PURIFICATION AND CHARACTERIZATION OF LEUKOTRIENE SYNTHESIZING ENZYMES FROM SHEEP UTERUS

Thesis **submitted** for **the** degree of
DOCTOR OF PHILOSOPHY

by

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DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Purification and characterization of leukotriene synthesizing enzymes from sheep uterus"** has been carried out by me under the supervision of **Dr. P. Reddanna** and that this has not been submitted for any degree or diploma of any other university.

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CERTIFICATE

This is to certify that **Mr. B. Muralidhar Reddy** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this university. We recommend his thesis **"Purification and characterization of leukotriene synthesizing enzymes from sheep uterus"** for submission for the degree of **Doctor of Philosophy** of this university.

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ABBREVIATIONS

AA	Arachidonic acid
BSTFA	*bis* (trimethyl silyl) trifluoro acetamide
CDNB	1 -chloro-2,4-dinitrobenzene
DEAE	Diethyl aminoethyl
FA	Free acid
GSH	Glutathione reduced
GST	Glutathione S-transferase
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High performance liquid chromatography
kDa	Kilo dalton
Lox	Lipoxygenase
LT	Leukotriene
Me	Methyl ester
PAGE	Poly acrylamide gel electrophoresis
PG	Prostaglandin
PMP	Penta methyl piperidine
PMSF	Phenylmethylsulfonyl fluoride
PUFA	Polyunsaturated fattyacid
RP	Reverse phase
RT	Retention time
SDS	Sodium dodecyl sulfate
SP	Straight phase
TBS	Tris buffered saline
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethane sulfonic anhydride
THF	Tetrahydrofuran
Tris	*tris* (hydroxymethyl) amino methane

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Busireddy Muralidhar Reddy

Introduction

1.0.0. Introduction

Progress of biochemical understanding has depended on the isolation of cellular biomolecules, determination of their structure and analysis of their function and metabolism. Many approaches from the level of whole animal to that of the isolated gene are being used to investigate the structure, function and metabolism of biomolecules. Each type of biomolecule possesses a distinct chemical property that suits it for the functions it serves in the cell.

In the early 1900's scientists discovered many essential compounds by observing symptoms that occurred after certain substances are excluded from diet and relieved when one of these substances added. Such studies led to the understanding that certain fatty acids are necessary for normal health. Based on these observations Burr and Burr (1930) demonstrated the importance of the fatty acids in health and disease.

Fatty acids are classified as saturated, monounsaturated and polyunsaturated fatty acids (PUFAs). About 40% of the total fatty acids in the fat are saturated. Naturally occurring PUFAs with *cis* unsaturation have essential fatty acid (EFA) activity where as the fatty acids with *trans* unsaturation do not have the EFA activity.

There are four distinct families of PUFAs, such as ω-3, ©-6, ω -7 and ω-9, of which ω-6 and co-3 are more common. The co-3 and co-6 fatty acids cannot be synthesized by animals and must be obtained from the diet. Animals derive these essential fatty acids such as linoleic acid (18:2, co-6) and α-linolenic acid (18:3, co-3) from plants. The ω-7 and w-9 fatty acids are considered non-essential because animals can synthesize them from saturated fatty acids. These fatty acids are extensively modified in animals by combination of desaturation and chain elongation and these processes result in the production of longer chain PUFAs

(Fig.1). However, all four families share the enzymes for the desaturation and elongation reactions by which the parent fatty acids are converted to their derived fatty acids. The rates of desaturation and elongation differ among the families. Interconversions among these four families of unsaturated fatty acids do not occur in humans. In animals the double bonds can be introduced between A^4 to A^9 but never beyond the A^9 and are with *cis* configuration. During the past four decades, number of scientists have focused attention on delineating the reasons for the existence of a great variety of fatty acids in biological systems.

Several studies have established a link between the PUFAs and pathological conditions in animals/humans. Numerous biochemical abnormalities are associated with the ω-6 fatty acid deficiency, which include scaly dermatitis. impaired growth and reproduction and poor wound healing and co-3 fatty acid deficiency failed to show any effect on growth, reproduction and general health in rats (Tinco *et al.,* 1978). The co-6 fatty acids are essential and beneficial, elevated intake may contribute to the pathogenesis associated with a number of chronic diseases. While ω-3 fatty acid deficiency did not seem to have any particular effect and proved to be useful in inhibiting the excess production of co-6 fatty acid metabolites and reduce the incidence of diseases (Lands, 1986). Epidemiological studies showed that the populations with low incidence of cardiovascular and immune related inflammatory diseases could be ascribed to their high consumption of diet rich in co-3 PUFAs (Bang and Dyerberg, 1972). It has been shown that the ω-3 fatty acids reduce cardiac arrhythmia, coronary artery ligation, and thrombosis (Culp *et al.,* 1980). Infants fed on imbalanced co-6/co-3 fatty acid diet have altered retinal composition, which results in abnormal visual function. In view of its biomedical importance, PUFAs have become the subject of intense study in recent years.

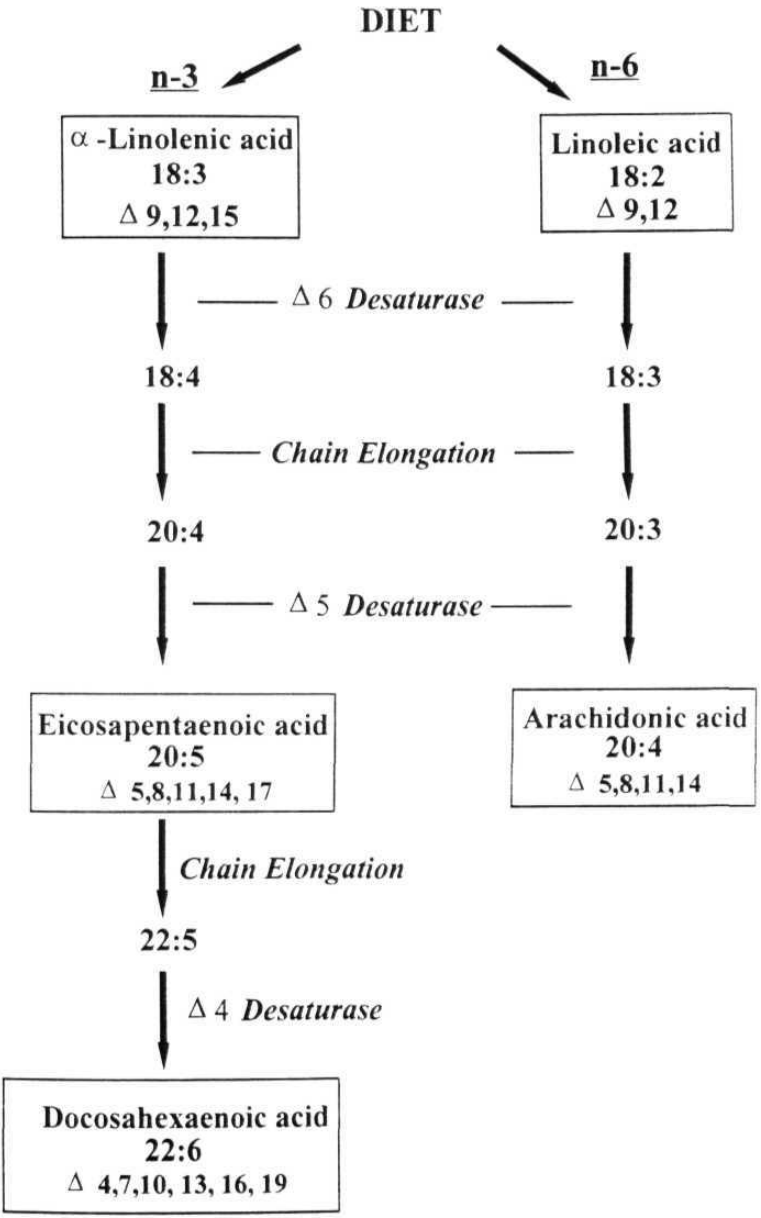


Fig.l Biosynthetic Pathway of Polyunsaturated Fatty acids
(In Animal Tissues)

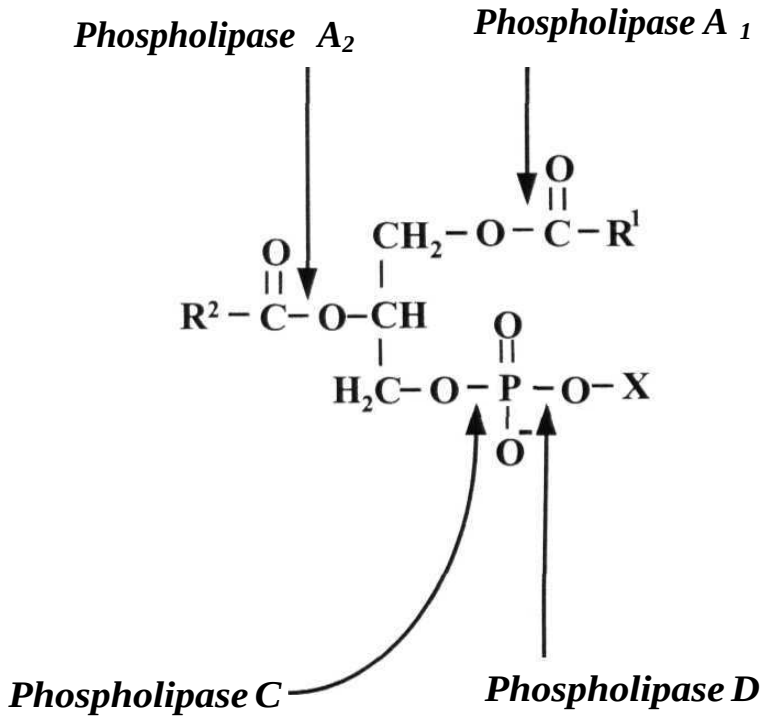


Fig.2 Release of arachidonic acid from membrane phospholipids

The different effects that fatty acids can have on human health are the consequences of the processes by which oxygenated derivatives are formed. Arachidonic acid (AA) is the most abundant PUFA found in the phospholipids of mammalian tissues. usually attached by an ester linkage to the second carbon of the glycerol backbone. As free AA is required for the action of cyclooxgenase and lipoxygenase enzymes, the release of AA is the critical step in the biosynthesis of eicosanoids. the oxygenated metabolites of AA. The phospholipases play an important role in the release of AA from esterified stores of phospholipids and various agents have been shown to influence these enzymes (Fig. 2) (Schaad *et al.*. 1991). The release of AA from the *sn-2* position of cellular phospholipids is primarily mediated by phospholipase A_2 (Mayer and Marshall, 1993 & Dennis. 1994). The two most studied mammalian forms of PLA_2 are type II, a 14 kDa protein known to exist as both an extra-cellular & cell associated forms. and a cytosolic 85 kDa PLA_2. The coexistence of both the cytosolic PLA_2 (cPLA:) and cell associated type II PLA_2 has been reported (Marshall and Roshak. 1993). The $cPLA_2$ primarily supports the formation of prostaglandins (PGs) and the enzymes other than $cPLA_2$ (the 14 kDa PLA_2) possibly provide the substrate for the formation of leukotrienes (LTs) (Marshall *et al.,* 1997). Apart from AA. other unsaturated fatty acids with C-18, C-20 and C-22 chain length can also lead to the synthesis of PGs (Nugteren, 1986) and LTs (Hammarstrom, 1980 & Jakschik *et al,* **1983).**

One of the metabolic fates of AA is its oxidative transformation to a variety of compounds mainly by three enzymatic pathways: prostaglandin H synthase/cyclooxygenase pathway, lipoxygenase pathway and epoxygenase pathway (Fig. 3).

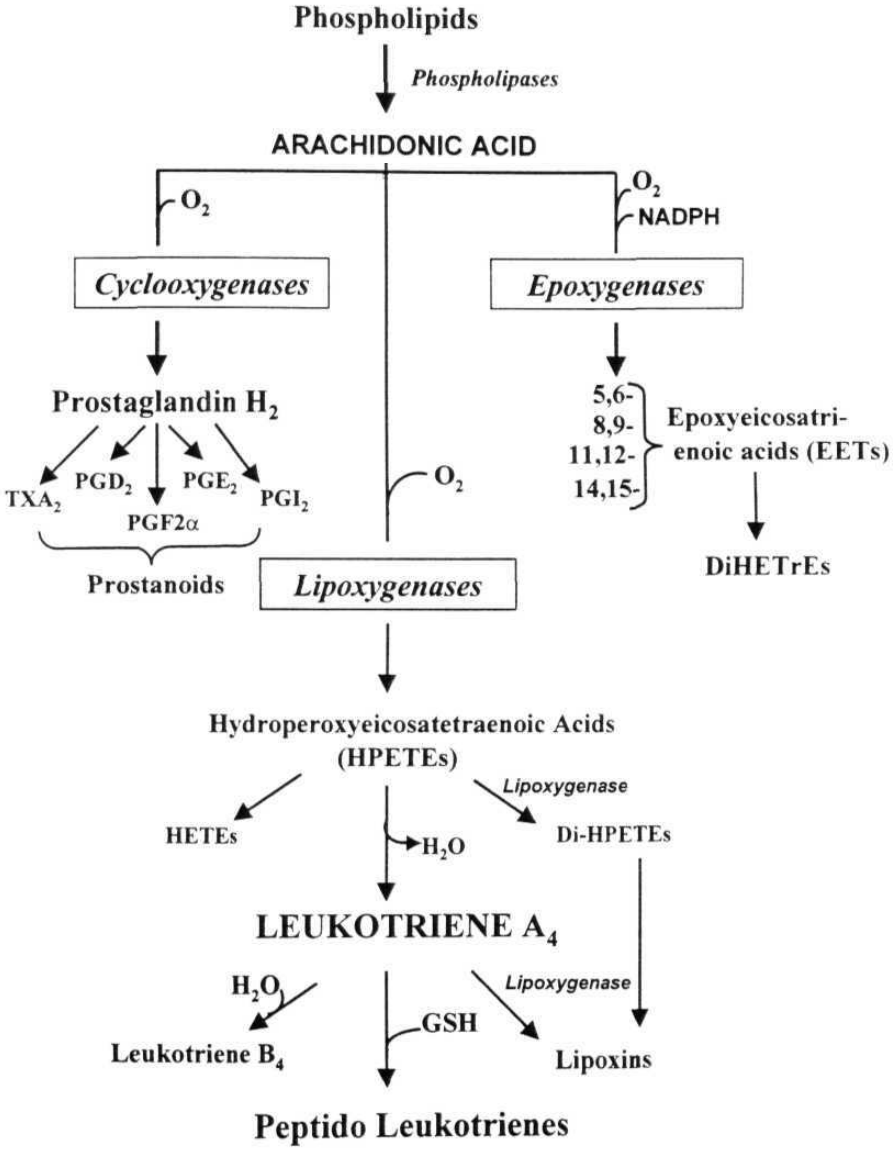


Fig.3 Major oxygenation pathways of arachidonic acid

1.1.0. Prostaglandin H Synthase Pathway

Prostaglandin H synthase (PGHS) pathway also known as cyclooxygenase (Cox) pathway, leads to the formation of PGs, thromboxanes (TXA_2) and prostacyclin. Two isozymes of PGHS have been described that are designated as PGHS-1 and PGHS-2, or Cox-1 and Cox-2. These isoforms have different biological functions. PGHS-1 is constitutively expressed in many tissues and is probably involved in the production of prostanoids which help in "housekeeping" functions of the cell, where as PGHS-2 often referred to as the inducible form, is expressed in fibroblasts, monocytes, and endothelial cells in response to mitogens, cytokines and tumor promoters. PGHS-2 is undetectable in most of the tissues. However, it is constitutively expressed in brain, kidney, testis and tracheal epithelium. These isoforms are interesting in the context of both structural biology and enzymology in that they are homodimeric, heme containing and glycosylated proteins with 2 catalytic sites. These enzymes have similar turnover number and K_m value for AA and O_2. The crystal structure of these two isozymes is essentially superimposable. All the fundamental catalytic properties of the isozymes are almost identical except for their susceptibility to pharmacological inhibition (Smith *et al*., 1996).

PGH synthase has both cyclooxygenase and peroxidase activities in the same protein. The PGHS catalyses the addition of two molecules of oxygen to one molecule of AA forming PG endoperoxides. PGH synthase reaction begins with the abstraction of the (13-S) hydrogen from A A to yield an arachidonyl radical and followed by sequential O_2 addition at C-11 and C-15 to yield endoperoxide PGG_2. The peroxidase activity of the enzyme reduces the 15-hydroperoxy group of PGG2 to its alcohol. PGH_2. The endoperoxides, PGG2 and PGH_2 are very unstable with a half-life of 4-5 minutes. PGH2, thus formed is the substrate for various isomerases.

each of them being responsible for the synthesis of specific PGs such as PGD_2. PGE_2 and $PGF_{2\alpha}$.

1.1.1. *Biological Effects of Prostaglandins*

PGs have been implicated in a wide variety of biological functions. They are complex and the degree of the response that a particular PG exerts varies with species and tissue (Oliw *et al.,* 1983). PGs have a variety of effects on several cell activities, including secretion, cell growth and differentiation. One important effect that many PGs exert is on smooth muscle. PGE and PG1 relax smooth muscle. where as PGF and TXA contract smooth muscle. PGs of E and I series are potent vasodilators, while PGF and TXA have opposite effect. PGD_2 and PGE: are involved in sleep regulation, one promotes sleep (Hayaishi, 1989) and the other wakefulness (Matsumura *et al.,* 1989). PGE_2 and PGI_2 increase the renal blood flow and provoke diuresis and natriuresis (Baer *et al.*. 1983). PGF increases the vascular permeability and also modulate the activity of histamine and bradykinin. The PGs involvement in asthma and inflammation has been reported. It has been reported that PGD_2 plays an important role in the inhibition of tumor cell growth or metastasis of various cancers.

PGs are involved in mediating several important processes in mammalian reproduction. PGE initiates follicular development by stimulating LH and FSH receptors. $PGF_{2\alpha}$ contract uterine smooth muscle (Goldberg and Ramwell. 1975) and help in parturition (Green *et al.,* 1982). $PGF_{2\alpha}$ causes rapid regression of the corpus luteum and hence is considered as the luteolytic hormone. The involvement of PGs in spermatogenesis has also been suggested. PGs are now known to act in many tissues by regulating the synthesis of the intracellular messenger molecules.

1.2.0. Epoxygenase Pathway

Cytochrome *P450* (Cyto.P450), the epoxygenase enzymes have been described as ubiquitous and involved in the oxygenation reactions of AA in addition to their involvement in xenobiotic metabolism (Fig. 4) (Capdevila *et al.*, 1981). The epoxygenase activity of Cyto.P450 enzyme produces four regioisomeric epoxyeicosatrienoic acids (EETs) by oxygenation of each of four double bonds of AA, such as 5,6-EET, 8,9-EET, 11.12-EET and 14.15-EET (Oliw *et al.*. 1982). 5,6-EET differs from other EETs of AA by having the 3 *cis* double bonds, which is one of the characteristics of the fatty acid substrates of cyclooxygenase and is metabolized to many polar products. These epoxides may appear superficially similar to the LTA4, however, LTA_4 epoxides are distinctly different from all the EETs The LT epoxides are allylic to a triene system and are extremely labile even at physiological pH, in contrast to the EETs which are homoallylic and relatively stable at physiological pH (Oliw *el al.*, 1982). LT epoxides have the *trans* configuration where as EETs have all *cis* configuration. The catalysis of GSH conjugation to EETs by various GST isozymes has been studied (Spearman *et al.*, 1985). The enantioselectivity of the epoxygenase is specific for the formation of products with (R) configuration (Capdevila *et al.*. 1986).

The EETs are further hydrogenated enzymatically to dihydroxy eicosatrienoic acids (DiHETrEs) (Chacos *et al.*, 1983). The rapid conversion of EETs to DiHETrEs has been proposed as a process, where by EETs are rendered biologically inactive. Cyto.P450s also act on both LTs & PGs, and are metabolized to hydroxylated compounds (Bosterling and Stier, 1983).

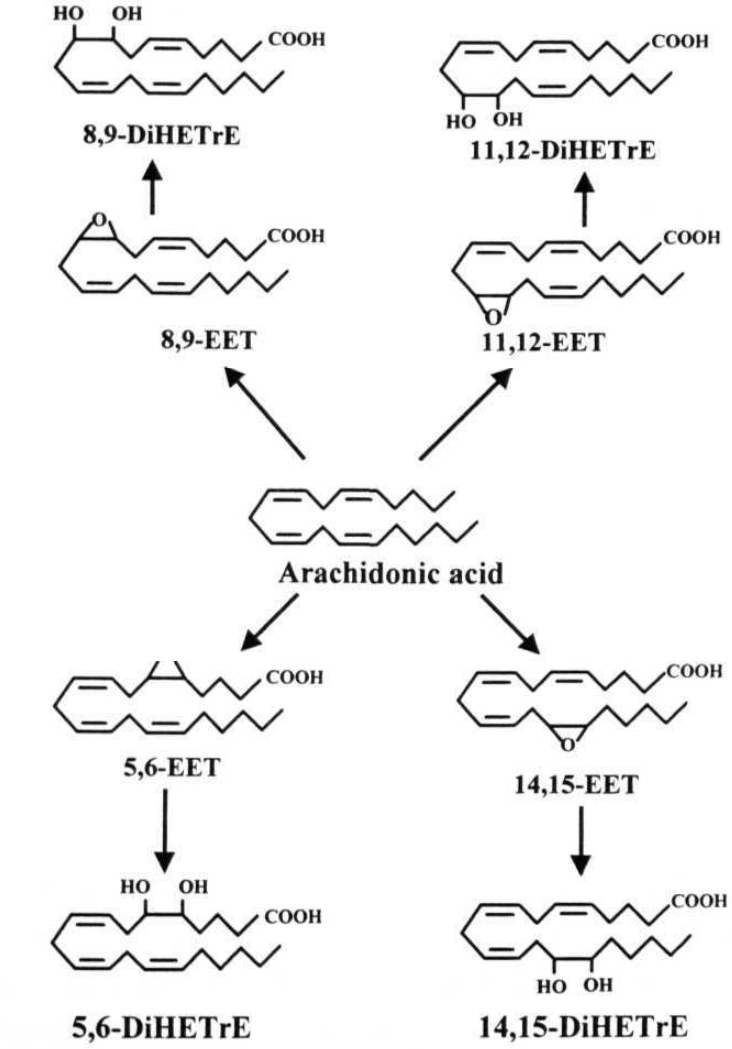


Fig.4 Epoxygenase pathway of arachidonic acid

1.2.1. *Biological Effects of EETs*

Several biologically active compounds are formed in epoxygenase pathway of AA and each of these metabolites has a spectrum of biological effects. 5.6-EET is implicated in the release of peptide hormones like somatostatin and leutinizing hormone (Capdevila *et al,* 1983 & Snyder *et al,* 1983). 11,12-EET activates the calcium dependent potassium channel through guanine nucleotide binding protein mediated mechanism (Li and Campbell, 1997). 14,15-EETs play an important role in the regulation of ion and water re-absorption in the kidney and thus in renal pathophysiology (Staudinger *et al.,* 1994). Also they have been shown to inhibit Na^+/K^+-ATPase (Schwartzman *et al.,* 1985) mobilization of calcium (Kutsky *et al*., 1983 & Snyder *et al*., 1986), vasodilation (Proctor *et al.,* 1987) and inhibition of platelet aggregation (Fitzpatrick *et al.,* 1986). Bums *et al.,* (1995) suggested a role for EETs in epidermal growth factor signalling. Reproductive tissues are known to release large quantities of EETs, but their functions are yet to be determined (Gonzalez *et al*., 1997)

1.3.0. Lipoxygenase Pathway

Lipoxygenase (Lox) pathway is a complex of pathways leading to the formation of hydroperoxyeicosatetraenoic acids (HPETEs), hydroxy eicosatetraenoic acids (HETEs), leukotrienes (LTs), and lipoxins from AA (Fig. 3). The metabolites of these pathways participate in various cellular functions. Among these, LTs are the most potent biologically active compounds and are formed from all the PUFAs with more than 3 *cis* double bonds.

