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Protocatechuate 3,4-dioxygenase from thermophilic *Geobacillus* sp. strain

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Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) catalyses the ring cleavage step in catabolism of aromatic compounds through the protocatechuate branch of the β-ketoadipate pathway. The protocatechuate 3,4-dioxygenase was purified to homogeneity from the thermophilic *Geobacillus* strain grown on naphthalene for the first time. The enzyme was purified about 24-fold with a specific activity of 34.2 U mg of protein^{-1} by a purification procedure including ammonium sulfate fractionation and column chromatographies on DEAE–cellulose and hydroxylapatite. The relative molecular mass of the native enzyme estimated on gel chromatography of Sephadex G-200 was 480 kDa. The pH and temperature optima for enzyme activity were 8 and 60 °C, respectively. A half-live of the protocatechuate 3,4-dioxygenase at the optimum temperature was 40 min. The kinetic parameters of the *Geobacillus* strain protocatehuate 3,4-dioxygenase were determinated. The enzyme gave typical saturation kinetics and had an apparent K_m of 7 μM for protocatechuate and 33 μM for catechol.

Key words: thermophilic bacteria, naphthalene, protocatechuate 3,4-dioxygenase, purification

INTRODUCTION

A number of thermophilic bacteria are capable of utilizing aromatic compounds as carbon and energy sources. Müller et al. [1] isolated microorganisms able to convert naphthalene, phenanthrene and anthracene under termophilic conditions. Their studies have indicated that metabolites differ significantly from those formed under mesophilic conditions. It is likely that thermozymes can differ from mesophilic microorganism enzymes. On the other hand, little is known on the metabolic pathways and enzymes involved in the degradation of aromatic hydrocarbons by thermophilic microorganisms.

Protocatechuate 3,4-dioxygenase is a key enzyme and a focal point in the aerobic mesophilic bacterial pathways for the metabolism of a large number of aromatic compounds, including p- hydroxybenzoate [2], phenanthrene [3], fluorene [4] and others. Naphthalene, the simplest fused polycyclic aromatic hydrocarbon, is utilized by mesophilic bacteria via two other central metabolites, catechol or gentisate. We identify the new metabolite and demonstrate the key enzymatic activity of protocatechuate 3,4-dioxygenase for metabolism of naphthalene by termophilic bacteria [5]. Protocatechuate dioxygenase has been isolated only from mesophilic microorganisms including *Brevibacterium fuscum* [6], *Streptomyces* sp. [7], *Pseudomonas putida* [8], *P. aeruginosa* [9].

In this study, we contribute to data on the degradation of naphthalene by thermophilic bacteria via a novel pathway, through protocatechuate by isolation of protocatechuate 3,4-dioxygenase from a thermophilic strain of *Geobacillius* grown on naphthalene as the sole carbon and energy source. This bacterial strain was able to utilize anthracene, phenanthrene, benzene-1.3-diol, benzene, phenol and phthalic acid. We give data on enzyme purification, some biochemical properties and thermal stability of the second enzyme in the naphthalene degradation pathway, the protocatechuate 3,4-dioxygenase (3,4-PCDase).

MATERIALS AND METHODS

Bacterium growth and extract preparation. Naphthalene using the bacterial strain *Geobacillius* sp. AY 946034 [5] was grown in a mineral salt medium. The medium contained (g l^{-1}): NH_4Cl – 1.0, $Na_2HPO_4 \cdot H_2O$ – 0.42, $NaH_2PO_4 \cdot H_2O$ – 0.38, $MgCl_2 \cdot 7H_2O$ – 0.1, $CaCl_2 \cdot 2\ H_2O$ – 0.1, KCl – 0.04, $FeSO_4 \cdot 7\ H_2O$ – 0.008 and supplemented by 0.03 g yeast extract and 2.5 mM naphthalene. The flasks were inoculated with 10% (v/v) of pre-culture grown overnight in a mineral salt medium at 60 °C to an OD_{590} of 0.7. Batch cultures were incubated in an Erlenmeyer flask in the dark at 60 °C until the late exponential growth phase without

