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Characterization of A Novel L-Histidine Oxidase

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A novel L-histidine oxidase produced by *Brevibacillus borstelensis* KAIT-B-022 was purified and characterized. The strain was cultivated for 18 h of incubation at 50℃ with the culture medium containing L-histidine, and the crude enzyme was extracted from the cells. L-Histidine oxidase was purified by ammonium sulfate fractionation, and column chromatographies on Octyl Sepharose, Q-Sepharose, Resource Q, Sephacryl S-100 and Sephacryl S-200. The enzyme was purified about 388-fold over the cell free extracts with 8.8% yield, and was homogeneous on native polyacrylamide gel-electrophoresis (NATIVE PAGE). The optimum pH for L-histidine oxidase activity was found to be at 7.0, and the enzyme was stable at pHs 6-11 and below 50℃. The molecular weight of L-histidine oxidase was estimated to be about 117,000 by Sephacryl S-200 gel filtration and NATIVE PAGE. L-Histidine oxidase showed powerful activity toward L-histidine, whereas the enzyme was inactive toward D-histidine, L-arginine, L-lysine, and the other L-amino acids. The enzyme possessed two heterogeneous subunits calculated to be 64,000 and 53,000, respectively.
Key words: L-Histidine oxidase, L-Amino acid oxidase, L-Histidine, *Brevibacillus borstelensis*, Substrate specificity

1. INTRODUCTION

L-Amino acid oxidase (EC 1.4.3.2, L-amino acid : oxygen oxidoreductase (deaminating)) has been known as the enzyme that catalyzes the oxidative deamination of L-amino acids. L-Amino acids are oxidized by L-amino acid oxidase to α-keto acid with the simultaneous production of ammonia and hydrogen peroxide, and its substrate specificity is generally low.

Recently, L-amino acid oxidases having high substrate specificity such as L-glutamate oxidase (EC 1.4.3.11) [1], L-lysine oxidase (EC 1.4.3.14) [2], L-phenylalanine oxidase (EC 1.4.3.-) [3], L-tryptophan oxidase (EC 1.4.3.-) [4] have been reported. However, to date, L-amino acid oxidase having high substrate specificity toward L-histidine has not been known at all.

We found a novel L-histidine oxidase (Histidine: oxygen oxidoreductase) in cell of *B. borstelensis* KAIT-B-022 isolated from soil, which oxidized specifically L-histidine to form hydrogen peroxide, ammonia, and imidazolepyruvate [5].

$$\text{L-Histidine} + O_2 + H_2O \xrightarrow{\text{L-histidine oxidase}} \text{Imidazolepyruvate} + NH_3 + H_2O_2$$

This paper describes the purification and characterization of L-histidine oxidase from the cell-free extracts of *B. borstelensis* KAIT-B-022.

2. MATERIALS AND METHODS

2.1 Materials

L-Histidine oxidase was produced in our laboratory by fermentation of *B. borstelensis* KAIT-B-022. The enzyme was purified from cell-free extracts of KAIT-B-022. Peroxidase (EC 1.11.1.7 ; Type I , 116 purpuro-gallin U/mg; POD) was purchased from Sigma Chemicals Co., Ltd. Q Sepharose, Octyl Sepharose, Resource Q, Sephacryl S-100, Sephacryl S-200 columns and blue dextran 2000 were purchased from Amersham Pharmacia Biotech. Co., Ltd. L-Histidine, sodium dodecyl sulfate (SDS) and 4-aminoantipyrine (4-AA) were purchased from Kanto Chemical Co., Inc. *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxy-aniline (DAOS) were purchased from Dojindo Laboratories. Beef extract, pepton and agar were purchased from Kyokuto, Difco and Wako Pure Chemical Industry Co. Ltd, respectively. Protein molecular weight markers were purchased from Daiichi Pure Chemicals Co. Ltd. Precast-gel (10-20% ; native poryacrylamide gel) was purchased from NIKKYOU TECHNOS Co., Ltd. The kit for protein determination was purchased from Nippon Bio-Rad Laboratories. All other reagents used were commercial products of the highest.

