Note

Purification and Characterization of an Esterase from Micrococcus sp.
YGJ1 Hydrolyzing Phthalate Esters

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Received January 31, 2001; Accepted March 8, 2001

An esterase hydrolyzing phthalate esters has been purified from Micrococcus sp. YGJ1. The enzyme, a monomeric protein (Mr=56 kDa) with a pI of 4.0, hydrolyzes various aliphatic and aromatic carboxylic esters. The medium chain (C8-C14) esters are the most preferred substrates. The enzyme is inhibited by HgCl2 and p-chloromercuribenzoate but not by phenylmethylsulfonyl fluoride.

Key words: esterase; phthalate esters; Micrococcus

Phthalate esters (PEs) are environmental pollutants due to their ubiquitous distribution in environment.10 They are industrial raw materials for synthesis of various chemicals. Especially, dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate are widely used as plasticizers for poly(vinyl chloride) resin. Release from chemical products may be a possible source of PEs in environment.20 By reason of the injurious effects of PEs on human health,3 several mammalian liver esterases involved in the metabolism of PEs have been purified and studied in detail.4-6 In the natural environment, various microorganisms are mainly responsible for the degradation of PEs.7-10 In vivo and in vitro studies so far reported have suggested that PEs are hydrolyzed to phthalate and further degraded via protocatechuate for mineralization.7 However, to our knowledge, there has been few reports on the detailed molecular and catalytic properties of bacterial enzymes hydrolyzing PEs. Krane et al. purified a lipase catalyzing the hydrolysis of PEs from Nocardia erythropolis.10 This paper deals with the purification and characterization of a Micrococcus esterase, which can hydrolyze PEs due to its broad specificity.

The enzyme reaction was done at 30°C for 1-5 min in a mixture (1 ml) containing 50 mM CHES-NaOH, pH 9.0, 4 mM dialkyl phthalate (dissolved in 100 μl of ethanol), and the enzyme. In the routine assay, DBP was used as the substrate. The reaction was stopped by adding 20 μl of 3 M H2SO4 and the solution was extracted with ether. The ether extract was evaporated and the residue was dissolved in 0.5 ml of 80% methanol. A portion (20 μl) of the solution was put through HPLC to measure the monoalkyl phthalate formed. HPLC was done at 45°C with a Shimadzu Shim-pack C18 reversed-phase column (0.6 × 15 cm) at a flow rate of 0.6 ml/min of methanol-H2O (4:1, v/v) as the eluent, and was monitored by measuring A234. The hydrolytic activity toward p-nitrophenyl (p-NP) ester was measured at 25°C from the increase in Anu (E = 13.6 mM-1 cm-1).11 The reaction mixture (3 ml) contained 50 mM Tris-H2SO4, pH 7.5, 2 mM p-NP ester, and the enzyme. Usually, the enzyme activity was first measured with p-NP acetate, and then with DBP. One unit (U) of enzyme activity was defined as the amount which degraded 1 μmol of substrate per min under the assay conditions, and the specific activity was defined as units per mg of protein. Protein was measured by the method of Bradford12 with bovine serum albumin as a standard.

The PE-assimilating bacteria, strain YGJ1, was isolated from mud on a road by enrichment culture with a mineral base medium3 with 0.05% yeast extract and 0.1% (v/v) DBP added. Strain YGJ1, a Gram-positive coccus, formed yellow colonies and was obligatory aerobic, catalase positive, non-motile, non-spore-forming, and non-fermentative. Thus, strain YGJ1 was tentatively identified as a Micrococcus sp. Phthalate, DBP, glucose, and succinate were effective carbon sources for bacterial growth. When the bacteria were grown with DBP, the consumption of DBP was correlated to the bacterial growth and no accumulation of monobutyl phthalate (MBP) and phthalate was found. Since butanol did not serve as growth substrate, the bacteria seemed to use only phthalate. There was no considerable variation of the hydrolytic activity on DBP in the crude extracts of phthalate-, DBP-, glucose-, and succinate-grown cells. For enzyme purification, the bacteria was cul-

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Abbreviations: PE, phthalate ester; DBP, dibutyl phthalate; MBP, monobutyl phthalate; p-NP, p-nitrophenyl; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; CHES, 2-(cyclohexylamino)ethanesulfonic acid
tured in a liquid medium of 6 g of phthalic acid, 24 g of (NH₄)₂SO₄, 9 g of K₂HPO₄, 3 g of KH₂PO₄, 1.2 g of MgCl₂·6H₂O, and 4.8 g of yeast extract in 6 liters of tap water. The pH of the medium was adjusted to 7.2 with KOH. The culture flasks were shaken at 100 rpm and 30°C for 24 h. The cells were harvested by centrifugation, washed once with 50 mM Tris-HCl, pH 7.5, and stored at −25°C until needed.

