ORIGINAL ARTICLE

Isolation and partial characterization of catechol 1,2-dioxygenase of phenol degrading yeast *Candida tropicalis*

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Submitted: 2009-07-14 Accepted: 2009-08-28 Published online: 2009-11-15

Key words: **phenol; biodegradation; yeast; *Candida tropicalis*; catechol 1,2-dioxygenase**

Neuroendocrinol Lett 2009; **30**(Suppl 1): 80–87 **PMID:** 20027149 NEL300709A12 © 2009 Neuroendocrinology Letters • **www.nel.edu**

Abstract

OBJECTIVES: *Candida tropicalis* yeast is a microorganism that possesses high tolerance for phenol and shows strong phenol degrading activity. This yeast is capable of utilizing phenol as the sole carbon and energy source. While the enzyme participating on the first step of phenol biodegradation, NADPH-dependent phenol hydroxylase, has already been characterized, information on the enzyme participating in the second step of its degradation, catechol 1,2-dioxygenase, is scarce. The development of the procedure suitable for catechol 1,2-dioxygenase isolation and partial characterization of this enzyme are the aims of this study.
METHODS: Combination of chromatography on DEAE-Sepharose and gel-permeation chromatography on Sephadex G-100 was used for isolation of cytosolic catechol 1,2-dioxygenase from *C. tropicalis* yeast. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel chromatography on Sephadex G-100 were used to evaluate the molecular mass of the enzyme. The enzyme activity was followed by HPLC (catechol consumption and/or *cis,cis*-muconic acid formation).
RESULTS: Using the isolation procedure consisting of chromatography and re-chromatography on a column of DEAE-Sepharose and gel filtration on Sephadex G-100, catechol 1,2-dioxygenase was purified from *C. tropicalis* cytosol to homogeneity. Catechol 1,2-dioxygenase was found to be a homodimer with a subunit molecular mass of 30000 ± 5000. The enzyme oxidized catechol producing *cis,cis*-muconic acid. The optimal temperature and pH were 30°C and 7.7, respectively.
CONCLUSIONS: The data are the first report showing the isolation of eukaryotic catechol 1,2-dioxygenase from *C. tropicalis* to homogeneity and its partial characterization.

Abbreviations & Units:

BSA	– bovine serum albumin
°C	– centigrade grades
Da	– Dalton
DEAE-Sepharose	– diethylaminoethyl-Sepharose
HPLC	– high performance liquid chromatography
h	– hour
µl	– microliter
mg	– milligram
min	– minute
M	– mol/liter
mM	– milimol/liter
µmol	– micromole
Mw	– molecular weight
Nm	– nanometer
NADPH	– nicotinamide adenine dinucleotide phosphate (reduced)
r.t.	– retention time
SDS-PAGE	– sodium dodecyl sulfate-polyacrylamide gel electrophoresis

INTRODUCTION

Phenol and its derivatives are found in a 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 & Wigmore, 1973; Yang & Humphrey, 1975). In addition, several other mesophilic bacteria are able to degrade phenol, including *Alcaligenes spp.* and *Spreptomyces setonii* and also the thermophile, *Bacillus stearothermophilus* (Gurujeyalakshmi & 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 & Straube, 1986; Chang *et al.* 1998; Bastos *et al.* 2000; Komarkova & Paca, 2000; Paca *et al.* 2002; Komarkova *et al.* 2003; Stiborova *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. 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 1***), which is converted in further enzymatic steps *via* 3-oxoadipate to succinate and acetyl-coenzyme A. These products enter the central metabolism of the cell (Krug *et al.* 1985, Krug & Straube, 1986; Bastos *et al.* 2000; Komarkova and Paca, 2000; Paca *et al.* 2002; Komarkova *et al.* 2003; Ahuatzi-Chacon *et al.* 2004; Tsai *et al.* 2005). Although examples are known in which the yeast *C. tropicalis* utilizes phenol for growth or metabolism (Krug *et al.* 1985, Krug and Straube, 1986, Stephenson, 1990, Chang *et al.* 1998, Bastos *et al.* 2000, Komarkova and Paca, 2000, Paca *et al.* 2002, Komarkova *et al.* 2003, Ahuatzi-Chacon *et al.* 2004), much less information on the nature of the phenol-oxidizing enzymes in this microorganism are known.

