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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.

fragment as a probe, and it was revealed that the ALOD gene was contained in a DNA fragment digested with *Sal* I. The *Sal* I fragment (about 4.0 kb) from chromosomal DNA was then ligated to a pT 7 blue cloning vector digested with *Sal* I, and the resulting plasmids were transformed into *E. coli* JM 109. The successful clones selected on colony hybridization were used in the following studies.

Sequence analysis of the ALOD gene Fig. 1 shows the nucleotide sequence containing the ALOD gene, in which an ORF of 888 bp encodes a 295 amino acid polypeptide. The predicted molecular mass of the ORF was in agreement with that of a subunit of ALOD from *Pseudomonas* sp. AIU 362. In addition, the deduced amino acid sequence of the N-terminal region of the ORF is identical to the amino acid sequence of the *Pseudomonas* ALOD. We therefore concluded that the ORF of 888 bp is the complete structural gene of ALOD from *Pseudomonas* sp. AIU 362, and the *Sal* I fragment was used in the following studies. The sequence data of 888 nucleotides in the ORF correspond to the ALOD gene in the DDBJ nucleotide sequence databases (accession number AB436915).

The deduced amino acid sequence of the ALOD polypeptide was used to search for homologous sequence using the BLAST (Basic Local Alignment Search Tool) program. Approximately 95% residues of the deduced amino acid sequence of the ALOD from *Pseudomonas* sp. AIU 362 was identical to that of 3-HIBDH from *P. putida* E23 (9) and *P. putida* KT2440 (10) (Fig. 2). The amino acid sequence of the *Pseudomonas* ALOD also exhibited high similarity to that of 3-HIBDH from *Ralstonia eutropha* H16 (12), *Acinetobacter baumannii* (13) and *Pseudomonas aeruginosa* PAO (14), but not to that of microbial ALODs.

Overexpression of the gene encoding ALOD in *E. coli* cells The *E. coli* BL21/pET-GOOX transformant cells, which are *E. coli* BL21 cells harboring a pET-21a(+) plasmid containing the ALOD gene, were incubated in the LB medium as described in Materials and Methods. The ALOD protein was expressed in a soluble histidine-tagged form and 3.6 units of ALOD activity was obtained from the cells in 1 ml of culture broth. This productivity is 20,000 times higher than that of *Pseudomonas* sp. AIU 362 in total units per culture broth volume.

Purification of recombinant ALOD The recombinant ALOD protein was purified to a homogeneous state by a Ni-charged resin column chromatography as described in Materials and Methods (Fig. 3). The purified enzyme solution exhibited no absorbance maximum in visible region (data not shown). The molecular mass of the recombinant enzyme is approximately 27 kDa (deduced by SDS-PAGE), which is in agreement with that of the ALOD protein from *Pseudomonas* sp. AIU 362. In addition, the 30 amino acid residues from the N-terminus were identical to those of ALOD from *Pseudomonas* sp. AIU 362. The purified enzyme exhibited

