ala557Phe mutant

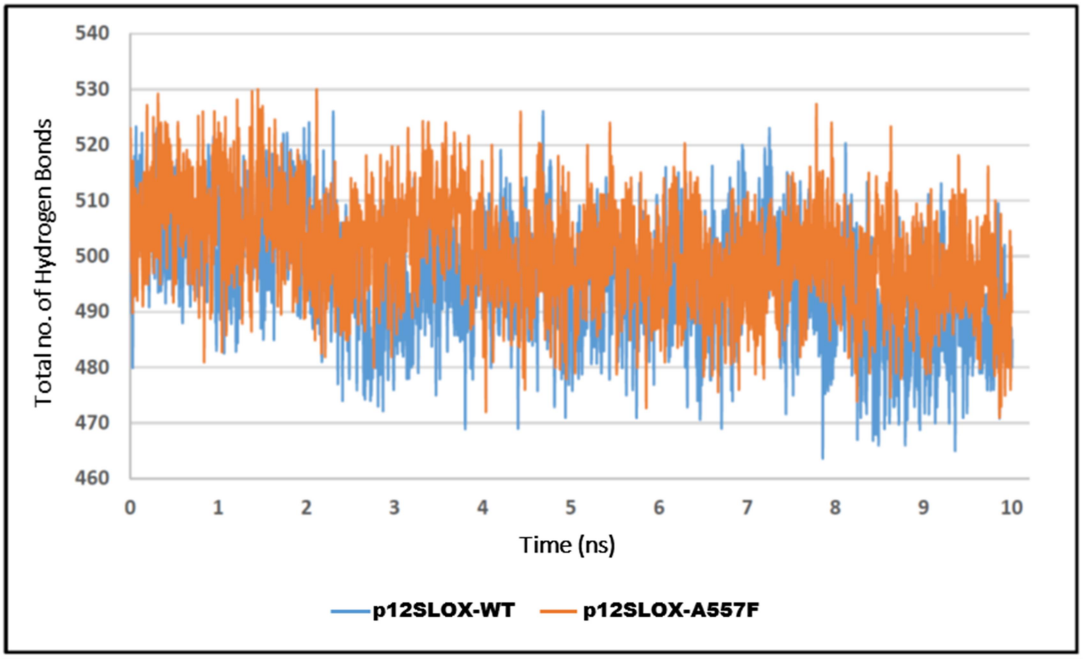

Figure 4.48: Total number of hydrogen bonds of the p12SLOX wild-type and p12SLOX Ala557Phe mutant over 10ns simulation

4.3.3.3 Human 5-Lipoxygenase Ala567Phe

The conversion of alanine to phenylalanine at position 567 in h5LOX resulted in complete loss of activity in the mutant. In order to understand the probable mechanism involved for this affect, we performed 10ns molecular dynamic simulation for the mutant and compared the results with wild-type. The h5LOX Ala567Phe mutant generated *in silico* showed similar RMSD profile over the simulation compared to h5LOX wild-type and the values are within the threshold values indicating no significant structural distortions (Figure 4.44). RMSF analysis of mutant in comparison with wild-type showed 4 regions with considerable differences. The amino acid regions 147-150, 295-301, and 424 showed higher fluctuations while amino acid region 73-79 showed fewer fluctuations in the mutant. Amino acids 73-79 belong to the N-terminal domain whereas the other regions belong to catalytic domain of the h5LOX. Amino acid 424 which showed higher fluctuations is a sequence determinant of h5LOX and conversion of the same resulted shift in the positional specificity of the enzyme [Schwarz et al., 2001]. The amino acid regions 147-150 and 295-301 are located close to the site of mutation (Ala567Phe) and contribute for the binding site topology. Total number of hydrogen bonds were are quite similar throughout the simulation for h5LOX wild-type and h5LOX Ala567Phe mutant (Figure 4.50). In conclusion, introduction of phenylalanine altered the topology of the binding site that can be a probable reason for the observed loss of activity in h5LOX Ala567Phe mutant.

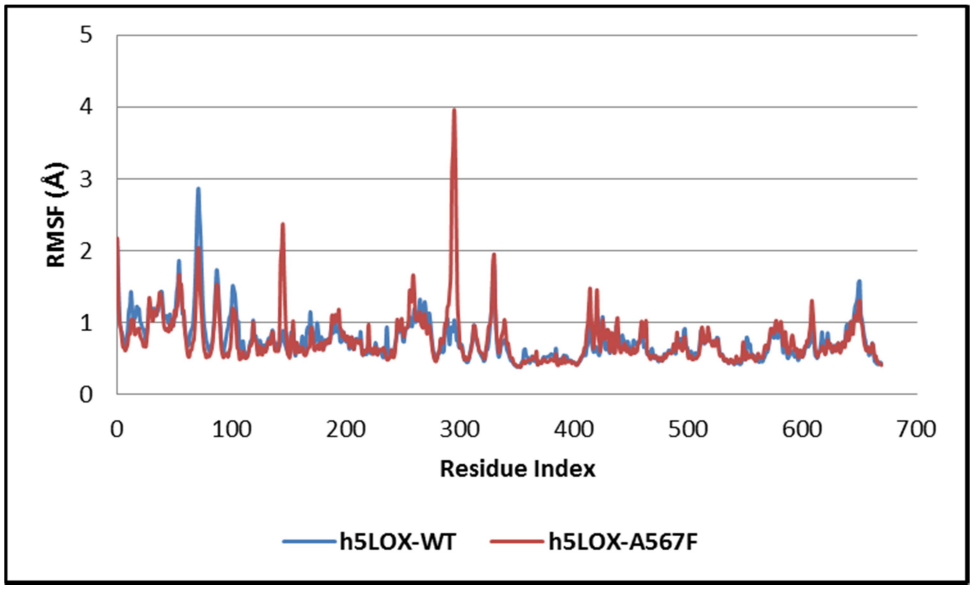

Figure 4.49: Root mean square fluctuations of the backbone atoms of the h5LOX wild-type and h5LOX Ala567Phe mutant

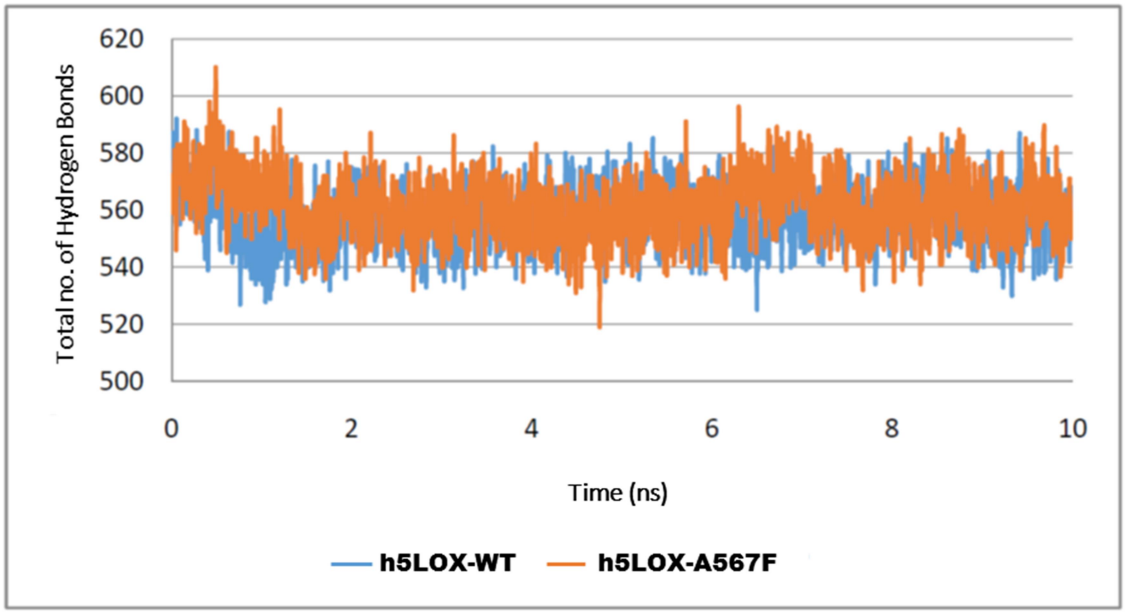

Figure 4.50: Total number of hydrogen bonds of the h5LOX wild-type and h5LOX Ala567Phe mutant over 10ns simulation

Molecular dynamic simulation studies for the exchange of alanine to phenylalanine at positions 558, 557, and 567 in r15LOX, p12SLOX, and h5LOX respectively indicated mutation induced structural modifications for the binding site. In case of p12SLOX and r15LOX, exchange altered the functionality of a helix that plays a crucial role upon ligand/substrate binding. Whereas mutation in h5LOX induced modifications in the structural flexibility of the binding site amino acids which will lead alterations in topology that is very critical for any enzyme. The observed effects could be due to steric inference caused by bigger phenylalanine. Based on these results, it can be concluded that exchange of alanine to phenylalanine will affect the binding site orientation leading to impaired functionality of the enzyme.

4.3.4 Molecular Dynamic Simulations of r15LOX Enzyme/Arachidonic acid Complexes

The effect of amino acid exchange on the substrate binding and its conformational changes in the active site was monitored by performing molecular dynamics simulation analysis of enzyme and substrate complex. There are no crystal coordinates to any of the mammalian LOXs with substrate in the active site and there is lack of proper force field parameters for iron to generate accurate molecular models. Hence, we have performed molecular dynamics simulation analysis only for r15LOX catalytic domain containing substrate arachidonic acid.

