Purification and Characterization of tert-Butyl Ester-hydrolyzing Lipase from *Burkholderia* sp. YY62

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An intracellular novel lipase which can hydrolyze tert-butyl octanoate (TBO) was purified to homogeneity from crude cell-free extracts of *Burkholderia* (formerly *Pseudomonas*) sp. YY62 with 9% overall yield. Seventy-four-fold purification was achieved by ammonium-sulfate precipitation, three consecutive open-column chromatographies (DEAE anion-exchange, Sepharose CL-6B gel-filtration, and the second DEAE anion-exchange columns), and two HPLCs (TSK G2000SWXL gel-filtration and phenyl SPW hydrophobic interaction columns). Enzymes hydrolyzing p-nitrophenyl acetate were separated into two peaks (peak I and II) on the hydrophobic HPLC, and only peak II was found to have TBO-hydrolyzing activity. The peak preparation showed a single band of 40 kDa on SDS-PAGE and a molecular mass of 39 kDa on gel-filtration under non-denatured conditions, indicating the monomeric nature of the TBO-hydrolyzing lipase. The lipase showed maximum activity at pH 7.0 and 28°C. The N-terminal 15 amino acid residues were determined as Met-Asp-Phe-Tyr-Asp-Ala-Asn-Glu-Thr-Arg-His-Pro-Glu-Gln-Arg, which showed no homology to known proteins, suggesting that the purified enzyme may belong to a novel class of hydrolase.

**Key words:** *Burkholderia* sp.; tert-butyl octanoate; esterase; lipase

Lipases (glycerol ester hydrolases, EC 3.1.1.3), which are hydrolases acting on the carboxyl ester bonds in acylglycerols to liberate fatty acids and glycerol, are widely distributed in organisms ranging from animals to bacteria. Because lipases are active and stable in both aqueous and nonaqueous solvent systems, they have considerable application in industry and medicine, and many microbial lipases with different characteristics have been screened. Although these lipases show a wide variety of properties with respect to substrate specificity, optimum pH, or thermostability, they are not as useful as expected when used in the organic synthesis of compounds with some structural complexity. This is because known lipases cannot hydrolyze esters containing a bulky substituent near the ester carbonyl, such as tert-alcohol esters. Tertiary alcohols and their derivatives containing tertiary C—O bonds are useful building blocks for many drugs and natural products. In comparison with the great progress in preparing optically active secondary alcohols, synthesis of enantiomerically pure tertiary alcohols is still a challenging problem. As reported in our previous paper, to overcome this limitation, we screened for a new lipase using tert-butyl octanoate as a model substrate for bulky esters, and isolated *Burkholderia* sp. YY62 as the producer of a lipase which can hydrolyze TBO into tert-butanol and octanoic acid (Fig. 1). The hydrolytic activity was dependent on the acyl chain length of the substrate, and the C₅ acid moiety gave the highest activity. Using the activity ratio between TBO and p-nitrophenyl acetate as a measure for preference to bulky esters, we confirmed that the lipase of strain YY62 was 100-fold superior to commercial lipases in TBO-hydrolyzing activity. As a first step for providing pure TBO-hydrolyzing lipase in large quantities and using it in the organic synthesis of natural complex compounds, we purified the enzyme and characterized it.

**Materials and Methods**

*Burkholderia* sp. YY62 was cultivated in NB medium consisting of (per liter) 10 g of beef extract, 10 g of polypeptone, and 5 g of NaCl (pH 7.5). An inoculum was prepared by cultivating *Burkholderia* sp. YY62 for 5 h at 30°C on a rotating shaker at 120 rpm. TBO was added to the NB medium at a concentration of 0.1% (w/v). Main cultivation was done with 60 l of NB containing 0.1% TBO in a 100-liter jar fermentor (MPF type, Marubishi Laboratory Equipment Co., Ltd. Tokyo, Japan) by inoculating 1.0% (v/v) of the seed culture under the conditions of 30°C for 39 h at 200 rpm of impeller speed with aeration of 601/min. The pH was maintained at 7.5 with 3 N
Purification of TBO-hydrolyzing lipase. (i) Preparation of crude cell extract. Cells were collected (750 g wet cells from 60 liters of culture broth) by continuous centrifugation (No-6, Kansai Centrifugal Separator Manufacturing Co., Ltd. Osaka, Japan), and suspended in 350 ml of 0.05 M potassium phosphate buffer (KPB) (pH 7.5). The suspension was sonicated for 10 min (30 sec × 20) at 4°C to disrupt cells, and the cell debris was removed by centrifugation at 6000 x g (Tomy SRX-201, Tomy Tech Inc., Tokyo, Japan) for 20 min at 4°C. Solid ammonium sulfate was added with stirring to the supernatant on ice to give 50% saturation. After 1 h, the precipitate was collected by centrifugation at 6000 x g for 20 min at 4°C and dissolved in 500 ml of 0.05 M KPB (pH 7.5), and the solution (380 ml) was dialyzed for 12 h against a 30-fold volume of 0.05 M KPB (pH 7.5) at 4°C with three changes.

