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Characterization of a novel L-amino acid oxidase with protein oxidizing activity from *Penicillium steckii* AIU 027

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An enzyme exhibiting oxidase activity for β-lactoglobulin, myoglobin, and L-lysine-containing peptides was found from a newly isolated fungal strain, *Penicillium steckii* AIU 027. The enzyme also oxidized L-amino acids, N^{α}-benzyloxycarbonyl-L-lysine (N^{α}-Z-L-lysine) and N^{ε}-Z-L-lysine, but not D-amino acids and amines. Thus, the enzyme was classified into a group of L-amino acid oxidases (L-AAOs). However, characteristics of this L-AAO were significantly different from those of other L-AAOs as follows. The L-AAO from *P. steckii* AIU 027 oxidized both the α-amino group and the ε-amino group in L-amino acids and L-lysine-containing peptides, and the K_m values for L-lysine-containing polypeptides were lower than those for N^{α}-Z-L-lysine and L-lysine-containing dipeptides. The enzyme contained flavin and iron, and composed of four identical subunits with molecular mass of 75.3 kDa. The N-terminal amino acid sequence, ENIADVADAMGPWFDGVAYMKSKKN, was different from that of other L-AAOs. Thus, the L-AAO with protein oxidase activity was first reported here from *P. steckii* AIU 027.

[**Key words:** L-Amino acid oxidase; Protein oxidase; L-Lysine; Lysyl oxidase; *Penicillium steckii*]

A wide variety of L-amino acids oxidases (L-AAOs), which contain flavin as a prosthetic group and catalyze oxidative deamination of L-amino acids but not of D-amino acids and amines, have been found from many fungal and bacterial strains (1–11), and registered in the different EC numbers by the unique substrate specificity of the L-AAOs. However, the L-AAOs are also classified into three groups by the regiospecificity for deamination of L-lysine and/or L-lysine derivatives. The first group enzymes are specific to the α-amino group of L-amino acids (1–9). The second group enzymes oxidize both the α-amino group of L-amino acids or N^{ε}-acyl-L-lysine and the ε-amino group of N^{α}-acyl-L-lysine or L-lysine-containing peptides (10). The third group enzymes preferably oxidize the ε-amino acid group of L-lysine (11). In those enzymes, the L-AAOs with limited substrate specificity such as L-glutamate oxidase (EC 1.4.3.11) and L-lysine-α-oxidase (EC 1.4.3.14), which are belonging to the first group, were utilized for selective assay of L-glutamic acid and L-lysine, respectively (12,13). The L-AAO with broad substrate specificity (EC 1.4.3.2) from *Rhodococcus* sp. AIU Z-35-1, which is belonging to the second group, was applied to development of new enzymatic methods for production of N^{α}-benzyloxycarbonyl-L-aminoadipic δ-semialdehyde (N^{α}-Z-L-AASA) from N^{α}-Z-L-lysine and D-amino acids from DL-amino acids (14). This enzyme also oxidized the ε-amino group of lysine-containing dipeptides, but oxidation of polypeptides was not demonstrated (10). The L-lysine-ε-oxidase from *Marinomonas mediterranea*, which is belonging to the third group, oxidized the ε-amino group of L-lysine, but did not oxidize the ε-amino group of lysine derivatives (11). The enzyme was also utilized for selective assay of L-lysine (15). Thus, oxidation of the ε-amino acid group of lysine residues in proteins by the L-AAOs has not been reported yet, although certain enzymes of the second and third groups were able to oxidize the ε-amino acid group of L-lysine or L-lysine derivatives.

In the studies of microbial L-AAOs, we newly isolated a fungal strain, which produced an enzyme exhibiting oxidase activity for L-amino acids, L-lysine-containing peptides and proteins. We therefore purified the enzyme and revealed some properties of the enzyme. The present paper describes identification of isolated strain, enzyme production and some remarkable properties of the L-AAO produced by the newly isolated strain.

MATERIALS AND METHODS

Chemicals β-Lactoglobulin from bovine milk, myoglobin, poly-L-lysine and L-lysine-containing peptides were purchased from Sigma–Aldrich Japan (Tokyo, Japan). L-Amino acids were from Wako Pure Chemicals (Osaka, Japan). N^{α}-Z-L-lysine, N^{ε}-Z-L-lysine, and N^{α}-Z-L-ornithine were from Watanabe Chemicals (Hiroshima, Japan). Fish collagen peptide and salmon gelatin (enzymatic hydrolysate) were from Maruha Nichiro Food (Tokyo, Japan) and Ihara (Sapporo, Japan), respectively. Peroxidase was gift from Amano Enzyme (Nagoya, Japan). All other chemicals used were of analytical grade and commercially available. Aminooctyl–Toyopearl resins, which were used for enzyme purification, were prepared using Toyopearl AF-Epoxy-650M and 1,8-diaminooctane as follows. Toyopearl AF-Epoxy-650M (10 g of wet weight) was stirred with 30 ml of 0.5 M 1,8-diaminooctane solution adjusted to pH 12 at 30°C for 24 h. Then, the resins were collected by filtration and washed with 1.0 M NaCl solution and water.