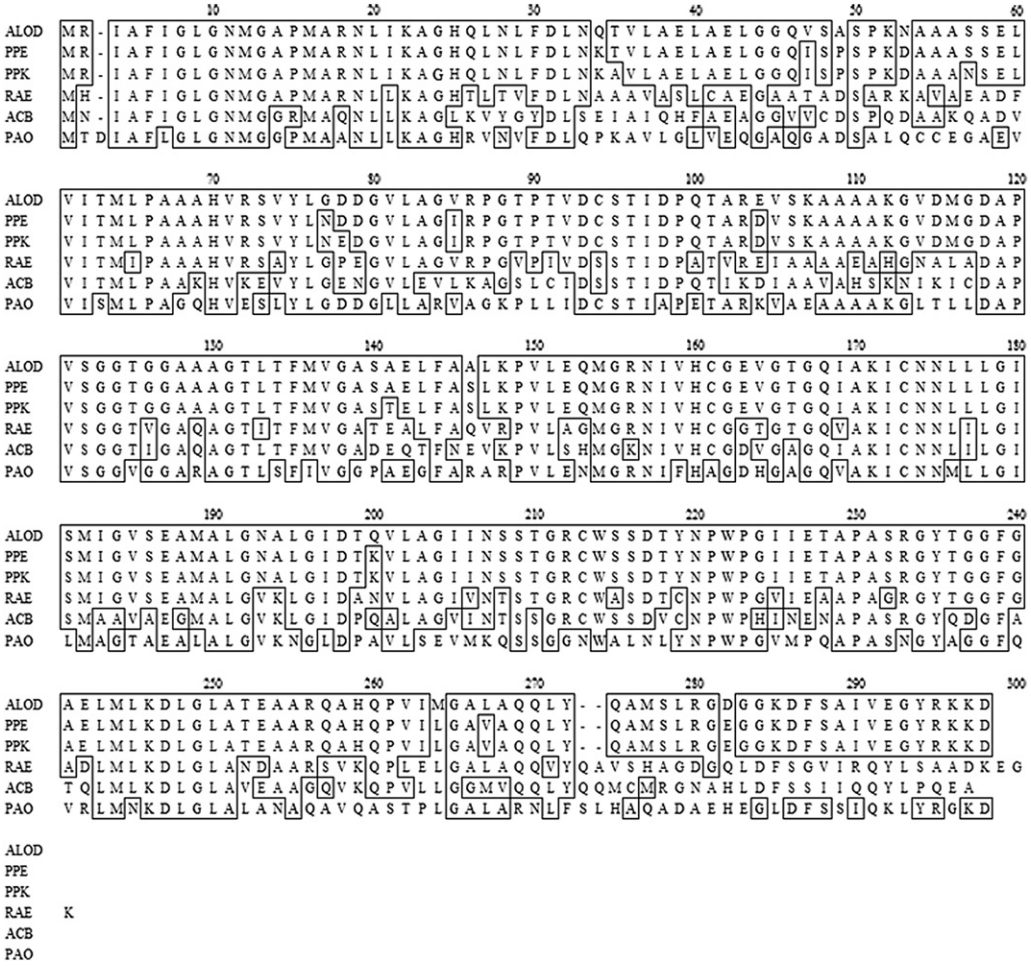

FIG. 2. Alignment of the amino acid sequences of the ALOD protein from *Pseudomonas* sp. AIU 362 and 3-HIBDHs from bacterial strains. The deduced amino acid sequence of ALOD from *Pseudomonas* sp. AIU 362 (ALOD) was compared to the sequences of 3-HIBDH from *Pseudomonas putida* E23 (PPE) (9), *P. putida* KT2440 (PPK) (10), *Ralstonia eutropha* H16 (RAE) (12), *Acinetobacter baumannii* (ACB) (13) and *Pseudomonas aeruginosa* PAO (PAO) (14). Amino acids identical to that of ALOD from *Pseudomonas* sp. AIU 362 are depicted by boxes.

14.5 units of glyoxal oxidase activity per mg of protein, which was also in agreement with the specific activity of the *Pseudomonas* ALOD protein (1).

Substrate specificity of recombinant ALOD The substrate specificity of the recombinant ALOD was analyzed using the purified enzyme. The recombinant enzyme oxidized glyoxal, aliphatic aldehydes and aromatic aldehydes, but did not oxidize glyoxylic acid and alcohols (data not shown). These results of substrate specificity and aldehyde oxidation speed were similar to those of the *Pseudomonas* ALOD protein (1). Then, dehydrogenase activity of the ALOD for 3-hydroxyisobutyric acid, its methyl ester and serine was assayed under standard assay conditions. When NAD^+ was used as a cofactor, highest dehydrogenase activity was obtained with L-3-hydroxyisobutyric acid (948 units per mg of protein), and the K_m values for L-3-hydroxyisobutyric acid and NAD^+ were estimated to be 0.24 mM and 0.10 mM, respectively. This enzyme also exhibited dehydrogenase activity for L-3-hydroxyisobutyric acid methyl ester and L-serine, but not for D-3-hydroxyisobutyric acid, D-3-hydroxyisobutyric acid methyl ester and D-serine. Thus, the dehydrogenase activity was specific to the L-form substrate, and the dehydrogenase activities for the L-form substrates were much higher than glyoxal oxidase activity (the relative value of reaction speed for glyoxal oxidation was 1.5% of that for L-3-hydroxyisobutyric acid dehydrogenation). When $NADP^+$ was used as a cofactor, the dehydrogenase activity for L-3-hydroxyisobutyric acid was also obtained, but the activity was less than 3% of the activity with NAD^+. The dehydrogenase activity for other five compounds tested above was not detected using $NADP^+$ (Table 1). Thus, the dehydrogenase activity was specific to NAD^+

