Chapter-2
S.O.O.O.Introduction

Most of the mammalian lipoxygenases are functionally similar and were even found to share some structural similarities in the conserved regions of their amino acid sequences. Platelet 12-lipoxygenase was found to have more than 80% similarity with the 12-lipoxygenase of porcine leukocytes and the reticulocyte 15-lipoxygenase and up to 62% with human 5-lipoxygenase. These lipoxygenases were even found to be similar up to 40% with their counterparts of plant kingdom such as soybean and pea lipoxygenases (Funk et al, 1990). Most of the lipoxygenases exhibit overall hydrophobicity which aids in attraction and attachment of the substrate fatty acid (Ponder et al, 1987). The conserved sequences of all the lipoxygenases share many acidic and basic residues including the putative iron binding domain (Funk et al, 1989). These conserved sequences are fundamental blocks responsible for the structure-function properties of the enzyme. Out of these conserved sequences, methionine was found to be the primary determinant for the positional specificity of the lipoxygenases (Solane et al, 1990).

The replacement of amino acid methionine with valine in 15-lipoxygenase was found to oxygenate at 15- and 12- positions of arachidonic acid equally (Solane et al, 1991). This methionine was found to be monoxygenated by aerobic inactivation of the lipoxygenase (Rapport et al, 1984). The oxygenation of methionine at the active center and the reduction of the putative iron bound to the histidine moiety, from Fe$^{3+}$ to Fe$^{2+}$ oxygenate the substrate that has been attracted and attached to the enzyme by the hydrophobic methionine and valine (Degroot et al., 1975).

Changes in the conserved amino acid sequence of the lipoxygenase alters the position of the 1,4-butadiene systems of the substrate from C-13 to C-10 changing the substrate specificity. Such changes in oxidation leads to the formation of dual lipoxygenation capacity seen in case of 5-lipoxygenase of PMNLs, 12-lipoxygenase of platelets or 15-lipoxygenase of reticulocytes. (Rouzer et al, 1986; Murray et al, 1988, Brash et al, 1989).

The primary oxygenation of arachidonic acid with lipoxygenase involves an antarafacial relationship between the hydrogen abstraction and reorientation of the
molecular oxygen (Corey et al., 1983). This antarafacial relationship is seen only in enzymatic conversions and were not found to be present in case of diHETEs formed by the hydrolysis of the epoxide *viz* the formation of 5,12- and 8,15-diHETEs (Walstra et al., 1987). The mechanism of hydrogen abstraction from the primary oxygenated metabolite of arachidonic acid, as in the case of 8-lipoxygenase with 5-HPETE, was found to be analogous with 12-lipoxygenase in 15-HPETE for the formation of epoxide, leukotrienes (Ueda et al., 1988, Mass et al, 1983). Dual lipoxygenation of the enzyme gives it an intrinsic ability to catalyze the formation of leukotrienes or it bestows the epoxide synthase ability as seen in cases of 5-, 12- and 15-lipoxygenases. Leukotrienes are formed from the transformation of arachidonic acid through a hydroperoxide intermediate, 5-HPETE in the case of 5,6-LTA₄ and 15-HPETE in case of 14,15-LTA₄ pathway. The hydroperoxy intermediate is acted upon by a dehydrase to form the 5,6 epoxide or 14,15 epoxide derivative. This dehydrase was later shown to be 8-lipoxygenase activity in case of 5,6-LTA4 pathway and 12-lipoxygenase activity in case of 14,15-LTA₄ pathway. These enzymes act at the C-10 position of the arachidonic acid in both the cases. Due to the conjugated diene system in the intermediate (5-HPETE or 15-HPETE) a 1-5 free radical movement results in LTA₄ formation. This mechanism is found to be common in both the lipoxygenase pathways (Fig 7 and 10).

5,6-LTA₄ synthase was first demonstrated by the dual regiospecificity of the 5-lipoxygenase of both potato and PMNLs (Shimizu et al, 1984 and Rouzer et al., 1985). However later studies have shown the operation of 14,15-LTA₄ pathway in leukocytes by Mass et al. (1981) and Lundberg et al. (1981). The operation of 11,12-LTA₄ pathway was also demonstrated by Westlund et al. (1988). The formation of 5,6-LTA₄, 8,9-LTA₄, 11,12-LTA₄ and 14,15-LTA₄ by the potato lipoxygenase was demonstrated by Reddanna et al. (1988).

The operation of 14,15-LTA₄ pathway was found to be catalyzed both by the synthase activities of leukocyte 12-lipoxygenase (Yokoyoma et al., 1986) and reticulocyte 15-lipoxygenase (Bryant et al., 1985). The operation of this leukotriene pathway was also
found to be temperature dependent and oxygen sensitive (anaerobic) i.e it gives various products at different reaction temperatures (Bryant et al., 1985).

