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Characterization of L-Phenylalanine Oxidase from *Aspergillus fumigatus* KAIT-M-002

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L-Phenylalanine oxidase (EC 1.4.3.-.) was found in cells of *Aspergillus fumigatus* isolated from soil. The enzyme was purified about 3,300-fold with a yield of 4.9 % by ammonium sulfate fractionation and column chromatographies on Q Sepharose, Octyl Sepharose, Sephacryl S-300, Resouce Q and hydroxyapatite. The purified enzyme was homogeneous on native polyacrylamide gel-electrophoresis. The optimum pH for the enzyme activity was found to be at 7.0, and its enzyme was stable at pH 6-9 and below 37°C. The molecular weight of the purified enzyme was estimated to be about 110,000. The enzyme showed powerful activity toward L-phenylalanine and a little activity L-tyrosine and L-methionine. Whereas the enzyme was inactivity toward D-phenylalanine. L-Phenylalanine oxidase of *A. fumigatus* catalyzed the oxidative deaminating reaction to form hydrogen peroxide from L-phenylalanine.
Key words : L-Phenylalanine oxidase, L-Phenylalanine, *Aspergillus fumigatus*

1. INTRODUCTION

L-Amino acid oxidase has been known as the enzyme that catalyzes the oxidative deamination of L-amino acids [1]. L-Amino acids are oxidized by L-amino acid oxidase to α-keto acid with the simultaneous production of ammonia and hydrogen peroxide, but substrate specificity of L-amino acid oxidase is generally low.

Recently, L-amino acid oxidases having high substrate specificity, that is, the enzyme such as L-glutamate oxidase (EC1.4.3.11.), L-lysine α-oxidase (EC 1.4.3.14.), L-phenylalanine oxidase [2-5] have been reported.

L-Phenylalanine oxidase is particularly important as the enzyme for diagnosis of phenylketonuria. To date, L-phenylalanine oxidase which form β-phenylpyruvate from L-phenylalanine by the oxidative deaminating reaction have been known [4,5], but the enzymes that form simultaneously hydrogen peroxide from L-phenylalanine have not been almost reported except for L-phenylalanine oxidase from *Pseudomonas* sp. P-501 [3] and *Trichoderma* sp. [5].

We found L-phenylalanine oxidase in cells of *A. fumigatus* KAIT-M-002 isolated from soil. The enzyme formed oxidative deaminationally hydrogen peroxide from L-phenylalanine. This paper describes the characterization and purification of L-phenylalanine oxidase from *A. fumigatus* KAIT-M-002.

2. MATERIALS AND METHODS

2.1 Materials

L-Phenylalanine oxidase (phenylalanine : oxygen oxido-reductase) was produced in our labolatory by fermentation of *A. fumigatus* KAIT-M-002. The enzyme was purified from cell-free extracts of KAIT-M-002. Peroxidase (EC 1.11.1.7. ; Type I, 116 purpurogallin U/mg ; POD) was purchased from Sigma Chemical Co., Ltd. Q Sepharose, Octyl Sepharose, Sephacryl S-300, Resouce Q and Hitrap Desalting column were purchased from Amersham Pharmacia Biotech. Co., Ltd. Hydroxyapatite (Econo-Pac CHT-II) was purchased from Bio-Rad Labolatories Co., Ltd. *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5dimethoxyaniline (DAOS) was purchased from Dojindo Laboratories. Precast-gel (10-20 % ; native polyacrylamide gel) was purchased from NIKKYO TECHNOS Co., Ltd. Phenylmethylsulfonyl fluoride (PMSF) was purchased from Wako Pure Chemical Industries. All other reagents used were commercial products of the highest quality.

2.2 Organisms

Aspergillus fumigatus KAIT-M-002 was used throughout this study.

2.3 Medium and culture condition

The microorganism was grown on potato dextrose agar slant at 30°C for 3 days. A loopful seed of microorganisms on an agar slant was inoculated into 100 ml of medium (pH 7.0) containing 0.1 % L-phenylalanine, 0.1 % yeast extract, 1 % glucose, 0.2 % $NaNO_3$, 0.2 % K_2HPO_4, 0.001 % $MgSO_4 \cdot 7H_2O$ and 0.001 % $FeSO_4 \cdot 7H_2O$ in a 500

ml shaking flask, and then kept at 30°C with shaking for 60 h on a rotary shaker operating at 200 rpm. The medium was sterilized at 121°C for 20 min.

