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Characterization of Copper-Containing Enzyme Searched from Molds with Oxidocondensation Activity

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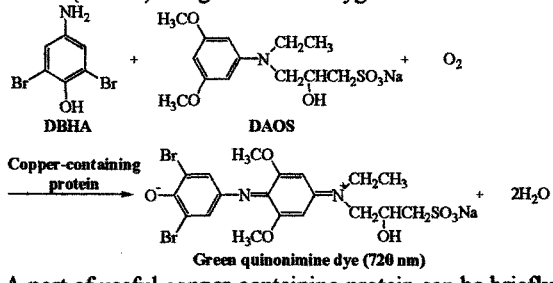
L-Ascorbate utilization molds were isolated from soils on an agar plate containing L-ascorbate as the sole carbon source. Copper-containing enzyme were searched from these isolated molds by using the oxidocondensation activity (OCA) of 3, 5-dibromo-4-hydroxyaniline (DBHA) and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3, 5-dimethoxyaniline (DAOS), and strain KAIT-M-161 containing the highest OCA was chosen from 178 L-ascorbate utilization strains. The strain KAIT-M-161 was identified as *Aspergillus* sp. The protein containing OCA was partially purified until about 90-fold by a series of chromatography steps on DEAE-Sepharose, Q-Sepharose, and Sephacryl S-100 with an overall yield of 32 %. The OCA of the protein had optimal temperature at 60 ℃, thermostable below 60 ℃ and pH stable between pH 6 and pH 9. The OCA of the protein was inactivated to 33 % and 0 % with ethylenediaminetetraacetate (EDTA) and *N*, *N*-diethyldithiocarbamate (DDTC) as chelating agent, respectively. The OCA of the inactivated protein was reactivated by only copper ion. The purified protein reacted with L-ascorbate and hydroquinone.

Key words: copper-containing enzyme, mold, oxidocondensation

1. INTRODUCTION

Copper-containing enzymes, such as L-ascorbate oxidase (EC 1.10.3.3), laccase (EC 1.10.3.2) and histamine oxidase (EC 1.4.3.6), have been reported and applied. L-Ascorbate oxidase is useful to eliminate ascorbate interference in the electrode-based biosensors, because L-ascorbate can be converted to an electrochemically inert form, dehydro-L-ascorbate [1]. Laccase is a family of multicopper oxidases that can catalyze the oxidation of wide range of aromatic substrates. Because of their high capability of catalyzing the oxidation of aromatic compounds, laccase attracts considerable attention as potentially available industrial enzymes in various fields, such as pulp delignification [2], dye or stain bleaching [3], and bioremediation [4-5]. Histamine oxidase can use the determination of histamine as an allergy-like food poisoning [6].

A port of copper-containing protein catalyses oxidocondensation of 3, 5-dibromo-4-hydroxyaniline (DBHA) and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3, 5-dimethoxyaniline (DAOS) using molecular oxygen as follows:



A port of useful copper-containing protein can be briefly searched by measuring the oxidocondensation activity (OCA). However, it is generally difficult that the protein indicates the copper-containing protein. The copper atom in the protein can measure by using inductively coupled plasma (ICP) or atomic absorption spectroscopy. The assay-using protein loses to burn, and it needs pretreatment before assay. The copper in protein can be also imagined by the catalytic activity of inactivation for chelating agent and reactivation for copper ion. This method doesn't seem screening of copper-containing protein, because it needs pretreatment.

Here, we report to search copper-containing protein from molds using the OCA, and indicate that the protein is copper-containing protein.

2. MATERIAL AND METHOD

2-1. Isolation of L-Ascorbate Utilization Molds

L-Ascorbate utilization molds were obtained from 52 soil samples, which collected from various regions of Japan, such as Aomori, Iwate, Miyagi, Tochigi, Fukushima, Saitama, Kanagawa, Hiroshima, Kagoshima and Okinawa. L-Ascorbate utilization molds were isolated by picking up a single colony formed on an agar (1.5 %) plate in isolation medium at 30 ℃ for 4 days. The isolation medium (pH 5) contained the following (in grams per liter of water): NH_4NO_3 (1.0), NaH_2PO_4 (1.0), K_2HPO_4 (2.0), $CuSO_4 \cdot 5H_2O$ (0.01), $MgSO_4 \cdot 7H_2O$ (0.01), $FeSO_4 \cdot 5H_2O$ (0.01), chloramphenicol (0.001) and ascorbate (0.1). The isolated molds were maintained on potato dextrose agar (PDA) medium slant.

