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Purification and Properties of Dibutyl Phthalate Esterase from *Arthrobacter* sp. ZJUTW

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In the natural environment, various microorganisms are mainly responsible for the degradation of phthate acid esters. phthate acid esterase is one of key enzymes in the degradation pathway. A dibutyl phthalate esterase from *Arthrobacter* sp. ZJUTW was purified at 45-70% saturation with ammonium sulfate, ion-exchange chromatography (IEC) on Sepharose Q-XL column and hydrophobic interaction chromatography (HIC) on Hiprep Octyl FF column. The purified enzyme appeared homogeneous on SDS-PAGE, and its molecular weight was estimated to be 56.17 KDa. The optimal pH and temperature were pH 9.0 and 35 ℃, respectively. The enzyme was stable in a pH range from 7.0 to 9.0 and below 25 ℃. The enzyme activity was stimulated by Mg^{2+}, but inhibited by several metals such as Cu^{2+}, Zn^{2+}, Ba^{2+}, EDTA, and strongly inhibited by Fe^{3+}. The K_m for DBP of the enzyme was 0.487 mmol/l, V_{max} was 223.3 ìmol/min·l.

Key words: *Arthrobacter* sp; dibutyl phthalate; esterase; purification.

Phthalic acid esters (PAEs) are major industrial products used as plasticizers in polyvinyl chloride (PVC) plastics. In recent years, phthalic acid diesters have appeared as important pollutants in environmental samples as endocrine disrupting chemicals which influence the genitals. Among rats and adult men, phthalate exposure resulted in decreased testicular weight, seminiferous tubular atrophy and increased DNA damage in sperm[1,2]. In the natural environment, various micro- organisms are mainly responsible for the degradation of phthate acid esters[3]. A review of this microbial degradation was published by Staples *et al.,*[4] showing that numerous different microbes degrade phthalates. The metabolism of phthalate esters is initiated in bacteria by their hydrolysis to mono-phthalate esters, which is further degraded by ester-hydrolysis to phthate acid. Phthalate is further metabolized in aerobic bacteria by two different dioxygenase-initiated pathways through the common intermediate, protocatechuate(3,4-dihydroxybenzoate). Protocatechuate is degraded into organic acids further through either ortho- or meta-cleavage pathway by ring cleavage enzymes, which eventually converted to CO_2 and H_2O through Krebs cycle[5-9]. Hydrolysis of the ester bond is a common key initial step in the microbial degradation of phthalate esters. Phthate acid esterase is one of key enzymes in the degradation pathway. However, to our knowledge, there have been few reports on the detailed molecular and catalytic properties of bacterial enzymes hydrolyzing PAEs[10,11]. The present study describes the purification and some general properties of the dibutyl phthalate (DBP) ester hydrolyzing enzyme from *Arthrobacter* sp. ZJUTW, which isolated from sludge in Shangtang River of Hangzhou City.

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MATERIALS AND METHODS

Chemicals

DBP was product of Sinopharm Chemical Reagent Co., Ltd. Methanol (HPLC grade) was product of Tianjin Shield Specialty Chemical Co., Ltd. Other chemicals were purchased form local company.

Strain and culture conditions

Arthrobacter sp. ZJUTW was isolated from sludge in Shangtang River of Hangzhou City, using dibutyl phthalate as sole carbon and energy source. The strain was deposited at the China Center for Type Culture Collection (CCTCC) under the accession number CCTCC M2012246. The medium contained 250 mg/l DBP, 1.0 g/l $K_2HPO_4 \cdot 3H_2O$, 1.0 g/l NaCl, 0.5 g/l $(NH_4)_2SO_4$, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 0.0755 g/l $CaCl_2$, 0.0143 g/l $FeCl_3 \cdot 6H_2O$. The initial pH of the above medium was adjusted to 7.0 by 2mol/l NaOH. All above medium was then transferred into 250 or 500 ml Erlenmeyer flask and autoclaved at 115°C for 20-30 min. All experimental cultures were incubated at 180 rpm and 30°C.