Lipoxygenases comprise a family of non-heme iron containing proteins and are ubiquitously present in plants and animals (Schewe and Kuhn, 1991). These enzymes oxidize both unesterified and esterified fatty acids such as those in phospholipids (Jung *et al.,* 1985). The stereospecific action of these enzymes

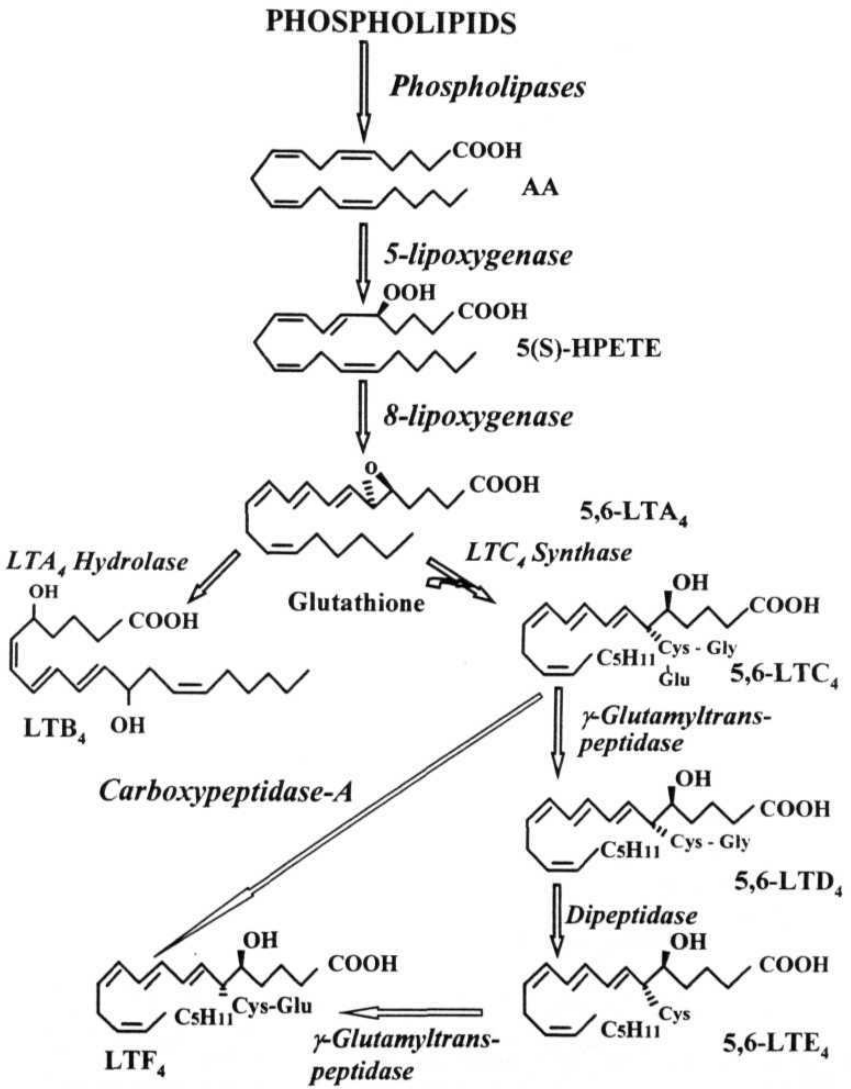


Fig. 5 Biosynthetic pathway of 5,6-leukotrienes

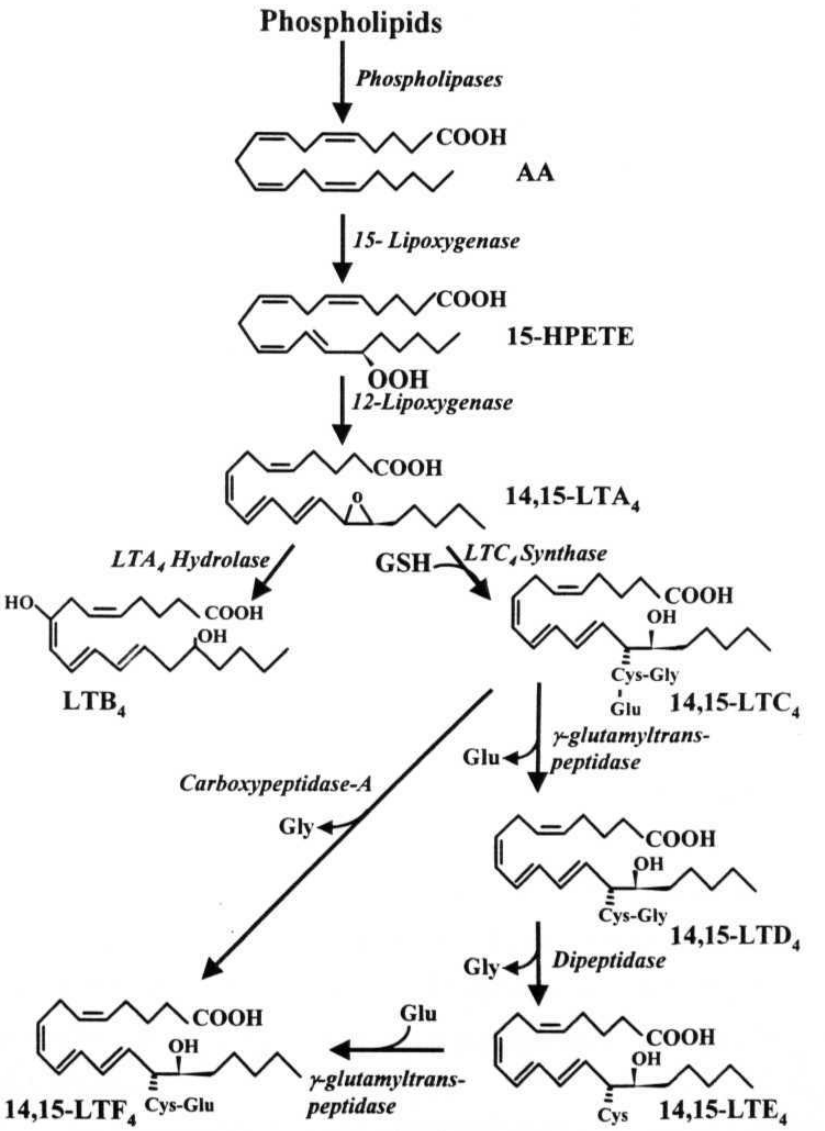


Fig.6 Biosynthetic pathway of 14,15-leukotrienes

require an 1,4-*cis*, *cis*- pentadiene structure in the substrate molecule, which is transformed into 1-hydroperoxy-2,4-*trans*, *cis*- pentadiene product, generating the corresponding hydroperoxy derivative of the fatty acid. These enzymes catalyze the stereospecific insertion of molecular O_2 into PUFA substances, probably by a radical mechanism (Turk *et al.,* 1981). Antarafacial addition of O_2 with respect to hydrogen atom removal has been shown to be a characteristic feature of lipoxygenase reactions (Hamberg and Hamberg, 1980). The enantioselectivity of lipoxygenase is specific for the formation of products having (S) configuration.

Lipoxygenases have been conveniently named in terms of the position (5-,8-,9-,11-,12-,15-) at which molecular O_2 is introduced into the fatty acid substrate, AA. For example, if O_2 is incorporated at C-12 position of AA, the product is 12-HPETE and the enzyme is designated as 12-lipoxygenase. In animal systems the 5-, 12- and 15-lipoxygenases have been isolated and well characterized. Most of the animal Loxs are cytosolic and require Ca^{2+} for their activity (Jakschik *et al.,* 1980).

In the bisynthesis of leukotrienes, 5- lipoxygenase is the key enzyme. This enzyme abstracts a specific hydrogen atom at C-7 of AA, resulting in the formation of carbon centered radical and the rearrangement of the free radical, with a shift to the C-5 carbon along with the rearrangement of double bond to form a conjugated diene. A molecule of O_2 is then added to the free radical and the product formed in the 5-Lox catalysis reaction is 5-HPETE (Panossian *et al.,* 1982). Oxygen incorporation at the C-15 position in 15-Lox catalyzed reaction produces 15-HPETE and so on.

The biosynthesis of LTs starts with HPETE, the Lox reaction product. which gets transformed on dehydration into an unstable allylic epoxide, LTA_4 (Maas *et al.,* 1982). The enzyme activities catalyzing the HPETE production and

the subsequent formation of LTA_4 have been shown to reside in a single protein (Shimizu *et al,* 1984 & Rouzer *et al,* 1986). It is also of interest that incubation of various hemoproteins with HPETEs results in the formation of LTA4 (Radmark *et al,* 1984a), which is the pivotal reactive intermediate in the biosynthesis of leukotrienes. LTA4 undergoes enzymatic and non-enzymatic reactions and the products formed non-enzymatically are biologically inactive (Borgeat and Samuelsson, 1979a). It has been shown that albumin protects/stabilizes LTA_4 from spontaneous non-enzymatic hydrolysis (Fitzpatrick *et al,* 1982).

The LTA_4, is enzymatically hydrolyzed by LTA_4 hydrolase to form biologically active dihydroxy acid LTB_4 (Borgeat and Samuelsson, 1979b). Another pathway is the formation of LTC_4 from LTA4 by an enzymatic conjugation with glutathione (GSH). This enzymatic conversion of LTA4 to LTC_4 is catalyzed by the enzymes involved in the GSH detoxification pathway, like glutathione S-transferases (Hammarstrom *et al.,* 1979) and by a specific GSH transferase, the LTC_4 synthase (Jakschik *et al.,* 1982 & Bach *et al.*. 1984). Successive elimination of glutamic acid from LTC_4 by y-glutamyl transpeptidase (Orning *et al.,* 1980) and glycine by dipeptidase gives LTD_4 and LTE_4 respectively (Sok *et al.,* 1981 & Lee *et al,* 1983). LTE_4 on further action by y-glutamyl transpeptidase results in the formation of LTF_4 (Anderson *et al.,* 1982). LTF4 can be formed directly from LTC4 by the action of carboxypeptidase-A (Fig. 5 and 6) (Reddanna *et al,* 1988). Upon non-enzymatic hydrolysis, 5,6-LTA_4 produces two isomers each of 5,6- and 5,12- dihydroxy compounds while 14,15-LTA_4 produces. 14,15-, and 8,15- dihydroxy compounds (Fig. 7).

1.3.1. Leukotrienes

The LTs were originally discovered in 1938 by Feldberg and Kellaway as smooth muscle contracting factor in the perfusate from guinea pig lung and were

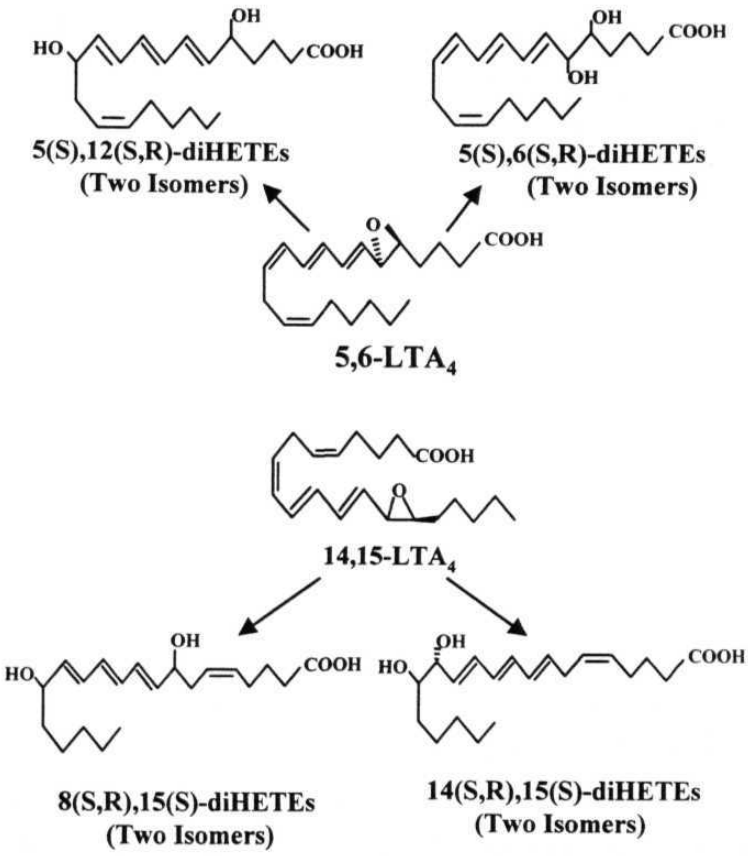


Fig.7 Non-Enzymatic hydrolysis of 5,6- and 14,15-LTA_4s

referred to as slow reacting substances (SRS). They were subsequently shown to be associated with anaphylaxis and termed as slow reacting substances of anaphylaxis (SRS-A) (Brocklehurst, 1953). Later Samuelsson's group defined the molecular structure of SRS-A as fatty acid component derived from AA linked to cysteine containing peptide (Murphy *et al.*, 1979). LTs, the family of highly bioactive substances derived from AA, first described by Borgeat and Samuelsson (1979a) in leukocytes and was determined to contain a conjugated triene structure. In view of these features and cells of origin this family of compounds has been named leukotrienes (Samuelsson *et al.*, 1979).

LTs can also be formed from PUFAs other than AA, 3-series of LTs are derived from eicosatrienoic acid (ω-6) (Hammarstrom, 1980 & Jakschik *et al.*. 1983), whereas 5-series are derived from eicosapentaenoic acid (ω-3) (Hammarstrom, 1981). Added to the complexity, other biosynthetic pathways of LTs have been described in addition to 5,6-LTA_4 from AA itself (Maas *et al.*. 1981). Jubiz *et al.*, (1981) reported the formation of 14,15-LTA_4 epoxide, a second pathway of LT biosynthesis in leukocytes. Westlund *et al.*, (1988) proposed the formation of 11,12-LTA_4 *via* 9/12- lipoxygenase pathway and the direct evidence for this epoxide has not been demonstrated due to its extremely unstable nature. The operation of 8,9-LTA_4 pathway has not been identified in any biological system. Potato Lox has been shown to produce 5,6-LTA_4, 8,9- LTA_4, 11,12- LTA4. and 14,15- LTA_4 (Whelan, 1988) (Fig. 8) and Corey *et al.*, (1980) reported the chemical synthesis of LTs.

The synthesis and release of LTs have been demonstrated in a variety of cellular sources and tissues from humans, monkeys, guinea pigs, etc. LTs are mainly classified into two types based on their chemical structure i.e. **dihydroxy**

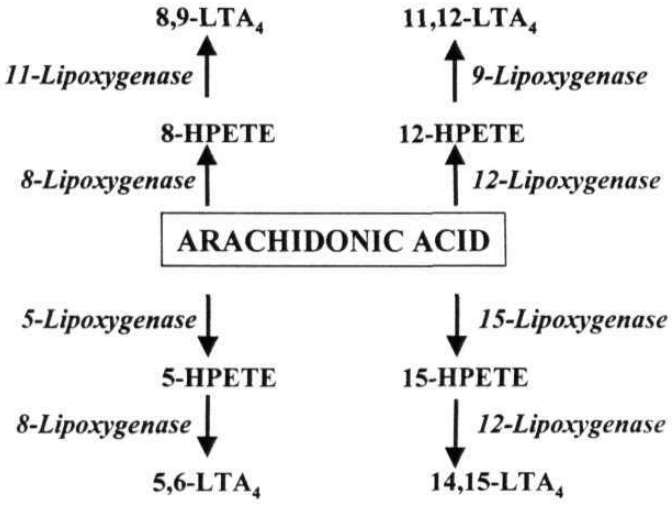


Fig.8 Formation of different possible **leukotrieneA_4s** from arachidonic acid

leukotrienes (LTB_4) and **peptido-leukotrienes** (LTC_4, LTD_4, LTE_4). These two differ from each other in their biological and chemical properties.

1.3.1.0. ***Dihydroxy Leukotrienes***

The biosynthesis of non-peptido-LTs biosynthesis occurs mainly in polymorphonuclear leukocytes like neutrophils, monocytes/ macrophages & mast cells and LTB_4 has even been detected in the cells that do not express the Lox activity such as erythrocytes, endothelial cells and fibroblasts (McGee and Fitzpatrick, 1985).

1.3.1.1. Biological Effects of Dihydroxy Leukotrienes

Elevated tissue levels of LTB_4 have been demonstrated in diseases such as inflammatory bowel disease, psoriasis and rheumatoid arthritis. LTB_4 is a pro-inflammatory mediator (Samuelsson *et al.,* 1987) and is capable of stimulating circulating leukocytes to adhere to the vascular endothelium and directs their migration to the site of inflammation (Ford-Hutchinson *et al.,* 1980). It also helps in the release of lysosomal enzymes, superoxide anion production and enhancement of complement-dependent cytotoxic reactions (Ford-Hutchinson. 1990). Apart from its chemotactic activity, LTB4 is known to involve in many other cellular functions like activation of phospholipase C, Ca^{2+} influx, and reorganization of cytoskeleton. It has a weak effect on smooth muscle but it also induces prolonged bronchial narrowing by stimulating the cyclooxygenation of A A and generation of contractile thromboxanes. LTB_4 also promotes local edema formation and evokes increased secretion of airway mucus (Piper and Samhoun. 1981). However, its role in bronchial asthma still remains to be fully elucidated.

1.3.2.0. ***Peptido Leukotrienes***

The peptido leukotrienes (PLTs) are found in variety of tissues like lung. brain, heart, and blood vessels and are synthesized in many cells such as

leukocytes, macrophages, mast cells, and eosinophils. Reports have demonstrated that the PLT formation may be metabolically regulated by a mechanism involving protein kinase C (Lin *et al.,* 1992 & Ali *et al.,* 1994).

1.3.2.1. Biological Effects of Peptido Leukotrienes

The actions of PLTs, however, appear to be exclusively pro-inflammatory in nature. It has been shown that SRS-A consists of a mixture of LTs, in which LTC_4 as the primary product and depending on the type of tissue and experimental conditions, varying amounts of LTD_4 and other LT metabolites are formed. LTC_4 is 1000 times more potent than histamine in causing contraction of isolated human bronchi. $PGF_{2\alpha}$ is recognized as an effective bronchoconstrictor, which is almost 500 times less potent than LTC4. LTC_4 and D_4 are equipotent as bronchoconstrictors in guinea pig lung although it has been suggested that LTD_4 is more potent than LTC_4 in guinea pig parenchymal strips (Lewis *et al.,* 1980).

The PLTs produce effects that are characteristic of asthma, such as potent bronchoconstriction, increased endothelial membrane permeability leading to airway edema and enhanced secretion of mucus. In addition, LTs have been linked to the development of inflammation. Many critical features of asthma and inflammation can be reproduced by the administration of PLTs.

These compounds constrict not only the smooth muscles of airway but also the myometrium (Carraher *et al.,* 1983). These compounds can stimulate uterine contractions and also play an important role in uterine preparation for implantation (Weichman and Tucker, 1982) and its role in parturition is well established (Walsh, 1991). Their pathophysiological effects in kidney have been described in bilateral obstruction, renal transplant rejection and nephritis (Petric *et al,* 1995).

The inactivation of LTs is achieved by N-acetylation of the cysteine moiety of LTE_4 to form N-acetyl-LTE_4 in liver. Indeed, it is difficult to follow the

extensive current literature on the role of these compounds thoroughly enough to remain completely informed of these advances.

1.4.0. Lipoxins

The trihydroxytetraene derivatives of AA were first isolated from human neutrophils (Serhan *et al.*, 1984) and were named as lipoxins (LXs). These bioactive compounds can be formed by the action of different lipoxygenases (Fig. 9). The conjugated tetraene epoxide is generated from 15-HPETE by the sequential action of 5- and 8-lipoxygenases. The second pathway of LXs biosynthesis from 5,6-LTA4 is by the action of 12/15-lipoxygenase. The lipoxin A_4 (LXA4) and lipoxin B_4 (LXB_4) are the major products formed *in vivo* and each possesses a unique spectrum of biological activities. (Serhan, 1997).

SCOPE OF THE PRESENT STUDY

Much is known and more is to be learned about the role of oxygenated derivatives of AA, especially the LTs, the potent biologically active compounds involved in cellular functions like hypersensitivity and inflammatory diseases. In spite of rapid progress on the effects of LTs, there is limited progress on the regulatory mechanisms associated with their production. A better understanding of the enzymes involved in leukotriene biosynthesis is required to regulate the production of LTs. Modulation of the enzyme activities provides a new therapeutic approach to treat the LT mediated afflictions and should offer an important means to further understand the role of LTs in pathophysiological events.