aeration. Bacterial growth was determined turbidimetrically at 590 nm. Cells were harvested with a refrigerated centrifuge at 5000 × g for 10 min at 4 °C, washed twice with 5 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 10% (v/v) glycerol and resuspended in the same volume of the buffer. The cells were disrupted by ultrasonic treatment for 3 min at 220 kHhz by using a sonicator (YZDH-2T, Russia). The residue was removed by centrifugation at 4 °C for 25 min at 14000 × g.

Enzyme assays. Enzyme activities were assayed by monitoring the disappearance of substrate or appearance of products with a UV-visible spectrophotometer (SF-46, LOMO, St. Petersburg, Russia) with a thermojacketed cuvette holder at 60 °C. The following enzymes were assayed according to the reported method given in the references: catechol 1,2-dioxygenase [10], catechol 2,3-dioxygenase [11], protocatechuate 2,3-dioxygenase [12], protocatechuate 3,4-dioxygenase [13], protocatechuate 4,5-dioxygenase [7] and gentisate 1,2-dioxygenase [14]. One unit of enzyme activity was defined as that catalyzing either the degradation of 1 μmol of substrate or the formation of 1 μmol of degradation product per minute.

Protein concentrations were determined spectrophotometrically from the absorbance at 280 nm during purification procedure and by the standard Bradford method [15] for a pure enzyme.

Initial velocities used in determining enzyme kinetic constants were measured spectrophotometrically with air-saturated 50 mM sodium phosphate buffer at 60 °C. The kinetic constants were determined graphically from double reciprocal plots.

Enzyme purification. The purification procedure was carried out in 50 mM sodium phosphate buffer containing 10% (v/v) glycerol, pH 7.0 at 4 °C.

Ammonium sulfate fractionation. The powdered ammonium sulfate was added to 30% of saturation. After 2 hours, the precipitate was removed by a 60 min centrifugation at 15 000 × g. A 30% supernatant was brought into 70% ammonium sulfate and centrifuged as before. The pellet was spooned into dialysis tubing and dialyzed for 20 h against 250 mM sodium phosphate buffer.

DEAE cellulose fractionation. A dialyzed 30–70% ammonium sulfate cut was applied to a DEAE cellulose column (1 × 10 cm) (Sigma) equilibrated with 50 mM sodium phosphate buffer, and the enzyme was eluted with a linear gradient from 0.1 to 0.6 M NaCl in 200 ml of the same buffer. The flow rate was maintained at 1 ml min^{-1}; 3 ml fractions were collected.

Streptomycin sulfate. Streptomycin sulfate was added at a final concentration of 1% with stirring. The solution was stirred for 30 min and centrifuged (15 000 × g, 45 min).

Hydroxylapatite fractionation. Streptomycin sulfate supernatant was applied to a (2.5 × 1.5 cm) Bio-Gel HTP hydroxylapatite column (Bio-Rad) equilibrated with 50 mM (pH 7) and fractions were eluted at a flow rate of 30 mlh $^{-1}$. The enzyme was eluted by a 60 ml linear gradient of phosphate buffer (10 mM – 0.3 M), and 2 ml fractions were collected.

Determination of pH and temperature optimums. The effect of pH on enzyme activity was measured at various pH values within the range of 4.0 to 11 by using sodium acetate, sodium phosphate and TRIS/HCl buffer systems. The pH values were equilibrated at 60 °C.

The temperature dependence of protocatechuate oxidation reaction at pH 7.0 was investigated in the range 20–90 °C by means of thermostated reaction cell. The enzyme and substrate solutions were preincubated for 10 min, mixed, and the enzymatic reaction was then carried out at the same temperature.

Temperature stability. The thermal stability of the enzymes was determined by incubating their solutions at 60 °C for 2 h and measuring activity under standard conditions.