Saam et.al [Saam et al., 2007] has developed force field parameters for the non-heme iron in r15LOX by employing density functional theory for the coordination complex. The developed parameters were then used for performing molecular dynamics simulation to identify oxygen channels that are crucial for oxygenation in LOXs. Prior to simulations, they have generated the enzyme/substrate complex of r15LOX catalytic domain & arachidonic acid and equilibrated the solvated complex for 50ps for simulation analysis. They excluded the N-terminal domain as r15LOX was shown to be enzymatically active without this domain and removal of the same will reduce computational power [Saam et al., 2007]. The same equilibrated complex (C-terminal domain and arachidonic acid) was taken for performing simulation studies in the present study. The same methodologies and protocols used in previous simulations were followed except that the simulation was only for 5ns. The changes in RMSF, RMSD and number of hydrogen bonds over 5ns simulation were analysed and compared with wild type r15LOX. The RMSD profile of the arachidonic acid in the wild-type and mutants was also analysed. The effect of mutation on the interaction profile of arachidonic acid with the protein was analysed and the type of interactions analysed are hydrogen bonds, water bridges and hydrophobic interactions.

4.3.4.1 Rabbit 15-Lipoxygenase C-terminal domain

The crystal structure for r15LOX was solved at a resolution of 1.4 Å by Choi J et.al in the year 2008 [Choi et al., 2007]. The protein crystal was a dimer of which one of the monomer contained inhibitor RS7 and other with none. Comparison of the binding sites revealed that the inhibitor bound protein is 1.4 times bigger than that of substrate/inhibitor free enzyme and small angle X-ray scattering experiments of rLOX15 showed the possibility of inter domain movement [Choi et al., 2007]. Hence, prior to studies on the effect of mutation on substrate binding and its conformational changes, we analysed the effect of substrate binding on the protein. To check this, we have performed 5ns simulation calculation for arachidonic acid bound and apo enzyme. The catalytic domain of r15LOX with arachidonic acid developed by Saam et.al was taken [Saam et al., 2007], pre-processed, solvated, and relaxed prior to calculation. Two simulation calculations of 5 ns were performed for the catalytic domain of r15LOX with and without arachidonic acid.

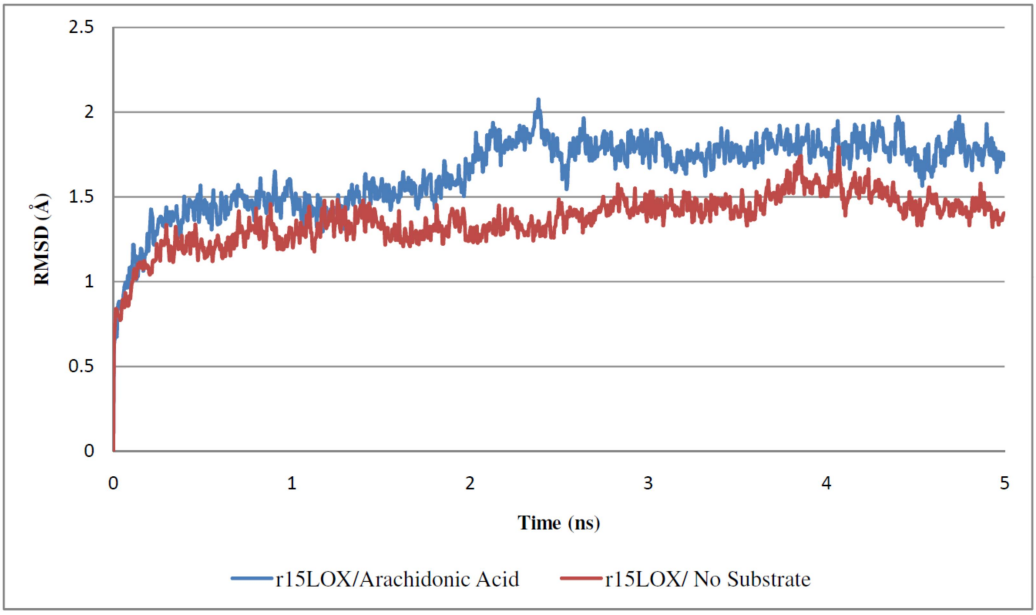

Figure 4.51: Root mean square deviations of backbone atoms for the C-terminal domains of r15LOX apo-enzyme and r15LOX/arachidonic acid complex

The RMSD profiles of both the calculations are shown in the above Figure 4.51. The catalytic domain r15LOX showed RMSD values in the range of 1 and 1.5 Å whereas for substrate bound complex the RMSD range between 1.5 and 2 Å (Figure 4.51). The difference in RMSD can be correlated with the structural modifications r15LOX undergo due to substrate binding. Comparison of RMSF profile of both the simulations did not show any significant differences except for few residues (Figure 4.52). The amino acids 120-126, 162-164, 201-207, and 573-575 showed higher fluctuations in substrate bound r15LOX in comparison with substrate free r15LOX. On the other hand, amino acids 188-195 & 355-359 showed fewer fluctuations. Interestingly, amino acids of substrate bound r15LOX which showed higher fluctuations won't contribute for binding site whereas amino acids showing lesser fluctuations are binding site amino acids. This analysis suggests that substrate binding may stabilize the binding site leading to lesser fluctuations of the binding site amino acids.

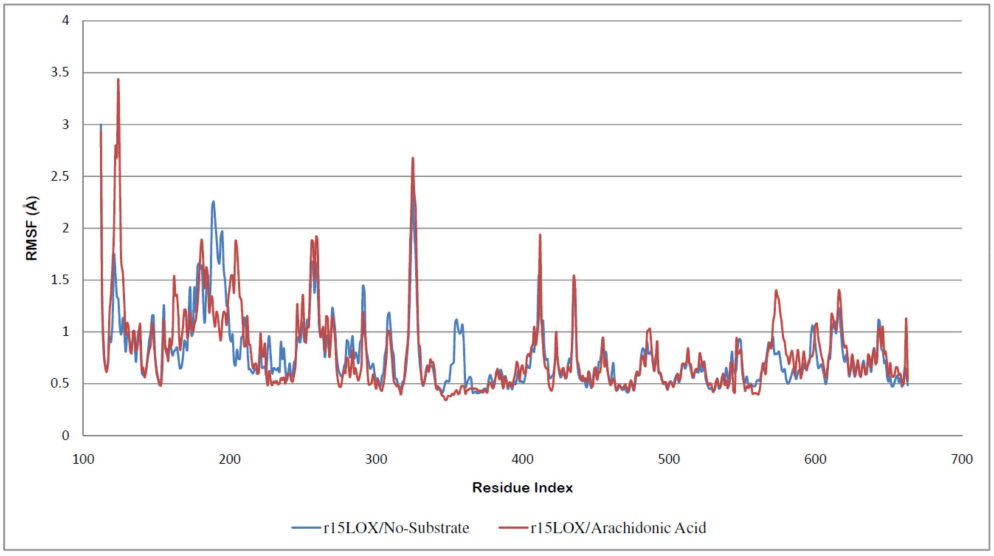

Figure 4.52: Root mean square fluctuations of backbone atoms for the C-terminal domains of r15LOX apo-enzyme and r15LOX/arachidonic acid complex

Further, we monitored the interactions of r15LOX with arachidonic acid over the 5 ns simulation. Maximum interactions observed were hydrophobic interactions followed by water bridges and hydrogen bonds (Figure 4.53). The only amino acid contributed for hydrogen bond is Arg403. Amino acids Leu358, Leu362, Ile400, Ala404, Leu408, Val409, Phe412, Ile414, Phe415, Met418, Ile593, Gln596 and Leu597 formed hydrophobic interactions with arachidonic acid. However, none of these interactions stayed throughout the simulation indicating that ligand may have large conformational landscape. This analysis led to the identification of crucial amino acids that interacts with the substrate arachidonic acid in r15LOX.

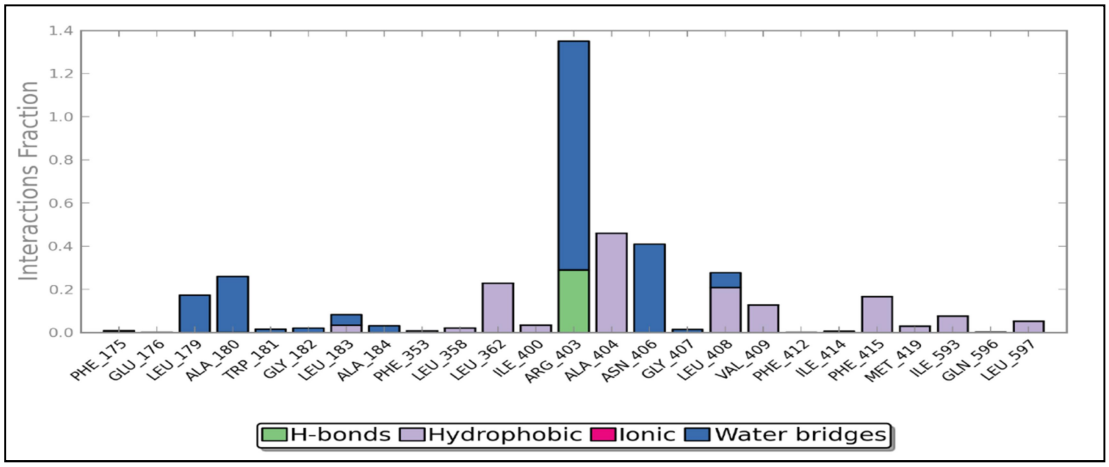

Figure 4.53: Fraction of various interactions formed by arachidonic acid with the binding site amino acids of r15LOX catalytic domain over 5 ns simulation

4.4.2 Rabbit 15-Lipoxygenase Phe415Ser

The effect of conversion from phenylalanine to serine on the substrate alignment and conformation changes of arachidonic acid in r15LOX were monitored by generating *in silico* mutation and by performing molecular dynamics. All the comparisons were done with respect r15LOX wild type/arachidonic acid complex. The RMSD values are less than 2 Å indicating there are no significant distortions from the native structure. The RMSD profile of wild-type and F415S mutant were shown in Figure 4.54. Wild-type r15LOX showed a significant distortion after 2 ns simulation which was not observed in the mutant. This could be due to mutation induced effect leading to reduced structural flexibility in the mutant variant.

