

Characterization of endogenous pyridoxal 5'-phosphate-dependent alanine racemase from *Bacillus pseudofirmus* OF4

Jiansong Ju,^{1,2} Shujing Xu,² Jianxin Wen,³ Gang Li,¹ Kouhei Ohnishi,⁴
Yanfen Xue,¹ and Yanhe Ma^{1,*}

State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100101, China,¹ Life College of Science and Engineering, Shaanxi University of Science and Technology, Xi'an, 710021, China,² College of Life Sciences, Fujian Normal University, Fuzhou, 350108, China,³ and Research Institute of Molecular Genetics, Kochi University, Kochi 783-8502, Japan⁴

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An open reading frame of 1100 bp in the partially sequenced genome sequence of alkaliphilic *Bacillus pseudofirmus* OF4 was identified as a putative alanine racemase gene (*dadX_{OF4}*), which was cloned and expressed in *Escherichia coli* BL21 (DE3). The encoded protein DadX_{OF4} was purified to homogeneity by His₆-tag affinity column, gel filtration and ion-exchange chromatography. The amino acid sequence has highest identity with the known alanine racemase from *Oceanobacillus ihayensis* HTE831 (48%). The protein was a dimeric, endogenous PLP-dependent enzyme, which was demonstrated by absorption spectra and enzyme activity with or without PLP. The racemization temperature optimum was 40 °C and the optimal pH was 10.5. The kinetic parameters K_m and V_{max} at 40 °C of alanine racemase, determined by HPLC analysis, were 41.79 mM, 10,500 units/mg for L-alanine and 14.91 mM, 3708 units/mg for D-alanine, respectively.

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D-Alanine is an essential component of the cell wall peptidoglycan and is required for the biosynthesis of spore cortex peptidoglycan (1). D-Alanine is converted from L-alanine by a specific enzyme, alanine racemase (EC 5.1.1.1). Alanine racemase is a pyridoxal 5'-phosphate (PLP)-dependent enzyme, catalyzing the interconversion of L-alanine and D-alanine. In some bacteria, such as *Escherichia coli* and *Salmonella*, two independent alanine racemase genes have been identified in each microorganism (2–4). For example, in *E. coli* the *alr* gene is constitutively expressed in the cells for peptidoglycan biosynthesis. The expression of the other gene, *dadX*, is induced when cells are grown in high concentrations of L- or D-alanine (2, 5). The enzyme encoded by the *dadX* gene catalyzes the conversion of L-alanine to the directly oxidizable D-alanine, which is required in the use of L-alanine as a carbon and energy source (6).

Alanine racemase is ubiquitous among bacteria, but is generally absent in higher eukaryotes with some exceptions: enzymes for D-alanine metabolism in yeast (7), the muscle of black tiger prawn (8) and alfalfa seedlings (9). Structure and properties such as amino acid sequence, quaternary structure, pH-dependency and kinetic parameters of eukaryotic enzymes seem to be distinct from those of bacterial alanine racemases.

Based on their apparent molecular masses analyzed by gel filtration or analytical ultracentrifugation, alanine racemases are classified into two types of subunit structure. The monomeric enzymes are those from crayfish (10), marine gastropod *Cellana grata* (11), and *Thermus thermophilus* (12). The homodimeric enzymes are those from *Bacillus psychrosaccharolyticus* (13), *Penaeus monodon* (8) and *Streptococcus pneumoniae* (14). Recent progress in crystal structural analyses has demonstrated that all analyzed alanine racemases from *Geobacillus stearothermophilus* (15), *Pseudomonas aeruginosa* (16), *Streptomyces lavendulae* (17), and *Mycobacterium tuberculosis* (18) are in dimeric form. Moreover, intermolecular complementation between two defective mutants of *P. aeruginosa* and *E. coli* alanine racemases clearly indicates that these enzymes function exclusively as homodimers (3).