Uterus, the seat of implantation, growth and development of embryo under continuous physiological stress, is known to metabolize the free AA. There is an extensive literature on the PGHS pathway of AA in uterus with often conflicting and contradictory reports. It was observed that the luteolysis was delayed when Lox pathway was inhibited in uterus, suggesting the importance of Lox pathway in

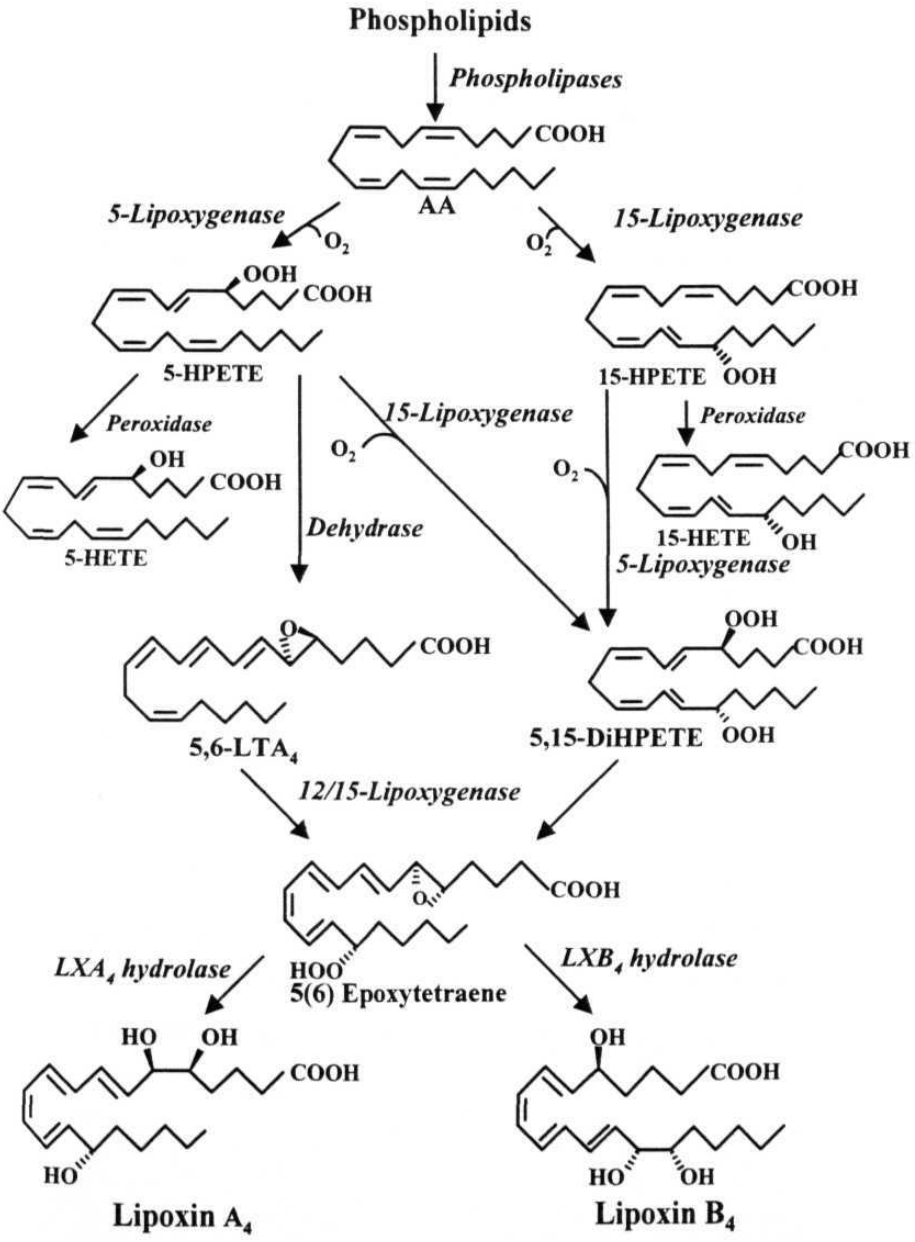


Fig.9 Biosynthetic pathway of lipoxins

female reproduction (Hamilton *et al.,* 1990). Flatman *et ed.,* (1986) reported the partial purification of 12-Lox from human uterine cervix. However there is limited information on the Lox pathway enzymes involved in LT biosynthesis in uterus. The discovery of similarities between lung, skin and uterus ultrastructurally and their resemblances in AA metabolism in their mast cells have opened up the Lox pathway in uterus (Massey *et al.,* 1991). Sailesh *et al.,* (1994) have shown that the sheep uterus is a rich source for Lox with LTA_4 synthase activity, leading to the formation of 14,15-LTA_4. In the present study an attempt is made to know the metabolic fate of LTA_4 in sheep uterus.

The aim of the present study is to identify the enzymes involved in the metabolism of LTA_4 in sheep uterus. Further, it is aimed to purify and characterize the LTA_4 metabolizing enzymes identified.

LTA_4 formed in the biological systems gets converted into either LTB_4 (non-peptido leukotriene) or LTC_4 (peptido leukotriene). In the conversion of LTA_4 to LTC4, at least two different enzymes have been identified so far, one acting only on the methyl esters of LTA4 (also known as classical GSTs) and the other which acts both on Me as well as FA forms of LTA_4 (also known as LTC_4 synthase). In the present study the involvement of both GSTs as well as LTC4 synthase in the metabolism of LTA4 was analyzed. Also the purification and characterization of these enzymes were undertaken.

Methodology

2.1.0.0. Materials

Arachidonic acid (AA) (99% pure), soybean lipoxygenase, fat free bovine serum albumin (BSA), ethylene diaminetetracetate (EDTA), glutathione reduced (GSH), S-hexyl glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), 3-[(3-cholamidopropyl) dimethylammonio]-l-propane-sulfonate (CHAPS), dithiothreitol (DTT), probenecid, Q-Sepharose, epoxy-activated Sepharose 6B, 4-(2-hydroxyethyl)-l-piperazine ethanesulfonic acid (HEPES), phenylmethylsulfonyl fluoride (PMSF), L,α-phosphatidyl choline, magnesium chloride, calcium chloride, Tris. glycine, L-serine, boric acid, potassium carbonate, lithium hydroxide monohydrate, taurocholate, deoxycholate, Tween-20, Triton X-100, glycerol, N-ethylmaleimide, sodium chloride, protein-A agarose, protein-A biotin, prostaglandin B_2 (PGB2), Freund's complete and incomplete adjuvant were purchased from Sigma Chemical Company (St. Louis, USA).

Pentamethyl piperidine (PMP), triethylamine (TEA), trifluoromethanesulfonic anhydride (TFMSA), bis(trimethylsilyl)trifluoro acetamide (BSTFA), boron trifluoride (BF_3), were purchased from Aldrich Chemical Company (MO, USA).

Acrylamide (99.9%), N,N`-methylene-bis-acrylamide,N,N,N`,N`-tetramethyl-ethylenediamine (TEMED), 2-mercaptoethanol, natriumlauryl-sulfat (SDS), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), coomassie brilliant blue G-250, coomassie brilliant blue R-250, bio-lyte ampholines, and bromophenol blue were purchased from Bio-Rad laboratories (Richmond, USA).

Low molecular weight markers for SDS-PAGE were purchased from Pharmacia Biotech (Uppsala, Sweden). Ultrafiltration units were procured from Amicon (MA, USA). Nitrocellulose membranes for immunoblotting were

purchased from Amersham (England, UK). C_{18} and silica cartridges were purchased from Waters, India.

LTC_4 synthase-specific peptides (MKDEVALLAAVTLLGVLLQ & GRLRTLLPWAC) conjugated to keyhole limpet hemocyanin (KLH) were procured from MBT, USA. The LTC_4 synthase and specific LTC_4 synthase anti-peptide antibodies were gift from Dr. John F. Penrose (Harvard Medical School. USA). Anti-rabbit IgG-alkaline phosphatase was purchased from Genei. Bangalore, India. HPLC solvents like hexane, methanol, propane 2-ol. tetrahydrofuran (THF), methylene chloride and acetic acid were procured from Spectrochem India Ltd. All other chemicals procured were from the local companies and were of high quality.

2.1.1.0. Sheep *(Ovis aries)* Uterus

Sheep uterine tissues were collected from local slaughter houses immediately after sacrifice and transported on ice to the laboratory.

Sheep belongs to the phylum-chordata, class-mammalia, order-artiodactyla (hooved, even footed), family-bovidae, genus-*ovis* and species-*aries*. Within the species many breeds exist. Sheep is a classical example of follower species i.e.. young tend to follow their mothers from birth. They are poly estrus, the estrous cycle is 14-19 days with a mean of 17 days and is divided into 4 stages i.e., estrus. metestrus, diestrus and proestrus. The main events in the estrous cycle of the ewe can be divided into those associated with the growth of follicle (follicular phase) and those associated with the growth of corpus luteum (luteal phase). The growth of the follicle takes place during proestrus and estrus. The period of corpus luteum can be divided into metestrus and diestrus.

Estrus is defined as the period of sexual receptivity during which ovulation occurs in most species and corpus luteum begins to form. The duration

of the estrus is 12-72 h with an average of 45 h and ovulation takes place about 12 h before the end of estrus. Metestrus phase lasts for about two days. The corpus luteum organizes during this period and becomes functional, the progesterone levels rise rapidly.

Diestrus phase is referred to as the phase of the corpus luteum. Metestrus and diestrus are referred to as the **luteal phase,** during this period large amounts of progesterone enters general circulation resulting in the development of endometrium. Corpus luteum, a temporary endocrine organ reaches maximum size by third day after ovulation, at which time it is reddish pink in color.

Proestrus is the period, which begins after the lysis of corpus luteum and the progesterone level drops. During this phase follicle stimulating hormone release takes place, which stimulates follicular growth and raises estrogen level. Proestrus is followed by estrus. Proestrus and estrus are often referred to as the **follicular phase.**

In sheep the uterus is of bipartite type. Ewes have a septum that separates the two horns and a prominent uterine body. Both sides of the uterus are attached to the pelvic and abdominal walls by the broad ligament. The uterus receives the blood and nerve supply through the broad ligament. The size of the uterus, ovarian follicles and corpus luteum were used to identify different stages of the uteri.

2.2.0.0. General Methods

2.2.1.0. *One Dimensional Gel Electrophoresis*

SDS-PAGE analysis of proteins was performed according to the method of Laemmli (1970). Electrophoresis was carried out in 10/12% acrylamide gels with 5% stacking gel. Samples were treated with buffer containing 1% SDS (w/v). 5% P-mercaptoethanol (v/v), 0.01% bromophenol blue (w/v) and 10% glycerol (v/v) in 0.063 M Tris-HCl, pH 6.8, for 5 min in boiling water. Proteins were

electrophoresed at constant voltage (100 V) in 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS (w/v), pH 8.3-8.6 and was stopped when the tracking dye reaching the bottom. Molecular weight marker proteins were also run simultaneously with the samples.

2.2.1.1. Molecular Weight Determination

Molecular weight of the proteins was determined from the calibration curve generated using Pharmacia low molecular weight (LMW) markers using gel documentation system (UVP, San Gabriel Inc. UK). The LMW markers included phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa). carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

2.2.2.0. *Native Gel Electrophoresis*

Tris-glycine gradient gel (4-12%) without SDS was prepared but with 0.1% deoxycholic acid and 0.1% Triton X-100 in the gel. The gel was subjected to pre-run with non-SDS buffer of 100 mM Tris (pH 9.0-9.2) containing 1 mM EDTA, 2 mM thioglycolic acid, 0.1% Triton X-100 and 0.1% deoxycholate (w/v) at 4°C at 25 mA for 30 min. Sample buffer without SDS, but with 2 mM thioglycolate was added to the equal volume of concentrated protein sample and was loaded onto the gel. The gel was electrophoresed in same buffer (thioglycolate in anode buffer) at 4°C at 25 mA for 6 h. Native molecular weight marker proteins were run simultaneously with the samples. Molecular weight of the proteins was determined from the calibration curve generated from molecular weight markers.

2.2.3.0. *Two-Dimensional (2-D) Electrophoresis*

Two-dimensional electrophoresis was performed as described by O`Farrell (1975). Isoelectric focusing gels were cast in glass tubes (130 X 2.5 mm). To setup the pH gradient the ampholyte polyacrylamide gels were prefocused at 200 V for

15 min, at 300V for 30 min and at 400 V for 30 min. The samples were loaded and the gels were run at 400V for 12 h and 800V for 1 h with 0.01 M H_3PO_4 as the anolyte and 0.02 M NaOH as the catholyte. The gels were extruded into 5 ml of 0.0625 M Tris-HCl (pH 6.8) buffer containing 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS and equilibrated for 2 h at room temperature with shaking. The gels were stored frozen at -20°C till use. The second dimension separation was carried out using 3.3% stacking gel and 12% resolving gel. Pharmacia LMW markers were run at the acidic end of the gel. The gels were run at 25 mA until the dye (bromophenol blue) reached the bottom of the gel. The gels were fixed and silver stained. To determine the pH gradient of the IEF gels. parallel gels were cut into pieces of 0.5 cm length and incubated for 2 h in 0.5 ml degassed distilled water. The pH of the eluant was determined electrometrically and was taken as isoelectric point (pI) of the protein present in the gel.

2.2.4.0. *Native Isoelectric Focusing*

The native isoelectric focusing gels were prepared and IEF was performed as described by Robertson *et al*., (1987). Mini gels (1.5 mm thick) were prepared (8 x 7 cm) with 7 ml water, 2 ml acrylamide mixture [(30% w/v) acrylamide. (1% w/v) bis-acrylamide], 2.4 ml glycerol (50% v/v) and 0.6 ml ampholytes (pH range 3-10). The gels were allowed to polymerize for 1 h. The cathode solution was 25 mM NaOH and the anode solution 20 mM acetic acid. The anolyte and catholyte were cooled to 4°C prior to electrophoresis. The samples were prepared by mixing equal volume of 60% (v/v) glycerol and 4% (v/v) ampholytes (pH 3-10). Electrophoresis was performed at room temperature at 200 V for 1.5 h and additional 1.5 h at 400V. After the run, the gels were placed in 10% trichloroacetic acid for 10 min to remove the ampholytes. After a brief rinse with distilled water.

the gels were fixed and stained with 0.25% (w/v) Coomassie blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid.

2.2.5.0. ***Silver Staining of Polyacrylamide Gels***

The silver staining of the proteins separated on polyacrylamide gels was carried out according to the procedure of Blum *et al*. (1987), with minor modifications. Gels were incubated first with 50% methanol, 12 % acetic acid and formaldehyde (50 µl/100 ml) for 1 h followed by three washings in 50% ethanol of 5 min each. Gels were treated with sodium thiosulphate (20 mg/100 ml) for 1 min. rinsed three times with distilled water (20 sec each) and impregnated in 0.2% silver nitrate solution containing formaldehyde (50 µl/100 ml). After 30 min the gels were rinsed with distilled water and color was developed with 6% sodium carbonate (w/v) containing formaldehyde (50 ul/100 ml). The color development was stopped with 1% acetic acid. The stained gels were preserved in 50% methanol after thorough washing with distilled water.

2.2.6.0. ***Production of Antiserum***

Purified protein/peptides (Peptide-1 MKDEVALLAAVTLLGVLLQ and peptide-2 GRLRTLLPWAC) conjugated to keyhole limpet hemocyanin (KLH) were used for immunization. Rabbits (New Zealand white male, 2 months old) were injected subcutaneously with sample emulsified in Complete Freund's adjuvant in 1:1 ratio. The booster injections were given with the sample in incomplete Freund's adjuvant after 15 days and continued for 3 months giving booster for every 15 days. Rabbits were bled a week after the final booster injection, serum was collected, purified using the protein-A agarose and stored in aliquots at -20°C as immune sera (primary antibody).

The rabbits were bled one week before starting the immunization schedule. serum collected, pooled and stored as pre-immune sera.

2.2.7.0. ***Western Blotting***

The proteins separated on SDS-PAGE were transferred on to nitrocellulose membrane by the method of Towbin *et al.* (1979), at 70 V for 3-4 h using Bio-Rad Trans Blot apparatus. After the transfer the membrane was air dried and incubated with Tris buffered saline (TBS) (10 mM Tris, 150 mM NaCl, pH 7.5) containing 3% bovine serum albumin (BSA) at 4°C for overnight to block non-specific binding. The membrane washed in TBS containing 0.05% Tween-20 (TBST) was incubated with affinity purified polyclonal antibodies (primary antibody) in TBST containing 3% BSA for 2 h or more depending on the titre of the antibody at 37°C with shaking. The membrane was washed with TBST (three times of 5 min each) and the blot was incubated with anti-rabbit IgG linked to alkaline phosphatase (ALP) (secondary antibody) at 37°C for 1 h. The membrane was again washed thoroughly with TBS (six times of 5 min each) and the color was developed with ALP buffer (10 mM Tris, 5 mM $MgCl_2$ and 100 mM NaCl, pH 9.5) containing 0.033% nitroblue tetrazolium (NBT) and 0.0165% 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Reaction was stopped by washing the membrane in distilled water, dried and stored.

2.3.0.0. Biomimetic Synthesis of 14,15- and 5,6-Leukotriene A_4

Leukotriene A_4, the highly unstable epoxide, is the precursor for all the biologically important leukotrienes (LTs). This epoxide gets enzymatically transformed into LTC4 by a specific enzyme, LTC_4 synthase and to LTB4 by LTA_4 hydrolase. In view of the exorbitant price (one mg costs US $ 3000) and its highly thermo-labile nature of the epoxide, an attempt is made to synthesize the epoxide using the published reports (Chang *et al,* 1987a) with few modifications (Fig. 10).

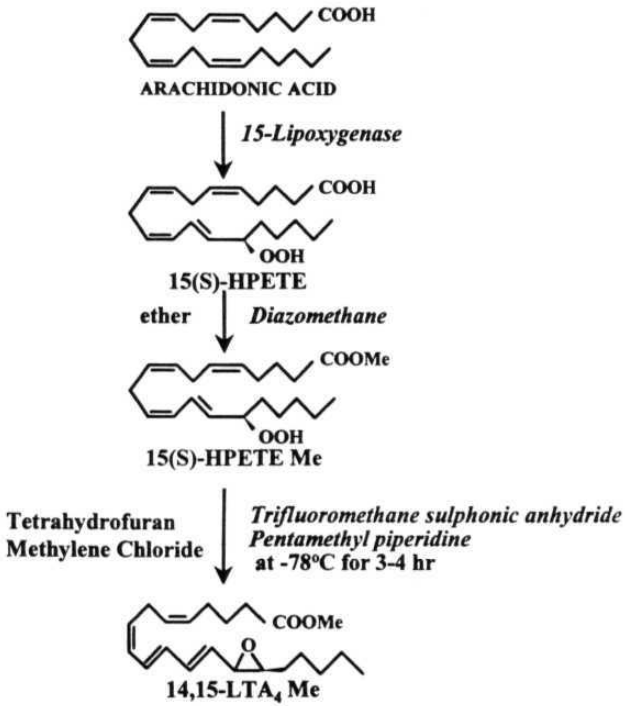


Fig.lO Biomimetic synthesis of 14,15-leukotrienes

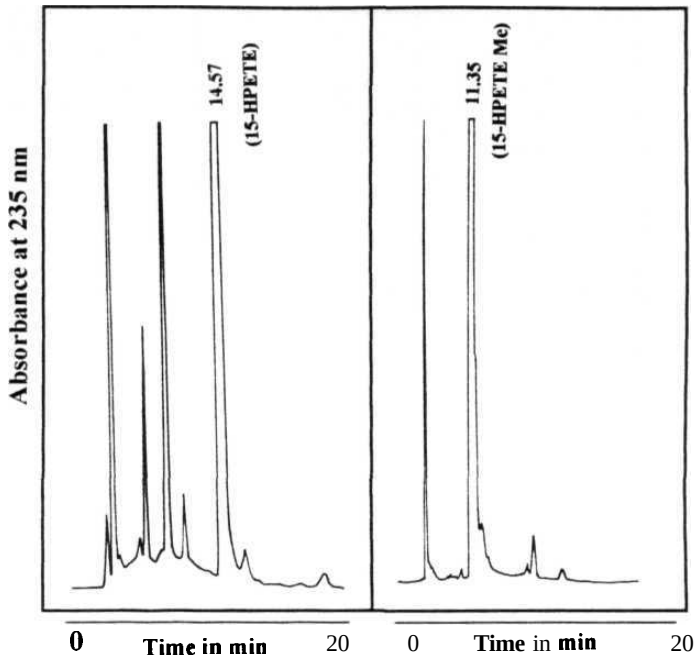
2.3.1.0. ***Preparation of 15- and 5-hydroperoxyeicosatetraenoic acids***

Large amounts of 15-HPETE was prepared by reacting commercially available soybean lipoxygenase with arachidonic acid, as per the method described by Reddanna *et ed.*, (1990). The soybean lipoxygenase was incubated with 150 µM AA in 0.1 M Tris-HCl buffer (pH 9.0) at room temperature for three min and the reaction was terminated by acidifying the reaction mixture to pH 3.0 with 6 N HC1. The products formed were extracted twice with equal volumes of hexane: ether (1:1, v/v). The organic phase separated from the aqueous layer was passed through the anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in straight phase HPLC (SP-HPLC) mobile phase consisting of hexane: propane 2-ol: acetic acid (100:2:0.1, v/v/v) and loaded on to the HPLC on a normal phase silica column equilibrated with the HPLC solvent, and products were separated by isocratic elution at a flow rate of 1ml/min (Fig. 11a). The effluent was continuously monitored at 235 nm. The individual peaks were collected and checked for the presence of conjugated diene spectrum. The peak with retention time (RT) 14.57 min showed the characteristic spectrum of HPETE with maximum absorption at 234 nm (Fig. 11b). The HPETE thus generated was used in the biomimetic synthesis of epoxide. the LTA4.

To prepare 5-HPETE using potato lipoxygenase the same procedure was employed except that the buffer used was 150 mM potassium phosphate (pH 7.0) and hexane: propane 2-ol: acetic acid (100:4:0.1, v/v/v) as mobile phase. The peak with RT 19.02 min, with characteristic conjugate diene spectrum was taken as 5-HPETE(Fig. 12a).