2.2 Microorganisms

Brevibacillus borstelensis KAIT-B-022 was used throughout this study.

2.3 Media and culture conditions

The microorganisms were grown on bouillon agar slant at 50℃ for 1 day. Medium containing 1.0% beef extract, 1.0% pepton, 0.5% sodium chloride and 1.5% agar was employed. A hundred ml of the production medium was placed in a 500 ml shaking flask and was sterilized at 121℃ for 20 min. A loopfull seed of microorganisms on a slant culture was inoculated into the flask and incubated for 18 h at 50℃ on a rotary shaker operating at 200 rpm. Medium containing 0.1% L-histidine, 0.1% L-arginine, 0.15% yeast extracts, 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.001% $Fe_2SO_4 \cdot 7H_2O$ (pH 7.0) was used for cultivation.

2.4 Preparation of cell-free extracts

All operations for the purification procedure were

performed below 10℃ unless otherwise specified. Cells were harvested from the culture of KAIT-B-022 by centrifugation at 10,000 *g* for 20 min at 4℃, washed with a large volume of 0.1 M phosphate buffer (pH 7.0) and suspended in the same buffer. Then, the cell suspension was subjected to an ultrasonication for 90 sec at a power setting of 20 kHz. The cells and debris were removed by centrifugation at 10,000 *g* for 20 min at 4℃ and discarded.

Purification of the enzyme was performed by a procedure involving ammonium sulfate fractionation, column chromatographies on Octyl Sepharose, Q-Sepharose, Resource Q, and gel filtration with Sephacryl S-100 and S-200.

2.5 Determination of cell

Cell concentration was photometrically determined by measuring the turbidity of cell at 600 nm with a spectrophotometer (model UV-1200 SHIMADZU Co., Ltd.).

2.6 Enzyme assay

L-Histidine oxidase activity was colorimetrically determined by measuring the amount of hydrogen peroxide produced from L-histidine as substrate. The reaction mixture containing 0.3 ml of 10 mM L-histidine, 0.5 ml of 5 mM DAOS, 0.5 ml of 7.5 mM 4-AA, 0.5 ml of POD (25 U/ml) in 20 mM phosphate buffer (pH 7.0) was incubated at 37℃ for 5 min. Then the reaction was stopped by addition of 5.0% SDS. The absorbance was read at 600 nm with a spectrophotometer. One unit of activity was defined as the amount of enzyme which liberated 1μmol of hydrogen peroxide per min under the specified conditions.

2.7 Protein determination

Protein concentration was determined by the method of Bradford or spectrophotometrically by measurering absorbance at 280 nm. A standard curve was drawn using bovine serum albumin.

2.8 Determination of molecular weight by gel filtration

The molecular weight (*Mw*) was estimated according to the method of Andrews. The 0.5 mg of each protein sample dissolved in 1.0 ml of 20 mM phosphate buffer (pH 7.0) was applied to a column (16 x 600 mm i.d.) of Sephacryl S-200 previously equilibrated with the same buffer at 4℃. The column was eluted with same buffer at a flow rate of 0.2 ml/min, and fractions of 3 ml each were collected. The void volume (V_0) was determined from the elution volume of blue dextran. The following proteins were used as standards ; cytochrom *c* (*Mw* : 12,400), myokinase (*Mw* : 32,000), enolase (*Mw* : 67,000), and lactate dehydrogenase (*Mr* : 142,000).

2.9 Electrophoresis

Polyacrylamide gel-electrophoresis was carried out according to the method of Davis with Tris-Glycine buffer (pH 8.5) at a current of 40 mA per gel at room temperature. Then the gels were stained with 0.1% coomassie brilliant blue in 10% acetic acid containing 50% methanol. After stain, the gels were destained by standing in 7.5% acetic acid containing 25% methanol. The molecular weight of the purified enzyme was estimated from their mobilities on PAGE. SDS PAGE was carried out according to the method of Laemmli. The following proteins were used as standards : carbonic anhydrase (*Mw* : 30,000), aldolase (*Mw* : 42,000), albumin (*Mw* : 66,000), β -galactosidase (*Mw* : 116,000) and myosine (*Mw* : 200,000).