2.4 Preparation of cell-free extracts

All operations for the purification procedure were performed below 10°C unless otherwise specified. Cells were harvested from the culture of KAIT-M-002 by filtration, washed with a large volume of 0.1 M phosphate buffer (pH 7.0) and weighed. The harvested cells were disrupted with the same amount of a sea sand and suspended in ten-times volume of 0.1 M phosphate buffer (pH7.0) containing 1 mM PMSF toward the weight of cells. The cells and debris were removed by centrifigation at 15,000 rpm for 10 min at 4°C and discarded.

2.5 Enzyme assay

L-Phenylalanine oxidase activity was assayed colorimetrically by measuring the amount of hydrogen peroxide produced from L-phenylalanine. The reaction mixture contained 0.3 ml of 30 mM L-phenylalanine, 0.5 ml of 5 mM DAOS , 0.5 ml of 7.5 mM 4-aminoantipyrine, 0.5 ml of POD (25 U/ml) in 0.1 M phosphate buffer (pH 7.0) and 0.3 ml of the enzyme solution. After the mixture was incubated at 37°C for 15 min, and then the reaction was stopped by addition of 0.5 ml of 3 % sodium dodecyl sulfate solution. The absorbance was read at 600 nm with a spectrophotometer (model UV-1200, SHIMAZU Co.,). One unit of activity was defined as the amount of enzyme which liberated 1 μmol of hydrogen peroxide per min under the specified conditions.

2.6 Determination of molecular weight by polyacrylamide gel-electrophoresis

The molecular weight of the purified enzyme was roughly estimated by a native polyacrylamide gel-electrophoresis (NATIVE-PAGE). NATIVE-PAGE was carried out with Tris-Glycine buffer system (pH 8.5) at room temperature for 50 min at 40 mA per gel. Protein was stained with 0.1 % coomassie brilliant blue in 0.1 M Tris-HCl buffer (pH 8.4) and then the gel was destained by standing in 7.5 % acetic acid containing 25 % methanol. The following proteins were used as standards ; trypsin inhibitor (MW : 20,100), bovine serum albumin (MW : 66,267), lactate dehydrogenase [EC 3.1.4.22. ; MW : 139, 850] and ferritin (MW : 443,000).

3. RESULT

3.1 Enzyme purification

The purified enzyme was prepared from the cell-free extracts of KAIT-M-002 according to the following procedures.

Step 1. Ammonium sulfate fractionation :

Solid ammonium sulfate was added slowly to the cell-free extracts with constant stirring so as to give 35 % saturation. The resulting precipitate was removed by centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant fraction was further brought to 70 % saturation with solid ammonium sulfate. The precipitated protein was collected by centrifugation and dissolved in 20 mM phosphate buffer (pH 7.0) containing 1 mM PMSF. The solution was desalted with Hitrap Desalting column (1.6 X 2.5 cm) equilibrated with 20 mM phosphate buffer (pH 7.0) containing 1 mM PMSF at a rate of 1 ml/min.

Step 2. Column chromatography on Q-Sepharose :

The desalted enzyme solution was applied to a column (1.6 X 2.5 cm) of Q-Sepharose equilibrated with 20 mM phosphate buffer (pH 7.0) containing 1 mM PMSF. The column was eluted with the same buffer containing 0.5 M sodium chloride. Then solid ammonium sulfate was added slowly to the active fraction so as to give 1 M ammonium sulfate.

Step 3. Column chromatography on Octyl-Sepharose :

The active fraction containing 1 M ammonium sulfate was applied to a column (0.7 × 2.5 cm) of Octyl-Sepharose equilibrated with 20 mM phosphate buffer (pH 7.0) containing 1M ammonium sulfate. Then the column was eluted with a linear gradient of ammonium sulfate from 1 M to 0 M in 20 mM phosphate buffer (pH 7.0).

Step 4. Gel filtration on Sephacryl-S 300 :

The active fraction was concentrated and a 5 mililiter aliquot of it was applied to a column (1.6 X 60 cm) of Sephacryl S-300 equilibrated with 20 mM phosphate buffer (pH 7.0) containing 0.5 M sodium chloride. Then the column was eluted with the same buffer.

Step 5. Column chromatography on Resouce Q :

The active fraction was applied to a column (0.64 X 3.0 cm) of Resource Q equilibrated with 20 mM phosphate buffer (pH 7.0). Then the column was eluted with the same buffer containing 0.5 M sodium chloride.