Electrophoretic analysis. The purified enzyme was monitored for non-reducing conditions in vertical gel containing 7.5% acrylamide [16]. Proteins were stained with silver.

Measurement of molecular mass. The native enzyme molecular mass was measured by gel filtration on Sephadex G-200 with thyroglobulin (670 kDa), ferritin (450 kDa), catalase (240 kDa), aldolase (160 kDa) as standards. The columns were calibrated by determining the elution volumes of standard proteins and then calculating the elution volume of each protein with respect to the elution volume of Blue Dextran.

RESULTS AND DISCUSSION

Protocatechuate 3,4-dioxygenase (protocatechuate : oxygen 3,4-oxidoreductase) catalyzes the intradiol addition of dioxygen to protocatechuate, cleaving the aromatic ring and forming β-carboxy-cis, cis-muconic acid. This enzyme is an important component of the bacterial metabolism of various aromatic compounds found in the environment, including aromatic monomers that arise during the decay of lignin or other vascular plant components.

Approximately 10 g of *Geobacillus* sp. cells, wet weight, were obtained from 3 liters of mineral salt medium supplemented with naphthalene for purification procedure.

The results of purification are summarized in Table 1. The pure protocatechuate 3,4-dioxygenase with the specific activity of 34 U mg^{-1} was obtained after a 24-fold enrichment after fractionation with ammonium sulfate, DEAE cellulose and hydroxyapatite. Specific activities, ranging from 7 to 47 U mg^{-1}, were observed from most purified protocatechuate 3,4-dioxygenase from mesophilic bacteria such as *P. putida* [8], *P. aeruginosa* [9], *Acinetobacter calcoaceticus* [17], *Thiobacillus* sp. [18], *Roseabacter* sp. [19].

The homogeneity of the enzyme preparation was examined by PAGE. The purified enzyme preparations

Table 1. **Purification of protocatechuate 3,4-dioxygenase from *Geobacillus* sp.**

Step	Volume, (ml)	Total protein, (mg)	Activity, (U)	Specific activity, (U/mg)	Purification fold
Crude extract	15	72.0	102.3	1.4	1
30–60 % saturated ammonium sulfate	6	26.9	97.9	3.6	3
DEAE cellulose	11	2.4	67.4	28.1	20
Streptomycin sulfate	8	2.3	67.0	29.1	21
Hydroxylapatite	5	0.5	17.1	34.2	24

migrated as a single protein band when subjected to electrophoresis on polyacrylamide gel (Fig.1). The molecular mass of the enzyme, measured by gel filtration, was about 480 ± 10 kDa and was eluted as a single symmetrical peak from the Sephadex G-200. Results of the gel filtration procedure are shown in Fig. 2. Ohlendorf et al. [20] in their work reported a large enzyme protocatechuate 3,4-dioxygenase from *P. aeruginosa* with a relative molecular mass about 587 kDa. The molecule was a dodecamer. The enzyme from *Acinetobacter calcoaceticus* was found to be 700 kDa [17].

