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Partial Purification and Properties of Putrescine Oxidase from *Candida guilliermondii*

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Abstract

Putrescine oxidase ([PO]; E.C. 1.4.3.4), which catalyzes the oxidative deamination of putrescine into γ-aminobutyraldehyde, has been partially purified from *Candida guilliermondii*. Among the substrates tested, putrescine has the highest reaction rate, followed by spermidine and cadaverine. The K_m values for putrescine, spermidine, and cadaverine were 20, 200, and 1.1 m*M*, respectively. The optimum pH and the temperature for PO were 8.0 and 37°C, respectively. Growth of *Candida* species on putrescine as the sole nitrogen source induced the synthesis of PO that converts putrescine into Δ^1-pyrroline and γ-aminobutyric acid. These two products were detected and identified from the culture medium. The enzyme was not activated by divalent cations. Among the species of *Candida* tested, the highest enzyme activity was found in cell-free extracts of *C. guilliermondii*. The pathway of putrescine degradation was identified by substrate analysis to be along the nonacetylated pathway in *C. guilliermondii*.

Index Entries: *Candida* sp.; enzyme purification; spermidine; polyamines.

Introduction

The diamine putrescine (1,4-diamine) and its aminopropyl derivatives, spermidine and spermine, found in various mammalian tissues and many microorganisms, have been implicated in a wide variety of biological reactions including synthesis of DNA, RNA, and protein *(1)*. Specifically, in fungi, spermidine and putrescine have been reported to be important nitrogen sources and regulators of growth *(2,3)*; however, the exact function of the polyamines has not been defined. The biosynthesis of these polyamines has been studied and reviewed *(4)*. Nevertheless, pathways

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of putrescine degradation in microorganisms have not been surveyed in detail.

Earlier studies indicate that there are two distinct pathways of putrescine degradation *(5)*. In the nonacetylated pathway, putrescine is converted into Δ^1-pyrroline and γ-aminobutyric acid (GABA). The Δ^1-pyrroline is then oxidized by either diamine aminotransferase or Δ^1-pyrroline dehydrogenase into γ-aminobutyrate, which is converted to succinate semialdehyde. This compound is subsequently converted into succinate by semialdehyde dehydrogenase. Succinate can then be metabolized by normal cell metabolism *(5)*. The other pathway of putrescine degradation, the acetylated pathway, proceeds through a different series of intermediates but eventually yields GABA, which is again converted to succinate. In this pathway, the polyamine is initially acetylated and then undergoes a series of enzymatic reactions, in which the nitrogen atoms are removed by a series of aminotransferases and oxidases. Specifically, in fungi, most of the oxidation is attributed to acetylputrescine oxidase. Finally, the molecule is deacetylated by acetamidobutyrate deacetylase and converted into γ-aminobutyrate. Haywood and Large *(3)* reported that putrescine degradation in *Candida boidinii* occurred through this acetylated pathway.

In this report, we show that putrescine degradation in an opportunistic yeast pathogenic to humans, *Candida guilliermondii*, occurred by oxidative deamination of putrescine through the nonacetylated pathway. The optimal conditions and properties of partially purified putrescine oxidase (PO) are also presented.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma, St. Louis, MO, and from Aldrich, Milwaukee, WI [1-4-^{14}C] putrescine dihydrochloride (specific activity, 72.50 mCi/mmol) was obtained from New England Nuclear, Boston, MA.

Cultural Conditions

C. guilliermondii (American Type Culture Collection [ATCC] #6260) originally isolated from a cancer patient was grown in a liquid medium containing the following ingredients (grams per liter of deionized water): 20 g/L glucose; 3.5 g/L KH_2PO_4; 2.5 g/L $MgSO_4 \cdot 7H_2O$; 2.5 g/L $CaCl_2$; and 30 mg biotin. The nitrogen source used in various experiments was either 2.5 g of ammonium sulfate, or 1.0 g putrescine chloride per liter of medium. Flasks (500 mL) containing 100 mL of synthetic medium were stoppered with foam plugs, autoclaved, and inoculated with yeast suspension from a 16-h old culture of *C. guilliermondii*. After incubation for the desired period of time at 37°C in a rotary shaker, the cells were harvested by centrifugation at 7000*g* for 15 min. Cells were washed with sterile saline and centrifuged again. Cell pellets were frozen or immediately used for enzyme extraction.