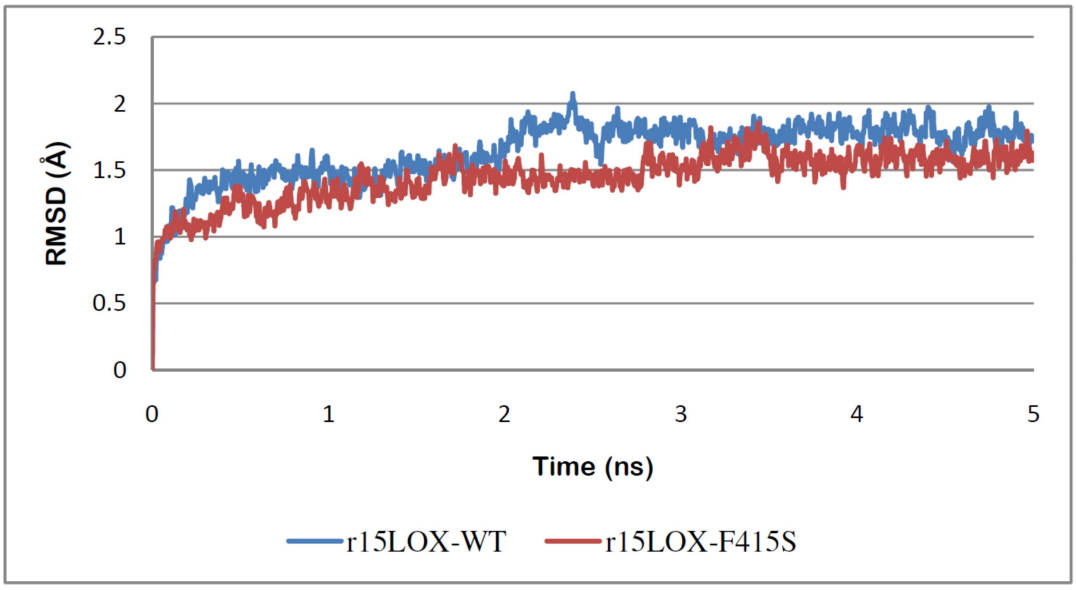

Figure 4.54: Root mean square deviations of backbone atoms for the C-terminal domains of r15LOX wild-type and r15LOX Phe415Ser mutant

Analysis of RMSF profile showed that residues of wild-type r15LOX had more fluctuations in comparison to that of mutant (Figure 4.55). More fluctuations indicate higher structural flexibility of the protein. The differences at n-terminal sequence of the catalytic domain were higher. The other significant differences observed were at the site of mutation. Interestingly, serine of mutant and phenylalanine of wild-type had similar fluctuation profile whereas neighbouring amino acids i.e. residues 410-414 showed reduced fluctuations in the mutant. These amino acids are upstream from 415 towards the carboxylic end of the substrate. In

addition, these amino acids formed hydrophobic interactions with the arachidonic acid in the wild-type for short fraction of the 5 ns simulation.

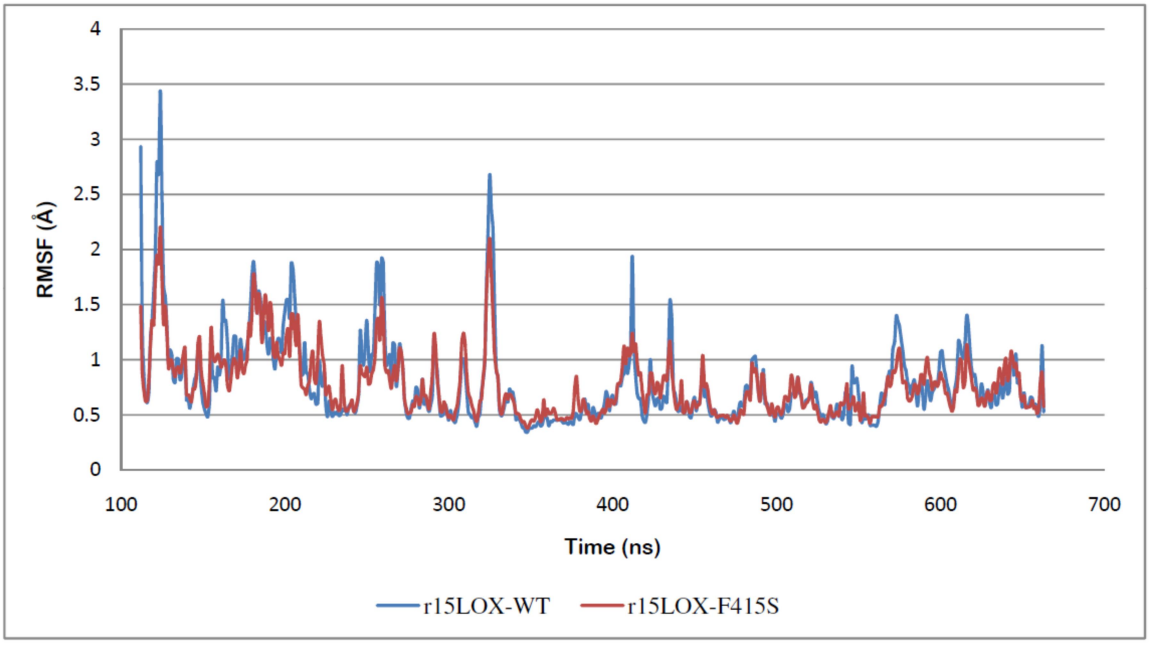

Figure 4.55: Root mean square fluctuations of backbone atoms for the C-terminal domains of r15LOX wild-type and r15LOX Phe415Ser mutant

Total numbers of hydrogen bonds are in between 450 and 500 throughout the simulation in both wild-type and mutant with no substantial changes. Further, the effect of mutation on binding conformation of the arachidonic acid in mutant was compared with that of wild-type. The RMSF profile of the arachidonic acid for the simulation showed higher fluctuations for the mutant compared to the wild type (Figure 4.56). Higher fluctuation implies that arachidonic acid took more conformational changes which may have affected the optimum favourable conformation for the catalysis.

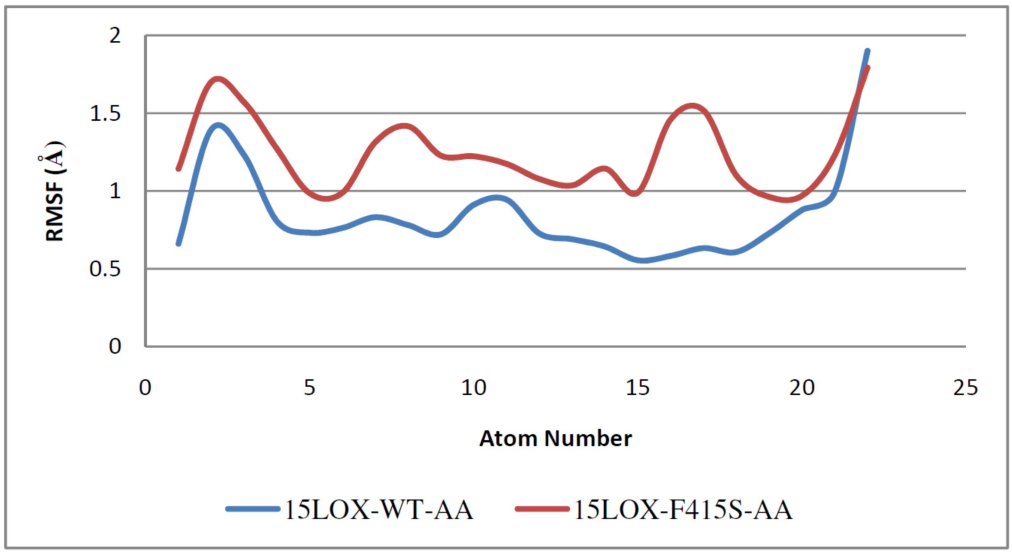

Figure 4.56: Root mean square fluctuations of arachidonic acid backbone atoms in the C-terminal domains of r15LOX wild-type and r15LOX Phe415Ser mutant

Further, we monitored the interactions formed by different amino acids with the arachidonic acid over the calculation in F415S mutant (Figure 4.57). The hydrogen bond formed by Arg403 persisted longer in the mutant compared to that of wild type. However, in wild-type the interaction with Arg403 was shuffled between water-bridge and hydrogen bond and stayed throughout the simulation. In case of the mutant, the interaction was only for a fraction of 0.65. The amino acids Leu408 and Val409, the positional determinants of 15LOX showed increased fractions of hydrophobic interactions. The phenylalanine 415 of wild-type formed hydrophobic interactions with arachidonic acid and the corresponding serine in mutant did not form any interactions. Overall, significant differences in the fractions of interactions formed by different amino acids were observed. The change in interacting amino acids could be one of the reasons for the distribution of favourable binding of the substrate.

