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Purification and Characterization of LTC_4 Synthase from Sheep Uterus

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Abstract

Eicosanoids, the oxygenated metabolites of eicosapolyenoic fatty acids such as arachidonic acid via the cyclooxygenase (COX), lipoxygenase (LOX) and epoxygenase (EPOX) pathways, are generated in response to specific stimuli, elicit the response and are then quickly metabolized. Hence, these are rightly termed as "local hormones" or "autocoids". They are involved in the regulation of a variety of physiological as well as pathological processes, including reproduction. While there are extensive studies on the role of COX metabolites, such as prostaglandins, in reproduction, not much is known on the role of LOX metabolites in reproduction. Earlier, we have identified abundant LOX activity in sheep uterus and the highly purified enzyme was found to be a homo-dimeric protein with a molecular weight of 66 kDa. When incubated with arachidonic acid, the enzyme showed two lipoxygenase activities producing both 12- and 15-Hydroxyeicosatetraenoic acid (15-HPETEs) at the optimum pH of 5.5. The relative concentration of 12- and 15-HPETEs, however, changed with the pH of the reaction, 12-Hydroxyeicosatetraenoic acid (HETE) being higher in the alkaline range and 15-HETE being the abundant in the acidic range. Furthermore, the enzyme showed the dual lipoxygenase based 14,15-LTA4 synthase activity as evidenced by the formation of 8,15-diHETEs, the hydrolysis products of 14,15-LTA4. In the present study, leukotriene C4 synthase (LTC_4S) enzyme was purified on Q-Sepharose column after solubilization of microsomes utilizing a combination of CHAPS and taurocholate. The purified enzyme showed activity with 5, 6-LTA_4 and 14, 15-LTA_4, with slight preference towards the latter, and converting them to corresponding LTC_4s. Both methyl esters and free acids of LTA_4 served as substrates, though the activity was more with methyl esters. However, the enzyme showed no activity with I-chloro-2, 4-dinitrobenzene (CDNB), the conventional substrate of glutathione S-transferases. Western blot analysis of sheep uterine microsomal proteins with LTC_4 synthase specific-peptide as well as whole protein antibodies showed strong cross reactivity with two closely migrating 70 kDa proteins. While showing similarity with the known LTC_4 synthases, sheep uterine LTC_4 synthase thus appears to be quite different in terms of molecular weight, as most LTC_4 synthases reported are 18 kDa proteins. In view of its association with the microsomal membranes and involvement in eicosanoid and glutathione metabolism, sheep uterine LTC_4 synthase may form a member of MAPEG (Membrane Associated Proteins in Eicosanoid and Glutathione metabolism) superfamily.

Keywords: Eicosanoids, Lipoxygenase, Sheep Uterine LTC_4 Synthase, MAPEG Superfamily

1. Introduction

Leukotrienes are a family of eicosanoid inflammatory mediators that are synthesized from arachidonic acid via a series of enzymes of 5-lipoxygenase (5-LOX) pathway, which have role in different pathophysiological conditions such as cancer and inflammatory-associated disorders (atherosclerosis, rheumatoid arthritis, uterine inflammation, etc.)[1-7]. 5-LOX converts the arachidonic acid to 5-hydroperoxyeicosatetraenoicacid (5- HPETE) which, on dehydration, gets transformed to an unstable allylic epoxide 5(S)- trans-S,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid, leukotrieneA_4 (LTA_4)[8]. LTA_4 is then conjugated to reduced glutathione (GSH) by LTC_4

