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A Rapid Radioassay and Some Properties for Leukotriene C_4 Synthase

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Abstract Incubation of glutathione, containing ^{35}S-glutathione (GSH), and leukotriene (LTA_4) in the presence of LTC_4 synthase produced ^{35}S-LTC_4. The LTC_4 was ether extracted and detected by ultraviolet absorbance (280 nm) after reverse phase-high performance liquid chromatography (RP-HPLC) separation. The ^{35}S-LTC_4 was quantitated by scintillation counting. LTC_4 production increased in a dose-dependent manner for GSH concentrations from 10^{-8} to 10^{-2} M. On the contrary, the ^{35}S detected in the ether phase and the ^{35}S-LTC_4 recovered from RP-HPLC decreased inversely with the concentration of unlabeled GSH added but did not with the addition of L-cysteine. This decrease in ^{35}S-LTC_4 production versus increased GSH levels results from a direct competition between unlabeled GSH and ^{35}S-GSH reacting with the LTA_4.

Incubation of GSH (0.5 mM), containing ^{35}S-GSH (0.3 uCi, 10 nM), and LTA_4 (10 uM) with LTC_4 synthase produced ^{35}S-LTC_4 at a linear rate over 5 min. The ^{35}S-LTC_4 increased in proportion to the LTC_4 synthase added. Therefore, we have demonstrated that LTC_4 synthase activity can be monitored by a simple radioassay procedure that measures ^{35}S counts in ether extracts from incubation mixtures. This reliable and rapid assay procedure promises to facilitate characterization and purification of LTC_4 synthase, a membrane-bound GSH S-transferase of unique specificity.

Introduction

Sulfidopeptide leukotrienes (LTC_4, D_4, E_4) are major constituents of the slow reacting substance of anaphylaxis (SRS-A) and are considered major mediators of acute inflammation and immediate hypersensitivity[9)15)]. Leukotriene A_4 is an unstable epoxide and pivotal intermediate in the 5-lipoxygenase pathway of arachidonic acid metabolism. The epoxide intermediate LTA_4 is transformed selectively to either LTC_4 or LTB_4 by specific cellular enzymes[1)10)14)18)]. In the case of LTC_4 formation the enzyme is a membrane-bound LTC_4 synthase[11)13)].

Glutathione (GSH) S-transferase activity can be assayed readily by spectrophotometric methods using relatively stable substrates[8)], whereas LTC_4 synthase activity is assayed by much more laborious procedures due mainly to the instability of the substrate LTA_4 and to the difficulty in quantifying the product, LTC_4. A bioassay for LTC_4 utilizing column chromatography on Dowex 50 was developed by Bach et al[4)]. A radioimmunoassay (RIA) for LTC_4 was developed using protein precipitation and reverse phase-high performance liquid chromatography (RP-HPLC) as reported by Yoshimoto et al.[17)] or using RP-HPLC following organic extraction according to Abe and Hugli[1)]. An advantage of the RIA for LTC_4 is its sensitivity; however the disadvantages are that the degradation products (LTD_4 or LTE_4) may interfere with accurate quantitation. The

primary advantage of the radioassay procedure is that product degradation to LTD_4 or LTE_4 will not influence the results. Also, the assay is more rapid than the RIA, a distinct advantage when monitoring purification procedures during enzyme isolation or screening for inhibitors of the synthase. Our goal was to develop a rapid procedure specific for detecting LTC_4 synthase activity in complex mixtures such as cellular lysates.

LTC_4 synthase catalyzes the conjugation of GSH, a water soluble reactant, with the lipid LTA_4 to produce LTC_4 which is soluble in organic solvents. The ^{35}S-GSH incorporated in LTC_4 can be separated by simple organic extraction from free ^{35}S-GSH in the aqueous layer.

Materials and Methods

All organic solvents used were HPLC quality. The water was distilled and filtered using a NANO-pure system (Barnstend). Synthetic leukotrienes C_4, D_4, E_4, B_4, and A_4 methyl ester were gifts or Dr. Rokach (Merck Frost Canada, Inc.). Other chemicals were of reagent grade.

High Performance Liquid Chromatography

A Novapak C18 (Waters Associate, 0.39×15 cm) 5 um column was equilibrated with the mobile phase of acetonitrile/methanol/water/acetic acid (33.6 : 5.4 : 61 : 1, v/v) adjusted to pH 5.6 with triethylamine[2]. Retention times for LTC_4, D_4, E_4 and B_4 were approximately 6, 8, 10 and 17 min, respectively. The operating flow rate was 1 ml/min and leukotrienes were monitored by ultraviolet absorbance at 280 nm.

