Journal of Molecular Catalysis B: Enzymatic 96 (2013) 89–95

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

# Characterization of a new enzyme oxidizing ω-amino group of aminocarboxyric acid, aminoalcohols and amines from *Phialemonium* sp. AIU 274

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ARTICLE INFO

*Article history:*
Received 11 April 2013
Received in revised form 21 June 2013
Accepted 22 June 2013
Available online 5 July 2013

*Keywords:*
Medium-chain amine
Aminocarboxylic acid
Aminoalcohol
ω-Amino compound
*Phialemonium*

ABSTRACT

A new enzyme exhibiting oxidase activity for ω-aminocarboxylic acids, ω-aminoalcohols, monoamines and diamines was found from a newly isolated fungal strain, *Phialemonium* sp. AIU 274. The enzyme also oxidized aromatic amines, but not L- and D-amino acids. The $V_{max}/K_m$ value for hexylamine was higher than those for 6-aminoalcohol and 6-aminhexanoic acid in the aliphatic $C_6$ substrates. In the aliphatic amines, the higher $V_{max}/K_m$ values were obtained by the longer carbon chain amines. Thus, the enzyme catalyzed oxidative deamination of the ω-amino group in a wide variety of the ω-amino compounds and preferred medium- and long-chain substrates. The oxidase with such broad substrate specificity was first reported here. The enzyme contained copper, and the enzyme activity was strongly inhibited by isoniazid, iproniazid and semicarbazide, but not by clorgyline and pargyline. The enzyme was composed of two identical subunits of 75 kDa.



## 1. Introduction

6-Aminohexanoic acid (6-AHA) is a derivative and analog of L-lysine, and used for treatment of excessive postoperative bleeding. The compound is also known as an intermediate in the polymerization of Nylon-6, and used for production of poly(ester amide)s, which have recently been developed as degradable materials with application as commodities. The enzymes for degradation of 6-AHA-containing polymers or 6-AHA dimer were reported [1–4], but those for degradation of 6-AHA have not been reported yet.

In the studies of microbial and enzymatic oxidation of L-lysine derivatives and analogs, we isolated some microorganisms, which produced an enzyme catalyzing oxidation of 6-AHA. Furthermore, we found that certain microorganisms in these isolates produced an enzyme catalyzing oxidation of ω-aminocarboxylic acids, ω-aminoalcohols, aliphatic monoamines and diamines. The oxidase exhibiting such broad substrate specificity has not been reported yet, although it was known that some microbial amine oxidases catalyzed oxidation of aliphatic monoamines, diamines and/or aromatic amines [5–8]. We therefore purified the enzyme from a fungal strain belonging to the genus of *Phialemonium* and revealed that the enzyme was a new copper-containing oxidase. The present paper describes production, purification and some remarkable properties of the oxidase produced by a newly isolated strain, *Phialemonium* sp. AIU 274.

## 2. Materials and methods

### 2.1. Chemicals

Monoamines from $C_2$ to $C_8$, diamines from $C_2$ to $C_{10}$, ω-aminocarboxylic acids from $C_2$ to $C_8$, ω-aminoalcohols from $C_2$ to $C_8$, benzylamine, tyramine, 2-phenylethylamine, 4-phenylbutylamine, L-lysine, D-lysine and 2-aminoadipic acid were purchased from Wako Pure Chemicals (Osaka, Japan). $N^{\alpha}$-Benzyloxycarbonyl-L-lysine ($N^{\alpha}$-Z-L-lysine) and $N^{\alpha}$-Z-D-lysine were from Watanabe Chemical Industries (Hiroshima, Japan). Peroxidase was gift from Amano Enzyme (Nagoya, Japan). All other chemicals used were of analytical grade and commercially available.

### 2.2. Screening of microorganism

Microorganisms were isolated by triple-enrichment culture using a 6-AHA medium, pH 7.0, consisting of 0.3% 6-AHA, 0.5%

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http://dx.doi.org/10.1016/j.molcatb.2013.06.012

glucose, 0.2% $KH_2PO_4$, 0.1% $Na_2HPO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$. The isolates were then incubated in a test tube containing 5 ml of the 6-AHA medium at 30 °C for 3 days with shaking (115 strokes/min), after which a cell-free extract was prepared by disrupting the cells using a Multi-beads shocker (Yasui Kikai, Osaka, Japan) below 5 °C for 8 min. In the next step, the oxidase activity for 6-AHA was assayed using the cell-free extract from each isolated strain, and five strains were selected as producers of oxidase for 6-AHA. Then, each crude enzyme solution from the five selected strains was applied to a DEAE-Toyopearl column and the enzyme was eluted by a linear gradient with 10 mM potassium phosphate buffer, pH 7.0 and 0.3 M NaCl. The enzyme activity of the eluates was then assayed using 6-AHA, hexylamine, 6-aminohexanol and 1,6-diaminohexane as substrates. The fungal strain, which produced the enzyme exhibiting high activity for these four substrates, was selected and used in this study.