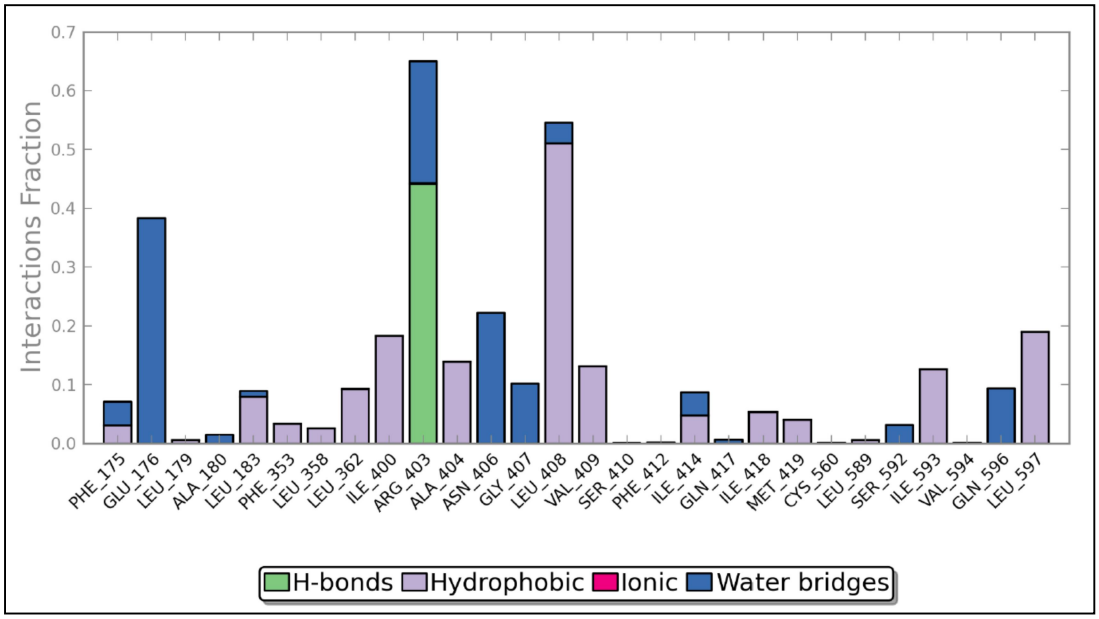

Figure 4.57: Interactions fraction of arachidonic acid binding amino acids of r15LOX Phe415Ser catalytic domain over 5 ns Simulation

Molecular dynamic simulation studies give valuable insights on the structural flexibility, conformational flexibility accessible and the interaction profile of the binding partners of the biological molecules [Hospital et al., 2015]. Recently, the molecular dynamic simulation calculations are being widely used to study the structural re-arrangements [Gebremichael et al., 2008], conformational changes, modifications in the chemical environment of catalytic domain upon ligand binding [Young et al., 2007], allosteric regulation [Vettoretti et al., 2016], and mutation-induced structural changes [Linder et al., 2015] of the proteins and nucleic acids. However, there are very few studies that exploited the advantage of molecular dynamics in understanding the structural and functional properties of LOXs. This could be due to improper force field parameters available for catalytic iron present in LOXs which will mediate the initial step of enzyme catalysis. In the present study, we used molecular dynamics to address the molecular mechanisms involved in phenylalanine to serine (h15LOX Phe414Ser, h12SLOX Phe414Ser, and h5LOX Phe421Ser) and alanine to phenylalanine (h15LOX Ala557Phe, h12SLOX Ala557Phe, h5LOX Ala567Phe) conversions that showed significant differences with wild-types.

Molecular dynamic simulation studies for the exchange of phenylalanine to serine at positions 415, 414, and 421 in r15LOX, p12SLOX, and h5LOX respectively showed no effects

on the binding site topology while the exchange of alanine to phenylalanine at positions 558, 557, and 567 in r15LOX, p12SLOX, andh5LOX respectively indicated mutation induced structural modifications in the binding site. Based on these studies, loss of function can be associated with absence of phenylalanine interaction (Phe414, Phe414, and Phe421 in h15-, h12S-, h5-LOXs respectively) that is essential for substrate binding while change in the binding site topology by the mutated phenylalanine (Phe557, Phe557, and Phe567 in h15-, h12S-, h5-LOXs respectively). Further studies of r15LOX wild-type and r15LOX Phe415Ser mutant with AA in the binding sites showed increased fluctuations in the substrate binding suggesting the importance of phenylalanine at positions 414, 414, and 421 in h15LOX, h12SLOX, and h5LOX respectively. The orientation of the substrate plays a very important role in LOXs as it defines the positional specificity and catalysis of the reaction [Newcomer and Brash, 2015]. Exchange of phenylalanine with serine will provide more space for the AA leading to more conformational landscape and also the observed hydrophobic interactions by phenylalanine was absent by serine in the mutant. The exchange of phenylalanine with tryptophan resulted in increased relative enzyme activity while with isoleucine resulted in reduced enzyme activity [Gan et al., 1996]. Taken together, it is evident that phenylalanine plays a very critical role in orienting the substrate that is suitable for catalysis.

CHAPTER 5

CONCLUSIONS

LOXs are non-heme iron containing dioxygenases that catalyse PUFAs to produce respective hydroperoxy derivatives. This leads to the production of different classes of lipid mediators that are responsible for various cellular functions and pathological implications. As these enzymes metabolize same substrates to different type of mediators that have contradictory roles in the disease milieu. LOX-derived metabolites are associated with auto-immune diseases, cancers, allergic diseases, and asthma. In addition, genetic variations in LOXs have been attributed for increased risk in many diseases such as coronary artery disease, breast cancer, osteoporosis, bronchial asthma, schizophrenia, and autosomal recessive congenital ichthyosis. Hence, characterizing the active site amino acids that are responsible for enzyme catalysis and specificity is essential. Various studies in this line have been performed and the sequence determinants that are responsible for positional specificity and regio-specificity have been reported. However most of those studies concentrated on the bottom of the active site but not at the core or top of the active site. In the present study, in order to identify the crucial amino acids that are located in different regions of the active sites of the human LOXs, we employed molecular modelling approaches along with site-directed mutagenesis strategies. Sequence analysis and receptor based pharmacophore models for h5LOX, p12SLOX and r15LOX binding sites resulted in identification of unique and different amino acids specific for each LOXs. Further, the identified important amino acids were validated for their roles by generating mutations in h5LOX, h12SLOX, h12RLOX and h15LOX. A total of 16 mutants were generated and were evaluated for their effects on enzyme catalysis and positional specificity.

Change in charge by mutating amino acid glutamic acid at positions 356 and 356 at the core of the active sites in h15LOX and h12SLOX respectively to glutamine didn't alter the positional specificity of both the mutants indicating charged amino acid won't play any role in the specificity of LOXs. The amino acid glycine 364 and threonine 364 of h15LOX

and h12SLOX respectively didn't affect the positional specificity and catalytic efficiency of the enzymes. Phenylalanine at positions 414, 414, and 421 in h15LOX, h12SLOX, and h5LOX respectively and serine at position 451 in h12RLOX are very critical for enzyme catalysis and positional specificity. Molecular dynamic simulation studies of r15LOX Phe414Ser, p12SLOX Phe414Ser, and h5LOX Phe421Ser and their respective wild-type suggested probable role of phenylalanine in controlling the optimal orientation of the arachidonic that is crucial for catalysis. Conversion of alanine to phenylalanine at 557, 557, 567 positions in h15LOX, h12SLOX, and h5LOX respectively appear to interfere with enzyme catalysis leading to complete loss of activity in the mutants. Conversely, conversion of phenylalanine to alanine at corresponding position (595) in h12RLOX had no effect on the enzyme functionality of the mutant. Molecular dynamics studies showed mutation induced variations in the structural topology of the binding site amino acids that play a critical role for substrate binding and catalysis. Amino acids glycine at position 597 in h15LOX and amino acid serine at position 598 in h12SLOX will not affect the positional specificity or enzyme catalysis when the polarity of them is altered. Changes in the volume of valine 659 and isoleucine 660 of h15LOX and h12SLOX respectively won't affect the positional specificity but may affect the enzyme catalysis and stereospecificity. In conclusion, the present study led to identification of critical amino acids that play imperative role in enzyme catalysis and positional specificity of human LOXs that are key layers in inflammation in cancer.

CHAPTER 6

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RESEARCH ARTICLE Open Access

Exploration of binding site pattern in arachidonic acid metabolizing enzymes, Cyclooxygenases and Lipoxygenases

Kakularam Kumar Reddy[1], Veena Kumari Vidya Rajan[2], Ashish Gupta[3], Polamarasetty Aparoy[3*] and Pallu Reddanna[1,4*]

Abstract

Background: Cyclooxygenase (COXs) and Lipoxygenase (LOXs) pathways are the two major enzymatic pathways in arachidonic acid (AA) metabolism. The term eicosanoid is used to describe biologically active lipid mediators including prostaglandins, thromboxanes, leukotrienes and other oxygenated derivatives, which are produced primarily from AA. Eicosanoids generated in a tissue specific manner play a key role in inflammation and cancer. As AA is the substrate common to variety of COXs and LOXs, inhibition of one pathway results in diversion of the substrate to other pathways, which often is responsible for undesirable side effects. Hence there is need for development of not only isozyme specific inhibitors but also dual/multi enzyme inhibitors. Understanding the interactions of AA and characterizing its binding sites in these enzymes therefore is crucial for developing enzyme specific and multi enzyme inhibitors for enhancing therapeutic efficacy and/or overcoming side effects.

Results: AA binding sites in COXs and LOXs are identified and compared by the development of receptor based pharmacophore using MultiBind. Physico chemical properties were compared to understand the details of the binding sites in all the enzymes and to elucidate important amino acids that can be targeted for drug design. The alignment of AA binding sites in the seven enzymes COX-1, COX-2, 5-LOX, 12-LOX, 15-LOX and plant soybean LOX-1 and LOX-3 indicated a common pattern of five common interacting groups. In the same way, comparison of AA binding sites was done pair wise and by multiple alignment in various combinations. It has been identified that aliphatic and aromatic interactions are the most common in all the enzymes. In addition interactions unique to each one of these enzymes were identified.

Conclusion: The complete analysis of AA binding sites in the seven enzymes was performed; 120 combinations for the seven enzymes were studied in detail. All the seven enzymes are structurally quite different, yet they share AA as the common binding partner. Comparisons in various combinations showed how they are similar and dissimilar with each other. This information will be helpful in designing specific as well as common inhibitors.

Keywords: Cyclooxygenase, Lipoxygenase, Arachidonic acid, Specific inhibitors, Receptor based pharmacophore, Drug design

Background

Arachidonic acid (AA), the major polyunsaturated fatty acid (PUFA) present in mammalian systems, is oxygenated by three important pathways – the cyclooxygenase (COX), the lipoxygenase (LOX) and the epoxygenase, to form biologically active molecules such as prostaglandins (PGs), leukotrienes (LTs), and epoxyeicosatrienoic acids (EETs), collectively called as eicosanoids [1].

