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Expression of human leukotriene A_4 hydrolase cDNA in *Escherichia coli*

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The cDNA clone encoding human leukotriene A_4 hydrolase was inserted into a vector pUC9 and expressed in *Escherichia coli* as a fusion protein containing the first 10 amino acid residues derived from a vector. The leukotriene A_4 hydrolase activity was recovered in the soluble fraction of the transformants. The purified enzyme showed kinetic properties similar to the native enzyme, including inactivation by the substrate and sulfhydryl-modifying reagents. The results demonstrate that a protein with an M_r of 70000 was expressed in *Escherichia coli* with a full enzyme activity and structural fidelity. Acquisition of the expression system makes it feasible to elucidate the reaction mechanism of the enzyme.

Leukotriene A_4 hydrolase; Leukotriene B_4; cDNA; DNA expression; (*E. coli*)

1. INTRODUCTION

Leukotrienes are a group of bioactive compounds involved in inflammation and hypersensitivity reactions [1,2]. An allylic epoxide intermediate, LTA_4, is synthesized from arachidonic acid by the action of 5-lipoxygenase [3–8]. LTA_4 hydrolase which synthesizes a chemotactic compound, LTB_4, was purified from various sources [9–12]. The enzyme is inactivated by its substrate (suicide-type inactivation) and also by SH-modifying reagents [12]. Recently, a cDNA for LTA_4 hydrolase was cloned and characterized from human spleen and placental libraries [13,14]. The mature enzyme consists of 610 amino acids, and the calculated molecular mass is 69153 Da [13,14]. To elucidate the reaction mechanism of the enzyme, the cDNA for LTA_4 hydrolase was expressed in *Escherichia coli*. The protein product possessed a full enzyme activity and various properties identical to the native enzyme.

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Abbreviations: LT, leukotriene; LTA_4, (5*S*)-*trans*-5,6-oxide-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTB_4, (5*S*,12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; bp, base pair; IPTG, isopropyl-1-thio-β-D-galactoside; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes were purchased from Takara Shuzo Co. (Kyoto). The Klenow fragment of DNA polymerase I, and an expression vector pUC9 were obtained from Amersham. *E. coli* YA 21 was a kind gift of Dr H. Furukawa (Sankyo Co., Ltd, Tokyo). Other reagents were of the highest grade available.

2.2. *Construction of the vector for expression*

The expression plasmids were constructed from cDNA recombinant clones pLTA851 and pLTA858 as described in fig.1. The cDNA insert of pLTA851, which corresponds to the N-terminal 350 amino acid residues of human LTA_4 hydrolase [13], was cut out with *Ava*I and *Eco*RI, and then ligated to a pUC9 plasmid vector digested with both *Hin*cII and *Eco*RI to obtain a clone (referred to as pEX851) according to the method of Maniatis et al. [15]. pLTA858, a clone encoding the residual C-terminal region of the enzyme (351–610 residues) [13], was digested with *Eco*RI, and the resulting 827 bp-fragment was in-

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serted into pEX851 digested with *Eco*RI to obtain an expression plasmid referred to as pEX85 (fig.1).

2.3. *Purification of LTA_4 hydrolase expressed in* E. coli

pEX85 was used to transform *E. coli* YA 21, by the method of Hanahan [16]. The transformant was cultured in the M9 medium containing 0.2% casamino acid and 75 μg/ml ampicillin at 37°C for 1 h. Then, IPTG was added to a final concentration of 1 mM, and the incubation was continued for another 2 h. Cells were collected by centrifugation (10000 × *g*, 10 min) and suspended in buffer A (50 mM Tris-HCl, 5 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml leupeptin, pH 8). The suspension was sonicated three times at output control 3 for 1 min with a Branson sonifier model 185. To the supernatant (centrifugation at 10000 × *g*, 15 min), solid ammonium sulfate was added. The precipitate formed between 40 and 70% saturation was dissolved in buffer A and dialyzed three times against 250 volumes each of the same buffer. The dialyzed sample was purified by Mono-Q and Mono-P column chromatographies (Pharmacia, Uppsala), as described [13].

2.4. *N-terminal sequence analysis*

Active fractions from a Mono-P column were applied to a Phenyl-5PW column (0.75 × 5 cm, Tosoh, Tokyo), and eluted with a linear gradient consisting of 20 ml each of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile. The N-terminal sequence of the expressed product was determined using a gas-phase amino acid sequencer model 470A (Applied Biosystems).