The enzymes responsible for the first step of degradation (the formation of catechol) in *C. tropicalis* yeast are: (i) cytochrome P450 (EC 1.14.15.1), the enzyme of the mixed function monooxygenase system localized in the membrane of endoplasmic reticulum (Stiborova *et al.* 2003, 2004; Sulc *et al.* 2008) and (ii) cytoplasmic NADPH-dependent phenol hydroxylase (EC 1.14.13.7) (Krug *et al.* 1985, Krug & Straube, 1986, Xu *et al.* 2001, Stiborova *et al.* 2004, Paca Jr *et al.* 2007). Indeed, recently, we have found that microsomal cytochrome P450 and cytosolic NADPH-dependent phenol hydroxylase are expressed in *C. tropicalis* grown on phenol and are capable of hydroxylation of phenol to form catechol (Stiborova *et al.* 2003; Paca Jr *et al.* 2007). Cytosolic NADPH-dependent phenol hydroxylase seems to be the predominant enzyme responsible for the first step of phenol biodegradation in the *C. tropicalis* yeast; its activity is more than two orders of magnitude higher than that found in the microsomal fraction of this microorganism (Stiborova *et al.* 2003; Paca Jr *et al.* 2007). NADPH-dependent phenol hydroxylase has already been purified from the cytosolic fraction of *C. tropicalis* and partially characterized (Paca Jr *et al.* 2007; Vilimkova *et al.* 2008).

During the second step of phenol degradation in *Candida* yeast, intra-diol cleavage of catechol to *cis,cis*-muconic acid (Figure 1) occurs (Bastos *et al.* 2000; Paca *et al.* 2002; Ahuatzi-Chacon *et al.* 2004; Tsai *et al.* 2005; Tsai and Li, 2007), being catalyzed by cytosolic catechol 1,2-dioxygenase (EC.1.13.11.1), the enzyme found in several microorganisms (Nakai *et al.* 1990; Eck & Bettler, 1991; Briganti *et al.* 1997; Shen *et al.* 2004), including yeast *C. albicans* (Tsai & Li, 2007). However, information on catechol 1,2-dioxygenase of *C. tropicalis* is still scarce. Even though the activity of this enzyme was detected in *C. tropicalis* cytosol (Ahuatzi-Chacon *et al.* 2004), its successful isolation from this microorganism has not been described yet (Vilimkova *et al.* 2008). The development of the procedure suitable for isolation of catechol 1,2-dioxygenase from cytosolic fraction of *C.*

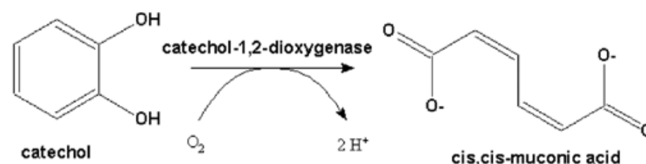

Figure 1. Catechol intra-diol cleavage to *cis,cis*- muconic acid

tropicalis and its partial characterization of this enzyme are the aims of this study.

MATERIAL & METHODS

Chemicals.: Nicotinamide adenine dinucleotide phosphate (reduced) (NADPH), catechol, *cis,cis*-muconic acid, bicinchoninic acid (2,2´-biquinoline-4,4´-dicarboxylic acid) were obtained from Sigma Chemical Co. (St. Louis, MO); DEAE-Sepharose, Sephadex G-100 were obtained from Pharmacia (Uppsala, Sweden). Other chemicals were obtained from Pliva-Lachema (Brno, Czech Republic). All these and other chemicals were of reagent grade purity or better.

