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2.1.0.0. Materials

Arachidonic acid (AA) (99% pure), soybean lipoxygenase, fat free bovine serum albumin (BSA), ethylene diaminetetraacetate (EDTA), glutathione reduced (GSH), S-hexyl glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), dithiothreitol (DTT), probenecid, Q-Sepharose, epoxy-activated Sepharose 6B, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), phenylmethanesulfonyl 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 B2 (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)trifluoroacetamide (BSTFA), boron trifluoride (BF3), were purchased from Aldrich Chemical Company (MO, USA).

Acrylamide (99.9%), N,N′-methylene-bis-acrylamide,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\textsubscript{18} and silica cartridges were purchased from Waters, India.

**LTC\textsubscript{4} synthase-specific** peptides (MKDEVALLAAVTLLGVLLQ & GRLRTLLPWAC) conjugated to keyhole limpet hemocyanin (KLH) were procured from MBT, USA. The LTC\textsubscript{4} synthase and specific LTC\textsubscript{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
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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
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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$_3$PO$_4$ 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\textsubscript{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\textsubscript{4}

Leukotriene A\textsubscript{4}, the highly unstable epoxide, is the precursor for all the biologically important leukotrienes (LTs). This epoxide gets enzymatically transformed into LTC\textsubscript{4} by a specific enzyme, LTC\textsubscript{4} synthase and to LTB\textsubscript{4} by LTA\textsubscript{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.10  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 al., (1990). The soybean lipoxygenase was incubated with 150μM 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 HCl. 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 A4

14,15-LTA4 was prepared according to the modified procedures of Corey and Barton, (1982) & Chang et 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 lipoygenase*
**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 LTA₄ Me. The presence of conjugated triene spectrum with maximum absorption at 278.5 nm shows the formation of LTA₄ (Fig. 13). The product formed was quantified by taking the molar extinction coefficient of LTA₄ Me (40,000 cm⁻¹ M⁻¹). For the preparation of 5,6-LTA₄ Me the same procedure was followed except that 5-HPETE was employed as the starting material (Table 1).

2.3.3.0. Preparation of LTC₄ from LTA₄ 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 μl 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 μl of 6 N HCl and the contents were diluted 10 fold with water and passed through the pre-
Fig. 13 UV-Scanning spectrum of leukotrieneA₄ Me.
Table 1. Biomimetic synthesis of LTA₄s.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>HPETE (mg)</th>
<th>LTA₄ (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₂Cl₂: THF (1:1)</td>
<td>5-HPETE(12.0)</td>
<td>5,6-LTA₄ Me (02.88)</td>
<td>24</td>
</tr>
<tr>
<td>CH₂Cl₂: THF (1:1)</td>
<td>15-HPETE(34.0)</td>
<td>14,15-LTA₄ Me (16.00)</td>
<td>47</td>
</tr>
</tbody>
</table>
a. RP-HPLC analysis of LTC₄

<table>
<thead>
<tr>
<th>Column</th>
<th>Waters μ Bondapak C₁₈ (0.39x 30 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>Methanol: Water: Acetic acid (65:35:0.08; pH 5.7)</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1 mL / Min.</td>
</tr>
<tr>
<td>Detection</td>
<td>280 nm</td>
</tr>
</tbody>
</table>

b. UV-Scanning spectrum of LTC₄

Fig.14 Preparation of standard 14,15-LTC₄.
equilibrated C\textsubscript{18} cartridges. The cartridge was washed with water and the adsorbed reaction products were eluted with methanol. The LTC\textsubscript{4} in methanol was dried and later re-suspended in HPLC solvent, methanol: acetic acid, pH 5.7 (65:35:0.08, v/v/v) and separated on reverse phase HPLC (RP-HPLC) using uBondapak (Waters India) C\textsubscript{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\textsubscript{4} with maximum absorption at 280 nm (Fig. 14b) and was used as the standard for co-chromatography.

2.3.4.0. Hydrolysis of LeukotrieneA\textsubscript{4}Me

Hydrolysis was carried out by modifying the method described by Wynalda et al., (1982). To LTA\textsubscript{4} Me dissolved in methanol: TEA, 6 \textmu l of 1 M LiOH was added and the reaction was carried with periodic shaking at 25\textdegree 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\textdegree 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 LTA\textsubscript{4} 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.
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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 KCl** till the absorbance at 280 nm 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 KCl** 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 C18 (0.39x30 cm) column (Ostlund et al., 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.

<table>
<thead>
<tr>
<th>Time</th>
<th>Solvent-B Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10min</td>
<td>0-15%</td>
</tr>
<tr>
<td>10-20 min</td>
<td>15-23 %</td>
</tr>
<tr>
<td>20-33 min</td>
<td>23-24%</td>
</tr>
<tr>
<td>33-40 min</td>
<td>24-26%</td>
</tr>
<tr>
<td>40-48 min</td>
<td>26-29%</td>
</tr>
<tr>
<td>48-80 min</td>
<td>29-33%</td>
</tr>
</tbody>
</table>

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 \(\mu\) mole of thioester formed or substrate consumed per min and the specific activity was expressed as units per mg protein.
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### 2.4.6.0. \textit{L} TC\textsubscript{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 HCl 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\textsubscript{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 \textit{et al.} (1985), with some modifications. Incubation mixture consisted of 25 mM KH\textsubscript{2}PO\textsubscript{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°C 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

Fig. 15 GSH affinity chromatography of rat liver cytosolic 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 LTC₄ 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

LTC₄ 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 LTC₄ synthase activity were pooled and concentrated using Amicon spin concentrators with 10 kDa cutoff size by centrifugation at 5,000 x g at 4°C.

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 \( \beta \)-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 and assayed for LTC\(_4\) synthase activity.

### 2.6.4.0. LTC\(_4\) Synthase Assay

LTC\(_4\) Synthase activity was assayed by the conversion of LTA\(_4\) to LTC\(_4\) by the method of Nicholson \textit{et al}, (1992a). The typical reaction mixture consisted of 10 mM GSH, 50 mM serine-borate complex (\( \gamma \)-glutamyl transpeptidase inhibitor). 20 mM magnesium chloride in 50 mM HEPES in a final volume of 500 ul (0.1 mg of L-\( \alpha \)-phosphatidylcholine was included in the reaction mixture for LTC\(_4\) synthase assay involving purified fractions). An initial concentration of LTA\(_4\) (10 times the final incubation concentration) was prepared by evaporating an aliquot of LTA\(_4\)/LTA\(_4\) Me stock under argon, re-suspending LTA\(_4\) 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 LTA\(_4\) Me (20/40 \( \mu \)M 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 LTC4. 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.