Enzyme purification

Arthrobacter sp. ZJUTW was cultured in the medium of 250 mg/l DBP at 180 rpm and 30°C.The cells were harvested by centrifugation at 5000×g and 4°C for 10 min, washed once with 0.02 mol/l phosphate buffer saline (PBS), pH7.2, and stored at -20°C until needed. The bacterial cells were suspended in 20 ml PBS and disrupted by ultrasonication. The solution was centrifuged for 30min at 20000 ×g. The supernatant (crude extract) was treated with solid $(NH_4)_2SO_4$. The precipitate obtained between 45% and 70% saturation was dissolved in 20 ml PBS, dialyzed overnight against the buffer, and put on Sepharose Q-XL column (1.6×25 cm) (GE Healthcare, USA) previously equilibrated with the PBS. The enzyme was eluted with a linear gradient of 0-1mol/l NaCl in the same buffer at a flow rate of 3 ml/min. The active fractions were pooled, and solid ammonium sulfate was added to a final concentration of about 1.7 M. Then, the solution was loaded onto a Hiprep Octyl FF column (1.0×20 cm) (GE Healthcare, USA) previously equilibrated with PBS containing 1.7 M ammonium sulfate. After equilibrated with the buffer, a linear gradient established with 1.7-0 mol/l $(NH_4)_2SO_4$ in the PBS at a flow rate of 3 ml/min, then eluted with 20% ethanol. The fractions of gradient elution were pooled separately, dialyzed against PBS, and the fractions eluted by 20% ethanol were ultra-filtered with 15ml-10KDa centrifugal ultrafilter tube (Millipore, USA) to remove ethanol. The activity solution in these fractions was determined, and stored at -20°C.

Some properties of the purified enzyme

The effect of pH and temperature on enzyme activity and stability was measured on DBP using HPLC for relative activity, which was expressed as a percentage of the initial activity. The optimum pH was determined by measuring the activity at 30°C over the pH region 5.0-10.0. The stability at different pH (3.0-10.0) was determined after incubating the enzyme for 30min and by measuring the relative activity at 30°C and pH 7.2. The optimum temperature was determined by assaying the enzyme activity at various temperatures (10-50°C) for 20 min in 0.02 mol/l potassium phosphate buffer (pH 7.2). The thermostability was determined by measuring the DBP residual rate at 30°C and pH 7.2, after incubation of the purified esterase between 15°C and 55°C and pH 7.2 for 0-30 min. The effect of some metals and reagents on enzyme activity was determined by measuring the relative activity in the reaction mixture contained various metal ions or reagents (10mmol/l of final concentration). The kinetic constants were determined by measuring the initial rate of enzymic reaction as a function of DBP concentration over the 12.5-100mg/l range. Parametric identification of maximum velocity (V_{max}) and Michaelis-Menten constants (K_m) was used from the equation for initial reaction velocity.

Determination of Enzyme Activity

The reaction mixture (4 ml) contained 0.02 mol/l potassium phosphate buffer (pH 7.2), 125 mg/ml DBP and appropriate amount enzymes. The reaction was carried out at 30°C for 20 min, and stopped by the addition of boiling water bath for 3min. The mixture was extracted with 5 ml of dichloromethane, 3.5 ml of the extract was evaporated, and the residue was dissolved in 1 ml methanol. Part of this sample (10 ìl) was subjected to HPLC (Type Agilent 1100, Agilent ,USA) which was performed on a Diamonsil C_{18} reversed-phase column (250×4.6mm) (Dikma Technologies Inc, USA) and monitored at A_{235}. A mixture of methanol and H_2O (90:10 by volume) was used as the mobile

phase at a flow rate of 1.0 mL min^{-1}.One unit of enzyme activity was defined as that amount catalyzing the degradation of 1 ìmol of substrate per min under the assay conditions. The specific activity was defined as units per mg of protein. Protein was determined by the method of Bradford[12] using bovine serum albumin as a standard.