### 2.3. Taxonomic studies of selected strain

Identification of a newly isolated strain was performed at TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan) as follows. The isolated strain was incubated on a potato-dextrose agar plate (Nihon seiyaku, Tokyo, Japan), 2% malt agar plate and an oatmeal agar plate (Becton Dickinson, MD, USA) at 25 °C in the dark, and the morphological characteristics were observed with both a compound microscope and a stereomicroscope. The sequence of 28S rDNA-D1/D2 was analyzed using an ABI BigDye Terminator v3.1 Kit (Applied Biosystems, Foster City, CA, USA), and a ABI PRISM 3100 Genetic Analyzer System (Applied Biosystems, Foster City, CA, USA). The sequence alignment and calculation of the homology levels were carried out using the database of Gen Bank, DDBJ and EMBL.

### 2.4. Cultivation of selected strain

A newly isolated strain, *Phialemonium* sp. AIU 274, was first incubated in a 500-ml shaker flask containing 150 ml of the 6-AHA medium, pH 7.0, at 30 °C for 4 days with shaking (115 strokes/min). The culture (100 ml) was then transferred into a 3-l culture flask containing 2 l of the 6-AHA medium. After the cultivation was carried out at 30 °C for 30 h, the mycelia were harvested by filtration, washed with 10 mM potassium phosphate buffer, pH 7.0, and then stored at −30 °C until use.

### 2.5. Assay of enzyme activity

The oxidase activity for 6-AHA was assayed by measuring the rate of hydrogen peroxide formation at pH 7.0 as follows, because the enzyme was stable at pH 7.0. The standard reaction mixture contained 20 μmol of 6-AHA, 0.6 μmol of 4-aminoantipyrine (4-AA), 1.94 μmol of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt dihydrate (TOOS), 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 assay of enzyme activity was started by addition of enzyme solution, and 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. The molar absorptivity value of $16.5 \times 10^3\ M^{-1}\ cm^{-1}$ was used for calculation of the enzyme activity.

### 2.6. Purification of enzyme

Purification of enzyme was carried out at 5–10 °C using mycelia obtained from 32 l culture broth of the 6-AHA medium.

In the first step, mycelia (48 g of wet weight) were suspended with 600 ml of 10 mM potassium phosphate buffer, pH 7.0, and disrupted by a Multi-beads shocker (Yasui Kikai, Osaka). The supernatant solution was obtained by centrifugation at 10,000 × *g* for 10 min. The cell pellets were resuspended with 500 ml of a newly prepared 10 mM potassium phosphate buffer, pH 7.0, and the supernatant solution was obtained by centrifugation. The cell pellets were suspended again with 500 ml of a newly prepared 10 mM potassium phosphate buffer, pH 7.0, and the cell-disruption was carried out two times under the same conditions as described above. The each supernatant solution obtained was mixed and used as a crude enzyme solution for purification. To the 1730 ml of supernatant solution, 419 g of solid ammonium sulfate was added to reach 40% saturation, and allowed to stand for 12 h. The resulting precipitate was collected by centrifugation at 10,000 × *g* for 10 min, dissolved with 10 mM potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer solution, pH 7.0.

The deionized enzyme solution was applied to a GigaCap Q-Toyopearl column (20 cm × 2.5 cm diameter) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, and the column was washed with same buffer solution. The adsorbed enzyme was then eluted by a linear gradient with 10 mM potassium phosphate buffer, pH 7.0, and 10 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl (500 ml each). The active fractions were collected, and solid ammonium sulfate was added to 0.5 M.

The enzyme solution was then applied to a Phenyl-Toyopearl column (20 cm × 2.5 cm diameter) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 0.5 M ammonium sulfate. After the column was washed with same buffer solution, the adsorbed enzyme was eluted by a linear gradient with 10 mM potassium phosphate buffer, pH 7.0, containing 0.5 M ammonium sulfate and 10 mM potassium phosphate buffer, pH 7.0 (500 ml each). The active fractions were collected, and deionized by ultrafiltration.

The deionized enzyme solution was applied to a DEAE-Toyopearl column (20 cm × 2.5 cm diameter) equilibrated with 10 mM potassium phosphate buffer, pH 6.3, and the column was washed with same buffer solution. The adsorbed enzyme was then eluted by a linear gradient with 10 mM potassium phosphate buffer, pH 6.3, and 10 mM potassium phosphate buffer, pH 6.3, containing 0.06 M NaCl (500 ml each). The active fractions were collected, and the purity was analyzed.

### 2.7. Identification of reaction products

The purified enzyme (13.6 mU) was incubated with 0.1 mmol of 6-AHA, hexylamine, 6-amino-1-hexanol or 1,6-diaminohexane in 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.0, at 30 °C for 1 h, and the reaction was stopped by boiling for 3 min. The reaction productions were analyzed using following methods.