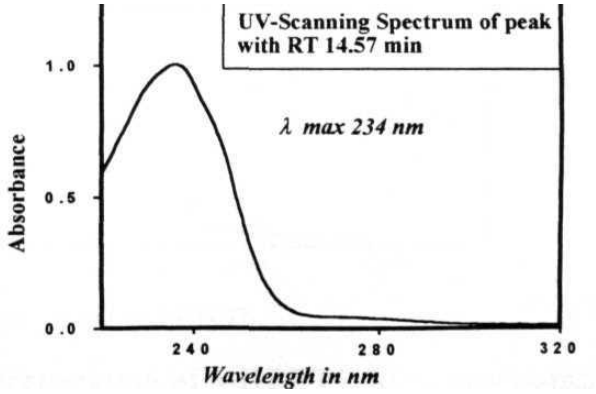
2.3.2.0. ***Preparation of 14,15- and 5,6-Leukotriene A_4***

14,15-LTA_4 was prepared according to the modified procedures of Corey and Barton, (1982) & Chang *el al.*, (1987a). 15-HPETE synthesized using soybean



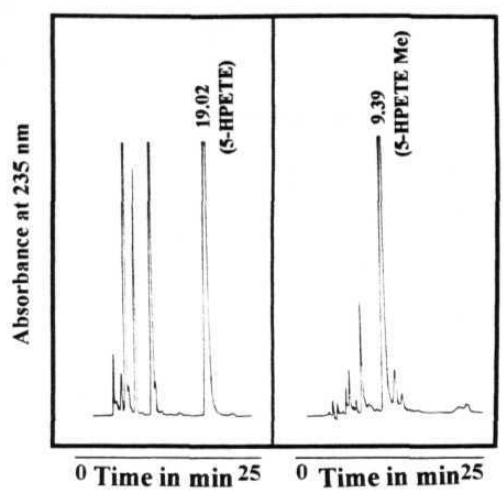
a. SP-HPLC analysis of i) Soybean Lox products ii) 15-HPETE Me Purification

Column	:	CLC-SIL
Solvent	:	Hexane: Propane 2-ol: Acetic acid (100:2:0.1)
Flow Rate	:	1 mL/min
Detection	:	235 nm



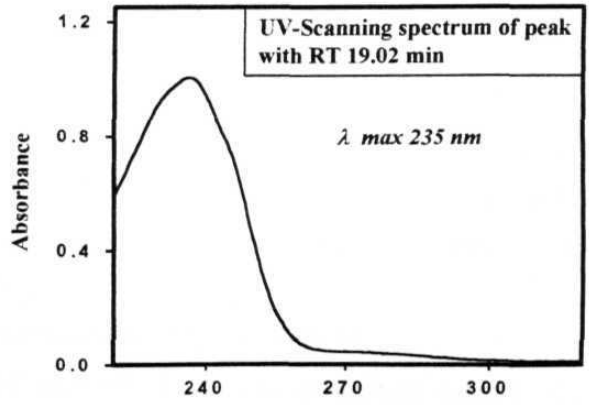
b. UV-Scanning spectrum of 15-HPETE

Fig.11 Preparation of 15-HPETE Me using soybean lipoxygenase



a. SP-HPLC *analysis of i) potato Lox products* ***ii)*** *purification of 5-HPETE Me*

Column	:	**CLC-SIL**
Solvent	:	**Hexane: Propane 2-ol: Acetic acid (100:4:0.1)**
Flow Rate	:	**1 mL/min**
Detection	:	**235 nm**



b. UV-Scanning spectrum of 5-HPETE

Fig.12 Preparation of 5-HPETE Me using potato lipoxygenase

lipoxygenase was methylated with diazomethane and purified on SP-HPLC (Fig.11b). The Pure 15-HPETE Me (~1000 AU) was dissolved in 50 ml of anhydrous methylene chloride and tetrahydrofuran (1:1, v/v) under inert conditions. The contents were cooled to -78°C while stirring in an ethanol-dry ice bath. Pentamethyl piperidine (0.9 ml) was added and after 15 min of vigorous shaking 0.45 ml of trifluoromethane sulphonic anhydride (TFMSA) was added. The reaction mixture was allowed to react for 3-4 h with constant stirring while maintaining the bath temperature at -78°C. The reaction was terminated by the addition of triethylamine (TEA) and the temperature was slowly raised to -10°C by transferring the flask onto ice. The contents were then diluted with 100 ml of ice cold hexane: ether: TEA (50:50:1, v/v/v) followed by 100 ml of saturated sodium chloride solution. The organic layer was separated, dried and evaporated under argon. The syrupy liquid finally obtained was redissolved in 10 ml of hexane: TEA (99:1, v/v) and passed through pre-equilibrated silica cartridges and the flow through was checked for LTA_4 Me. The presence of conjugated triene spectrum with maximum absorption at 278.5 nm shows the formation of LTA_4 (Fig. 13). The product formed was quantified by taking the molar extinction coefficient of LTA_4 Me (40,000 cm^{-1} M^{-1}). For the preparation of 5,6-LTA_4 Me the same procedure was followed except that 5-HPETE was employed as the starting material (Table 1).

2.3.3.0. *Preparation of LTC_4 from LTA_4 Me*

LTA4 Me (~100 AU) was dissolved in 0.5 ml methanol: TEA (2:1, v/v) and was reacted with 60 mg of reduced glutathione (GSH). To this 250 ul of 1 M Potassium carbonate (K2CO3) was added and the reaction mixture was incubated in dark. After 2 h the reaction was terminated by the addition of 250 ul of 6 N HCl and the contents were diluted 10 fold with water and passed through the pre-

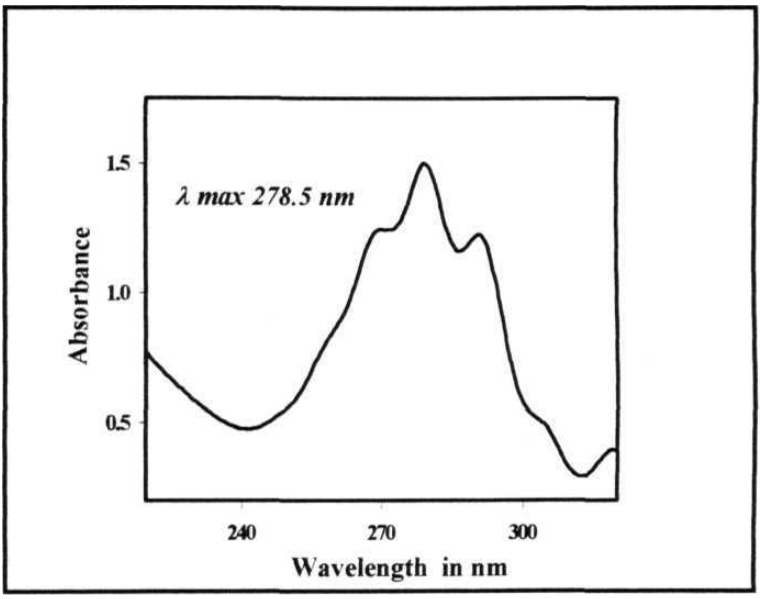
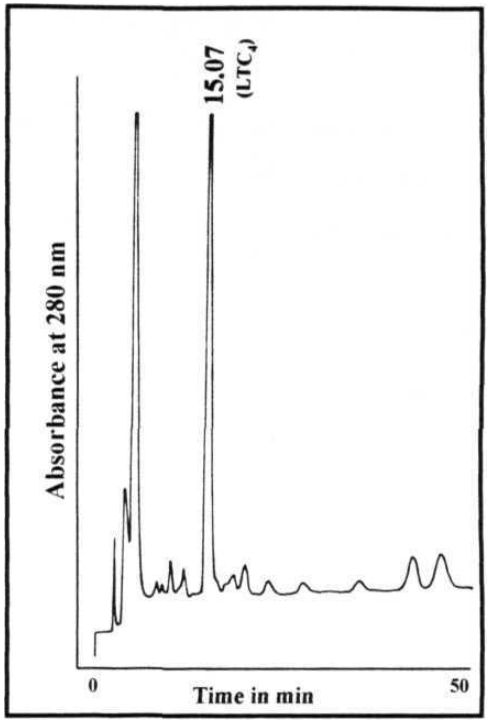


Fig. 13 UV-Scanning spectrum of leukotriene A_4 Me.

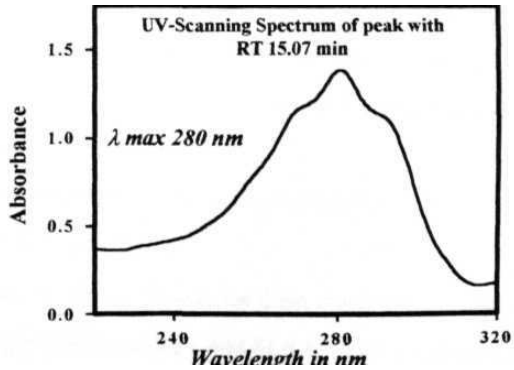
Table 1. Biomimetic synthesis of **LTA_4s.**

Solvent	**HPETE (mg)**	**LTA_4 (mg)**	**Yield (%)**
CH_2Cl_2: THF (1:1)	5-HPETE(12.0)	5,6-LTA_4 Me (02.88)	24
CH_2Cl_2: THF (1:1)	15-HPETE(34.0)	14,15-LTA_4 Me (16.00)	47



a. RP-HPLC analysis of LTC_4

Column	Waters μ Bondapak C_{18} (0.39x 30 cm)
Solvent	MethanolrWater: Acetic acid(65:35:0.08;pH 5.7)
Flow Rate	1 mL / Min.
Detection	280 nm



b. UV-Scanning spectrum of LTC_4

Fig.14 Preparation of standard 14,15-LTC_4.

equilibrated C_{18} cartridges. The cartridge was washed with water and the adsorbed reaction products were eluted with methanol. The LTC4 in methanol was dried and later re-suspended in HPLC solvent, methanol: water: acetic acid, pH 5.7 (65:35:0.08, v/v/v) and separated on reverse phase HPLC (RP-HPLC) using u Bondapak (Waters India) C_{18} column (0.39 x 30 cm). The column was equilibrated with the HPLC solvent, and LTs were separated by isocratic elution at a flow rate of 1ml/min (Fig. 14a). The effluent was monitored at 280 nm and the peaks were collected and were subjected to UV scanning. The peak with retention time (RT) 15.07 min showed the characteristic spectrum of LTC_4 with maximum absorption at 280 nm (Fig. 14b) and was used as the standard for co-chromatography.

2.3.4.0. ***Hydrolysis of Leukotriene A_4 Me***

Hydrolysis was carried out by modifying the method described by Wynalda *et al*., (1982). To LTA4 Me dissolved in methanol: TEA, 6 µl of 1 M LiOH was added and the reaction was carried with periodic shaking at 25°C for 16 h in dark. The solvent was removed by flushing argon and the residue was dissolved in ethanol to give 4 mM stock solution. The stock solution was stored at -80°C under argon until use. To make working solution the stock solution was diluted 10 times with 100 mM phosphate buffer pH 7.4 containing BSA (5mg/ml) to give 0.4 mM daily use LTA4 solution.

2.4.0.0. Purification of Glutathione S-Transferases

2.4.1.0. *Preparation of Crude Extract*

20% homogenate was prepared with the fresh sheep uterine tissue in 10 mM phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM PMSF and 0.25 M sucrose.

The tissues were washed with the buffer after removing all the extraneous adherent tissues and blood vessels. The tissues were minced, processed in a mixer at 4°C and homogenized on ice using Potter-Elvejhen homogenizer with 10 strokes of 30 seconds each at intervals of 3 min. The homogenate was centrifuged at 10,000 x g for 25 min in Kubota centrifuge using RA-5 rotor and the resulting supernatant was then centrifuged at 105,000 x g for 1 h to obtain cytosol in a Hitachi ultracentrifuge using P50AT2 rotor. This cytosolic fraction was used as the enzyme source for further purification.

2.4.2.0. *Affinity Chromatography*

2.4.2.1. Preparation of GSH Affinity Matrix

Affinity matrix was prepared by the method of Simons and Vander Jagt (1977). Epoxy-activated Sepharose 6B (8g) was washed with 500 ml of distilled water and 100 ml of 44 mM phosphate buffer, pH 7.0. The slurry was transferred to 100 ml conical flask and the volume adjusted to 40 ml with same buffer. Argon gas was passed through the flask for 5 min and 8 ml of GSH (100 mg per ml in water, adjusted to pH 7.0/KOH) was added to suspension. Coupling was allowed to proceed at 37°C for 36 h with shaking. To the thoroughly washed gel, 1 M ethanolamine was added to block the unreacted active groups and allowed the suspension at 37°C for 4 h. The suspension was washed successively with 200 ml each of distilled water, 0.5 M KCl in 0.1 M sodium acetate (pH 4.0), 0.5 M KCl in 0.1 M sodium borate (pH 8.0), and 0.15 M KCl in 10 mM potassium phosphate (pH 7.0). Finally the gel was transferred to 10 mM potassium phosphate buffer (pH 7.0) containing 0.15 M KCl, 0.01% azide and stored at 4°C until use.

2.4.2.2. Purification of GSTs on GSH Affinity Column

The cytosol was filtered through a plug of glasswool and dialyzed at 4°C for overnight against 20 mM phosphate buffer to remove the endogenous GSH.

The dialyzed cytosol after centrifugation at 10,000 x g for 30 min was loaded onto the affinity column pre-equilibrated with 20 mM phosphate buffer (pH 7.0). The column was washed thoroughly with 50 mM phosphate buffer (pH 7.0) containing 0.15 M KC1 till the absorbance at 280 run dropped to zero. GST activity was eluted with 50 mM Tris-HCl buffer (pH 9.6) containing 5 mM GSH and 3 ml fractions were collected using Pharmacia fraction collector. Fractions showing transferase activity were pooled and concentrated to 5 ml by ultrafiltration (Centricon-10). The protein in concentrate was estimated by the method of Bradford (1976), as modified by Stoschek (1990).

2.4.3.0. ***Anion Exchange Chromatography***

DEAE- Cellulose (~25 g) was suspended in 250 ml of 100 mM potassium phosphate buffer (pH 7.4) and was washed and degassed thoroughly. The gel was finally washed and suspended in 50 mM phosphate buffer (pH 7.0) and the column was prepared (2 x 14 cm). The affinity purified GSTs were dialyzed against 10 mM Tris-HCl (pH 8.0) containing 1 mM β-mercaptoethanol and the dialyzed enzyme was passed through the anion-exchange column equilibrated with 10 mM Tris-HCl (pH 8.0) buffer containing 1 mM β-mercaptoethanol (equilibration buffer). The column was washed until the absorbance reached zero at 280 nm and the enzyme was eluted with linear salt gradient of 0-0.1 M KC1 in equilibration buffer. Fractions of 2 ml each were collected using fraction collector and monitored for GST activity and protein. The active fractions were pooled and concentrated by ultrafiltration.

2.4.4.0. ***Reverse Phase High Performance Liquid Chromatography***

The subunit composition of purified GSTs was analyzed by reverse- phase HPLC on a Shimadzu 6A HPLC system using Waters u Bondapack C_{18} (0.39x30 cm) column (Ostlund *et ah,* 1987). The solvents employed were 0.1%

trifluoroacetic acid in 35% acetonitrile (solvent-A) and 0.1% trifluoroacetic acid in 85% acetonitrile (solvent-B). The affinity purified GST protein was injected in 100% solvent-A and eluted using the following gradient.

Time	Solvent -B **Conc.**
l-10min	0-15%
10-20 min	15-23 %
20-33 min	23-24%
33-40 min	24-26%
40-48 min	26-29%
48-80 min	29-33%

The effluent was continuously monitored at 214 nm for the detection of the polypeptides and their relative abundance was determined from integrated peak areas of CR6A. The peaks were collected and analyzed for molecular weight determination.

2.4.5.0. ***GST*** *Assay*

GST activity was determined spectrophotometrically as described by Habig and Jakoby (1981) using 1 mM CDNB and 1 mM GSH as substrates. The thioester formation was determined by reading the absorbance at 340 nm and quantified using an extinction coefficient of 9.6 mM^{-1} cm^{-1}. The values were corrected with non-enzymatic reaction value.

2.4.5.1. Definition of Enzyme Activity

One unit of enzyme activity was defined as one u mole of thioester formed or substrate consumed per min and the specific activity was expressed as units per mg protein.

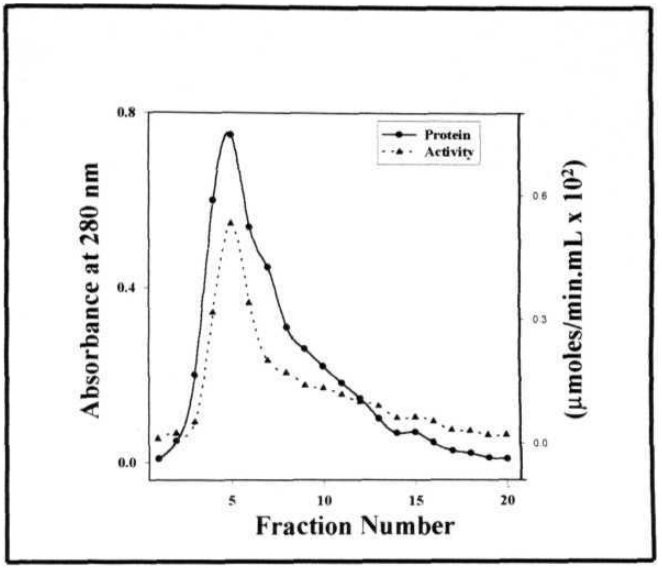
2.4.6.0. *L TC_4 Synthesis Assay of GSTs*

Assay mixture consisted of 75 mM Tris-HCl (pH 8.0) containing 5 mM GSH and purified GST in a final volume of 1 ml. The reaction was initiated with 40 uM LTA4 Me (final concentration) and incubated at 30°C for 10 min. The reaction was terminated using 6 N HC1 and ice cold methanol was added to the reaction mixture to adjust to the mobile phase composition. The reaction mixture was centrifuged at 15,000 x g for 15 min to remove precipitated proteins. The products were analyzed on reverse phase HPLC using u Bondapak (Waters India) C_{18} column (0.39 x 30 cm) with a mobile phase consisting of methanol: water: acetic acid (65:35:0.04) (pH adjusted to 5.8 with TEA) at a flow rate of 1 ml/min. The eluant was continuously monitored at 280 nm and the product LTC4 Me was identified based on its UV characteristic spectrum and by co-chromatography with an authentic standard and was quantified using both CR4A data.

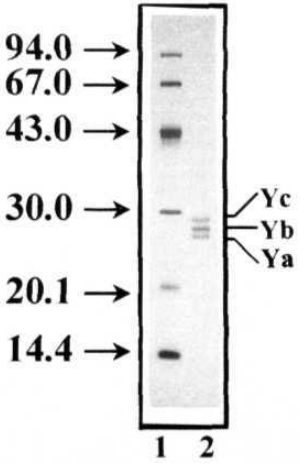
LTC4 synthase activity of GSTs with LTA4 free acid as the substrate was determined as per the method described by Soderstrom *et al.* (1985), with some modifications. Incubation mixture consisted of 25 mM KH_2PO_4 (pH 7.4), 0.5 mM EDTA, 5 mM GSH, 20 uM LTA4 (final concentration) and GST protein. The mixture was pre-incubated at 30°C for 2 min before the addition of LTA4 and was incubated at 30^0C for 10 min. Reaction was terminated by the addition of ice cold methanol. The reaction products were separated on RP-HPLC and analyzed as described above.

2.5.0.0. Purification of Rat Liver GSTs

Rat liver GSTs were purified using GSH affinity chromatography (Fig. 15a). Conditions used for the purification of rat liver GSTs are same as used in sheep uterine GST purification. The purified GSTs were used as molecular weight markers (Fig. 15b). The affinity purified GST were analyzed on RP-HPLC (Fig.



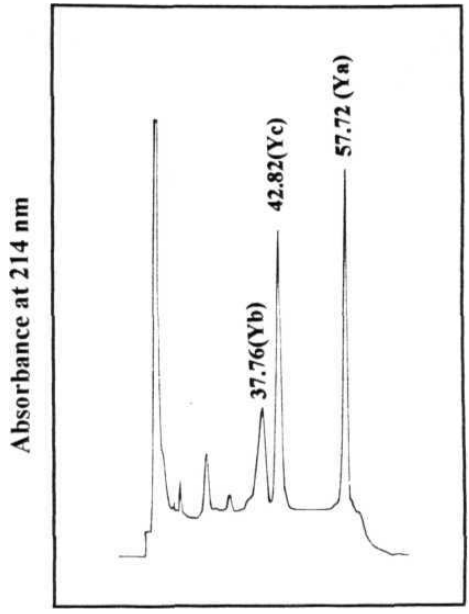
Elution profile of rat liver GSTs on GSH affinity column



1. Molecular weight markers (kDa)
2. Affinity GSTs (0.5 μg)

b. SDS-PAGE analysis of purified GSTs

Fig. 15 GSH affinity chromatography of rat liver **cytosolic** GSTs



Column	:	Waters μ Bondapak C_{18} (0.39 x 30 cm)
Solvent	:	0.1% TFA in 35% Acetonitrile **(Solvent-A)**
		0.1% TFA in 85% Acetonitrile **(Solvent-B)**
Gradient	:	Step
Flow rate	:	1 **mL/min**
Detection	:	214.....
Sample	:	50 **μg** of affinity GSTs

Fig.16 RP-HPLC analysis of affinity purified rat liver cytosolic GSTs

16) using step gradient (15-20%-solvent-B for a period of 15 min, 20-30 % solvent-B for 30 min, 30-35% solvent-B for 10 min and 35-45% solvent-B for 10 min) over a period of 65 min. The affinity purified GSTs and the individual GST subunits were used to raise antibodies.

2.6.0.0. Purification of LTC_4 Synthase

2.6.1.0. *Preparation of Microsomes*

With Fresh Sheep uterine tissues obtained from slaughter house, 20% homogenate was prepared in 50 mM HEPES buffer, pH 7.6, containing 2 mM EDTA, 2 mM PMSF and 0.25 M sucrose. The tissues were minced, processed in a omnimixer at 4°C and homogenized on ice using Potter-Elvejhen homogenizer with 10 strokes of 30 seconds each at intervals of 3 min. The homogenate was centrifuged at 1000 x g for 15 min and the supernatant was again centrifuged at 5,000 x g for an additional 25 min. The resulting supernatant was then centrifuged at 105,000 x g for 1 h to obtain microsomal pellet. The microsomal pellets thus obtained were washed 3 times with the same buffer followed by centrifugation at 105,000 x g for 1 h each time. The washed microsomal pellet was then suspended in phosphate buffer containing 1 mM EDTA to a final concentration of 15-20 mg protein/ml, snap frozen in liquid nitrogen and stored at -80°C until use.

2.6.1.1. Solubilization of Microsomes

LTC_4 synthase being microsomal protein and the solubilization of microsomal proteins has been the key step in its purification. Various detergents like taurocholate, deoxycholic acid, CHAPS and etc. have been employed for the solubilization of microsomes by different investigators.

In the present study LTC4 synthase was solubilized with CHAPS/taurocholate (1% final concentration) by combining microsomal membrane suspension with an equal volume of two fold concentrated detergent in

50 mM phosphate buffer, pH 7.4 containing 2 mM EDTA. The solubilized microsomes were centrifuged at 200,000 x g at 4°C for 1 h. The resulting supernatant was clarified by passing through 0.45 µm filter and the filtrate was used for further purification.

2.6.2.0. *Anion Exchange Chromatography*

The taurocholate/CHAPS solubilized microsomal extract was applied on to Q-Sepharose column (1.5 x 10 cm) equilibrated with 20 mM Tris-HCl containing 1 mM EDTA, 2 mM GSH, 1 mM DTT, 0.1% taurocholate 0.5% n-octyl glucoside. 0.5% CHAPS, pH 7.6 at 4°C. The column was washed with 100 ml of buffer and the bound proteins were eluted with a linear gradient of sodium chloride (0-1 M. 100 ml gradient volume) and 3 ml fractions were collected. The fractions containing LTC_4 synthase activity were pooled and concentrated using Amicon spin concentrators with 10 kDa cutoff size by centrifugation at 5,000 x g at 4^0C.

