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Purification and characterization of an L-amino acid oxidase from *Pseudomonas* sp. AIU 813

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An L-amino acid oxidase was found from a newly isolated strain, *Pseudomonas* sp. AIU 813. This enzyme was remarkably induced by incubation with L-lysine as a nitrogen source, and efficiently purified using an affinity chromatography with L-lysine as ligand. The enzyme oxidized L-lysine, L-ornithine and L-arginine, but not other L-amino acids and D-amino acids. The oxidase activity for L-lysine was detected in a wide pH range, and its optimal was pH 7.0. In contrast, the oxidase activity for L-ornithine and L-arginine was not shown in acidic region from pH 6.5, and optimal pH for both substrates was 9.0. The enzyme was a flavoprotein and composed of two identical subunits with molecular mass of 54.5 kDa. The N-terminal amino acid sequence was similar to that of putative flavin-containing amine oxidase and putative tryptophan 2-monooxygenase, but not to that of L-amino acid oxidases.

[**Key words:** L-Amino acid oxidase; L-Lysine; L-Arginine; L-Ornithine; *Pseudomonas*]

L-Amino acids are constituent of protein and peptides, and are widely being used as nutrients of foods, raw materials for pharmaceuticals and other fields. Therefore, enzymes concerning to metabolism of L-amino acids were widely investigated, and different type of enzymes such as oxidase, monooxygenase, dehydrogenase and transferase, have been isolated and their characteristics have been revealed. In those enzymes, L-specific amino acid oxidases (L-AAOs) have been obtained from many fungal and bacterial strains, and those are separated into two groups. One group contains L-AAOs, which are specific to one or a few amino acids, such as L-glutamate oxidase (EC 1.4.3.11) (1,2), L-lysine oxidase (EC 1.4.3.14) (3,4), L-aspartate oxidase (EC 1.4.3.16) (5,6) and L-phenylalanine oxidase (7). Another group contains L-AAOs with broad substrate specificity produced by *Bacillus carotarum* 2Pfa (8), *Rhodococcus opacus* DSM 43250 (9) and *Rhodococcus* sp. AIU Z-35-1 (10). In these enzymes, L-glutamate oxidase and L-lysine oxidase in the former group are used for assay of L-glutamic acid and L-lysine, respectively (11,12). The enzymes in the latter group are useful for production of D-amino acids from racemic amino acids (13). In the studies of microbial L-AAOs, we found an L-AAO, which is specific to L-lysine, L-ornithine and L-arginine, from a newly isolated strain. The present paper describes production, purification and some remarkable properties of the L-AAO produced by a newly isolated strain, *Pseudomonas* sp. AIU 813.

MATERIALS AND METHODS

Chemicals L-Lysine, L-ornithine, L-arginine and other L-amino acids were purchased from Wako Pure Chemicals (Osaka). Peroxidase was gift from Amano Enzyme (Nagoya). All other chemicals used were of analytical grade and commercially available. L-Lysine–Toyopearl resins were prepared using Toyopearl AF-Epoxy-650M and L-lysine as follows. Toyopearl AF-Epoxy-650M (10 g of wet weight) was stirred with 30 ml of 0.5 M L-lysine solution adjusted to pH 12 at 30°C for 24 h. Then, the resins were collected by filtration and incubated with 30 ml of 1.0 M ethanolamine solution to inactivate the remaining epoxy groups of the resins at 30°C for 15 h with shaking. The resins were then collected by filtration and washed with 1.0 M NaCl solution and water.

Identification of isolated strain Identification of a newly isolated strain was performed at TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan).

Cultivation of microorganism A newly isolated strain, *Pseudomonas* sp. AIU 813, was first incubated in a test tube containing 5 ml of an L-lysine medium, pH 7.0, consisting of 0.2% KH_2PO_4, 0.1% Na_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% glucose, and 0.5% L-lysine at 30°C for 2 days with shaking (120 strokes/min). The culture (1.5 ml) was then inoculated into a 500-ml of shaker flask containing 150 ml of the L-lysine medium, and it was incubated at 30°C for 2 days with shaking. Then, 20 ml of the second culture was transferred into a 3-l of culture flask containing 2 l of the L-lysine medium. After the third culture was carried out at 30°C for 3 days, the cells were harvested by centrifugation at 20,000 ×g for 10 min, washed with 10 mM potassium phosphate buffer, pH 7.0, and then stored at −20°C until use.