3. RESULT

3.1 Purification of L-histidine oxidase

The purified enzyme was prepared from the cell-free extracts of KAIT-B-022 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 30% saturation (adjusting at pH 7.0 with 1 M sodium hydroxide) and after stirring for an additional 30 min, the precipitate was removed by centrifugation at 10,000 *g* for 40 min. The supernatant fraction was further brought to 70% saturation with solid ammonium sulfate and then the solution was stirred for 30 min. The precipitated protein was collected by centrifugation and dissolved in 20 mM phosphate buffer (pH 7.0). The solution was equilibrated with 20 mM phosphate buffer (pH 7.0) containing 1.0 M ammonium sulfate by using dialysis for 12 h.

Step-2. Column chromatography on Octyl Sepharose :

The equilibrated enzyme solution was applied to a column (16 x 200 mm i.d.) of Octyl Sepharose, previously equilibrated with 20 mM phosphate buffer (pH 7.0) containing 1.0 M ammonium sulfate. Then the adsorbed proteins were eluted with 20 mM phosphate buffer (pH 7.0) containing 1.0 M ammonium sulfate. Fractions 25 to 34 were collected, and dialyzed overnight against 0.1 M phosphate buffer (pH 7.0). One fraction volume was 5 ml.

Step-3. Column chromatography on Q-Sepharose :

The active fraction was applied to a column (26 x 100 mm i.d.) of Q Sepharose previously equilibrated with 20 mM phosphate buffer (pH 7.0). Then the column was subjected to elution with a linear gradient of sodium chloride concentrations from zero to 1.0 M in the same buffer. Fractions 48 to 52 were combined and used for further purification. One fraction volume was 5 ml.

Step-4. Gel filtration on Sephacryl S-200 :

The active fraction was concentrated to 1 ml by ultrafiltration and applied onto a column (16 x 600 mm i.d.) of Sephacryl S-200 previously equilibrated with 20 mM phosphate buffer (pH 7.0). Elution was performed with the same buffer. Fractions 18 to 20 were pooled and concentrated by the ultrafiltration. One fraction volume was 2 ml.

Step-5. Column chromatography on Resource Q :

The active fraction was applied onto a column (6.4 x 30 mm i.d.) of Resource Q previously equilibrated with 20 mM phosphate buffer (pH 8.0). Then elution was perfomred with the same buffer containing 1.0 M sodium chloride.

Step-6. Gel filtration on Sephacryl S-100 :

The active fraction was concentrated to 1 ml by ultrafiltration and applied onto a column (16 x 600 mm

i.d.) of Sephacryl S-100 previously equilibrated with 20 mM phosphate buffer (pH 8.0). Elution was performed with the same buffer. Fractions 29 to 44 were pooled. One fraction volume was 1 ml.

The purification procedure of L-histidine oxidase from *B. borstelensis* KAIT-B-022 is summarized in Table I. The enzyme was purified about 388-fold over the cell-free extracts.

Table I. Purification of L-histidine oxidase from *Brevibacillus borstelensis* KAIT-B-022. The enzymatic activity was assayed colorimetrically under the specified conditions.

Step	Total activity (U)	Total protein (mg)	Specific acitivity (mU/mg)	Recovery of activity (%)
Crude extract	2.68	1,088	2.5(1)	–
Ammonium sulfate	2.64	592	4.5(2)	98
Octyl Sepharose	2.70	219	12.4(5)	101
Q Sepharose	2.46	56.1	43.8(17)	92
Sephacryl S-200	0.83	6.43	129 (52)	31
Resource Q	0.38	0.55	679 (272)	14
Sephacryl S-100	0.24	0.25	970 (388)	9

3.2 Purity of the enzyme

To check the purity of the enzyme, the final enzyme preparation was submitted to NATIVE PAGE. L-Histidine oxidase showed a single band of enzymatic activity on gel, coincident with the single band of protein. The results are shown in Figure I. The purified enzyme was homogeneous on NATIVE PAGE.

