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Cloning and sequencing of a gene encoding of aldehyde oxidase in *Pseudomonas* sp. AIU 362

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We have cloned a gene encoding an aldehyde oxidase (ALOD) oxidized glyoxal but not glyoxylic acid from *Pseudomonas* sp. AIU 362. The ALOD gene contained an open reading frame consisting of 888 nucleotides corresponding to 295 amino acid residues. The deduced amino acid sequence exhibited a high similarity to those of 3-hydroxyisobutyrate dehydrogenases (3-HIBDHs). We expressed the cloned gene as an active product in *Escherichia coli* BL21 cells. The productivity (total units per culture broth volume) of the recombinant ALOD expressed in *E. coli* BL21 was 20,000-fold higher than that of ALOD in *Pseudomonas* sp. AIU 362. The recombinant ALOD exhibited ALOD activity and 3-HIBDH activity. The 3-HIBDH from *Pseudomonas putida* KT2440 also exhibited ALOD activity. Thus, the ALOD from *Pseudomonas* sp. AIU 362 and 3-HIBDH from *P. putida* KT2440 were classified into the same enzyme group.

[**Key words:** Aldehyde oxidase; 3-Hydroxyisobutyrate dehydrogenase; Glyoxal; Glyoxylic acid; *Pseudomonas*]

Recently, we isolated *Pseudomonas* sp. AIU 362 as a producer of an aldehyde oxidase (ALOD) using a methoxyethanol medium (1). This enzyme oxidized not only glyoxal but also short-chain aliphatic aldehydes and aromatic aldehydes. Thus, the enzyme was classified into the ALOD group. However, the enzyme was composed of four identical subunits, whereas other microbial ALODs are composed of three hetero subunits (2–5), and ALODs from plant and animals are composed of two identical subunits (6,7). The N-terminal sequence of the ALOD from *Pseudomonas* sp. AIU 362 also showed no similarity to that of glyoxal oxidase from *Phanerochaete chrysosporium* (8). We, therefore, concluded in our previous report that the ALOD from *Pseudomonas* sp. AIU 362 is a new aldehyde oxidase. Our previous report also demonstrated that the N-terminal sequence of the ALOD from *Pseudomonas* sp. AIU 362 exhibited high similarity to that of 3-hydroxyisobutyrate dehydrogenase (3-HIBDH; EC 1.1.1.31) from *Pseudomonas putida* E23 (9) and *P. putida* KT2440 (10), although it has not been reported that both 3-HIBDHs have the ALOD activity. Therefore, we studied the cloning and sequence analysis of the ALOD gene from *Pseudomonas* sp. AIU 362. The expression of the ALOD gene in *Escherichia coli* and characteristics of the recombinant enzyme are also studied.

MATERIALS AND METHODS

Chemicals Glyoxal, glyoxylic acid sodium salt, L-3-hydroxyisobutyric acid sodium salt, D-3-hydroxyisobutyric acid sodium salt, L-serine and D-serine were purchased from Sigma Aldrich Japan (Tokyo). L-3-hydroxyisobutyric acid methyl ester and D-3-hydroxyisobutyric acid methyl ester were from Tokyokasei (Tokyo). Horseradish peroxidase (EC 1.11.1.7) was obtained from Amano Enzymes (Nagoya). Ni-charged resin was purchased from Bio-Rad Laboratories Japan (Tokyo). All other chemicals used were commercial products of the highest grade available.

Medium and culture conditions *Pseudomonas* sp. AIU 362, which is a source of chromosomal DNA, was incubated in a methoxyethanol medium consisting of 1% 2-methoxyethanol, 0.2% NH_4NO_3, 0.1% K_2HPO_4, 0.1% NaH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% $CaCl_2 \cdot 2H_2O$, and 0.1% yeast extract, pH 5.5, at 30°C for 3 days with shaking at 120 strokes per min.

A transformant carrying the ALOD gene was incubated in 5.0 ml of a Luria–Bertani (LB) medium consisting of 1% peptone, 0.5% yeast extract, and 1% NaCl, pH 7.0, containing ampicillin (50 μg/ml of medium) at 30°C for 5 h, and then isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM) was added to the culture medium and further incubated at 30°C for 20 h with shaking at 120 strokes per min.

Preparation of DNA from *Pseudomonas* sp. AIU 362 Cells from a 100 ml of culture broth were suspended in a 20 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 0.01 M EDTA-Na_2, 25% sucrose and 300 mg lysozyme. To the cell suspension, 2.0 ml of 0.5 M EDTA-Na_2 was added and incubated at 37°C for 4 h with shaking at 20 strokes per min. Then, 2.0 ml of 10% SDS solution containing 20 mg protease K was added and the mixture was incubated at 50°C for 4 h with shaking. The DNA was extracted with phenol/chloroform (50/50, volume/volume)

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and precipitated with 3 M sodium acetate (1/10th volume of the mixture) and ethanol (2 volumes of the mixture). The extracted DNA was rinsed with 80% ethanol.

