Purification and Characterization of Cyclohexanone 1,2-Monoxygenase from *Exophiala jeanselmei* strain KUFI-6N

Yoshie HASEGAWA,* Yuka NAKAI, Tai TOKUYAMA, and Hiroaki IWAKI

Department of Biotechnoloy, Faculty of Engineering and High Technology Research Center, Kansai University, Yamate-cho, Suita 564-8680, Japan

Received May 29, 2000; Accepted August 9, 2000

Baeyer-Villiger cyclohexanone 1,2-monoxygenase (CHMO) was purified 17.1-fold from cell extracts of the fungus *Exophiala jeanselmei* grown on cyclohexanol to electrophoretically homogeneity by serial chromatographies. The molecular mass of the native enzyme was approximately 74 kDa by gel filtration and SDS-PAGE. Some enzymic characterizations were studied. The N-terminal amino acid residues were Ala-Lys-Ser-Leu-Asp-Val-Leu-Ile-Val-Gly-Ala-Gly-Phe-Gly-Gly-Ile-Tyr-Gln-Leu-, with similarity to the bacterial CHMOs of FAD-binding and NADPH-dependent type Baeyer-Villiger monoxygenases.

Key words: Baeyer-Villiger oxidation; cyclohexanone monoxygenase; cyclohexanol-degradation; *Exophiala jeanselmei*

*Exophiala jeanselmei* strain KUFI-6N, a fungus, uses cyclohexanol as a sole carbon source and metabolizes it by two pathways, a lactone formation pathway and an aromatization pathway. In the lactone formation pathway, *E. jeanselmei* has been shown to convert cyclohexanol to cyclohexanone, which is then oxygenated by the action of a Baeyer-Villiger type cyclohexanone 1,2-monoxygenase (CHMOs).

The Baeyer-Villiger monoxygenases (BVMOs), typified by the cyclohexanone monoxygenase (CHMO) of *Acinetobacter* sp. strain NCIMB 9871, are versatile biocatalysts in chiral lactone production and other asymmetric synthetic applications. CHMOs and cyclopentanone monoxygenase (CPMO) studied to date have been found in microorganisms, including bacteria of the genera *Acinetobacter*, *Nocardia*, *Xanthobacter*, and *Pseudomonas*. CHMOs from *Acinetobacter* sp. strain NCIMB 9871 and *Nocardia globerula* strain CL1 were shown to be single polypeptides, using one FAD cofactor and being NADPH dependent. On the other hand, the CPMO from *Pseudomonas* sp. strain NCIMB 9872 was shown to be made up of three or four identical subunits, each one using an FAD equivalent, and to be NADPH dependent. On the contrary, the CHMO from *Xanthobacter* sp. used FMN as the prosthetic group instead of FAD. CHMOs of these strains accept cyclobutanone, cyclopentanone, cycloheptanone, and several bicyclic ketones as substrates to produce the corresponding lactones.

However, no fungus CHMO had been purified and characterized. In this paper we report the purification of *Exophiala* CHMO and the results of studies with the purified CHMO.

*Exophiala jeanselmei* strain KUFI-6N was grown at 30°C in YPD medium (yeast extract 10 g/l; peptone 20 g/l; dextrose 20 g/l, pH 6.0) containing 0.3% cyclohexanol. Cells were harvested by centrifugation at 8,000 × g for 20 min when the absorbance reached 1.0 at 660 nm. For preparing cell extracts, cell paste was suspended in 10 mM KH2PO4-Na2HPO4 buffer (pH 7.5) and disintegrated by passage through a French pressure cell at 1300 lb/in². Unbroken cells and debris were removed by centrifugation at 100,000 × g and 0°C for 1 h. The activity and substrate specificity of CHMO were assayed by measuring the rate of the decrease in absorbance at 340 nm (ε = 6270 M⁻¹ cm⁻¹) due to the oxidation of NADPH to NADP⁺. The reaction mixture contained 1.0 µmol of substrate, 42.5 µmol of KH2PO4-Na2HPO4 buffer (pH 8.0), 0.2 µmol of NADPH, and enzyme in a final volume of 1 ml. Incubation was done at 25°C in a 1-cm light-path cuvette. One unit of CHMO was defined as the amount of enzyme needed to disappear 1 µmol of NADPH per min under these assay conditions. Protein was measured by the method of Bradford using crystalline bovine serum albumin as the standard. Protein from the column effluents was monitored by measuring the absorbance at 280 nm. Polyacrylamide slab gel electrophoresis (PAGE) was done by the method of Davis 10% polyacrylamide gel with Tris-glycine buffer, pH 8.3. Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) was done with 12.5% polyacrylamide and 0.1% SDS, by the method of Laemmli. Electrophoretic gel was stained with Coomassie blue R-250. The protein bands were visualized by staining with Coomassie blue R-250 and destained with water. Protein concentration of the fractions of eluted was measured by the method of Bradford using crystalline bovine serum albumin as the standard.