Cell Preparation and Crude LTC_4 synthase isolation

Male Hsd: BR mice (Albino ICR, body weight=20−25 g) were used. Two and one half milliliters of 2.5 % thioglycollate medium was injected into the peritoneal cavity. The animals were sacrificed on day three after injection and peritoneal cells were harvested by washing the peritoneal cavity with phosphate buffered saline (PBs, Gibco) containing 10 unit/ml heparin (Elkines-Sinn, Inc.). The harvested cells were washed with PBS and centrifuged at 220×g for 10 min at 4°C. After the hemolysis of red blood cells the macrophages were then counted. The cells were suspended at a density of $0.9\text{-}1.0\times10^8$ cells/ml with Hepes buffer (137 mM NaCl, 2.6 mM KCl, 0.36 mM NaH_2PO_4, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes), 1 mM EDTA at pH 7.5.

The cells were sonicated on ice for 30 sec at an intensity of 5 at a 40% duty cycle (sonicator W-375 (Ultrasonics)), this procedure was repeated 5 times with 30-60 sec pauses. The crude sonicate was centrifuged at 1,500×g for 20 min at 4°C. The supernatant was collected and centrifuged at 100,000×g for 60 min at 4°C. This pellet contains LTC_4 synthase and could be stored at −70°C. Under this condition, no apparent loss of enzyme activity was observed over a 2 months period.

Saponification of LTA_4 methyl ester

Aliquots of the LTA_4 methyl ester (100-300 nmol) were stripped of solvent under a stream of argon and treated with 95% ethanol solution with 0.2 N NaOH. The LTA_4 sample was suspended in pH 7.5 buffer containing 10 mg/ml of fatty acid-free bovine serum albumin (BSA, Sigma) and stored on ice until used.

Incubation conditions for enzyme assay

Enzyme solution (65 ul) and various concentrations of GSH ($10^{-8}\text{-}10^{-2}$ M) containing 20 ul of ^{35}S-GSH were suspended in 400 ul of the Hepes buffer and preincubated for 1 min at 37 °C. Once the LTA_4 was added (65 ul) total volume of incubation mixture was 650 ul, each sample was incubated for 10 min and the reaction was terminated by addition of 520 ul isopropyl alcohol at 4°C. Four hundred pmol of PGB_1 was added to each tube as an internal standard. Leukotrienes were extracted by diethyl ether after acidification. The ether was evaporated and the residue was resuspended in 100 ul of HPLC carrier solvent. A 25 or 50 ul aliquot was injected onto the HPLC column. Quantitation of LTC_4 was based on peak height

ratios between the internal standard (PGB_1) and the recovered LTC_4[1].

The protein content of the enzyme solutions was determined by the method of Bradford[5] using bovine serum albumin as a standard.

Radiolabeled Leukotrienes

Radiolabeled LTC_4 (14,15-^{3}H-LTC_4) (39 Ci/mmol), LTA_4 methyl ester (14,15-^{3}H-LTA_4) (47 Ci/mmol), and glutathione (^{35}S-GSH) (46.1 Ci/mmol) were purchased from New England Nuclear.

Eluate from the RP-HPLC column was manually collected, one fraction every 1 minute, and 10 ml of aqueous scintillant (ACS, Amersham) was added to each fraction. Isotope was counted for 10 min using a dual channel liquid scintillation counter (Beckman, LS 7500).

Radioassay Procedure for LTC_4 synthase activity

The reaction mixture contained crude LTC_4 synthase and 0.1 mM GSH (final concentration) with ^{35}S-GSH (0.3 uCi, 10 nM) which was preincubated for 1 min at 37°C. A 50 ul aliquot of LTA_4 solution (pH 7.5) containing 10 mg/ml BSA (total volume=650 ul) was added to the incubation mixture and the reaction continued for 10 min. The reaction was terminated by addition of 520 ul isopropyl alcohol. The ^{3}H-LTC_4 (0.02 uCi) was added to each tube as an internal stantard. Leukotrienes were extracted from the aqueous phase by diethyl ether under acidic conditions (pH adjusted to 3.0-3.5 with formic acid). The ether phase was decanted into another tube and the pH was adjusted to pH 7.0-7.5 by ammonium hydroxide (15 N). After evaporation under a stream of nitrogen, the residue was resuspended in 650 ul of pH 7.5 Hepes buffer. Isopropyl alcohol (520 ul) was added and the leukotrienes were extracted by diethyl ether after acidification. The ether was evaporated and thereafter 10 ml of ACS was added to each tube. Both ^{3}H and ^{35}S were counted at the same time.