Formation of hydrogen peroxide was assayed using 50 μl of the above reaction mixture by the color development method with 4-AA, TOOS and peroxidase as described in the section of assay of enzyme activity.

Formation of ammonia was assayed by the glutamate dehydrogenase method under the following conditions. The above reaction mixture (50 μl) was incubated with 3 μmol of α-ketoglutarate, 0.36 μmol of NADPH and 20 units of glutamate dehydrogenase at pH 8.0, in a final volume of 1.0 ml. The formation of glutamic acid, which is compatible with ammonia amounts released from substrate in the previous reaction, was spectrophotometrically followed at 30 °C by measuring the absorbance at 340 nm.

The oxidation product from 6-AHA was analyzed by HPLC with a TSKgel DEAE-5PW column (Tosoh, Tokyo, Japan). The product was eluted by a linear gradient (0–100%) with water and 0.3 M NaCl solution for 10 min, and followed by 0.3 M NaCl solution for 10 min at a flow rate of 0.8 ml/min. The elution was monitored at 210 nm.

Identification of aldehyde group was carried out by the method of Paz et al. [9] using 3-methyl-2-benzothiazolinone hydrazone (MBTH) as follows. Derivative 1; The 50 μl of reaction mixture or eluate from a TSKgel DEAE-5PW column was incubated with 1.0% MBTH solution (0.3 ml) and 0.2 M glycine–HCl buffer, pH 4.0 (0.75 ml), at 25 °C for 20 min. Derivative 2; The 50 μl of reaction mixture or eluate from a TSKgel DEAE-5PW column was incubated with 1.0% MBTH solution (0.30 ml) and 0.2 M glycine–HCl buffer, pH 4.0 (0.15 ml) at 25 °C for 20 min. Then, 0.75 ml of 0.2 M $FeCl_3$ solution was added into the reaction mixture and incubation was continued at 25 °C for 10 min. The absorption spectra of derivative 1 and derivative 2 were analyzed.

### 2.8. Other analytical methods

Protein concentration was spectrophotometrically determined by measuring the absorbance value at 280 nm. The $E^{1\%}_{1\,cm}$ value of 10. 0 was used throughout this work.

SDS-PAGE was performed according to the method of Laemmli [10]. Proteins were stained with Coomassie Brilliant Blue R-250. Molecular mass of denatured enzyme was estimated by SDS-PAGE using standard markers of molecular mass (Sigma, Japan, Tokyo). Molecular mass of intact enzyme was estimated by gel filtration on a TSK gel G3000$SW_{XL}$ column equilibrated with 50 mM potassium phosphate buffer, pH 7.0 containing 0.3 M NaCl.

The copper concentration was analyzed using Inductively Coupled Plasma-Atomic Emission Spectrometry (Shimadzu ICPE spectrometry-9000) under the following conditions. Radio frequency power, 1.2 kW; plasma gas flow rate, 10.0 l/min.

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

## 3. Results

### 3.1. Isolation of microorganisms

In the first step, five strains exhibiting oxidase activity for 6-AHA were isolated from soil samples after triple-enrichment culture using the 6-AHA medium containing 0.3% 6-AHA as a sole nitrogen source. Then, substrate specificity of the enzymes from these five strains was analyzed using each partially purified enzyme solution eluted from a DEAE-Toyopearl column. Of these strains selected, an enzyme from a fungal strain, No. 274, exhibited high activity for 6-AHA, hexylamine, 6-aminohexanol and 1,6-diaminohexane. We, therefore, selected this strain and used in the following studies.

### 3.2. Identification of isolated strain

The sequence of 28S rDNA-D1/D2 of the selected strain was 99.1% identical to that of *Phialemonium* aff. *dimorphosporum* II-056.3b (AY188371). When the selected strain was incubated at 25 °C for 10 days, it grew well on the agar plates of potato-dextrose, malt and oatmeal. The colonies were velvet with a color of grayish yellow-yellowish white on the potato-dextrose agar plate, grayish yellow-yellowish gray on the malt agar plate and olive brown-yellowish gray on the oatmeal agar plate. The hyphae had septa and conidiophores did not develop or very short conidiophores arose from the vegetative hyphae. Adelophialides were mainly recognized. Conidia were phialidic and oval, and its surface was smooth. On the basis of above genetic and morphological characteristics, we concluded that the selected strain is imperfect fungus belonging to the genus *Phialemonium* and named *Phialemonium* sp. AIU 274.