2-2. Measurement of OCA

OCA was measured spectrophotometrically at 37 ℃ as the increase in the absorbance (720 nm). Protein solution (0.5 ml) was added to 1.5 ml of 0.1 M sodium

phosphate buffer (pH 7), and mixed with 1 ml of assay mixture containing 2.5 mM DBHA and 2.5 mM DAOS. The mixture was placed into a test tube at 37 ℃ for 20 min. After incubation, the solution was placed into a glass cuvette (1-cm light path) to measure the increase in the absorbance with a spectrophotometer (UV-1200; Simadzu). One arbitrary unit (AU) of OCA was defined as the amount of enzyme capable of one absorbance increase at 720 nm per a minute. The protein concentration was calculated using the specific absorption coefficient at 280 nm (1.0 $ml \cdot mg^{-1}cm^{-1}$). Bovine serum albumin was used as a standard protein for calibration.

2-3. Screening of Molds containing OCA

For the detection of OCA, isolated molds were cultured in 15-ml test tubes containing 5 ml of isolation medium on a rotary shaker (200 rpm) at 37 ℃ for one week. Cells were harvested by filtration, washed twice in 0.1 M sodium phosphate buffer (pH 7), and weighed. The harvested cells were grated with the same amount of sea sand and suspended in ten-fold volume of 0.1 M sodium phosphate buffer (pH 7). The supernatants were obtained by centrifugation at 20,000×*g* for 20 min at 4 ℃. The OCA of the supernatants was measured, and the strain containing the highest OCA was chosen.

2-4. Identification of the Mold Containing the Highest OCA

The identification of mold containing the highest OCA was based on conventional morphological characteristics. For the morphological characterization of the mold culture, the strain was grown on PDA media slant at 30 ℃ for one week. Observations were made under a light microscope.

2-5. Culture condition

The mold containing the highest OCA was grown on PDA medium at 30 ℃ for 3 days. A loopful seed of mold on an agar slant was inoculated into 100 ml of isolation medium (pH 5) in a 500-ml Erlenmeyer flask, and then kept at 30 ℃ with shaking for one week on a rotary shaker operating at 200 rpm.

2-6. Purification Steps

The protein containing OCA was purified by the procedures described below.

Step 1. Crude extracts:

The harvested cells (about 20 g) were washed with a cool 0.1 M phosphate buffer (pH 7.0), and grated with 20 g of sea sands on an ice-cooling mortar. The grated cells were suspended in 200 ml of 0.1 M phosphate buffer (pH7.0). The crude extracts (supernatant) was obtained by centrifugation at 20,000×*g* for 20 min at 4 ℃.

Step 2. DEAE-Sepharose chromatography:

The crude extracts was loaded onto a column of DEAE-Sepharose (1.6×10 cm; Amersham Pharmacia Biotech) equilibrated with 3 column volumes of 0.1 M phosphate buffer (pH 7.0). The column was washed with 0.1 M phosphate buffer (pH 7.0), and oxidocondensation activity was eluted with a linear gradient of NaCl (0 to 1 M in a total volume of 200 ml). Fractions with high activity were combined. For desalination and buffer exchange, the supernatant was subjected to ultrafiltration in an Amicon stirred cell unit (model 8400) fitted with a Diaflo ultrafilter UK-10 (Advantec; 10,000-molecular-weight cut-off) under 5 kgf/cm^2 pressure.

Step 3. Resource-Q chromatography:

After desalination by ultrafiltration with three exchanges of 0.1 M phosphate buffer (pH 7.0), the supernatant from Step 2 was loaded onto a column of Resource-Q (1.6×1cm: Amersham Pharmacia Biotech) equilibrated with 3 column volumes of 0.1 M phosphate buffer (pH 7.0). The column was washed with 0.1 M phosphate buffer (pH 7.0). The column was washed with 0.1 M phosphate buffer (pH 7.0), and oxidocondensation activity was eluted with a linear gradient of NaCl (0 to 1 M in a total volume of 40 ml). Fractions with high activity were combined. For concentration, the supernatant was subjected to ultrafiltration as described in Step 2.

Step 4. Sephacryl S-100 chromatography:

The enzyme solution from Step 3 was loaded onto a Sephacryl S-100 (1.6×60 cm: Amersham Pharmacia Biotech) equilibrated with 0.1 M phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min. The protein containing oxidocondensation activity was eluted with the same buffer, and the active fractions were collected.