The ^{35}S counts in ether extracts were corrected based on ^{3}H-LTC_4 recovery rates. LTC_4 production was estimated by using following equation (I):

$$\frac{(\text{net } ^{35}\text{S-incorporation}) \times (\text{GSH}) \times \text{incubation volume}}{\text{total } ^{35}\text{S-GSH added}}$$

Net ^{35}S-incorporation equals ^{35}S with enzyme added minus ^{35}S without enzyme added. GSH concentration in the incubation mixture is denoted as (GSH).

Radioimmunoassay for LTC_4

Radioimmunoassay for LTC_4 was performed using RIA kits (^{3}H-LTC_4) purchased from New England Nuclear. Cross-reactivities of LTC_4 antibody with LTD_4, E_4, and B_4 are 11.6, 3.3 and 0.03%, respectively.

Inhibitor experiment

Sulfasalazine and the analog CL 42A were supplied by Dr. Goran Smedegard (Pharmacia). Both drugs were solubilized in saline (pH 7.5). Sulfasalazine (final concentration=0.005-0.5 mM) and CL 42A (0.01-1.0 mM) were added to each tube including 0.5 mM GSH with 0.6 uCi ^{35}S-GSH and 60 ug of the crude LTC_4 synthase by 10% volume of the final incubation mixture. After preincubation for 1 min at 37°C, 33 uM LTA_4 was added to each tube and incubation was continued for 10 min. Analysis of LTC_4 synthase activity was performed by the radioassay procedure.

Results

Leukotriene A_4 was converted to the potent mediator LTC_4 in the presence of reduced glutathione by an enzyme in the 100,000×g pellet obtained from mouse peritoneal macrophages (i. e. crude LTC_4 synthase). LTC_4 production increased in proportion to the concentration of GSH (10^{-8}-10^{-2} M) (Fig. 1).

When GSH containing ^{35}S-GSH was incubated with both LTA_4 and the crude LTC_4 synthase, ^{35}S counts were recovered in the ether phase. Increasing the ^{35}S-GSH (0.1-10 uCi) level in the incubation mixture increased the ^{35}S recovered in the ether phase in a linear fashion (data not shown). On the contrary, when cold GSH concentrations were varied from 0-10 mM

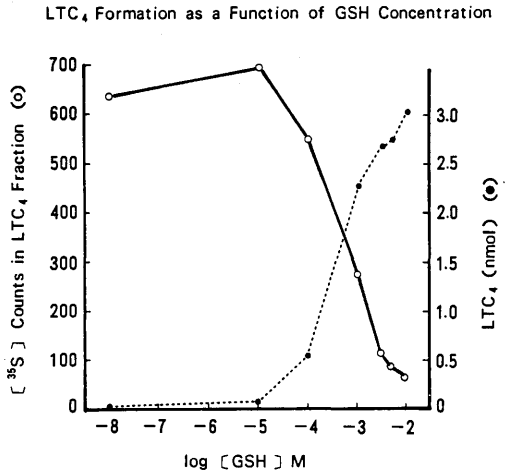

Fig. 1 Influence of varying the GSH concentration on LTC_4 (●) produced by LTC_4 synthase using constant levels of LTA_4 (10 uM). The ^{35}S counts (○) measured (i. e. LTC_4 fraction) were recovered from C18 RP-HPLC and reflect incorporation of ^{35}S-GSH (0.3 uCi) into LTC_4 as a function of unlabeled GSH added.

with constant levels of ^{35}S-GSH, the ^{35}S in the ether phase decreased in an inverse manner with increasing concentrations of cold GSH added. As shown in Fig. 1, the ^{35}S counts in the recovered LTC_4 fraction from RP-HPLC decreased inversely with cold GSH concentrations, corresponding to the ^{35}S counts in the ether phase. When the GSH concentration was increased from 10^{-5} to 10^{-2} M, LTC_4 production was enhanced from 73 pmol to 3 nmol; however the conversion rate of ^{35}S-GSH to ^{35}S-LTC_4 decreased inversely with the GSH concentration. Plots of ^{35}S counts and LTC_4 production versus the GSH concentration intersect between 0.1 and 1.0 mM of GSH. LTC_4 production was estimated by the following equation (II):

$$\frac{^{35}\text{S-GSH incorporated into LTC}_4 \times (\text{GSH}) \times \text{volume}}{\text{total } ^{35}\text{S-GSH added}}$$

On the other hand, when L-cysteine (from 0-10 mM) was added to fixed quantities of ^{35}S-GSH and incubated with both LTA_4 and crude LTC_4 synthase, ^{35}S counts recovered in the ether phase were not significantly changed (Fig. 2). These data indicate that ^{35}S counts decrease as a result of competition between ^{35}S-labeled and unlabeled GSH, but that L-cysteine cannot substitute for unlabeled GSH in this reaction.