Determination of protein molecular mass and detection of esterase activity on electrophoresis

The protein molecular masses of crude enzyme, dialysate, the active fraction purified with Sepharose Q-XL column and the purified enzymes with Hiprep Octyl FF column were estimated by sodium dodecylsulfate (SDS)-PAGE (12% polyacrylamide)[13]. Nondenaturing native polyacrylamide gel electrophoresis (PAGE) was done according to Mostafa *et al.,*[14], easterase activity was made visible with alfa-naphthyl acetate and Fast Blue BB as described by Higerd and Spizien[15].

RESULTS

Purification of dibutyl phthalate esterase

The results of the purification were summarized in Table 1. The elution pattern from the Sepharose Q-XL column was shown in Fig. 1A. The hydrophobic interaction chromatography (HIC) by Hiprep Octyl FF column was shown in Fig. 1B. The enzyme was purified by about 35.05-fold with a 5.02% recovery from the original crude enzyme solution. The specific activity of the final preparations of dibutyl phthalate esterase was 2.419 unit /mg. The purified enzyme preparation using HIC migrated as a single protein band on SDS-PAGE (Fig. 2A), and its molecular mass was found to be 56.17 kDa. Esterase activity on electrophoresis was shown in Fig.2B. The crude extract showed four activity bands, while the purified enzyme had a single activity band, at the same position as the major activity band observed in the crude extract.

Table 1. Purification of DBP esterase from *Arthrobacter* sp. ZJUTW

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	10.34	148.79	0.069	1	100
45-70% Ammonium sulfate	6.78	38.42	0.176	2.55	65.57
IEC by Sepharose Q-XL	1.84	1.27	1.449	20.99	17.79
HIC by Hiprep Octyl FF	0.52	0.215	2.419	35.05	5.02

Some general properties of dibutyl phthalate ester hydrolyzing enzyme

Effects of pH

Effects of pH on the activityand stability (Fig.3) of the enzyme were examined. The optimum pH for the activity was 9.0. The enzyme was stable in the pH range from 7.0 to 9.0.

Effects of temperature

As shown in Fig. 4, the enzyme was most active at about 35°C. The enzyme was stable between 15°C to 25°C. However, the enzyme was unstable above 45°C, and completely inactivated by incubation at 55°C for 20 min.

Effects of some metal ions and reagents

As shown in Table 2, The enzyme activity was stimulated by Mg^{2+}, but inhibited by several

Table 2. Effect of some metal ions and reagents on

Metal ions and reagents	Relative activity (%)
Control	100
K^{+}	99
Ca^{2+}	84
Cu^{2+}	33
Co^{2+}	81
Fe^{3+}	8.5
Zn^{2+}	52
Mn^{2+}	96
Mg^{2+}	112
Ba^{2+}	69
EDTA	61.9
Tween-80	102

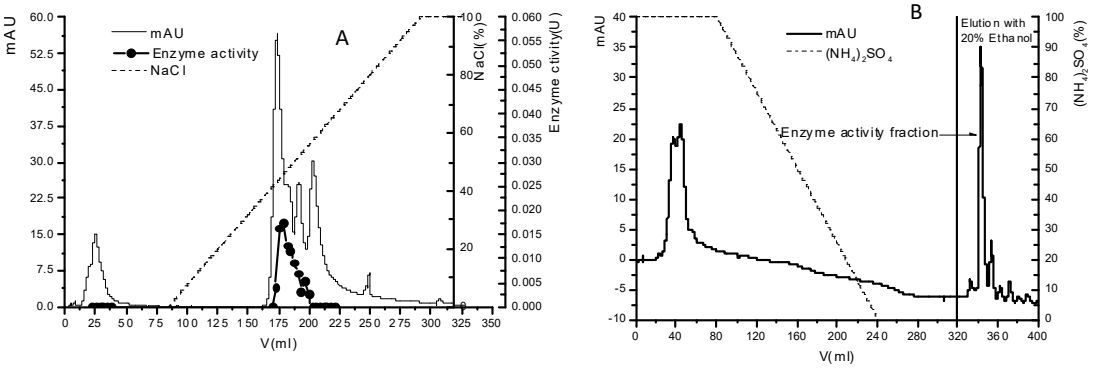
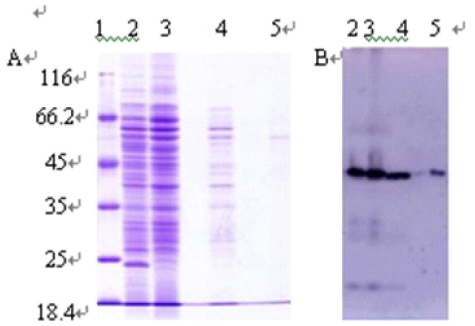