2-7. Effect of Temperature and pH

To investigate the effect of temperature on OCA, the reaction was carried out in thermo-controlled water bath at between 30 and 70 ℃ for 20 minutes in 0.1 M sodium phosphate buffer (pH 7). For the determination of thermostability, the protein solutions were pre-incubated for 10 minutes at various temperatures in 0.1 M sodium phosphate buffer (pH 7). Heating was stopped by cooling in an ice bath. Measurement of the residual activity was performed spectrophotometrically at 37 ℃ in 0.1 M sodium phosphate buffer (pH 7).

To determine the pH stability, the protein solutions were preincubated at 37 ℃ for 20 minutes in various buffers, such as 0.1 M sodium citrate buffer (pH 3-6), 0.1 M sodium phosphate buffer (pH 6-8) and 0.1 M borate buffer (pH 8-10). The protein solutions were diluted 50-fold with 0.5 M sodium phosphate buffer (pH 7), and the remaining activity was determined.

2-8. Substrate specificity

For the determination of substrate specificity, the purified protein solutions was incubated at 37 ℃ in 0.1 M sodium phosphate buffer (pH 7) containing 0.5 mM L-ascorbate or 0.5 mM hydroquinone in quartz cuvettes (1-cm light path). The activity was calculated to measure the decrease in the absorbance for 5 minutes. The extinction coefficient of L-ascorbate and hydroquinone used 10,000 $M^{-1}cm^{-1}$ at 245 nm and 1,840 $M^{-1}cm^{-1}$ at 290 nm, respectively.

2-9. Inactivation and Reactivation

To investigate the effect of chelating agents on OCA, the purified protein solutions were incubated at 37 ℃ for 3 hours in 0.1 M sodium phosphate buffer (pH 7) containing 1 mM ethylenediaminetetraacetate (EDTA) or 1 mM *N*, *N*-diethyldithiocarbamate (DDTC). After incubation, the solutions were dialyzed against 0.1 M sodium phosphate buffer (pH 7) with three changes of

buffer, and then the OCA of the proteins was measured.

For the determination of reactivation for metal ion, the EDTA- or DDTC-treatment solutions were incubated at 37 ℃ for one hour in 0.1 M sodium phosphate buffer (pH 7) containing 0.1 M metal ion, such as Cu^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, and Ca^{2+}. After incubation, the solutions were dialyzed against 0.1 M sodium phosphate buffer (pH 7) with three changes of buffer, and then the OCA of the proteins was measured.

3. RESULTS AND DISSCUSSION

3-1. Isolation of Soil Microorganisms

A total of 178 L-ascorbate-utilizing molds were isolated from 52 soil samples originating from various regions of Japan. Their colonies grew rapidly until on isolation medium at 30 ℃ attaining a size of 5-12 mm for 4 days. Their molds may have an "ascorbate and aldarate metabolism pathway" to metabolize L-ascorbate, and the pathway includes copper-containing L-ascorbate oxidase, glutathione-dependent dehydroascorbate oxidoreductase (EC 1.8.5.1), and NADH-dependent semidehydro-L- ascorbate oxidoreductase (EC 1.6.5.4) [7]. Some molds may produce L-ascorbate-oxidizing laccase, too. In this result, we will search L-ascorbate oxidase or laccase from molds by screening the isolation medium.

3-2. Screening of Molds containing OCA

The OCA was contained with the most of the isolated L-ascorbate-utilizing molds. Three strains (KAIT-M-161, KAIT-M-154, and KAIT-M-123) showed high activity, as shown in Table I. The best oxidocondensation protein producer, the strain KAIT-M-161 was chosen for further characterization.

Table I. **Oxidocondensation activities (OCA) in crude extracts of isolated molds.**

Strain	Total OCA (AU)	Origin of soil
KAIT-M-161	0.367	Kagoshima
KAIT-M-154	0.349	Okinawa
KAIT-M-123	0.329	Aomori

3-3. Identification of Mold Containing the Highest OCA

The strain KAIT-M-161 grew rapidly on PDA medium at 30 ℃ attaining a size of 35-45 mm for 4 days. The mycelium surface was white and generated soluble brown pigments and black conidia (Fig. 1A). Microscopic observation showed conidiomata consisting of brown conidiophore (0.2-1.0 mm long and 8-10 μm in diameter), a brown conidial head (80-100 μm in diameter). The conidial head was consisted by a terminal vesicle (40-50 μm in diameter) and single layer metula (20-30 μm long and 3-5 μm in diameter) covered all surface of terminal vesicle (Fig. 1B). The conidia were brown sphere (4-5 μm in diameter), that had black spines and single cell (Fig. 1C). According to the morphological data, we identified this strain as *Aspergillus* sp. KAIT-M-161. There are reports on laccase from the genus *Aspergillus*, such as *A. nidulans*, *A. oryzae*, *A. fmigatus*, *A. niger*, and *A.terreus*, but are no reports on L-ascorbate oxidase from the genus *Aspergillus*.

