Purification and some properties of a phthalate ester hydrolyzing enzyme from *Nocardia erythropolis*

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Summary. A phthalate ester hydrolyzing enzyme has been purified from the culture broth of *Nocardia erythropolis*, a Gram-positive bacterium capable of degrading phthalate esters rapidly. The purified enzyme appeared homogeneous on polyacrylamide gel disc-electrophoresis, and its molecular weight was estimated to be about 15,000. The optimal pH and temperature were pH 8.6 and 42 °C, respectively. The enzyme was stable in a pH range from 7.0 to 8.0 and below 30 °C. The enzyme activity was stimulated by Ca²⁺ and taurocholate, but inhibited by several metals such as Hg²⁺. Most of the phthalate esters tested were hydrolyzed to phthalate and alcohols regardless of the type of side-chain. In addition, the enzyme rapidly hydrolyzed olive oil and tributyrin. This enzyme from *N. erythropolis* may be a novel type of lipase with broad substrate specificity.

Introduction

In recent years, there have been many reports on the biodegradation of phthalate esters (Saeger and Tucker 1976; Engelhardt and Wainofer 1978; Ohta and Nakamoto 1979; Kurane et al. 1977a and b, 1978, 1979a, b, and c; Taylor et al. 1981) prompted by the increased production of plastic goods. Considerable attention is still being paid to biodegradation of phthalate esters utilized as plasticizers. However, little work has been reported on the enzymes hydrolyzing phthalate esters.

The authors have reported in previous papers (Kurane et al. 1980a and b) that *Nocardia erythropolis* rapidly degrades phthalate esters after hydrolysing them to free phthalic acid. We further discovered that the phthalic acid is then mineralized via the intradiol fission of protocatechuate, and that the production of enzymes for the metabolic pathway appears to be successively induced by the intermediates. In a previous paper (Kurane et al. 1980b), we also reported that the first-step enzyme, i.e., the phthalate ester hydrolyzing enzyme of *N. erythropolis* is a form of normal lipase (esterase) or a new characteristic esterase.

Following up these earlier reports, the present study describes the purification and some general properties of the phthalate ester hydrolyzing enzyme from *N. erythropolis*.

Materials and methods

Microorganism. *Nocardia erythropolis* S-1, previously characterized as a phthalate ester-utilizing microorganism (Kurane et al. 1977a and b), was employed.

Chemicals. Dextran T₃₀₀, DEAE-Sephadex A-50 and Sephadex G-100 were obtained from Pharmacia Fine Chemicals (Sweden). Di-2-ethylhexyl phthalate (DEHP) was obtained from Tokyo Kasei Co. (Tokyo). Other chemicals used in this study were of reagent grade.

Cultivation. Liquid cultures were grown in 500-ml flasks containing 100 ml of a complete, synthetic, defined medium (Kurane et al. 1977a) supplemented with 0.3% of DEHP. DEHP was uniformly dispersed with a homogenizer. Most particles were less than 0.5 μm in diameter. The organism was cultivated on a rotary shaker at 30 °C for 2–5 days. For the harvesting of large amounts of enzyme, cells of *N. erythropolis* preincubated in the above defined medium were inoculated into 51 jar-fermentor containing the above mineral medium supplemented with 0.5% of DEHP and 0.05% of soybean meal. Soybean meal has been reported as an activator for the production of lipase by Yamada et al. 1962. This organism was grown in the jar-fermentor at 30 °C for 2–3 days.

Enzyme assay

(i) Phthalate ester-hydrolyzing activity. The activity was determined by measuring the rate of decrease in DEHP or by base titration. The decrease in DEHP concentration was estimated with a gas chromatographically, as described previously (Kurane et al. 1977a).
A volume of 5.0 ml of the reaction mixture was made up of 2.5 ml of 5,000 ppm DEHP in Tris-HCl buffer (pH = 6.5, 0.5 ml of 0.5% CaCl₂ solution in the same Tris buffer, and 2.0 ml of the enzyme solution. DEHP was uniformly dispersed by homogenization. Most of the particles were less than 0.5 μm in diameter. The reaction mixture was incubated on a reciprocal shaker at 30°C for 18 h. Before and after the incubation, DEHP was extracted with an equal volume of n-hexane and its concentration determined. Controls without substrate or without enzyme solution were examined simultaneously. One unit of the activity was defined as that which produced 1 μmol of phthalic acid/min at 30°C. Phthalate esters were estimated gas chromatographically (Shimazu model GC-5A) as described previously (Kurane et al. 1977a). Duplicates usually differed by less than 1% from the mean, unless otherwise stated.

(ii) Lipase activity. Lipase activity was assayed by the modification of Nord’s method reported by Yamada et al. 1962.

Purification procedure. The phthalate ester-hydrolyzing enzyme was purified according to the following procedures. All processes were performed at 5°C, unless otherwise stated.