We examined the ^{35}S-containing products by RP-HPLC analysis. A reaction mixture of 26 uM LTA_4, containing ^{3}H-LTA_4 (0.7uCi, 23 nM), and 0.1 mM GSH, containing ^{35}S-GSH (2.0 uCi, 66 nM), were incubated with crude LTC_4 synthase for 10 min at 37°C. The ether extract of the incubation mixture was injected onto the RP-HPLC column and fractions were collected every minute. Fig. 3A shows a chromatogram of the enzyme-free mixture monitored by ultraviolet absorbance at 280 nm. Major peaks were identified as 6-trans LTB_4 diastereoisomers in addition to trace quantities of LTC_4 and LTB_4. On the other hand, in the presence of crude LTC_4 synthase (Fig. 3B), significant quantities of LTC_4 and 11-trans LTC_4 appeared in addition to the nonenzymatic metabolites of LTA_4, 6-trans LTB_4 diastereoisomers (Fig. 3B).

The upper panel in 3A shows a radiochromatogram of the enzyme-free fraction.

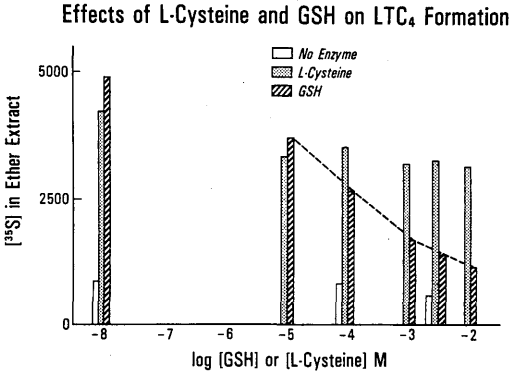

Fig. 2 Influence of GSH and L-cysteine on the incorporation of ^{35}S into LTC_4 by the LTC_4 synthase. The ^{35}S-LTC_4 is recovered in ether extracts of the incubates described in Materials and Methods. Only GSH has a significant competitive effect on ^{35}S-GSH incorporation into LTC_4 by the enzyme.

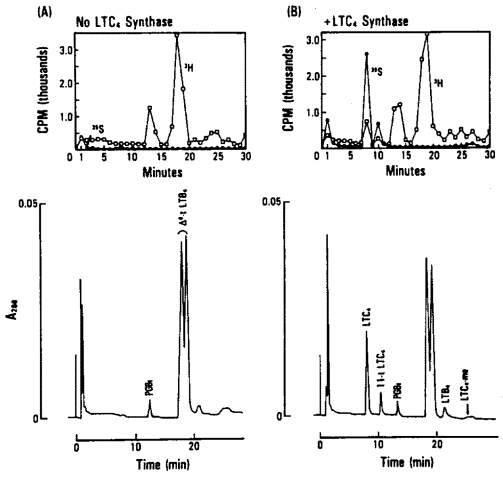

Fig. 3 RP-HPLC separation of ether extracts from mixtures containing 0.7 uCi ^{3}H-LTA_4 (23 nM) in 26 uM LTA_4 and 2.0 uCi ^{35}S-GSH (66 nM) in 0.1 mM GSH without (A) or with (B) the LTC_4 synthase added. The mixtures were incubated for 10 min at 37°C prior to extraction. Eluate from the RP-HPLC C18 column was collected and leukotrienes were monitored by uv absorbance at 280 nm and ^{35}S counts were also determined. Radiochromatography of ^{35}S and ^{3}H products in the reaction mixtures are shown in the upper panel of A and B respectively.

The ^{3}H peaks appear at retention times corresponding to 6-trans LTB_4 diastereoisomers, but no significant ^{35}S peaks appear. However, in the presence of LTC_4 synthase, ^{3}H and ^{35}S peaks appear at retention times corresponding to LTC_4 and 11-trans LTC_4 in addition to the peak at the solvent front. Moreover, small quantities of ^{35}S eluted at 25 to 27 min, possibly representing LTC_4 methyl ester because this peak coeluted with authentic LTC_4 methyl ester, as shown in Fig. 3B. The ^{35}S LTC_4 and 11-trans LTC_4 fractions were associated with ^{3}H counts as expected.