Microorganisms and their cultivation The yeast *C. tropicalis* was isolated from soil contaminated with aromatic hydrocarbons and identified using the culture collection in Research Center (Brno, Czech Republic) (Komarkova & Paca, 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 $g.l^{-1}$ K_2HPO_4, 3.4 $g.l^{-1}$ KH_2PO_4, 2 $g.l^{-1}$ $(NH_4)_2SO_4$, 0.34 $g.l^{-1}$ $MgCl_2.6\ H_2O$] containing 350 $g.l^{-1}$ 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; Paca & Martius, 1996; Stiborova *et al.* 2003; Paca Jr *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 cytosolic fraction from the *C. tropicalis* cell-free homogenate was carried out by differential centrifugation (Stiborova *et al.* 2003, Arlt *et al.* 2004, Paca Jr *et al.* 2007), the procedure used for isolation of such subcellular fractions from rat tissues (Stiborova *et al.* 1995; 2001a, 2001b, 2006, Arlt *et al.* 2004; Krizkova *et al.* 2008; Sistkova *et al.* 2008).

Purification of cytosolic enzymes. All operations were carried out at 4°C. Cytosolic fraction (200 ml) was applied to a DEAE-Sepharose column (2.6 × 22 cm) equilibrated with 50 mM sodium phosphate buffer pH 7.6. Linear gradient of 0–0.3 M NaCl in the same buffer was used for separation of the cytosolic proteins. Phenol hydroxylase eluted at 0.12-0.18 M NaCl, while catechol 1,2-dioxygenase at 0.16–0.22 M NaCl (***Figure 2***). Fractions showing phenol hydroxylase and catechol 1,2-dioxygenase activities were pooled separately and dialyzed against 50 mM sodium phosphate buffer pH 7.6. Dialyzed samples were re-chromatographed separately on a DEAE-Sepharose column (1 × 10 cm), previously equilibrated with the same buffer. Fractions showing catechol 1,2-dioxygenase activity were pooled, dialyzed against distilled water and lyophilized. Lyophilized catechol-1,2-dioxygenase was dissolved in 50 mM sodium phosphate buffer pH 7.6 and additionally purified by gel permeation chromatography on column of Sephadex G-100 (1 × 60 cm) using 50 mM sodium phosphate buffer pH 7.6 as elution buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the enzyme samples obtained by enzyme purification was carried out as described by (Laemmli, 1970). Protein concentrations were assessed using the bicinchoninic acid protein assay with the bovine serum albumin as a standard (Weichelman *et al.* 1988).

Determination of phenol hydroxylase and catechol-1-,2-dioxygenase activities. 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 x 250 mm), and 40% (v/v) methanol-water mobile phase as described previously (Stiborova *et al.* 2003; Paca Jr *et al.* 2007; Vilimkova *et al.* 2008). The catechol 1,2-dioxygenase activity was also followed by formation of the product of catechol oxidation, *cis,cis*-muconic acid, using the same HPLC procedure described above. Catechol and *cis,cis*-muconic acid were identified by co-chromatography with authentic standards. Catechol and *cis,cis*-muconic acid eluted with retention times of 8.7 and 5.66 minutes, respectively. The activity of purified catechol 1,2-dioxygenase was also determined spectrophotometrically (Hewlett-Packard 8453 diode array spectrophotometer), by measuring an increase in absorbance at 260 nm (Tsai & Li, 2007). The reaction mixture contained in a total volume of 480 µl 0.5 M of sodium phosphate buffer pH 7.6, 10 µl of 50 mM catechol and such an amount of the enzyme sample guaranteeing that an increase of absorbance at 260 nm is linear for at least 120 seconds. The reaction was started by addition of the enzyme and an increase in 260 nm was measured for 240 seconds. The enzyme activity was calculated from ΔA_{260} produced per 60 seconds using a molar absorption coefficient of *cis,cis*-muconic acid of ε_{260} = 18 500 $mM^{-1}cm^{-1}$.