Step 1 (ammonium sulfate precipitation). A saturated ammonium sulfate solution, adjusted to pH 7 with 1 N NaOH, was added to the centrifuged (28,000 g, 30 min) culture broth to bring its saturation to 75%; the solution was stirred for 15 min and allowed to stand for 1 h. The precipitate was collected then by centrifugation. The precipitate was dissolved in Na₂-phosphate buffer (4.2 × 10⁻² M, pH 7) and dialyzed thoroughly against the same buffer.

Step 2 (two-phase aqueous polymer method). The two-phase aqueous polymer method of Albertson (Albertson 1970) was then applied to the dialyze. A mixture of 30% Dextran T₅₀₀ and 30% polyethylene glycol 6000 was added to the dialyze to yield a final concentration of 4% (w/v). The mixture was stirred for 30 min and then centrifuged at 100,000 g at 10,000 rpm. The upper phase containing the enzyme activity was adjusted to 70% ammonium sulfate saturation, stirred for 30 min and allowed to stand for 4 h at 15°C. The precipitate was collected by centrifugation at 15,000 g for 10 min, dissolved in a phosphate buffer (pH = 6.8) and dialyzed thoroughly against the same buffer.

Step 3 (DEAE-Sephadex A-50 column chromatography). The dialyze above was loaded on a column of DEAE-Sephadex A-50 (Ø 2.5 × 15 cm) previously equilibrated with the same phosphate buffer. After washing with this buffer, the column was subjected to a linear gradient elution with the same buffer varying from μ = 0.05 to 1.0 M NaCl at a flow rate of 5 g/h.

Step 4 (gel filtration on Sephadex G-100). The active fractions from DEAE-Sephadex chromatography were concentrated at a Diaflo membrane (UM-2; Amicon Corp., U.S.A.), and loaded on to a column of Sephadex G-100 (Ø 2.8 × 28 cm) previously equilibrated with the above phosphate buffer, with which the column was eluted.

Gel electrophoresis. The active fraction (No. 24) from the Sephadex G-100 column was concentrated at a Diaflo membrane (UM-2; Aminco Corp., U.S.A.), and loaded on to a column of Sephadex G-100 (Ø 2.8 × 28 cm) previously equilibrated with the above phosphate buffer, with which the column was eluted.

Some properties of the purified enzyme. Duplicates usually differed by less than 2% from the mean.

(i) pH stability. One milliliter aliquots of the purified enzyme solution in distilled water were mixed with 1.0 ml of buffers of various pH values (μ = 0.1). The mixtures were stored overnight at 5°C. The pH of each mixture was adjusted back to 8.0 and the remaining enzyme activity was assayed by the standard method described above.

(ii) Thermal stability. The enzyme was incubated in Tris-HCl buffer (pH 8) for 30 min at various temperatures, after cooling to 30°C in ice-water, the remaining activity was assayed and expressed as a percentage of the initial activity.

(iii) Effects of some metals and reagents. One milliliter of the enzyme solution was mixed with 1.0 ml of various metal ions or reagents (final concentration: 1 × 10⁻³ M) and the mixture was allowed to stand for 15 min at room temperature. The remaining activity was assayed under the standard condition in the absence of Ca²⁺.

Properties of the esterase and lipase activities. The phthalate ester (DEHP)-hydrolyzing activity and lipase activity of the purified enzyme from N. erythropolis and of two kinds of commercial lipases were measured according to the standard methods described above. The initial DEHP concentration was 5,000 ppm. The O.D.₂₈₀ values of the enzymes used were about 0.01 in Tris-HCl buffer (μ = 0.05, pH 8.0).

Determination of the product of enzyme action. The enzyme reaction product (phthalate) was detected and determined gas chromatographically as dimethyl phthalate (DMP). The enzyme reaction mixture (total volume 5 ml as described in enzyme assay) was extracted with n-hexane and then with dichloromethane yielding dimethyl phthalate (DMP), which was estimated on a glass column of 3.0 mm internal diameter, 2 m in length, packed with Silicic OV-17 on 60/80 mesh Uniport B, employing anthrone as internal standard at a column temperature of 180°C. Its structure was confirmed by GC-MS on a JEOI D-300 gas-mass spectrometer operating with ionization energy, as previously described (Kurane et al. 1980a).

Results

Purification of phthalate-ester hydrolyzing enzyme

Purification. The phthalate-ester hydrolyzing enzyme was purified from the culture broth as described in the experimental section. The results are summarized in Table 1. The elution pattern from the DEAE-Sephadex column is shown in Fig. 1. The chromatogram of the gel filtration by Sephadex G-100 is shown in Fig. 2. The enzyme was purified by about 190-fold with a 2.6% recovery from the original crude enzyme solution.

Homogeneity and molecular weight. The purified enzyme was homogeneous according to polyacrylamide gel, as shown in Fig. 3. The molecular weight of the enzyme was estimated by the gel filtration on Sephadex G-100 to be about 15,000 (Fig. 4).

Some general properties of phthalate-ester hydrolyzing enzyme

Effects of pH. Effects of pH on the activity and stability of the enzyme were examined (Fig. 5). The