Note

Purification and Characterization of Pyrocatechase from the Catechol-assimilating Yeast *Candida maltosa*

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Pseudomonas and *Trichosporon* etc. can assimilate aromatic compounds such as toluene,¹⁾ naphthalene,²⁾ salicylate,³⁾ and phenol.⁴⁾ However, there are few microorganisms that can grown on catechol, though it is a central metabolite in the breakdown of various kinds of aromatic compounds by microorganisms. Pyrocatechase, catechol-1,2-dioxygenase, which catalyzes the incorporation of two atoms of molecular oxygen into catechol, has been purified from several bacteria and yeast which can grown on aromatic compounds.^{5~9)}

We searched for catechol-assimilating microorganisms, including bacteria and yeast, from the stock cultures of the ATCC as well as soil samples collected throughout Japan. As a result, several yeasts which grew on a catechol-containing medium were obtained. Especially, we found

new evidence that *Candida maltosa* (ATCC 20184) could grown on catechol very well and also accumulated muconic acid in the culture medium. This strain is useful for the production of muconic acid from catechol by bioprocess. It is necessary to characterize the pyrocatechase of this strain to establish the bioprocess for muconic acid production. This paper deals with the purification and some properties of the pyrocatechase from *Candida maltosa* (ATCC 20184).

The stock cultures and the soil samples were tested by shaking culture at 30°C for several days in a cultivation medium consisting of: 1 g of catechol, 4 g of NH_4NO_3 , 4 g of NaCl, 1.15 g of Na_2HPO_4 , 0.2 g of KH_2PO_4 , 0.1 g of KCl, 10 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 500 mg of yeast extract and 1 l of deionized water. Several *Candida* species grew on catechol, *Candida maltosa* (ATCC 20184) especially well and accumulated a product in the culture medium. This product was identified by GC-MS analysis and UV spectrum. The cultured medium (100 ml) of *C. maltosa* grown on catechol was centrifuged and pooled. The product was extracted with ethyl acetate from the supernatant after adjusting the pH to 2.0 with 2N HCl and the solvent was evaporated with a rotary vacuum evaporator. The sample was dissolved in a small amount of methanol, and put on a reversed phase HPLC column (Cosmosil 5C18, Nakarai Chemicals LTD.). HPLC analysis was done using a Shimadzu LC 4A with a three-dimensional chromatographic analyzer (Multi-320, Japan Spectroscopic Co., Ltd.), eluting with methanol containing 0.05% acetic acid: water containing 0.05% acetic acid = 70:30, at a flow rate of 1.0 ml/min. The chromatogram gave a single major peak with a retention time of 4.8 min and maximum absorbance at 260 nm but it was different from that of *t, t*-muconic acid (retention time 4.4 min) as shown in Fig. 1. The sample

FIG. 1. Reversed Phase HPLC of the Product in the Cultured Medium of *C. maltosa* Grown on Catechol. *t, t*-Muconic acid was added to the sample solution as a standard.

FIG. 2. Chromatography on DEAE-5PW Column of Pyrocatechase from *C. maltosa*.TABLE I. PURIFICATION OF PYROCATECHASE FROM *Candida maltosa*

	Activity (u/ml)	Prot. conc. (mg/ml)	Specific act. (u/mg)	Fold	Yield (%)
Crude enzyme	2.60	5.70	0.456	1	100
DEAE-5PW	1.72	0.055	31.2	68	86
G3000-SW	1.14	0.012	95.0	208	55

was methylated by diazomethane and analyzed by diazomethane and analyzed by GC-MS (QP-1000 Shimadzu Co. Ltd.) with a temperature gradient from 100 to 200°C using a silicon OV-1 column (3 mm × 1 m) and He as a carrier gas (flow rate 30 ml/min). Spectrum analysis revealed a molecular ion peak at 170. Accordingly GC-MS analysis of the methylated derivative was done after UV irradiation in alcohol. The fragment pattern was identified as that of methylated *t,t*-muconic acid at this time, therefore, it was concluded that the product in the cultured medium was *cis, cis*-muconic acid.