COX is a bifunctional heme containing enzyme that catalyzes the biosynthesis of PGs from AA. It is bifunctional enzyme and exhibits cyclooxygenase and peroxidase activities. It introduces two molecules of oxygen into AA to form PGG_2, a cyclic hydroperoxy endoperoxide, which is subsequently reduced by peroxidase to give hydroxy endoperoxide, PGH_2 [2]. There are three COX isoforms, COX-1, COX-2, and COX-3 [1-3]. COX-1,

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Understanding the Dual Inhibition of COX-2 and Carbonic Anhydrase-II by Celecoxib and CG100649 Using Density Functional Theory Calculations and other Molecular Modelling Approaches

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Abstract: Recent developments in the dual inhibition studies of cyclooxygenase-2 (COX-2) and carbonic anhydrase (CA-II) imply a promising platform for the development of new generations of non-steroidal anti-inflammatory drugs (NSAIDs). CG100649 is such a substance that got recently approved by Korean Ministry of Food and Drug safety (MFDS) and is being marketed by the name polmacoxib for the treatment of osteoarthritis. CG100649 significantly inhibits CA-II in blood and COX-2 in inflammatory tissues. However, the mechanism of CG100649 dual inhibition of COX-2/CA-II is not well understood. In this study, we employed well known methods like pharmacophore modelling, A DFT based quantum chemical descriptors analysis, and molecular docking to explore the chemical features and to understand the binding behaviour of CG100649 along with other COX-2/CA-II dual inhibitors. The HOMO-LUMO and docking results indicated the prominent role of aryl sulphonamide in CG100649. The aryl sulphonamide moiety formed T-shaped Π…Π interactions with His94 in the CA-II active site, which was not observed in the case of celecoxib. Other crucial interactions were also observed which may aid in further understanding the action of dual inhibitors of this class.

Keywords: CA-II, COX-2, Docking, HOMO, Ligand based Pharmacophore, LUMO, Receptor based pharmacophore.

1. INTRODUCTION

Cyclooxygenases (COXs) are a group of enzymes that catalyze the rate limiting step in the conversion of arachidonic acid to various prostaglandins (PGs) and thromboxane [1]. There are two major isoforms of COXs, constitutively expressed COX-1, which helps in stomach mucous production, regulation of gastric acid secretion, and kidney water excretion, and inducible COX-2, which is associated with pain and various inflammatory diseases [2]. Few reports also suggest that COX-2 is constitutively expressed in kidneys [3, 4]. Most of the traditional non-steroidal anti-inflammatory drugs (NSAIDs) are non-selective and act on both COX-1 and COX-2, which causes gastrointestinal and renal side effects. The gastrointestinal erosions reported by the use of first generation NSAIDs were shown to be due to the inhibition of both COX-1 and COX-2. The discovery of COX-2 as the key player in various inflammatory disorders led to the development of specific COX-2 inhibitors called "COXIBs", which contain a sulfonamide group. Some studies associate COX-2 selective inhibitors with an increased risk of cardiovascular side effects [5, 6], which has resulted in the withdrawal of some COXIBs [4-8]. The cardiovascular side effects have been attributed to the shift of arachidonic acid (AA) towards LOX pathway [7], which initiated a growing interest in developing COX-2/5-LOX dual inhibitors (CLOXIBs) along with other alternative approaches to develop safer NSAIDs. Recently, nitric oxide (NO) and hydrogen sulfide (H_2S) releasing NSAIDs and NSAIDs releasing have been reported as effective alternatives [8].

One of the recent crucial findings in the development of anti-inflammatory drugs is the discovery that COXIBs (sulfonamide COX-2 inhibitors) also inhibit carbonic anhydrase (CA) isoforms, especially CA-II. Celecoxib inhibited CA-II at the nanomolar level. Furthermore, a X-ray crystal structure was reported for CA-II complexed with celecoxib [9]. The binding of COXIBs to both COX-2 and CA-II is very interesting, because the inhibition of enzyme CA-II, which is critical for the development and invasion of cancer cells, may constitute an important mechanism of antitumor action of such sulfonamide compounds [10]. Nanomolar affinity of sulfonamide containing COX-2 inhibitors for CA-II might cause diuretic effects that could minimize COX-2 induced renal hypertension but not yet confirmed in clinical trials [11, 12]. The inhibition of α-carbonic anhydrase by drugs with sulfonamide moiety was studied earlier [13].

CG100649, a dual inhibitor for COX-2 and CA-II, is a novel NSAID drug candidate proposed with a new mode of "tissue-specific" activity intended to deliver sustained levels of drug to inflamed tissues and also responsible to maintain low systemic exposure by binding to CA in red blood cells.

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Review

Evolutionary aspects of lipoxygenases and genetic diversity of human leukotriene signaling

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ABSTRACT

Leukotrienes are pro-inflammatory lipid mediators, which are biosynthesized via the lipoxygenase pathway of the arachidonic acid cascade. Lipoxygenases form a family of lipid peroxidizing enzymes and human lipoxygenase isoforms have been implicated in the pathogenesis of inflammatory, hyperproliferative (cancer) and neurodegenerative diseases. Lipoxygenases are not restricted to humans but also occur in a large number of pro- and eucaryotic organisms. Lipoxygenase-like sequences have been identified in the three domains of life (bacteria, archaea, eucarya) but because of lacking functional data the occurrence of catalytically active lipoxygenases in archaea still remains an open question. Although the physiological and/or pathophysiological functions of various lipoxygenase isoforms have been studied throughout the last three decades there is no unifying concept for the biological importance of these enzymes. In this review we are summarizing the current knowledge on the distribution of lipoxygenases in living single and multicellular organisms with particular emphasis to higher vertebrates and will also focus on the genetic diversity of enzymes and receptors involved in human leukotriene signaling.

Contents

Abbreviations: AA, arachidonic acid; EPA, 5,8,11,14,17-eicosapentaenoic acid; DHA, 4,7,10,13,16,19-docosahexaenoic acid; LOX, lipoxygenase; COX, cyclooxygenase; PG, prostaglandins; Tx, thromboxane; cPLA2, cytosolic phospholipase 2; LTA4H, leukotriene A4 hydrolase; LTC4S, leukotriene C4 synthase; GGT, gamma-glutamyltranspeptidase; DPEP, dipeptidase; Mya, million years ago; Bya, billion years ago; LX, lipoxins; RS, resolvins.

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Leukotriene signaling in the extinct human subspecies *Homo denisovan* and *Homo neanderthalensis*. Structural and functional comparison with *Homo sapiens*

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ABSTRACT

Mammalian lipoxygenases (LOXs) have been implicated in cell differentiation and in the biosynthesis of pro- and anti-inflammatory lipid mediators. The initial draft sequence of the *Homo neanderthalensis* genome (coverage of 1.3-fold) suggested defective leukotriene signaling in this archaic human subspecies since expression of essential proteins appeared to be corrupted. Meanwhile high quality genomic sequence data became available for two extinct human subspecies (*H. neanderthalensis*, *Homo denisovan*) and completion of the human 1000 genome project provided a comprehensive database characterizing the genetic variability of the human genome. For this study we extracted the nucleotide sequences of selected eicosanoid relevant genes (ALOX5, ALOX15, ALOX12, ALOX15B, ALOX12B, ALOXE3, COX1, COX2, LTA4H, LTC4S, ALOX5AP, CYSLTR1, CYSLTR2, BLTR1, BLTR2) from the corresponding databases. Comparison of the deduced amino acid sequences in connection with site-directed mutagenesis studies and structural modeling suggested that the major enzymes and receptors of leukotriene signaling as well as the two cyclooxygenase isoforms were fully functional in these two extinct human subspecies.

Introduction

Leukotrienes (LTs)[2] are inflammatory lipid mediators, which have been implicated in the pathogenesis of cardiovascular, hyperproliferative and neurodegenerative disorders [1,2]. They are formed from free arachidonic acid [2] *via* a cascade of consecutive enzymatic reactions and ALOX5 plays a major role as key enzyme in this process. After LTs are released from activated inflammatory cells they are delivered into the blood and bind to specific receptors on the surface of target cells. There are two principle types of LTs. (i) The cysteinyl LTs (LTC4, LTD4, LTE4) have been implicated as potent bronchoconstrictors in the pathogenesis of bronchial asthma [3] and LT synthesis inhibitors [4] as well as LT receptor antagonists [5] have been approved as anti-asthmatics. (ii) The dominant cysteinyl-free LT (LTB4) is a potent chemoattractant for neutrophils and plays a major role in innate immunity [6]. In addition to ALOX5, which constitutes the key enzyme in leukotriene biosynthesis, the *Homo sapiens* genome involves five other genes encoding for functional LOX-isoforms (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOXE3). In plants [7], lower marine organisms [8], fungi [9] and bacteria [10] LOX-isoforms have also been identified but little is known about the functionality of these ALOX-isoforms [11]. Three years ago a draft sequence of the *Homo neanderthalensis* genome has been published [12] and on the basis of their sequence data the authors suggested compromised leukotriene signaling in this archaic human subspecies since the gene encoding for the cysteinyl leukotriene receptor 2 (cysLTR2) was corrupted [12]. When we compared the amino acid sequence of the six human LOX isoforms with the primary structure of the *H. neanderthalensis* orthologs [13] we found that four of the six *H. sapiens* orthologs appeared to be functional

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[2] *Abbreviations used:* ALOX5, arachidonate 5-lipoxygenase; ALOX15, arachidonate 15-lipoxygenase; ALOX12, arachidonate 12-lipoxygenase; ALOX15B, arachidonate 15-lipoxygenase-2; ALOX12B, arachidonate 12R-lipoxygenase; ALOXE3, arachidonate epidermal lipoxygenase-3; COX1, cyclooxygenase-1; COX2, cyclooxygenase-2; LTA4H, leukotriene A4 hydrolase; LTC4S, leukotriene C4 synthase; ALOX5AP, arachidonate 5-lipoxygenase activating protein; CYSLTR1, cysteinyl leukotriene receptor 1; CYSLTR2, cysteinyl leukotriene receptor 2; BLTR1, leukotriene B4 receptor 1; BLTR2, leukotriene B4 receptor 2; LT, leukotrienes; LOX, lipoxygenase; HETE, hydroxyeicosatetraenoic acid.