2.5. *Miscellaneous*

The assay of LTA_4 hydrolase and the identification of the reaction product were carried out as described in [12]. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [17]. The protein concentration was determined by the method of Lowry et al. [18] with bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

E. coli YA 21 transformed with pEX85 overexpressed a protein with an M_r of 70000 (hereafter referred to as EX85). As shown in fig.2, EX85 was recovered in the supernatant obtained by centrifugation (10000 × *g*, 10 min) of the sonicated suspension of the transformant (lane 2). The amount of EX85 in the supernatant increased up to 2 h after IPTG induction, but thereafter remained constant, while EX85 in the precipitate increased time-dependently, as assessed by SDS-polyacrylamide gel electrophoresis (not shown). This finding suggests that LTA_4 hydrolase is relatively resistant to proteolytic digestion or encapsulation to the inclusion bodies of the bacteria, for at least 2 h. Since the supernatant possessed the LTA_4 hydrolase activity, it was further purified according to the methods described in section 2. From a 3 l culture, 3 mg of EX85 was obtained using Mono-Q and Mono-P column chromatographies (lanes 3 and 4). The expressed protein (EX85) has a similar electrophoretic mobility to the native enzyme from human lung (lane 5). Overall purification was about 100-fold with a yield of 120–250%. A prominent increase in the total activity (2–5-fold) was observed after the ammonium sulfate fractionation. This might be due either to the removal of inhibitory factor(s) present

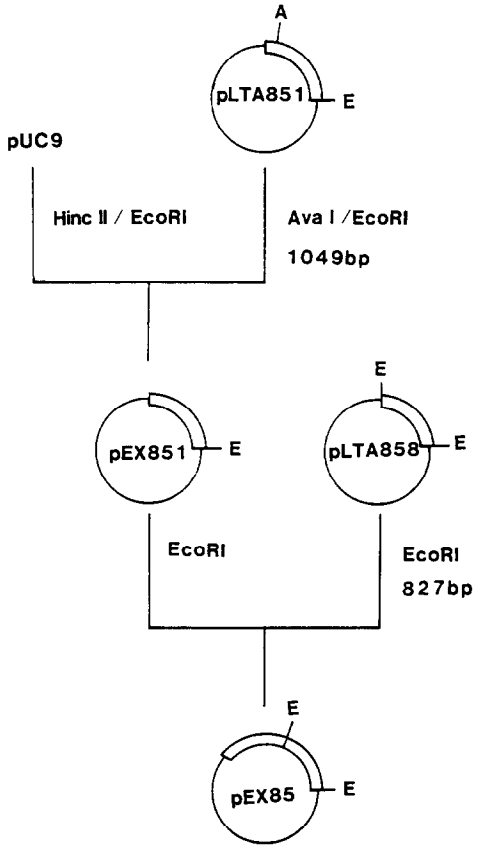

Fig.1. Construction of plasmid pEX85 for the synthesis of LTA_4 hydrolase in *E. coli*. pLTA851, a clone encoding the N-terminal 350 amino acid residues of human LTA_4 hydrolase [13] was cut out with *Eco*RI after being digested with *Ava*I and filled-in with the Klenow fragment. The *Eco*RI fragment was ligated to a pUC9 plasmid vector digested with both *Hinc*II and *Eco*RI to obtain pEX851. pLTA858, a clone encoding the residual C-terminal region of the enzyme (351–610 residues) [13], was digested with *Eco*RI, and the resulting 827 bp fragment was inserted into the *Eco*RI-digested pEX851 to obtain pEX85. A and E are *Ava*I and *Eco*RI sites abbreviated, respectively.