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synthase. LTC_4 synthase (EC 2.5.1.37) is the terminal enzyme of 5-LOX pathway required for the biosynthesis of5(S)-hydroxy-6(R)-S-glutathionyl-7,9-trans-11,14-cis-eicosatetraenoic acid (LTC_4), the parent compound of cysteinyl leukotrienes. LTC_4 synthase is a membrane-bound homodimer with a subunit molecular mass of 18 kDa. The conjugation of LTA_4 to GSH to form LTC_4 can also be catalyzed by cytosolic and microsomal glutathione S-transferases which act only on methyl esters of LTA_4 (LTA_4Me)[9]. The microsomal LTC4 synthase, on the other hand, acts on methyl esters as well as free acids of LTA_4,[10]. These microsomal enzymes, along with others involved in eicosanoid and glutathione metabolism, were included in a superfamily designated as MAPEG (Membrane Associated Proteins in Eicosanoid and Glutathione metabolism)[11]. LTC_4synthase is expressed at low levels in a limited number of hematopoietic cells including mast cells, eosinophils, basophils, monocytes, macrophages and platelets[12-17]. In addition, LTC_4 synthesizing ability was also observed in endothelial cells and vascular smooth muscle cells[18, 19], human lung tissue[20] and rat renal microsomes[21]. So far LTC_4 synthase has been cloned and characterized from transformed cell lines but not much has been defined from tissue source. In the present study we report the presence of LTC_4 synthase activity in sheep uterine microsomes. Isolation and characterization of LTC_4 synthase revealed that it is a unique protein with molecular weight 70 kDa and with slightly higher affinity towards 14,15- LTA_4.

2. Materials and Methods

2.1 Chemicals

Arachidonic acid (AA) (99% pure), GSH, and Freund's complete and incomplete adjuvants were purchased from Sigma Chemical Company (St. Louis, Mo, USA). Acrylamide (99.9%), TEMED, 2-mercaptoethanol, Coomassie brilliant blue, G-250 and bromophenol blue were purchased from Bio-Rad laboratories (Richmond, USA). Ultrafiltration units were obtained from Amicon (MA, USA), nitrocellulose membranes for immunoblotting from Amersham Pharamacia Inc., USA, and C_{18} cartridges were purchased from Waters, India. LTC4 synthase-specific peptides (MKDEVALLAAVTLLGVLLQ and GRLRTLLPWAC) conjugated to keyhole limpet hemocyanin (KLH) were purchased from Molecular Biotechnologies (MBT), USA. The human LTC_4 synthase 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 methanol, propane2-ol, tetrahydrofuran, methylene chloride and acetic acid were procured from Spectrochem India Ltd. All other chemicals were procured from local companies.

2.2 Methods

2.2.1 Preparation of Sheep Uterus Homogenates

Sheep *(Ovis aries)* uterine tissues were collected from local slaughter houses immediately after sacrifice and transported on ice to the laboratory. The sizes of the uterus, presence of ovarian follicles and corpus luteum were used as markers to identify different stages of uteri. Sheep uterus homogenate (20%) was prepared in 50 mM HEPES buffer (pH 7.6), containing 2 mM EDTA, 2 mM PMSF and 0.25 M sucrose. The homogenate was centrifuged at 1000xg for 15 min and the supernatant was again centrifuged at 5,000xg for 25 min. The resulting supernatant was centrifuged at 1,05,000 xg for 1hour to obtain microsomal pellet. The microsomal pellet thus obtained was washed three times with the same buffer and then suspended in phosphate buffer (pH 7.6) containing 1 mM EDTA to a final concentration of 15-20 mg protein/mL.Microsomes were solubilized with CHAPS and taurocholate (1 % final concentration) by combining microsomal membrane suspension with an equal volume of detergent in 50 mM phosphate buffer, pH 7.4, containing 2 mM EDTA. The solubilized microsomes were centrifuged at 200,000xg at 4°C for 1h. The supernatant was clarified by passing through 0.45 micron filter and the filtrate was used for further purification.