Ether extracts of the reaction mixture were suspended in 100 ul of RP-HPLC solvent and 50 ul were injected on the C18 RP-HPLC column. After separation and collection of one minute fractions (0-30 min), ^{35}S counts for all collected fractions were combined (^{35}S fractions). Summation of ^{35}S fractions (0-30 min) included 97-100% of the total ^{35}S count over wide range of cold GSH concentrations used (0-5.0 mM). These chromatography results suggest that the majority of counts are recovered after C18 RP-HPLC and that products having less polarity than the LTC_4 methyl ester, are apparently not generated. On the basis of the combined data, it is speculated that the major ^{35}S constituent in the ether extract is LTC_4, although the extract also contains ^{35}S-labeled 11-trans LTC_4 and LTC_4 methyl ester as well as unreacted ^{35}S-GSH.

We studied dependency of the radioassay

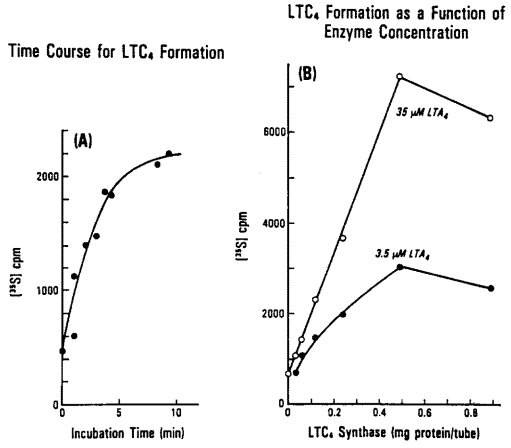

Fig. 4 (A) A time course is shown for LTC_4 production by LTC_4 synthase from GSH (0.1 mM) containing 0.3 uCi of ^{35}S-GSH and LTA_4 (10 uM). Reaction conditions were similar to those described in the enzyme assay section of Materials and Methods. The content of ^{35}S in the ether extracts increases in a linear fashion over approximately 5 min of incubation time at 37°C. (B) The influence of the enzyme concentration on incorporation of ^{35}S-GSH in LTC_4 was examined. Two LTA_4 concentrations were employed (3.5 uM and 35 uM) and in each experiment ^{35}S incorporation was corrected for efficiency of extraction by monitoring ^{3}H-LTC_4 recovery as an internal standard. Non-linearity appears to be a function of the enzyme perhaps indicating aggregation.

procedure on incubation time and protein content of crude LTC_4 synthase. As shown in Fig. 4A, the ^{35}S counts increased in a linear manner over 10 min. When LTA_4 and ^{35}S-GSH were incubated at 37°C for 10 min in the presence of various quantities of crude LTC_4 synthase, the ^{35}S level in the ether extract increased in proportion to the protein content using two concentration of LTA_4 (3.5 and 35 uM). Incubation with the higher concentration of LTA_4 gave a steeper slope than that with the lower level of LTA_4. Recovery of ^{35}S-LTC_4 was calculated by adding ^{3}H-LTC_4 to each tube before ether extraction and counting ^{3}H recovered in the ether phase. Consequently, ^{35}S counts in the ether extract could be corrected according to the recovery of ^{3}H counts determined in each tube and these corrected ^{35}S values were plotted in Fig. 4.

The optimal GSH concentration for this assay was determined by examining the effect of GSH concentration on the net ^{35}S incorporation using three concentrations of cold GSH (0.1, 0.5 and 1.0 mM). The incorporation of ^{35}S-GSH into LTC_4 by LTC_4 synthase decreased inversely to an increase of the unlabeled GSH concentration, the highest net ^{35}S incorporation occurred at 0.5 mM GSH (data not shown).

Comparison of three different LTC_4 synthase assay methods

The three different assay methods (RP-HPLC, RIA and ^{35}S radioassay) were compared for correlation over variable enzyme concentrations. Variable quantities (0-0.7 mg protein) of the crude LTC_4 synthase were incubated with 29 uM LTA_4 and 0.5 mM GSH for 10 min at 37°C. Each tube was divided into two portions, one sample was used to estimate LTC_4 production by radioimmunoassay and the other using RP-HPLC, respectively (Fig. 5A). Enzyme dependency curves from the two methods showed a good correlation ($r=0.9608$, $p<0.01$).

Next, variable quantities (0-0.43 mg protein) of the crude LTC_4 synthase were incubated with 24 uM LTA_4 and 0.5 mM GSH containing 0.3 uCi ^{35}S-GSH. LTC_4 synthase activity was estimated by using ^{35}S incorporation and RP-HPLC for LTC_4 production (Fig. 5B). In the case of ^{35}S incorporation, LTC_4 production was estimated by the equation (I). The enzyme dependency curves from these two methods also exhibited good correlation ($r=0.9878$, $p<0.01$).