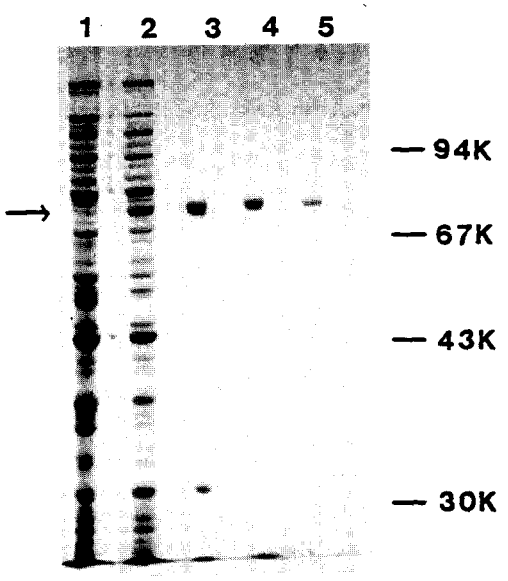

Fig.2. SDS-polyacrylamide gel electrophoresis of the fusion protein EX85. Protein preparations obtained from *E. coli*, transformed with a pUC9 or pEX85 (lanes 1 and 2, respectively); Mono-Q column chromatography (lane 3); Mono-P column chromatography (lane 4); and the purified LTA_4 hydrolase from human lung (lane 5). The arrow indicates the position of EX85.

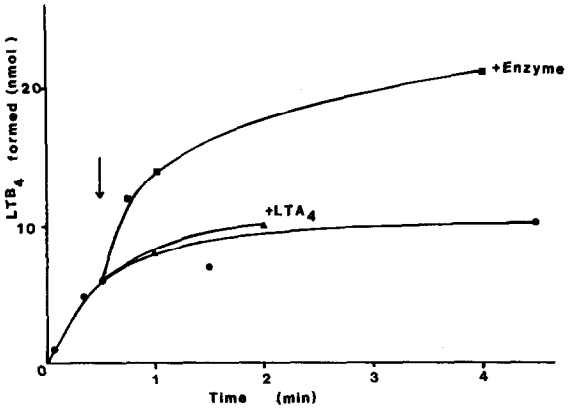

Fig.3. The time course of the enzymic hydration of LTA_4 by the fusion protein EX85. EX85 (2 μg/10 μl) was incubated with LTA_4 (20 μM) in 50 μl of 0.1 M Tris-HCl (pH 7.8) containing 2 mg/ml bovine serum albumin at 37°C for 5 min (●—●). After the 30 s incubation (indicated by the arrow), EX85 (■) or LTA_4 (▲) was further added to the reaction mixture.

in *E. coli* extracts or to a conformational adjustment during this step. Human LTA_4 hydrolase has been purified from neutrophils [9] and lung [12] by 4 steps of column chromatography with yields of 12% and 10%, respectively [9,12]. Therefore, LTA_4 hydrolase is more efficiently obtained with a much higher yield from the transformant. The N-terminal sequence analysis of EX85 revealed that it contained 10 amino acids derived from pUC9 (Thr-Met-Ile-Thr-Pro-Ser-Leu-Ala-Ala-Gly), followed by the N-terminal sequence (Pro-Glu-Ile-Val-Asp-Thr) reported for the enzymes from human neutrophils and lung [9,12].

The K_m value of EX85 for LTA_4 was 27 μM, and the V_{max} value was 4.3 μmol LTB_4/min·mg. These values are comparable with those of human neutrophils [9], lung [12] and rat neutrophils [10]. Fig.3 shows the time course of the LTB_4 synthesis by the purified enzyme (EX85). The reaction almost reached a plateau in 30 s. The rapid deceleration of the enzyme reaction was not due to the depletion of substrate LTA_4, since the initial velocity of the reaction was recovered by the addition of the enzyme, and not of the substrate. This phenomenon is known as the suicide-type inactivation of LTA_4 hydrolase, caused by its substrate, LTA_4 [11,12].

SH-modifying reagents, NEM, PCMB and $HgCl_2$, inhibited the activity of LTA_4 hydrolase. The IC_{50} values of NEM, PCMB and $HgCl_2$ were 1 mM, 0.02 μM and 0.026 μM, respectively. Inhibition by those SH-modifying reagents has already been noted for the native LTA_4 hydrolase purified from human lung [12].

All these results provide direct evidence that clone LTA85 [13] encodes human LTA_4 hydrolase. It was also demonstrated that a protein with an M_r of 70000 was expressed in *E. coli* with full enzyme activity and high structural fidelity to the native enzyme. The acquisition of the expression system makes it feasible to elucidate the reaction mechanism of the enzyme, and to synthesize a substantial amount of LTB_4, a biologically active compound.

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