Transactions of the Materials Research Society of Japan 28 [3] 837-840 (2003)

The Cofactor of a New Thermostable Histamine Oxidase Produced by *Arthrobacter crystallopoietes* KAIT-B-007

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Histamine oxidase (EC 1.4.3.-) was found in cells of *Arthrobacter crystallopoietes* KAIT-B-007 isolated from soil. The enzyme was purified about 462-fold with 23% yield by ammonium sulfate fractionation column chromatographies on DEAE-Sepharose and so on. The purified enzyme was homogeneous on native polyacrylamide gel-electrophoresis (NATIVE-PAGE). The catalytic activity was inhibited by *N,N*-diethyldithiocarbamate (DDTC). The inactivated enzyme was restored by Cu^{2+}, but Cu^{+} and the other metal ions did not show restoring effect on it. The enzyme had one atom of copper per mol of the enzyme protein as a result from atomic absorption analysis. The hydrazine reagents inhibited the enzyme activity, too. The enzyme catalyzed the nitroblue tetrazolium (NTB)/glycinate reaction. These results strongly suggested that histamine oxidase from *A. crystallopoietes* KAIT-B-007 contains copper and quinone as a cofactor.

Key words: histamine oxidase, cofactor, quinoprotein, copper ion, *Arthrobacter*

1. INTRODUCTION

Copper-containing amine oxidases (EC 1.4.3.6) are widely in microorganisms, plants, and animals. These enzymes catalyze the oxidative deamination of various biogenic primary amines to generate the corresponding aldehydes, ammonia, and hydrogen peroxide. The cofactor covalently bound with copper-containing amine oxidase from bovine plasma was identified as 6-hydroxydopa (2,4,5-trihydroxyphenyl-$_L$-alanine;topa) by J.P.Klinman *et al.* [1]. Afterwards, the copper-containing amine oxidases have been found in bacteria. They were methylamine oxidase from *Arthrobacter* strain P1 [2], amine oxidase from *Escherichia coli* [3], phenylethylamine oxidase [4] and histamine oxidase [5] from *A. globiformis* IFO 12137. These copper-containing enzymes have been shown to contain the same cofactor.

We found a new thermostable histamine oxidase in the cells of *A. crystallopoietes* KAIT-B-007[6]. Its enzymatic properties were similar to histamine oxidase from *A. globiformis* IFO 12137 except for thermal stability. The enzyme from KAIT-B-007 strain was stable at pH 7.0 up to 70℃. This paper describes the effect of copper ion to histamine oxidase from *A. crystallopoietes* KAIT-B-007, and the reactions of NTB/glycinate and phenylhydrazine with this enzyme. Moreover, we discuss similarities or differences of a cofactor by comparing them in histamine oxidases from KAIT-B-007 and IFO 12137 strains.

2. MATERIALS AND METHODS

2.1 Materials

Histamine oxidase was produced in our laboratory by fermentation of *A. crystallopoietes* KAIT-B-007. The enzyme was purified from cell-free extracts of KAIT-B-007. Peroxidase (EC 1.11.1.7 ; Type Ⅰ , 116 purpurogallin U/mg; POD) was purchased from Sigma Chemicals Co., Ltd. Q-Sepharose, DEAE-Sepharose, Octyl Sepharose, Resource Q, and Sephacryl S-100 columns were purchased from Amersham Pharmacia Biotech. Co., Ltd. Histamine dihydrochloride, sodium dodecyl sulfate (SDS) and 4-aminoantipyrine (4-AA) were purchased from Wako Pure Chemical Industries. *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (DAOS) and NTB were purchased from Dojindo Laboratories. Bovine serum albumin was purchased from INTERGEN 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

A. crystallopoietes KAIT-B-007 was used throughout this study.

2.3 Media and culture conditions

The microorganisms were grown on bouillon agar slant at 30 ℃ for one day. Medium containing 1.0% beef extract, 1.0% peptone, 0.5% NaCl 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 17 h at 30℃ on a rotary shaker operating at 200 rpm. Medium containing 0.1% histamine, 0.1% NaCl, 0.03% yeast extracts,

0.03% peptone, 0.2% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.0005% $CuSO_4 \cdot 5H_2O$ (pH 7.0) was used for cultivation.

