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Expression of human 5-lipoxygenase cDNA in *Escherichia coli*

Masato Noguchi, Takashi Matsumoto, Motonao Nakamura and Masana Noma

Life Science Research Laboratory, Japan Tobacco Inc., Midori-ku, Yokohama 227, Japan

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A cDNA for human 5-lipoxygenase (5LO) was inserted into the vector pKC (constructed from pKK223-3 by replacing its replication origin with that of pUC18) and expressed in *Escherichia coli*. The enzyme expressed was purified to homogeneity from the cellular soluble fraction. The purified enzyme showed both 5LO and leukotriene A_4 synthase activities, which were stimulated by Ca^{2+} and ATP. Its molecular mass (78 kDa) and NH_2-terminal sequence were identical with those of 5LO purified from human leukocytes. The availability of the expression system will facilitate further studies on its regulation and the reaction mechanism of the enzyme.

Lipoxygenase, 5-; Hydroperoxyicosatetraenoic acid, 5-; Leukotriene A_4; cDNA expression; (*E. coli*, Human)

1. INTRODUCTION

5-Lipoxygenase (5LO) catalyzes the conversion of arachidonic acid to 5-hydroperoxy-6,8,11,14-icosatetraenoic acid (5-HPETE) and the formation of leukotriene A_4 (LTA_4) from 5-HPETE [1–4]. LTA_4 is further metabolized to LTB_4, peptidoleukotrienes, LTC_4, LTD_4 and LTE_4, all of which exhibit biological activities related to inflammatory and allergic responses [5,6]. 5LO has been purified from sources such as human leukocytes [1,7], porcine leukocytes [2], rat basophilic leukemia cells [3,8], and mouse mastocytoma cells [4]. It requires Ca^{2+} and ATP for maximal activity [1,4–7]. Human 5LO is also stimulated by unidentified cytosolic and membrane-bound proteins [7,9] and by sonicated phosphatidylcholine [10]. Recently, 5LO cDNAs derived from human placenta [11], dimethyl sulfoxide-differentiated HL-60 cells [12], and rat basophilic leukemia cells [13] have been isolated. The human enzyme consists of 673 amino acid residues and its calculated molecular mass is about 78 kDa [11,12]. Here, we report the expression of human 5LO cDNA in *Escherichia coli*.

Correspondence address: M. Noguchi, Life Science Research Laboratory, Japan Tobacco Inc., 6-2 Umegaoka, Midori-ku, Yokohama 227, Japan

Abbreviations: 5LO, 5-lipoxygenase; HPETE, hydroperoxyicosatetraenoic acid; LT, leukotriene; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; IPTG, isopropyl-β-thiogalactoside; HPLC, high-performance liquid chromatography; PGB_1, prostaglandin B_1

2. MATERIALS AND METHODS

2.1. *Materials*

A human placental cDNA library in λgt11 was purchased from Clontech (Palo Alto, CA), restriction enzymes and other DNA-modifying enzymes from Takara Shuzo (Kyoto), Nippon Gene (Toyama), and IBI (New Haven, CT), oligonucleotide-directed in vitro mutagenesis system from Amersham (Tokyo), pUC18 and pUC118 from Takara Shuzo (Kyoto) and pKK223-3 from Pharmacia (Tokyo).

2.2. *Construction of expression vector*

Screening of the human placental cDNA library by plaque hybridization using a synthetic human 5LO DNA corresponding to 120 bases upstream from the terminator codon of 5LO cDNA resulted in the isolation of three positive clones. Of these, λplL5 (insert, 2.6 kbp) contained a 2073 bp open reading frame, in which two tandem 51 bp repeating units were found. λplL6 (2.2 kbp) and λplL7 (1.6 kbp) lacked one of the repeating units. As discussed in [11], the repeat in λplL5 was concluded to be due to a cloning artifact. A 2.3 kbp cDNA was cut out of λplL5 with *Bcl*I and *Eco*RI and ligated to pUC118 that had been digested with *Eco*RI and *Bam*HI. This construct, pH5LOU1, contained the tandem repeat between two *Xma*I sites (434 bp). λpL7 was digested with *Xma*I and the resulting