Fig. 1. Purification of DBP esterase by (A) Sepharose Q- XL and (B) Hiprep Octyl FF column



Lane 1: Marker protein, Lane 2: crude extract, Lane 3: dialysate, Lane 4: enzyme purified by IEC on Sepharose Q-XL column, Lane 5: enzyme purified by HIC on Hiprep Octyl FF column.

Fig. 2. Electrophoretic pattern of DBP esterase on SDS-PAGE (A) and esterase staining activity on Native-PAGE (B)

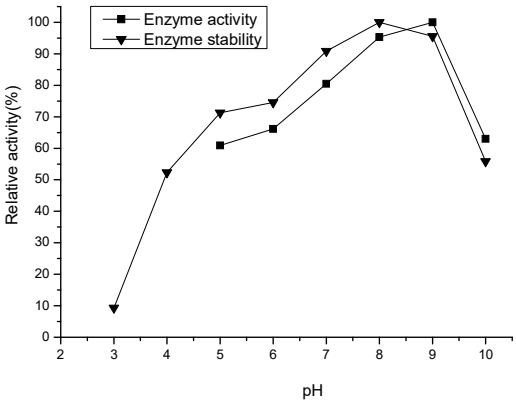


Fig. 3. Effects of pH on the activity and stability of DBP esterase

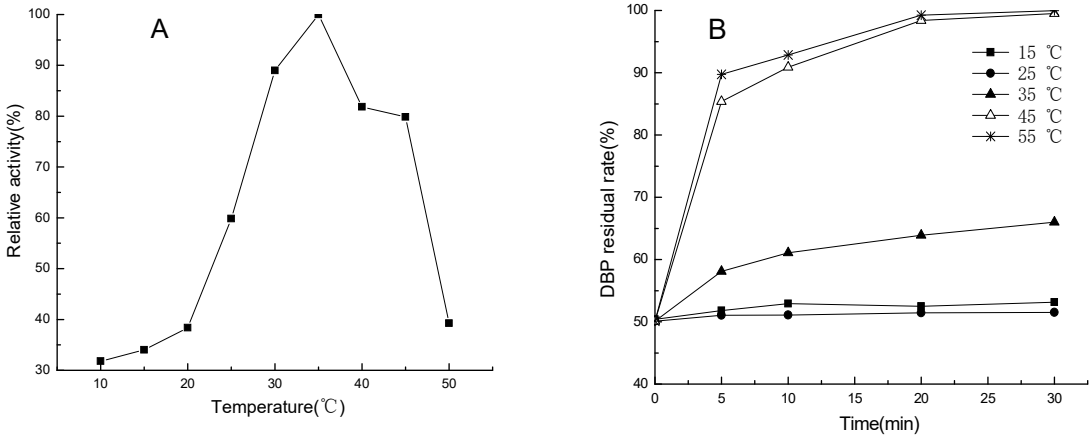


Fig. 4. Effects of temperature on the activity (A) and stability (B) of DBP esterase and strongly inhibited by Fe^{3+}. K^{+}, Mn^{2+} and Tween-80 did not affect the enzyme activity