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RESEARCH ARTICLE

The Augmenting Effects of Desolvation and Conformational Energy Terms on the Predictions of Docking Programs against mPGES-1

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Abstract

In this study we introduce a rescoring method to improve the accuracy of docking programs against mPGES-1. The rescoring method developed is a result of extensive computational study in which different scoring functions and molecular descriptors were combined to develop consensus and rescoring methods. 127 mPGES-1 inhibitors were collected from literature and were segregated into training and external test sets. Docking of the 27 training set compounds was carried out using default settings in AutoDock Vina, AutoDock, DOCK6 and GOLD programs. The programs showed low to moderate correlation with the experimental activities. In order to introduce the contributions of desolvation penalty and conformation energy of the inhibitors various molecular descriptors were calculated. Later, rescoring method was developed as empirical sum of normalised values of docking scores, LogP and Nrotb. The results clearly indicated that LogP and Nrotb recuperate the predictions of these docking programs. Further the efficiency of the rescoring method was validated using 100 test set compounds. The accurate prediction of binding affinities for analogues of the same compounds is a major challenge for many of the existing docking programs; in the present study the high correlation obtained for experimental and predicted pIC_{50} values for the test set compounds validates the efficiency of the scoring method.

Introduction

Microsomal prostaglandin E synthase-1 (mPGES-1) belongs to the membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) super family [1]. It is the terminal enzyme in the metabolism of arachidonic acid (AA) via the cyclooxygenase (COX) pathway (particularly COX-2), responsible for the conversion of prostaglandin H_2 (PGH_2) to a more stable product prostaglandin E_2 (PGE_2). As PGE_2 is a key mediator of pain and

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Functional characterization of genetic enzyme variations in human lipoxygenases ☆

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ABSTRACT

Mammalian lipoxygenases play a role in normal cell development and differentiation but they have also been implicated in the pathogenesis of cardiovascular, hyperproliferative and neurodegenerative diseases. As lipid peroxidizing enzymes they are involved in the regulation of cellular redox homeostasis since they produce lipid hydroperoxides, which serve as an efficient source for free radicals. There are various epidemiological correlation studies relating naturally occurring variations in the six human lipoxygenase genes (SNPs or rare mutations) to the frequency for various diseases in these individuals, but for most of the described variations no functional data are available. Employing a combined bioinformatical and enzymological strategy, which included structural modeling and experimental site-directed mutagenesis, we systematically explored the structural and functional consequences of non-synonymous genetic variations in four different human lipoxygenase genes (ALOX5, ALOX12, ALOX15, and ALOX15B) that have been identified in the human 1000 genome project. Due to a lack of a functional expression system we resigned to analyze the functionality of genetic variations in the hALOX12B and hALOXE3 gene. We found that most of the frequent non-synonymous coding SNPs are located at the enzyme surface and hardly alter the enzyme functionality. In contrast, genetic variations which affect functional important amino acid residues or lead to truncated enzyme variations (nonsense mutations) are usually rare with a global allele frequency $< 0.1\%$. This data suggest that there appears to be an evolutionary pressure on the coding regions of the lipoxygenase genes preventing the accumulation of loss-of-function variations in the human population.

Introduction

Lipoxygenases (LOXs) are lipid-peroxidizing enzymes that catalyze the dioxygenation of poly-unsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene structure [1]. Together with various oxygen activating enzymes LOXs increase the cellular oxidizing potential by biosynthesizing lipid hydroperoxides, which serve as an efficient source for free radicals unless they are reduced by peroxide reducing enzymes to the corresponding alcohols [2,3]. LOXs are widely distributed in terrestrial life since they occur in bacteria, fungi, lower marine organisms, plants and higher animals [4,5]. In humans six functional LOX genes (ALOX5, ALOX12, ALOX15, ALOX15B, ALOX12B, and ALOXE3) have been identified [1]. The crystal structures of three different mammalian LOX isoforms (human ALOX5, rabbit ALOX15, porcine ALOX15) have been solved [6–8] and despite subtle structural differences there is a high degree of structural similarity between different LOX isoforms. Human LOXs (hALOX) have been implicated in the pathogenesis of cardiovascular and inflammatory diseases as well as in various forms of cancer but the detailed mechanistic basis for their pathophysiological roles is still a matter of discussion [9–12].

In different epidemiological correlation studies genetic variations in hALOX genes have been related to an increased risk of colorectal cancer [13,14], stroke [15], bronchial asthma [16] and

Abbreviations: LOXs, lipoxygenases; ALOX, arachidonate lipoxygenase; 5-H(p)ETE, (6E,8Z,11Z,14Z)-5-hydroperoxyeicosa-6,8,11,14-tetraenoic acid; 15-H(p)ETE, (5Z,8Z,11Z,13E)-15-hydroperoxyeicosa-5,8,11,13-tetraenoic acid; 12-H(p)ETE, (5Z,8Z,10E,14Z)-12-hydroperoxyeicosa-5,8,10,14-tetraenoic acid; 8-H(p)ETE, (5Z,9E,11Z,14Z)-8-hydroperoxyeicosa-5,9,11,14-tetraenoic acid; IPTG, Isopropyl-β-D-thiogalactopyranosid; HETE, hydroxyeicosatetraenoic acid; H(p)ETE, hydroperoxyeicosatetraenoic acid; LTA_4, 4-[(2S,3S)-3-[(1E,3E,5Z,8Z)-tetradeca-1,3,5,8-tetraen-1-yl]oxiran-2-yl]butanoic acid; LTB_4, 5(S),12(R)-dihydroxy-6,8,10,14-(Z,E,E,Z)-eicosatetraenoic acid; LTC_4, (5S,6R,7E,9E,11Z,14Z)-6-{[(2R)-2-[(4S)-4-amino-4-carboxy-butanamido]-2-[(carboxymethyl) carbamoyl]ethyl]sulfanyl}-5-hydroxyeicosa-7,9,11,14-tetraenoic acid; UTR, untranslated region

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Conversion of pro-inflammatory murine Alox5 into an anti-inflammatory 15S-lipoxygenating enzyme by multiple mutations of sequence determinants

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ABSTRACT

5-Lipoxygenase (ALOX5) is a key enzyme in biosynthesis of pro-inflammatory leukotrienes whereas 15-lipoxygenases (ALOX15) have been implicated in the formation of pro-resolving eicosanoids (lipoxins, resolvins). Although mammalian LOX-isoforms share a high degree of structural similarity X-ray coordinates indicated that the substrate-binding pocket of ALOX5 is some 20% bigger than that of ALOX15 suggesting the possibility of interconverting the two isoenzymes. To test this "space-based" hypothesis we reduced the volume of the substrate-binding pocket of mouse Alox5 by introducing space-filling amino acids at critical positions and found that multiple mutations at Phe359, Ala424, Asn425 and Ala603 of Alox5 led to gradual increase in 15-HETE formation. The Phe359Trp + Ala424Ile + Asn425Met Alox5 triple mutant was a major (67 ± 2%) 15-lipoxygenating enzyme and similar data were confirmed for human ALOX5. Structural modeling on the basis of the X-ray coordinates of ALOX5 indicated that the volume of the substrate-binding pocket inversely correlates with the share of 15-HETE biosynthesis for the human ($r^2 = 0.79$, $p < 0.05$) and the mouse ($r^2 = 0.59$, $p < 0.01$) enzyme. This data proves the principle possibility of converting pro-inflammatory 5-lipoxygenases to anti-inflammatory 15-lipoxygenases by reducing the volume of the substrate-binding pocket.

Introduction

Lipoxygenases (LOXs)[1] constitute a heterogeneous family of lipid-peroxidizing enzymes that catalyze the dioxygenation of free and/or esterified polyunsaturated fatty acids to their corresponding hydroperoxy derivatives [1–3]. Originally, mammalian LOXs have been categorized with respect to their positional specificity of arachidonic acid oxygenation but recently sequence-based classification systems have been introduced [1,4]. In humans six functional LOX-genes (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOX5, eLOX3) exist which encode for six LOX-isoforms [4]. Knockout experiments on Alox12B as well as epidemiological studies on naturally occurring human mutants [5,6] implicated these LOX-isoforms in epidermal differentiation. In contrast, Alox15 [7], Alox12 [8] and Alox5 [9] knockout mice do not show major phenotypic defects unless challenged in special ways.

Many LOX isoforms such as ALOX12 [10] or ALOX15B [11] oxygenate fatty acid substrates to a single chiral product isomer (singular specificity). Other enzymes, such as ALOX15 from different species, exhibit dual [12,13] or even multiple [14] positional specificity. The structural basis for the reaction specificity of various LOX isoforms has been studied in the past and a number of specificity determinants have been identified [2,4]. However, there is no concept explaining the reaction specificity of all LOX isoforms.