2.4 Preparation of Enzyme

All operations for the purification procedure were performed below 10℃ unless otherwise specified. Cells were harvested from the culture of KAIT-B-007 strain 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 Q-Sepharose, DEAE-Sepharose, Octyl Sepharose, Resource Q, and gel filtration with Sephacryl S-100.

2.5 Enzyme assay

Histamine oxidase activity was calorimetrically determined by measuring the amount of hydrogen peroxide produced from histamine as substrate. The reaction mixture containing 0.3 ml of 10 mM histamine, 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 0.5 ml 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.

2.6 Protein determination

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

2.7 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 NATIVE-PAGE. SDS-PAGE was carried out according to the method of Laemmli.

2.8 Spectrophotometric analysis

The spectrophotometric determination was carried out using the model UV-2400 P (SHIMAZU Co.,).

2.9 Activation or inhibition test of various compounds on enzyme activity

The enzymes (2.0 μg as protein) was incubated at 30℃ for 30 min in 0.1M phosphate buffer (pH 7.0) containing 0.5 mM each compounds given. Then a treated enzyme activity was assayed.

2.10 Reactivation test of metal ions on enzyme activity

One ml of enzyme solution (0.36 mg/ml) was incubated at 30℃ for 1h with 0.5 M DDTC in 0.1 M phosphate buffer (pH 8.0) . Then the DDTC-treated enzyme was dialyzed thoroughly against a large volume of 20 mM phosphate buffer (pH7.0). After dialysis, 1 ml of the treated enzyme was incubated at 30℃ for 3 h with 1 ml of 0.5 mM CuCl, $CuSO_4$, $FeSO_4$, $MgSO_4$, $ZnSO_4$ and $CaCO_3$, respectively. Therefore, the enzyme activity was assayed.

2.11 Atomic absorption analysis

The copper in the enzyme protein were analyzed with a model AA-680 (SHIMAZU Co.,) atomic absorption spectrophotometer (acetylene/air frame) at 324.8 nm.

2.12 NBT/Glycinate reaction

The 1.5ml of the reaction mixture containing 100 μl each enzyme solution, 300 μl of 2M potassium glycinate (pH 10), 100 μl of 1.0 mg/ml sodium borohydride-reduced serum albumin and 1ml of 0.24mM NBT in 2M potassium glycinate (pH 10). Reagents were pipetted into the test tubes immersed in an ice bath. The reaction was started by incubating at 25℃ in the dark and the absorbance at 530 nm was monitored. The amount of enzyme protein added were 15.8, 31.6 , 47.4 and 94.8 μg, respectively. A reduced serum albumin was prepared by incubating 560 mg of serum albumin and 200 mg of sodium borohydride in 20 ml of water for 15 h at room temperature.

The quinones and related compounds catalyzed the oxidation of glycine with oxygen in the presence of NBT, and this reaction was coupled with the formation of a formazan dye. The quinone compounds in methylamine oxidase, amine oxidase from bovine plasma and diamine oxidase from pig kidney have been detected by this method [7].

2.13 Titration with phenylhydrazine

The 1 ml of enzyme solution (8.38 mM as protein) was titrated with a freshly prepared solution of 1mM phenylhydrazine (carbonyl reagents) to final concentration of 1.7, 4.2, 5.6, 8.0 and 15.0 μM, respectively. The titration with phenylhydrazine was accompanied by the formation of a yellow hydrazine adduct with an absorption maximum at 440 nm. Each spectrum was recorder after a 15 min incubation at 30℃ and the residual activity was determined.

3. RESULTS AND DISCUSSION

3.1 Purification of histamine oxidase

The purification procedure of histamine oxidase from *A. crystallopoietes* KAIT-B-007 is summarized in Table 1.

The enzyme was purified about 462-fold with 23% yield over the cell-free extracts.