The pyrocatechase of this strain was a inducible enzyme because its activity was not detected in cells or cell-free extract which was cultivated in a nutrient-rich medium such as YM Broth (Difco Laboratories) or glucose-containing medium. Therefore, pyrocatechase was purified from the cells cultured in a catechol-containing medium. The enzyme activity was monitored by measuring the rate of increase in absorbance at 260 nm in 50 mM Tris-HCl buffer (pH 8.0) containing 200 μM catechol as substrate. Five grams of wet cells were suspended in 15 ml of 100 mM Tris-HCl buffer (pH 9.0) and homogenized with 40 g of glass beads (0.45 mm) by a Braun Cell Homogenizer at 4000 rpm for 1 min with constant cooling by blowing CO₂ gas. The crude enzyme solution was obtained from the supernatant after centrifugation for 30 min (10,000 × g) at 4°C. It was concentrated by ultrafiltration using a polysulfon membrane which excludes molecules less than 10,000

daltons, and put directly on a HPLC column (TSK-DEAE 5PW, Toyo Soda Manufacturing Co., Ltd.). HPLC was done under the following conditions: apparatus, LKB HPLC system; detection, absorbance at 280 nm; eluate: 50 mM Tris-HCl (pH 9.0), same buffer containing 0.5 M NaCl = 100:0 to 0:100; flow rate, 0.75 ml/min. The enzyme was developed by a linear gradient of NaCl from 0 to 0.5 M. The pyrocatechase activity was detected at the position of about 0.23 M of NaCl as shown in Fig. 2. The active fractions were collected and put on another HPLC column (TSK G-3000 SW, Toyo Soda Manufacturing Co., Ltd.) after concentration using the above apparatus. As a result, enzyme activity was detected at the point corresponding to a molecular weight of 65,000, which was estimated from the calibration curve using a standard kit purchased from Pharmacia Inc. Then the purity was checked by SDS polyacrylamide gel electrophoresis, which gave a single major band with a molecular weight of 33,000. This suggested that pyrocatechase was present in a native state as a dimer of homogeneous subunits with a molecular weight of 33,000. It was reported by Itoh that the molecular weight of pyrocatechase from *Trichosporon* was 105,000 and it was present as a tetramer of identical subunits with a molecular weight of 35,000.⁹⁾ Table I summarizes the purification. Ultimately, 18 μg of purified pyrocatechase was obtained from 5 g wet cells of *Candida maltosa* and it was purified to 208-fold, as calculated from the crude enzyme

solution. Its yield was 55% and the specific activity of the purified enzyme was 95 u/mg protein. The enzyme was found to be stable between pH 8.0 and 9.0 and up to 35°C and its optimal reaction temperature and pH were 37°C and pH 8.5.

REFERENCES

- 1) M. J. Worsey and P. A. Williams, *J. Bacteriol.*, **124**, 7 (1975).
 - 2) N. W. Dunn and I. C. Gunsalus, *J. Bacteriol.*, **114**, 974 (1973).
 - 3) A. M. Chakrabarty, *J. Bacteriol.*, **112**, 8125 (1972).
 - 4) J. M. Varga and H. Y. Neujahr, *Eur. J. Biochem.*, **13**, 37 (1970).
 - 5) H. Nakagawa, H. Inoue and Y. Takeda, *J. Biochem.*, **54**, 65 (1963).
 - 6) C. Nakai, H. Kagamiyama, Y. Saeki and M. Nozaki, *Arch. Biochem. Biophys.*, **195**, 12 (1979).
 - 7) R. N. Patel, C. T. Hou, A. Felix and M. O. Lillard, *J. Bacteriol.*, **127**, 536 (1976).
 - 8) E. Dorn and H. J. Knackmuss, *Biochem. J.*, **174**, 73 (1978).
 - 9) M. Itoh, *Agric. Biol. Chem.*, **45**, 2787 (1981).
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