Enzyme Extracts

Crude enzyme preparations were obtained by disrupting cells suspended in 0.01 *M* potassium phosphate buffer, pH 7.2. Approximately 10 g of glass beads (0.45–0.5 mm) were added to 2 g (wet weight) of cells in prechilled 50-mL Braun glass homogenizing flasks. Cells were disrupted in a Braun disintegrator (Braun, Melsunger, Germany) using two 30-s bursts with a 1-min interval between bursts. The temperature of the flask chamber was maintained at approx 4°C by a continuous flow of carbon dioxide. The disrupted cell mass was centrifuged at 20,000*g* for 20 min to remove cell debris, unbroken cells, and glass beads. The supernatant fluid was dialyzed in the cold against 100 vol of buffer for 18 h with three changes of buffer. The dialyzed extracts were stored at –20°C. Protein content was measured by the Bradford method using serum albumin as the standard *(6)*.

PO Assay

The release of Δ^1-pyrroline from putrescine by cell-free extracts was assayed by modifying the method of Shimizu et al. *(7)* for PO. The reaction mixture contained 1.0 mL of 0.2 *M* Tris-HCl buffer, pH 8.0, 0.5 mL of 20 m*M* putrescine, 0.3 mL of 0.1% *O*-aminobenzaldehyde, and 0.5 mL of enzyme in a total volume of 3.0 mL. The assay was carried out at 37°C with continuous shaking and stopped with 0.5 mL of 50% trichloroacetic acid and 2.0 mL of absolute ethanol. The reaction mixture was filtered and the absorbency of the filtrate was measured at 435 nm. Enzyme activity was expressed as nanomoles of Δ^1-pyrroline produced per minute per milligram of protein.

Enzyme Purification

PO was partially purified from yeast cells of *C. guilliermondii* by modifying the methods of Yamada *(8)* and DeSa *(9)*. The cells were ground and the enzyme was extracted with 0.01 *M* phosphate buffer, pH 7.0. The cell-free extract was then fractionated with ammonium sulfate (40–60% saturation). The precipitate was suspended in phosphate buffer and then dialyzed for 48 h against four changes of 1 L of phosphate buffer. The dialyzed enzyme was then applied to a DEAE-cellulose column (7 × 50 cm) and equilibrated with phosphate buffer. The enzyme was eluted with 0.1 *M* phosphate buffer, pH 7.0, containing 0.1 *M* NaCl. The fractions were pooled and concentrated by the addition of ammonium sulfate (70% saturation) and dialyzed again. The dialyzed enzyme was then applied to a DEAE-Sephadex (A-50) column (5.5 × 6.5 cm) and equilibrated with phosphate buffer. After removal of impurities, the enzyme was eluted with 0.1 *M* phosphate buffer, containing 0.3 *M* NaCl. The fractions were then pooled and concentrated by addition of ammonium sulfate (70% saturation). The enzyme was finally dialyzed for 48 h against four changes of 5 L each of 0.01 *M* phosphate buffer, pH 7.0. The entire purification process was carried out at 4°C.

Determination of Optimum Conditions for PO from C. guilliermondii

The effect of temperature on PO activity was determined by incubating the reaction mixtures at various temperatures ranging from 15 to 50°C. The influence of pH on enzyme activity was measured by adjusting the pH of the substrate (putrescine) using three types of buffers (citrate-phosphate, Tris-maleate, and Tris-HCl) over a pH range from 4.0 to 9.0 under standard enzyme assay conditions.

Identification of PO Reaction Products

Using [1-4-^{14}C] putrescine, the products of the PO reaction were separated by thin-layer chromatography *(3)*. The reactive spots on the thin-layer plates were carefully removed with a razor blade, mixed with liquid scintillation fluid, and counted in the ^{14}C channel of a Beckman LS 801 liquid scintillation counter.

All experiments were repeated at least three times and the mean values were given. Samples were analyzed in duplicates. The assays were reproducible within 10% of the standard deviation of the mean values.

Results and Discussion

Although their mechanism of action still remains unclear, polyamines are widely regarded as the central role in the cellular growth process *(4)*. Putrescine, in particular, has been shown to be an important nitrogen source and growth factor in fungi *(10)*. Because of the central role polyamines play in cell proliferation, there have been numerous studies on polyamine synthesis and ornithine decarboxylase, as well as polyamine degradation and polyamine oxidases *(1,4,10)*. Only recently, however, have studies been done on putrescine degradation and PO in fungal systems *(2,3)*.

