Low temperature cultivation of *Escherichia coli* carrying a rice lipoxygenase L-2 cDNA produces a soluble and active enzyme at a high level

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Using at T7 RNA polymerase promoter system, rice lipoxygenase L-2 cDNA was expressed in *E. coli* as a fusion protein with 18 amino acid residues at the amino terminal end of the original enzyme. Incubation at 37°C for 3 h in the presence of the inducer resulted in the production of inactive lipoxygenase. However, when induction was carried out at 15°C for 16 h, active lipoxygenase, amounting to 3% of the total soluble protein, was produced. The enzyme was purified by ammonium sulfate precipitation and Mono-Q column chromatography to homogeneity at a yield of 80%. Expression of this protein should permit future site-directed mutagenesis of the gene and crystallization of the enzyme.

Lipoxygenase; Inclusion body; Low temperature cultivation of *E. coli*; T7 RNA polymerase promoter

1. INTRODUCTION

Lipoxygenases catalyze the incorporation of molecular oxygen into fatty acids containing a *cis,cis*-pentadiene moiety, to form hydroperoxides. The discovery of highly potent physiological effectors formed via lipoxygenase-catalyzed reactions in mammalian tissues have attracted considerable attention [1].

Since the complete primary structure of soybean lipoxygenase L-1 was determined [2], other full-length cDNAs have been isolated from plants [3–5] and animals [6–9], resulting in the identification of a conserved coding region among lipoxygenase genes. As mentioned by Shibata et al. [3], a histidine cluster found in the conserved region could be an iron-binding site of lipoxygenase. Funk et al. [10] reported expression of human 5-lipoxygenase in a baculovirus/insect cell culture system and also showed that site-directed mutation of two histidine residues within the putative iron-binding domain did not alter the enzyme activity. Therefore, we have undertaken further investigation in order to determine the role of the conserved amino acid residues.

We have cloned a full-length cDNA coding for lipoxygenase L-2 from a library made from rice seedlings germinated for 3 days [11]. Here we report high level expression of an active form of the enzyme in *E. coli*.

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

2.1. *Expression in* E. coli

A rice lipoxygenase L-2 cDNA clone (pRLC11) [11] was digested by both *Ase*I and *Eco*RI, and then filled in using Klenow enzyme. The fragment was inserted into a pET3a expression vector [12] at a filled-in *Bam*HI site. The plasmid DNA carrying the rice gene in the appropriate orientation was designated pET3a/RLOX2 and purified from *E. coli* HB101. pET3a/RLOX2 was used to transform *E. coli* BL21(DE3), which carries the gene for T7 RNA polymerase under control of the *lac* UV5 promoter on its chromosome [13]. The cells were grown at 37°C in 50 ml of LB medium containing 50 μg/ml of ampicillin to a cell density of A_{600} = 1.0. The culture was incubated in the presence of 0.4 mM IPTG at 37°C for 3 h or at 15°C for 16 h. The cells were collected, washed with 50 ml of water, suspended in 25 ml of 50 mM Tris-HCl, 10% glycerol, 0.1% Tween 20, 1 mM EDTA, 0.5 M NaCl (pH 7.5), to stabilize the rice lipoxygenase L-2 [13] and then disrupted using a French press. The particulate material was removed by centrifugation (5000 × g for 15 min). Cells cultivated at 37°C were collected and washed with 20 ml of 1% Triton X-100, 1 mM EDTA in order to isolate inclusion bodies [14]. Ammonium sulfate (9.4 g in 20 ml) was added to the supernatant of the disrupted cells. The precipitate was dissolved in 2 ml of 10 mM sodium phosphate (pH 6.8), 10% glycerol, 0.1% Tween 20, 0.1 mM EDTA (buffer A). Ammonium sulfate was removed using a PD-10 column (Pharmacia) with buffer A. 10% of the ammonium sulfate-free solution (a 0.4-ml aliquot) was applied to a Mono-Q column (Pharmacia) equilibrated with buffer A. Lipoxygenase was eluted with a NaCl gradient of 40 mM to 60 mM in buffer A using an FPLC system (Pharmacia). The amount of protein was estimated by the Bradford method using bovine plasma gamma globulin as a standard [15].

2.2. *Assay of lipoxygenase activity*

The activity of lipoxygenase was determined using a Clark oxygen electrode [16]. The reaction mixture contained 7.5 mM linoleic acid and 0.25% (w/v) Tween 20 in 0.1 M piperazine-*N,N'*-bis(ethane)sulfonic acid (Pipes) (pH 6.0). Rice lipoxygenase L-2 showed anomalous rate dependence on enzyme concentration [11]. Therefore, 1 unit of activity was defined arbitrarily as 10-times the quantity of enzyme catalyzing the consumption of 0.10 μmol of O_2 per min at 30°C.