Determination of molecular mass of catechol 1,2-dioxygenase. Molecular mass of catechol 1,2-dioxygenase was estimated using a gel permeation chromatography and SDS-PAGE. The molecular mass of the native protein was determined using gel filtration on a Sephadex G-100 column (1 × 60 cm) and a series of standard proteins (BSA, ovalbumin, chymotrypsin, ribonuclease A). The molecular mass of the subunit of catechol 1,2-dioxygenase was determined using SDS-PAGE as described by (Laemmli, 1970). „Wide range" molecular mass markers (Sigma Chemical Co., St. Louis, MO, USA) were used as standards.

RESULTS

Purification of catechol 1,2-dioxygenase from C. tropicalis cytosol. In our previous study, we have detected catechol 1,2-dioxygenase that cleaves the catechol ring derived from the phenol degradation in the cytosolic

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Table 1. Purification of catechol 1,2-dioxygenase from *C. tropicalis*

Step	Fraction	Volume (ml)	Protein (mg/ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)
1	cytosol	200	25	13250	2.65	100
2	eluate, DEAE Sepharose chromatography	130	0.7	6288	69.10	47.45
3	eluate, DEAE Sepharose re-chromatography	46	0.98	5175	114.8	39
4	eluate, Sephadex G-100 chromatography	10	0.7	840	120.0	6.34

fraction isolated from *C. tropicals* and separated it partially from NADPH-dependent phenol hydroxylase by chromatography on a DEAE-Sepharose column (Vilimkova *et al.* 2008). In the present study, we extended our previous findings by purifying and partial characterizing this dioxygenase. Using the isolation procedure consisting of chromatography and re-chromatography on DEAE-Sepharose, followed by a gel filtration on a column of Sephadex G-100, the cytosolic catechol 1,2-dioxygenase enzyme of *C. tropicalis* was purified to homogeneity (***Table 1***).

One unit of catechol 1,2-dioxygenase activity is the amount of enzyme converting 1 nmol of catechol per one minute

Catechol 1,2-dioxygenase was separated from phenol hydroxylase using chromatography on a column of DEAE-Sepharose, by elution with gradient of NaCl (0–0.3 M) (Figure 2A). Phenol hydroxylase eluted at 0.12 – 0.18 M NaCl, while catechol 1,2-dioxygenase at 0.16 – 0.22 M NaCl (Figure 2). Fractions containing phenol hydroxylase and catechol-1,2-dioxygenase were pooled separately, dialyzed to remove NaCl, and catechol 1,2-dioxygenase was further purified by re-chromatography on DEAE-Sepharose (Figure 2B). Nevertheless, during such re-chromatography, this catechol 1,2-dioxygenase preparation was still contaminated with traces of phenol hydroxylase (***Figure 3***). Using a final purification step, gel-permeation chromatography on a column of Sephadex G-100 (***Figure 4***), a homogeneous preparation of catechol 1,2-dioxygenase was obtained (Figure 3). Yields for each step of the purification process are summarized in Table 1. A 45.3-fold purification of catechol 1,2-dioxygenase was achieved with yield of 6.3 %. The specific activity of the purified enzyme was 120 units per mg protein.

The catechol 1,2-dioxygenase activity of individual fractions obtained during its purification was estimated by HPLC. We found that catechol is oxidized with individual fractions of purified catechol 1,2-dioxygenase to a major reaction product having the retention time of 5.66 minutes. This metabolite was identified using co-chromatography with an authentic standard, *cis,cis*-muconic acid, having the same retention time, 5.66 min (Figure 5).

Characterization of catechol 1,2-dioxygenase. Gel-filtration analysis using a Sephadex G-100 column showed that the purified catechol 1,2-dioxygenase eluted with a volume corresponding to a molecular mass of about 63 000 ± 5 000 Da (Figures 4 and 6). Using SDS-PAGE, the purified enzyme appeared as a single band protein having a molecular mass of 30 000 ± 2 000 Da (Figure 3), suggesting that the enzyme is a dimeric protein consisting of two identical subunits.

Oxidation of catechol to *cis,cis*-muconic acid catalyzed by catechol 1,2-dioxygenase is influenced by pH and temperature. The optimal pH and temperature were pH 7.7 (Figure 7) and 30 °C (Figure 8), respectively.