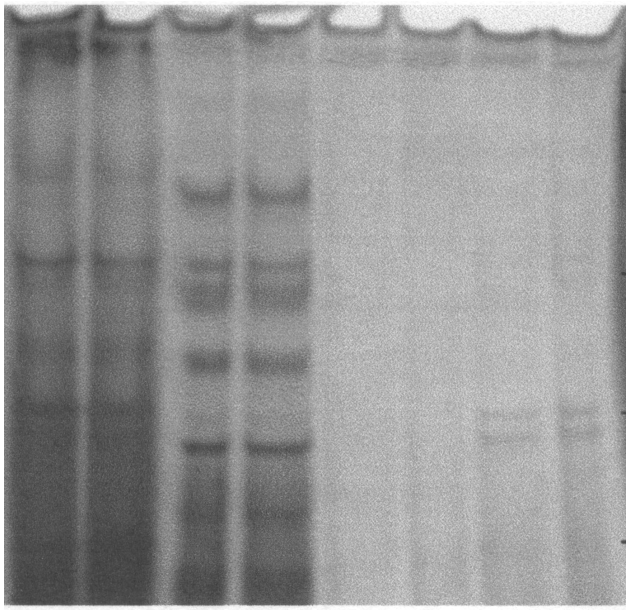

Fig. 1. Electrophoresis on nondenaturing conditions
Lane 1 – crude extract of *Geobacillus* sp.; lane 2 – after ammonium sulfate fractionation, lane 3 – purified 3,4-PCDase after purification procedure, and lane 4 – after DEAE cellulose chromatography.

The kinetic constants of the purified enzyme are presented in Table 2. Protocatechuate 3,4-dioxygenase from thermophilic *Geobacillus* sp. has a high specificity for protocatechuate with the K_m value of 7 μM. Among the compounds tested, catechol was oxidized at rates 25% that of protocatechuate. Gentisate was not oxidized.

The effects of pH and temperature on enzyme activity were examined. The optimal pH for enzyme activity was found to be about 8 (Fig. 3A). The enzyme lost its activity at pH below 4 and above 11 after heating at 60 °C for 10 min. The optimum temperature

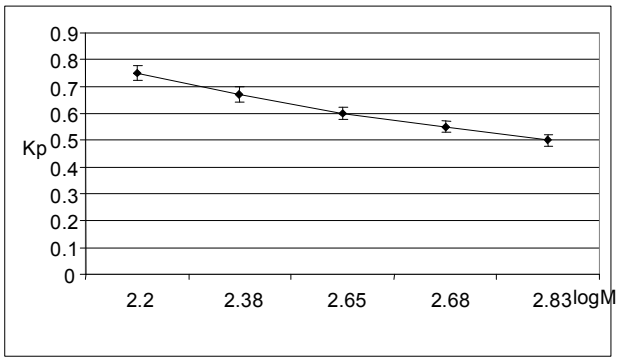

Fig. 2. A semilogarithmic plot of molecular mass as a function of the distribution coefficient, K_p (from left to right – aldolase, catalase, ferritin, 3,4-PCDase and thyroglobulin)

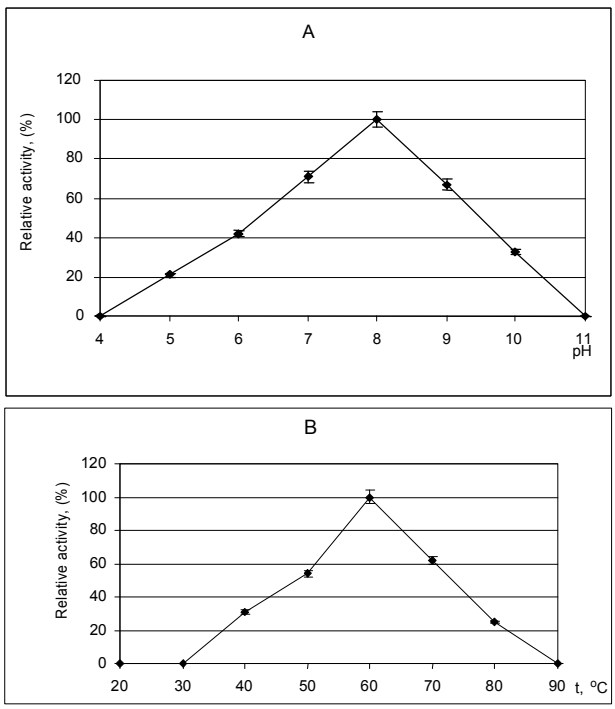

Fig. 3. Effects of pH (A) and temperature (B) on the activity of 3,4-PCDase

for enzyme activity was 60 °C (Fig. 3B). No catalytic activities were detected at 20 and 90 °C. The temperature optimum of 60 °C for the enzyme was the same as the growth optimum of our thermophilic bacteria. The thermal stability of the enzyme was analyzed at 60 °C (Fig. 4). However, at this temperature the enzyme was not very stable. The half-live of the purified enzyme at