Screening of microorganism Microorganisms were first isolated by triple-enrichment culture using a N^{α}-Z-L-lysine medium, pH 7.0, consisting of 0.5% N^{α}-Z-L-lysine, 0.5% 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 N^{α}-Z-L-lysine

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medium at 30°C for 3 days, after which a cell-free extract was prepared by disrupting the cells using a Multi-beads shocker (Yasui Kikai, Osaka, Japan) at below 5°C for 8 min, and the oxidase activities for N^{α}-Z-L-lysine, β-lactoglobulin and gelatin were assayed using the cell-free extract from each isolated strain. In these steps, five strains were selected as producers of enzyme catalyzing oxidation of above three compounds. Then, each crude enzyme solution from the five selected strains was applied to a DEAE—Toyopearl column and the adsorbed enzymes were eluted by a linear gradient with 10 mM potassium phosphate buffer, pH 7.0, and 0.2 M NaCl. The enzyme activity of the eluates was assayed using β-lactoglobulin, gelatin and N^{α}-Z-L-lysine as substrate. The strain, which produced an enzyme exhibiting high activity for β-lactoglobulin and gelatin, was finally selected, and used in this study.

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) 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 sequences of 28S rDNA-D1/D2 and ITS-5.8S rDNA were analyzed using a PrimeSTAR HS DNA polymerase (TakaraBio, Otsu, Japan), an ABI BigDye Terminator v3.1 Kit (Applied Biosystems, Foster City, CA, USA), and a ABI PRISM 3130x1 Genetic Analyzer System (Applied Biosystems). The sequence alignment and calculation of the homology levels were carried out using the database of Gen Bank, DDBJ and EMBL.

Cultivation of selected strain A newly isolated strain, *Penicillium steckii* AIU 027, was first incubated in a 500-ml shaker flask containing 150 ml of a L-lysine medium, pH 7.0, consisting of 0.5% L-lysine, 0.5% glucose, 0.2% KH_2PO_4, 0.1% Na_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, at 30°C for 2 days with shaking (115 strokes/min). The culture (200 ml) was then transferred into a 3-l culture flask containing 2 l of the L-lysine medium. After the cultivation was carried out at 30°C for 3 days, the mycelia were harvested by filtration, washed with 10 mM potassium phosphate buffer, pH 7.0, and then stored at −30°C until use.

Assay of enzyme activity The oxidase activity for β-lactoglobulin was assayed by measuring the rate of hydrogen peroxide formation as follows. The standard reaction mixture contained 4 mg of β-lactoglobulin, 0.6 μmol of 4-aminoantipyrine (4-AA), 1.94 μmol of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt dehydrate (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 absorbance at 555 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing 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.

Purification of enzyme Purification of enzyme was carried out using mycelia obtained from 38 l culture broth of the L-lysine medium. All procedures were done at 5—10°C. The buffer used was potassium phosphate buffer, pH 6.0.

In the first step, 66 g of mycelia (wet weight) were suspended in 680 ml of 10 mM buffer solution, and disrupted by a Multi-beads shocker. The supernatant solution was obtained by centrifugation at 10,000 ×*g* for 10 min, and the cell pellets were suspended again in 600 ml of a newly prepared 10 mM buffer solution. The cell-disruption was carried out three times under the same conditions described above, and the each supernatant solution obtained was mixed. To the 1900 ml of supernatant solution, 460 g of solid ammonium sulfate was added to reach 40% saturation, and resulting precipitate was discarded by centrifugation at 10,000 ×*g* for 10 min.

The supernatant solution was then applied to a Phenyl—Toyopearl column (20 cm × 2.5 cm diameter) equilibrated with 10 mM buffer solution containing 2.0 M ammonium sulfate. After the column was washed with same buffer solution, the adsorbed enzyme was eluted with 10 mM buffer solution containing 1.5 M ammonium sulfate. The active fractions were collected, and deionized by ultrafiltration.