Assay of enzyme activity L-Amino acid oxidase activity was assayed by measuring the rate of hydrogen peroxide formation as follows. The standard reaction mixture contained 60 μmol of L-lysine, L-ornithine or L-arginine, 0.6 μmol of 4-aminoantipyrine, 1.94 μmol of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt dihydrate, 6.7 units of peroxidase, 0.1 mmol of potassium phosphate, pH 7.0, and an appropriate amount of enzyme, in a final volume of 1.0 ml. The formation of hydrogen peroxide was spectrophotometrically followed at 30°C for 5 min by measuring the absorbance at 555 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole of hydrogen peroxide per min.

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Buffer for measurement of optimal pH To determine the optimal pH of the enzyme activity, potassium phosphate buffer, pH 5.0 to pH 8.5 and H_3BO_3—KCl—NaOH buffer, pH 8.0 to pH 10.0, were used.

Purification of enzyme Purification of enzyme was carried out using cells obtained from 6 l culture broth of the L-lysine medium. All procedures were done at 5—10°C. The buffer used was potassium phosphate buffer, pH 7.5.

In the first step, 20 g of wet cell weight were suspended in 300 ml of 10 mM buffer solution, and disrupted by a Multi-beads shocker (Yasui Kikai, Osaka) at 2200 rpm for 8 min (2 min × 4). After the cell disruption, 250 ml of the supernatant solution was obtained by centrifugation at 20,000 ×g for 20 min. The cell pellets collected by the centrifugation were resuspended in 250 ml of a newly prepared 10 mM buffer solution, and the cell disruption was carried out again under the same conditions as described above. The cell disruption was carried out four times, and each supernatant solution obtained was mixed (total volume, 1020 ml). Then, the pH of the supernatant solution was adjusted to 5.2 with 0.5 M HCl solution, and the resulting precipitate was discarded by centrifugation at 20,000 ×g for 10 min. The pH of the supernatant solution was readjusted to 7.5 with 0.5 M NaOH solution.

The enzyme solution adjusted to pH 7.5 was then applied to a DEAE—Toyopearl column (23 cm × 2.4 cm diameter) equilibrated with 60 mM buffer solution. The column was washed with 60 mM buffer solution, and the adsorbed enzyme was eluted by a linear gradient with 60 mM buffer solution and 60 mM buffer solution containing 0.1 M NaCl (500 ml each). The active fractions obtained by this column chromatography were collected and solid ammonium sulfate was added to 2.0 M.

The enzyme solution was further applied to a Phenyl—Toyopearl column (11 cm × 2.4 cm diameter) equilibrated with 10 mM buffer solution containing 2.0 M ammonium sulfate. After the column was washed with 10 mM buffer solution containing 1.3 M ammonium sulfate, the adsorbed enzyme was eluted by a linear gradient with 10 mM buffer solution containing 1.3 M ammonium sulfate and 10 mM buffer solution containing 0.3 M ammonium sulfate (250 ml each). The active fractions were collected, and dialyzed against 10 mM buffer solution.

The dialyzed enzyme solution was finally applied to an L-Lysine—Toyopearl column (25 cm × 1.4 cm diameter) equilibrated with 30 mM buffer solution, and the column was washed with the same buffer solution. The adsorbed enzyme was eluted by a linear gradient with 30 mM buffer solution and 30 mM buffer solution containing 0.1 M NaCl (200 ml each), followed with 50 ml of the same buffer solution. The active fractions were collected, and the purity was analyzed.

Protein measurement Protein concentration was measured with a Protein Quantification Kit (Dojindo Laboratories, Tokyo).

SDS-PAGE and molecular mass SDS-PAGE was performed according to the method of Laemmli (14). Proteins were stained with Coomassie Brilliant Blue R-250. Molecular mass was estimated by gel filtration on a TSK gel G3000SW$_{XL}$ column and by SDS-PAGE using standard markers of molecular mass (Sigma Japan, Tokyo).

Isoelectric point The isoelectric point was determined with an isoelectric focusing apparatus (Nippon Eido, Tokyo) under conditions of 1% Pharmalyte, pH 3.5—10 (GE Healthcare Japan, Tokyo), with a sucrose gradient at 400 V for 2 days at 4°C. One-milliliter fractions were collected, and the pH was measured at 4°C.

N-terminal amino acid sequence The amino acid sequence of the intact enzyme was determined using Applied Biosystems gas-phase protein sequencer equipped with an on-line reverse-phase chromatography system for identification of PTH-amino acids.