2.6.3.0. *S-hexyl GSH Sepharose Chromatography*

S-hexyl GSH Sepharose 6B matrix was prepared by the method of Reddy *et al.*, (1983). Epoxy activated Sepharose 6B (6g) was suspended in 30 ml of deionized water. The gel was washed thoroughly with water followed by 500 ml of 0.1 M sodium phosphate buffer, pH 10.0 (coupling buffer). S-hexyl GSH was dissolved (130 mg/ g dry gel) in 6 ml of coupling buffer and the pH was adjusted to 10 with dilute NaOH. The washed gel was suspended in 30 ml of coupling buffer, mixed with S-hexyl GSH solution and incubated at 37°C in a water bath for 24 h under continuous shaking. The coupled gel was washed with water to remove excess unbound S-hexyl GSH. To block unreacted epoxy groups, the gel was suspended in 40 ml of 1 M ethanolamine and allowed to stand at 37°C for 4 h with occasional stirring. Then the gel was washed with 250 ml of coupling buffer

followed successively by an equal volume of water, 0.1 M bicarbonate buffer (pH 10), 0.1 M acetate buffer (pH 4.0) and water.

The affinity matrix was equilibrated with 50 mM HEPES (pH 7.6) containing 10% glycerol, 5 mM β-mercaptoethanol, 1 mM EDTA, 0.1% deoxycholate and 0.1% Triton X-100. The solubilized microsomal protein was loaded onto the column. After washing the column with 0.2 M NaCl, the proteins were eluted with 10 mM GSH followed by 10 mM probenecid. The fractions were collected were concentrated and assayed for LTC_4 synthase activity.

2.6.4.0. *LTC_4 Synthase Assay*

LTC4 Synthase activity was assayed by the conversion of LTA4 to LTC_4 by the method of Nicholson *et al*, (1992a). The typical reaction mixture consisted of 10 mM GSH, 50 mM serine-borate complex (γ-glutamyl transpeptidase inhibitor). 20 mM magnesium chloride in 50 mM HEPES in a final volume of 500 ul (0.1 mg of L-α-phosphatidylcholine was included in the reaction mixture for LTC_4 synthase assay involving purified fractions). An initial concentration of LTA4 (10 times the final incubation concentration) was prepared by evaporating an aliquot of LTA_4/LTA_4 Me stock under argon, re-suspending LTA4 in ethanol and diluted with fat free BSA (5mg/ml). Before initiating the reaction, the reaction mixture was incubated at 37°C for 1 min. LTAV LTA4 Me (20/40 uM final concentration) was added to the reaction mixture and incubated at 37°C for 10 min. The reaction was terminated by the addition of an equal volume of ice cold methanol. The resulting mixture was allowed to stand at -20°C for one hour and the proteins precipitated were removed by centrifugation at 15,000 x g for 15 min. The extract of reaction mixture was then analyzed by isocratic RP- HPLC on Waters u Bondapak C_{18} column (0.39 x 30 cm), with a mobile phase consisting of methanol/water/acetic acid (65:35:0.08) (pH 5.7) or acetonitrile/ methanol/water/acetic acid (47:15: 30:1)

(pH 5.7), at a flow rate of 1 ml/ min. The product LTC4 was identified based on the RT, the typical triene spectrum and co-chromatography with standard LTC_4. The LTC4 synthase activity is expressed as nano mole of LTC4 formed /mg protein per 10 min. All the reactions were carried out in duplicate and blanks were run with denatured protein i.e. heat killed enzyme.

2.6.4.1. Definition of Enzyme Activity

One unit of enzyme activity was defined as one nano mole of LTC4 formed per 10 min and the specific activity was expressed as units per mg protein.

2.6.5.0. *Protein Estimation*

Protein content in the crude preparations was estimated by Bardford method modified by Stoschek (1990) and in chromatographic fractions the protein content was determined by taking absorption at 260 nm and 280 nm on Beckman DU-64 spectrophotometer using 10 mm path length quartz cuvette.

Part-I

Purification and characterization of GSTs from sheep uterus

3.1.0.0. Introduction

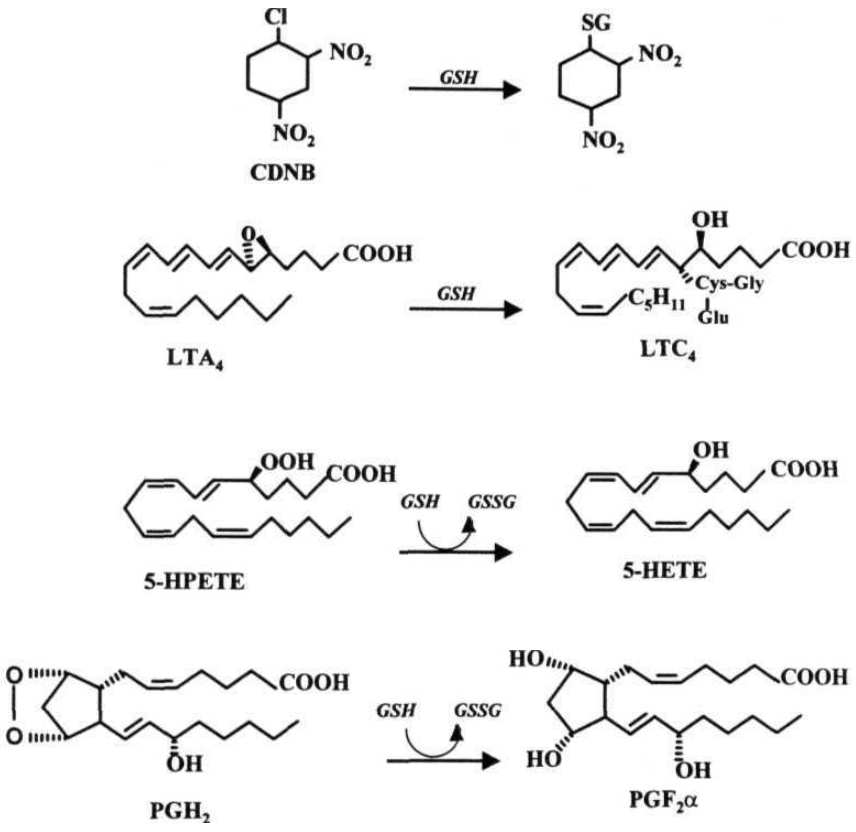
Glutathione S-transferases (GSTs, EC 2.5.1.18) are important family of proteins classified under class II detoxifying enzymes. GSTs are multifunctional proteins with ubiquitous in distribution and have been found in almost every organism studied to date. These proteins catalyze a variety of reactions that include conjugation, reduction, isomerization and thiolysis (Fig. 17). In all these reactions GSH functions as an obligate nucleophilic primary reactant for a number of electrophilic compounds produced/formed from the biotransformation of xenobiotics and also endogenous substances (Coles and Ketterer, 1990 & Tsuchida and Sato, 1992). The GSH conjugates thus formed are further metabolized by γ-glutamyl transpeptidase and dipeptidase to cysteine-S-conjugates, which are acetylated by N-acetyl transferases to form corresponding mercapturic acids, the chief/classical excretion products of xenobiotics.

GSTs were first identified by Booth *et al.*, (1961) and since then, they have been studied extensively. Because of their central role in the biotransformation of xenobiotics, this group of enzymes has attracted attention of many scientists working in all areas of biosciences. GSTs exist as dimers in the case of cytosolic GSTs and trimers in microsomes. Each subunit in a dimer has two domains, the smaller domain contains GSH binding site (G-site) and the larger domain features the binding site for electrophilic substrates (H-site). GSTs are generally found to occur in the molecular weight range of 17-29 kDa (Mannervik and Danielson. 1988).

3.1.1.0. *Classification*

Seven gene families responsible for the expression of GSTs have been identified so far, five encode for cytosolic GSTs (a, u, π, 0 and σ) and two for microsomal GSTs (Table 2) (Ketterer and Christodoulides, 1994). The cytosolic

1. **Conjugation**



3. **Isomerization**

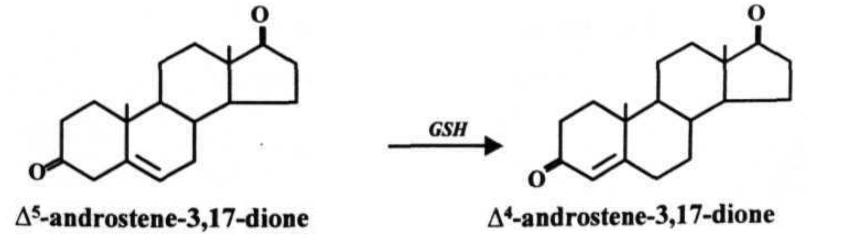


Fig.17 Reactions catalyzed by glutathione S-transferases

Table 2. Classification of rat GSTs. *

Gene Family	GST subunits
a	**1-1 (Ya)** 2-2 **(Yc)** 8-8 **(Yk)** **10-10(Yc2)**
μ	3-3 **(Yb1)** 4-4 **(Yb2)** 6-6 **(Yn1)** 9-9 **(Yn2)** **11-11(Yo)**
	7-7 (Yp)
e	5-5 12-12 (Yrs)

**** (Ketterer and Christodoulides, 1994)***

GSTs characterized from different mammalian tissues share common structural and catalytic properties. Evolutionarily the cytosolic GSTs are derived from common origin as illustrated by their considerable homology (>70%) according to the primary structure within the gene family and 20-30% homogeneity between gene families (Hayes and Pulford, 1995).

Most of the mammalian cytosolic GSTs fall into three classes according to their primary structure (a, μ, and n) and are distinguished on the basis of distinctive biochemical properties (Mannervik *et al*., 1985).

All the cytosolic GSTs are dimeric proteins and exist either as homodimers or heterodimers. There is some evidence that the heterodimers form between the products of allelic genes and also been the products of genes that are from different but closely related loci. All the heterodimers characterized so far are formed from the subunits with in the same evolutionary class.

3.1.2.0. *GST Isozymes*

The existence of multiple forms of GSTs in an organism is a predominant feature. The presence of several isozymes of GST probably signifies an important biological function, since all species carefully examined so far exhibit multiple forms of GST. These isozymes have broad and overlapping substrate specificities and also differ in their physical, chemical, enzymatic and immunological properties.

Several isozymes of GSTs have been demonstrated in each of the animal species investigated and new GST isozymes and new subunits are still being reported. However, when different organs in the same animal are examined, it is generally found that not all of the multiple forms of enzyme are present in every tissue. Numerous studies documented differences in isoenzyme pattern between one organ and another. The high degree of heterogeneity of the GSTs appears to be

largely determined genetically and results from the differential expression of multiple genes between tissues, stages of development and individuals.

The establishment of such multiplicity has, in most cases, been based on chromatographic and electrophoretic separations combined with activity measurements using CDNB as the electrophilic substrate (Mannervik, 1985). The use of this compound as a general substrate for GSTs has been of utmost importance in recognizing the multitude of isozymes in various sources. However, it has been noted from the literature that some enzyme forms display low specific activity with CDNB and the exclusive use of this substrate may impede detection of some isozymes that exist (Hiratsuka *et al*., 1990).

The isozymes have also been characterized with respect to substrate specificity, sensitivity to inhibitors, immunological cross-reactivity, isoelectric points and primary structure as well. GST isozymes within the same class are shown to have almost similar physical, catalytic, immunological and primary structure. On the other hand, homologies among the inter class GST isozymes are comparatively less. It is very difficult to characterize each isoform because of the low yields resulting from the multiple purification steps employed in purifying individual GST isoforms. Rat liver GSTs and their antibodies are generally used in characterization of different GST isoforms.

3.1.3.0. *Importance of GSTs*

Glutathione S-transferases play a significant role in protecting the cell from a variety of exogenous and endogenous challenges. They share a common feature in that all of them catalyze the nucleophilic attack of GSH on electrophilic centers in a wide spectrum of organic compounds. Despite their multiplicity, these enzymes from different tissues and species display a characteristic function. One such function would be their involvement in enzymatic oxidation of arachidonic

acid via Cox pathway to prostaglandins and via Lox pathway to biologically active compounds like leukotrienes.

For the first time Cagen *et al.,* (1975) and Christ-Hazelhof *et al.,* (1976) reported the involvement of GSTs in arachidonic acid metabolism. Apart from its detoxification reactions, some GSTs have been shown to have peroxidase activity referred to as non-selenium glutathione peroxidase, which ascribes a significant role for GSTs in PG biosynthesis as well as protection against oxidative damage from lipid peroxides. GSTs belonging to a and 8 class are known to have high peroxidase activity.

It is well established that the endogenous compounds as well as xenobiotics form an important group of GST substrates. Among these the epoxides have received considerable attention. GST mediated GSH conjugation is the significant route in their biotransformation. Thus LTA_4 epoxide can also act as a substrate for the GSTs. There are reports suggesting GSTs involvement in the formation of leukotrienes (LTs), the mediators of immediate hypersensitivity reactions (Samuelsson, 1982 & Mannervik *et ed.,* 1984). GST Yn_1-Yn_1 exhibits the highest LTC4 synthase activity among the nine cytosolic GST isozymes of rat brain. GST-M_T (6-6) and GST 4-4, immunologically related to GST Yni-Yni, also showed high LTC_4 synthase activities (Tsuchida *et al.,* 1987). However, knowledge of specific GST isozymes responsible for LTC4 biosynthesis and the tissue specific expression of such isozymes are still very limited. In this connection it should be pointed that individual isozymes show differences in specific activity with a given epoxide.

It is known from the literature that the GSTs are expressed in every tissue studied and are quite heterogeneous. There exists variation in GST expression

between tissues, between individuals, between different stages of development and also under different stress conditions.

Uterus is a unique organ, which gets subjected to oxidative stress due to infiltration by immune effector cells. Earlier studies from the our laboratory have reported abundant Lox with LTA_4 synthase activity in sheep uterus (Sailesh *et al.*, 1994). The present study is aimed at the isolation and characterization of enzymes involved in the metabolism of LTA_4 in sheep uterus. Since GSTs are known to conjugate GSH to epoxides and exhibit LTC_4 synthase activity, in the present study an attempt is made to analyze the involvement of GSTs in the metabolism of LTA4. Also individual GST isozymes/subunits were separated and the isozyme with LTC_4 synthase activity identified.

3.2.0.0. Results

Cytosolic and microsomal fractions of sheep uterus were tested for LTC4 synthase activity using both 5,6-LTA4, 14,15-LTA_4 Me and FA as the substrates (Table 3). The cytosolic fraction when incubated with 5,6-LTA4 Me/14,15-LTA_4 Me in the presence of GSH, substantial 5,6-LTC_4 Me/14,15-LTC_4 Me was formed. However no conversion was recorded when LTA4 FAs were used as the substrates. In contrast, the microsomal fraction converted both LTA4 Me as well as LTA4 FAs to the corresponding LTC_4s and the rate of conversion being much higher than the cytosolic fraction. This conversion of LTA4 to LTC_4 in the cytosolic and microsomal fractions of sheep uterus was thermolabile as the heat denatured samples showed no activity. These observations indicate that the conversion of LTA4 to LTC4 by the cytosol and microsomes is an enzymatic reaction.

It is known from the literature that the GSTs are involved in the LTC_4 synthesis. Activity levels of GSTs were measured in the cytosolic fractions of sheep uteri at of various stages of estrous cycle with CDNB and LTA_4 as the

Table 3. LTC_4 synthase activity of cytosolic and microsomal fractions of sheep uterus.

Fraction	5,6-LTA_4		14,15-LTA_4	
	Me	FA	Me	FA
Cytosol	16782	Nil	15793	Nil
Microsomes	73923	39752	78054	37348

LTA_4 Me/ FA (40 μM/20 μM) was incubated with the cytosolic/microsomal fractions in the presence of GSH and the products formed were extracted and then separated on RP-HPLC. LTC4 Me formed was quantified based on the peak areas from RP-HPLC chromatograms and the activity expressed in arbitrary units. Equal amount of protein was used in all the assays.

substrates and the data is presented in Table 4. As shown in the table, maximum GST activity with CDNB was recorded in the uteri at luteal phase (~13 units/g tissue) followed by follicular > pregnant (early) > nulliparous. Similarly LTA_4 to LTC4 conversion rate was also maximum in the uteri at luteal phase. These observations indicate the possible involvement of GSTs in conversion of LTA4 to LTC_4. In order to probe the involvement of GSTs in the LTC_4 formation. GSTs from cytosolic fraction of sheep uterus at luteal phase were purified and further analyzed for isozymes, subunit composition and their involvement in LTA_4 to LTC_4 formation.

3.2.1.0. *Purification of GST Isozymesfrom Sheep Uterus Cytosol*

Glutathione S-transferases have been purified to electrophoretic homogeneity from sheep uterine cytosol by employing a combination of chromatographic techniques like GSH-affinity chromatography, ion-exchange chromatography and RP-HPLC.

The cytosol was obtained by centrifuging the 20% homogenate (prepared from luteal phase sheep uterine tissue) initially at 10,000 x g for 25 min and later (supernatant) at 105,000 x g for 1 h. The cytosol obtained was dialyzed against 20 mM phosphate buffer (pH 7.0) for overnight to remove the endogenous GSH. The dialyzed cytosol was re-centrifuged at 10,000 x g for 30 min at 4°C to remove precipitated proteins, and later applied to the GSH-Sepharose affinity column. The column was washed thoroughly with 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M KG to remove the proteins bound non-specifically. The bound proteins with GST activity were eluted with 50 mM Tris-HCl buffer (pH 9.6) containing 5 mM GSH, which eluted in a single sharp peak. The typical elution profile of GST from affinity column was represented in Fig. 18a. Active fractions were pooled and concentrated to 5 ml by ultrafiltration using Amicon

Table 4. GST activity of cytosolic fractions of sheep uteri at different stages of reproductive cycle.

Stage	GST activity* (units/ g tissue)
Nulliparous	07.18
Follicular	11.25
Luteal	12.70
Pregnant (early)	09.04

The cytosolic fractions were tested for GST activity with CDNB as the substrate.

concentrators. About 90% of the GST activity assayed using CDNB as substrate was bound to the affinity matrix. The GST activity in the flow-through fraction of the column matrix (~10%) was not characterized.

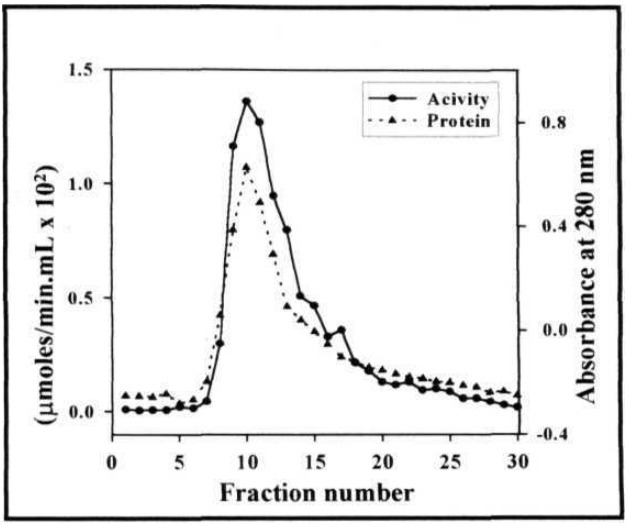
The affinity purified GSTs, when subjected to SDS-PAGE were resolved into three bands with molecular weights of 26.5, 25.5 and 24.0 kDa (Fig. 18b). The gels were calibrated with a mixture of affinity purified rat liver GSTs containing Yc (27.5 kDa) Yb (26.5 kDa) and Ya (25.5 kDa) subunits and molecular weight markers. The purification achieved was about ~270 folds with an overall yield of 56% in one step of affinity chromatography (Table 5).

The affinity purified GSTs on two-dimensional gel electrophoresis (isoelectric focusing/SDS-electrophoresis) resolved into four prominent spots (Fig. 19a). Native isoelectric focussing analysis of affinity purified GSTs (Fig. 19b) reveals that the p/ values of the polypeptides ranged between pH 5-7.

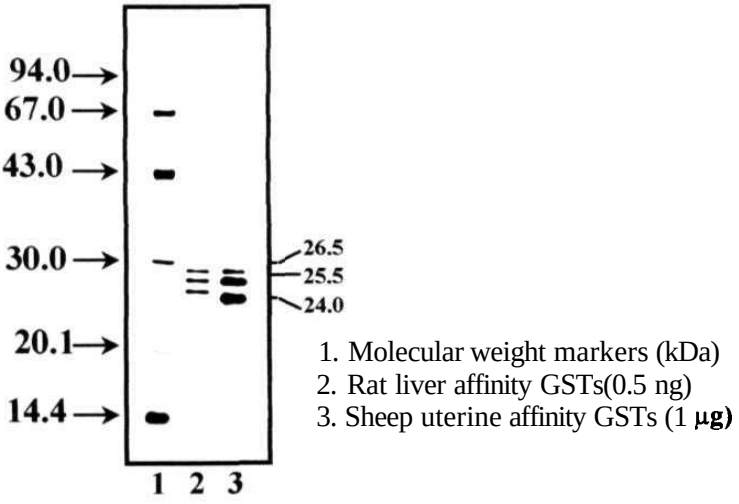
The affinity purified GSTs were further characterized on RP-HPLC. On RP-HPLC, GSTs were clearly separated into four prominent peaks with RTs 23.37. 32.22, 43.02 and 52.81 min and a minor peak at RT 15.47 min (Fig. 20a). The RP-HPLC fractions having similar retention times from different runs were pooled and analyzed on SDS-PAGE (Fig. 20b). Further, the relative subunit concentration of affinity purified GSTs was calculated based on CR4A data of the individual peaks on RP-HPLC analysis (Table 6). The peak with RT 23.37 min showed a molecular weight of 24.0 kDa on SDS-PAGE. Similarly the peaks with RT 32.22, 43.02 and 52.81 min showed molecular weights of 24.0, 25.5, and 25.5 kDa respectively.