Among mammalian LOX isoforms 5-LOX has drawn particular attention because of its critical role in leukotriene biosynthesis [15]. In contrast, 12/15-LOX has been implicated in biosynthesis of resolving eicosanoids [16] and thus, may have anti-inflammatory potential. Although the crystal structure of a stabilized chimeric version of human ALOX5 has recently been solved [17] and X-ray coordinates for an arachidonic acid complex of the Ser663Asp mutant of this protein [18] have been released, the structure of the productive enzyme–substrate complex still remains a matter of discussion. For the time being, there are two hypotheses rationalizing the mechanistic differences between arachidonic acid

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[1] Abbreviations used: LOX, lipoxygenase; ALOX15, human 12/15-lipoxygenase; ALOX5, human 5-lipoxygenase; Alox15, mouse 12/15-lipoxygenase; Alox5, mouse 5-lipoxygenase; 5(±)-HETE, 5(±)-hydroxy-6,8,11,14(E,Z,Z,Z)-eicosatetraenoic acid; 8(±)-HETE, 8(±)-hydroxy-5,9,11,14(Z,E,Z,Z)-eicosatetraenoic acid; 9(±)-HETE, 9(±)-hydroxy-5,7,11,14(E,Z,Z,Z)-eicosatetraenoic acid; 11(±)-HETE, 11(±)-hydroxy-5,8,12,14(Z,Z,E,Z)-eicosatetraenoic acid; 12(±)-HETE, 12(±)-hydroxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid; 15(±)-HETE, 15(±)-hydroxy-5,8,11,13(Z,Z,Z,E)-eicosatetraenoic acid; RP-HPLC, reverse phase high performance liquid chromatography; SP-HPLC, straight phase high performance liquid chromatography; IPTG, isopropyl-â-D-1-thiogalactopyranoside.

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Molecular basis for the catalytic inactivity of a naturally occurring near-null variant of human ALOX15

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ABSTRACT

Mammalian lipoxygenases belong to a family of lipid-peroxidizing enzymes, which have been implicated in cardiovascular, hyperproliferative and neurodegenerative diseases. Here we report that a naturally occurring mutation in the hALOX15 gene leads to expression of a catalytically near-null enzyme variant (hGly422Glu). The inactivity may be related to severe misfolding of the enzyme protein, which was concluded from CD-spectra as well as from thermal and chemical stability assays. *In silico* mutagenesis experiments suggest that most mutations at hGly422 have the potential to induce sterical clash, which might be considered a reason for protein misfolding. hGly422 is conserved among ALOX5, ALOX12 and ALOX15 isoforms and corresponding hALOX12 and hALOX5 mutants also exhibited a reduced catalytic activity. Interestingly, in the hALOX5 Gly429Glu mutants the reaction specificity of arachidonic acid oxygenation was shifted from 5S- to 8S- and 12R-H(p)ETE formation. Taken together, our data indicate that the conserved glycine is of functional importance for these enzyme variants and most mutants at this position lose catalytic activity.

1. Introduction

Lipoxygenases (LOX) are non-heme iron containing enzymes that catalyze the dioxygenation of poly-unsaturated fatty acids containing a *cis*/*cis* 1,4-pentadiene structure to their corresponding hydroperoxides [1,2]. LOX genes occur in a few bacteria, fungi, plants and animals but are lacking in archae [3–6]. According to the positional specificity of arachidonic acid oxygenation mammalian LOXs can be classified as 5-LOX, 8-LOX, 12-LOX and 15-LOX [1,2]. The 3D-structures of various mammalian LOXs have been solved [7–10] and despite subtle structural differences the published X-ray coordinates indicate a high degree of structural similarity between the different isoforms.

Human arachidonate lipoxygenases (hALOX) have been implicated in the pathogenesis of cardio-vascular [11–14] and neurodegenerative diseases [15] but the mechanistic basis for their patho-physiological role is controversially discussed. In fact, pro- [16–19] and anti-atherogenic [20–23] activities have been reported for hALOX15 in different animal atherosclerosis models and thus, the precise role of the human enzyme in atherogenesis remains to be clarified. In an attempt to shed a light on this question, different case–control studies have been carried out in which single nucleotide polymorphisms (SNPs) in the ALOX15 gene were correlated with different read out parameters of cardio-vascular diseases such as the frequency of ischemic stroke [24], coronary artery disease [25,26] and myocardial infarction [27]. Unfortunately, the functional consequences of these mutations, especially for those localized in the 3′-UTR (rs916055) and intronic gene regions (rs7217186 + rs2619112) have not been explored in detail. Functional studies for the non-synonymous hALOX15 SNP Thr560Met (rs34210653) in exon 13 have demonstrated that this mutation may cause partial destruction of the hydrogen bonding network connecting hThr560 with active site residues [28] and these structural alterations

Abbreviations: LOXs, lipoxygenases; ALOX, arachidonate lipoxygenase; 15-H(p)ETE, (5Z,8Z,11Z,13E)-15-hydroperoxyeicosa-5,8,11,13-tetraenoic acid; 13-H(p)ODE, (9Z,11E,13S)-13-hydroperoxyoctadeca-9,11-dienoic acid; 12-H(p)ETE, (5Z,8Z,10E,14Z)-12-hydroperoxyeicosa-5,8,10,14-tetraenoic acid; 8-H(p)ETE, (5Z,9E,11Z,14Z)-8-hydroperoxyicosa-5,9,11,14-tetraenoic acid; IPTG, Isopropyl-β-D-thiogalactopyranoside; HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; UTR, untranslated region

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Structure and Ligand Based Drug Design Strategies in the Development of Novel 5-LOX Inhibitors

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Abstract: Lipoxygenases (LOXs) are non-heme iron containing dioxygenases involved in the oxygenation of polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA). Depending on the position of insertion of oxygen, LOXs are classified into 5-, 8-, 9-, 12- and 15-LOX. Among these, 5-LOX is the most predominant isoform associated with the formation of 5-hydroperoxyeicosatetraenoic acid (5-HpETE), the precursor of non-peptido (LTB_4) and peptido (LTC_4, LTD_4, and LTE_4) leukotrienes. LTs are involved in inflammatory and allergic diseases like asthma, ulcerative colitis, rhinitis and also in cancer. Consequently 5-LOX has become target for the development of therapeutic molecules for treatment of various inflammatory disorders. Zileuton is one such inhibitor of 5-LOX approved for the treatment of asthma.

In the recent times, computer aided drug design (CADD) strategies have been applied successfully in drug development processes. A comprehensive review on structure based drug design strategies in the development of novel 5-LOX inhibitors is presented in this article. Since the crystal structure of 5-LOX has been recently solved, efforts to develop 5-LOX inhibitors have mostly relied on ligand based rational approaches. The present review provides a comprehensive survey on these strategies in the development of 5-LOX inhibitors.

Keywords: Arachidonic acid, 5-LOX, asthma, drug design, pharmacophore, QSAR, scaffold hopping, pseudoreceptor.

INTRODUCTION TO DRUG DESIGN

5-LOX and its Importance

Arachidonic acid (AA) is normally found esterified to cell membrane glycerophospholipids. Activation of phospholipase A_2 (PLA_2) results in the release of AA from membrane phospholipids and makes it available for oxidative metabolism by several enzyme systems. AA can be metabolized by three pathways: cyclooxygenase (COX), lipoxygenase (LOX) and epoxygenase (EPOX) as depicted in Fig. (**1**).

COXs (prostaglandin-endoperoxide synthase, EC 1.14.99.1) catalyze the production of prostaglandins (PGs), prostacyclins and thromboxanes (TXs). The COX activity introduces two molecules of oxygen into AA to form the cyclic hydroperoxy endoperoxide (PGG_2), which is subsequently reduced by the peroxidase to the hydroxy endoperoxide, PGH_2 [1]. There are three isoforms COX-1, COX-2, and COX-3 [2]. COX-1, constitutively expressed in most tissues and involved in the synthesis of prostaglandins (PGs) at low levels, is presumed to function primarily in the maintenance of physiological functions [3-5]. COX-2, the inducible isoform of COX, is induced by several mitogenic and proinflammatory stimuli and plays a direct role in tumor cell growth and various other diseases. COX-3 is recently identified isozyme and is a splice variant of COX-1.

LOXs (linoleate: oxygen oxido reductase, EC 1.13.11.12) are a group of closely related non-heme iron containing dioxygenases. These enzymes catalyze the addition of molecular oxygen into Poly Unsaturated Fatty Acids (PUFAs) containing cis, cis 1-4 pentadiene structures to give their hydroperoxy derivatives [6]. All LOXs have a two domain structure, the small N-terminal β-barrel domain and larger catalytic domain containing non-heme iron atom. They contain a ‘‘non-heme’’ iron per molecule in the active site as high-spin Fe(II) in the native state, and high-spin Fe(III) in the activated state [7-8]. Iron is ligated in an octahedral arrangement by three conserved histidines, one His/Asn/Ser, and a conserved isoleucine at the C-terminus of the protein [9]. LOX proteins have a single polypeptide chain with a molecular mass of 75–80 kDa in animals and 94–104 kDa in plants and the highest sequence identity between these LOXs is in the portion of the catalytic domain near the iron atom [10].

LOXs are classified on the basis of site of arachidonate oxygenation into 5-, 8-, 9-, 11-, 12- and 15-LOX. Though most of the lipoxygenases insert molecular oxygen stereospecifically at ‘S’, recently ‘R’ lipoxygenases also have been reported [11-15]. The prominent animal LOXs are 5-LOX, 8-LOX, 12-LOX and 15-LOX, while the plant LOXs are mostly 5-LOX and 15-LOX. Among these, 5-LOX is the most predominant isoform associated with the formation of 5-hydroperoxyeicosatetraenoic acid (5-HpETE) and other bioactive lipid mediators [16]. Cellular activation by immune complexes and other inflammatory stimuli result in an increase in intracellular calcium and the translocation of Cytosolic Phospholipase A_2 ($cPLA_2$) and 5-LOX from the cytosol to the nuclear membrane and association with 5-lipoxygenase activating protein (FLAP), an 18-kDa integral membrane protein essential for Leukotriene (LT) biosynthesis in intact cells. FLAP selectively transfers AA to 5-LOX and enhances the sequential oxygenation of AA to 5-HpETE and dehydration to LTA_4 [17-21]. LTA_4 can be further metabolized to LTB_4 by LTA_4 hydrolase or to LTC_4 by conjugation of glutathione at the sixth carbon by the action of LTC_4 synthase [20]. Additional studies established that LTC_4 and its extracellular metabolites LTD_4 and LTE_4 are the constituents of slow-reacting substance of anaphylaxis, but they are now more properly termed as cysteinyl leukotrienes. The cysteinyl leukotrienes have been recognized to mimic many of the clinical manifestations of asthma. LTE_4 is further metabolized to inactive LTF_4 by the action of c-glutamyl transpeptidase. Studies have also shown that LTF_4 was formed directly from LTC_4 by the action of carboxypeptidase [22]. LTB_4 is a potent chemotactic and chemokinetic agent for a variety of leukocytes, the cysteinyl leukotrienes C_4, D_4 and E_4 cause vascular permeability and smooth muscle contraction [23].