The enzyme purification was done at 0-4°C with 50 mM Tris-HCl buffer, pH 7.5, unless otherwise noted. The bacterial cells (24 g, wet weight) were suspended in 120 ml of the buffer and disrupted by a French press at a pressure of 600 kg/cm². The solution was centrifuged for 15 min at 25,000 × g. The esterase activity was found predominantly in the supernatant (crude extract) but not in the precipitate. The supernatant was treated with solid (NH₄)₂SO₄. The precipitate obtained between 40 and 60% saturation was dissolved in 15 ml of the buffer, dialyzed overnight against the buffer, and put on a column (3 × 30 cm) of Bio-Gel A-1.5m (medium), previously equilibrated with the buffer, at a flow rate of 13 ml/h. The active fractions were pooled and put on a DEAE-cellulose column (1.5 × 20 cm) previously equilibrated with the buffer. The column was washed with 100 ml of the buffer. Then, the enzyme was eluted with a linear gradient of NaCl, established with 100 ml of the buffer and 100 ml of the buffer containing 1 M NaCl, at a flow rate of 20 ml/h. The active fractions were pooled and dialyzed twice against 25 mM buffer and once against H₂O. The solution was put on the isoelectric focusing at 900 V for 45 h with a 110-ml glass column as described by Vesterberg. The pH gradient was formed with 2.5% Pharmalyte (pH 4-6.5). The active fractions obtained at pH 3.9-4.1 were pooled, dialyzed against the buffer, and filtered on a gel as above to remove the residual Pharmalyte. The solution was concentrated by ultrafiltration and stored at −25°C. The result of enzyme purification is summarized in Table 1, showing about 51-fold purification with a yield of 2.0%. The ratio of the specific activity toward DBP to that toward p-NP acetate became nearly constant as the advance of enzyme purification and remained constant under any conditions accompanied by the alteration of enzyme activity. Since p-NP acetate may serve as substrate for various hydrolases such as esterases, lipases, and proteases, these results suggest that there is no other hydrolase than the objective esterase in the purified enzyme preparation.

Disc polyacrylamide gel electrophoresis was done as described by Davis. Protein was stained with Coomassie Brilliant Blue R-250, and activity was made visible with α-naphthyl acetate and Fast Blue BB as described by Higerd and Spizizen. The crude extract showed three activity bands, while the purified enzyme had almost single band, as stained either for protein or for activity, at the same position as the major activity band observed in the crude extract (Fig. 1). When SDS-PAGE was done by the method of Laemmli, the purified enzyme gave a single band with an Mr of 56 kDa (Fig. 1). The pro-

![Fig. 1. Polyacrylamide Gel Electrophoreses of Enzyme Fractions.](image)

The crude extract (100 µg, A) and the purified enzyme (10 µg, B) were put on disc polyacrylamide gel electrophoresis at pH 8.3 and 4°C with 7.5% gel. Electrophoresis was done until the tracking dye reached the bottom of the gel. Then, the gels were stained for protein (left) and for activity (right). The purified enzyme (10 µg, C) was put on SDS-PAGE using a 12.5% gel. The Mr marker proteins used were muscle myosin (Mr = 200 kDa), E. coli β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21 kDa).

### Table 1. Purification of the PE-Hydrolyzing Esterase from *Micrococcus* sp. YGJ1

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity(U)</th>
<th>Total protein(mg)</th>
<th>Specific activity(U/mg)</th>
<th>Purification(fold)</th>
<th>Yield(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1) DBP</td>
<td>(2) p-NPA</td>
<td>(1)/(2)</td>
</tr>
<tr>
<td>Crude extract</td>
<td>495</td>
<td>756</td>
<td>0.655</td>
<td>3.78</td>
<td>0.173</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>364</td>
<td>343</td>
<td>1.06</td>
<td>6.24</td>
<td>0.170</td>
</tr>
<tr>
<td>Bio-Gel A-1.5m</td>
<td>326</td>
<td>27.9</td>
<td>11.7</td>
<td>51.2</td>
<td>0.229</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>76.4</td>
<td>3.25</td>
<td>23.5</td>
<td>101</td>
<td>0.233</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>9.95</td>
<td>0.298</td>
<td>33.4</td>
<td>153</td>
<td>0.218</td>
</tr>
</tbody>
</table>