In this chapter the regiospecificity of the purified sheep uterus lipoxygenase with various PUFA substrates and reaction conditions such as optimum pH are presented. Also the involvement of uterine lipoxygenase in the synthesis of leukotrienes was analyzed.

5.1.0.0. Results

5.1.1.0. Regiospecificity of sheep uterine lipoxygenase

Sheep uterus lipoxygenase was incubated with arachidonic acid at pH 5.5 in citrate-phosphate buffer and the compounds were extracted into hexane: ether (1:1). The products were reduced with sodium borohydride under inert cold conditions and the reduced products were analyzed on SP-HPLC system. The separation of the oxidized metabolites is shown in Fig. 36. The major peaks with retention times 7.21 min and 8.43 min gave a typical conjugated diene spectra (Fig. 15) on the UV/VIS scanning spectrophotometer. The peak with retention time 7.21 min when injected with standard 12-HETE coeluted as a single peak. The peak with retention time of 8.43 min coeluted with authentic 15-HETE suggesting that the two peaks are 12- and 15-HETEs. The positional specificity of the uterine dual lipoxygenase is shown in Fig. 37.

5.1.2.0. GC-MS analysis

The two major peaks of Fig. 36 with retention times 7.21 min and 8.43 min were collected from a number of runs and pooled. These were concentrated individually and methylated with diazomethane. The methyl esters after purification on straight phase HPLC, were trimethylsilylated using BSTFA. The trimethylsilylated derivatives were then analyzed on Hewlett Packard GC/MS. The fragmentation pattern of the peak 7.21 min is shown in Fig. 38 and the fragmentation pattern of peak 8.43 min is shown in Fig. 39. The peak with RT 7.21 min gave fragmentations at M/Z 391, 375, 295, 173 and 73 as expected for 12-HETE (Hamberg et al., 1974a). Peak with retention time 8.43 min was identified by the M+ 406 and the fragmentation pattern of M/Z 391, 335, 225, 173 and 73.
Fig. 36: SP-HPLC analysis of AA products generated with uterine lipoxygenase. AA was incubated with uterine LOX and the products were extracted into hexane : ether, the organic extract was evaporated and redissolved in SP-II PI.(' solvent system. The analysis conditions employed for separation were:

- **Column**: CLC-SIL
- **Solvent**: Hexane:propane 2-ol:acetic acid (1000:14:1)
- **Flow rate**: 1mL/min
- **Detection**: 235nm
Absorbance at 235 nm
Fig. 37: Scheme showing the possible carbon atoms of A A that can be oxygenated with uterine lipoxygenase.
Fig. 38: GC/MS analysis of Peak I (RT 7.21 min) of Fig. 36.
Fig. 39: GC/MS analysis of Peak II (RT 8.43 min) of Fig. 36.
as 15-HETE (Hamberg et al., 1974b) The fragmentation analysis of both the peaks were compared with standards from the data bank and then confirmed as 12-HETE (peak with RT 7.21 min) and 15-HETE (peak with RT 8.43 min).

5.1.3.0. Effect of pH

The effect of pH on the regiospecificity of the uterus lipoxygenase was also checked and the HPLC profiles of the reduced products at different pH are presented in Figs. 40, 41, and 42. As shown in the chromatogram 15-HETE was formed in major quantity compared to 12-HETE at highly acidic conditions such as pH 3.5 (Fig. 40). However under alkaline conditions i.e at pH 9.0 the relative concentration of 12-HETE was higher than 15-HETE (Fig. 42) At its optimum pH i.e at 5.5 both the HETEs were formed in equal proportions (Fig. 41). On either side of the optimum pH the total oxygenated products formed were decreased coinciding with decreased activities of the enzyme.

5.1.4.0. Uterus lipoxygenase with y-linolenic acid

The regiospecificity of the uterus lipoxygenase was also studied with y-linolenic acid (GLA) as the substrate which has three double bonds GLA was incubated with sheep uterus lipoxygenase at pH 5.5 in citrate-phosphate buffer and the products were extracted into hexane:ether (1:1) and reduced with sodium borohydride. The reduced products were analyzed on SP-HPLC system as described in the methodology. The HPLC separation of the y-linolenic acid products is shown in Fig. 43. Two major peaks were obtained with retention times 8.73 min (peak I) and 10.37 min (peak II). These peaks were found to contain absorption max of 234 nm on UV/VIS spectral analysis. Peak I with retention time of 8.73 min co-eluted with 13-hydroxy octadecatrienoic acid (13-HOTrE). The peak II with retention time of 10.37 min was identified as 10-HOTrE based on the retention time and co-elation with standard 10-HOTrE. The possible carbon atoms that could be oxidized by the lipoxygenase action on y-linolenic acid is shown in Fig. 44.
Fig. 40: SP-HPLC analysis of A A products generated with uterine lipoxygenase at pH 3.5.
AA was incubated with uterine LOX and the products were extracted into hexane : ether, the organic extract was evaporated and redissolved in SP-HPLC solvent system. The analysis conditions employed for separation were.