Alanine racemase has also attracted much interest as a possible target for antibacterial drugs. Not only is D-alanine a vital component of the bacterial cell wall, but recent studies also indicate that alanine racemase, which is accessible in the exosporium, plays a key role in inhibition of germination in *Bacillus* spore (19). With *Bacillus cereus* spores, L-alanine is an effective germination-promoting compound and D-alanine is an effective inhibitor of L-alanine-induced germination (20).

Alkaliphilic *Bacillus pseudofirmus* OF4 were obligately aerobic, spore-forming, gram-positive, motile rods with the optimal pH 10.5 and temperature optimum at 30 °C (21, 22). Recently, we initiated a project to sequence the genome sequence of alkaliphilic *B. pseudofirmus* OF4. According to the partially sequenced genome sequence, an

* Corresponding author. Tel.: +86 10 64807618; fax: +86 10 64807616.

E-mail address: mayanhe@im.ac.cn (Y. Ma).

open reading frame encodes a protein which shows amino acid sequence identity to the alanine racemases from *Oceanobacillus iheyensis* HTE831 (48%, accession NP_694071), *Carnobacterium* sp. AT7 (45%, accession ZP_02184389), and *Methanococcus maripaludis* C7 (42%, accession YP_001329976). Herein we report the gene cloning, purification and characterization of the putative alanine racemase from *B. pseudofirmus* OF4.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media *E. coli* DH12S was used for cloning. MB2795 (*alr::frit, dadX::frit*) is a D-alanine auxotroph (23). *B. pseudofirmus* OF4 was routinely grown at 30 °C and pH 10.5 in malate-containing medium (21). D-alanine was added to LB medium at 0.5 mM when necessary. Ampicillin was added at 100 µg/ml. Expression vectors, pET-22b (+) (Novagen, Darmstadt, Germany), and TA cloning vector, pMD18-T (Takara, Japan), were used.

Cloning of alanine racemase gene Chromosomal DNA of *B. pseudofirmus* OF4 was isolated using the TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instruction. From the partially sequenced genome sequences (Ma, unpublished data), a pair of primers were designed: OF4-F01, 5'-GCCCATATGAAGACG-AGCAGTTTAGA-3'; OF4-R01, 5'-GGCTCGAGGTTCTCTTCGTAA-TATCTC-3', which introduced an *NdeI* restriction site at the 5' end and an *XhoI* restriction site at the 3' end of the alanine racemase gene.

The OF4 alanine racemase gene was PCR-amplified by *TaKaRa Ex Taq*TM (Takara, Japan) with the primers OF4-F01 and OF4-R01. PCR was carried out at 94 °C for 4 min, followed by 25 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min. The reaction was stopped after a final incubation step for 10 min at 72 °C.

The amplified *dadX* gene fragments were separated on 0.8% agarose gels, purified using the E.Z.N.A. Gel Extraction kit (Omega Bio-tek, Doraville, GA, USA), and cloned into a TA cloning vector pMD18-T according to the manufacturer's instructions to construct plasmid pMDOFdadX. The plasmid was digested with restriction endonucleases, *NdeI* and *XhoI*, and cloned into pET-22b (+) (pOFdadX). The plasmids were isolated from the recombinant cells using the Plasmid Mini kit 1 (Omega Bio-tek, Doraville, GA, USA).

Enzyme and protein assays The activity of alanine racemase was assayed as described previously (23) with some modification. The standard racemization mixture contained PLP (10 µM) when necessary, sodium hydrogen carbonate-sodium hydroxide buffer (12.5 mM, pH 10.5), L-alanine (50 mM), and cell lysate or appropriate protein in a final volume of 200 µl. The reaction was started by addition of cell lysate or protein and stopped by addition of 25 µl of 2 M HCl at 40 °C. Incubation time was 10 min. Following cooling on ice for 2 min, reaction mixture was spun down at 20,000×g for 10 min at 4 °C. An aliquot (180 µl) was withdrawn and mixed with 20 µl of 2 M NaOH for neutralization. D-Forms of amino acids were measured with D-amino acid oxidase (24). The amino acid oxidase reaction mixture contained Tris-HCl