Earlier studies postulated that there are two pathways of putrescine degradation in different organisms, namely, acetylated and nonacetylated. Both pathways finally create 4-aminobutyrate, but through different processes. In most higher eukaryotic organisms, catabolism of polyamine has been shown to proceed through the nonacetylated pathway *(5)*. Recently, however, studies have shown that catabolism of putrescine in two species of *Candida, C. boidinii* and *C. nagoyaensis*, have occurred through the acetylated pathway *(11)*. In our study, the pathway of putrescine degradation by PO in *C. guilliermondii* was studied by using the ^{14}C-labeled putrescine as a substrate and measuring the production of GABA, a product in the nonacetylated pathway. Our investigation revealed that putrescine degradation does indeed occur through the nonacetylated pathway. GABA was labeled with ^{14}C and was measured in the culture medium and in cell-free extracts of *C. guilliermondii*. Table 1 presents the data. Over half of the putrescine added was utilized by the cells. Nearly 20% of the putrescine was converted into GABA. Note that the count of GABA in the culture

Table 1
Isolation of ^{14}C-Labeled GABA from Culture Medium and Cell-Free Extracts of *C. guilliermondii*

Putrescine added	5.00×10^6 cpm
Putrescine left in the medium	2.07×10^6
Putrescine utilized	2.93×10^6
Total counts from cell-free extracts	4.30×10^5
Counts in GABA	1.10×10^5
Total counts from culture filtrates	20.70×10^5
Counts in GABA	8.00×10^5
Total counts in GABA	9.10×10^5
Conversion	18.2%

Table 2
The Specific Activity of PO from Different *Candida* Species with Putrescine as the Substrate

Organism	Growth (g)	Specific activity[a]
C. albicans	2.65	241
C. guilliermondii	1.95	409
C. krusei	2.35	307
C. tropicalis	2.55	398
C. parapsilosis	2.45	246
C. stellatoidea	2.15	125

[a]Δ^1-pyrroline (nmol · min^{-1} · mg protein^{-1}).

filtrate was eight times larger than that in the cell. This is consistent with other studies in fungi that have reported a similar pathway for putrescine breakdown *(11,12)*.

Furthermore, the activity of PO was surveyed in six different *Candida* species and purified and characterized from *C. guilliermondii*. The specific activity of PO was measured in six species of *Candida*—*C. albicans*, *C. guilliermondii*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, and *C. stellatoidea*—and the data are presented in Table 2. The growth of the organisms was predominantly the same; interestingly enough, *C. guilliermondii* did have strikingly less growth than the other organisms. However, in terms of the specific activity of PO, *C. guilliermondii* did have far greater activity than the other organisms. The activity of *C. tropicalis* was nearly as high as in *C. guilliermondii*; the other four organisms showed significant activities as well. From these data, it seems quite possible that putrescine degradation in these other organisms may proceed through the nonacetylated pathway also. Further investigation is necessary to confirm this hypothesis.

PO from *C. guilliermondii* was also partially purified and characterized. The optimum pH for enzyme activity was 8.0 (Fig. 1), and the optimum temperature was 37°C (Fig. 2). Again, the data were consistent with previous studies that reported optimum pH and temperature to be in that