Table 1. Purification of histamine oxidase from *A. crystallopoietes* KAIT-B-007.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)		Recovery of activity (%)
Crude extract	223	7716	28	(1)	—
Ammonium sulfate	216	5082	43	(2)	97
Q-Sepharose	152	353	431	(16)	68
DEAE-Sepharose	131	152	862	(31)	58
Octyl Sepharose	104	22	4727	(171)	47
Phenyl Sepharose	76	11	6909	(250)	34
Resource Q	59	6	9833	(356)	26
Sephacryl S-200	51	4	12751	(462)	23

3.2 Purity of the enzyme

To check the purity of the enzyme, the final enzyme preparation was submitted to NATIVE-PAGE and SDS-PAGE. Histamine oxidase showed a single band of enzymatic activity on gel, coincident with the single band of protein. The molecular weight of histamine oxidase was calculated to be about 81,000. The results are shown in Figure 1.

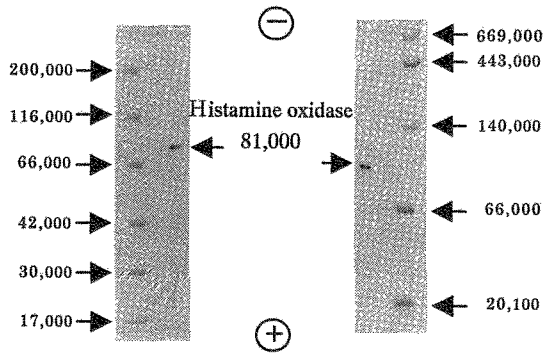

Fig.1. Polyacrylamide gel-electrophoresis of the purified enzyme. Right: NATIVE-PAGE, Left: SDS-PAGE

3.3 Effect of compounds on enzyme activity

The effect of compounds on the enzyme activity are summarized Table 2. The carbonyl group-blocking compounds such as hydrazine, methylhydrazine and phenylhydrazine inhibited the enzyme activity. These reagents have no structural feature in common with other than reactivity with carbonyl groups. These results strongly suggest that these compounds modified the carbonyl group in the enzyme protein. Also the Cu-chelators, DDTC inhibited the 76% of enzyme activity. The other metal ions had not significant effect on the enzyme activity.

3.4 Effect of compounds on enzyme activity

The reactivation effect of metal ions on the enzyme activity are summarized in Table 3. The dialyzed enzyme showed a relative activity of 5%. An inactive enzyme was restored with $CuSO_4$ (Cu^{2+}) to 65% of the relative activity. The other metal ions did not enhance the enzyme activity.

It was indicated that the enzyme is dissociated in part into the apoenzyme and Cu^{2+} by the action of DDTC, and a part of the apoenzyme is reconstituted to the holo-enzyme in the presence of Cu^{2+}. Namely, it was suggested that Cu^{2+} is essential to expression of histamine oxidase activity, but Cu^{+} is not essential.

Table 2. Effect of compounds on the enzyme activity

Compounds (0.5 mM)	Relative activity (%)
None	100
Cu^{+}	100
Cu^{2+}	100
Ca^{2+}	100
Ze^{2+}	98
Mg^{2+}	114
Fe^{2+}	100
EDTA	82
DDTC	24
FAD	100
Hydrazine	5
Methylhydrazine	0
Phenylhydrazine	0
8-Hydroxyquinone	76
α,α'-dipyridyl	78
1,10 - Phenanthroline	53
2 - Mercaptoethanol	74
Dithiothreitol	88

Table 3. Reactivation effect of copper ion on the enzyme activity

Compounds (0.5 mM)	Relative activity (%)
None	100
DDTC treatment	5
Cu^{2+}	65
Cu^{+}	5
Ca^{2+}	5
Ze^{2+}	5
Mg^{2+}	5
Fe^{2+}	5

3.5 Analysis of copper in enzyme

The copper content of an enzyme protein was measured by atomic absorption spectrophotometer. The concentration of copper in the enzyme protein was 15.3 mM per 16.4 mM of the enzyme protein.

3.6 Estimation of quinone in enzyme

The quinone content in the enzyme protein was estimated by NBT/glycinate reaction. The absorbance of the dye at 530 nm increased linearly for 30 min. The rate of the dye formation was proportional to the amount of the enzyme added up to 47.4 μM. The results are shown in Figure 2.