Substrate specificity of 3-HIBDH from *P. putida* KT2440 The ALOD activity of the 3-HIBDH protein from *P. putida* KT2440 was determined under standard assay conditions using 2.11 units of L-3-hydroxyisobutyrate dehydrogenase activity. This enzyme also oxidized glyoxal, aliphatic aldehydes and aromatic aldehydes, but did not oxidize glyoxylic acid and alcohols (Table 2). In addition, the apparent reaction speed for glyoxal oxidation was 1.2% of that for L-3-hydroxyisobutyric acid dehydrogenation. Thus, this 3-HIBDH exhibited similar substrate specificity to the *Pseudomonas* ALOD, although the aldehyde oxidation speed of this 3-HIBDH was slightly different from that of the *Pseudomonas* ALOD.

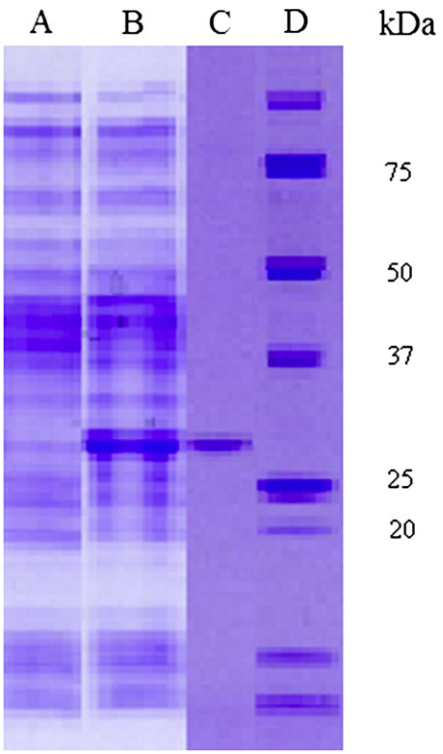

FIG. 3. SDS-PAGE of recombinant ALOD. Lane A, cell-free extract from *E. coli* BL21; lane B, cell-free extract from *E. coli* BL21/pET-GOOX; lane C, purified recombinant ALOD; lane D, standard proteins.

TABLE 1. Dehydrogenase activity of the recombinant ALOD from *Pseudomonas* sp. AIU 362.

Substrate	Enzyme activity (%)	
	NAD^+	$NADP^+$
L-3-Hydroxyisobutyric acid	100*	2.8
D-3-Hydroxyisobutyric acid	0.3	0
L-3-Hydroxyisobutyricacidmethylester	39	0
D-3-Hydroxyisobutyricacidmethylester	0	0
L-Serine	13	0
D-Serine	1.8	0

The dehydrogenase activity was assayed at 340 nm under standard assay conditions using 1.724 μg of enzyme (50 μl of the ALOD solution of 0.5 unit/ml as glyoxal oxidase activity). The enzyme activity is expressed by relative rate to the activity with L-3-hydroxyisobutyric acid and NAD^+ (*32.7 units/ml). Data are mean values of three independent measurement, S.D. <10%.