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 (Krug *et al.* 1985, Krug & Straube, 1986, Bastos *et al.* 2000, Komarkova & Paca, 2000, Paca *et al.* 2002; Komarkova *et al.* 2003; Ahuatzi-Chacon *et al.* 2004; Tsai *et al.* 2005; Tsai & Li, 2007; Vilimkova *et al.* 2008).

NADPH-dependent phenol hydroxylase of *C. tropicalis*, catalyzing the first step of phenol degradation, has already been characterized (Paca Jr *et al.* 2007; Vilimkova *et al.* 2008). Since catechol 1,2-dioxygenase catalyzing the intra-diol cleavage of catechol has not yet been isolated in sufficient amounts and purity (Vilimkova *et al.* 2008), it has also not been characterized. Nevertheless, this enzyme plays a key role in the degradation pathways of various aromatic compounds, including phenol in several fungi (Tsai *et al.* 2005; Tsai & Li, 2007). Although catechol 1,2-dioxygenase has recently been successfully isolated from yeast *C. albicans* TL3 (Tsai & Li, 2007), and its activity was detected in *C. tropicalis* yeast (Ahuatzi-Chacon *et al.* 2004; Vilimkova *et al.* 2008), the enzyme has not been isolated from this microorganism as yet. The enzyme was found to be

Figure 2: Chromatography of cytosolic proteins of *C. tropicalis* on a column of DEAE-Sepharose (A) and re-chromatography of catechol 1,2-dioxygenase on a column of DEAE-Sepharose (B). Experimental conditions are described in Material and methods.

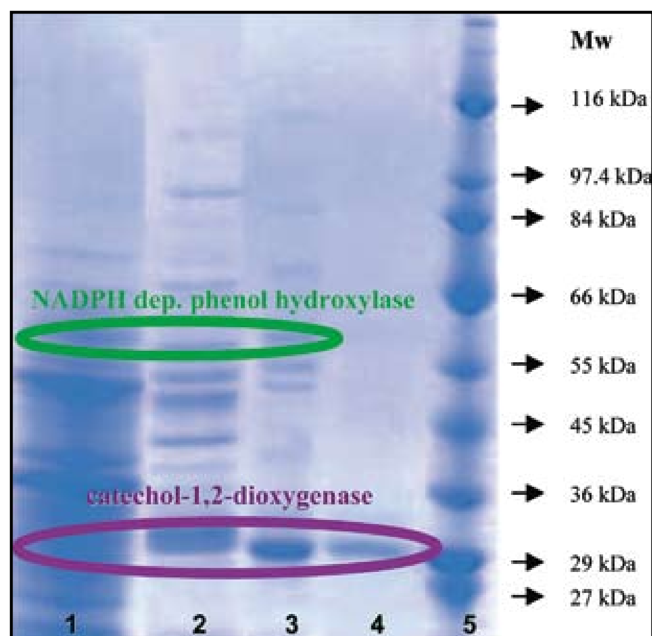

Figure 3: SDS-PAGE analysis of purification of catechol 1,2-dioxygenase of *C. tropicalis*. Lane 1, cytosolic fraction, lane 2, pooled fractions obtained using the first chromatography on a DEAE-Sepharose column, containing phenol hydroxylase and catechol 1,2-dioxygenase, lane 3, fractions of catechol 1,2-dioxygenase obtained by re-chromatography on a column of DEAE-Sepharose, lane 4, catechol 1,2-dioxygenase obtained by gel-permeation chromatography on a column of Sephadex G-100. Lane 5, molecular weight markers (standards) (Wide Range, Sigma Aldrich, USA). The gel shown was a 1.5-mm-thick, 10% polyacrylamide slab gel according to Laemmli (1970). Proteins were stained with Coommassie Brilliant Blue R-250. Samples were 20 µg protein per lane.

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located in the cytosolic fraction of *C. tropicalis* (Vilimkova *et al.* 2008). Therefore, we isolated catechol 1,2-dioxygenase from this subcellular fraction of *C. tropicals*. The enzyme was purified to homogeneity and its fundamental enzymatic properties were characterized. To the best of our knowledge, this is the first report on the purification and partial characterization of catechol 1,2-dioxygenase from *C. tropicalis* yeast.

