Isolation of cytoplasmic NADPH-dependent phenol hydroxylase and catechol-1,2-dioxygenase from *Candida tropicalis* yeast

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ABSTRACT

The efficiencies of NADPH-dependent phenol hydroxylase (EC 1.14.13.7) and catechol 1,2-dioxygenase (EC 1.13.11.1) in biodegradation of phenol in the cytosolic fraction isolated from yeast *Candida tropicalis* were investigated. Enzymatic activities of both NADPH-dependent phenol hydroxylase and catechol 1,2-dioxygenase were detected in the cytosolic fraction of *C. tropicalis* grown on medium containing phenol. Using the procedure consisting of chromatography on DEAE-Sepharose, fractionation by polyethylene glycol 6000 and gel permeation chromatography on Sepharose 4B the enzyme responsible for phenol hydroxylation in cytosol, NADPH-dependent phenol hydroxylase, was isolated from the cytosolic fraction of *C. tropicalis* close to homogeneity. However, fractionation with polyethylene glycol 6000 lead to a decrease in catechol 1,2-dioxygenase activity. Therefore, another procedure was tested to purify this enzyme. Gel permeation chromatography of proteins of the eluate obtained by chromatography on a DEAE-Sepharose column was utilized to separate phenol hydroxylase and catechol 1,2-dioxygenase. Among gel permeation chromatography on columns of Sephadex G-100, Sephacryl 5-300 and Sepharose 4B tested for their efficiencies to isolate phenol hydroxylase and catechol 1,2-dioxygenase, that on Sephacryl 5-300 was found to be suitable for such a procedure. Nevertheless, even this chromatographic method did not lead to obtain catechol 1,2-dioxygenase in sufficient amounts and purity for its further characterization. The data demonstrate the progress in resolving the enzymes responsible for the first two steps of phenol degradation by the *C. tropicalis* strain.

KEY WORDS: environmental pollutants; phenol; biodegradation; yeast; *Candida tropicalis*; NADPH-dependent phenol hydroxylase; catechol-1,2-dioxygenase

Introduction

Phenol and its derivatives are found in wide variety of wastewaters including those from the oil refining, petrochemical, coke and coal gasification industries. Removal of phenol from such wastewaters can be achieved through aerobic biodegradation in well-run activated sludge plants. *Pseudomonas* is a bacterial genus commonly found in such plants and *Pseudomonas putida* is a species capable of using phenol as a major source (Bayly and Wigmore, 1973; Yang and Humphrey, 1975). In addition, several other mesophilic bacteria are able to degrade phenol, including *Alcaligenes spp.* and *Sphingomyces setonii* and also the thermophilic, *Bacillus stearothermophilus* (Gurujevalakshmi and Oriel, 1989). Although bacteria are most likely to be responsible for aerobic breakdown of phenol in activated sludge, fungi including *Trichosporon cutaneum*, *Candida albicans* TL3 and *Candida tropicalis* are also capable of utilizing phenol as the major carbon source (Krug et al., 1985; Krug and Straube, 1986; Chang et al., 1998; Bastos et al., 2000; Komárková and Páca, 2000; Páca et al., 2002; Komárková et al., 2003; Stiborová et al., 2003; Ahuatzi-Chacon et al., 2004; Tsai et al., 2005). The aerobic degradation pathways in bacteria and yeast involve the occurrence of vicinal diols as substrates of ring-cleaving enzymes. Thus, the first step of phenol degradation is a hydroxylation of phenol to catechol (Figure 1). Catechol can undergo fission either by an intra-diol or an extra-diol type of cleavage (*ortho*- or *meta*-fission). *Meta*-fission leads to 2-hydroxymuconic semialdehyde and further to formate, acetaldehyde, and pyruvate. Such a catechol cleavage was not found in yeast. *Ortho*-fission, found in yeast such as *T. cutaneum*, *C. albicans* TL3 and *C. tropicalis*, gives rise to cis,cis-muconic acid (Figure 2), which is converted in further enzymatic steps via 3-oxoadipate to succinate and acetyl-CoA. These products enter the central metabolism of the cell (Krug et al., 1985; Krug and Straube,
The aim of the present study was to develop the procedure to isolate both phenol hydroxylase and catechol 1,2-dioxygenase from the cytosolic fraction of C. tropicalis. Materials and methods

Chemicals

Chemicals were obtained from the following sources: NADPH, catechol and bicinchoninic acid (2,2’-biquinoline-4,4’-dicarboxylic acid) from Sigma Chemical Co., (St. Louis, MO), DEAE-Sepharose, Sephacryl S-300, Sephadex G-100 and Sepharose 4B from Pharmacia (Uppsala). Other chemicals were supplied by Lachema (Brno). All chemicals were of reagent grade purity or better.