(200 mM, pH 8.0), 4-aminoantipyrine (0.1 mg/ml), *N*-ethyl-*N*-(2-hydroxy-3-sulfo-propyl)-*m*-toluidine sodium salt (TOOS, 0.1 mg/ml), 2 units of peroxidase, 0.1 unit of D-amino acid oxidase, and D-forms of amino acids solution in a final volume of 200 µl. The reaction was carried at 37 °C for 20 min for D-alanine. The absorbance at 550 nm was measured by SpectraMax 190 Microplate Reader (Molecular Devices Corp. USA). One unit (U) of the enzyme was defined as the amount of the enzyme that catalyzed the formation of 1 µmol of D-alanine from L-alanine per min.

Protein concentration in the cell lysate was measured by Bio-Rad Protein Assay Reagent (Bio-Rad, USA). Protein concentration of the purified enzyme was determined by BCA Protein Assay Reagent Kit (Pierce). In both methods, bovine serum albumin was used as a protein standard.

DNA sequencing Plasmid pMDOFdadX was used as template for DNA sequencing with a set of primers, M13-47 (5'-CGCCAGGTTTCCAGTCACGAC-3'), and RV-M (5'-GAGCGGATAACAATTTCACACAGG-3'). Nucleotide sequences were determined using an ABI 3730xl DNA sequencer (Applied Biosystems).

Purification of alanine racemases Plasmid pOFdadX was transformed into *E. coli* BL21 (DE3). The pre-cultured recombinant cells (2 ml) were inoculated into 200 ml of fresh media and incubated at 37 °C. When the cell density at OD₆₀₀ reached 0.6, isopropyl thio-β-D-galactoside (IPTG) was added at the final concentration of 1 mM, and incubation was continued at 28 °C overnight. The protein was purified as follows.

First step: Ni-NTA agarose column chromatography. Cells were harvested, resuspended in 20 ml of lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, and 10 mM imidazole), and sonicated for 4 min several times until the solution became clear. Sonicated cells were centrifuged to remove cell debris, and the supernatant was mixed with 2 ml of 50% Ni-NTA agarose slurry (Qiagen, Germany) and incubated with gentle mixing at 4 °C for 1 h. Proteins not bound to the Ni-NTA were removed by three washes with 4 ml of wash buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, and 20 mM imidazole). Bound proteins were eluted four times with 0.5 ml of elution buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, and 250 mM imidazole).

The elution were combined, concentrated and dialyzed against the standard buffer [10 mM phosphate buffer (pH 8.0), 0.5 mM EDTA, 10 µM PLP when necessary, and 0.01% 2-mercaptoethanol] with 10% glycerol by ultrafiltration with an Amicon Ultra-15 Centrifugal Filter Devices (30K MWCO, Millipore).

Second step: Gel filtration chromatography. Protein was put on a Superdex 200 10/300 GL (1.0×30 cm; Amersham Biosciences) at 4 °C and eluted with 20 mM potassium phosphate buffer (pH 8.0) containing 150 mM NaCl, 0.5 mM EDTA, 20 µM PLP, and 0.01% (v/v) 2-mercaptoethanol at a flow rate of 0.6 ml/min. Fractions of 1.0 ml were collected. Cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa) and alcohol dehydrogenase (150 kDa) were used as molecular mass standard (Sigma, St. Louis, MO, USA). The fractions with alanine racemase activity were combined, concentrated and dialyzed against the standard buffer with 10% glycerol by ultrafiltration with an Amicon Ultra-15 Centrifugal Filter Devices.