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383 (434–51) bp fragment was ligated between the two *Xma*I sites of pH5LOU1 to obtain pH5LOU2. This construct contains the true human 5LO cDNA lacking the repeat. The 5′-noncoding segment between the *Eco*RI site and the initiator ATG codon of pH5LOU2 (see fig.1) was then deleted by oligonucleotide-directed in vitro mutagenesis to place 10 bases between the Shine-Dalgarno sequence and the initiator ATG codon of 5LO cDNA. The 49-mer primer used was designed such that one half is complementary to 25 bases upstream of the *Eco*RI site of pH5LOU2 and the other half to 24 bases downstream from the ATG codon. This primer was hybridized with single-stranded DNA of pH5LOU2 and, after second-strand synthesis, the double-stranded product was used to transform *E. coli* MV1184. A mutant clone was digested with *Eco*RI and *Hin*dIII, and the resulting *Eco*RI-*Hin*dIII fragment containing 5LO cDNA, from which part of the 5′-noncoding region had been deleted, was inserted between the *Eco*RI and *Hin*dIII sites of pKC to obtain the expression plasmid pH5LOKC. pKC was constructed from pKK223-3 by replacing the replication origin with that of pUC18. The construction of the expression plasmid is outlined in fig.1.

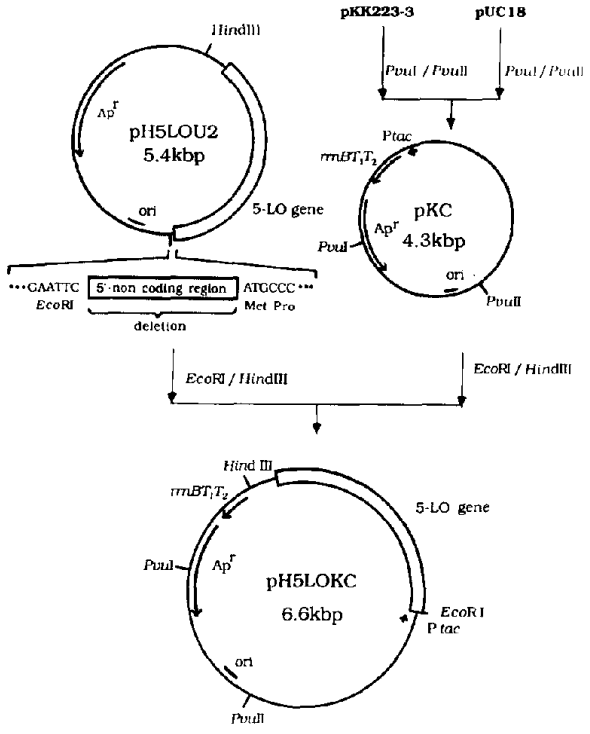

Fig.1. Construction of plasmid pH5LOKC for expression of human 5LO cDNA in *E. coli*. The clone, pH5LOU2, contains a 5′-noncoding region, nucleotide sequence corresponding to 674 amino acid residues between the initiator ATG codon and terminator TGA codon, and 3′-noncoding region of human 5LO cDNA. After deletion of the 5′-noncoding region downstream from the *Eco*RI site of pH5LOU2 by using an oligonucleotide-directed in vitro mutagenesis system, the modified 5LO DNA was cut out with *Eco*RI and *Hin*dIII from pH5LOU2. The *Eco*RI-*Hin*dIII fragment was ligated into the vector pKC which was constructed from pKK223-3 by replacing the replication origin with that of pUC18. The resulting pH5LOKC was used for expression of the human 5LO gene in *E. coli*.

2.3. *Purification of 5LO expressed in* E. coli

pH5LOKC was used to transform *E. coli* MV1184 as described by Hanahan [14]. The transformant was grown at 20°C for 10 h in 1.8 l of TYSG medium (1% Bactotryptone/0.5% yeast extract/2% NaCl/2% glycerol; pH 7.8). IPTG (final concentration, 0.2 mM) was then added and incubation continued for 15 h. Cells were harvested by centrifugation (6000 × *g*, 10 min) and suspended in KP-1 buffer (50 mM potassium phosphate/100 mM NaCl/2 mM EDTA/2 mM DTT/0.5 mM PMSF/60 μg/ml of soybean trypsin inhibitor; pH 7.1). After addition of lysozyme (10 mg/ml), the suspension was placed on ice for 30 min and then sonicated in a Biomic model 7250B ultrasonic processor at power level 4. A total sonication time of 100 s was achieved using five 20-s periods at intervals of 30 s. The sonicate was centrifuged at 10000 × *g* for 10 min at 4°C.