3.2.1.1. Ion-exchange Chromatography

Virtually all species studied so far have been found to contain multiple forms of GSTs. The affinity purified GSTs of sheep uterus also represent a mixture of different isozymes, which is evident from 2-D, native IEF and RP-HPLC



a. Elution profile of sheep uterine cytosolic GSTs on GSH affinity column



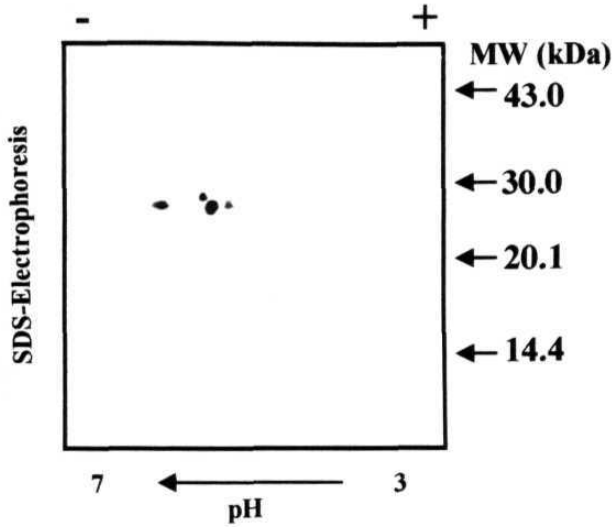
b. SDS-PAGE analysis of affinity purified GSTs

Fig.18 GSH affinity chromatography of sheep uterine GSTs

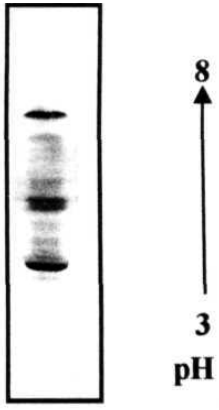
Table 5. Purification profile of sheep uterine cytosolic GSTs.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification Fold	Yield (%)
Homogenate (10,000 xgSupt.)	4500	781	0.17	1	100
Cytosol (105,000 xgSupt.)	3552	666.6	0.19	1.08	85
GSH affinity chromatography	9.32	440	47.20	272.04	56

- *GST activity was calculated using CDNB as the substrate.*

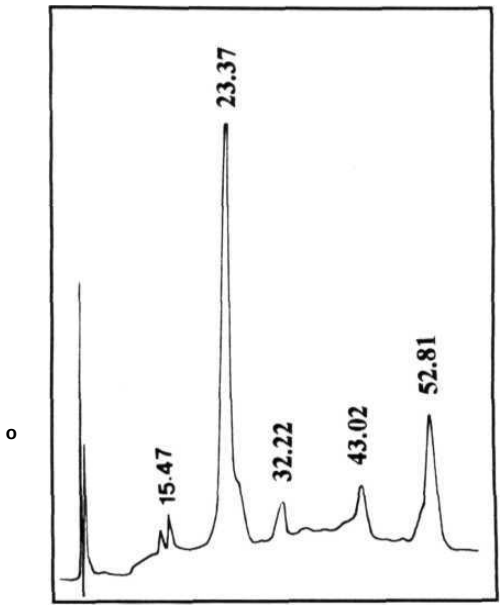


a. 2-D Electrophoresis of affinity purified sheep uterine GSTs



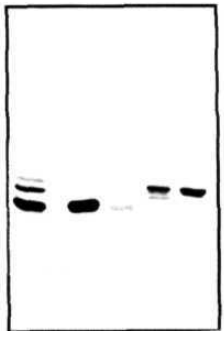
b. Native Isoelectric focusing of affinity purified GSTs

Fig.19 2-D and native IEF of affinity purified sheep uterine cytosolic GSTs



a. RP-HPLC of sheep affinity GSTs

Column : Waters μ Bondapak C_{18} (0.39 x 30 cm)
Solvent : 0.1% TFA in 35% Acetonitrile (Solvent-A)
0.1% TFA in 85% Acetonitrile (Solvent-B)
Gradient : Step
Flow rate : 1 mL/min
Detection : 214 nm
Sample : 60 μg of sheep affinity GSTs



1. Sheep affinity GSTs
2. Peak with RT 23.37 min
3. Peak with RT 32.22 min
4. Peak with RT 43.02 min
5. Peak with RT 52.81 min

b. SDS-PAGE analysis

Fig.20 RP-HPLC analysis of affinity purified sheep uterine cytosolic GSTs

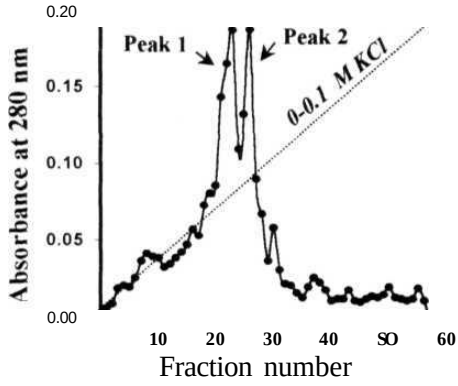
Table 6. Relative subunit composition of affinity purified sheep uterine cytosolic GSTs.

Retention time (min)	**Subunit Mol. Weight (kDa)**	**Subunit** (%)
15.47	26.5	01.50
23.37	**24.0**	55.00
32.22	**24.0**	02.50
43.02	25.5	06.70
52.81	25.5	20.40

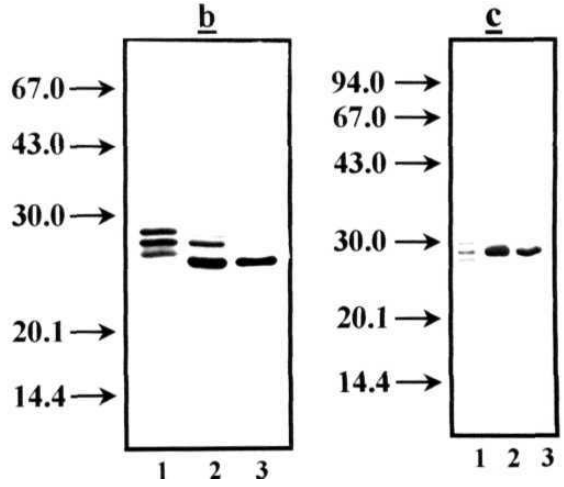
- *Arbitrary units calculated basing on RP-HPLC analysis data.*

analyses. The affinity purified GSTs were hence subjected to ion-exchange chromatography to separate individual isozymes. The affinity purified GSTs were loaded on to CM-cellulose column (2 x 15 cm) after dialyzing against 10 mM phosphate buffer (pH 6.5). It was noticed that no GST activity was retained on the column (data not shown). The protein eluted in the flow through fractions was loaded on to DEAE-cellulose column after dialyzing against 10 mM Tris-HCl (pH 8.0) containing 1 mM β-mercaptoethanol. Only ~40% GST activity was retained on the column and the remaining protein with GST activity appeared in the flow through fractions. The bound GSTs were eluted using the 0-0.1 M KC1 gradient. As shown in the Fig. 21a, GSTs were resolved into two distinct peaks, which were designated as peak 1 and Peak 2 in the order of their elution from anion exchange column. The GSTs, which appeared in flow-through fraction, showed molecular weight of 24.0 kDa on SDS-PAGE analysis (Fig. 21b), where as both peak 1 and peak 2 GSTs showed a molecular weight of 25.5 kDa (Fig. 21c). The peak fractions of each peak demonstrated that all of them were electrophoretically pure. The minor band with molecular weight of 26.5 kDa, which appeared on SDS-PAGE analysis of affinity purified GSTs, could not be traced on ion-exchange column.

To characterize the subunit structure of individual isozymes purified. proteins were analyzed on RP-HPLC using a complex step gradient starting with 35% acetonitrile with 0.1% TFA to 85% acetonitrile with 0.1% TFA. The flow-through fraction from anion-exchange column, eluted as a single peak on RP-HPLC with RT 22.77 min (Fig. 22a). Similarly the GST isozymes eluted from DEAE column chromatography (peak 1 and peak 2) were also analyzed on RP-HPLC. The peak 1 and peak 2 GSTs on RP-HPLC were eluted as single peaks with RT 53.08 min and RT 43.21 min respectively (Fig. 22b and c). On SDS-PAGE



*a. DEAE-Cellulose Chromatography of **affinity purified** GSTs*



1 Rat liver GSTs (0.5 µg)
2 Sheep uterine GSTs (1 µg)
3 DEAE flow-through fraction (0.3 µg)

1. Rat liver GSTs (0.5 µg)
2. Peak 1 (0.4 µg)
3. Peak 2 (0.25 µg)

*b. **SDS-PAGE** analysis of **DEAE-cellulose** flow-through fraction*
*c) **SDS-PAGE** analysis of Peak 1 and 2*

Fig.21 DEAE-Cellulose chromatography of affinity purified sheep uterine cytosolic GSTs

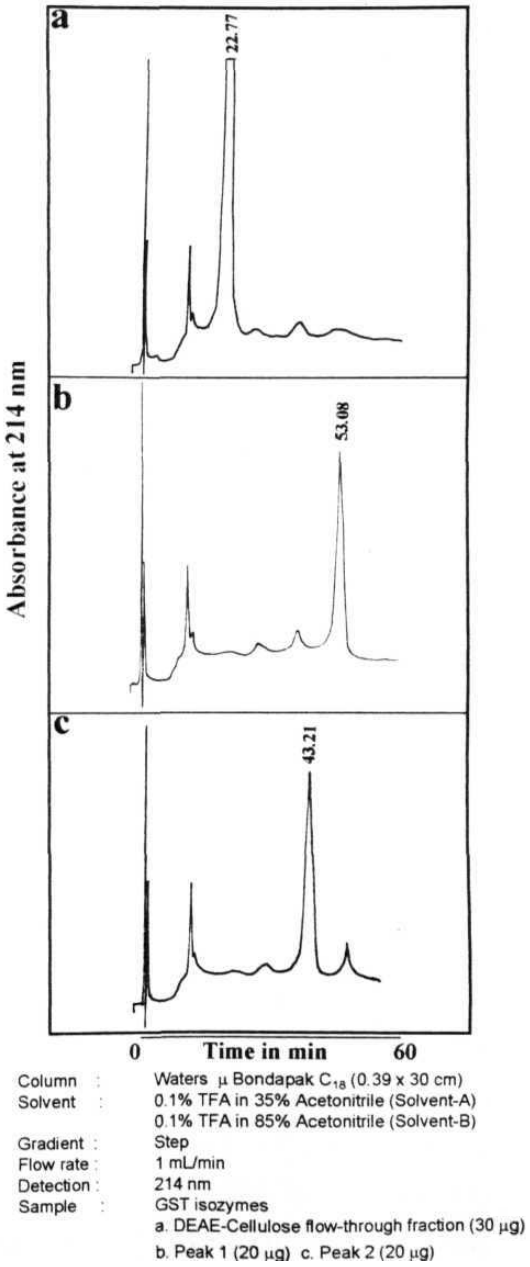


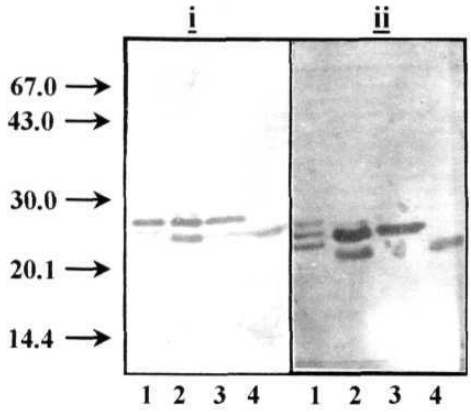
Fig.22 RP-HPLC Analysis of affinity purified sheep uterine cytosolic GST isozymes

analysis, the peak with RT 22.77 min showed a molecular weight of 24.0 kDa. However, both the peaks with RT 43.21 and RT 53.08 min showed molecular weight of 25.5 kDa. When these RP-HPLC polypeptides were subjected to two-dimensional electrophoresis all of them showed a single prominent band only. These studies show that all the GST isozymes of sheep uterus are homodimers. Although significant batch variation occurred in the elution profile of GSTs on RP-HPLC, the relative order of elution of different subunits did not change. The RP-HPLC chromatogram shown in the present study is a typical separation out of 5 analyses. Minor GST isoforms with 24.0 and 26.5 kDa could not be characterized further due to their limited quantities.

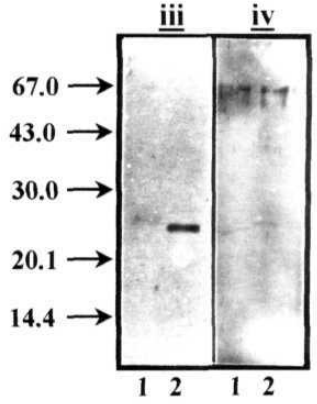
3.2.1.2. Western blot Analysis

Polyclonal antibodies were raised against sheep uterine affinity GSTs, rat liver affinity GSTs and to the subunits like Ya, Yb and Yc of rat liver and used for characterizing the sheep uterine GSTs. Further, the class specific human GST antibodies (gift from Dr.Yogesh C. Awasthi, University Texas Medical branch. Galveston. USA) were also employed for analyzing structural similarity between sheep and human GSTs.

Antibodies raised against sheep uterine affinity GSTs were found to cross-react with sheep uterine as well as rat liver GSTs (Fig. 23a). The sheep uterine GST antibodies readily recognized the Yb subunit of rat liver. Sheep uterine GSTs separated on SDS-PAGE, when probed with rat liver GST antibodies, all the subunits were recognized, more prominently the 25.5 kDa protein, the subunit with molecular weight similar to Yb GST of rat liver (Fig. 23b). When human a class GST specific antibodies were used only the sheep uterine GST subunit with 24.0 kDa was recognized (Fig. 23c). Human u class specific GST antibody, on the other hand recognized the 25.5 kDa protein only (Fig. 23d).



1. Rat liver GSTs
2. Sheep uterus GSTs
3. GST fraction bound to DEAE-cellulose
4. DEAE-cellulose flow-through fraction



1. Rat liver GSTs
2. Sheep uterus GSTs

Immunoblot analysis of sheep uterine GSTs probed with

i. antisera raised against affinity purified rat liver GSTs
ii. antisera raised against affinity purified sheep uterine GSTs
iii. human α-class specific antisera
iv. human μ-class specific antisera

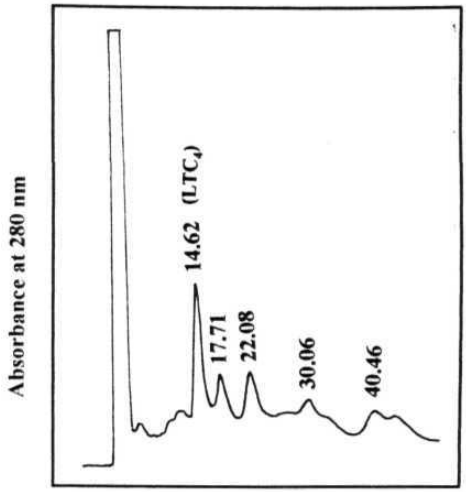
Fig.23 Immunological cross reactivity of sheep uterine GSTs with rat and human polyclonal antibodies

3.2.1.3. LTC_4 Synthase Activity of GSTs

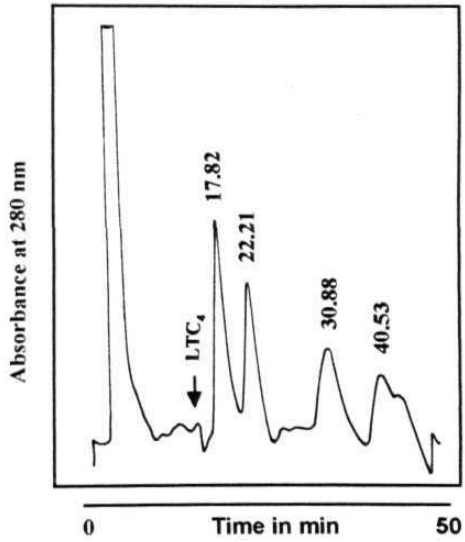
Several anionic GST isozymes with Yb related subunits are known to exhibit appreciable activity towards either 5,6-LTA_4 Me or 14,15-LTA_4 Me. In the present study also purified GSTs showed appreciable LTC_4 synthase activity with 5,6-LTA_4 Me as well as 14,15-LTA_4 Me. (Fig. 24). Further studies were carried by employing 14,15-LTA_4 Me only.

14,15-LTA_4 Me was incubated with affinity purified sheep uterine GSTs and the reaction was allowed at 37^0C for 10 min. The reaction was terminated with cold methanol, centrifuged at 15,000 x g for 15 min to remove precipitated proteins and the products were separated isocratically on RP-HPLC using methanol: water: acetic acid (65: 35: 0.04, pH 5.8) as the solvent system (Fig. 24a). The effluent was monitored at 280 nm and peaks were collected and their UV scanning spectra recorded. The peak with RT 14.62 min alone showed LTC_4 characteristic spectrum with maximum absorption at 280 nm and shoulders at 270 and 290 nm (Fig. 14b). Based on the typical UV scanning spectrum and co-chromatography with standard LTC_4 Me, the peak with RT 14.62 min was identified as LTC_4 Me. Typical 8,15-di-HETE spectrum (Fig. 26b) was obtained to the peaks with RT 17.71 and 22.08 min when subjected to UV scanning with maximum absorption at 268 nm and shoulders at 258 nm and 278 nm. The other two peaks with RT 30.06 and 40.46 min showed typical 14,15-di-HETE spectrum with absorption maximum at 272 nm. In the experiments with heat killed enzyme (Fig. 24b), the peak with LTC_4 spectrum (RT 14.62 min) was absent.

Sheep uterine GST isozymes were further screened for LTC_4 synthase activity. The GST isozyme with molecular weight 24.0 kDa (DEAE flow-through fraction) showed very little LTC_4 synthase activity (Fig. 25a). The peak 1 and peak 2, on the other hand, showed substantial LTC_4 synthase activity when



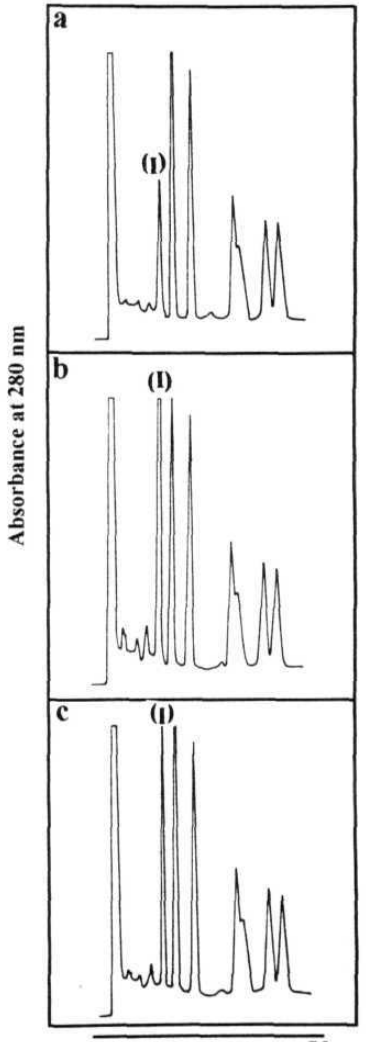
a. ***Enzymatic reaction products separated on RP-HPLC***



b. Non-enzymatic Reaction (heat killed) reaction products

Column : Waters μ Bondapak C,, (0 39 x 390 cm)
Solvent : Methanol : Water: Acetic acid (65:35:0 04 pH 5.8)
Flow Rate 1 mL / Min
Detection : 280 nm
Sample Affinity purified sheep uterine GSTs (200 μg)

Fig.24 **LTC_4** synthesis by affinity purified sheep uterine GSTs



1. (LTC_4 Me)

Column : Waters μ Bondapak C_{18} (0.39 x 390 cm)
Solvent : Methanol : Water: Acetic acid (65:35:0.04 pH 5.8)
Flow Rate : 1 mL / Min.
Detection : 280 nm
Sample : a. DEAE-Cellulose flow-through fraction (40 μg)
b. **Peak 1** (20 μg) c. **Peak 2** (20 μg)

Fig.25 **LTC_4** synthesis by sheep uterine GST **isozymes**

Table 7. LTC4 Synthase activity of sheep uterine cytosolic GSTs.

GST fraction/ isozyme	LTC_4 Me*	Protein
Affinity purified GSTs	72532	200 μg
GST isozyme with 24.0 KDa	12143	40 μg
GST isozyme with 25.5 KDa (Peak 1)	58569	20 μg
GST isozyme with 25.5 KDa (Peak 2)	36201	20 ug

*14,15-LTA_4 Me (40 uM) was incubated with the GSTs in the presence of GSH and the products formed were extracted and then separated on RP-HPLC. LTC4 Me formed was quantified based on the peak areas from RP-HPLC chromatograms and the activity expressed in arbitrary units.

incubated with 14,15-LTA_4 Me, the peak 1 being the most potent (Fig. 25b and c) (Table 7). However, none of the isozymes showed detectable activity with LTA_4 FA as the substrate.

3.3.0.0. Discussion

The present study is a part of continuing interest in the purification and characterization of GST isozymes from different mammalian sources for further identification of specific physiological functions associated with them. In the present study the main focus is on the role of GSTs from sheep uterus in the metabolism of LTA_4.

The cytosolic GSTs are a group of enzymes existing either as heterodimers or homodimers and the heterodimers characterized so far are formed from subunits within the same evolutionary class. Considerable differences exist in the sequences between isozymes of different classes and these differences may result in sufficient modification in the tertiary structure to preclude/prevent the formation of inter class heterodimers. Extrahepatic tissues, with the exception of kidney, contain predominantly acidic enzymes and are homodimers.

Several forms of GSTs differing in their structural, functional and immunological properties have been purified from a number of tissues. In spite of extensive knowledge on the properties of the multiple forms of GSTs, the possible relationship between different classes of enzymes in one species to the classes of other species and different isoforms within the animal in different tissues remained obscure. Most of the tissues contain more than one isozyme of GST and each of the tissue investigated so far appears to have somewhat unique enzyme/isozyme pattern. Virtually all species studied so far have been found to contain multiple forms of GSTs. The technique of isoelectric focusing and RP-HPLC has high

resolving power and has been used to separate various isozymes and subunits of GSTs respectively.

Purification of GST isozymes from sheep liver was reported by Reddy *et al.*, (1983) and found to have seven distinct cationic isoforms and five overlapping anionic forms. The subunit analysis revealed that cationic forms are composed of dimers of 24.0 kDa, whereas 26 kDa subunit forms the anionic GSTs. Isozyme C-4 was found to be the most abundant GST in sheep liver.

The protective role of the GSTs as detoxifying system against elctrophilic compounds is widely accepted and their presence in uterine tissues may contribute to the prevention of fetal demise during pregnancy. DiIlio *et al.*, (1987) reported the purification of GSTs from human uterine tissue obtained from patients operated for fibrodenoma and found to have five isozymes (GST-I, II, III, IV and V) with apparent isoelectric points at pH 4.5, 4.9, 5.5, 6.1 and 6.8. The GST V accounts for about 85-90% of the activity. All the GST isozymes of human uterus are similar to the anionic isoforms of human placenta. It was reported that the GSTs are localized in endometrium and they were known to be a part of an important defense system (Murakoshi *et al.*, 1990). GSTs purified from rat uterus showed five isoforms and the major GST isozyme (π) accounts to 90% of the total GSTs (Singhal *et al.*. 1996). The rat uterus contains an α-class GST (p/9.8), a *n* class GST (p/ 8.1), and two μ class GSTs (p*I* 6.7 and rGST 8-8). The present study forms the first detailed study on sheep uterine GSTs.

In the present study the purification and characterization of sheep uterine GSTs was attempted. The purification of GSTs from sheep uterus resulted in specific activity of 47.2 units/mg protein with overall yield of 56%. Overall, the GSTs of sheep uterus constituted 0.03-0.06% of total cytosolic proteins. The

purified sheep uterine GSTs were found to contain three subunits with molecular weights of 24.0, 25.5 and 26.5 kDa, the 24.0 kDa subunit being the most abundant.