Third step: DEAE superose column chromatography. The obtained protein dadX_{OF4} was subjected to a DEAE superose column (Amersham Biosciences) at 4 °C with standard buffer with 0 or 1 M NaCl at a flow rate of 3 ml/min and fractionated in 1.2 ml

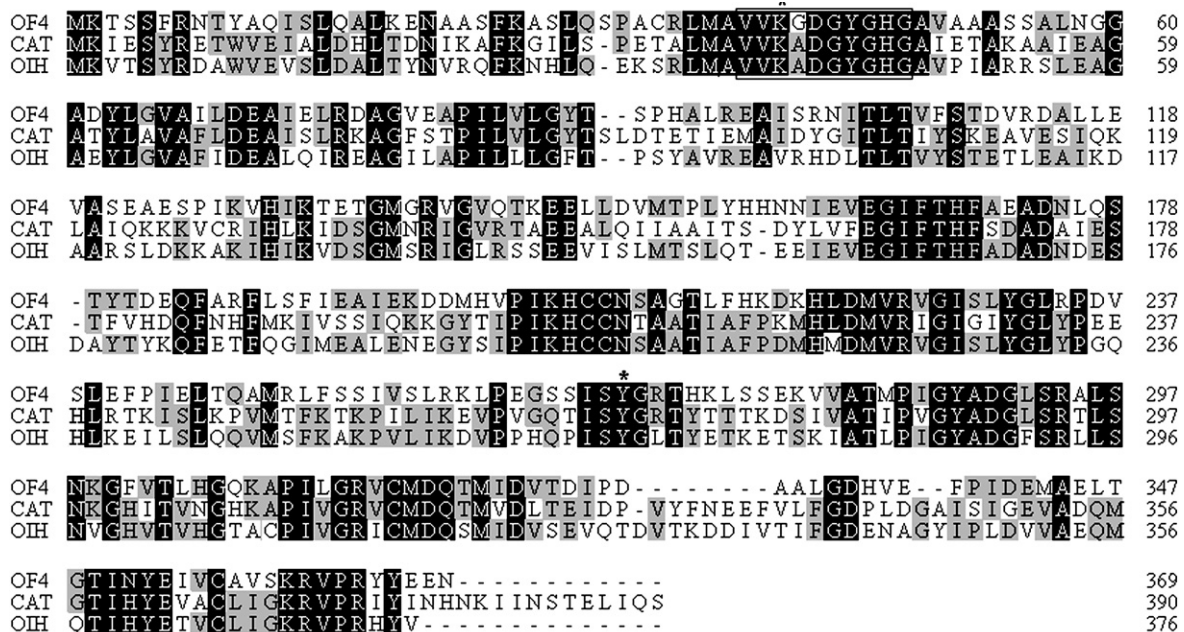


FIG. 1. ClustalW multiple sequence alignment of alanine racemases. Alanine racemases from *B. pseudofirmus* OF4 (OF4), *Carnobacterium* sp. AT7 (CAT) and *O. iheyensis* HTE831 (OIH) were compared. The conserved pyridoxal 5'-phosphate binding site is boxed. Asterisk indicates the two catalytic residues, K41 and Y266.