2.2.2 Anion Exchange Chromatography

The solubilized microsomal extract was applied onto Q-Sepharose anion exchange chromatography. Q-Sepharose (1.5 x 10 em) column was equilibrated with 20 mM Tris-HCI containing 1 mM EDTA, 2 mM GSH, 1 mM DTT, 0.1% taurocholate, 0.5% n-octylglucoside, 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 (from 0-1.0 M, 100 mL gradient volume) and 3 mL fractions were collected. The fractions that contained LTC_4 synthase activity were pooled and concentrated using Amiconspin concentrators with 10 kDa cutoff size by centrifugation at 5,000 xg at 4°C.

2.2.3 Biomimetic Synthesis of 14,15 and 5,6-leukotriene A_4

14,15 LTA_4 and 5,6-LTA_4 methyl esters as well as free acids were prepared in the lab, as per the biomimetic synthesis described (9), from 15-HPETE and 5-HPETEs, respectively. 15-HPETE and 5-HPETEs were prepared employing soybean and potato lipoxygenases as per the methods described earlier (22).

2.2.4 LTC_4 Synthase Assay

LTC_4 synthase activity was assayed by the conversion of LTA_4 (5,6-&14, 15-LTA_4) to LTC_4 (5,6-&14,15-LTC_4) by the method of Chang et al.[9]. The typical reaction mixture consisted of 10 mM GSH, 50-mM serine-borate complex (γ-glutamyltranspeptidase inhibitor), and 20-mM magnesium chloride in 50 mM HEPES in a final volume of 500 µL (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 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 diluted with fat-free BSA (5 mg/mL). Before initiating the reaction, the reaction mixture was incubated at 37°C for 1 min. LTA_4/LTA_4 Me (20/40 µM final concentration) was added to the reaction mixture and incubated at 37°C for 10 min. The reaction was terminated by 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 that precipitated were removed by centrifugation at 15,000xg for 15 min. The LTC_4 that formed was extracted into organic solvent and the extract was then analyzed by isocratic RP-HPLC on Schimadzu HPLC system using Waters µ Bondapak CIS column (0.39 x 30 cm). The mobile phase consisted of methanol: water: acetic acid (65:35:0.8) (pH 5.7) at a flow rate of 1 mL/min. The product LTC_4 was identified based on the retention time (RT). The synthase activity was expressed as units/mg protein where one unit is defined as one nanomole of LTC_4 formed per 10 min. All the reactions were carried out in duplicate, and blanks were run with denatured protein i.e., heat-killed enzyme.

2.3 Protein Estimation

Protein content in the crude preparations was estimated by Bradford method modified by Reddanna et al. (23) and in chromatographic fractions the content was determined by finding absorption at 260 nm and 280 nm on Beckman DU-64 spectrophotometer using 10-mm path length quartz cuvettes.

2.4 Preparation of Standard LTC_4 from LTA_4 Me

LTA_4Me (~100 AU), dissolved in 500 µL methanol and 250 µL triethanolamine (TEA), was allowed to react with 60 mg of GSH. To this 250 µL of 1M potassium carbonate (K_2CO_3) 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-equilibrated C_{18} cartridges. The cartridge was washed with water and the adsorbed reaction products were eluted with methanol. The LTC_4Me in methanol was dried under argon gas 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 µ Bondapak (Waters India) C_{18} column (0.39 x 30 cm). The effluent was monitored at 280 nm and the peaks collected were subjected to UV VIS scanning. The peak with characteristic UV VIS spectrum of LTC_4, with absorption maximum at 280 nm and shoulders at 270nm and 290nm, was taken as the standard LTC_4Me.