LTs are involved in a variety of inflammatory and allergic diseases such as asthma, ulcerative colitis and rhinitis [14]. 5-LOX pathway is also associated with gastroesophageal reflux disease (GERD) and Crohn's disease [24]. The potential role of leukotrienes in atherosclerosis, another chronic inflammatory disease has been recently discussed [25]. 5-LOX plays an important role in distinct types of cancers like colon, esophagus, prostate, lung, etc. [26-30]. Recently it has also been shown that 5-LOX (ALOX5) is critical regulator for leukemia cancer stem cells (LSCS) in chronic myeloid leukemia (CML) [31].

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CoMFA and CoMSIA studies on 5-hydroxyindole-3-carboxylate derivatives as 5-lipoxygenase inhibitors: Generation of homology model and docking studies

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ABSTRACT

In this study, comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) were performed on a series of 2-substituted 5-hydroxyindole-3-carboxylate derivatives as potent 5-LOX inhibitors with IC_{50} values ranging from 0.031 to 13.4 μM. Two datasets of same molecules were prepared with two different partial atomic charges; one with Gasteiger–Huckel and another with the ESPFIT charges obtained from the GAUSSIAN package. CoMFA and CoMSIA models were generated for both the datasets and the results were analysed. With regard to the non-cross validated r^2 values (r^2_{ncv}) and cross-validated q^2 values (q^2_{cv}) of the resulting QSAR models, the dataset with ESPFIT charges yielded higher values; hence it was further used in the study. The CoMFA and CoMSIA models have been further validated for their stability and robustness using group validation and bootstrapping techniques and for their predictive abilities using an external test set of ten compounds. The predictive power of the CoMSIA model was higher than the CoMFA model, the high predictive r^2 values of the test set reveals that the models prove to be useful tools for activity prediction of newly designed 5-LOX inhibitors. The ESPFIT-derived charges yielded better models than those based on charges calculated from Gasteiger–Huckel charges. We generated a homology model for human 5-LOX and identified the key residues at the binding site. The 3D-QSAR models were compared with the interactions at the active site to further elucidate the accuracy of the models. The data generated from 3D-QSAR study was used to design potential 5-LOX inhibitors.

Lipoxygenases (LOXs) (linoleate: oxygen oxido reductase, EC 1.13.11.12) are a group of closely related non-heme iron containing dioxygenases. These enzymes catalyze the addition of molecular oxygen into Poly Unsaturated Fatty Acids (PUFAs) containing cis, cis 1–4 pentadiene structures to give their hydroperoxy derivatives.[1] LOXs are classified according to their positional specificity of arachidonate oxygenation into 5-, 8-, 9-, 11-, 12- and 15-LOXs.[2] One of the LOX pathways of arachidonic acid metabolism, the 5-LOX pathway, is the source of potent pro-inflammatory mediators.[3] LOX metabolites are potent physiological effectors in a variety of cellular responses, associated with normal host defense and inflammation. In particular, leukotrienes (LTs), the mediators of allergy and asthma, are produced through the 5-LOX pathway. Products of the 5-LOX pathway are thus important mediators of inflammation. Inhibitors of the 5-LOX pathway, therefore, have therapeutic potential in a variety of inflammatory and allergic diseases. 5-LOX plays a key role in gastroesophageal reflux disease (GERD), rheumatoid arthritis and Crohn's disease.[4] High expression of 5-LOX was found in prostate, lung and other cancer cell lines.[5–8] Recently it has been shown that 5-LOX (ALOX5) is critical regulator for leukemia cancer stem cells (LSCS) in chronic myeloid leukemia (CML).[9] Currently an emerging strategy of therapeutic value consists of creating molecules with specific 5-LOX inhibition activity.

In our earlier studies, a theoretical 3D model of potato 5-LOX was elaborated by homology modeling.[10] The 5-LOX active site was then characterized from a structural point of view and used to study the docking of selected inhibitors. This shed new light on the binding features of the enzyme. In a more recent study, chemical feature based pharmacophore modeling of inhibitors of 5-LOX have been carried out by using HypoGen module within Catalyst program package.[11] The fact that 5-LOX inhibitors can be successfully identified by employing pharmacophore based virtual screening explains its usefulness in predicting activities of large datasets of molecules. Thus, our earlier studies provided homology and pharmacophore models which help in designing the novel 5-LOX inhibitors.

In this study, we have performed three dimensional quantitative structure–activity relationship (3D-QSAR), using the comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) techniques.[12,13] The study

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Pharmacophore modeling and virtual screening for designing potential 5-Lipoxygenase inhibitors

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ABSTRACT

Inhibitors of the 5-Lipoxygenase (5-LOX) pathway have a therapeutic potential in a variety of inflammatory disorders such as asthma. In this study, chemical feature based pharmacophore models of inhibitors of 5-LOX have been developed with the aid of HipHop and HypoGen modules within Catalyst program package. The best quantitative pharmacophore model, Hypo1, which has the highest correlation coefficient (0.97), consists of two hydrogen-bond acceptors, one hydrophobic feature and one ring aromatic feature. Hypo1 was further validated by test set and cross validation method. The application of the model shows great success in predicting the activities of 65 known 5-LOX inhibitors in our test set with a correlation coefficient of 0.85 with a cross validation of 95% confidence level, proving that the model is reliable in identifying structurally diverse compounds for inhibitory activity against 5-LOX. Furthermore, Hypo1 was used as a 3D query for screening Maybridge and NCI databases within catalyst and also drug like compounds obtained from Enamine Ltd, which follow Lipinski's rule of five. The hit compounds were subsequently subjected to filtering by docking and visualization, to identify the potential lead molecules. Finally 5 potential lead compounds, identified in the above process, were evaluated for their inhibitory activities. These studies resulted in the identification of two compounds with potent inhibition of 5-LOX activity with IC_{50} of 14 μM and 35 μM, respectively. These studies thus validate the pharmacophore model generated and suggest the usefulness of the model in screening of various small molecule libraries and identification of potential lead compounds for 5-LOX inhibition.

Lipoxygenases (LOXs-linoleate: oxygen oxido reductase, EC 1.13.11.12) are a group of closely related non-heme iron containing dioxygenases. Polyunsaturated fatty acids containing a series of cis–cis double bonds act as suitable substrates for LOXs. LOXs are classified according to their positional specificity of arachidonate oxygenation into 5-, 8-, 9-, 11-, 12- and 15-LOXs.[1] LOX metabolites are potent physiological effectors in a variety of cellular responses. Particularly, leukotrienes (LTs), the mediators of allergy and asthma, are produced through the 5-LOX pathway. It has been also reviewed that 5-LOX plays a key role in Gastro Esophageal Reflux Disease (GERD).[2] Elevated levels of LTB_4 have been found in blood and joint fluid from patients with rheumatoid arthritis[3] and in colonic mucosa from patients with ulcerative colitis or Crohn's disease.[4,5] LOX and their products are shown to play important role in tumor formation and cancer metastasis.[6–8] High expression of 5-LOX was found in prostate, lung and other cancer cell lines.[9,10] Inhibitors of the 5-LOX pathway, therefore, have a therapeutic potential in a variety of inflammatory and allergic diseases. These efforts have resulted in the release of Zileuton (5-LOX inhibitor) and Montelukast (LT receptor antagonist) into the market for the treatment of asthma. Recently, the arachidonate 5-LOX gene (*Alox5*) has been identified as a critical regulator of leukemia stem cells (LSCs) in BCR-ABL-induced chronic myeloid leukemia (CML). It has been also reported that the treatment of CML mice with a 5-LOX inhibitor prolonged survival.[11]

As 5-LOX is implicated in many inflammatory disorders, there is growing emphasis by many pharmaceutical companies and academic research groups on the development of effective 5-LOX inhibitors. The novel inhibitors thus developed provide a good basis for elucidating the structure–activity relationship, which will aid in the identification of more potent inhibitors. Lack of crystal structure information of 5-LOX, however, has been an obstacle for the application of structure based drug design strategies. As an alternative homology modeling strategy was employed to generate 3-D models of various LOXs, which were used in various drug design strategies.[12–18] Ligand based drug design is an alternative in such cases. In a ligand-based design, identification of a pharmacophore is one of the most important steps.

Pharmacophore model is widely employed to quantitatively explore common chemical characteristics among a considerable number of structures with great diversity. Such a model could also be used as a query for searching chemical databases and find new chemical entities.[19–22] In this Letter, we identified pharmacophore

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PRIMARY SOURCES

1	bmcresnotes.biomedcentral.com Internet Source	%1
2	Saura, Patricia, Laura Masgrau, Dagmar Heydeck, Hartmut Kühn, JoséMaría Lluch, and Àngels González-Lafont. "Is regioselectivity of arachidonic acid enzyme-catalyzed hydroperoxidation necessarily determined by the hydrogen abstraction? The case of rabbit Leu597Ala/Ile663Ala ALOX15 mutant", ChemPhysChem, 2016. Publication	%1
3	Aparoy, P.. "Computational analysis of R and S isoforms of 12-Lipoxygenases: Homology modeling and docking studies", Journal of Molecular Graphics and Modelling, 200902 Publication	%1
4	L. M. HultÃ©n. "15-Lipoxygenase-2 is expressed in macrophages in human carotid plaques and regulated by hypoxia-inducible	<%1