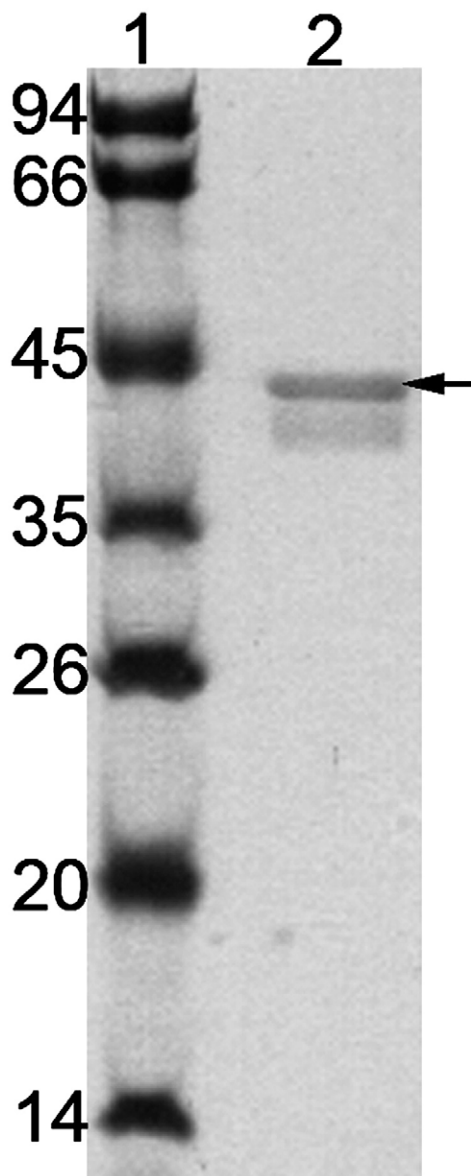


FIG. 2. SDS-PAGE profile of purified enzyme. Alanine racemase with six histidines on C-termini was purified from BL21 (DE3) cells expressing *dadX_{OF4}* (lane 2) and analyzed by 12.5% SDS-PAGE. Molecular mass standards were shown on the lane 1 in kDa. Arrow shows the protein alanine racemase.

fractions. The fractions with alanine racemase activity were combined, concentrated and dialyzed against the standard buffer with 10% glycerol by ultrafiltration with an Amicon Ultra-15 Centrifugal Filter Devices. The purity of the enzyme was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Kinetic parameters Alanine racemase activity was determined by measuring the amounts of D- and L-alanine by HPLC. The reaction mixture (40 μ l) was composed of 10 mM NaHCO₃-NaOH buffer (pH 10.5), 10 μ M PLP, and various amounts of D- or L-alanine. The enzyme reaction was started by the addition of the purified enzyme or NaHCO₃-NaOH buffer as a negative control, continued for 10 min at 40 °C, and terminated by adding 40 μ l of 2 M HCl. After neutralization by NaOH, D- and L-alanine in the reaction mixture were derivatized for 2 min at 25 °C with a 0.28 M borate solution (pH 9.0), containing 0.2% *N*-tert.-butyloxycarbonyl-L-cysteine (Boc-L-Cys) (Fluka, Switzerland) and 0.2% *o*-phthalaldehyde (OPA) (Sigma, Germany). A 10 μ l aliquot of the resulting mixture was separated using a Wakopak wakosil-PTH (5 μ m, 4.6 \times 250 mm; Shimadzu, Japan), and the alanine derivatives were detected by a spectrofluorometer (RF-10A, Shimadzu, Japan). Other conditions were described previously (25). One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μ mol of L- or D-alanine from either enantiomer per min.

Nucleotide sequence accession number The nucleotide and amino acid sequences of DadX_{OF4} are available in the GenBank under the accession numbers EU751624.

RESULTS AND DISCUSSION

Cloning of alanine racemase gene A 1110 bp DNA fragment was amplified and cloned into a TA cloning vector pMD18-T for plasmid pMDOFdadX construction. The plasmid pMDOFdadX was sequenced and the nucleotide sequence was identical (100%) with the putative alanine racemase gene from the partial genome sequence of *B. pseudofirmus*. This gene encodes a polypeptide of 370 amino acids with a calculated molecular mass of 40,322 Da. The deduced protein showed amino acid sequence similarity to the known alanine racemases from *O. iheyensis* HTE831 (identity: 48%), *Carnobacterium* sp. AT7 (45%), and carried the expected motifs such as the characteristic pyridoxal phosphate binding site (VVKGDGYGHG) near the N-terminus, and the two catalytic amino acid residues (K41, Y269) of the active center (Fig. 1) (18).

The plasmid pMDOFdadX was double digested by restriction enzymes and ligated into the expression vector pET22b (+) (pOFdadX). Before purification, the plasmid pOFdadX was put into an *E. coli* *alr dadX* double mutant MB2795 and vector pET22b (+) was