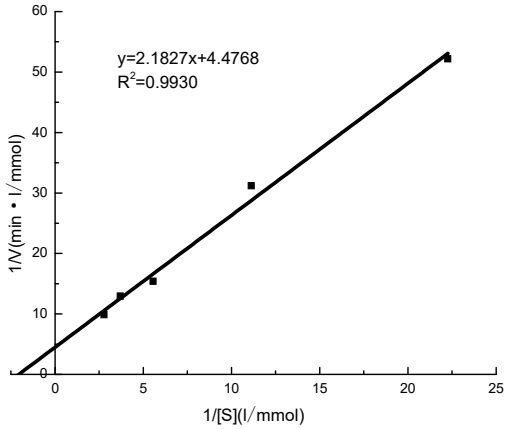


Fig. 5. Lineweaver-Burk plot of DBP esterase activity

metals such as Cu^{2+}, Zn^{2+}, Ba^{2+}, EDTA, and strongly inhibited by Fe^{3+}. K^{+}, Mn^{2+} and Tween-80 did not affect the enzyme activity.

Determination of kinetic constants

The enzyme kinetic constants were determined by measuring the initial rate of enzymic reaction as a function of DBP concentration over the 12.5-100mg/l range. Lineweaver-Burk plot of DBP esterase activity was shown in Fig. 5. The Michaelis-Menten constants (K_m) the enzyme was 0.487 mmol/l, and the V_{max} was 223.3 ìmol/min·l.

DISCUSSION

Since the rates of photolysis and chemical hydrolysis of phthalate esters are very slow, metabolic breakdown by microorganisms is considered to be one of the major routes for the environmental degradation of these widely spread pollutants in aquatic and terrestrial systems. In recent years, numerous studies have demonstrated the microbial bio- degradation pathways of phthalate esters. Hydrolysis of the ester bond is a common key initial step in the microbial degradation of phthalate esters. Phthate acid esterase is one of key enzymes in the degradation pathway. However, there are only a few reports on the detailed molecular and catalytic properties of the enzymes, for example, esterases catalyzing the hydrolysis of phthalate esters from *Nocardia erythropolis* S-1[10] and *Micrococcus* sp.YGJ1[11].In this study, the dibutyl phthalate esterase obtained, from *Arthrobacter* sp. ZJUTW which corresponds to the enzyme catalyzing the first step in the metabolism of the phthalate esters. The purified enzyme preparation migrates as a single protein band on SDS-PAGE, and its molecular mass was found to be 56.17 kDa, which similar to esterase from *Micrococcus* sp.YGJ1 hydrolyzing phthalate esters (56KDa), but different from that of *Nocardia erythropolis* S-1 (15KDa). In respect of the enzyme properties, the optimum pH (9,0), temperature (35°C) , pH stability (7.0-9.0), thermostability (15-25°C) and the effects of metal ions (such as strongly inhibited by Fe^{3+}) of the purified enzyme were different from that of *Micrococcus* sp.YGJ1 (such as Fe^{3+}, Zn^{2+}, Mg^{2+}, EDTA did not affect the enzyme activity) and *Nocardia erythropolis* S-1 (such as the optimum pH of 8.6 and the optimum temperature of 42°C). In addition, an esterase hydrolyzing monoalkyl phthalates (MAP) and monobutyl phthalates (MBP) purified from *Micrococcus* sp. YGJ1 was reported by Kiyofumi M. *et al.,*[16]. The metabolism of phthalate esters is initiated in bacteria by their hydrolysis to mono-phthalate esters, which is further degraded by ester-hydrolysis to phthate acid. However, in our further experiments, *Arthrobacter* sp. ZJUTW could not degraded MBP (data not shown), suggesting that the metabolism of phthalate esters in *Arthrobacter* sp. ZJUTW may be different from the previous reports. Further experiments are currently being performed in order to better characterize the present enzyme in terms of catalytic properties and substrate specificities. Furthermore the catalytic ability of trans-esterification of the present enzyme will be the subject of additional investigations.

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