The affinity purified sheep uterine GSTs on two-dimensional electrophoresis and on RP-HPLC analysis showed 5 bands, showing the existence of multiple forms of GSTs. The uterine GSTs were further separated to isozymes on ion-exchange column. The major GST isoform, which appeared in flow-through fraction of anion-exchanger, accounted to 55-60% of total GSTs. This is in contrast with rat and human uterus, where the major isozyme accounted to ~90% of the total GSTs (Singhal *et al,* 1996 & Dillio *et al,* 1987). The other GST isoforms retained on the column were eluted as two peaks (peak 1 and & 2). The flow-through fraction showed a 24.0 kDa band on SDS-PAGE analysis where as the peak 1 and 2 showed a molecular weight of 25.5 kDa. All the purified GST isozymes showed a single band on SDS-PAGE, RP-HPLC and 2-D analysis. These studies clearly demonstrate that all the GST isoforms of sheep uterus are homodimers. Most of the sheep uterine GST isoforms are anionic forms and the p/ value spans between 5-7, which is comparable with human uterine GSTs (p*I* ranging from 4.8 to 6.8) (Dillio *et al,* 1987). However, rat uterine GST isozymes are basic, p/ ranging between 6.8-9.8 (Singhal *et al,* 1996).

Polyclonal antisera raised against rat liver affinity GSTs were tested against sheep uterine cytosolic GSTs. Rat liver GST antibodies readily recognized all the sheep GST isozymes, with higher affinity towards 25.5 kDa protein followed by 24.0 kDa subunit. The sheep uterine GST antibodies, on the other hand, detected only Yb subunit of rat liver GSTs. Human α-class specific GST antibodies recognized only the 24.0 kDa subunit of sheep uterine GSTs. The human |i-class specific antibodies, on the other hand, recognized only the 25.5 kDa subunit of sheep uterine GSTs.

Apart from detoxification reactions, GSTs play an important role in the biosynthesis of physiologically active compounds like LTs and PGs (Chang *et al*.. 1987a & Burgess *et al*., 1987). It has been demonstrated that cytosolic GSTs from different tissues catalyzed the conjugation of GSH with LTA4 (Cagen *et al.,* 1975 & Mannervik *et al*., 1984). GST isoforms differ significantly in their catalytic activity with LTA_4 (Chang *et al.,* 1987a). Among various rat liver GST isozymes tested, the anionic isozymes, homodimer of Yb subunit, showed the highest specific activity (Chang *et al.,* 1987). In general, the isozymes containing the Yb subunit showed better activity than the isozymes containing the Ya &/or Yc subunits. Anionic GSTs exhibited detectable catalytic activity with LTA4 free acid. While GST *n* form showed a limited reaction with LTA4 Me, the near neutral (μ) isoform catalyzed significant conversion of the substrate into LTC_4 Me. The most active forms of rat GSTs with LTC_4 synthase activity are those containing Yb subunit (Mannervik, 1984). GST isomers exhibit differential reaction rates in the formation of LTC_4 from LTA4 Me. This latter phenomenon is more interesting to understand the tissue specific expression of GSTs as well as to explore hitherto unidentified physiological functions in addition to their role in detoxification.

Affinity purified sheep uterine GSTs in the present study showed substantial LTC4 synthase activity when LTA4 Me was used as the substrate. However, no detectable activity was observed when LTA4 free acid was used as the substrate. In order to identify the isozyme(s) with LTC_4 synthase activity. individual isozymes were screened for activity with LTA4 Me as well as LTA_4 FA. Of all the isozymes screened, the isozyme with 25.5 kDa (peak 1) showed highest LTC_4 synthase activity followed by the isozyme with 25.5 kDa (peak 2). Both these isoforms with molecular weight of 25.5 kDa showed structural similarity to Yb subunit of rat liver. It is well known that GST subunits having immunologically

cross-reaction with Yb subunit exhibit very high LTC_4 synthase activity (Mannervik, 1984 & Tsuchida *el al*., 1987).

From these experiments it can be concluded that GSTs account for much of the LTC_4 synthase activity recorded in the cytosolic fraction of sheep uterus. Further it is demonstrated that GST isozymes with 25.5 kDa specifically is involved in the conversion of LTA4 Me to LTC4 Me. However, no activity is recorded with LTA_4 FA as the substrate. Sheep uterine GSTs were equally effective in converting both 5,6-LTA4 Me as well as 14,15-LTA_4 Me.

Part-II

Purification and characterization of LTC_4 synthase from sheep uterus

4.1.0.0. Introduction

The discovery of the leukotrienes (LTs) and the finding that some of the major derivatives with strong physiological activities originate from conjugation with glutathione (GSH) has opened a new field of investigation (Samuelsson, 1982).

It has been demonstrated that the cytosolic GSTs catalyze the conjugation of LTA4 with GSH, which results in the formation of LTC_4 (Cagen *et al,* 1975& Mannervik *et al,* 1984) and GST isozymes differ significantly in their catalytic activity with LTA4 (Chang *et al.*, 1987a). Acidic GSTs (π) share a limited reaction with LTA4 Me where as the near neutral (μ) GSTs catalyze the conversion of LTA4 Me into LTC_4 Me.

In the earlier chapter, it was shown that sheep uterine GSTs with close similarity to μ class of GSTs catalyze the conversion of LTA4 Me to LTC4 Me. However, no detectable activity was observed with LTA4 FA as substrate.

In addition to cytosolic GSTs, a specific microsomal enzyme known as LTC4 synthase is known to catalyze the conversion of LTA4 to LTC_4. This enzyme acts on both LTA4 Me as well as FA. In the present study an attempt was made to detect, isolate and characterize LTC4 synthase from sheep uterus.

LTC4 synthase activity was first demonstrated in particulate material of RBL cells (Jakschik and Kuo, 1983 & Bach *et al.*, 1984). Yoshimoto *et al.,* (1985) isolated a glutathione S-transferase from microsomes of RBL cells, which is distinct from cytosolic GSTs and appears to be exclusively committed to LTC_4 synthase. The expression of LTC_4 synthase has been described with concomitant expression of Lox in eosinophils, basophils, mast cells and certain phagocytic mononuclear cells (Du *et al.*, 1983; Weller *et al,* 1983; MacGlashan *et al.,* 1982 & MacGlashan *et al,* 1986).

However, in human endothelial, vascular smooth muscle cells and platelets LTC4 synthase expression is without concomitant expression of Lox. Therefore, the formation of LTC_4 is dependent on the trans-cellular metabolism of LTA4 (Feinmark and Cannon, 1986; Claesson and Haeggstrom, 1988 & Maclouf and Murphy, 1988). The LTC_4 synthase has been characterized from certain leukemia cell lines such as KG-1, THP-1, U-937 and HL-60 (Penrose *et al.*, 1992; Nicholson *et al.,* 1992b; Ali *et ed.,* 1994 & Nicholson *et al.,* 1993). Wu., (1986) for the first time demonstrated LTC_4 synthase activity in guinea pig lung tissue. The guinea pig lung LTC4 synthase showed high specificity towards 5,6-LTA4 and no activity with CDNB (the universal substrate for cytosolic GSTs), $14,15\text{-}LTA_4$ and its methyl ester (Izumi *et al.,* 1988). Penrose *et al.,* (1992) purified LTC4 synthase from KG-1 cells, myeloid cell line and reported a molecular mass of 18 kDa. Photoaffinity labeling studies of LTC4 synthase also revealed that the enzyme is an 18 kDa protein (Ali *et al.,* 1993).

LTC4 synthase catalyzes the committed step in the biosynthesis of all of the peptido LTs, which are implicated as important mediators of various human diseases. Reports indicate that the LTC4 synthase is a member of mGST family with restricted substrate specificity and limited cellular distribution. In contrast to previous observations, it is evident from the recent studies that the LTC4 synthase activity is different from those of cytosolic and microsomal GSTs and is more related to the proteins of lipid binding family, 5-lipoxygenase activating protein (FLAP), another protein involved in the LT biosynthesis. LTC_4 synthase amino acid sequence shows 31% identity and 53% similarity with FLAP and no homology with GSTs (Lam *et ed.,* 1994 & Welsch *et al.,* 1994). It has been suggested that LTC_4 synthase undergoes

protein kinase C dependent phosphorylation as a means of regulating its activity (Ali *et al.,* 1994 & Tornhamre *et al.,* 1995).

Among the Lox pathway enzymes the LTC4 synthase is the least studied. Though LTC4 synthase activity has been detected in all blood cells, endothelial cells, vascular smooth muscle and certain leukemia cell lines, not much is known about the tissue source except the one from lung. The complete purification and characterization of LTC_4 synthase has been hindered owing to the apparent instability of the enzyme, limited availability of the substrate and lack of a rich source (Izimu *et al.,* 1989). An abundant tissue source of the enzyme should alleviate the problems of insufficient material for purification and characterization.

4.2.0.0. Results

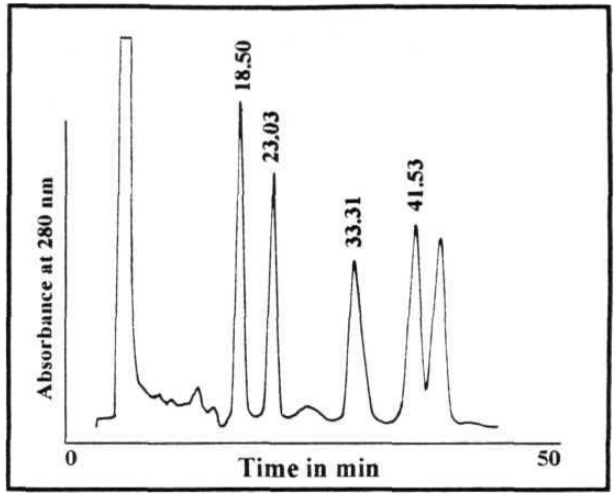
LTC_4 synthase activity was detected in the microsomes of sheep. Microsomes were prepared from sheep uterine tissue at different phases of reproductive cycle. Among the uteri at all the stages tested, the microsomes prepared from the luteal stage showed maximum LTC4 synthase activity (Fig. 26) (Table 8). Hence for further studies on LTC4 synthase purification, uteri at luteal stage alone were taken. The microsomes were prepared from luteal phase sheep uterine tissues, solubilized and were further purified using Q-sepharose anion exchange chromatography (Fig. 30).

The sheep uterine tissues were homogenized and the microsomes were obtained by centrifuging initially at 5,000 x g for 25 min and later at 105,000 x g for 1 h. The microsomes were washed thoroughly and tested for LTC4 synthase activity (Fig. 27) by employing 14,15-LTA_4 Me as the substrate.

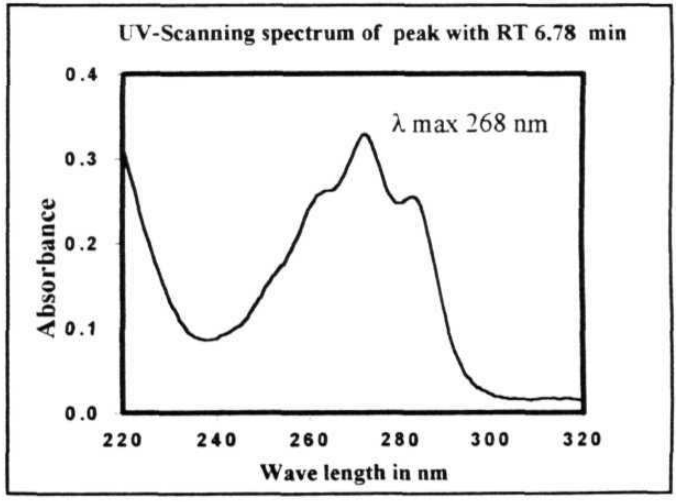
The reaction mixture without microsomes served as the control, which yields LTA4 hydrolyzed products. The LTA4 hydrolysis products were analyzed on RP-

HPLC with methanol: water: acetic acid (65:35:08, pH 5.7) as the solvent system (Fig. 26a) and the individual peaks were collected and scanning spectra recorded. Typical 8,15-di-HETE spectrum was obtained to the peaks with RT 18.50 and 23.03 min when subjected to UV scanning with maximum absorption at 268 nm and shoulders at 258 nm and 278 nm (Fig. 26b). The other two peaks with RT 33.31 and 41.53 min showed typical 14,15-di-HETE spectrum with absorption maximum at 272 nm.

In a typical reaction mixture, 100 ug of microsomal proteins were incubated with 14,15-LTA_4 Me (40 μM) and the reaction was allowed at 37°C for 10 min. The reaction was terminated by the addition of ice cold methanol. The resulting mixture was allowed for one hour at -20°C and centrifuged at 15,000 x g for 15 min. The reaction products were analyzed on RP-HPLC with methanol: water: acetic acid (65:35:08, pH 5.7) as the solvent system (Fig. 27a). The effluent was monitored at 280 nm and all the peaks were collected and the scanning spectra recorded. As shown in the chromatogram (Fig. 27a) the peak with RT 14.98 min alone showed the characteristic spectrum of LTC4 with absorption maximum at 280 nm, shoulders at 270 nm and 290 nm (Fig. 14b). Further the peak with RT 14.98 min coeluted with the authentic standard, indicating its identity as LTC4. While other peaks with RT 17.81, 22.12, 32.76 and 40.41 min, showed typical spectra of hydrolysis products of LTA_4 (Fig. 26b). LTC_4 peak was abolished when heat denatured (90°C, 10 min) microsomes were used in the assay (Fig. 27b). Similarly when GSH was omitted in the reaction mixture, no LTC_4 peak was observed (Fig. 28b). The formation of peak with RT 14.98 min increased with increasing amounts of enzyme. From these experiments it can be concluded that the transformation of LTA4 to LTC_4 carried by the microsomes of



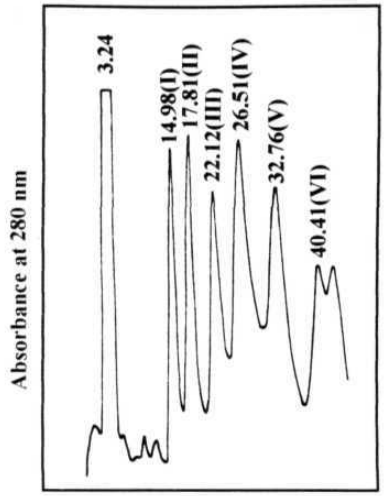
a. LTA_4 Me hydrolysis products



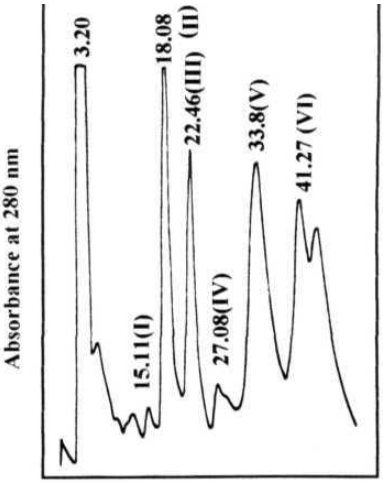
b. UV-Scanning Spectrum of di-HETEs

Column : Waters μ Bondapak C_{18} (0 39 x 390 cm)
Solvent : Methanol : Water Acetic acid (65:35:0.08, pH 5.7)
Flow Rate : 1 mL / Min.
Detection : 280 nm

Fig.26 RP-HPLC analysis of non-enzymatic products of LTA_4 Me



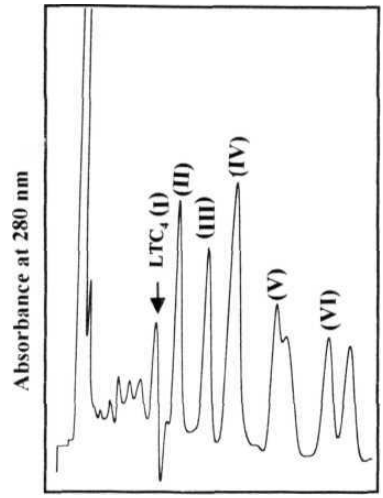
a. *enzymatic reaction*



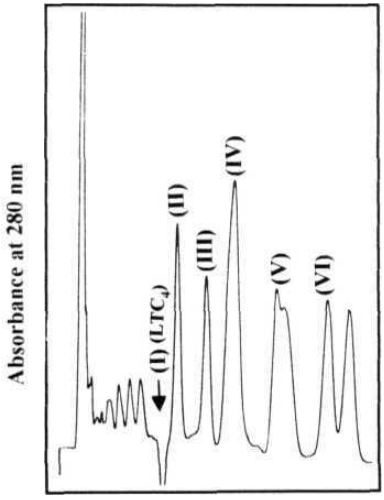
b. *Non-enzymatic reaction (heat killed) products*

Column : Waters μ Bondapak C_{18} (0.39 x 390 cm)
Solvent : MethanolWaterAcetic acid (65:35:0 08; pH 5.7)
Flow Rate : 1 mL / Min
Detection : 280 nm

Fig.27 **LTC_4** synthase activity in sheep uterine **microsomes**



a. *with glutathione*



I. LTC_4
II,III, V,VI } di-HETEs
IV. LTB_4

b. without glutathione

Column : Waters μ Bondapak C_{18} (0.39 x 390 cm)
Solvent : Methanol:Water:Acetic acid (65:35:0 08: pH 5.7)
Flow Rate : 1 mL /Min.
Detection : 280 nm

Fig.28 **LTC_4** synthase activity of sheep uterine microsomes with and without glutathione

sheep uterus is an enzymatic process and is GSH dependent. LTC_4 formation was not affected when 10 µM S-hexyl GSH, a GST inhibitor was included in the reaction mixture. This observation further indicates that a specific LTC4 synthase, other than GST, is involved in the transformation of LTA_4 to LTC_4 in sheep uterine microsomes.

4.2.1.0. LTC_4 Synthase Activity of Uteri at Different Stages of Sexual Cycle

Sheep uteri at different phases of sexual cycle (Nulliparous, follicular, luteal and early pregnant stages) were analyzed for LTC_4 synthase activity as per the methods described above. As shown in the table (Table 8), uteri at luteal phase showed maximum LTC_4 synthase activity (4.67 nmole/mg protein per 10 min). Further purification and characterization of LTC4 synthase was carried by taking sheep uteri at luteal phase only.

4.2.2.0 ***LTC_4 Synthase Activity with 5,6- and 14-15- LTA_4 as Substrates***

Sheep uterine microsomes when incubated with 5,6-LTA4 Me/FA also showed significant formation of the corresponding 5,6-LTC4S. The activity levels of LTC4 synthase in the microsomes of sheep uterus with 5,6-LTA4 and 14,15-LTA_4 as substrates were almost similar with slight preference towards 14,15-LTA4 (Table 3). This observation assumes importance in the light of reported 14,15-LTA_4 formation in the sheep uterus (Sailesh *et al.*, 1994). Hence LTC_4 synthase assays during the course of purification were carried by employing 14,15-LTA_4 only.

Table 8. LTC_4 synthase activity levels in the microsomes of sheep uteri at different stages of reproductive cycle.

Stage	LTC_4 synthase activity (units /mg protein)*
Nulliparous	**1.08**
Follicular Stage	2.32
Luteal stage	**4.67**
Early pregnant	**3.34**

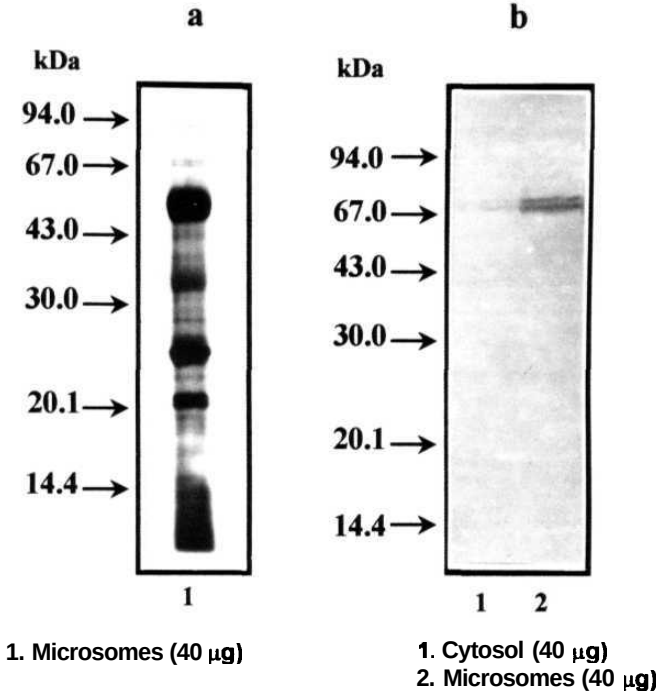
- On unit is defined as one nmole of LTC_4 formed per 10 min.

4.2.3.0. *Immuno Assays*

The custom made N-terminal (MKDEVALLAAVTLLGVLLQ) and C-terminal (GRLRTLLPWAC) LTC_4 synthase specific peptides (Welsch *et a!.,* 1994) coupled to KLH were procured from MBT, Philadelphia, USA. These peptides were used for raising polyclonal antibodies in rabbit. The antibodies raised in the rabbits were purified using protein-A sepharose affinity column. Anti-peptide antibodies were used for immunoblot analysis. The microsomal proteins separated on SDS-PAGE were electroblotted onto the nitrocellulose membrane and the proteins were probed with LTC_4 specific peptide antibodies (Fig. 29a). As shown in Fig. 29b, a ~69-70 kDa protein was detected on immunoblot, when probed with LTC_4 synthase specific peptide antibodies. To confirm further, the antibodies raised against the pure human LTC_4 synthase protein were used for immunoblot analysis (antibodies were gift from Dr. John F. Penrose, Harvard Medical School, USA). With whole protein antibodies also, a ~ 69-70 kDa protein alone was recognized on western blots. Both peptide as well as whole protein antibodies recognized two closely migrating proteins (doublet) on immunoblots when sheep microsomal proteins were employed.