**Table 1**
Effect of nitrogen source on production of ω-amino compound-oxidizing enzyme by *Phialemonium* sp. AIU 274.

| Nitrogen | pH of culture broth | Cell growth (mg/100 ml broth) | Enzyme activity (mU/100 ml broth) |
|---|---|---|---|
| 6-Aminohexanoic acid | 6.44 | 73 | 20.8 |
| L-Lysine | 6.21 | 395 | 0.8 |
| Ammonium sulfate | 4.23 | 376 | 0 |

The spore suspension (1.0 ml) of *Phialemonium* sp. AIU 274 was inoculated into 150 ml of the medium containing 0.3% indicated nitrogen source and it was incubated at 30 °C for 4 days. Mycelia of 150 ml culture broth were disrupted by a Multi-beads shocker at 2200 rpm for 8 min. The oxidase activity was assayed under standard assay conditions using crude enzyme solution and 20 mM 6-AHA. *Phialemonium* sp. AIU 274 did not grow with the medium containing 6-aminohexanol, 1,6-diaminohexane or hexylamine as a sole nitrogen source.

### 3.3. Enzyme induction

Effects of nitrogen sources on enzyme production were investigated using 150 ml of the medium containing 6-AHA, 6-aminohexanol, 1,6-diaminohexane, hexylamine, L-lysine or ammonium sulfate as a sole nitrogen source in a 500-ml shaker flask. *Phialemonium* sp. AIU 274 grew well in the medium containing 6-AHA, L-lysine or ammonium sulfate, but not in the medium containing 6-aminohexanol, 1,6-diaminohexane or hexylamine. The oxidase activity for 6-AHA was detected in the mycelia grown in the medium containing 6-AHA or L-lysine, and enzyme productivity of the 6-AHA medium was higher than that of the L-lysine medium. No oxidase activity for 6-AHA was detected in the mycelia grown with the ammonium sulfate medium (Table 1). These results indicated that compounds containing the amino group and carboxyl group might be essential for induction of the enzyme. Then, the strain was incubated with 2 l of the 6-AHA medium in a 3-l culture flask at 30 °C for 4 days according to the method described in the section of cultivation of selected strain, and the oxidase activity for 6-AHA was assayed each day. The oxidase activity reached maximum between 24 and 36 h of cultivation (Fig. 1). Therefore, the mycelia harvested after 30 h of incubation were used for purification of the enzyme.

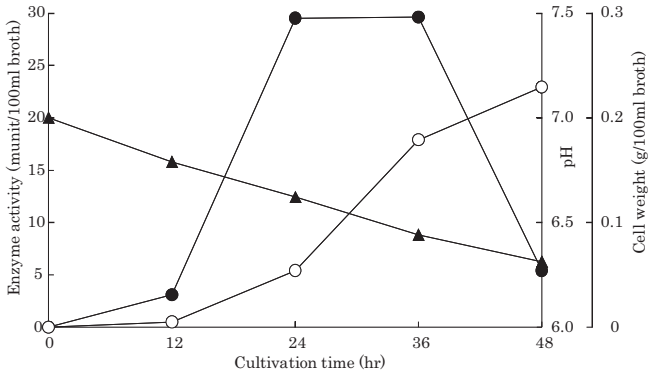


**Fig. 1.** Effect of cultivation time on enzyme production by *Phialemonium* sp. AIU 274. *Phialemonium* sp. AIU 274 was incubated in a 3-l culture flask containing 2 l of the 6-AHA medium at 30 °C with shaking (115 strokes/min). The 6-AHA oxidase activity was assayed each day under standard assay conditions at pH 7.0. Closed circles, oxidase activity for 6-AHA; open circles, cell growth; closed triangles, pH of culture broth.

**Table 2**
Summary of purification of ω-amino compound-oxidizing enzyme from *Phialemonium* sp. AIU 274.

| Step | Activity (unit) | Protein (mg) | Specific activity (unit/mg protein) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|
| Cell-free extract | 14.4 | 8650 | 0.0017 | 100 | 1.0 |
| Ammonium sulfate | 11.5 | 4640 | 0.0025 | 80 | 1.5 |
| GigaCap Q-Toyopearl | 9.11 | 60.1 | 0.152 | 63 | 89 |
| Phenyl-Toyopearl | 7.78 | 9.90 | 0.786 | 54 | 462 |
| DEAE-Toyopearl | 4.05 | 3.93 | 1.03 | 28 | 606 |

Purification of enzyme was carried out using mycelia obtained from 32 l culture broth of the 6-AHA medium. Enzyme activity was assayed under standard assay conditions using 20 mM 6-AHA. Specific activity was expressed as units per milligram of protein.

### 3.4. Purification and molecular mass

A 600-fold purification of the enzyme was achieved by a procedure summarized in Table 2. The purified enzyme showed a single protein band on native-PAGE (line A of Fig. 2). The denatured enzyme also showed a single protein band on SDS-PAGE, and the molecular mass was estimated to be 75 kDa (line B of Fig. 2). The molecular mass of the native enzyme was estimated to be 150 kDa by a TSK gel G3000SW$_{XL}$ column chromatography. These results indicated that the enzyme consisted of two identical subunits.