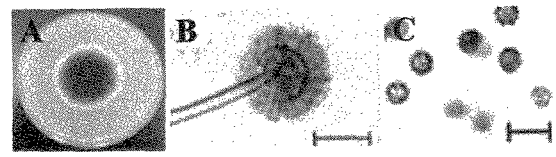


Fig. 1. **Strain KAIT-M-161.** (A) Culture on a PDA medium after 4 days incubation. (B) Conidiomata on a PDA medium viewed under a light microscope. (C) Conidia from conidiogenous cells on a PDA medium viewed under a light microscope. Bars: B=50μm; C=10μm.

3-4. Purification of the protein containing OCA

The protein containing OCA produced in *Aspergillus* sp. KAIT-M-161 was purified by several chromatogramphic steps as described in "MATERIALS AND METHODS". A typical overall purification is summarized in Table II. The final protein preparation was purified about 90-fold over the crude extracts, and the overall yield was 32 %. In this series of chromatogram, isozyme of oxidocondensation protein was not isolated. The molecular mass of the protein was determined to be around 11 kDa by gel filtration. The molecular mass of reported laccase and L-ascorbate oxidase are a range of 35-390 kDa and 130-150 kDa, respectively. The purified protein was comparatively small.

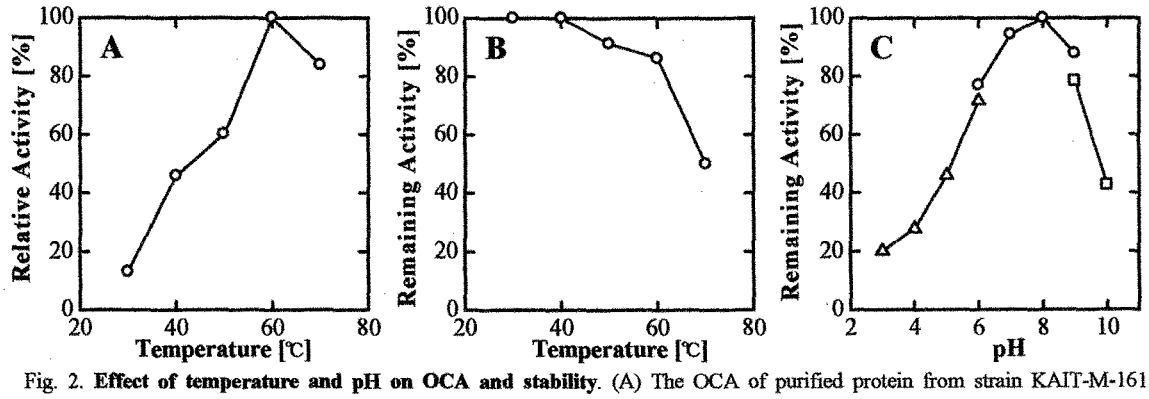


Fig. 2. **Effect of temperature and pH on OCA and stability**. (A) The OCA of purified protein from strain KAIT-M-161 was measured as described in "MATERIALS and METHOD" at the temperatures indicated. (B) Protein stability was determined by measuring the remaining activity of OCA from strain KAIT-M-161 after incubation for 10 minutes at the temperatures indicated. (C) Protein stability was determined by measuring the remaining activity of OCA from strain KAIT-M-161 after incubation at 37 ℃ for 20 minutes in 0.1 M sodium citrate buffer (pH 3-6) (△), 0.1 M sodium phosphate buffer (pH 6-8) (○) and 0.1 M borate buffer (pH 8-10) (□).

Table II. **Purification of the protein containing OCA from strain KAIT-M-161.**

Step	Total Protein (mg)	Total Activity (AU)	Specific Activity (AU/mg)	Yield (%)	Purification (fold)
Crude extracts	756	167	0.2	100	1
DEAE-Sepharose	68	135	2.0	81	10
Resource-Q	11	109	10.4	65	50
Sephacryl S-100	3	54	17.5	32	90

3-5. Effect of Temperature and pH

The optimal temperature for the OCA was determined to be 60 ℃ in 0.1 M sodium phosphate buffer (pH 7), as shown in Fig. 2A. Thermal stability of the protein containing OCA was assessed in 0.1 M sodium phosphate buffer (pH 7), after heating for 20 minutes at various temperature (30-70 ℃). The protein was stable at temperatures below 60 ℃, as shown in Fig. 2B. The protein had comparatively high stability.