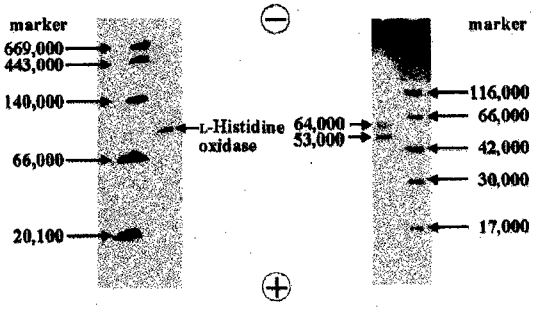

Fig. I. Polyacrylamide gel-electrophoresis of the purified enzyme. Left: NATIVE PAGE, Right: SDS PAGE (see "MATERIALS AND METHODS").

3.3 Molecular weight of the enzyme

1) Estimation by NATIVE PAGE

The molecular weight of L-histidine oxidase was caluclated to be 117,000 by NATIVE PAGE.

2) Estimation by gel filtration

The molecular weight of L-histidine oxidase was calculated to be 117,000 by gel filtration on Sephacryl S-200.

3.4 Subunit of the enzyme

L-Histidine oxidase showed two heterogeneous subunits of on SDS PAGE. The results are shown in Figure I. The molecular weight of two subunits were calculated to be 64,000 and 53,000, respectively.

3.5 Enzymatic properties of L-histidine oxidase

1) Action of the enzyme on various substrates

The activities of L-histidine oxidase from *B. borstelensis* KAIT-B-022 on various substrates are summarized in Table II. The enzyme showed powerful activity toward L-histidine, whereas the enzyme was inactive toward D-histidine and other L-amino acids.

2) Thermal Stability

The enzyme in 0.1 M phosphate buffer (pH 7.0) was incubated at different temperatures (30-60℃) for various time (15-60 min), and the residual activity was assayed. The results are shown in Figure II. L-Histidine oxidase from *B. borstelensis* KAIT-B-022 was stable at pH 7.0 up to 50℃.

3) pH stability

The enzymes were kept in 0.1 M buffers of different pH values (4.0-13.0) at 37℃ for 1 h. Then they were adjusted to pH 7.0 and the enzyme activity was assayed. The results are shown in Figure III. L-Histidine oxidase from *B. borstelensis* KAIT-B-022 was stable in the range of pH's between 6-11.

4) pH optimum

The effect of pH on the activity of L-histidine oxidase was determined in various buffers. The results are shown in Figure IV. The optimum pH for the activity of L-histidine oxidase from *B. borstelensis* KAIT-B-022 was found to be at 7.0.

5) Temperature optimum

The reaction mixture was incubated at different temperature (30-70℃) for 5 min, and then the residual activity was assayed. The optimum temperature for the activity of L-histidine oxidase from *B. borstelensis* KAIT-B-022 was found to be at 55℃.

Table II. Substrate specificity of L-histidine oxidase from *Brevibacillus borstelensis* KAIT-B-022

Substrate	Relative activity (%)
L-His	100
D-His	0
1-Methyl-L-His	0
L-Arg	0
L-Lys	0
L-Orn	0
L-Cit	0
Gly	0
L-Ala	0
L-Val	0
L-Leu	0
L-Ile	0
L-Phe	0
L-Tyr	0
L-Glu	0
L-Gln	0
L-Asp	0
L-Asn	0
L-Ser	0
L-Thr	0
L-Trp	0
L-Pro	0