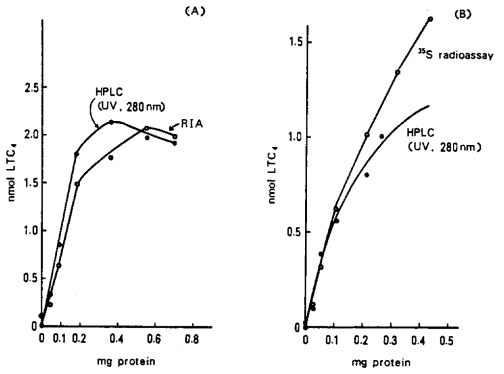

Fig. 5 Estimation of LTC_4 synthase activity was compared using three different assay methods. The LTC_4 synthase activity was estimated as ^{35}S-incorporation in LTC_4 after ether extraction, as LTC_4 production quantitated by radioimmunoassay, or LTC_4 quantitated by uv absorbance after RP-HPLC. (A) Variable quantities (0−0.7mg of protein) of the crude LTC_4 synthase were incubated for 10 min, with 29 uM LTA_4 and 1 mM GSH at 37°C. LTC_4 production was estimated by radioimmunoassay (○) or by uv absorbance after RP-HPLC (●). (B) Variable quantities (0−0.43mg of protein) of the crude LTC_4 synthase were incubated for 10 min with 24 uM LTA_4 and 0.5mM GSH containing 0.3 uCi of ^{35}S-GSH at 37°C. The enzyme activity based on ^{35}S-incorporation was estimated based on LTC_4 production (○) as calculated by the equation described in Discussion section.
Each point denotes the mean value of duplicate experiments in both (A) and (B). GSH concentrations used were different between (A) and (B).

Application of ^{35}S radioassay to inhibitors experiments

The radioassay was used to evaluate effects

of several inhibitors[3] on LTC_4 synthase (Fig. 6). Negative and positive control experiments were performed under the conditions of 33 uM LTA_4, 0.5 mM GSH containing 0.6 uCi ^{35}S-GSH and with or without the crude LTC_4 synthase (60 ug protein), respectively. Sulfasalazine or a derivative CL 42 A was added to individual tubes containing the LTC_4 synthase in a volume of 10 percent incubation mixture. As shown in Fig. 6, only the highest concentration of sulfasalazine (0.5 mM) and CL 42 A (1.0 mM) significantly inhibited LTC_4 synthase activity.

The radioassay method for LTC_4 synthase was applied to examine whether or not LTC_4 synthase is controlled by its product LTC_4. Either 0.5 or 5.0 nmol LTC_4 was added to separate tubes. Fig. 7 shows dose-dependent inhibition for LTC_4 synthase activity by preaddition of LTC_4. Prior addition of 5 nmol LTC_4 reduced the LTC_4 synthase activity to approximately 60% of the control activity at 10 min of incubation time. This experiment was repeated

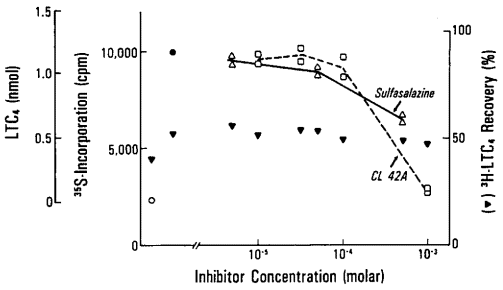

Fig. 6 Evaluation of two possible inhibitors of LTC_4 synthase. Enzyme activity was estimated by the ^{35}S-incorporation method under the condition of 33 uM LTA_4, 0.5 mM GSH with 0.6 uCi ^{35}S-GSH and 60 ug of the crude LTC_4 synthase (positive control, ●) incubated for 10 min at 37°C. The negative control (○) did not contain enzyme (n=2). Sulfasalazine (△) and CL42 -A (□) were added before the incubation. The product formed in the positive control was estimated to be 1.2 nmol of LTC_4. The ^{35}S-incorporation denotes corrected values for ^{35}S (c. p. m.) detected in the ether extracts by recovery rates of ^{3}H-LTC_4 (▼).