Purification of catechol 1,2-dioxygenase from cytosol of *C. tropicalis* was performed using chromatography and re-chromatography on DEAE-Sepharose, followed by a gel filtration on Sephadex G-100. The enzyme was found to be a homodimer with a subunit molecular mass of 30 000. The dimeric nature of this enzyme protein is similar to that of catechol 1,2-dioxygenase of *C. albicans* (Tsai and Li, 2007) and to that of prokaryotic bacteria, which have molecular masses of 30 500–34 000 Da per subunit (Neidle *et al.* 1988; Eck & Bettler, 1991).

Oxidation of catechol by catechol 1,2-dioxygenase leads to formation of *cis,cis*-muconate. This finding confirmed the intra-diol cleavage activity of isolated catechol 1,2-dioxygenase and showed its similarity to the enzyme of both eukaryotic microorganism *C. albicans* (Tsai *et al.* 2005) and the enzymes of prokaryotes such as *Acinetobacter* sp. (Caposio *et al.* 2002; Kim *et al.* 2003), *Pseudomonas arvilla* C-1 (Nakai *et al.* 1990) and *Frateuria* sp. ANA-18 (Aoki *et al.* 1984). Likewise, the pH and temperature optima of catechol 1,2-dioxygenase from *C. tropicalis* are similar to those of the enzyme isolated from *C. albicans* (Tsai & Li., 2007), *Acinetobacter* sp. (Kim *et al.* 2003) and *Pseudomona*s sp. (Nakai *et al.* 1990; Briganti *et al.* 1997).

In conclusion, the results found in the present study are the first report on purification of homogeneous catechol 1,2-dioxygenase from *C. tropicalis* and its partial characterization.

Acknowledgment

Supported by the Grant Agency of the Czech Republic (grant 303/09/0472) and the Ministry of Education of the Czech Republic (grants MSM0021620808, 1M0505 and RP MSMT 14/63).

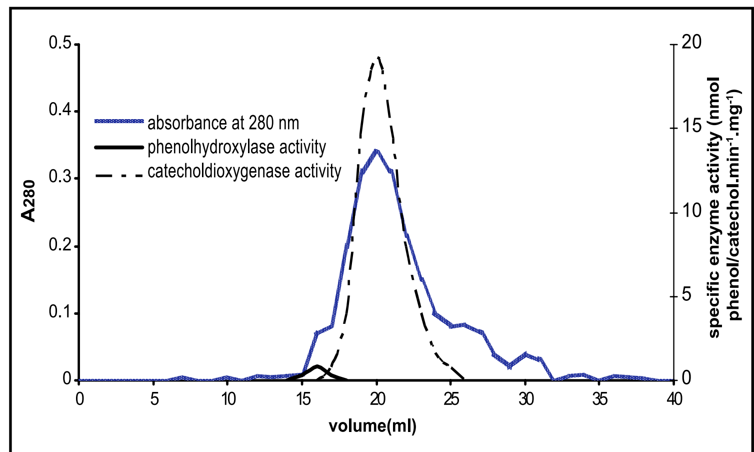

Figure 4: Gel permation chromatography of catechol 1,2-dioxygenase of *C. tropicalis* on Sephadex G-100. Experimental conditions are described in Material and methods.

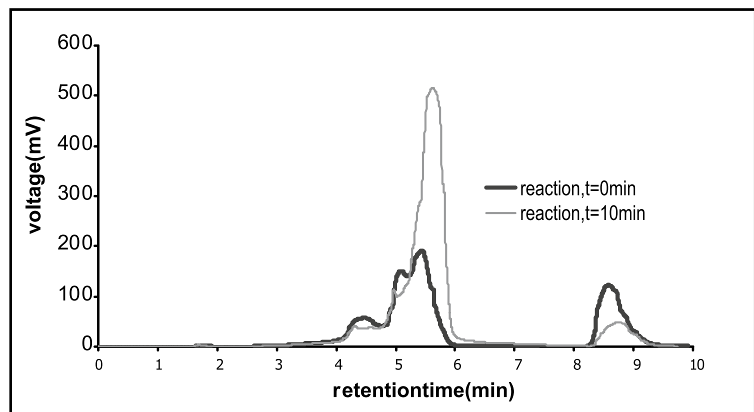

Figure 5: HPLC of reaction mixture used to study oxidation of catechol to *cis,cis*-muconic acid. Experimental conditions are described in Material and methods. Catechol and *cis,cis*-muconic acid eluted at the retention times of 8.7 and 5.66 minutes, respectively.