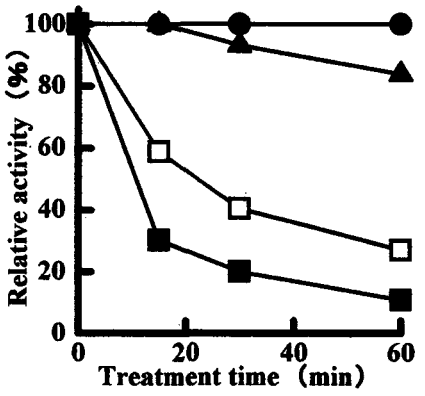

Fig. II. The thermal stability of L-histidine oxidase from *Brevibacillus borstelensis* KAIT-B-022 (see "MATERIALS AND METHODS").
●: 45℃, ▲: 50℃,□: 55℃, ■: 60℃

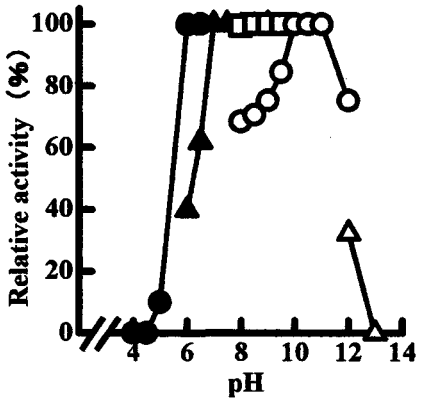

Fig. III. The pH stability of L-histidine oxidase from *Brevibacillus borstelensis* KAIT-B-022 (see "MATERIALS AND METHODS").
●: Acetate buffer, ▲: Phosphate buffer,□: Tris-HCl buffer, ○ : Borate buffer, △: Phosphate-NaOH buffer

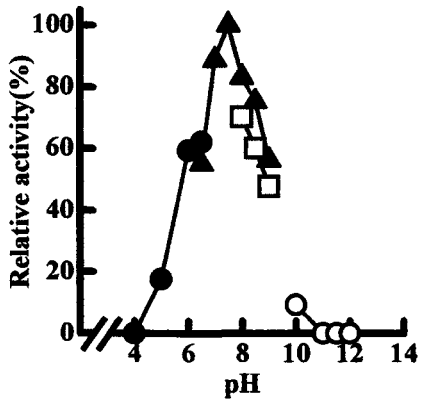

Fig. IV. The effect of pH on L-histidine oxidase activity from *Brevibacillus borstelensis* KAIT-B-022 (see "MATERIALS AND METHODS").
●: Acetate buffer, ▲: Phosphate buffer,□: Tris-HCl buffer, ○ : Borate buffer

4. DISCUSSION

In this study, the L-histidine oxidase of *B.borstelensis* KAIT-B-022 was purified by ammonium sulfate and various column chromatographies. The enzyme was purified about 388-fold over the cell-free extracts, and shown to be homogeneous on NATIVE PAGE.

L-Histidine oxidase from *B. borstelensis* KAIT-B-022 showed powerful activity toward L-histidine, whereas the enzyme was inactive toward D-histidine and other L-amino acids. These properties were different to it of L-amino acid oxidase from *Proteus rettgeri* [6] in the substrate specificity. L-Amino acid oxidase from *P. rettgeri* showed powerful activity toward basic L-amino acids and the relative activities for L-histidine, L-arginine, L-lysine and L-ornithine were 100%, 144%, 61% and 81%, respectively.

To date, little has been known about the L-amino acid oxidase having two heterogeneous subunits. Now we are investigating the structure and function of L-histidine oxidase from *B. borstelensis* KAIT-B-022 by using technologies of gene recombination and protein engineering.

It has been reported that L-phenylalanine oxidase from *Pseudomonus* sp. P-501 [3] and L-tryptophan oxidase from *Coprinus* sp. SF-1 [4] have simultaneously oxidase and oxygenase activities. L-Histidine oxidase from *B. borstelensis* KAIT-B-022 produced oxidative deaminationally hydrogen peroxide from L-histidine to form simultaneously imidazolepyruvate and ammonia [5]. Therefore, the presence or absence of oxygenase activity in L-histidine oxidase will be reported later.

Futhermore, L-histidine oxidase can be applied to assay L-histidine in fish meat extracts for diagnosis of an allergy-like food poisoning. The enzymatic assay method of L-histidine with L-histidine oxidase will be reported in a subsequent paper.

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