* The enzyme activity was measured both with DBP and with p-NP acetate (p-NPA). Total activity, purification, and yield were calculated using the activity obtained with DBP.
tein band was electroblotted onto a polyvinylidene difluoride membrane and the N-terminal amino acid sequence of MLNPVLTQTG was identified by Edman degradation. Protein sequence similarity indicated 70% sequence identity with bovine ubiquitin carboxyl-terminal hydrolase (UCH-L1) [EC 3.1.2.15]. The enzyme showed an $M_\text{s}$ of 50 kDa by analytical gel filtration on Sephadex G-200, indicating that the enzyme is a monomeric protein. Isoelectric focusing gave the pI of 4.0. The purified enzyme contained no carbohydrate as tested by periodic acid Schiff staining, and showed a typical protein UV-absorption spectrum.

The product of enzymatic hydrolysis of DBP was identified to be MBP by silicagel TLC, 1H-NMR, and GC-MASS. The stoichiometric amount of MBP was formed from DBP and no formation of phthalic acid was detected. The purified enzyme was heat-labile. At pH 9.0 and 7.5, the enzyme lost half of its activity after 10-min incubation at 28°C and 37°C, respectively. Dithiothreitol (10 mM) could not prevent the inactivation. The optimum pH was around 9 in CHES-NaOH buffer. Ethanol (10%, v/v) increased the enzyme activity by about 3-fold, possibly due to solubilization of DBP, but was rather inhibitory at above 15%. HgCl$_2$ (0.01 mM), p-chloromercuribenzoate (0.01 mM), N-ethylmaleimide (5 mM), and diethylpyrocarbonate (1 mM) inhibited the activity more than 78%. The inhibition of the former two reagents were partially reversed by the subsequent incubation with 10 mM dithiothreitol. Unlike the general carboxylesterases (serine hydrolases), the enzyme was scarcely inhibited by phenylmethysulfonyl fluoride (1–5 mM), a typical inhibitor for serine hydrolases. Mercaptoethanol, diithiothreitol, cysteine, EDTA, Ca$^{2+}$, Mg$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Fe$^{2+}$, and Zn$^{2+}$ (each 1 mM) did not affect the enzyme activity.

The broad substrate specificity of the enzyme was shown by measuring the released acid at pH 7.5 and 25°C with a pH-Stat. The reaction mixture (5.0 ml) contained 5 mM KCl, ester (100 µl of liquid ester or 40 mM ethanol solution of DBP or MBP) and the enzyme. NaOH (10 mM) was used for continuous titration. The activity relative to that toward butyl butyrate were as follows: PE$_2$s [DBP (30.6); MBP (0)], butyrate esters [methyl (18.8); ethyl (22.4); propyl (55.3); butyl (100); amyl (41.1); hexyl (3.5); benzyl (101); cyclohexyl (77.6); 2-ethylhexyl (9.4)], butyl esters [acetate (35.3); propionate (90.6); butyrate (100); caproate (11.8); benzoate (11.8)], triacylglycerols [tricetin (99.7); tripalmitin (124); tributyrin (26.6); tricaprin (2.3); triolein ($\leq 0.4$)], lactones [3-hydroxybutyrolactone (0)]; 4-hydroxyvalerolactone (0)]. The enzyme could not hydrolyze the thioester bond of acetyl-CoA and malonyl-CoA as tested by CoA formation, and showed no endopeptidase activity as tested by casein digestion. Thus, the enzyme appeared to belong to the non-specific esterases preferentially hydrolyzing the medium chain (C$_7$-C$_{10}$) esters. The specificity for chain length was further confirmed by the kinetic constants for substrate homologs found by double reciprocal plots (Table 2). The enzyme showed the highest $V_{\text{max}}$ on PE of C$_7$-alkyl chain and on p-NP ester of C$_7$-acyl chain, and showed the lower $K_m$-values for the esters of the longer chain length. The $V_{\text{max}}$-values of the esterase in hydrolyzing PE$_2$s are higher than those of the purified liver microsomal esterases, which are serine hydrolases with subunit $M_\text{s}$s of about 47–60 kDa and preferably hydrolyze the short chain (C$_{7}$-C$_{10}$) PE$_2$s.

Since the purified esterase shows no activity on monoalkyl phthalates, it is expected that Micrococcus sp. YGJI has another enzyme to degrade monoalkyl phthalates. Experiments are now in progress to purify this enzyme.

Acknowledgments

The authors wish to thank Profs. Y. Takesue and N. Yoshizaki for their valuable suggestions, and Prof. F. Suzuki for his useful advice about operating a protein sequencer. This study was partially supported by the special research fund of Gifu University.

References

Purification of Micrococcus Esterase