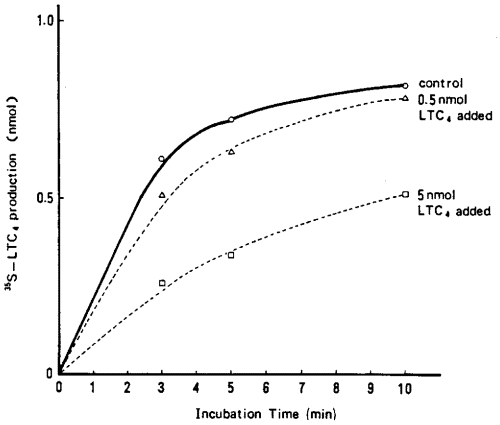

Fig. 7 The ^{35}S radioassay for LTC_4 was used to examine product inhibition of LTC_4 synthase.

by adding 1 nmol of LTC_4, LTD_4, LTB_4, and 5 nmol of LTC_4 to separate tubes (in triplicate). Statistical analysis to compare results with the control experiment was performed using the student t-test. Control tubes without added leukotrienes exhibited 0.612 ± 0.025 (s. e. m.) nmol of ^{35}S-LTC_4. On the other hand, tubes containing 1 nmol of LTC_4, LTD_4, or LTB_4 reduced ^{35}S-LTC_4 production to 0.538 ± 0.038 ($p<0.05$), 0.529 ± 0.042 ($p<0.05$), and 0.546 ± 0.053 (not significant) nmol ^{35}S-LTC_4, respectively. Moreover, addition of 5 nmol LTC_4 reduced ^{35}S-LTC_4 production to 0.350 ± 0.055 nmol ($p<0.001$, approximately 60% of control activity) at 10 min of incubation time. These results suggest that LTC_4 synthase is partially controlled by product inhibition.

Discussion

LTC_4 synthase is a unique particulate GSH S -transferase which appears to specifically utilize only leukotriene A_4 as a primary lipid substrate. The reaction between LTA_4 and GSH is mediated by LTC_4 synthase and can be monitored by measuring the GSH that is incorporated in LTC_4 and extracts with organic solvent. Therefore, organic extraction separates the fraction of GSH that reacts with

LTA_4 from the unreacted GSH pool which remains in the aqueous phase. Therefore, it was predicted that LTC_4 synthase activity could be assayed by extracting LTC_4 in the organic phase and measuring relative levels of conjugated ^{35}S-GSH by quantitating radioactivity.

The plots of ^{35}S-GSH incorporation into ^{35}S-LTC_4 and of total LTC_4 production versus GSH concentration intersect at 0.4 mM near the estimated Km value (0.38 mM)[1]. Therefore, an optimal GSH concentration was selected for the radioassay based on the net ^{35}S incorporation as measured using a narrower range of GSH concentrations (0.1, 0.5 and 1.0 mM). The 0.5 mM GSH concentration was considered optimal because it gave the highest net value of ^{35}S incorporation and was within the same range as the Km for GSH[1]. The higher Km value for GSH in comparison with that for LTA_4 (5 uM)[1] and dissociation of ^{35}S-GSH by unlabeled GSH indicates that GSH has a lower affinity for the enzyme in this reaction than does the other substrate LTA_4.

The RP-HPLC analysis of a reaction mixture containing ^{3}H-LTA_4 and ^{35}S-GSH indicated that only the ^{35}S counts eluted at the solvent front (i. e., unreacted GSH) in the absence of LTC_4 synthase. On the other hand, when LTC_4 synthase was added ^{35}S-GSH was incorporated in LTC_4, 11-trans LTC_4 and LTC_4 methyl ester in addition to eluting as GSH at the solvent front. It has been speculated that 11-trans LTC_4 originates from isomerization of LTC_4 and moreover that LTC_4 methyl ester comes from the conjugation of unsaponified LTA_4 methyl ester with GSH by the action of LTC_4 synthase[6)12)16)]. Consequently, all three ^{35}S-GSH products except for the unreacted ^{35}S-GSH are formed by LTC_4 synthase. These results suggest that the radioassay is specific for LTC_4 synthase activity and that the product LTC_4 can be estimated by the equation (I). Application of ^{3}H-LTC_4 as an internal standard corrects the variation of recovery rates in this radioassay procedure.

The ^{35}S-LTC_4 level increased in proportion to time over a 10 min period. Incorporation of ^{35}S-LTC_4 was also proportional to the LTC_4 synthase level. Better linearity was obtained using the higher concentration of LTA_4 (i. e. 35 uM). However, at higher enzyme levels the activity seems to be inhibited to some extent. Such an inhibition at the higher enzyme concentration was also observed using RIA and HPLC methods. Therefore, inhibition may be explained by aggregation of the particulate enzyme, LTC_4 synthase, at the higher protein concentrations.