- **Column**: CLC-SIL
- **Solvent**: Hexane:propane 2-ol:acetic acid (1000:14:1)
- **Flow rate**: 1mL/min
- **Detection**: 235nm

Fig. 41: SP-HPLC analysis of AA products generated with uterine lipoxygenase at pH 6.0
AA was incubated with uterine LOX and the products were extracted into hexane : ether, the organic extract was evaporated and redissolved in SP-HPLC solvent system. The analysis conditions employed for separation were:

- **Column**: CLC-SIL
- **Solvent**: Hexane:propane 2-ol:acetic acid (1000:14:1)
- **Flow rate**: 1mL/min
- **Detection**: 235nm
Absorbance at 235 nm

Absorbance at 235 nm
Fig. 42: SP-IIPLC analysis of AA products generated with uterine lipoygenase at pH 9.0

AA was incubated with uterine LOX and the products were extracted into hexane:ether, the organic extract was evaporated and redissolved in SP-IIPLC solvent system. The analysis conditions employed for separation were:

- **Column**: CLC-SIL
- **Solvent**: Hexane:propane 2-ol:acetic acid (1000:14:1)
- **Flow rate**: 1mL/min
- **Detection**: 235nm
Absorbance at 235 nm
Fig. 43: SP-HPLC analysis of GLA products generated with uterine lipoxygenase.

GLA was incubated with uterine LOX and the products were extracted into hexane : ether, the organic extract was evaporated and redissolved in SP-HPLC solvent system. The analysis conditions employed for separation were:

- **Column**: CLC-SIL
- **Solvent**: Hexane:propane 2-ol:acetic acid (1000:14:1)
- **Flow rate**: 1mL/min
- **Detection**: 235nm
Absorbance at 235 nm

Peak I (8.73)
Peak II (10.37)
**Fig. 44**: Scheme showing the possible carbon atoms of GLA that could be oxidized with uterus lipoxygenase.
In order to analyze the peaks on GC/MS, the products were generated in large quantities and after methylation and trimethylsilylation, they were subjected to GC-MS analysis as described in the methodology. The fragmentation pattern of peak I, i.e., retention time of 8.73 min is shown in Fig. 45. The fragmentation pattern with $M^+$ at 380 and M/Z ions at 309, 290, 251, 243, 225, 173, and 73 confirm the peak as 13-HOTrE. The fragmentation pattern of peak II with retention time of 10.37 min is shown in Fig. 46. The $M^+$ at 380 and M/Z ions at 365, 269, 173, and 73 confirm this as 10HOTrE.

Thus sheep uterus lipooxygenase exhibits dual regiospecificity both with arachidonic acid and $\gamma$-linolenic acid. As a result of dual lipooxygenase activity, the enzyme should be able to exhibit LTA4 synthase activity similar to that observed with leukocyte and potato lipooxygenases (Shimizu et al., 1984).