3. Results

3.1 Assay of LTC_4 Synthase Activity

LTC_4 synthase activity was measured in sheep uterine microsomes by RP-HPLC analysis of the products formed on incubation with 14,15-LTA_4Me as the substrate. In a typical assay the reaction products were extracted and analyzed on RP-HPLC. As shown in the chromatogram (Figure. l) the peak with RT 14.98 min alone showed the characteristic spectrum of LTC_4 with absorption maximum at 280 nm, shoulders at 270 and 290 nm. Further, the peak with RT 14.98 min co-eluted with the authentic standard, indicating its identity as LTC_4 and the formation of peak with RT 14.98 min increased with increasing amounts of enzyme (data not shown). The other peaks with RT 17.81, 22.12, 32.76 and 40.41 min, showed typical spectra of hydrolysis products of LTA_4. The peak with RT 26.51 min, on the other hand, showed absorption maximum at 270 nm and shoulders at 260 nm and 280 nm, which is typical of LTB_4. LTC_4 (peak I) as well as LTB4 (peak IV) peaks were abolished when heat denatured (boiling water bath, 10 min) microsomes were used in the assay (Figure 2). Similarly, when GSH was omit-

Fig.1

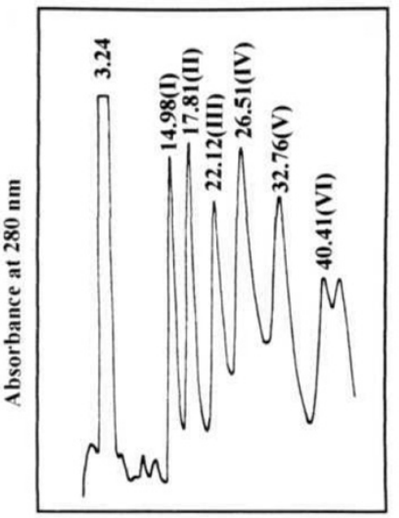

Figure 1. RP-HPLC analysis of the products generated, when sheep uteine microsomes were incubated with 14,15-LTA_4 Me and GSH. Peak I: LTC_4, Peak II, III, V, VI: di-HETEs, Peak IV: LTB_4. Column: C18 Waters μ bondapak; Mobile phase: Methanol: water: acetic acid in a ratio of 65 : 35 : 0.8; Flow rate: 1 mL/min; Detection: 280 nm.

Fig.2

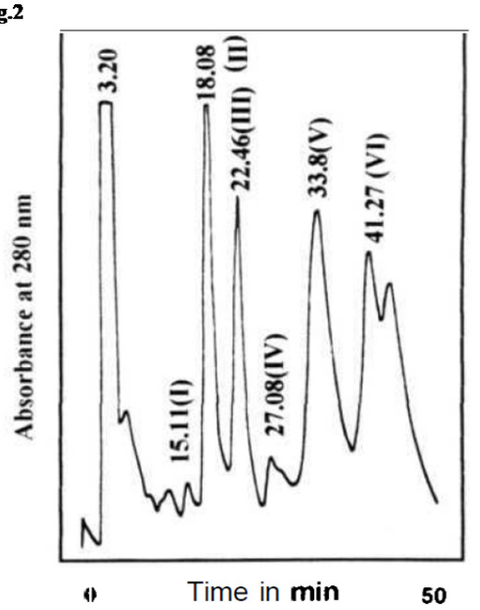

Figure 2. RP-HPLC analysis of the products generated, when heat-denatured microsomes were incubated with 14, 15-LTA_4Me and GSH. Note the disappearance of the peaks 1 and IV, compared to the products generated from sheep uterine microsomes, separated on RP-HPLC (Fig. 1).

ted in the reaction mixture, no LTC_4 peak was observed (Figure 3). However, the LTB_4 peak IV was still observed in the products. From these experiments it is evident that the transformation of LTA_4 to LTC_4 carried out by the microsomes of sheep uterus is an enzymatic process and is GSH-dependent. The formation of LTB_4 is also an enzymatic process but is GSH-independent.

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

Sheep uterine microsomes when incubated with 5, 6-LTA_4Me/FA also showed significant formation of the corresponding 5, 6-LTC_4s (Table 1). The cytosolic fraction, on the other hand, showed activity only with LTA_4 Me. Also, the activity was much lower compared to that of microsomal fraction. The activity levels of microsomal LTC_4 synthase when measured with 5, 6-LTA_4Me and 14,15-LTA_4Me as substrates showed slight preference towards 14,15-LTA_4 Me.