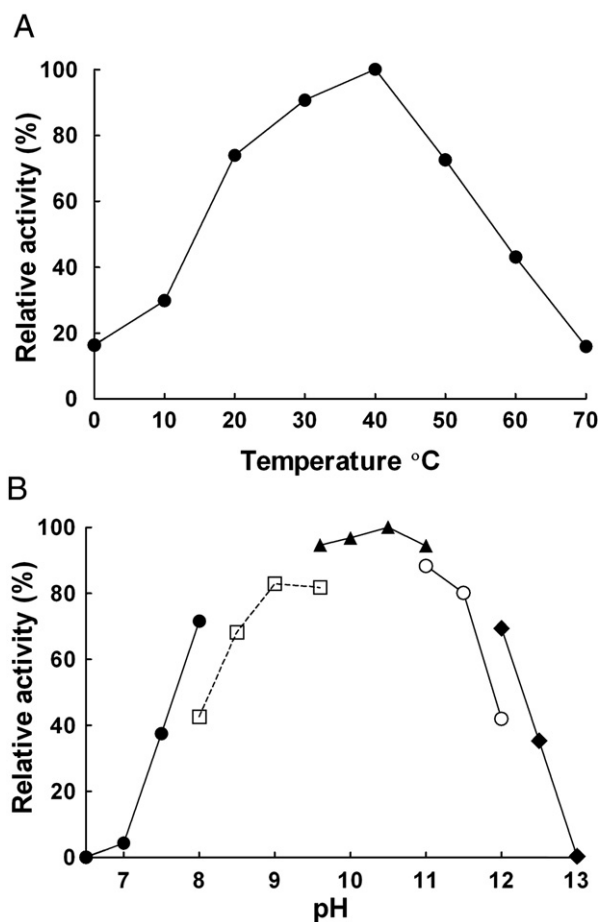


FIG. 3. Optimal temperature and pH of alanine racemase. (A) Optimal temperature. The reaction time was 10 min at each temperature. Enzyme activities for L to D direction were determined in 12.5 mM NaHCO₃-NaOH buffer at pH 10.5. (B) Optimal pH. The activity of alanine racemase was assayed in the presence of one of the following buffers: closed circle, 12.5 mM potassium phosphate buffer (pH 6.5–8.0); open square, 12.5 mM barbitone-HCl buffer (pH 8.0–9.6); closed triangle, 12.5 mM NaHCO₃-NaOH buffer (pH 9.6–11.0); open circle, 12.5 mM Na₂HPO₄-NaOH buffer (pH 11.0–12.0); or closed square, 12.5 mM KCl-NaOH buffer (pH 12.0–13.0). The reactions were carried out for 10 min at 40 °C.

used as a negative control. While MB2795/pET-22b (+) did not grow on the LB media without D-alanine, the recombinant MB2795/pOFdadX grew well on the same medium (data not shown), indicating the cloned *B. pseudofirmus* gene encodes a functional alanine racemase. The gene organization surrounding the alanine racemase gene was alanine dehydrogenase gene, putative alanine: Na⁺ symporter gene and *N*-carbamoyl-L-amino acid amidohydrolase gene (data not shown). Genes for alanine dehydrogenase and alanine racemase are located side by side and form an operon (2, 26). The catabolic alanine racemases (e.g. *DadX* in *E. coli*) are commonly part of an operon (16), suggesting that the alanine racemase of *B. pseudofirmus* OF4 might be a catabolic alanine racemase.

Purification and aggregate structure of the alanine racemase Alanine racemase with C-terminal histidines was overexpressed in BL21 (DE3)/pOFdadX cells and purified to electrophoretic homogeneity (Fig. 2). The apparent molecular mass of purified alanine racemase was about 41 kDa by SDS-PAGE, which is in good agreement with the predicted molecular mass of 41,387 Da. Oligomerization states of protein DadX_{OF4} was analyzed by dynamic light scattering and gel filtration chromatography. When the dynamic light scattering was performed using the Superdex purified protein at 1.6 mg/ml, a multi-disperse profile was observed and the peaks had hydrodynamic radius of 2.9 nm, 3.4 nm and 3.7 nm. These radii corresponded to the molecular mass of 40 kDa, 58 kDa and 73 kDa, respectively, suggesting that in solution the protein DadX_{OF4} was in a monomer-dimer equilibrium. This observation is consistent with the alanine racemases from *Pseudomonas fluorescens* LBR3W1 and TM5-2 (26). However, the molecular mass of the protein determined by gel filtration on a Superdex 200 10/300 GL column was ca. about 87 kDa, corresponding to a dimeric aggregate. Even at low quantities of proteins (2.8 μg, 1.4 μg and 0.7 μg), the molecular mass was ca. about 83 kDa. These results indicated that the enzyme DadX_{OF4} existed as a dimer.