The purified enzyme solution exhibited absorption maximum at 275 nm and a gentle absorption curve without marked absorbance maximum in visible region (Fig. 3). These results indicated that the enzyme did not contain a flavin as a prosthetic group. The presence of one copper atom per enzyme protein was identified by a Shimadzu ICPE spectrometry-9000, which was similar to that of amine oxidase-I from *Aspergillus niger* AKU 3302 [6].

### 3.5. Substrate specificity and kinetic values

The enzyme oxidized ω-aminocarboxylic acids, ω-aminoalcohols, aliphatic monoamines, diamines and aromatic amines, but did not oxidize L-ornithine, L-lysine, D-lysine, $N^{\varepsilon}$-Z-L-lysine and $N^{\varepsilon}$-Z-D-lysine (Table 3). Thus, the enzyme was specific to the ω-amino compounds. In addition, the oxidation rates became faster by increasing the carbon chain length in all substrates tested, and oxidation speeds of monoamines were faster than those of aminocarboxylic acids, aminoalcohols and diamines in the aliphatic substrates from $C_4$ to $C_6$. The enzyme also oxidized aromatic amines such as benzylamine, 2-phenylethylamine and 4-phenylbutylamine, and the reaction rates were also affected by the side-chain length. Thus, the enzyme oxidized a wide variety of the ω-amino compounds with different functional groups, and the reaction speed was affected by the functional groups and carbon chain length of substrates.

Effect of substrate concentration on enzyme activity was analyzed under standard assay conditions using 6-AHA, 6-amino-1-hexanol, hexylamine and 1,6-diaminohexane, and the kinetic values for these aliphatic $C_6$ substrates were calculated by a plot of $s/v$ against $s$. The $K_m$ value for hexylamine was lowest in these substrates, while that for 1,6-diaminohexane was not calculated, because the speed of 1,6-diaminohexane oxidation exhibited a sigmoidal curve against its concentrations. The $K_m$ value for 6-AHA was higher than those for 6-aminohexanol and hexylamine (Table 4). Thus, the binding affinity between enzyme and substrate was strongly affected by another functional group in the ω-amino compounds.

Effect of carbon chain length of substrate on the binding affinity to enzyme was analyzed using aliphatic amines from $C_2$ to $C_8$. In these aliphatic amines, the $K_m$ values became lower by increasing

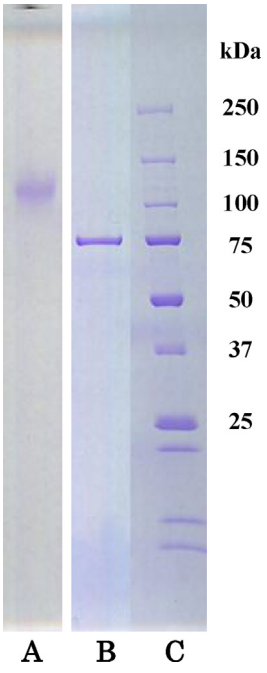


**Fig. 2.** Native- and SDS-PAGE of purified enzyme. Line A, native-PAGE of intact enzyme; Line B, SDS-PAGE of denatured enzyme; Line C, SDS-PAGE of standard proteins.

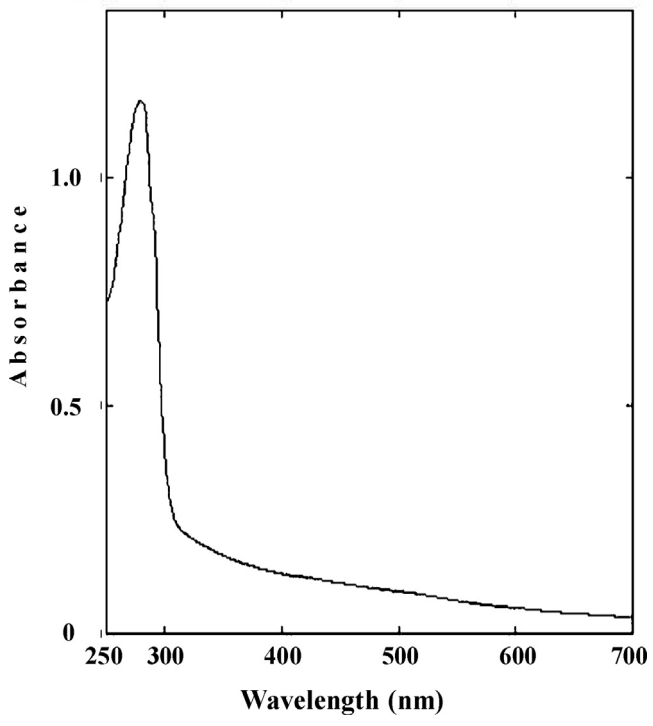


**Fig. 3.** Absorption spectrum of purified enzyme solution. The purified enzyme solution with specific activity of 1.03 units/mg protein was used. UV/visible spectrum of the enzyme was measured with a Shimadzu UV-2450 spectrophotometer.

