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

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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 A₄ synthase activities, which were stimulated by Ca²⁺ and ATP. Its molecular mass (78 kDa) and NH₂-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 A₄; 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 A₄ (LTA₄) from 5-HPETE [1-4]. LTA₄ is further metabolized to LTB₄, peptidoleukotrienes, LTC₄, LTD₄ and LTE₄, 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 Ca²⁺ 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*.

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, AplL5 (insert, 2.6 kbp) contained a 2073 bp open reading frame, in which two tandem 51 bp repeating units were found. AplL6 (2.2 kbp) and AplL7 (1.6 kbp) lacked one of the repeating units. As discussed in [11], the repeat in AplL5 was concluded to be due to a cloning artifact. A 2.3 kbp cDNA was cut out of AplL5 with *Bgl*II and *Eco*RI and ligated to pUC18 that had been digested with *Eco*RI and *Bam*HI. This construct, pHSLOU1, contained the tandem repeat between two *Xma*I sites (434 bp). AplL7 was digested with *Xma*I and the resulting...
The construction of the expression plasmid is outlined in fig.1. 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 a 3'-noncoding region of human SLO cDNA. After deletion of the 5'-noncoding region downstream from the EcoRI site of pH5LOU2 by using an oligonucleotide-directed in vitro mutagenesis system, the modified SLO DNA was cut out with EcoRI and HindIII from pH5LOU2. The EcoRI-HindIII fragment was ligated into the vector pKC which was constructed from pKK223-3 by replacing the replication origin with that of pUC18. The resulting plasmid pH5LOKC was used for expression of the human SLO gene in E. coli.

2.3. Purification of SLO 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 x 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 Bioronic 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 100,000 x 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% NaCl/2% glycerol; pH 7.8) and applied to a MonoQ HR 5/5 (Pharmacia) column (5 x 50 mm) equilibrated with TEG as in [7]. Upon elution with a potassium phosphate gradient from 10 mM to 0.25-0.35 M KCl, the enzyme was obtained at 0.3-0.33 M and used for NH2-terminal sequence analysis.

2.4. Enzyme assays

The standard mixture for SLO assay (total volume, 100 µl) contained 0.1 M Tris-HCl buffer (pH 7.5), 2 mM CaCl2, 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 x 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 LTA₄ synthase activity was the same as above, the reaction being stopped by adding 2 ml cold ethanol containing 1 nmol prostaglandin B₃ (PGB₃). 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 (0.5:0.8:1.2:0.025:0.025, v/v) as solvent. The flow rate was 1.0 ml/min, with detection of absorbance at 270 nm. LTA₄ synthase activity was determined from the peak area ratio of LTB₄ isomers to PGB₃.

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 NH₂-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).

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 Ca²⁺ 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 LTA₄ synthase activity in the presence of both Ca²⁺ and ATP (not shown). The synthesis of LTA₄ was estimated from the sum of the areas of peaks of 6E,5(S),12(R)-LTB₄ (retention time, 11.0 min) and 6E,5(S),12(S)-LTB₄ (retention time, 11.7 min) divided by 269.
that of the PGB₁ peak (retention time, 9.2 min). Assuming that the two LTB₄ 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 LTA₄ amounted to 30 and 35%, respectively. No proteinaceous stimulatory factors were required for both 5LO and LTA₄ synthase activities.

NH₂-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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REFERENCES