Step 6. Column chromatography on hydroxyapatite

The buffer concentration in the active fraction was exchanged with Hitrap desalting column in order to give the concentration of 10 mM as phosphate buffer(pH 7.0). The buffer-exchanged active fraction was applied to a column of hydroxyapatite equilibrated with 10 mM phosphate buffer (pH 7.0). Then the column was eluted with 0.4 M phospate buffer (pH 7.0).

The purification procedure of L-phenylalanine oxidase from *A. fumigatus* is summarized in Table 1. The enzyme was purified about 3,300-fold over the cell-free extracts.

3.2 Native polyacrylamide gel-electrophoresis

L-Phenylalanine oxidase showed a single band of enzymatic activity on NATIVE-PAGE, coincident with the single band of protein. The molecular weight of the purified enzyme was estimated from its mobility on NATIVE-PAGE. The molecular weight of L-phenylalanine oxidase from *A. fumigatus* was calculated to be about 110,000 as shown in Figure 1.

3.3 Enzymatic Properties of L-phenylalanine oxidase

1) Action of the enzyme on various substances:

The activities of L-phenylalanine oxidase from *A. fumigatus* on various substances are summerized in Table 2. The enzyme showed powerful activity toward L-phenylalanine and a little activity L-tyrosine and L-methionine. Whereas the enzyme was inactivity toward D-phenylalanine.

2) Thermal stability:

The enzyme in 0.1 M phosphate buffer (pH 7.0) was incubated at different temperatures (30-100°C) for 10 min, and the residual activity was assayed. The results are shown in Figure 2. L-Phenylalanine oxidase from *A. fumigatus* was stable at pH 7.0 up to 37°C.

3) pH stability:

The enzymes were kept in 0.1 M buffers of different pH values (4.0-12.0) at 25°C for 10 min. Then they were adjusted to pH 7.0 and the enzyme activities were assayed. The results are shown in Figure 3. L-Phenylalanine oxidase from *A. fumigatus* was stable in the range of pH' s between 6-9.

4) pH optimum:

The effect of pH on the activity of L-phenylalanine oxidase was determined in various buffers. The results are shown in Figure 4. The optimum pH for the activity of L-phenylalanine oxidase from *A. fumigatus* was found to be at 7.0.

5) Temperature optimum:

The reaction mixture was incubated at different temperature (30-70°C) for 10 min, and then the residual activity was assayed. The optimum temperature for the activity of L-phenylalanine oxidase from *A. fumigatus* was found to be 37°C.

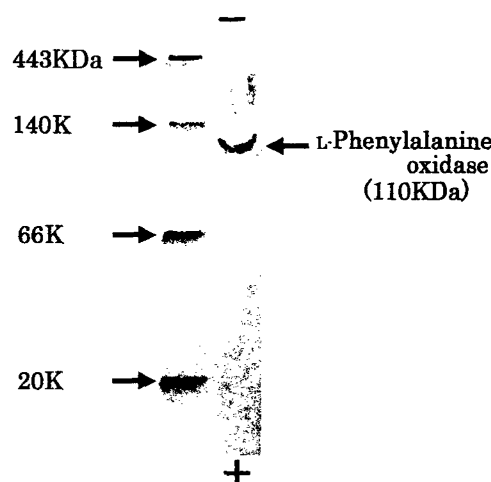

Fig. 1. Native polyacrylamide gel-electrophoresis of the purified enzyme. Right : purified enzyme, Left : standard proteins (see "MATERIALS AND METHODS").

Table 1. Purification of L-phenylalanine oxidase from *Aspergillus fumigatus* KAIT-M-002. The enzyme activity was assayed colorimetrically under the specified conditions.

Step	Total activity (mU)	Total protein (A_{280})	Specific activity (mU/A_{280})	Recovery of activity (%)
Crude extract	2686	15818	0.2 (1)	100
Ammonium sulfate fractionation	2680	9299	0.3 (2)	99.8
Q Sepharose	1424	376	3.8 (19)	53.1
Octyl Sepharose	1170	31.2	37.5 (188)	43.6
Sephacryl S-300	521	6.6	78.9 (395)	19.4
Resource Q	464	1.0	464 (2320)	17.3
Hydroxyapatite (Econo Pac CHT-II)	132	0.2	660 (3300)	4.9

4. DISCUSSION

In this study, the L-phenylalanine oxidase of *A. fumigatus* KAIT-M-002 was purified by ammonium sulfate and various column chromatographies. The enzyme was purified about 3,300-fold over the cell-free extracts, and shown to be homogeneous on NATIVE PAGE.