RESULTS

Identification of isolated strain Strain was identified by phylogenetic analysis and biochemical properties. In the analysis of 16S rDNA sequence (DDBJ accession number, AB646255), 99.3% of the sequence of this strain was identical to that of *Pseudomonas putida* ATCC12633 and *Pseudomonas oryzihabitans* IAM1568. This isolated strain was mesophilic, no fermentative, motile, no spore-forming, rod-shaped (0.8—0.9 × 1.5—2.0 μm), gram-negative, catalase-positive, oxidase-positive and reduction of NO_3-positive. These results indicate that the isolated strain belongs to the genus *Pseudomonas*. Therefore, we named the isolated strain *Pseudomonas* sp. AIU 813.

Production of enzyme When the isolated strain, *Pseudomonas* sp. AIU 813, was incubated with the L-lysine medium containing 0.5% glucose in a 3-l of culture flask at 30°C for 2 days with shaking as describe in Materials and Methods, product amount of the L-AAO was much lower than that of a test tube (data not shown). Since it was presumed that a significant decrease of the product amount of the L-AAO was affected by the pH change of culture medium to around 4.0 during cell-growth, 20 g of calcium carbonate was added into a 2-l of the L-lysine medium and incubated at 30°C for 2 days with shaking. The pH of culture medium was kept in the neutral pH range, and the product amount of L-AAO was approximately 10 times higher than that of no addition of calcium carbonate (Table 1). The production of L-AAO was further improved by reduction of the glucose concentration of the L-lysine medium from 0.5% to 0.1% (Table 1).

TABLE 1. Effects of glucose and calcium carbonate on L-AAO production by *Pseudomonas* sp. AIU 813.

Glucose (%)	pH of culture broth	Cell growth (OD$_{660}$)	Enzyme activity (mU/100 ml broth)
0.5 (−$CaCO_3$)	4.10	1.29	0.20
0.5 (+$CaCO_3$)	6.95	1.24	1.86
0.1 (−$CaCO_3$)	6.63	1.87	6.05

Pseudomonas sp. AIU 813 was incubated in the L-lysine medium at 30°C for 2 days. Cells of 100 ml culture broth were disrupted by a Multi-beads shocker at 2200 rpm for 8 min, and resulting cell debris was discarded by centrifugation at 20,000 ×g for 10 min. Enzyme activity was assayed under standard assay conditions using the supernatant and 60 mM L-lysine.

Effect of nitrogen sources on the L-AAO production was also investigated by replacing L-lysine of the culture medium containing 0.1% glucose with L-argine, L-ornithine, L-glutamic acid, 6-aminohexanoic acid or ammonium sulfate. The L-lysine oxidase activity was also detected in the cells incubated with the medium containing L-ornithine, L-argine or L-glutamic acid, but their enzyme activities were lower than that of the L-lysine medium. No L-lysine oxidase activity was detected in the cells incubated with 6-aminohexanoic acid or ammonium sulfate (Table 2). Thus, the L-lysine was the best nitrogen source in those compounds for the L-AAO production. Then, we incubated the strain with the L-lysine medium containing 0.1% glucose as carbon source in a 3-l of culture flask at 30°C for 4 days with shaking, and the product amount of L-AAO was assayed each day. The L-lysine oxidase activity reached maximum after 2—3 days of cultivation (Fig. 1), and it was approximately 30 times higher than that of the L-lysine medium containing 0.5% glucose. Therefore, the strain was incubated with the L-lysine medium containing 0.1% glucose at 30°C for 2—3 days to obtain the L-AAO.

Purification and molecular mass The purification procedure is summarized in Table 3. The L-AAO was purified to an electrophoretically homogeneous state by approximately 400-fold, although contaminants that interfere with assay of L-lysine oxidase activity might be contained in crude enzyme solution. The purified enzyme showed a single protein band on SDS-PAGE (Fig. 2). The molecular mass of the native and denatured enzymes was estimated to be 110 kDa on a TSK gel G3000SW$_{XL}$ column and 54.5 kDa on SDS-PAGE, respectively (data not shown). These results indicate that the enzyme consisted of two identical subunits.

TABLE 2. Effect of nitrogen source on L-AAO production by *Pseudomonas* sp. AIU 813.

Carbone source	Cell growth (OD$_{660}$)	Enzyme activity (mU/100 ml broth)
L-Lysine	1.75	5.46
L-Arginine	1.36	0.99
L-Ornithine	1.47	0.40
L-Glutamic acid	1.38	0.42
Aminohexanoic acid	1.29	0
Ammonium sulfate	0.64	0

Pseudomonas sp. AIU 813 was incubated in the medium containing the indicated L-amino acids, aminohexanoic acid or ammonium sulfate as a sole nitrogen source, at 30°C for 3 days. The cell-free extract was prepared by disrupting the cells of 100 ml culture broth, and L-AAO activity was assayed under standard assay conditions using the cell-free extract and 60 mM L-lysine.