The resultant supernatant was fractionated with ammonium sulfate. The precipitate formed between 30 and 60% saturation was dissolved in 40 ml TEG (50 mM Tris-HCl/2 mM EDTA/2 mM DTT/20% glycerol; pH 8.0) and dialyzed three times (3 h each) vs 100 vols TEG. The dialyzed sample was applied to a Q-Sepharose (Pharmacia) column (26 × 300 mm) equilibrated with TEG, elution being conducted with a linear KCl gradient in TEG. Enzyme-containing fractions (eluates at 0.25–0.35 M KCl) were combined, concentrated to 20 ml by ultrafiltration (PM10, Amicon), and then dialyzed vs KP-2 buffer (10 mM potassium phosphate/2 mM DTT/20% glycerol; pH 7.6). The dialyzed material was applied to a Blue Sepharose CL-6B (Pharmacia) column (16 × 500 mm), equilibrated with KP-2 buffer, and eluted isocratically. The pooled enzyme fractions (elution volume 75–120 ml) were next subjected to chromatography on a hydroxyapatite (8010G, Mitsui Toatsu) column (8 × 100 mm) equilibrated with KP-2. When carrying out elution with a potassium phosphate gradient from 10 mM to 0.15 M, the enzyme was eluted at about 0.1 M. For further purification, the enzyme was diluted 4-fold with TEA (25 mM triethanolamine acetate/2 mM EDTA/2 mM DTT/30% glycerol; pH 7.8) and applied to a MonoQ HR 5/5 (Pharmacia) column (5 × 50 mm) equilibrated with TEA as in [7]. Upon elution with a sodium acetate gradient, the enzyme was obtained at 0.3–0.33 M and used for NH_2-terminal sequence analysis.

2.4. *Enzyme assays*

The standard mixture for 5LO assay (total volume, 100 μl) contained 0.1 M Tris-HCl buffer (pH 7.5), 2 mM $CaCl_2$, 2 mM ATP, 16 μg sonicated phosphatidylcholine, and enzyme. After preincubation (30°C, 2 min), the reaction (30°C, 10 min) was initiated by addition of 8 nmol arachidonic acid and 20 μM nonenzymatically generated mixed HPETEs prepared as described [7] and terminated by addition of 0.3 ml cold (−20°C) methanol containing 0.2 nmol of 13-hydroxylinoleic acid as an internal standard and 1 μl of 1 N acetic acid. Following centrifugation, the supernatant was analyzed by HPLC on a Capcellpak C18 column (4.6 × 150 mm, Shiseido) using methanol/water/acetic acid (75:25:0.01, v/v) as solvent, with

detection of the absorbance being at 233 nm. 5LO activity was determined from the peak area ratio of 5-HPETE to the internal standard. The assay mixture for LTA_4 synthase activity was the same as above, the reaction being stopped by adding 2 ml cold ethanol containing 1 nmol prostaglandin B_1 (PGB_1). The mixture was then processed via the Sep-Pak procedure [1], and analyzed by HPLC on a Nucleosil 3C18 (Chemco) column (4.6 × 100 mm) using methanol/acetonitrile/water/acetic acid/triethanolamine, pH 5.6 (0.5:0.8:1.2:0.025:0.028, v/v) as solvent. The flow rate was 1.0 ml/min, with detection of absorbance at 270 nm. LTA_4 synthase activity was determined from the peak area ratio of LTB_4 isomers to PGB_1.

2.5. *Other methods*

The oligonucleotides were synthesized in an Applied Biosystems 381A DNA synthesizer. DNA sequencing was carried out via the dideoxy chain-termination method [15]. The NH_2-terminal amino acid sequence was determined in an Applied Biosystems 477A liquid-phase protein sequencer. SDS-PAGE was performed according to Laemmli [16]. Protein was evaluated as described by Bradford [17] with bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

The expression vector pKC was constructed from pKK223-3 by replacing its replication origin with that of pUC18, which displays a temperature-dependent copy number phenotype [18]. Then human 5LO cDNA was placed 10 bases downstream from the Shine-Dalgarno sequence of pKC to obtain the final expression plasmid pH5LOKC (fig.1).

When grown at 37°C, *E. coli* transformed with pH5LOKC expressed an SDS-PAGE-detectable protein of 78 kDa, which was recovered in the insoluble fraction upon centrifugation of the sonicated cell suspension. The amount of this protein, presumably 5LO, increased time-dependently after IPTG induction (not shown). Light microscopy indicated the presence of inclusion bodies in these cells, suggesting that 5LO synthesized under these conditions was encapsulated into the inclusion bodies. The insoluble fraction including the inclusion bodies was solubilized with 6 M guanidine hydrochloride and then subjected to a protein renaturation procedure [19], however no 5LO activity could be detected. Nevertheless, when the copy number of the expression plasmid was decreased by growing transformed cells at 20°C for 15 h after IPTG induction, the soluble fraction showed 5LO activity and upon SDS-PAGE displayed a protein band of 78 kDa (fig.2, lane 1).