Optimal reaction temperature and pH The enzyme activity was maximal at approximately 40 °C in the direction of L-alanine to D-alanine with the standard racemization buffer (pH 10.5) (Fig. 3A). The optimal pH of the enzyme was approximately 10.5 in the direction of L-alanine to D-alanine at 40 °C (Fig. 3B). These data were consistent with the enzyme of *Bifidobacterium bifidum* (amino acid sequence identity: 33.5%) (27). Because of the high activity of the enzyme, the activity could be detected at a wide range of temperature and pH. It was about 28% at 0 °C and 20% at 70 °C of the activity at 40 °C; 37.5% at pH 7.5 and 35.4% at pH 12.5 of the activity at 40 °C.

Coenzyme Alanine racemases require PLP as a cofactor to form a Schiff base between PLP and ε-amino group of the lysine residue in the active site (28). The enzyme DadX_{OF4}, which was purified without PLP addition during the purification procedure, showed same activity as that in the presence of PLP (data not show). The enzyme showed a maximum absorption at 420 nm as well as 280 nm (Fig. 4). The absorption peak at 420 nm is probably derived from an internal Schiff-base (8, 29). It indicates that alanine racemase from the *B. pseudofirmus* OF4 does not require exogenous PLP, which is similar to the alanine racemase from the *Thalassiosira* sp. (30). The PLP content of the enzyme was 2.16 mol/mol of the enzyme by the fluorometric method (31).

To obtain the apoenzyme, the enzyme was incubated with 10 mM hydroxylamine at 37 °C water bath for 30 min (27), followed by dialysis against 10 mM potassium phosphate buffer (pH 8.0) containing 0.01% 2-mecaptoethanol. The apoenzyme still showed more than 60% of the activity of the holoenzyme and low absorption maximum at 420 nm (Fig. 4). When the mutant at PLP binding site (Lys 41 → Ala 41) was prepared, even if the activity of mutant enzyme could not be detected (data not show), the maximum absorption at 420 nm still existed. These results indicate that the PLP binds strongly to the protein not only with a covalent bond to lysine 41 but also other residues (15).

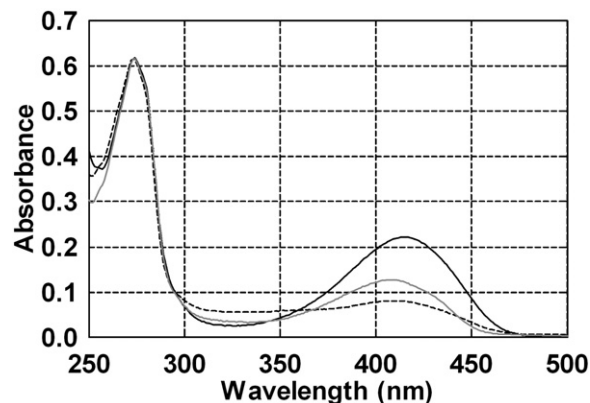


FIG. 4. Absorption spectra of alanine racemase from *B. pseudofirmus* OF4. Absorption spectra were taken in 10 mM potassium phosphate buffer (pH 8.0). Black line, the holoenzyme (the native form of the enzyme); gray line, the mutant enzyme at residue 41 (K41 → A41); and dotted line, the apoenzyme.

Kinetic parameters of purified alanine racemase The apparent velocity of the enzymatic alanine racemization was measured against various concentrations of both enantiomers at 40 °C. The K_m value for L-alanine was higher than that for D-alanine. K_m value was 41.79 mM for L-alanine and 14.91 mM for D-alanine. The catalytic activity of DadX_{OF4} was 10,500 units/mg for L-alanine and 3708 units/mg for D-alanine. The calculated $K_{eq(L/D)}$ for alanine racemization was 1.01, in good agreement with the theoretical value for the chemically symmetric reaction (32).

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