Table 2. **Determination of kinetic constants of protocatechuate 3,4-dioxygenase from *Geobacillus* sp.**

Substrate	K_m, (μM)	V_{max}, (μM min^{-1})	V_{max} / K_m (min^{-1})
Protocatechuate	7	200	28.6
Catechol	33	50	1.5
Gentisate	0	0	0

60 °C was 40 min. *Meta*-cleaving oxygenase (catechol 2,3-dioxygenase) from the phenol-degrading thermophilic *Bacillius thermoleovorans* was less stable, with a half-life of 1.5 min [21]. Thermostable *meta*-cleaving oxygenases such as catechol 2,3-dioxygenase and protocatechuate 2,3-dioxygenase were purified from the extremely thermophilous *Thermus thermophilus* [22]. The enzyme purified in this study is the first *ortho*-cleaving oxygenase, protocatechuate 3,4-dioxygenase purified from thermophilic microorganisms and discovered in the naphthalene oxidation pathway. The protocatechuate 3,4-dioxygenase is an inducible enzyme in the strain *Geobacillus*, since no activity of the enzyme was observed when this organism was grown in a medium containing glucose instead of naphthalene as the major carbon source. When this strain was grown in a medium containing α-naphthol as a sole source of carbon, catechol 1,2-dioxygenase was formed, whereas no protocatechuate 3,4-dioxygenase activity was observed.

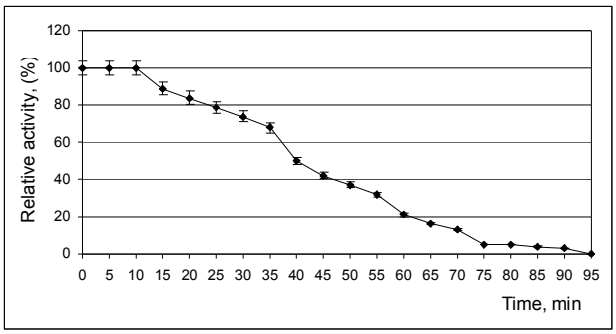

Fig. 4. Thermal stability of 3,4-PCDase at 60 °C

The purified enzyme can be used in bioremediation of polluted groundwater or soil contaminated with various aromatic compounds ranging from monocyclic to polycyclic. Its properties such as substrate specificity, temperature–pH optimum and stability are important determinants to consider when considering microorganisms to be used in remediation of contaminated sites.

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GEOBACILLUS SP. PROTOKATECHUATO 3,4-DIOKSIGENAZĖ

Santrauka

Protokatechuato 3,4-dioksigenazė (EC 1. 13. 11. 3), kuri katalizuoja aromatinio žiedo skėlimo reakciją, įtraukiant į ją du deguonies atomus, buvo išskirta iš termofilinio *Geobacillus* sp. izoliato, auginto mineralinių druskų terpėje su anglies šaltiniu – naftalenu. Fermentas išgrynintas 24 kartus panaudojus amonio sulfato frakcionavimo ir kolonėlės chromatografijos metodus (DEAE-celiuliozę ir hidroksiapatitą). Išgryninto fermento specifinis aktyvumas siekia 34,2 U/mg baltymo. Gelfiltracijos metodu nustatyta termofilinio fermento santykinė molekulinė masė yra 480 kDa. Protokatechuato 3,4-dioksigenazės specifinio aktyvumo temperatūros ir pH optimumai yra atitinkamai 60 °C ir 8. Fermentas netenka pusės savo aktyvumo 60 °C temperatūroje po 40 minučių.

Buvo nustatytos pagrindinės fermento kinetinės konstantos K_m reikšmės: kai substratas yra protokatechuatas – 7 μM, kai katecholis – 33 μM.