There are four methods (^{35}S incorporation, HPLC analysis, RIA and bioassay[1)4)6)17)]) available for measuring LTC_4 synthase activity. When three of the methods (except for the bioassay) were compared they showed good correlation with one other. HPLC clarifies the metabolic pattern for the products, but it is timeconsuming. On the contraty, ^{35}S incorporation and RIA are relatively rapid assays. Comparison between ^{35}S incorporation and the RIA is summarized in Table 1. The radioassay may be more promising in overcoming the difficulty of a conversion of LTC_4 to LTD_4 and LTE_4 during an incubation than the latter method because LTC_4, D_4, and E_4 each contain the ^{35}S-thioether bond and can be measured collectively.

Sulfasalazine is a well known anti-inflammatory drug for ulcerative colitis[7)]. When the ^{35}S incorporation method was applied to evaluate the effect of sulfasalazine and its derivative CL 42A on the crude LTC_4 synthase, only high doses of both drugs inhibited the enzyme activity ($EC_{50}=0.2-0.3$ mM). Bach et al.[3)] reported that the EC_{50} for sulfasalazine against solubilized, particulate LTC_4 synthase from RBL cells is approximately 0.4 mM. Therefore, the inhibitory profile of sulfasalazine appears to be consistent using various assay methods and different species as enzyme sources.

Table 1 Comparisons Between the Radioassay and Radioimmunoassay for Leukotriene C_4 Synthase

	RADIOASSAY (^{35}S-Incorporation)	RADIOIMMUNOASSAY (RIA)
Methodology	simple and direct[a]	simple and direct[a]
Steps	2 (extraction & counting)	4 (dilution, Ag-Ab reaction, centrifugation and counting)
Data	qualitative or quantitative	quantitative
Time/procedure	1 hr	1 day
Sensitivity	50 pmol LTC_4	0.05 pmol LTC_4
Materials	^{35}S-GSH, LTA_4	anti-LTC_4 Ab, ^3H-LTC_4, LTA_4
Analysis	direct	dilutions required
Expense	relatively low	relatively high
Applications	measures all products (LTC_4, D_4, E_4 and 11-t-LTC_4, D_4 and E_4 products)	specific for only LTC_4
Miscellaneous	applicable for sulfidopeptide LTs derived from 8, 12, 15 lipoxygenase pathway[b], in addition to 5-lipoxygenase pathway	LTC_4
Interference	None	LTA_4 and LTC_4 catabolites, GTP[c] and dipeptidase[d]

[a] Facile when compared with identification and characterization of products using HPLC separation and spectroscopic techniques.
[b] Hammarstrom, S. (1983); [c]Gamma-glutamyl transpeptidase; [d]Bach et al. (1984)

In conclusion, the two-step radioassay (i.e. extraction and counting) procedure for LTC_4 synthase is a rapid and useful tool for monitoring LTC_4 synthase isolation or for determining effects of drugs and inhibitors on the enzyme in either pure or crude form.

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(和文抄録)

ロイコトリエン C_4 合成酵素の迅速アッセイ法と酵素の性質

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ロイコトリエン C_4 (LTC_4) 合成酵素の存在下で LTA_4 と放射標識したグルタチオン (^{35}S-GSH) を反応させると ^{35}S-LTC_4 が生成された．LTC_4 はエーテルで抽出し，RP-HPLC で分離後，紫外部吸収計(280 nm)で検出した．同時に ^{35}S-LTC_4 をシンチレーションカウンターで測定した．LTC_4 生成は GSH の濃度を 10^{-8} から 10^{-2} M に変化させると濃度依存的に増加した．一方，エーテル相の ^{35}S と RP-HPLC からの ^{35}S-LTC_4 は添加された無標識の GSH 濃度の増加と逆に減少したが，L-cysteine の添加では影響されなかった．この ^{35}S-LTC_4 生成の減少は LTA_4 と反応する ^{35}S-標識 (^{35}S-GSH) 並びに無標識-GSH との間の直接的な拮抗作用の結果である．

^{35}S-GSH (0.3 uCi, 10 nM) を含んだ 0.5 mMGSH と LTA_4 (10 nM) を LTC_4 合成酵素の存在下で種々の時間反応させると，反応開始後，5 分迄直線的に ^{35}S-LTC_4 の生成が増加した．また ^{35}S-LTC_4 の生成は添加した LTC_4 合成酵素の量に比例して増加した．したがって，LTC_4 合成酵素活性は反応液からエーテル抽出される ^{35}S の放射線量を測定する，簡単な放射アッセイ法により測定され得ることが示された．この迅速アッセイ法は特異的な模型グルタチオン抱合酵素である LTC_4 合成酵素のキャラクタリゼイション並びに精製への応用が期待される．