**Table 3**
Substrate specificity of ω-amino compound-oxidizing enzyme from *Phialemonium* sp. AIU 274.

| Substrate | Relative activity (%) | Substrate | Relative activity (%) |
|---|---|---|---|
| *Aminocarboxylic acids* | | *Monoamines* | |
| 2-Aminoacetic acid | 0 | Ethylamine | 7 |
| 3-Aminopropanoic acid | 0 | Propylamine | 42 |
| 4-Aminobutyric acid | 0 | Butylamine | 112 |
| 5-Aminopentanoic acid | 2 | Pentylamine | 188 |
| 6-Aminohexanoic acid | 100 | Hexylamine | 192 |
| 7-Aminoheptanoic acid | 146 | Heptylamine | 299 |
| 8-Aminooctanoic acid | 140 | Octylamine | 359 |
| *Aminoalcohols* | | *Diamines* | |
| 2-Aminoethanol | 10 | 1,2-Diaminoethane | 0 |
| 3-Amino-1-propanol | 83 | 1,3-Diaminopropane | 1 |
| 4-Amino-1-butanol | 87 | 1,4-Diaminobutane | 9 |
| 5-Amino-1-pentanol | 138 | 1,5-Diaminopentane | 10 |
| 6-Amino-1-hexanol | 161 | 1,6-Diaminohexane | 25 |
| | | 1,7-Diaminoheptane | 31 |
| | | 1,8-Diaminooctane | 71 |
| | | 1,10-Diaminodecane | 131 |
| *Amino acids and derivatives* | | *Aromatic amines* | |
| L-Ornithine | 0 | Benzylamine | 54 |
| L-Lysine | 0 | 2-Phenylethylamine | 75 |
| $N^{\alpha}$-Z-L-lysine | 1 | 4-Phenylbutylamine | 188 |
| $N^{\varepsilon}$-Z-L-lysine | 0 | 4-Hydroxyphenyl ethylamine | 65 |
| D-Lysine | 0 | Tryptamine | 10 |
| $N^{\alpha}$-Z-D-lysine | 2 | 5-Hydroxytryptamine | 0 |
| $N^{\varepsilon}$-Z-D-lysine | 0 | Histamine | 12 |
| | | Dopamine | 0 |

Enzyme activity was assayed under standard assay conditions using 0.01 U/ml of enzyme activity for 6-AHA. Relative activity is repressed as percent of the oxidase activity for 6-AHA.

carbon chain length. In contrast, the $V_{max}$ values became higher in the longer carbon chain amines (Table 4). These results indicated that the enzyme preferred the medium- and long-chain amines in the ω-amino compounds tested.

### 3.6. Reaction products

When 0.1 mmol 6-AHA was incubated with 13.6 mU of purified enzyme at 30 °C for 1 h at pH 7.0, formation of 1.2 μmol of hydrogen peroxide and 0.98 μmol of ammonia was confirmed. Formation of oxidation product with the aldehyde group was confirmed by the MBTH method. Then, the incubation time was prolonged up to 3 h, and the oxidation product was analyzed by HPLC with a TSKgel DEAE-5PW column. The oxidation product was eluted at 17.1 min from the column, and the peak area comparably increased by the incubation time (data not shown). When 0.1 M hexylamine, 6-aminohexanol or 1,6-diaminohexane was incubated in the same conditions, formation of 0.70, 0.92 and 0.44 μmol of hydrogen peroxide and 0.70, 0.82 and 0.49 μmol of ammonia were determined, respectively. Thus, it was demonstrated that the enzyme catalyzed oxidative deamination of the ω-amino group of aminocarboxylic acids, aminoalcohols, monoamines and diamines according to schemes in Fig. 4.

**Table 4**
Kinetic properties of ω-amino compound-oxidizing enzyme from *Phialemonium* sp. AIU 274.

| Substrate | $K_m$ (mM) | $V_{max}$ (μmol/min/mg protein) | $V_{max}/K_m$ |
|---|---|---|---|
| Ethylamine | 113 | 0.334 | 0.00295 |
| Propylamine | 20.7 | 0.621 | 0.0301 |
| Butylamine | 3.01 | 0.924 | 0.307 |
| Pentylamine | 0.705 | 1.39 | 1.97 |
| Hexylamine | 0.550 | 1.39 | 2.53 |
| Heptylamine | 0.379 | 1.97 | 5.20 |
| Octylamine | 0.349 | 2.57 | 7.36 |
| 1,6-Diaminohexane | n.d. | n.d. | – |
| 6-Amino-1-hexanol | 2.31 | 0.579 | 0.251 |
| 6-Aminohexanoic acid | 31.0 | 0.915 | 0.0295 |

