Purification and Characterization of a Carboxylesterase from *Pseudomonas* sp. KW1-56

Akio Sugihara, Yuji Shimada, Toshihiro Nagao, Taro Iizumi, Koichi Nakamura, Tetsuro Fukase, and Yoshihio Tominaga

Osaka Municipal Technical Research Institute, 6-30, Morinomiya 1-chome, Joto-ku, Osaka, Osaka 536, Japan

* Kurita Central Laboratories, Kurita Water Industries Ltd., 7-1 Wakamiya, Morinosato, Atsugi, Kanagawa 243-01, Japan

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An intracellular carboxylesterase from *Pseudomonas* sp. was overproduced in *E. coli*, and purified to homogeneity by a combination of hydrogen bond chromatography, gel filtration, and hydrophobic interaction chromatography. Gel filtration and SDS-PAGE suggested that the purified enzyme consisted of two subunits of molecular mass of 28 kDa. Its isoelectric point was 5.9. The enzyme was thermolabile, and showed its maximum activity at 22°C (pH 7.5). Methyl propionate was hydrolyzed at the highest rate among the fatty acid methyl esters tested. PMSF, DFP, PCMB, and HgCl₂ inhibited the enzyme markedly, suggesting that serine and/or cysteine is or near the active site.

Carboxylesterases (EC 3.1.1.1) are a group of enzymes catalyzing the hydrolysis of carboxylic acid esters, and occur in most living organisms.¹⁻³⁰ With regard to the acyl residues, short chain esters are cleaved at the highest rate, the optimal length being 3 to 6 carbon atoms.¹² They have been widely used in the production of flavor compounds in food,³⁻⁷ and in the enantioselective hydrolysis and synthesis of various esters.⁸⁻¹⁰ The most prominent and best-studied carboxylesterases are those of mammalian tissues, especially liver. On the other hand, microbial esterases have recently been the focus of intense research interest because of their industrial applications. They include the enzymes from *Bacillus steaerothermophilus*,¹¹,¹² *Bacillus subtilis*,¹³,¹⁴ *Aspergillus niger*,¹⁵ *Aspergillus awamori*,¹⁶ *Pseudomonas cepacia*,¹⁷ *Pseudomonas fluorescens*,¹⁸,¹⁹ *Ochrobactrum anthropi*,¹⁰ baker's yeast,²¹ and *Arthrobacter globiformis*.²²

We previously reported purification and characterization of a thermostable lipase from *Pseudomonas* sp. KW1-56,²³ and cloning of the lipase gene.²⁴ During the course of the experiments we found that several forms of esterase, though in low quantities, were produced intracellularly by the strain. We have recently succeeded in cloning, nucleotide sequencing, and overexpression of one of the esterase genes in *E. coli*.²⁵ The enzyme was thermolabile. This paper deals with the purification and properties of the *Pseudomonas* esterase produced by this recombinant *E. coli*.

Materials and Methods

**Enzyme assay.** Esterase activity was assayed by titrating the released fatty acid with alkali. The standard assay mixture contained 2.5 ml of 50 mM phosphate (pH 7.5), 0.5 ml of methyl propionate ( Wako Pure Chemical Industries), and 30-100 µl of enzyme solution. The enzyme reaction was done at 40°C with stirring at 250 rpm, and was stopped by adding 20 ml of ethanol. Propionic acid released during the incubation was titrated with 50 mM KOH. One unit of esterase activity was defined as the release of 1 µmol of the fatty acid per min under these conditions.

**Protein measurement.** Protein in crude samples was estimated by measuring the absorbance at 280 nm assuming that the value of E₅₀₀ (280 nm) is 10. In the final stage of the purification, a bicinechonic acid assay kit (Pierce) was used with bovine serum albumin as a standard.

**Microorganism and cultivation.** *Pseudomonas* sp. KW1-56 was cultivated in a medium consisting of 2% peptone, 0.1% yeast extract, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, and 1% olive oil at 27°C for 2 to 4 days. Recombinant *E. coli* HB101[pPSJ], which bore the plasmid harboring the esterase gene referred to as pPE5,²⁵ was grown in LB medium supplemented with 100 µg of ampicillin per ml at 37°C. The esterase gene was expressed from the lacZ promoter. The cultivation was continued for 16 h, and the cells were harvested by centrifugation at 8000 rpm.

**Purification of esterase.** Three grams of the recombinant *E. coli* cell paste were suspended in 50 ml of 0.5M phosphate (pH 7.0) containing 10% saturated ammonium sulfate. The suspension was placed in an ice bath and sonicated for three 5-min bursts at maximum output with a sonicator (model 200M, Kubota). The cell debris was removed by centrifugation at 12,000 rpm for 10 min.