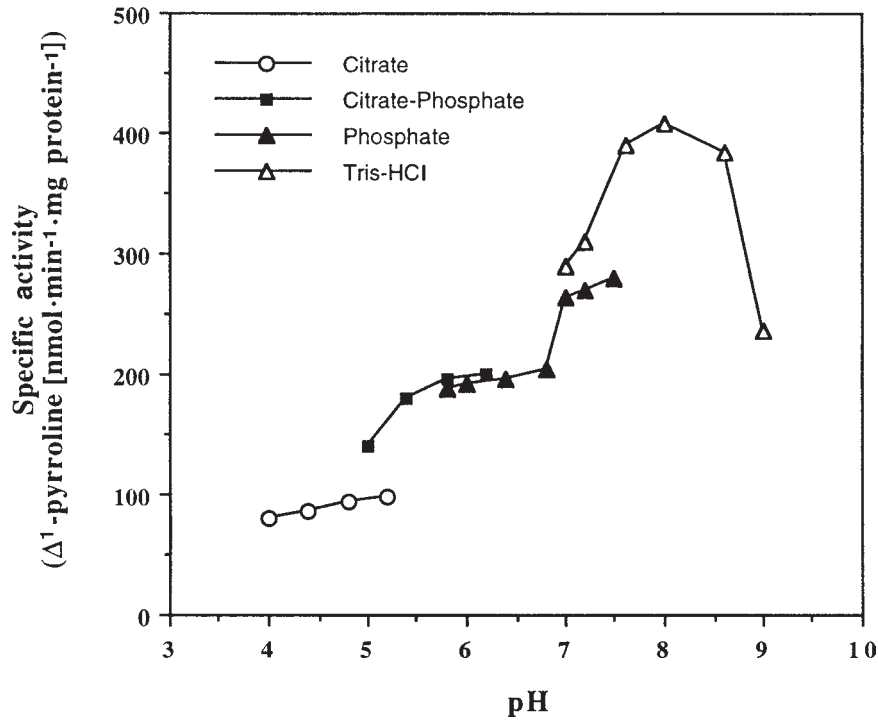

Fig. 1. Effect of pH on the PO activity from *C. guilliermondii*.

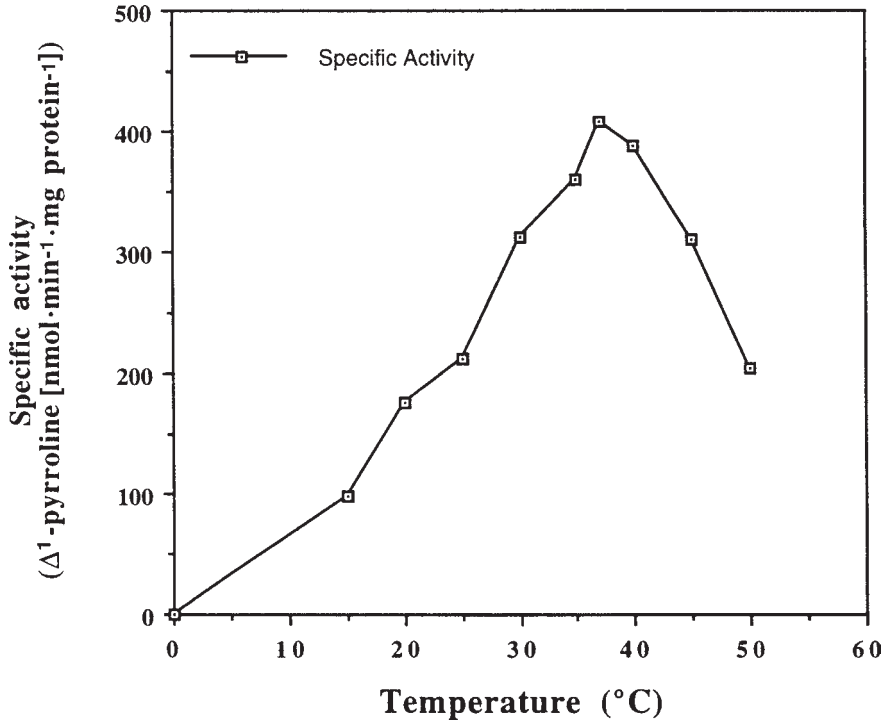

Fig. 2. Effect of temperature on the PO activity from *C. guilliermondii*.

Table 3
Specific Activity, Amount of Oxidation, and K_m of PO from *C. guilliermondii*, with Different Polyamine Substrates

Substrate	Specific activity[a]	Oxidation (%)	Apparent K_m (m*M*)[b]
Putrescine	407	100	0.02
Spermidine	210	52	0.20
Cadaverine	192	47	1.10
Spermine	7	2	ND
Histamine	0	0	ND

[a]Δ^1-pyrroline (nmol · min^{-1} · mg $protein^{-1}$).
[b]ND, not determined.

range *(12)*. The M_r of PO from *C. guilliermondii* was determined by gel filtration to be 50,000, which was also consistent with previous reports in fungi and higher organisms *(13)*. Prior studies had also reported that divalent cations had served to inhibit enzyme activity. We found that divalent cations had no significant effect on the activity of PO from *C. guilliermondii (12)*. The substrate specificity was also tested, using five different substrates—putrescine, spermidine, cadaverine, spermine, and histamine. Table 3 presents the data. The enzyme showed activity with only putrescine, spermidine, or cadaverine as a substrate; the activity with putrescine was twice as high as the activity with either spermidine or cadaverine. The K_m values for putrescine, spermidine, and cadaverine were 0.02, 0.20, and 1.10 m*M*, respectively. Spermine and histamine showed no activity. Based on these data, these two polyamines show potential competitive inhibitors of PO.

The data on breakdown and utilization of polyamines, such as putrescine, in fungal systems still remains extremely incomplete. Further work needs to be done to understand better the role of polyamines as growth factors and an organism's ability to utilize them.

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