(ii) First DEAE-Sephadex column chromatography. The dialyzed enzyme solution was applied to a DEAE-Sephacel column (6 × 75 cm, Pharmacia Biotechnology LKB, Uppsala, Sweden) pre-equilibrated with 0.05 M KPB (pH 7.5). After a wash with 2 bed volumes of 0.05 M KPB (pH 7.5), bound proteins were eluted with 5 bed volumes each of 0.05 M KPB containing 0.1%, 0.2%, 0.3%, 0.4% and 0.5 M NaCl. Active fractions eluted at 0.2 M NaCl were combined and concentrated to about 270 ml by ultrafiltration (UP-20; M. C. Cut 20,000, Advantec Toyo, Osaka, Japan).

(iii) Sepharose CL-6B Gel filtration chromatography. The concentrated solution from step (ii) was applied to a Sepharose CL-6B column (6 × 75 cm, Pharmacia Biotechnology LKB, Uppsala, Sweden) pre-equilibrated with 0.05 M KPB (pH 7.5), and developed with the same buffer. The active fractions containing the lipase activity (fraction 55–82, 670 ml) were pooled and concentrated to about 273 ml by ultrafiltration.

(iv) Second DEAE-Sephadex column chromatography. The concentrated solution from step (iii) was applied to a DEAE-Sephadex column (5 × 42 cm) pre-equilibrated with buffer A (0.02 M KPB containing 0.1% NaOC, pH 7.5). After a wash with 2 bed volumes of buffer A, adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M in 5 bed volumes of buffer A. Active fractions (fraction 38–62, 463 ml) eluted at 0.16 to 0.28 M NaCl were combined and concentrated to about 81 ml by ultrafiltration.

(v) Gel filtration HPLC. HPLC was performed with a Jasco model Tri Rotor-V with a UV detector (Jasco Uvidec-100-V, Tokyo, Japan). The concentrated sample from step (iv) was separated at ambient temperature on a TSK G2000 SWXL column (0.78 × 30 cm, Tosoh Co., Ltd. Osaka, Japan) equilibrated with buffer B (0.1 M KPB containing 0.2 M NaCl), at a flow rate of 0.3 ml/min with detection at 280 nm. Active fractions containing the lipase activity (retention time 28–32 min) were pooled and concentrated to 23 ml by ultrafiltration.

(vi) Phenyl SPW HPLC. The pooled active sample was finally purified by hydrophobic HPLC on a phenyl SPW column (0.75 × 7.5 cm, Tosoh Co., Ltd. Osaka, Japan) with two steps of a linear gradient of ammonium sulfate from 1 to 0 M (33.3 mM/min for 30 min and then 50 mM/min for 20 min) in buffer C (0.05 M KPB containing 0.02% NaOC, pH 7.5) at a flow rate of 0.5 ml/min with detection at 280 nm. Active fractions were combined (10 ml) and dialyzed against 11 (100-fold) of buffer C for 12 h at 4°C.

Determination of molecular weight. The molecular weight of the TBO-hydrolyzing lipase under native conditions was determined by gel filtration HPLC (TSK G2000SWXL, M. C. Cut 100,000) using buffer B at a flow rate of 0.3 ml/min. Fractions were collected every 30 sec and assayed for TBO-hydrolyzing activity to determine the elution position. A standard mixture (M. C. 12,400, 32,000, 67,000, 142,000, and 290,000, Oriental Yeast Co., Ltd. Tokyo, Japan) was used as a molecular weight marker. SDS-PAGE was performed with a ready-made 10–20% gradient gel ( Daiichi Pure Chemicals, Ltd. Tokyo, Japan) by using a mini-gel apparatus (Daiichi). Samples from phenyl SPW HPLC were dialyzed against distilled water at 4°C overnight with three changes to remove all traces of ammonium sulfate, lyophilized, and then dissolved in SDS buffer before being applied to SDS-PAGE gels. Proteins on the gels were stained with Coomassie Brilliant Blue. Standard proteins (M. C. 20,000, 30,000, 43,000, 67,000, and 94,000, Pharmacia Biotechnology LKB, Uppsala, Sweden) were used as markers.