Microorganisms, cultivation methods and preparation of microsomal and cytosolic fractions

The yeast C. tropicalis was isolated from soil contaminated with aromatic hydrocarbons and identified using the culture collection and Research Center (Brno, Czech Republic) (Komárková and Páca, 2000). The yeast culture was maintained on slope agar with mineral salts and glucose as a carbon and energy source at 4°C. The growth medium was BSM medium [4.3 g/l K₂HPO₄, 3.4 g/l KH₂PO₄, 2 g/l (NH₄)₂SO₄, 0.34 g/l MgCl₂, 6 H₂O] containing 350 g/l phenol as a sole carbon and energy source (phenol medium). Cell cultivations were carried out in shaking flasks using fed batch process with the growth medium containing phenol (see above) at 30°C and pH 5.2 as described previously (Martius et al., 1996; Páca and Martius, 1996; Stiborová et al., 2003; Páca et al., 2007).

After separation, the cells were washed three times with distilled water and disintegrated using mechanical disruption of the cells in the presence of liquid nitrogen to obtain the cell-free homogenate. The isolation of the microsomal and cytosolic fractions from the C. tropicalis cell-free homogenate was carried out by differential centrifugation (Stiborová et al., 2003; Páca et al., 2007) by the procedure used for isolation of such subcellular fractions from rat liver (Stiborová et al., 1995, 2001a,b).

Purification of NADPH-dependent phenol hydroxylase and catechol 1,2-dioxygenase from C. tropicalis cytosol

All operations were carried out at 4°C. For the first, the procedure which was used in our former study to isolate NADPH-dependent phenol hydroxylase was used (Páca et al., 2007). Briefly, cytosolic fraction of C. tropicalis (330 ml) was applied to a DEAE-Sepharose column (2.6×22 cm) equilibrated with 50 mM sodium phosphate buffer pH 7.6. The NADPH-dependent phenol hydroxylase was eluted using a linear gradient of 0–0.5 M NaCl in the same

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buffer. Phenol hydroxylase eluted at 0.1–0.15 M. Fractions containing phenol hydroxylase activities were pooled and precipitated by PEG 6000 (16% saturation). The precipitate was dissolved in 50 mM sodium phosphate buffer pH 7.6 (2 ml) and applied to a Sepharose 4B column (1.6×60 cm), previously equilibrated with the same buffer. The enzyme was eluted using 50 mM sodium phosphate buffer pH 7.6 and the fractions containing the phenol hydroxylase activity were pooled, frozen and stored at −20 °C until used.

However, using precipitation of proteins with PEG 6000 (16% saturation), no catechol 1,2-dioxygenase activity was detectable. Therefore, proteins of eluate obtained by DEAE-Sepharose chromatography were lyophilized and enzymes additionally purified by gel permeation chromatography on columns (0.5×50 cm, bed volume 20 ml) of Sephadex G-100, Sephacryl S-300 and Sepharose 4B, equilibrated with 50 mM sodium phosphate buffer pH 7.6. Lyophilized proteins (2 mg), re-suspended in 200 μl of 50 mM sodium phosphate buffer (pH 7.6), were applied on a column and eluted with the same buffer.

The phenol hydroxylase and catechol 1,2-dioxygenase activities were followed by formation and consumption of catechol, respectively, measured with HPLC, using a column of Nucleosil 100-5 C18 (4×250 mm) as described previously (Stiborová et al., 2003; Páca et al., 2007). The major product formed by phenol hydroxylation was identified by comparison of its retention time with an authentic standard of catechol, having the retention time of 7.9 min and by mass and UV/vis absorbance spectroscopy. Mass spectra were recorded on a FINNIGAN MAT INCOS 50 (electron impact, 70 eV, low resolution, direct inlet). UV/vis spectra were recorded on a Hewlett-Packard 8453 diode array spectrophotometer (Stiborová et al., 2003; Páca et al., 2007).

Results

The cytosolic fraction of C. tropicalis is able to oxidize phenol; a time-dependent decrease in phenol followed by an increase in formation of an oxidation metabolite, catechol, was found (data not shown). Non-Michaelian saturation curves were seen when the initial velocity of phenol oxidation catalyzed by the crude cytosolic fraction was plotted as a function of phenol concentrations (Figure 3A,B). The observed decrease in amounts of catechol generated in incubations containing the crude cytosolic fraction (Figure 3B), might be caused by its consumption with catechol 1,2-dioxygenase, whose activity was found by Ahuatzi-Chacon and collaborators (2004) in C. tropicalis cytosol. Therefore, these findings suggest the presence not only of NADPH-dependent phenol hydroxylase, but also catechol 1,2-dioxygenase in this subcellular fraction.