Fig.3

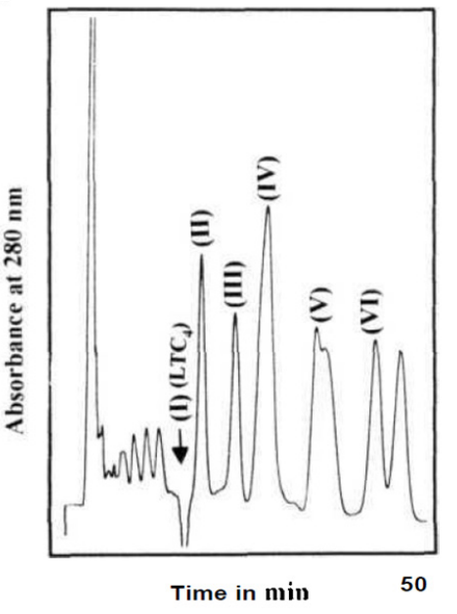

Figure 3. RP-HPLC analysis of the products generated when sheep uterine microsomes were incubated with 14, 15-LTA_4 but without GSH. The peaks corresponding to LTC_4 (peak I) was not formed. However, the peak corresponding to LTB_4 (peak IV) is still present.

LTA_4 Me/FA (40 μM) was incubated with the cytosolic/microsomal fractions of sheep uteri at luteal phase in the presence of GSH and the products formed were extracted and then separated on RP-HPLC. LTC_4Me

formed was quantified based on the absorbance at 280 nm of the peak collected from RP-HPLC. LTC_4 molar extinction coefficient is taken as 40000. *Activity expressed in units/mg protein. One unit is defined as one nanomole of LTC_4 formed per 10 min.

Table 1. 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	1.1	Nil	1.0	Nil
Microscomes	4.3	2.3	4.7	2.2

3.3 Microsomal Solubilization

In the present study a panel of detergents, deoxycholic acid, CHAPs and tween-20, were tried for solubilization of LTC_4 synthase from sheep uterine microsomes. Of all these detergents, CHAPs/taurocholate (1%) resulted in maximum recovery of the LTC_4 synthase.

Sheep uteri at different phases of sexual cycle were analyzed for LTC_4 synthase activity in the solubilized microsomes using 14,15- LTA_4Me as the substrate. Maximum activity was recorded in luteal phase uteri (4.7 units/ mg protein) followed by those at follicular phase (2.3 units/mg protein) and nulliparous phase (1.1 units/mg). Hence, uteri at luteal phase were employed for purification of LTC_4 synthase.

3.4 LTC_4 Synthase Purification

The solubilized microsomes of uteri at luteal phase were centrifuged at 200000 xg for 1 hr at 4°C and the supernatant was loaded onto the Q-Sepharose anion exchange column. The column was washed thoroughly and the bound enzyme was eluted with 0-1M KCl linear gradient (Figure 4a) and the fractions were analyzed for LTC_4 synthase activity by RP-HPLC. Of all the fractions screened, substantial activity was observed in fractions 6, 7 and 8 (data not shown). LTC_4 synthase protein in these fractions was confirmed again on immunoblot analysis (Figure 3b). Typical purification of LTC_4 synthase from sheep uterus resulted in specific activity of 30 units/mg protein with an overall yield of 44% (Table 2).