The pH characteristics of the purified protein were determined by OCA. Three buffers covered the range between pH 3 and 10. The protein retained ~80 % of its activity when stored at 37 ℃ for 20 minutes over a pH range of 6-9, as shown in Fig. 2C. When stored below pH 5 or above pH 10, the OCA was completely lost within 5 hours.

3-6. Substrate Specificity

The purified protein reacted with substrate including L-ascorbate and hydroquinone, as shown in Table III. The specific activity for L-ascorbate and hydroquinone was 63.2 and 47.0 nmol·min^{-1}mg^{-1}, respectively. The hydroquinone is exemplary substrate of laccase, and laccase catalyzes oxidation of L-ascorbate. The purified protein may belong to laccase or L-ascorbate oxidase. Signification homology was observed with multicopper oxidases, including laccase genes and ascorbate oxidase genes [8]. It is difficult that the purified protein identify either laccase or L-ascorbate oxidase.

Table III. **Substrate specificity of the protein containing OCA from strain KAIT-M-161.**[a]

Substrate	Oxidation rate (%)
L-Ascorbate	100
Hydroquinone	74.4

[a] The purified protein was estimated spectrophotometrically using 0.5 mM substrate in 0.1 M sodium phosphate buffer (pH 7) at 37 ℃. The linear absorbance decrease was follow for ~5 minutes with recording spectrophotometer, and the activity was calculated from the ΔA/min. The 100 % activity is 63.2 nmol of L-ascorbate oxidized per minutes per mg of protein.

3-7. Inactivation and Reactivation

The purified protein was metal-containing protein, because OCA or L-ascorbate oxidase activity of the protein was inactivated by chelating agents, EDTA and DDTC, as shown in Fig. 3. Inactivation by EDTA and DDTC was equilibrated within about 10 minutes, and remaining activity of EDTA- and DDTC-treatment protein was 34 % and 0% of the activity without treatment protein, respectively. The DDTC-treatment protein was reactivated by only copper ion, as shown in Table IV. The activity of both oxidocondensation and L-ascorbate oxidase was reactivated to about 35 % of the activity without chelating agent-treatment protein. The activity was not reactivated by other metal ion. It indicated that the protein was copper-containing protein, and the activity of the protein needed copper ion.

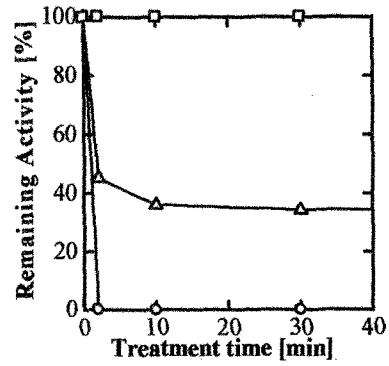


Fig. 3. **Effect of chelating agents on OCA.** The protein containing OCA was incubated at 37 ℃ for various treatment time (2-40 minutes) in 0.1 M sodium phosphate buffer (pH 7) containing 0.1 mM EDTA (△), 0.1 mM DDTC (○) or no chelating agent (□) and remaining activities were measured.

Table IV. **Reactivate activity of DDTC-treatment protein for metal ion.**

Metal ion	Relative OCA[a] (%)	Relative activity[b] (%)
Cu^{2+}	35	36
Ca^{2+}	0	0
Fe^{2+}	0	0
Mg^{2+}	0	0
Zn^{2+}	0	0

The DDTC-treatment protein was incubated in 0.1 M sodium phosphate buffer (pH 7) containing 20 mM metal ion at 37 ℃ for one hours, the OCA was measured in 0.1 M sodium phosphate buffer (pH 7). [a] The 100 % OCA is 1 AU, before DDTC-treatment. [b] The 100 % L-ascorbate oxidase activity is 63.2 nmol of L-ascorbate oxidized per minutes per mg of protein, before DDTC-treatment.

4. CONCLUSIONS

L-Ascorbate- and hydroquinone-oxidizing enzyme was selected by OCA from *Aspergillus* sp. KAIT-M-161. The enzyme was copper-containing protein, because it was inactivated by chelating agents and then reactivated by Cu^{2+}.

5. REFERENCES

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