Amplification of ALOD gene by PCR Since the N-terminal amino acid sequence (30 residues) of ALOD from *Pseudomonas* sp. AIU 362 was identical to that of 3-HIBDH from *P. putida* E23 (9) and *P. putida* KT2440 (10), two primers for PCR, sense primer (S1) 5′-GTTCACCCAGAGCGGCGCGGCA-3′ and antisense primer (A1) 5′-GGCAAAGGGCGTGGACATGGGGGAT-3′, were designed using the DNA sequence data of 3-HIBDH from *P. putida* KT2440 (10) (see Fig. 1). The PCR was carried out in a 50-μl volume consisting of 2.5 units of TaKaRa Ex *Taq* DNA polymerase, 20 nmol of dNTP mixture, 0.5 μmol of each primer, and 0.5 μg of the extracted chromosomal DNA. The reaction mixture was first heated at 94°C for 5 min, followed by 30 cycles of amplification (a denaturation step at 94°C for 30 s, an annealing step at 55°C for 30 s, and extension step at 72°C for 1 min).

Southern hybridization The chromosomal DNA from *Pseudomonas* sp. AIU 362 was digested with *Bam* HI, *Hin*d III, *Nde* I, *Pst* I, *Sac* I, *Sal* I, *Spe* I, *Sph* I or *Xba* I, and the digested DNA was subjected to gel electrophoresis on a 1% agarose gel in TEA buffer at 50 V for 3 h. The DNA fragments on the gel were transferred to a nitrocellulose membrane by capillary blotting, and incubated at 55°C for 24 h with the labeled amplified DNA fragment containing the ALOD gene described above.

Colony hybridization A ligation mixture (10 μl) consisting of 5 μl of Ligation Solution I (DNA Ligation Kit ver. 2.1, Takara Bio), 1 μg of a chromosomal DNA digested with *Sal* I and 100 ng of a cloning vector DNA (pT 7 Blue cloning vector, Novagen) digested with *Sal* I was incubated at 16°C overnight. The resulting plasmids were subsequently transformed into *E. coli* JM 109. The positive clones were selected on a LB-agar plate containing X-gal/IPTG and ampicillin by the blue-white selection, and then the successful clones were identified by colony hybridization with the labeled DNA fragment containing ALOD gene described above.

DNA sequence analysis The DNA sequences were determined by the dideoxy chain termination method using a DNA sequencer CEQ2000 XL (Beckman Coulter Inc., CA, USA) with a CEQ Dye Terminator Cycle Sequencing Kit (Beckman Coulter Inc.). Identification of the open reading frame and analysis of the deduced amino acid sequence were carried out using a GENETYX DNA sequence analysis software.

Expression of the *Pseudomonas* ALOD in *E. coli* On the basis of the sequence obtained from the *Sal* I fragment, two oligonucleotides, S2 (5′-CATATGCGTATCGCATTCATCGGCC-3′) and A2 (5′-GCGGCCGCGTCCTTCTTGCGGTAGC-3′), were designed as a sense primer containing a *Nde* I site in the upstream of initiation codon and an antisense primer containing a *Not* I site in the downstream of stop codon, respectively (see Fig. 1). The ALOD gene was amplified by PCR using these primers and ligated into *Nde* I-*Not* I site of the pET-21a(+) expression vector, containing a histidine-tag site. The pET-21a(+) plasmid containing the ALOD gene (pET-GOOX) was transformed into *E. coli* BL21 cells (*E. coli* BL21/pET-GOOX).

Purification of ALOD expressed in *E. coli* BL21 The recombinant cells (100 mg of wet weight) obtained at 25 h of cultivation from 20 ml of culture broth were suspended in 3 ml of 10 mM potassium phosphate buffer, pH 7.0, and disrupted with glass beads by a Multi-beads shocker (Yasui Kikai, Osaka) for 10 min. The crude enzyme solution (5.0 ml) was applied to a Ni-charged resin column (1.0 × 3.0 cm), equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.3 M NaCl. The adsorbed enzyme was then eluted with 50 mM potassium phosphate buffer, pH 7.0, containing 0.3 M NaCl and 0.25 M imidazole.

Preparation of recombinant 3-HIBDH of *P. putida* KT2440 Protein derived from the deduced 3-HIBDH gene of *P. putida* KT2440 was expressed in a soluble form in *E. coli* JM 109 according to the method of Chowdhury et al. (9). The recombinant 3-HIBDH protein was also purified to a homogeneous state according to the method of Chowdhury et al. (9).

Assay of enzyme activity ALOD activity was assayed by measuring the rate of hydrogen peroxide formation at 30°C according to our previous report (1).