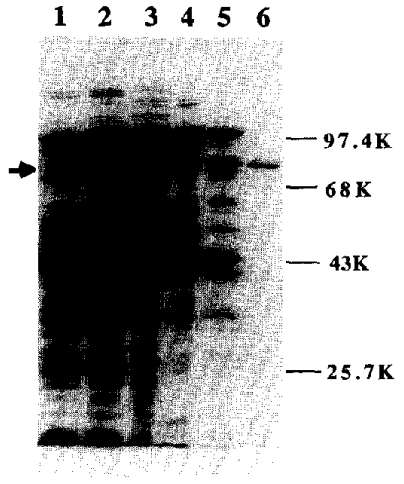

Fig.2. SDS-PAGE of fractions obtained from the purification of 5LO. Samples were loaded onto an SDS-10% polyacrylamide gel, electrophoresed and stained with Coomassie brilliant blue R250. Lanes: (1) 10000 × *g* supernatant, (2) 30–60% ammonium sulfate precipitate, (3) Q-Sepharose, (4) Blue Sepharose, (5) hydroxyapatite, (6) MonoQ HR 5/5.

An attempt was therefore made to purify 5LO from the soluble fraction of the cells grown at 20°C as described in section 2.3, with progress of purification being monitored by SDS-PAGE (fig.2, lanes 2–6). As can be seen, the 78 kDa protein was finally purified to homogeneity by ammonium sulfate fractionation followed by chromatography on Q-Sepharose, Blue Sepharose, hydroxyapatite and Mono Q columns. 5LO activity was thereby purified about 390-fold with an overall yield of 4.4%. About 160 μg purified 5LO was obtained from 1.8 l culture. The yield of 5LO from human peripheral blood leukocytes has been reported to be only 1.5% [7].

As shown in fig.3, the 5LO activity of the enzyme purified from transformed *E. coli* cells showed similar requirements for Ca^{2+} and ATP to those reported for human leukocyte 5LO activity [1,7]. Like the enzyme from human leukocytes [10], 5LO purified in this study was also activated by sonicated phosphatidylcholine (not shown). The purified enzyme also showed LTA_4 synthase activity in the presence of both Ca^{2+} and ATP (not shown). The synthesis of LTA_4 was estimated from the sum of the areas of peaks of 6*E*,5(*S*),12(*R*)-LTB_4 (retention time, 11.0 min) and 6*E*,5(*S*),12(*S*)-LTB_4 (retention time, 11.7 min) divided by

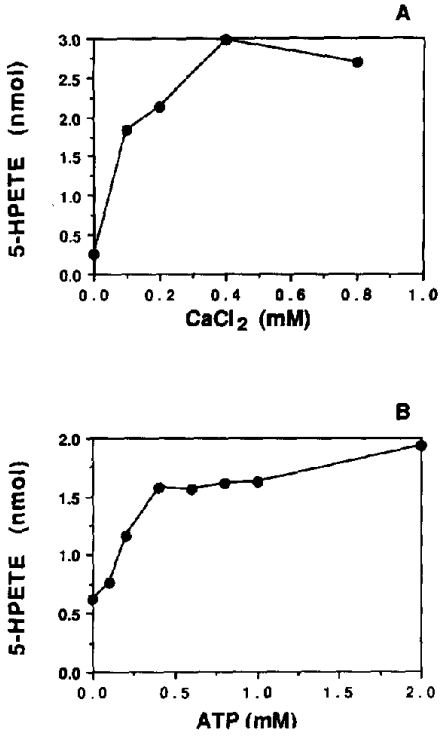

Fig.3. Effects of Ca^{2+} and ATP concentration on 5LO activities. (A) 1 μg purified 5LO was assayed for the activity of conversion to 5-HPETE in the presence of 2 mM ATP and the indicated concentration of Ca^{2+}. (B) Conditions as for A except that samples contained 2 mM Ca^{2+} and the ATP concentration was varied as indicated.

that of the PGB_1 peak (retention time, 9.2 min). Assuming that the two LTB_4 isomers have a molar extinction coefficient of 51 000 at 270 nm [20], it was calculated that conversion of arachidonic acid to 5-HPETE and of the latter to LTA_4 amounted to 30 and 35%, respectively. No proteinaceous stimulatory factors were required for both 5LO and LTA_4 synthase activities.

NH_2-terminal sequence analysis showed that the first 29 amino acid residues of the enzyme purified here were in complete agreement with those of purified human leukocyte 5LO [11]. Since this sequence started with proline, but not methionine, it was concluded that the processing of the initiator methionine residue had taken place in the *E. coli* system as in the leukocyte system.

The observations described above indicate that the 5LO purified from transformed *E. coli* cells was identical with that obtained from human leukocytes. This expression system thus makes it possible to produce human 5LO in sufficiently large quantities for detailed structural studies and development of anti-inflammatory chemotherapeutic agents.

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