To whom correspondence should be addressed.
done with a Bio-Rad blot cell in accordance with the manufacture’s instructions. The protein band was stained with Coomassie Brilliant Blue R-250 and excised from the membrane, and the NH₂-terminal amino acids were sequenced with an Applied Biosystems model 476A protein sequencer.

All purification procedures were done at 4°C unless otherwise specified. The cell extracts of E. jeanselmei harvested from 15 liters of culture were used as the starting material for the purification of CHMO. Solid ammonium sulfate was added to the cell-free extracts to a final 30% of saturation, and the pH was adjusted to 7.5 with ammonia. The precipitate formed was removed by centrifugation (8,000 × g, 30 min) and the supernatant was brought to 50% saturation by further addition of ammonium sulfate. The resultant precipitate was collected by centrifugation (8,000 × g, 30 min) and then dissolved in a small volume of 10 mM KH₂PO₄-Na₂HPO₄ buffer (pH 7.5). This enzyme solution was dialyzed against the same buffer for 12 h. The dialyzed solution was put on a DEAE-Cellulofine AH column (3 × 10 cm) equilibrated with 10 mM KH₂PO₄-Na₂HPO₄ buffer (pH 7.5). After it was washed with the same buffer, the enzyme was eluted using a 200-ml linear gradient of 0-0.2 M KCl in the same buffer, at a flow rate of 15 ml/h. The enzyme was eluted at 0.07-0.11 M KCl. The active fractions (total 20 ml) were combined and concentrated to 2.5 ml using polyethylene glycol 20,000. The concentrated enzyme was put on a Sephadex G-200 column (1.5 × 100 cm) pre-equilibrated with 10 mM KH₂PO₄-Na₂HPO₄ buffer (pH 7.5). The elution was done at a flow rate of 6 ml/h with the same buffer, and the active fractions (total 6 ml) were pooled.

As shown in Table 1, the enzyme was purified 17.3-fold with a 17.1% yield after 3 steps from the cell extracts, and the specific activity was 670 milliunits per mg of protein. The purified preparation gave a single band on SDS-PAGE and native PAGE (data not shown), indicating homogeneity of the protein.

The molecular mass of Exophiala CHMO was estimated to be approximately 74 kDa by gel filtration on Superose 12. A similar molecular mass, 74 kDa, was obtained by SDS-PAGE. These results indicate that the enzyme is a monomeric protein. The molecular mass of Exophiala CHMO is larger than Acinetobacter CHMO (59 kDa), Nocardia CHMO (53 kDa), or Xanthobacter CHMO (50 kDa) which has a single polypeptide chain.

Investigation into the stoichiometry of purified Exophiala CHMO demonstrated that the oxidation of 1 μmol of cyclohexanol was accompanied by the consumption of 1 μmol of O₂ and 1 μmol of NADPH in a similar manner to that of the cell extracts, which is close to that theoretically required for a mixed-function monooxygenase. NADPH cannot be replaced by NADH to supply electrons. Thus, the characterization of Exophiala CHMO is very similar to that of other bacterial Baeyer-Villiger type-CHMOs.