5.1.5.0. 14,15-LTA$_4$ synthase activity

In view of the dual lipooxygenase activity (12- and 15-) expressed by the purified sheep uterus lipooxygenase, its role in leukotriene bio-synthesis was analyzed by reacting the enzyme with 15-HPETE as the substrate. The products were extracted into hexaneether (1:1) and after evaporation analyzed on RP-HPLC. The effluent was monitored at 268 nm for the first 16 min and at 235 nm for the rest of the analysis period, so as to monitor the separation of both diHETEs and H(p)ETEs. The products were eluted as two peaks mainly with retention times 6.06 min and 7.82 min when monitored at 268 nm (Fig. 47). These two peaks on spectral analysis showed a typical conjugated triene spectrum with $\lambda_{\text{max}}$ at 268 nm and shoulders at 258 nm and 278 nm (Fig. 47 inset). The peaks were methylated with diazomethane and trimethylsilylated with BSTFA. The methyl esters were then analyzed on Hewlett-Packard GC/MS. The fragmentation pattern gave a molecular ion peak of $M^+$ 494 and M/Z peaks at 333, 263, 243, 173 and 73 as expected for 8,15-diHETE (Fig. 48, Bild et al., 1977). These two peaks were found to correspond with the hydrolyzed products of synthetic 14,15-LTA$_4$, thus confirming that the two peaks seen in Fig. 47 are the hydrolyzed products of 14,15-LTA$_4$ i.e 8(S),15(R)-diHETE (Rt 6.06 min) and 8(S),15(S)-diHETE (RT 7.82 min). The unreacted 15-HPETE with the
Fig. 45: GC/MS analysis of Peak I of Fig. 43 (13-HOTrE)
Fig. 46: GC/MS analysis of Peak 11 of Fig. 43 (10-HOTrE)
Fig. 47: RP-HPLC analysis of 15-HPETE product profile reacted with uterus lipoxygenase: Assay of 14,15-LTA_{4} synthase activity:
15-HPETE was incubated with uterine LOX in citrate-phosphate pH 5.5 buffer. The products were extracted and evaporated under nitrogen. The residue was redissolved in RP-HPLC solvent system and analyzed as follows. The individual peaks were collected and checked for spectral analysis on scanning spectrophotometre.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>CLC-ODS</td>
</tr>
<tr>
<td>Solvent</td>
<td>Methanol:water:acetic acid (68:32:0.1) pH 6.8</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1mL/min</td>
</tr>
<tr>
<td>Detection</td>
<td>268 and 235 nm</td>
</tr>
</tbody>
</table>
Fig. 48: GC/MS analysis of 8,15-dilTEEs.
Fig. 49: RP-HPLC analysis of endogenous 15-HPETE, made available with AA and uterine 15-lipoxygenase, demonstrating endogenous 15-HPETE to be a better substrate for 14,15-LTA4 synthase activity:
AA was incubated with uterine LOX in citrate-phosphate pH 5.5 buffer for 10 min with excess of enzyme. The products were extracted and evaporated under nitrogen. The residue was redissolved in RP-HPLC solvent system and analyzed as follows. The individual peaks were collected and checked for spectral analysis on scanning spectrophotometre.

Column : CLC-ODS
Solvent : Methanol:water:acetic acid (68:32:0.1) pH 6.8
Flow rate : 1 mL/min
Detection : 268 and 235 nm
sheep uterine lipoxygenase eluted as a single peak with retention time of 21.7 min. This peak was verified for its authenticity by co-chromatography with standard 15-HPETE and on scanning spectrophotometer.

5.2.0.0. Discussion

Lipoxygenases constitute a family of closely related non-heme iron containing dioxygenases that convert polyenoic fatty acids such as arachidonic acid or linolenic acid to their corresponding hydroperoxy derivatives. These enzymes are ubiquitously distributed both in animal and plants, to a higher degree in the latter. In mammalian cells most of the lipoxygenases are grouped into 4 types viz 5-lipoxygenases (leukocytes), two 12-lipoxygenases (platelet type and leukocyte type) and the 15-lipoxygenase (reticulocytes), (Yamamoto et al., 1992). However, it should be stressed that the positional specificity of the lipoxygenase is not an absolute property but depends on the substrate structure and the reaction conditions such as temperature, pH and the nature of the solvent (Gardener, 1989). It has been shown, that with 15-HPETE as the substrate soybean lipoxygenase which is incidentally, 15- type exhibits 8- and 5-lipoxygenase activities producing 8,15-and 5,15- diHPETEs (Bild et al., 1977). Potato lipoxygenase is known to exhibit multi regiospecificity with arachidonic acid as the substrate producing 5-HPETE as the major product and other HPETEs such as 8-,9-,11-, 15- and 12- in minor quantities (Reddanna et al., 1990). PMNL lipoxygenase exhibits 5- and 8-lipoxygenase activities by which the enzyme possesses LTA4 synthase activity (Rouzer et al., 1985). The reticulocyte 15-lipoxygenase exhibits a minor 12-lipoxygenase activity and hence LTA4 synthase activity (Bryant et al., 1985), whereas the leukocyte 12-lipoxygenase forms 15-HPETE as the minor product along with LTA4 synthase activity (Yokoyoma et al., 1986).

In the present study a cytosolic lipoxygenase from sheep uterus was isolated, that exhibits dual regiospecificity with arachidonic acid as the substrate, producing 12-and 15-HPETEs. The two products were formed in equal quantities at the optimum pH unlike other lipoxygenases reported so far (Murray et al., 1988). The sheep uterus lipoxygenase
also showed dual regiospecific with y-linolenic acid producing 10- and 13-HOTrEs. Platelet 12-lipoxygenase was also reported to produce two products with y-linolenic acid (Hamberg, 1983) and docosahexaenoic acid (Gardener 1989). This dual regiospecificity is also seen with reticulocyte 15-lipoxygenase on oxygenating with membranes producing 9- and 13-octadecadienoic acids (Kuhn et al., 1990).