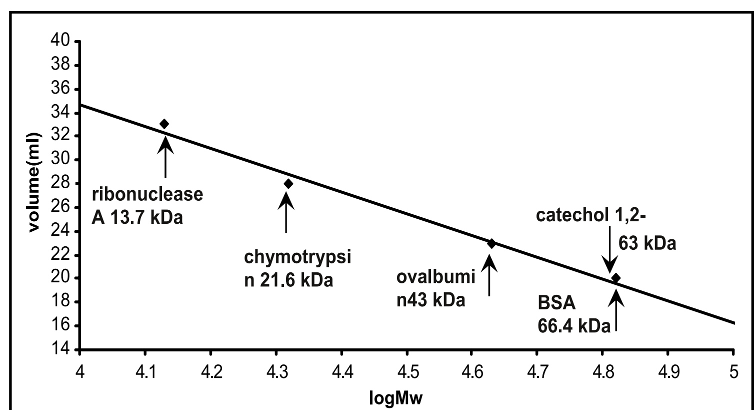

Figure 6: Molecular mass determination of catechol 1,2-dioxygenase of *C. tropicalis* by gel permeation chromatography on Sephadex G-100. Experimental conditions are described in Material and methods.

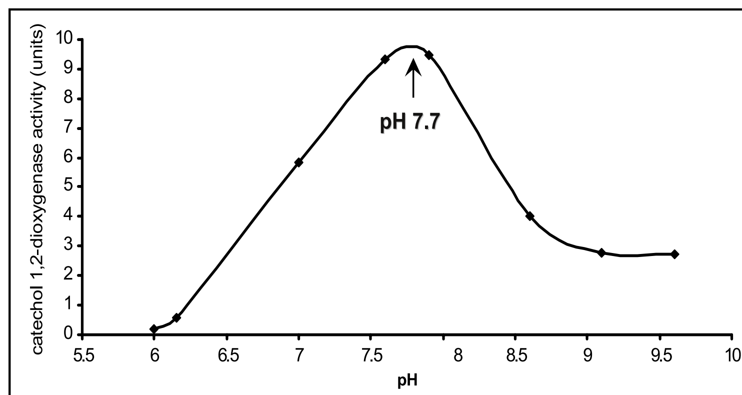

Figure 7: pH optimum of catechol 1,2-dioxygenase of *C. tropicalis*

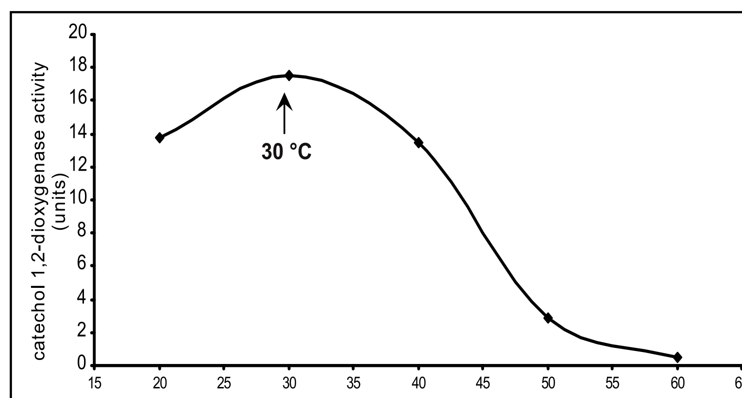

Figure 8: Temperature optimum of catechol 1,2-dioxygenase of *C. tropicalis*

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