3.7 Titration with phenylhydrazine

The quinone compounds containing in the enzymes was titrated with the carbonyl reagents phenylhydrazine, reacting with the quinone to form a hydrazine adduct. The titration with phenylhydrazine was accompanied by the formation of a yellow adduct with an absorption

maximum at 440 nm. The results are shown in Figure 3.

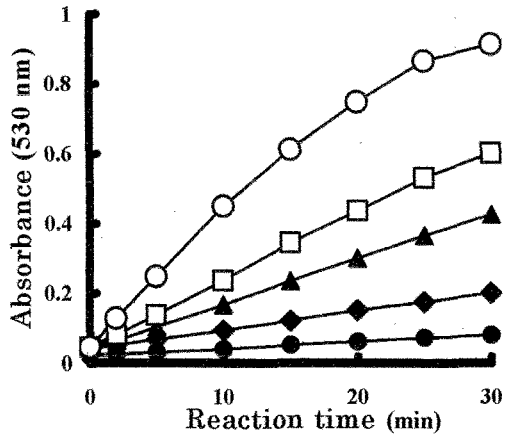

Fig. 2. NBT/Glycinate reaction catalyzed by histamine oxidase.
●: water, ◆ : 15.8 μg, ▲: 31.6 μg, □:47.4 μg, ○ : 94.8 μg

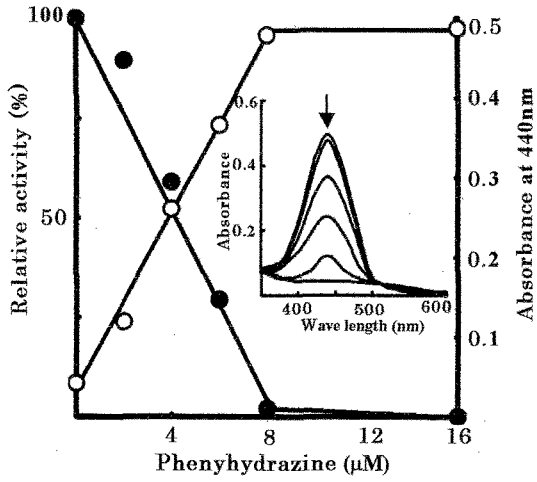

Fig. 3. Titration of the histamine oxidase with phenylhydrazine.
The arrow indicates the direction of spectral change. The titration curves for spectral change at 440nm (○) and residual activity (●) are shown in the *inset*.

The complete inactivation was obtained when 0.96 mol phenylhydrazine was added per mol of the enzyme protein. The increase in absorbance at 440 nm also reached to an end point where the phenylhydrazine : enzyme molar ratio was 0.96 : 1.

As a result of the NBT/glycinate reaction and titration test of the enzyme with phenylhydrazine, it was strongly estimated that the quinone cofactor is contained in this enzyme. These properties were similar to copper-containing amine oxidases such as methylamine oxidase of *A.* strain P1[2], phenylethylamine oxidase[4] and histamine oxidase [5] of IFO 12137 strain.

Histamine oxidase from KAIT-B-007 strain was found to contain one atom of copper and one quinone compound per mol of the enzyme protein. These results were similar to histamine oxidase from IFO 12137 strain.

Recently, the gene coding for histamine oxidase IFO 12137 strain has been cloned and sequenced [8]. Throughout the studies on this enzyme, the sequence of [-Asn-Tyr-Asp-] was consensus sequence for topa quinone in the gene coding for the enzyme, and the middle Tyr was the precursor to the quinone cofactor covalently bound to the enzyme [8]. The sequence of histamine oxidase from KAIT-B-007 strain contains the sequence of [-Asn-Tyr-Asp-], too (not shown).

The identification and localization of quinone in histamine oxidase from *A. crystallopoietes* KAIT-B-007 will be reported in a subsequent paper.

4. CONCLUTION

In this study, it is concluded that a new thermostable histamine oxidase from *A. crystallopoietes* KAIT-B-007 was found to contain one atom of copper and one quinone compound per mol of the enzyme protein as a cofactor, that is, the enzyme was shown to be a copper-containing quinoidprotein. This result was similar to histamine oxidase from IFO 12137 strain.

5. REFERENCES

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(Received December 21, 2002; Accepted March 7, 2003)