DISCUSSION

Recently, we demonstrated that ALOD from *Pseudomonas* sp. AIU 362 exhibited oxidase activity for aliphatic aldehydes and aromatic aldehydes, but the N-terminal amino acid sequence was not homologous to that of other microbial ALODs (1). We therefore cloned and sequenced the ALOD gene from *Pseudomonas* sp. AIU 362, and revealed the characteristics of the ALOD in more detail in this study. The ALOD gene had 888 nucleotides encoding 295 amino acids, and approximately 95% of the deduced amino acid sequence of the ALOD was identical to those of 3-HIBDH from *P. putida* E23 (9) and *P. putida* KT2440 (10). These results indicate that these ALOD and 3-HIBDHs have potential to catalyze two different reactions, oxidation of aldehydes and dehydrogenation of 3-hydroxyisobutyric acid. Then, we analyzed the substrate specificity using the purified recombinant ALOD and 3-HIBDH. The recombinant ALOD exhibited both enzyme activities of ALOD and 3-HIBDH, and the substrate specificities were similar to those of the previous reports of ALOD from *Pseudomonas* sp. AIU 362 (1) and 3-HIBDH from *P. putida* E23 (11), respectively. In these reactions, the K_m and V_{max} values for L-3-hydroxyisobutyric acid were much lower than those for glyoxal (1). Thus, main reaction of the ALOD might be dehydrogenation of L-3-hydroxyisobutyric acid. The recombinant 3-HIBDH from *P. putida* KT2440 also exhibited the

TABLE 2. Oxidase activity of the recombinant 3-HIBDH from *P. putida* KT2440.

Substrate	Enzyme activity (%)
Glyoxal	100*
Acetaldehyde	72
Propionaldehyde	100
Butyraldehyde	109
Valeraldehyde	138
Hexylaldehyde	171
Benzaldehyde	111
p-Hydroxybenzaldehyde	185
p-Methoxybenzaldehyde	130

The ALOD activity was assayed under standard assay conditions using 50 μl of the 3-HIBDH solution of 42.2 units/ml as L-3-hydroxyisobutyrate dehydrogenase activity. The enzyme activity is expressed by relative rate to the glyoxal oxidizing activity (*0.5 unit/ml). Data are mean values of three independent measurement, S.D. <10%.

ALOD activity with similar substrate specificity to the ALOD from *Pseudomonas* sp. AIU 362, and the glyoxal oxidase activity was 1.2% of the L-3-hydroxyisobutyric acid dehydrogenase activity, which was not big difference from that of the recombinant of ALOD (the ratio of the ALOD was 1.5%). The small difference of the ratio may be affected by difference of both sequences (16 amino acids are different in both enzymes). Because difference of amino acids in the deduced amino acid sequences of 3-HIBDHs from *P. putida* KT2440 and *P. putida* E23 is only 4 amino acids, 3-HIBDH from *P. putida* E23 probably also exhibit the ALOD activity. Thus, we concluded that the ALOD from *Pseudomonas* sp. AIU 362 and 3-HIBDHs from *P. putida* E23 and *P. putida* KT2440 might be classified into the same enzyme group.

In the measurement of the above ALOD and 3-HIBDH activities, the 3-HIBDH activity required NAD^+ as cofactor, but the ALOD activity did not require additional cofactors. It is known that FAD-containing oxidases have the motif of GXGXXG in the N-terminal region. The ALOD from *Pseudomonas* sp. AIU 362 also had this motif in the N-terminal region, but no absorption maxima were detected in the visible region of the purified enzyme solution. Chowdhury et al. also reported that no absorption peak was detected in the region from 300 to 500 nm of the 3-HIBDH from *P. putida* E23, although the enzyme contained the motif of GXGXXG in the N-terminal region (11). Guan et al. reported that the flavin-containing monooxygenase, which requires FAD and $NADP^+$ for the oxygenase reaction, contained the motif of GXGXXG in two regions (15). We therefore concluded that the GXGXXG near the N-terminal region may be a binding position of NAD^+ in the ALOD from *Pseudomonas* sp. AIU 362, and the other cofactors contribute to the ALOD activity. We intend to identify the prosthetic group of the ALOD from *Pseudomonas* sp. AIU 362 in a future study.

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