Amino acid sequence analysis. Purified protein from phenyl SPW HPLC was subjected to SDS-PAGE and electroblotted to an Immobilon-P® polyvinylidene difluoride membrane (Millipore Corp. Massachusetts, U.S.A.) using Trans-Blot SD (Bio-Rad) as recommended by the manufacturer. After being stained with Coomassie Brilliant Blue R-250, the protein band was excised and analyzed on a pulsed-liquid protein sequencer (model ABI 476 A, Applied Biosystems Co., Ltd. Tokyo, Japan) with an on-line phenylthiohydantoin-amino acid analyzer (model 610 A, Applied Biosystems Co., Ltd. Tokyo, Japan).

Assay of esterase activity. Esterase activity was routinely assayed by measuring the amount of p-nitrophenol formed from p-nitrophenyl acetate. The reaction mixture (3.0 ml) contained 50 mM potassium phosphate (pH 6.0), 1 mM MgCl₂, 2.5 mM p-nitrophenyl acetate, 5% CH₃CN, and enzyme. After preincubation for 1 min at 37°C, the reaction was initiated by the addition of p-nitrophenyl acetate (50 mM, 0.15 ml) dissolved in dry CH₃CN. After incubation at 37°C for 10 min, the reaction was stopped by the addition of an equal volume of ethanol (3 ml). Absorbance at 400 nm originating from p-nitrophenol (ε₄₀₀nm = 14,700) was measured. One unit of esterase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of p-nitrophenol per min.

Enzymatic hydrolysis of t-butyl octanoate (TBO). The hydrolysis reaction was carried out in 4 ml of reac-
tion mixture containing 50 mm potassium phosphate (pH 7.0), 1 mm MgCl₂ and 0.1% TBO. After 20 min of reaction at 28°C on a reciprocating shaker (120 rpm), the reaction mixture was extracted with 10-fold volumes of CH₂Cl₂. The CH₂Cl₂ layer was dried over Na₂SO₄, and concentrated in vacuo. The residue was dissolved in one ml of hexane containing 5 mg of diethyl phthalate as an internal standard. The amount of the produced acid and remaining t-butyl octanoate were analyzed by gas chromatography (G-5000, Hitachi) equipped with an Ultra Alloy (8H)-1 column (0.8 mm × 30 m; Hitachi, Co., Ltd. Tokyo, Japan) using N₂ as a carrier gas. Injector and detector temperatures were 250 and 300°C, respectively. The column temperature was kept at 75°C for 3 min, raised to 150°C at a rate of 18°C per min, kept at 150°C for 1 min, and raised to 200°C at a rate of 5°C per min. One unit of activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of acid per min.

**Protein assay.** The protein profiles during the purification were followed by measurement of the absorbance at 280 nm. Otherwise protein concentration was determined by a dye binding assay (Protein assay kit, Bio-Rad, Tokyo, Japan) using bovine serum albumin as a standard.

**Chemicals.** p-Nitrophenyl acetate was obtained from Tokyo Kasei Kogyo Co., (Tokyo, Japan). t-Butyl octanoate was prepared from t-butyl alcohol and octanoyl chloride. The non-ionic detergent Noigen HC was obtained from Daiicchi Kogyo Seiyaku Co., (Kyoto, Japan). All other chemicals used in this study were of reagent or HPLC grade.