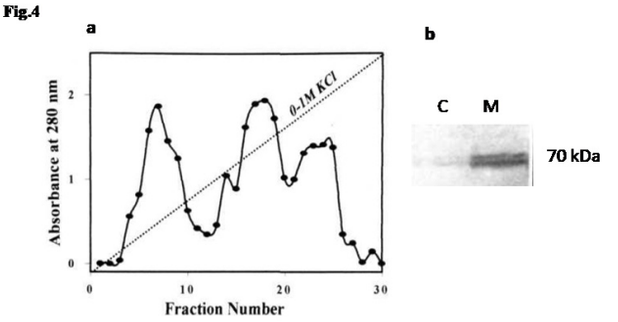

Figure 4a. Q-Sepharose anion-exchange chromatography of solubilized sheep uterine microsomal proteins. The solubilized microsomal proteins were loaded onto Q-Sepharose pre-equilibrated with buffer. The column was washed with the same buffer and the bound proteins were eluted with a liner gradient buffer with salt (from 0 to 1.0 M KCl). The eluted fractions were checked for protein activity and immunological cross reactivity with LTC_4 synthase specific peptide antibodies.

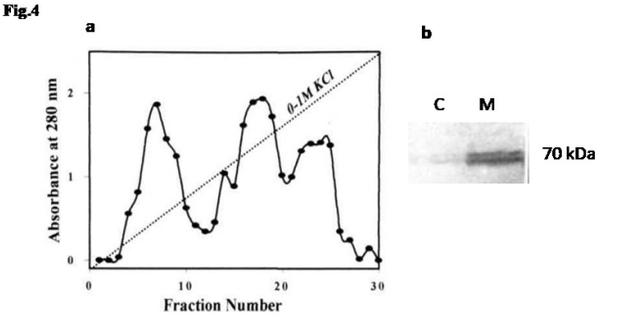

Figure 4b. Western blot analysis of microsomal proteins probed with LTC_4 synthase-specific antibodies. C: cytosol; M: microsomes

Table 2. Purification profile of LTC_4 synthase from sheep uterus (30 g fresh weight) at luteal phase

Purification Step	Total Protein (mg)	Total Activity (Units)	Specific Activity* (Units/ mg protein)	Fold Purification	Yield (%)
Microsomes	203	912	4.49	1.00	100
Solubilized microsomes	123	548	4.45	0.99	60.1
Q-sepharose Anion-exchange Chromatography	13.5	402	29.77	6.62	44.0

*Activity expressed in units/mg protein. One unit is defined as one nanomole of LTC_4 formed per 10 min

3.5 Immuno-Assays

The custom-made N-terminal (MKDEV ALLAA VTILLGVLLA) and C-terminal (GRLRTLLPWAC) LTC_4 synthase specific peptides coupled to KLH were used for raising polyclonal antibodies in rabbit (24). 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 nitrocellulose membrane and the proteins were probed with LTC_4synthase-specific peptide antibodies (Figure 4b). As shown in the figure (lane2), a doublet with MW around 70 kDa was detected on immunoblot analysis.

4. Discussion

Though LTC_4 synthase was detected in many human cells and leukemia cell lines, it was reported only in a few tissues like guinea pig lung[25], endothelial and vascular smooth muscle cells[19], human lung[20] and rat renal microsomes[21]. Several investigators have attempted to purify LTC_4 synthase from a variety of sources but the purification has been hampered by many factors including solubility, stability and difficult assay protocols. LTC_4 synthase activity was stimulated by the presence of divalent cations, Mg^{2+} and Ca^{2+}[26]. Low concentrations of reduced glutathione (GSH) stabilize LTC_4 synthase activity, whereas at higher concentrations GSH inactivates LTC_4synthase. Induction of high levels of LTC_4 synthase expression was observed in the presence of dimethyl sulfoxide (DMSO) and thioglycolate in certain cell lines[16,27].

Solubilization is an important step in the purification of any microsomal enzyme. Attempts were made to solubilize LTC_4 synthase by using a variety of detergents. Solubilization of LTC_4 synthase by using a combination of detergents like CHAPS and digitonin was described[10] and has shown that the enzyme lost its activity on treatment with sodium deoxycholate. Yashimoto et al.[28], on the other hand, used detergents such as deoxycholate and Triton X-100 for solubilizing LTC4 synthase. Solubilization was best achieved with detergents that have charged derivatives of bile acids and taurocholate. In the present study solubilization of microsomes with 1 % CHAPS and taurocholate resulted in maximum recovery of the enzyme activity.