The deionized enzyme solution was then applied to a DEAE—Toyopearl column (20 cm × 2.5 cm diameter) equilibrated with 40 mM buffer solution, and the column was washed with the same buffer solution. The adsorbed enzyme was then eluted by a linear gradient with 40 mM buffer solution and 40 mM buffer solution containing 0.12 M NaCl (500 ml each). The active fractions were collected, and dialyzed against 30 mM buffer solution.

The dialyzed enzyme solution was applied to an Aminooctyl—Toyopearl column (10 cm × 2.5 cm diameter) equilibrated with 50 mM buffer solution, and the column was washed with the same buffer solution. The adsorbed enzyme was then eluted by a linear gradient with 50 mM buffer solution and 50 mM buffer solution containing 0.2 M NaCl (250 ml each). The active fractions were collected, and dialyzed against 10 mM buffer solution.

The dialyzed enzyme solution was then applied to a Hydroxyapatite column (38 cm × 1.0 cm diameter) equilibrated with 20 mM buffer solution, and the column was washed with the same buffer solution. The adsorbed enzyme was then eluted by a linear gradient with 20 mM and 0.12 M buffer solutions (150 ml each). The active fractions were combined and concentrated to 0.8 ml by ultrafiltration.

The concentrated enzyme solution was applied to a Toyopearl HW-55 column (53 cm × 1.3 cm diameter) equilibrated with 50 mM buffer solution. The active fractions were combined and the purity was analyzed.

Identification of reaction products The purified enzyme (1.16 μg or 2.94 μg) was incubated with 180 μmol of N^{α}-Z-L-lysine or 10 mg of β-lactoglobulin in 1.0 ml of 0.1 M potassium phosphate buffer, pH 6.0, at 30°C for 1 h, and the reaction was stopped by boiling for 3 min. The reaction productions were analyzed by the following methods.

Hydrogen peroxide formed by the oxidation of N^{α}-Z-L-lysine or β-lactoglobulin was assayed by the color development method using peroxidase, 4-AA and TOOS as described in the section of oxidase activity measurement.

Ammonia formed by the oxidation of N^{α}-Z-L-lysine was assayed by the glutamate dehydrogenase method under the following conditions. The above reaction mixture (100 μl) was incubated with 3 μmol of α-ketoglutarate, 0.06 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.

Ammonia from β-lactoglobulin was assayed using Ammonia-Test Wako (Wako Pure Chemicals) after β-lactoglobulin was precipitated and discarded.

The oxidation product from N^{α}-Z-L-lysine was analyzed according to the method of Isobe et al. using a TSK-Gel DEAE-5PW column and 3-methyl-2-benzothiazolinone hydrazone (16).

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

Other analytical methods Protein concentration was spectrophotometrically determined by measuring the absorbance value at 280 nm, and the $E^{1\%}_{1\ cm}$ value of 10.0 was used throughout this work.

Native- and SDS-PAGE was performed according to the method of Laemmli (17) and proteins in the gel 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, Japan).

Metals in the enzyme were analyzed using Inductively Coupled Plasma — Atomic Emission Spectrometry (Shimadzu ICPE spectrometry-9000, Kyoto, Japan) 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—10.0 (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 value of each fraction was measured at 4°C.

RESULTS

Isolation of microorganisms Since we presumed that relatively high-molecular L-amino acid derivatives with the ω-amino group might be available for screening of enzymes, which catalyze oxidation of the ε-amino acid group of lysine residues in proteins, we used N^{α}-Z-L-lysine as a sole nitrogen source, and isolated five strains exhibiting oxidase activity for N^{α}-Z-L-lysine, β-lactoglobulin and salmon gelatin from soil samples by triple-enrichment culture using the N^{α}-Z-L-lysine medium. Then, substrate specificity of the enzyme produced by these five strains was analyzed using each partially purified enzyme solution obtained by a DEAE—Toyopearl column chromatography. Of these strains selected, one fungal strain, no. 027, produced an enzyme exhibiting high activity for gelatin and β-lactoglobulin. We therefore selected this strain and used in the following studies.

Identification of isolated strain The sequence of 28S rDNA-D1/D2 of the selected strain was 100% identical to that of *P. steckii* NRRL35367, NRRL35625, NRRL354633 and KUC1681-1 (DQ123665, EF200085, DQ123666, and HM469415, respectively), and 99.3% identical to that of *Penicillium tropicum* NRRL35470 (EU427292). The sequence of ITS-5.8S rDNA was also 100% identical to that of *P. steckii* NRRL354633 and KUC1681-1, and 99.5% identical to that of *P. steckii* NRRL35367.

When the selected strain was incubated at 25°C for 7 days, it grew well on the potato-dextrose agar plate and the oatmeal agar plate, and the colonies were velvet with a color of grayish green-pale green on the potato-dextrose agar plate and with a color of