**Results and Discussion**

**Purification of lipase**

Purification of the TBO-hydrolyzing lipase starting from 250 g of cells is summarized in Table 1. The enzyme was purified 74-fold with an overall yield of 8.8% after 6 steps. Because enzyme preparation during the purification tends to form aggregates, the addition of detergents such as Noigen HC was essential to achieve good separation and also to prevent activity loss. For monitoring the activity profile during purification, we measured p-nitrophenyl acetate (p-NPA) hydrolyzing activity (esterase activity) instead of TBO hydrolysis because preliminary purification revealed only one peak of esterase activity, which also coincided with TBO-hydrolyzing activity. Actually, during the ion-exchange chromatography (Fig. 2) and gel-filtration chromatography (Fig. 3), only one peak of esterase, which also showed TBO-hydrolyzing activity, was observed. However, by hydrophobic HPLC (Fig. 4), the esterase activity was separated into two peaks (peaks I and II), of which only peak II had TBO-hydrolyzing activity (data not shown). SDS-PAGE analysis of peak II revealed only one protein band of 40 kDa (Fig. 5-A, B), indicating that the TBO-hydrolyzing enzyme can hydrolyze both TBO and p-NPA. The molecular mass of the enzyme under native conditions was determined to be 39 kDa (Fig. 5-C), indicating that TBO-hydrolyzing enzyme is a monomeric protein of 39 kDa. The purified enzyme was stable for at least 5 months at −80°C (residual activity 92.1%). The pure enzyme preparations were stored at −80°C and used to study the properties of the lipase.

**Table 1.** Purification of the TBO-Hydrolyzing Lipase from Burkholderia sp. Y62

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>TBO activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (-fold)</th>
<th>Yield (%)</th>
<th>TBO/p-NPA ratio (×10⁻³)</th>
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<tbody>
<tr>
<td>Crude extract</td>
<td>23656</td>
<td>9.1</td>
<td>0.385</td>
<td>1</td>
<td>100</td>
<td>1.43</td>
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<tr>
<td>(NH₄)₂SO₄ precipitate</td>
<td>14220</td>
<td>7.2</td>
<td>0.510</td>
<td>1.32</td>
<td>79</td>
<td>1.38</td>
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<td>DEAE-Sephalce</td>
<td>9855</td>
<td>6.8</td>
<td>0.710</td>
<td>1.84</td>
<td>75</td>
<td>1.74</td>
</tr>
<tr>
<td>Sepharose CL-6B</td>
<td>3990</td>
<td>4.2</td>
<td>1.05</td>
<td>2.72</td>
<td>46</td>
<td>2.06</td>
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<tr>
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<td>858</td>
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<td>3.85</td>
<td>10.0</td>
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<tr>
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<td>17.7</td>
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<td>23</td>
<td>4.75</td>
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<tr>
<td>Phenyl 5PW</td>
<td>28</td>
<td>0.8</td>
<td>28.6</td>
<td>74.2</td>
<td>8.8</td>
<td>3.11</td>
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</table>

Fig. 2. Elution Profile for the Second DEAE-Sephalce Column Chromatography.

Pooled active fractions from the Sepharose CL-6B column were concentrated by ultrafiltration and divided into 3 portions. Each portion, containing 1330 mg of protein, was separated by the Second DEAE-Sephalce column and collected in 19.5-ml fractions. Esterase activity (●) was measured under the conditions described in Materials and Methods, and the protein (●) was monitored by absorption at 280 nm. The line in the upper box indicates the gradient profile for NaCl concentration. The line above the chromatogram indicates the combined fractions for the next step of purification.
Effects of pH and temperature on the activity
Figures 6 and 7 show the effects of pH and temperature on the purified enzyme for the hydrolysis of TBO and p-NPA, respectively. The optimum pH for TBO hydrolysis (Fig. 6) was narrow, with maximum activity at 7.0, while that for p-NPA was broad, with a maximum ranging from 7 to 10. The rapid decrease in TBO hydrolysis at alkaline pH was not due to inactivation of the enzyme, because it was known that the enzyme was

Fig. 3. Elution Profile for TSK G2000SW4H HPLC.
The concentrated active sample from the Second DEAE-Sephacel step was further purified by a TSK G2000SW4H column in portions of 800 µl per run (about 8.5 mg of protein). Other experimental conditions are described in Materials and Methods section. Esterase activity (●) and absorbance at 280 nm (○).

Fig. 4. Elution Profile of Phenyl SPW HPLC.
The pooled active solution was separated into portions of 3 ml per run (about 15.5 mg of protein). Other experimental conditions are described in Materials and Methods section. Esterase activity (●) and protein (○) measured by absorbance at 280 nm. The line in the upper box indicates the gradient profile for (NH₄)₂SO₄ concentration.