4.2.4.0. *LTC_4 Synthase Purification*

The microsomes prepared from the luteal stage of uterus alone were used for further purification of the LTC_4 synthase protein. Solubilization is an important step in the purification of microsomal proteins. For solubilizing the microsomal proteins a panel of detergents were used. In the present study maximum solubilization of the LTC4 synthase protein was achieved when CHAPS/taurocholate (1% final



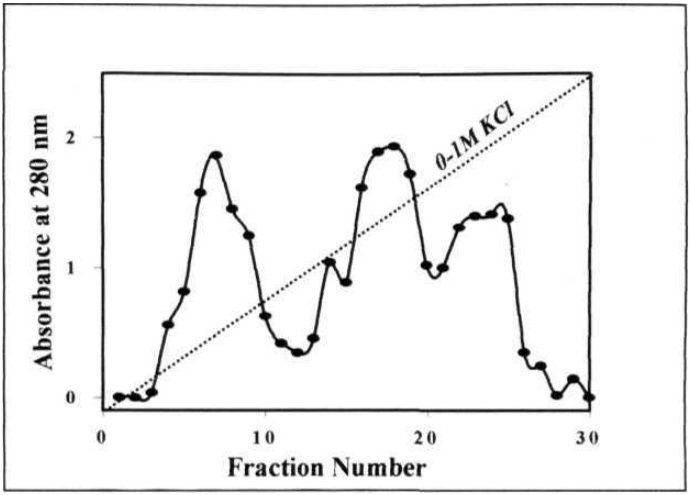
a. SDS-PAGE Analysis of sheep uterine microsomes
b. Immunoblot analysis of sheep uterine microsomes

Fig.29 Immunoblot analysis of sheep uterine microsomes using LTC_4 synthase-specific peptide antibodies

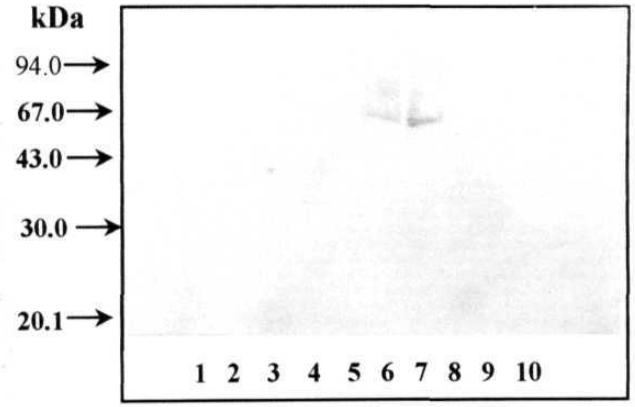
concentration) was used. Routinely the microsomes were solubilized using 1% CHAPS/taurocholate and centrifuged at 200,000 x g at 4°C for lh. The supernatant obtained (solubilized microsomal proteins) was loaded onto the Q-sepharose anion exchange column equilibrated with 20 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, 2 mM GSH, 1 mM DTT, 0.1% taurocholate, 0.5% n-octyl glucoside, 0.5% CHAPS. The column washed with 100 ml of buffer was eluted using 0-1 M NaCl/KCl (Fig. 30a), the fractions collected were analyzed for LTC_4 synthase activity, both by HPLC as well as immuno assays. Of all the fractions screened, substantial activity was observed with 6, 7 and 8 fractions on Q-sepharose column as evidenced by RP-HPLC assay (Fig. 31). This was confirmed again on immunoblot analysis (Fig. 30b). From these experiments, it is observed that LTC_4 synthase activity is associated with a ~69-70 kDa protein eluted in 6,7 and 8 protein fractions of anion exchange column.

In general, purification of LTC4 synthase from sheep uterus resulted in specific activity of 29.77 units/ mg protein with an overall yield of 44% (Table 9). Further purification, however, resulted in decreased yield and purification folds, indicating the possible denaturation of enzyme.

Goppelt-Struebe (1995) reported the purification of LTC4 synthase on S-hexyl GSH agarose chromatography followed by preparative native gel electrophoresis from murine mast cells. Procedures described by Goppelt-Struebe (1995) were employed for the purification of LTC4 synthase in the present study also. The microsomes were prepared as described earlier and solubilized in 0.4% deoxycholate, 0.4% Triton X-100 and 20 % glycerol. The solubilized mixture was centrifuged at 200,000x g at 4°C for 1 h and the supernatant obtained was loaded onto S-hexyl GSH affinity column

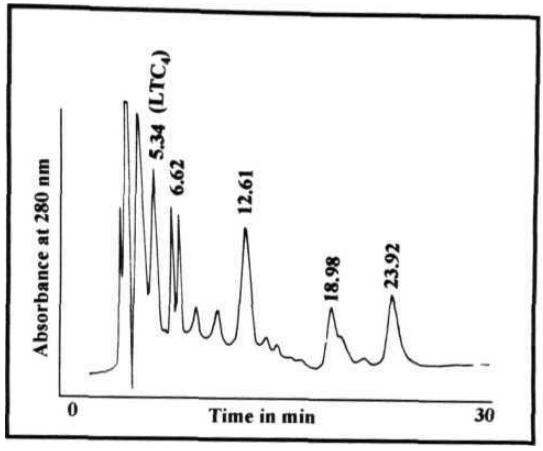


a. Anion-exchange (Q-Sepharose) Chromatography

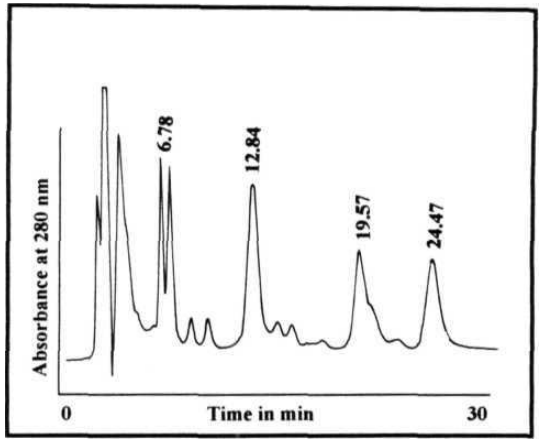


b. Immunoblot analysis of the fractions probed with LTC_4 synthase -specific peptide antibodies

Fig. 30 Anion-exchange chromatography of solubilized sheep uterine microsomal proteins



a. ***Enzymatic reaction***



b. Non-enzymatic reaction (Heat killed)

Column : Waters μ Bondapak C_{18} (0.39 x 390 cm)
Solvent : Acetonitrile Methanol : Water: Acetic acid (40:15:30:1; pH 5.71)
Flow Rate : 1 mL /Min.
Detection : 280 nm
Sample : Fraction # 7 from anion exchange column

Fig.31 LTC_4 synthase activity of anion-exchange purified fraction

Table 9. **Purification profile of LTC4 synthase from sheep uterus.**

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Fold Purification	Recovery (%)
Microsomes	203	912	04.49	1.00	100
Solubilized microsomes	123	548	04.45	0.99	60.1
Anion-exchange chromatography	13.5	402	29.77	6.62	44.0

- one unit of enzyme is defined as one n mole of LTC_4 formed per 10 min.

pre-equilibrated with 50 mM HEPES (pH 7.6) containing 10% glycerol, 5 mM p-mercaptoethanol, 1 mM EDTA, 0.1% deoxycholate and 0.1% Triton X-100. After washing the column with 0.2 M NaCl, the protein was eluted with 10 mM GSH and later by using 10 mM probenecid. The fractions collected were concentrated using Amicon centrifugal concentrators and checked for LTC_4 synthase activity (RP-HPLC assay) and protein (western blots). These studies, however, were unsuccessful as none of the fractions showed activity as well as the protein. As a result, LTC4 synthase purification from sheep uterus was carried as per the methods described earlier.

4.2.5.0. *LTB_4 Synthesis*

LTB_4 formation was also observed while analyzing LTC_4 synthesis in the microsomes of sheep uterine tissues (Fig. 27, peak-IV). When reaction products were analyzed on RP-HPLC, the peak with RT 26.5 min showed LTB_4 characteristic UV scanning spectrum (Fig. 32). This peak was absent when the reaction was carried with heat-killed enzyme preparation. In addition. LTB_4 formation was seen even after the deletion of GSH in the reaction mixture (Fig. 28, Peak-IV). From these experiments it is clear that the formation of LTB4 in sheep uterine microsomes is an enzymatic process and GSH is not required for this reaction. The conversion of LTA4 to LTB4 is generally catalyzed by LTA4 hydrolase, which is known to be mostly cytosolic in nature (Radmark *et al.*, 1984b). It is surprising that LTA4 hydrolase observed in the present study is microsomal in nature.

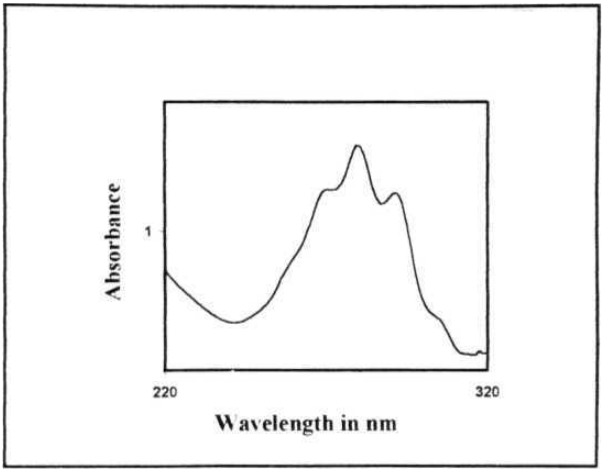


Fig.32 UV Scanning Spectrum of leukotriene B_4

4.3.0.0. Discussion

The conjugation of LTA_4 with reduced GSH, is known to be catalyzed by an enzyme (LTC4 synthase) distinct from GSTs. LTC4 biosynthesis is particularly prominent in certain cells like basophilic leukemia cells, mouse mastocytoma cells and eosinophilic leukemia cells and the discovery of LTC4 in high amounts in these cell lines has become the basis for the identification of a special/distinct enzyme in its biosynthesis. For the first time, LTC4 synthase activity was reported in the microsomal fraction of rat basophil leukemia (RBL) cells (Jakschik and Kuo, 1983 & Bach *et al.*, 1984). Later it was reported in many human cells and leukemia cell lines. Though LTC4 synthase activity was identified/detected in many human cells, it was not reported from the tissues except guinea pig lung (Wu, 1986). Identification of enzyme in tissue source alleviates the problems of insufficient material for the studies. In the present study an attempt is made to identify LTC4 synthase in sheep uteri at different phases of sexual cycle.

Several investigators have attempted to purify LTC4 synthase from a variety of sources, the purification of this enzyme has been hampered by many factors. LTC_4 synthase activity was stimulated by the presence of divalent cations, Mg^{2+} and Ca^{2+} (Nicholson *et al.*, 1992b). Low concentrations of GSH stabilize the enzyme activity, where as at higher concentrations, GSH inactivates LTC_4 synthase. Induction of high levels of LTC4 synthase expression was observed in the presence of dimethyl sulfoxide (DMSO) and thioglycolate in certain cell lines (Nicholson *et al.*, 1992a & Abe *et al.*, 1990). LTC_4 synthase protein is reported to have weak acidic pI value (~6), where as the cytosolic GSTs and microsomal GSTs were all basic proteins (pI > 8.5).

Solubilization is an important step in the purification of microsomal enzymes. Attempts were made to solubilize the LTC_4 synthase by using variety of detergents. Izumi *et al.,* (1988) described the solubilization of LTC_4 synthase by using a combination of detergents like CHAPS and digitonin and it was shown that the enzyme lost its activity on treatment with sodium deoxycholate. Yoshimoto *et al.* (1988), on the other hand, used detergents like deoxycholate and Triton X-100 for solubilizing the LTC_4 synthase. Solubilization of LTC_4 synthase was best achieved with detergents that have charged derivatives of bile acids and taurocholate. In the present study solubilization of microsomes with 1% CHAPS/taurocholate resulted in maximum recovery of the enzyme protein.

LTC4 synthase purified to homogeneity from myelomonocytic leukemia cell line was shown to have a molecular of 18 kDa (Penrose *et al.,* 1992). Evidence that an 18 kDa protein is associated with LTC4 synthase was obtained from the use of an LTC_4 photoaffinity label (Nicholson *et al.,* 1992b & Ali *et al.,* 1993). LTC_4 synthase was purified >25,000 fold from the taurocholate solubilized microsomal membranes of monocytic leukemia (THP-1) cells and reported that the enzyme exists as homodimer (39 kDa) with subunit molecular weight of 18 kDa. In guinea pig lung the LTC_4 synthase was found to be a tetramer with subunit molecular weight of 60 kDa (Jenny, 1995).

In the present study, LTC_4 synthase activity was identified in the microsomes of sheep uterus, the activity being maximum in the uteri at luteal stage. This conversion of LTA4 to LTC4 was shown to be an enzymatic reaction as no LTC_4 formation was observed when heat killed microsomes were employed. Further no

activity was recorded when GSH was deleted in the assay mixture. From these observations, it is concluded that microsomes from sheep uterus at luteal stage contain substantial LTC_4 synthase activity. The enzyme was equally effective in the conversion of 5,6-LTA4 as well as 14,15-LTA_4 to the corresponding LTC4S. Further. LTC_4 synthase activity of sheep uterus was observed when LTA4 Me and FAs were used as the substrates. LTC4 synthase activity was not inhibited when tested with GST inhibitors like S-hexyl GSH. However, no activity was observed with CDNB, the conventional substrate for GSTs, indicating that LTC_4 synthase activity observed in sheep uterus is catalyzed by a unique LTC_4 synthase and not by GSTs. The LTC_4 synthase in sheep uterus appears to be quite different from guinea pig lung LTC_4 synthase, which is active only on 5,6-LTA_4 but not on 14,15-LTA_4 (Izumi *et al.*. 1988).

Immunoblot analysis of sheep uterine microsomes using polyclonal antibodies raised against LTC_4 synthase specific peptides recognized two closely migrating proteins with molecular weight of ~70 kDa. This observation also supports the presence of LTC_4 synthase in sheep uterine microsomes. However, the molecular weight is much higher to the reported molecular weight (18 kDa) of LTC_4 synthase (Penrose *et al.*, 1992). To further confirm the presence of LTC_4 synthase, antibodies developed against LTC4 synthase native protein (gift from Dr. John F. Penrose) were used for probing the LTC_4 synthase of sheep uterine tissue. These results also confirmed the presence of LTC4 synthase in sheep uterus, but with a molecular weight of ~ 69-70 kDa. Penrose *et al.*, (1992) showed that LTC_4 synthase purified from myeloid cell line (KG-1) is a monomer with molecular weight of 18 kDa. Nicholson

et al., (1993) have reported that LTC4 synthase from human leukemia cell line (THP-1) is a homodimer with a subunit molecular weight of 18 kDa. In guinea pig lung LTC4 synthase was found to be a tetramer with subunit molecular weight of 60 kDa (Jenny, 1995). In the present study, sheep uterine LTC_4 synthase showed two closely migrating bands with molecular weight of ~69-70 kDa. These proteins may be two subunits of a high molecular weight native protein or two isoforms of LTC4 synthase. These studies revealed that sheep uterine LTC4 synthase is quite different from the reported LTC4 synthases in other animal systems, but still showing structural similarity. The reasons for the differences in the molecular weights of LTC4 synthases from different animal systems are not clear.

In an attempt to purify LTC4 synthase, sheep uterine microsomes were solubilized with taurocholate\CHAPS and loaded onto Q-Sepharose column. The enzyme was purified to 6.62 folds with an overall yield of 44% and a specific activity of 29.77 units/mg protein. The highly purified fractions from anion-exchange column on immunoblot analysis also showed a molecular weight of ~69-70 kDa when probed with peptide as well as protein antibodies. The very low fold purification and yield achieved in the present study indicates the highly unstable nature of the enzyme protein. As a result of this, further purification of the enzyme could not be carried.

Recently Jacobsson *et al.,* (1997) have classified microsomal GSTs, which are capable of conjugating electrophilic substrates to GSH, into microsomal GST-I, microsomal GST-II and microsomal GST-HI. The microsomal GST-I has much wider substrate specificities but with poor affinity towards LTA4. Microsomal GST-II forms a link between LTC_4 synthase and microsomal GST-I, since it acts both on LTA4 and

CDNB (Jacobsson *et al.,* 1996). Microsomal GST-Ill, on the other hand, is with LTC_4 synthase and peroxidase activities, but no activity on CDNB. (Jacobsson *et al.,* 1997). The LTC_4 synthase reported in the present study can be classified under microsomal GST-III group, as it shows no activity with CDNB. However, it is to be tested whether this enzyme from sheep uterus exhibits peroxidase activity.

4.3.1.0. *LTB_4 Synthesis*

The fate of LTA_4 formed in the tissues is its conversion to either LTC_4 or LTB_4. LTA4 to LTB4 is known to be catalyzed by a cytosolic enzyme, LTA_4 hydrolase (Radmark *et al.,* 1984b). The LTA4 hydrolase activity observed in the present study, on the other hand, is microsomal in nature. It will be interesting to probe further into the characteristics of this unique membrane bound LTA4 hydrolase.

Summary and Conclusions

Each component of a living organism has a specific function. This is true not only of microscopic structures but also of individual compounds. One of the fruitful approaches in understanding biological phenomena has been to purify individual components from living organism and to characterize their structure and function. When these molecules are isolated and examined individually they conform to all the physical and chemical laws that describe the behavior of living organisms.

Several studies have established a link between PUFAs and pathological conditions. The different effects that fatty acids can have on human health are the consequences of processes by which their oxygenated derivatives are formed. Of all the oxygenated derivatives, leukotrienes (LTs) are extremely potent biologically active compounds with bewildering variety of actions. Elevated levels of LTs have been demonstrated in immediate hypersensitive reactions like allergy, asthma and inflammation. A better understanding of enzymes involved in leukotriene biosynthesis is required to regulate the production of LTs and to have better insights into their role in health and disease.

Once AA is released from membrane phospholipids, it is metabolized *via* the cyclooxygenase or lipoxygenase pathways. The immediate oxygenated products of Lox pathway are hydroperoxyeicosatetraenoic acids (HPETEs). The hydroperoxides, intum, serve as substrates for LT biosynthesis. Two major LT biosynthetic pathways have been identified, 5-6-LT pathway and 14,15-LT pathway. In a typical 5,6-LT pathway, 5-HPETE is converted into an allelic epoxide, 5,6-LTA4, which inturn serves as substrate for peptido and non-peptido LTs. GSTs and LTC_4 synthases form the major enzymes in the conjugation of GSH to LTA4 to give rise to peptido LTs. LTA4 hydrolase, on the other hand, is involved in the formation of LTB_4, the non-peptido

LT. Typically the same steps are involved in the formation of 14,15-LTs, except that 15-HPETE forms the precursor for the 14,15-LT series of compounds.

In spite of rapid progress on the effects of LTs, there is limited progress on the enzymes involved in LT biosynthesis. Since peptido leukotrienes (LTC_4, LTD_4, LTE4 and LTF_4) and non-peptido LT (LTB_4) are known to play an important role in the mediation of allergy, asthma and inflammation, purification and characterization of LT biosynthetic enzymes assumed importance. Earlier studies have shown abundant dual Lox activity (12- and 15-Lox) in sheep uterus with 14,15-LTA_4 synthase activity (Sailesh *et al*., 1994). However, it is not known how exactly 14,15-LTA_4 is metabolized in sheep uterus. Hence in the present study an attempt is made to analyze the metabolism of 14,15-LTA_4 in sheep uterus and to characterize the associated enzymes.

1. Sheep uteri at different stages of reproductive cycle were collected from local slaughter houses, and the cytosolic and microsomal fractions checked for LTC4 synthase activity. Of all the stages studied uteri at luteal phase showed maximum LTC4 synthase activity. Further studies have revealed that glutathione S-transferases in the cytosol and LTC4 synthase in the microsomes were responsible for the conversion of LTA4 to LTC4 in sheep uterus.
2. GST activity when measured with CDNB as the substrate showed maximum activity in the cytosolic fraction with no activity was in the microsomal fraction. Of all the stages tested, uteri at luteal phase showed maximum GST activity (~13 units/ g wet tissue).
3. GSTs were purified from sheep uterine (luteal stage) cytosol using GSH affinity matrix in a single step to ~270 fold with an overall yield of 56%. Affinity purified

GSTs on SDS-PAGE analysis showed 3 bands with molecular weights of 24.0. 25.5 and 26.5 kDa. The highly purified enzyme showed specific activity of 47.2 units/mg protein with CDNB as substrate.

4. The affinity purified cytosolic GSTs catalyzed the conversion of LTA_4 Me to LTC_4 Me but no activity was observed with LTA4 free acid.
5. SDS-PAGE, 2-D, isoelectric focusing and RP-HPLC analyses of affinity purified GSTs revealed the presence of multiple forms of GSTs, which is the characteristic feature of GSTs.
6. Affinity purified GSTs when separated on ion exchange chromatography (DEAE-Cellulose) resolved into 3 major isoforms, one flow-through fraction with a pI value of ~7.0 (24.0 kDa) and two anionic isozymes (peak 1 and peak 2) (25.5 kDa). All these isozymes were found to be homodimers with pI values ranging between 5-7. Of the three isozymes, the flow-through fraction accounted for 55-60 % of the total cytosolic GSTs.
7. Both the anionic isozymes (peak 1 and 2) showed significant LTC_4 synthase activity when LTA_4 Me was used as the substrate, the activity being more with the peak 1 isozyme. The flow-through fraction, on the other hand, showed very low LTC_4 synthase activity.
8. Further immunological characterization of sheep uterine GSTs revealed that the anionic GSTs are more closely related to μ class of GSTs, which were shown to exhibit very high LTC_4 synthase activity.
9. Sheep uterine microsomes in the presence of GSH catalyzed the conversion of LTA_4 Me as well as LTA_4 free acid to their respective LTC_4s. This activity of microsomes was the highest in uteri at luteal phase. However, sheep uterine

microsomes showed very little or no GST activity with CDNB as the substrate. indicating that GSTs may not be involved in LTC_4 synthesis. Further studies have revealed that specific LTC4 synthase is responsible for the conversion of LTA_4 to LTC_4 in sheep uterine microsomes. This microsomal LTC_4 synthase showed equal preference to 5,6-LTA4 as well as 14,15-LTA4, and hence 14,15-LTA4 was used as substrate for further characterization of LTC4 synthase from sheep uterus.

10. In order to purify LTC_4 synthase sheep uteri, microsomes were solubilized by employing a variety of detergents. Of all the detergents tested 1% CHAPS and 1% taurocholate, recovered maximum LTC4 synthase activity.
11. LTC4 synthase from solubilized microsomes was further purified on Q-sepharose anion exchange column, which resulted in 6.62 fold purification and an overall yield of 44.0%. The purified enzyme showed specific activity of 29.77units/mg protein. Further purification of LTC_4 synthase could not be carried due to the highly unstable nature of the enzymes.
12. Western blot analysis of sheep uterine microsomal proteins with LTC_4 synthase specific peptide as well as protein antibodies showed strong cross reactivity with ~69-70 kDa protein.
13. Sheep uterine LTC4 synthase, thus appears to be unique protein with a molecular weight of 69-70 kDa as the molecular weight of most of the LTC4 synthases reported are of ~18 kDa. Further this enzyme showed equal affinity towards 5,6-LTA4 as well as $14,15\text{-}LTA_4$ as substrates unlike the well characterized LTC4 synthases, which showed preferential activity towards 5,6-LTA4.

From the present study it can be concluded that $14,15\text{-}LTA_4$ is metabolized into $14,15\text{-}LTC_4$ in the cytosol as well as in the microsomes of sheep uterus.

Glutathione S-transferases with molecular weight of 25.5 kDa and close similarity to μ class GSTs are responsible for the conversion of LTA_4 Me to LTC_4 Me in the cytosol. However in the microsomes, a unique LTC_4 synthase without GST activity, is responsible for the conversion of LTA4 Me as well as LTA4 free acid to the corresponding LTC_4s.

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