As a result of this dual regiospecificity of the sheep uterine lipoxygenase, the enzyme should exhibit 14,15-LTA₄ synthase activity as reported for other dual lipoxygenases (Yokoyama et al., 1986, Bryant et al., 1985, Rouzer et al., 1985). In the present study it was demonstrated that the purified lipoxygenase from sheep uterus exhibits 14,15-LTA₄ synthase activity when incubated with 15-HPETE as the substrate. 15-HPETE gets converted to 14,15-LTA₄ which immediately gets hydrolyzed into 8(S),15(R)-diHETE and 8(S), 15(S)-diHETEs (Sok et al., 1982). The hydrolysis of synthetic 14,15-LTA₄ also yields the same two isomers of 8,15-diHETEs confirming that the 8,15-diHETEs seen in the present study are infact the hydrolysis products of 14,15-LTA₄. The sequential action of 15- and 12-lipoxygenase activities of the uterine enzyme on arachidonic acid, thus could be responsible for the synthesis of 14,15-LTA₄ as per the mechanism shown in Fig. 9.

Apart from getting converted into 8,15-diHETEs a substantial amount of 15-HPETE was left over in the reaction with uterine lipoxygenase indicating, that not all the 15-HPETE provided externally is converted into 14,15-LTA₄. However, the 15-HPETE generated in situ was better utilized for the formation of 14,15-LTA₄ as evidenced by the formation of 8,15-diHETEs when uterine enzyme was incubated with arachidonic acid (Fig 49). From this it appears that the exogenous 15-HPETE is a poor substrate for the formation of 14,15-LTA₄. Similar observation was made in the case of 5,6-LTA₄ formation, where only 20% of the exogenous substrate (5-HPETE) was found to be utilized for 5,6-LTA₄ synthesis (Puustien et al., 1987 and Rouzer et al., 1986). The 14,15-LTA₄ synthase activity observed in the present study is exactly opposite to that of 5,6-LTA₄ synthase activity of leukocyte and potato lipoxygenase (Bryant et al. 1985, Mass et al. 1983; and Shimizu et al. 1984, Rouzer et al. 1985).
Although some lipoxygenases exhibit strict positional specificity multiple or **dual lipoxygenase regiospecificity** was also observed with several lipoxygenases including the present one (Gardner and Weisleder 1970, Bild et al, 1977, Yamamoto et al, 1980, Kuhn et al, 1985, 1990) But the mechanism of non-regiospecificity or **multiregiospecificity** of lipoxygenases is still largely unknown. It is known that the reduction of Fe$^{3+}$ to Fe$^{2+}$ allows the oxidation of the 1,4-butadiene systems of the substrate molecule by the abstraction of proton where antarafacial addition of O$_2$ molecule takes place and rearrange itself to a peroxy group giving 1 hydroperoxy 2,4-cis, trans pentadiene systems. It is also known that the reduced iron moiety dissociates itself from the histidine group and the protein gets degraded into smaller mol.wt peptides or fragments (Nelson et al., 1991 and Rouzer et al, 1986), there by making it impossible to assume that the enzyme molecule could have another lipoxygenation capacity. The dual regiospecificity of uterus lipoxygenase observed in the present study can only be explained in terms of dimeric nature of the enzyme. As pointed out in the first chapter, only the dimeric enzyme is catalytically **active** The two enzyme molecules are attached either by hydrophobic nature of the lipoxygenase (Funk et al, 1989; Sigal et al, 1990) or by electrostatic attractions of the charges present on the enzyme. The carboxylic or terminal group of one protein molecule might be attached to the **amino** or the terminal group of the other molecule in head to tail arrangement.

The cup like structure formed by the attraction of two molecules could give a neutral charge to the whole molecule as seen in case of the sheep uterine **lipoxygenase** This dimeric enzyme could oxidize two molecules of substrate at any given time simultaneously, one on either side. Further the orientation of the first substrate molecule might expose C-13 carbon atom for oxidation, possibly restricting the orientation of the 2nd molecule of the substrate to C-10 carbon leading to the production of 15-and 12-HPETEs and thus exhibiting dual regiospecificity. The proposed model to explain dimeric nature and dual regiospecificity is presented in Fig. 50.
Fig. 50: Hypothetical working model of uterine lipoxygenase to explain dual regiospecificity, dimeric form and neutral nature of the uterine LOX. (original model was from Solane et al., 1990)