Fig. 5. Molecular Weight Determination of the TBO-Hydrolyzing Enzyme by SDS-PAGE and Gel-filtration.
A, samples were run on a 10–20% linear gradient gel, and stained with Coomassie Brilliant Blue R-250. Lane 1, Marker proteins (phosphorylase b, M₉ = 94,000; bovine serum albumin, M₉ = 67,000; ovalbumin, M₉ = 43,000; carbonic anhydrase, M₉ = 30,000; soybean trypsin inhibitor, M₉ = 20,000). Lane 2, peak II (0.5 µg) from Phenyl SPW HPLC. B, log (M₉) versus relative mobility plot for SDS-PAGE. ●, marker proteins; arrow, position of purified enzyme. C, Gel-filtration HPLC on a TSK G2000 SW4H column. The elution position of the TBO-hydrolyzing enzyme is indicated by an arrow. The following proteins (●) were used as standards: glutamate dehydrogenase (M₉, 290,000), lactate dehydrogenase (M₉, 142,000), yeast enolase (M₉, 67,000), yeast adenylate kinase (M₉, 32,000), and cytochrome c (M₉, 12,400). Other experimental conditions are given in the Materials and Methods section.
stable between 7.0 and 10.0 for 20 min (data not shown). Most extracellular lipases have an acidic or neutral optimum pH,22,23 and very few have an alkaline optimum.22,23 The effects of temperature on the enzyme activity were examined, and the optimum temperature was about 28°C for both TBO and p-NPA hydrolysis as shown in Fig. 7, but the enzyme was completely inactivated at 50°C.

**Effects of metal ions and other reagents on the activity**

Because some lipases are known to require divalent metal ions,22,23-26 the effect of metal ions on the enzyme activity was investigated. Of the metal ions tested (Ca²⁺, Co²⁺, Cu²⁺, Fe²⁺, Hg²⁺, Mg²⁺, Mn²⁺, Zn²⁺, 1 mM as chloride), none showed any stimulatory effect on the hydrolysis of either TBO or p-NPA, which indicated, together with the absence of any activity loss in the presence of 1 mM EDTA or EGTA, that the enzyme does not require any metal ions for its activity. On the other hand, some ions (Zn²⁺, Cu²⁺, Hg²⁺) are inhibitors. Inhibition was stronger for TBO hydrolysis (65.2%, 79.3%, and 96.7% inhibition by ZnCl₂, CuCl₂, and HgCl₂, respectively) than for p-NPA hydrolysis (54.4%, 73.1%, and 68.8% inhibition by ZnCl₂, CuCl₂, and HgCl₂, respectively), probably reflecting the strict requirement in enzyme structure for catalyzing TBO hydrolysis: minor structural disturbances by metal ions seem to cause greater decreases in TBO hydrolysis.

Next, to characterize the enzyme more, the effects of various reagents (p-chloromercuribenzenoate (pCMB), phenylmethylsulfonyl fluoride (PMSF), diithiothreitol (DTT), iodoacetamide, and 2-mercaptoethanol, each at 1 mM) on the enzyme activity was investigated. Almost no effect was observed for iodoacetamide, DTT, or 2-mercaptoethanol on the hydrolysis of either TBO or p-NPA. The enzyme was strongly inhibited by pCMB (50% inhibition for TBO hydrolysis, 47.2% inhibition for p-NPA hydrolysis, respectively), and PMSF (100% inhibition for TBO hydrolysis, 73.7% inhibition for p-NPA hydrolysis). Because iodoacetamide or DTT showed no effect, the inhibitory effect of pCMB seems to arise from the non-specific denaturation of the enzyme rather than specific modification of —SH groups. On the other hand, clear inhibition by PMSF suggested that the enzyme probably contained active site Ser residue(s).

**NH₂-terminal amino acid sequence analysis of the TBO-hydrolyzing protein**

To isolate the corresponding gene by hybridization to an oligonucleotide probe, the NH₂-terminal region of the purified lipase was sequenced by automated Edman degradation. At each cycle of Edman degradation, only 1 amino acid signal was significantly stronger than baseline, confirming the homogeneity of the purified protein. By this procedure, the sequence of MDFYDANETRHPEQR was obtained. No protein was found to have any similarity to the N-terminus sequence, suggesting that the purified TBO-hydrolyzing enzyme may belong to a novel class of microbial lipases.

**References**

6) Brahim-Horn, M. C., Gugilelimo, M. L., Elling, L., and Sparrow, L. G., The esterase profile of a lipase from *Candida*