3-HIBDH activity was assayed by measuring the formation rate of NADH or NADPH at 30°C according to the method of Chowdhury et al. (11) as follow. The standard reaction mixture was consisted of 20 μmol of substrate, 4 μmol of NAD^+ or $NADP^+$, 200 μmol of CAPS buffer, pH 10.1, and enzyme in a final volume of 1.0 ml. The reaction was started by addition of NAD^+ or $NADP^+$ and the absorbance at 340 nm was monitored for 5 min.

One unit of the enzyme activity was defined as the amount catalyzing the formation of 1 μmol of H_2O_2, NADH or NADPH per min in the above reaction.

RESULTS

Cloning of the gene encoding ALOD from *Pseudomonas* sp. AIU 362 A DNA fragment of approximately 1.1 kb containing the ALOD gene was obtained by PCR using S1 and A1 primers. Southern hybridization was carried out using the amplified DNA

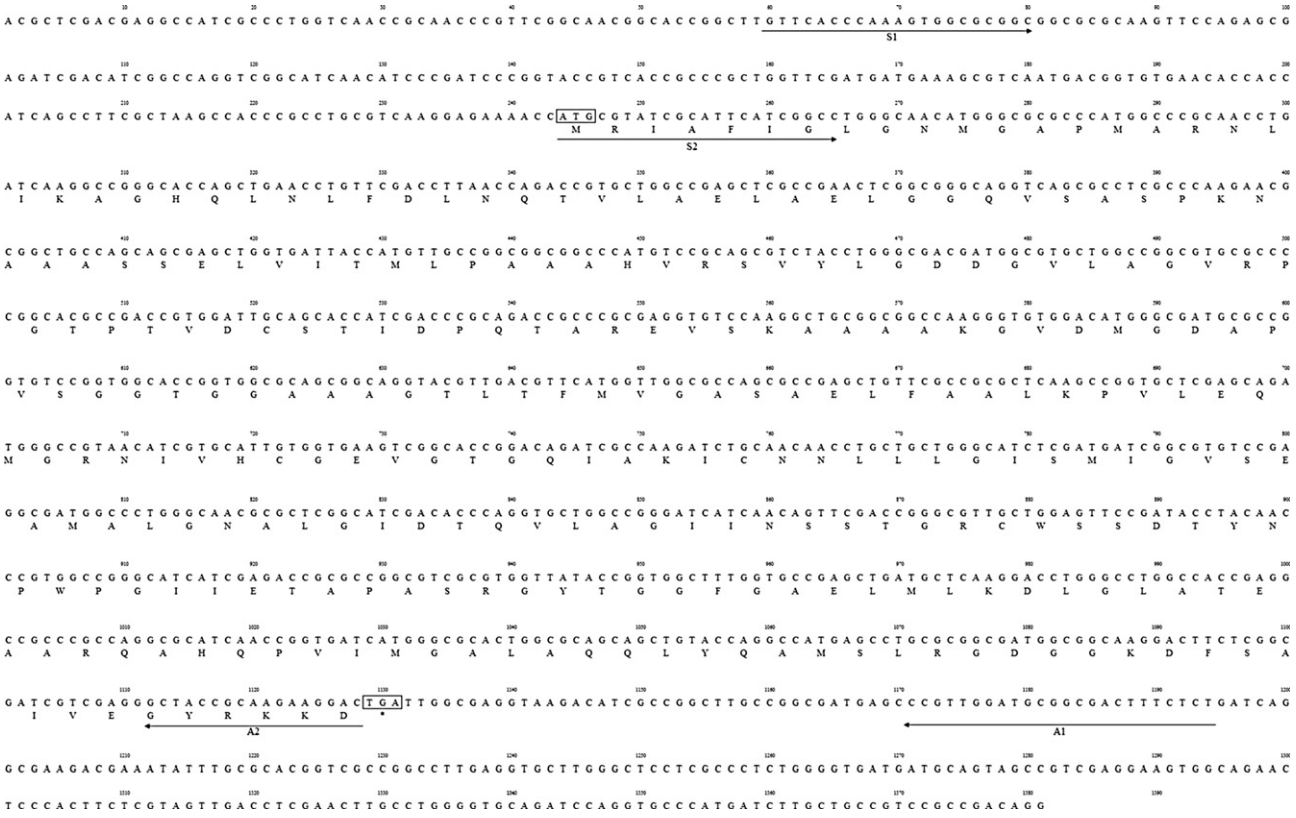

FIG. 1. Nucleotide sequence of a part of the *Sal* I fragment containing the ALOD gene from *Pseudomonas* sp. AIU 362. The initiation and stop codons are indicated by a box. S1 and A1 are the position of primers used for PCR. S2 and A2 are the position of primers used for expression of the *Pseudomonas* ALOD in *E. coli*. The deduced amino acids are also indicated under the DNA sequence.