To the crude extract was added solid ammonium sulfate to a final concentration of 30% saturation. The resulting insoluble material was removed by centrifugation. The supernatant was analyzed by hydrogen bond chromatography on a Sepharose CL-4B column (3 x 22 cm, Pharmacia LKB) equilibrated with 0.1 M phosphate (pH 7.0) containing 30% saturated ammonium sulfate. The column was washed with 350 ml of the same buffer, and then material was eluted with a linear gradient of 0-10% (w/v) sucrose in a total volume of 600 ml at a flow rate of 20 ml/h. Active fractions were pooled, concentrated by ultrafiltration, and dialyzed against 50 mM phosphate (pH 7.0) containing 35% saturated ammonium sulfate.

The dialyzed enzyme solution was put on a Ether Toyopearl 650M column (2 x 16 cm, Tosoh) equilibrated with 0.1 M phosphate (pH 7.0) containing 55% saturated ammonium sulfate. After washing the column with 100 ml of the same buffer, elution was done with a decreasing gradient of 35-10% saturated ammonium sulfate in a total volume of 200 ml, followed by isocratic elution with the buffer containing 10% saturated ammonium sulfate.

**Electrophoresis.** Nondenaturing polyacrylamide gel electrophoresis (PAGE) was done on a 7.5% polyacrylamide gel as described by Davis.²⁷ Esters were stained with a mixture of 0.03% α-naphthyl acetate and 1% Fast Blue Salt B dissolved in 20 mM phosphate (pH 7.0). SDS PAGE was done on a 15% gel by the method of Laemmli.²⁸ α₁-macroglobulin (170KDa), phosphorylase b (97.4KDa), glutamate dehydrogenase (55.4KDa), lactate dehydrogenase (36.5KDa), and trypsin inhibitor (20.1KDa) were used as molecular mass standards, and purchased from Boehringer. Isoelectric focusing was done on a 0.4-mm thick poly-

**Abbreviations:** PCMB: p-chloromercuribenzoate; PAGE, polyacrylamide gel electrophoresis.
acrylamide gel using a flat-bed cell (model 111, Bio-Rad). The gel was prepared with 5% acrylamide and 2% ampholytes, pH 3-10 (Bio-Lyte, Bio-Rad). pi markers used were phycocyanin (pi 4.65), beta-lactoglobulin B (5.1), bovine carbonic anhydrase (6.0), human carbonic anhydrase (6.5), equine myoglobin (7.0), human hemoglobin A (7.1), human hemoglobin C (7.5), lentil lectins (8.2, 8.4, 8.6), and cytochrome c (9.6), and were obtained from Bio-Rad. Protein bands on SDS PAGE and isoelectric focusing were stained with Coomassie Brilliant Blue.

Analytical gel filtration. For molecular weight estimation of the native enzyme, the purified enzyme solution was put on a Superose 10/30 column (1 x 30 cm, Pharmacia LKB) using an FPLC system (Pharmacia LKB). Elution was done at a flow rate of 0.5 ml/min with 10 mM phosphate (pH 7.0) containing 0.1 M NaCl.

Sequencing of N-terminal amino acids. N-terminal amino acids were sequenced with an automated protein sequencer (model 471A, Applied Biosystems) with a PTH amino acid analyzer.

DNA manipulation. Chromosomal DNA was extracted from Pseudomonas sp. KW1-56 as described in our previous paper.25 Oligonucleotides used as primers for the polymerase chain reaction (PCR) were 5'-GGACGCGAACGCGGCGGTTCG-3' and 5'-GGCCACGGCC-TTCTGACCTTCG-3', corresponding to the sequences from -380 to -356, and from 1052 to 1076, respectively, on the cloned esterase gene.25 The oligonucleotides were synthesized using a DNA synthesizer (model Oligo 1000, Beckman). PCRs were done using the chromosomal DNA or a plasmid (pRES) containing the cloned esterase gene as the target DNA, with these oligonucleotides as primers. Other techniques as well as PCR used in this study were those described by Sambrook et al.26

Results and Discussion

Purification of esterase

When prepared in the absence of salts, the supernatant of cell extracts after sonication became turbid due to aggregation of the esterase. Once aggregated, the enzyme was not molecularly dispersed even with the surfactants tested. For this reason, both preparation of the cell extracts and purification of esterase were done in the presence of salts. Ion exchange chromatography was therefore inapplicable. Hydrogen bond chromatography on a Sepharose CL-6B column was, however, effective to remove colored impurities in the crude extracts (Fig. 1). Subsequent gel filtration and hydrophobic interaction chromatography lead to 30-fold increase in specific activity, with a final activity yield of 43%. Table I summarizes the result of purification.

Isoelectric point and molecular mass

The enzyme preparation thus obtained appeared homogeneous as judged by SDS-PAGE and isoelectric focusing. SDS-PAGE showed a single band with an apparent molecular mass of 28 kDa (Fig. 2(A)). Isoelectric focusing in the pH range of 3–10 gave a band at pH 5.9 (Fig. 2(B)). Gel filtration on a Superose 10/30 column indicated that the esterase has an apparent molecular mass of about 50 kDa, suggesting that the active enzyme obtained is a dimeric protein.

Effects of temperature on activity and stability

The purified esterase showed maximum activity at 22°C (pH 7.5). When incubated at various temperatures for 30 min, the enzyme was stable up to 36°C (Fig. 3).