The enzyme had an optimum pH of 8.0. When the enzyme was incubated at different pHs for 1 h at 30°C, no remaining activity was observed below pH 5.5 or above pH 10.5. The enzyme was stable from pH 7.0 to 8.0. Substrates that oxidized NADPH at the same rate as cyclohexanol (relative activity = 100%) include cyclobutanone (98%), cyclopentanone (87%), and cycloheptanone (104%). Furthermore, cyclooctanone (64%), cyclocdecane (22%) and cycloundecanone (11%), on which the activity of the bacterial CHMOs is relatively insignificant or not

### Table 1. Summary of the Purification of Cyclohexanol 1,2-Monoxygenase from E. jeanselmei KUFI-6N

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity (mU)</th>
<th>Specific activity (mU/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>182</td>
<td>7100</td>
<td>39.0</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>30-50% (NH₄)₂SO₄</td>
<td>102</td>
<td>6810</td>
<td>67.0</td>
<td>95.0</td>
<td>1.7</td>
</tr>
<tr>
<td>DEAE-Cellulofine AH</td>
<td>16.3</td>
<td>3420</td>
<td>210</td>
<td>48.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>1.8</td>
<td>1220</td>
<td>678</td>
<td>17.1</td>
<td>17.3</td>
</tr>
</tbody>
</table>

### Binding motif for adenosine moiety of FAD

| Exophiala CHMO  | 1AKSL[DVL]IVGAGFPGI[IVQL] |
| Acinetobacter CHMO | 1MSQKMDF[DAI]IVGAGFPGLYAV |
| Rhodococcus CHMO | 1TAITHV[DAI]VAGAGFPGLYA |
| Pseudomonas CHMO  | 1TMTMTTTEQLG[MNSSVNDKL]DVLILIGAG |
| Rhodococcus SMO | 1MGQHPRSVVTADATGTTSYDDVTVVAGCAALYA |

### Fig. 1. Alignment of the NH₂-Terminal Amino Acid Sequence of Exophiala CHMO with Similar Sequences from the Bacterial BVMOs.

The NH₂-terminal amino acid sequence of Acinetobacter CHMO and Rhodococcus SMO was obtained from the nucleotide sequence. Amino acid residues shared by three, four, or five sequences were boxed. The location of the consensus sequences as described by Eppink et al., GXGXXG, are as indicated. CHMO; cyclohexane monooxygenase, CPMS; cyclopentanone monooxygenase, SMO; steroid monooxygenase.
detectable (Hasegawa, Y. et al., unpublished results), are also viable substrates for Exophiala CHMO. The substrate specificity of Exophiala CHMO shows a difference in the characteristics of the bacterial CHMOs which are mainly specific towards alicyclic rings with four to seven carbon atoms. Various types of chemical agents and metal ions (each at a final concentration of 1 mM) were examined for their inhibitory or stimulatory actions on Exophiala CHMO. The enzyme was inhibited completely by Cu2+ and 5,5'-dithiobis-(2-nitrobenzoate). The enzyme was 70 to 50% inhibited by flavin enzyme inhibitors such as quinine and quinacrine. Enzyme stimulation was not observed with any of the chemical agents and metal ions tested. The K_m for cyclohexanone and NADPH were 0.48 μM and 14.2 μM, respectively. The free prosthetic group was obtained by extraction with pyridine. The spectrum of the extract had maximal absorption at 374 nm and 450 nm. When the extract was chromatographed on cellulose thin-layer plate (DC-Fertigplatten cellulose plates, 5 × 20 cm, Merck) with the solvent system n-butanol/acetone/acidic acid/water (5/2/1/3, by vol.), it showed a single bright yellow fluorescent spot with a R_f of 0.07. With the same solvent system, FAD gave a single spot with an identical R_f. These results indicate that the Exophiala CHMO is a flavoprotein, the prosthetic group of which is FAD.

The NH2-terminal amino acid sequence of the Exophiala CHMO, 1-AKSLDV1IVAGFGG1YQ1-19, was obtained by using an automated protein sequence. We compared this sequence with those of the bacterial CHMOs. The Exophiala CHMO was found to show significant similarity to the bacterial CHMOs and a steroid monooxygenase from Rhodococcus rhodochrous strain IFO 3338 that belong to FAD-binding and NADPH-dependent type Baeyer-Villiger monooxygenase (Fig. 1). Furthermore, the consensus sequence of the putative FAD-binding site (GXGXG) is found within this sequence.

Cloning and DNA sequence analysis of an Actinobacter CHMO-encoding gene was pioneered by Chen et al. A second BVMO-encoding gene, that of a steroid monooxygenase from Rhodococcus was reported by Morii et al. Exophiala CHMO gene cloning is in progress to gain a better understanding of the structure.

Acknowledgments

Financial support for this study was provided by the Kansai University Grant-in Aid for the Faculty Joint Research Program, 2000 and by The Science Research Promotion Fund administered from the Japan Private School Promotion Foundation.

References