LTC_4 synthase purified to homogeneity from myelomonocytic leukemia cell line was shown to have a molecular weight of 18 kDa[15]. LTC_4 synthase was purified >25000-fold from the taurocholate-solubilized microsomal membranes of monocytic leukemia (THP-1) cells[29]. In guinea pig lung also the LTC_4 synthase activity has been reported[8]. 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. The conversion of LTA_4, to LTC_4 was shown to be an enzymatic reaction since no LTC_4formation was observed when heat-killed microsomes were employed. Further, no activity was recorded when GSH was deleted in the assay mixture. The enzyme was equally effective in the conversion of 5, 6-LTA_4 as well as 14,15 LTA_4 to corresponding LTC_4s. Earlier studies reported presence of abundant dual lipoxygenase activity with potential for 14, 15-LTA_4 formation in sheep uterus[30]. Further, LTC_4 synthase enzyme was active when either LTA_4Me or LTA_4FA was used as substrate. Thus, LTC4 synthase is different from GSTs, which are known to act only on LTA_4Me and not on LTA4FAs[10]. Also GST inhibitors like S-hexyl GSH did not inhibit LTC4 synthase activity. No activity was observed when LTC_4 synthase was incubated with CDNB, the conventional substrate for GSTs. From these observations it is evident that LTC_4 synthase activity observed in sheep uterine microsomes is catalyzed by a unique LTC_4 synthase and not by GSTs. The enzyme 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 [10].

Immunoblot analysis of sheep uterine microsomes using polyclonal antibodies raised against LTC_4 synthase-specific peptides as well as LTC_4 synthase whole protein recognized two closely migrating proteins with molecular weight ~70 kDa. These proteins may be two subunits of a high molecular weight native protein or two isoforms of LTC_4 synthase. However, the molecular weight is much higher compared to the reported molecular weight (18 kDa) of LTC_4 synthase purified from myeloid cell line. Nicholoson et al.[31] have reported that LTC_4 synthase from human leukemia cell line (THP-1), which is a homodimer with a subunit molecular weight of 18 kDa. In guinea pig lung LTC_4 synthase was found to be a tetramer with subunit molecular weight 60 kDa[32]. Sheep uterine LTC_4 synthase isolated in the present study is quite different from the one reported in other animal systems, but still shows immunological reactivity to the known LTC_4synthases.

In an attempt to purify LTC_4 synthase from sheep uterus the uterine microsomes were solubilized with taurocholate/CHAPS and loaded onto Q-Sepharose column.

The enzyme was purified with an overall yield of 44% and specific activity of 30-units/mg protein. The highly purified fractions from anion-exchange column on immunoblot analysis also showed a molecular weight of 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. As a result of this, further purification of the enzyme could not be followed through.

Jakobsson et al.[33] have classified microsomal GSTs, which are capable of conjugating electrophilic substrates to GSH, into microsomal GST-I, microsomal GST-II and microsomal GST-III. The microsomal GST-I has much wider substrate specificities but with poor affinity towards LTA_4. Microsomal GST - II forms a link between LTC_4 synthase and microsomal GST-I, since it acts both on LTA_4 and CDNB[34]. Microsomal GST-III, on the other hand, possesses LTC_4 synthase and peroxidase activities, but no activity on CDNB[33].

In view of the common structural features of membrane-associated proteins with highly divergent functions in eicosanoid and glutathione metabolism, a novel MAPEG (Membrane Associated Proteins in Eicosanoid and Glutathione metabolism) superfamily has been designated[11]. The other members of the MAPEG superfamily include FLAP (5-Lipoxygenase Activating Protein) andLTC_4 synthase, of which the latter exhibits only the LTC_4synthase activity. The enzyme isolated from sheep uterine microsomes, reported in the present study, can be classified under LTC_4 synthase group of MAPEG superfamily.

5. Acknowledgments

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