L-Phenylalanine oxidase from *A. fumigatus* showed activity toward L-phenylalanine, L-tyrosine and L-methionine, but little activity toward other amino acids. These properties were similar to it of L-phenylalanine oxidase from *Pseudomons* sp. P-501 [3] in the

substrate specificity. The optimum pH and the thermal stability of the enzyme from *A. fumigatus* KAIT-M-002 were found to be at 7.0 and below 37°C against those of *Pseudomonas* sp. P-501 [3] was pH 10.5 and 70°C, respectively.

L-Phenylalanine oxidase from *A. fumigatus* KAIT-M-002 formed oxidative deaminationally hydrogen peroxide from L-phenylalanine. This result shows that L-phenylalanine oxidase from *A. fumigatus* is comparable to it from *Psudomonas* sp. P-501 [3] and *Trichoderma* sp. [5].

It is reported that the enzyme from *Psudomonas* sp. P-501 [5] has simultaneously oxygenase activity in L-phenylalanine oxidase. Therefore, the presence or absence of oxygenase activity in L-phenylalanine oxidase from *A. fumigatus* KAIT-M-002 will be reported later.

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Table 2. Substrate specificity of L-phenylalanine oxidase from *A. fumigatus*.

Substrate	Relative activity (%)
L-Phenylalanine	100
L-Tyrosine	38
L-Methionine	26
L-Arginine	7
L-Leucine	4
L-Histidine	3
L-Serine	2
L-Asparagine	0
L-Asparatic acid	0
L-Isoleucine	0
L-Glutamine	0
L-Glutamic acid	0
L-Lysine	0
L-Threonine	0
L-Tryptophane	0
L-Alanine	0
L-Valine	0
L-Proline	0
D-Phenylalanine	0

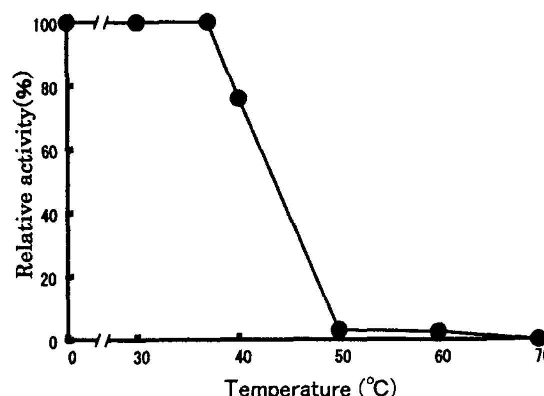

Fig. 2. The thermal stability of L-phenylalanine oxidase activity from *A. fumigatus* (see "MATERIALS AND METHODS").

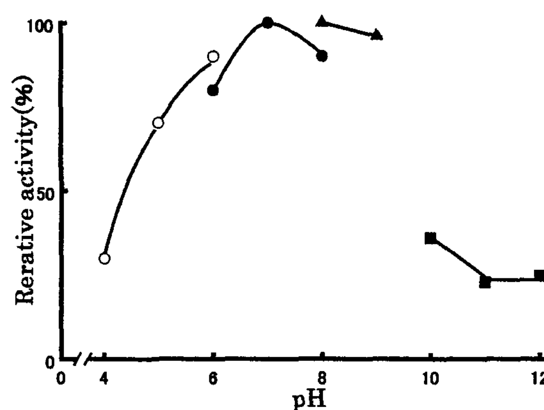

Fig. 3. The pH stability of L-phenylalanine oxidase activity from *A. fumigatus* (see "MATERIALS AND METHODS").
○ : acetate buffer, ● : phosphate buffer, ▲ : Tris-HCl buffer, ■ : borate buffer

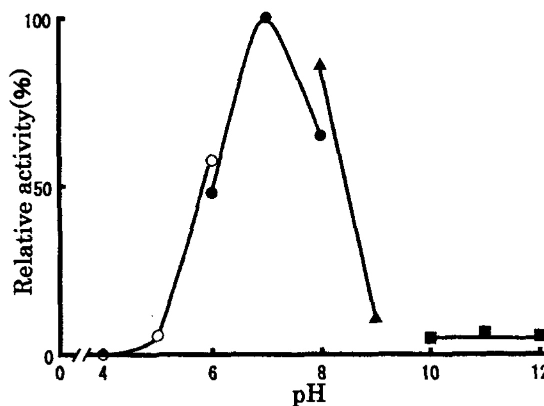

Fig. 34 The effect of pH on L-phenylalanine oxidase activity from *A. fumigatus* (see "MATERIALS AND METHODS").
○ : acetate buffer, ● : phosphate buffer, ▲ : Tris-HCl buffer, ■ : borate buffer

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