The oxidase activity was assayed under standard assay conditions using indicated substrates. The $K_m$ and $V_{max}$ values were calculated using 20–200 mM of ethylamine, 2.0–80 mM propylamine, 1.0–20 mM butylamine, 0.025–10 mM of pentylamine, 0.025–10 mM of hexylamine, 0.025–5.0 mM of heptylamine, 0.025–5.0 mM of octylamine, 1.0–20 mM of 6-amino-1-hexanol and 5.0–80 mM of 6-AHA. n.d., $K_m$ value was not determined, because the speed of 1,6-diaminohexane oxidation exhibited a sigmoidal curve against its concentrations.

**Table 5**
Effects of chemicals and metals on oxidase activity for 6-AHA.

| Chemical | Relative activity (%) |
|---|---|
| None | 100 |
| Hydroxylamine | 0 |
| Hydrazine | 0 |
| Phenylhydrazine | 0 |
| Semicarbazide | 0 |
| Iproniazid | 5 |
| Isoniazid | 0 |
| Clorgyline | 80 |
| Pargyline | 60 |
| *o*-Phenanthroline | 44 |
| α,α′-Dipyridyl | 57 |
| 8-Hydroxyquinoline | 6 |
| EDTA | 90 |
| $NaN_3$ | 80 |
| *N*-Ethylmaleimide | 22 |
| Monoiodo acetic acid | 90 |
| KCN | 64 |
| $MgCl_2$ | 72 |
| $NiCl_2$ | 65 |
| $CoCl_2$ | 90 |
| $MnCl_2$ | 90 |
| $CuCl_2$ | 39 |
| $FeCl_2$ | 24 |
| $FeCl_3$ | 82 |

Enzyme activity was assayed under standard assay conditions with 1 mM chemicals or metals, except for 0.1 mM clorgyline. Relative activity was obtained as percentage of enzyme activity without chemicals.

### 3.7. Effect of compounds on enzyme activity

Effect of compounds on enzyme activity was investigated under standard assay conditions by addition of 1 mM chemicals or metals. The enzyme activity was strongly inhibited by carbonyl reagents such as hydroxylamine, hydrazine or phenylhydrazine and inhibitors of copper-containing amine oxidase such as isoniazid or iproniazid. The enzyme activity was also strongly inhibited by semicarbazide but not by clorgyline and pargyline (Table 5). We further confirmed that the enzyme activity was completely inhibited by addition of 0.1 μM phenylhydrazine, 1 μM hydroxylamine or 10 μM hydrazine, and 97%, 95% and 90% of the enzyme activity were inhibited by addition of 0.1 mM of isoniazid, semicarbazide and iproniazid, respectively (Fig. 5). Chelating reagents such as 8-hydroxyquinoline, *o*-phenanthroline and α,α′-dipyridyl also inhibited the oxidase activity. In the metals tested, $FeCl_2$ and $CuCl_2$ inhibited the oxidase activity (Table 5). These results indicated that copper might play an important role in the oxidation reactions.

### 3.8. N-terminal amino acid sequence

The amino acid sequence of the intact enzyme was determined using Shimadzu gas-phase protein sequencer equipped with an on-line reverse-phase chromatography system for identification of PTH-amino acids. However, the N-terminal sequence of the intact protein was not identified, indicating that N-terminus of this enzyme might be blocked.

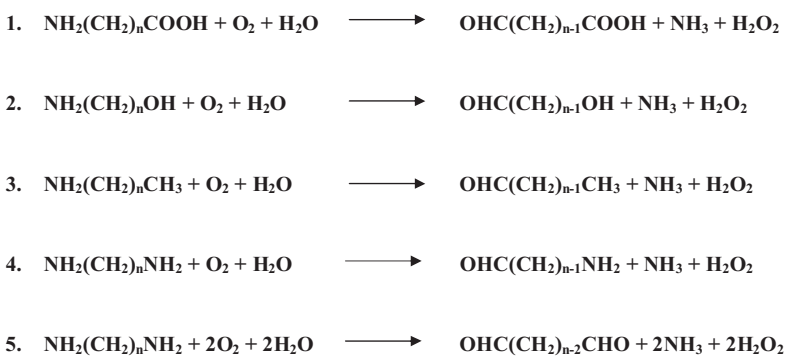


**Fig. 4.** Reaction scheme for oxidation of ω-amino compounds by enzyme from *Phialemonium* sp. AIU 274.

### 3.9. Isoelectric point

A single peak of the enzyme activity at pH 5.7 was observed after isoelectric focusing with carrier Pharmalyte, pH 3.5–10.