Using the procedure described in our previous work (Páca et al., 2007), consisting of the chromatography on a DEAE-Sepharose column, fractionation with PEG 6000 and a gel filtration on Sepharose 4B (Table 1A), NADPH-dependent phenol hydroxylase was purified from the cytosolic fraction of C. tropicalis close to homogeneity. Figure 4 shows the kinetics of phenol oxidation by the purified enzyme. Phenol consumption and formation of catechol was measured in the reaction medium, which contained isolated NADPH-dependent phenol hydroxylase, NADPH and various concentrations of phenol. The reaction measured by both phenol consumption and formation of catechol followed the Michaelis-Menten kinetics (Figure 4). The Michaelian kinetics measured by formation of catechol confirms that the preparation of purified phenol hydroxylase is free of catechol 1,2-dioxygenase utilizing catechol as substrate. The values of a maximal velocity (Vmax) and an
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The separation of both enzymes was reached only using chromatography on a column of Sephacryl S-300, whereas chromatography on Sephadex G-100 and Sepharose 4B was ineffective under the conditions used in the experiments.

**Discussion**

Several fungi including *C. tropicalis* are capable of utilizing phenol as the sole carbon and energy source. These organisms might therefore be useful for biotechnological applications such as decontamination of phenol in wastewaters. The hydroxylation of phenol to catechol, and its additional intra-diol cleavage are the initial and rate-determining steps in the phenol degradation pathways in *Candida* yeast. The question which of the yeast enzymes are responsible for these two steps of phenol degradation in *C. tropicalis* yeast has not been fully answered yet.

Here, we demonstrate that NADPH-dependent phenol hydroxylase capable of oxidation of phenol to catechol and catechol 1,2-dioxygenase are present in cytosol of *C. tropicalis* yeast. Non-Michaelian saturation curves were seen when the initial velocity of phenol oxidation catalyzed by the crude cytosolic fraction was plotted as a function of phenol concentrations. Here, we show that the observed decrease in amounts of catechol generated in incubations containing the crude cytosolic fraction, is caused by its consumption with catechol 1,2-dioxygenase.

**Table 1.** Purification of NADPH-dependent phenol hydroxylase and catechol 1,2-dioxygenase from *C. tropicalis*.

| Fraction               | Volume (ml) | Proteins (mg/ml) | phenol hydroxylase (nmol phenol/min/mg) | catechol 1,2-dioxygenase (nmol catechol/min/mg) |
|------------------------|-------------|-----------------|----------------------------------------|-----------------------------------------------|
| Cytosol                | 330.0       | 2.16            | 22.1                                   | not measured                                  |
| Eluate, DEAE-Sepharose | 70.0        | 1.73            | 28.4                                   | 23.1                                          |
| PEG 6000, ppt 0-16%    | 5.2         | 4.14            | 32.5                                   | not detectable                                |
| Eluate, Sepharose 4B   | 13.8        | 1.15            | 41.5                                   | not detectable                                |

*a* ppt – precipitate

| Fraction               | Specific activity |
|------------------------|-------------------|
| phenol hydroxylase     | catechol 1,2-dioxygenase |
| (nmol phenol/min/mg)   | (nmol catechol/min/mg) |
| Cytosol                | 22.1              | not measured |
| Eluate, DEAE-Sepharose | 28.4              | 23.1         |
| Lyophilisate           | not measured      | not measured |
| Eluate, Sephadex G-100 | 14.8              | 54.8         |
| Eluate, Sephacryl S-300| 45.9              | 100.0        |
| Eluate, Sepharose 4B   | 30.0              | 110.8        |

Experimental conditions are described in the Material and methods section.
NADPH-dependent phenol hydroxylase was purified from the cytosolic fraction of *C. tropicalis* by the procedure consisting of chromatography on DEAE-Sepharose, fractionation by polyethylene glycol 6000 and gel filtration on Sepharose 4B and partially characterized. The $K_m$ value for phenol was one order of magnitude higher than the value found for the enzyme from yeast *Trichosporon cutaneum* (Neujahr and Gaal, 1973). Although catechol 1,2-dioxygenase has recently been successfully isolated from *C. tropicalis* (Ahuatzi-Chacon et al., 2004), the enzyme has not been isolated from *C. tropicalis* as yet. Here, we tried to develop a procedure capable of separating catechol 1,2-dioxygenase from phenol hydroxylase, partially purified by chromatography on DEAE-Sepharose. The partial separation of both enzymes was reached using gel permeation chromatography on a column of Sephacryl S-300. Nevertheless, even this chromatographic method did not provide catechol 1,2-dioxygenase in sufficient amounts and purity for its further characterization. Therefore, additional procedures for its isolation are now tested in our laboratory.

In conclusion, the results presented in this paper demonstrate the ability of cytosolic NADPH-dependent phenol hydroxylase and catechol 1,2-dioxygenase of *C. tropicalis* to metabolize phenol, which is a contaminant of a wide variety of wastewaters. Here we assume that organisms rich in such enzymes might be able to degrade phenol and might be utilized in bioremediation technologies. The data shown in the paper are the first report showing isolation of both NADPH-dependent phenol hydroxylase and catechol 1,2-dioxygenase from *C. tropicalis* and demonstrate the progress in resolving the enzymes responsible for the first steps of phenol degradation by the *C. tropicalis* strain.

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