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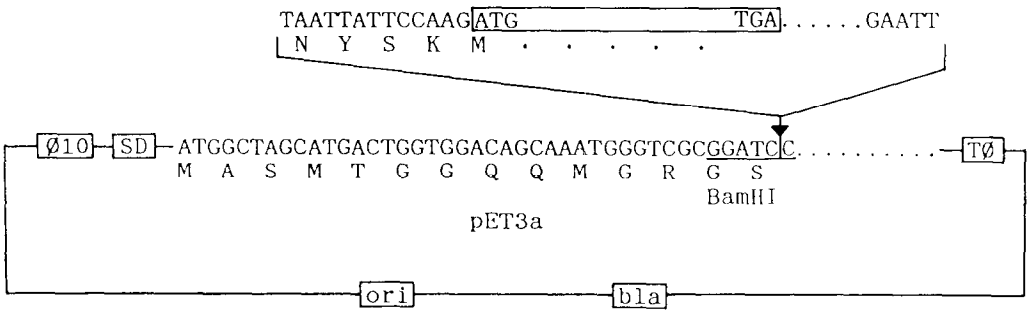

Fig. 1. Construction of the expression plasmid, pET3a/RLOX2. A rice lipoxygenase cDNA fragment (see section 2, the upper strand in this figure) was ligated with the gene *10* translation start site (s*10*) at the *Bam*HI site of pET3a, which is located between the Shine-Dalgarno sequence (SD) downstream from the T7 RNA polymerase promoter (ϕ*10*) and the transcription terminator (Tϕ). The locations of the coding region of the lipoxygenase L-2 gene (boxed), the origin of replication (*ori*), the *bla* of pBR322 are indicated.

2.3. *SDS polyacrylamide gel electrophoresis*

Proteins were separated on a 10% SDS polyacrylamide gel and stained with Coomassie blue R250 or blotted on a nitrocellulose filter using a semi-dry blotting apparatus (Biometra). The amount of protein on the Coomassie stained gel was estimated using a densitometer (Shimadzu CS-9000). The blotted filter was incubated with a rabbit antibody against rice lipoxygenase L-3 which crossreacts with the L-2 isozyme. The band was visualized using an immunoscreening kit, picoBlue (Stratagene).

3. RESULTS

To construct pET3a/RLOX2, we inserted the rice lipoxygenase L-2 gene into the vector such that transcription of the gene produced the following fusion protein. The lipoxygenase L-2 protein was attached to the carboxy terminus of a short peptide which consisted of 18 amino acid residues. The first 14 residues of the peptide were produced by the gene *10* translation start site of the vector [12] and the last 4 residues were encoded by a region 13 nucleotides upstream of the first methionine in the rice gene (Fig. 1).

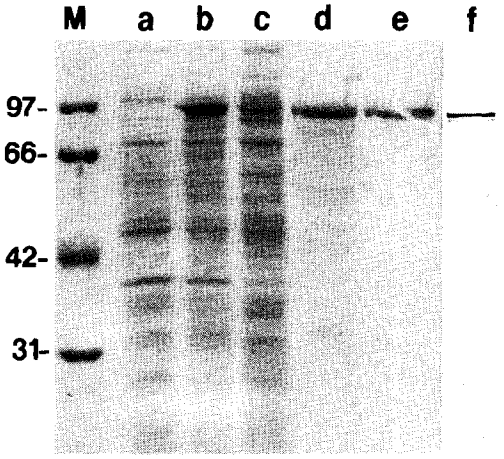

Fig. 2. Expression of the cloned rice lipoxygenase gene in *E. coli*. Gel electrophoresis of the crude extracts from cells carrying pET3a (control) (a) and pET3a/RLOX2 (b, c) cultured in the presence of IPTG at 37°C (a, b) or 15°C (c). Purified lipoxygenase from inclusion bodies (d), purified active enzyme (e) and Western blot of lane c (f) are also shown. Protein in lanes a, b and c, 7 μg; lanes d and e, 2 μg; lane f, 140 ng.

E. coli cells containing pET3a/RLOX2 which were grown at 37°C expressed the fusion product as 27% of the total protein, estimated by Western blot analysis (Fig. 1b). However, no lipoxygenase activity was detected in the supernatant of these cells after disruption. The expressed protein was recovered from the particulate fraction of the disrupted cells (inclusion bodies) in an insoluble form and was easily purified by washing this fraction with a Triton X-100 solution (Fig. 2d). The active enzyme was found in the supernatant of disrupted cells which had been cultivated at 15°C for 16 h in the presence of IPTG (11.9 units/ml cultured cells). This enzyme was purified to homogeneity (Fig. 2e, Table I), and was stable under the conditions described here. From the specific activity of the purified enzyme (450.7 units/mg protein) the active enzyme was estimated to be 3% of the soluble protein in the supernatant of the disrupted cells.

4. DISCUSSION

An active form of lipoxygenase has been expressed in *E. coli*, which should be useful for investigation of the enzyme by site-directed mutagenesis. High level expres-

Table I

Purification of expressed rice lipoxygenase from 50 ml of *E. coli* cell culture medium incubated at 15°C

	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	56.5	720.0	12.7	100
Ammonium sulfate fractionation	30.5	437.0	14.3	61
Mono-Q chromatography[a]	1.48	595	402.0	82

[a] 1/10 of the ammonium sulfate fraction was chromatographed. Values are adjusted to correspond to the total ammonium sulfate fraction.

sion of foreign genes in bacterial has often resulted in the production of inactive expression products which are found in inclusion bodies. In this work, low temperature incubation of the transformed cells prevented the formation of inclusion bodies and resulted in a high yield of the protein product in soluble form. Similar temperature dependence has been reported for the solubility of several other proteins (see [17] for review).

The extra 18 amino acid residues found at the amino terminus of the fusion protein (Fig. 1) did not alter the pH optimum, heat stability or regiospecificity (position of oxygen insertion into substrate) of rice lipoxygenase L-2 [11]. Therefore, this expression system may be useful for characterization of enzyme products produced by site-directed mutagenesis of the rice lipoxygenase L-2 gene.

The high level of expression of lipoxygenase in both active and inactive forms in *E. coli* may be related to the narrow codon usage of the rice gene which shows strong preference to G or C at the third codon position [11]. Most of the codons used in the gene were included in those which are frequently used by *E. coli* [18].

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