## 4. Discussion

The L-amino acid oxidases and amine oxidases are well known as enzymes capable of oxidizing certain compounds with amino group. In these enzymes, L-amino acid oxidases (EC 1.4.3.2) contain flavin as the prosthetic group, and catalyze oxidative deamnination of the α-amino group of α-amincarboxylic acids to the corresponding α-keto acids with the subsequent release of ammonia and hydrogen peroxide [11–15]. Some other L-amino acid oxidases also catalyze oxidative deamnination the ε-amino group of L-lysine and/or $N^{\alpha}$-acyl-L-lysine [16,17]. However, those L-amino acid oxidases do not oxidize ω-aminocarboxylic acids, ω-aminoalcohols and aliphatic amines. In contrast, amine oxidases, which are classified into two groups, flavin-containing amine oxidase (EC 1.4.3.4) and copper-containing amine oxidase (EC 1.4.3.21), catalyze oxidation of a wide range of amines to the corresponding aldehydes with the subsequent release of ammonia and hydrogen peroxide, but do not oxidize free L-amino acids. Recently, we demonstrated that copper-containing amine oxidase from *A. niger* AKU 3302 oxidized not only aliphatic amines but also $N^{\alpha}$-Z-L-lysine and $N^{\alpha}$-Z-D-lysine

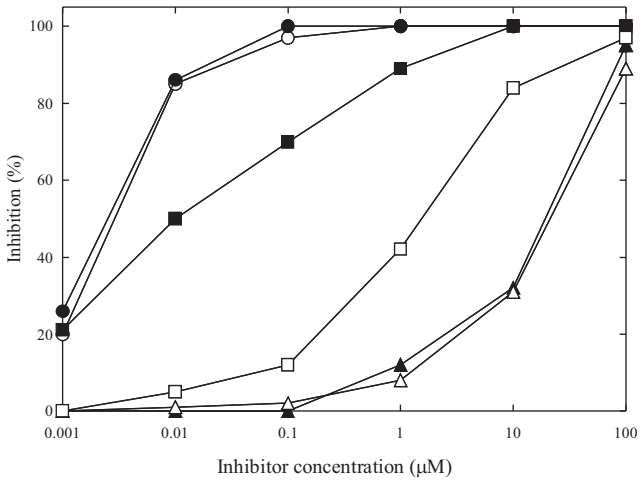


**Fig. 5.** Effect of inhibitor concentration on 6-AHA oxidizing activity. The enzyme activity was assayed under standard assay conditions by addition of 0.001–100 μM inhibitor. Closed circles, phenylhydrazine; open circles, hydroxylamine; closed squares, hydrazine; open squares, isoniazid; closed triangles, semicarbazide; open triangles, iproniazid.

[18]. However, oxidation of aliphatic ω-aminocarboxylic acids and ω-aminoalcohols by amine oxidases has not been reported yet.

Here, we first demonstrated that *Phialemonium* sp. AIU 274 produced a new enzyme catalyzing oxidative deamination of a wide variety of ω-amino compounds such as ω-amincarboxylic acids, ω-aminoalcohols, aliphatic amines, aliphatic diamines and aromatic amines. The enzyme efficiently oxidized medium- and long-chain ω-amino compounds, and the oxidation speeds of aliphatic amines were faster than those of aminoalcohols, aminocarboxylic acids and diamines. The $K_m$ value for aliphatic amine was lower than those for aminoalcohol and aminocarboxylic acid in the ω-amino $C_6$ compounds tested. In addition, the higher $V_{max}/K_m$ values were obtained by the longer carbon chain amines in alphatic amines tested. Thus, the *Phialemonium* enzyme efficiently oxidized medium- and long-chain amines in the ω-amino compounds. We further revealed that copper played an important role in the above oxidation reactions by the *Phialemonium* enzyme. In addition, the enzyme was strongly inhibited by semicarbazide but not by clorgyline (inhibitor of minoamine oxidase A) and pargyline (inhibitor of minoamine oxidase B). These results indicated that the *Phialemonium* enzyme might be classified into the semicarbazide-sensitive amine oxidase in the copper-containing amine oxidases. The purified enzyme solution from *Phialemonium* sp. AIU 274 did not exhibit the marked absorbance maximum in visible region, whereas that of the copper-containing amine oxidase I from *A. niger* AKU 3302 exhibited absorbance maximum at 490 nm [6]. These results indicated that the binding states of copper or existence of another cofactor might be different in both enzymes. The p*I* value and N-terminal amino acid were also different in both enzymes, although the molecular mass of subunit was similar to each other.

Studies of gene cloning and DNA sequence analysis of the *Phialemonium* enzyme are now in progress to reveal the unique characteristics of the enzyme in more detail. Studies of enzymatic production of aldehyde carboxylic acids, aldehyde alcohols, aldehyde amines are also underway using the *Phialemonium* enzyme and the ω-amino compounds.

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