Effects of pH on activity and stability

The enzyme had a pH optimum of 7.5 at 20°C, and was stable between pH 6–7.5 for 20 h at 25°C (Fig. 4).

Fig. 1. Hydrogen Bond Chromatography of Pseudomonas sp. Esterase.

The cell extracts of Pseudomonas sp. KW1-56 were put on a Sepharose CL-4B column equilibrated with 0.1 M phosphate (pH 7.0) containing 30% (NH₄)₂SO₄. Material was then eluted by an increasing gradient of sucrose. •, activity; ○, absorbance at 280 nm; ——, sucrose concentration (% w/v).

Table I. Purification of Pseudomonas sp. Esterase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Activity yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>1487</td>
<td>51,600</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>Sepharose CL-6B</td>
<td>162</td>
<td>43,500</td>
<td>268</td>
<td>84</td>
</tr>
<tr>
<td>Sepharacryl S-100</td>
<td>43</td>
<td>30,440</td>
<td>710</td>
<td>59</td>
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<tr>
<td>Ether Toyopearl 650 M</td>
<td>20</td>
<td>22,190</td>
<td>1110</td>
<td>43</td>
</tr>
</tbody>
</table>

Fig. 2. SDS-PAGE (A) and Isoelectric Focusing (B) of Pseudomonas sp. Esterase.

SDS-PAGE was done on a 15% gel. Isoelectric focusing was done on a 7.5% acrylamide gel with carrier ampholytes covering pH 3–10. Proteins were stained with Coomassie Brilliant Blue.

Fig. 3. Effects of Temperature on Activity and Stability of Pseudomonas sp. Esterase.

The effects of temperature were examined by hydrolyzing methyl propionate for 20 min at pH 7.5. Stability of the enzyme was explored by measuring the remaining activity after 30 min of incubation at pH 7.5. •, activity; ○, remaining activity.
Fig. 4. Effects of pH on Activity and Stability of *Pseudomonas* sp. Esterase.

The effects of pH on activity were examined by hydrolyzing methyl propionate for 20 min at 20°C. Stability of the enzyme was explored by measuring the remaining activity after 30 min of incubation at 25°C. ●, activity; ○, remaining activity.

<table>
<thead>
<tr>
<th>pH</th>
<th>Activity (%)</th>
<th>Remaining Activity (%)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
<td>90</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
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</tr>
<tr>
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<td>3</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 5. Fatty Acid Specificity of *Pseudomonas* sp. Esterase.

Fatty acid methyl esters and simple triglycerides were hydrolyzed by the enzyme for 20 min at 20°C, pH 7.5. Activities were expressed as per cent of that on methyl propionate. ■, fatty acid methyl esters; ●, simple triglycerides.

**Fatty acid specificity**

Figure 5 illustrates relative activities towards a variety of fatty acid methyl esters and simple triglycerides. Methyl propionate and tributyryl were hydrolyzed at the highest rate. Water-insoluble methyl esters having a fatty acid carbon number of 8 or more were not hydrolyzed. Also Span and Tween were not hydrolyzed at all, though they were water-soluble. It seemed peculiar that the enzyme did not hydrolyze methylacetate. This differentiated the enzyme from other *Pseudomonas* sp. esterases. Comparison of the fatty acid specificity of *Pseudomonas* sp. KWI-56 esterase with those of the other esterases reported earlier indicates that the enzyme in this study is a novel esterase.

**Effects of metal ions and chemicals on activity**

Table II shows effects of various metal ions and chemicals on activity. Organophosphorus compounds such as PMSF, DFP, and eserine, and sulphydryl reagents such as PCMB and HgCl₂ inhibited the esterase markedly. These results suggested that serine and/or cysteine are in or near the active site of the enzyme. Captopril esterases isolated to date have been serine hydrolases, as concluded from the inhibition studies using organophosphorus compounds.¹⁰,¹¹ Since all the cysteine residues in *Pseudomonas* sp. KWI-56 esterase are not conserved among the esterases the amino acid sequences of which were examined, they would not be directly involved in catalysis.

**Table II. Effects of Metal Chlorides and inhibitors on Activity**

<table>
<thead>
<tr>
<th>Compounds (1 mM)</th>
<th>Residual activity (%)¹</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>PbCl₂</td>
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<tr>
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<td>HgCl₂</td>
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<td>Eserine</td>
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<td>DFP</td>
<td>44</td>
</tr>
<tr>
<td>PMSF</td>
<td>21</td>
</tr>
</tbody>
</table>

¹ After the incubation for 1 h at 25°C.

Fig. 6. Nondenaturing PAGE of *Pseudomonas* sp. Esterases.

The cell extracts of *Pseudomonas* sp. KWI-56 cultured for 2-4 days, and of the recombinant *E. coli* HB101[pPE5] were separated by nondenaturing PAGE on a 7.5% gel, followed by activity staining. Lane 1, 2 days cultured *Pseudomonas* sp.; lane 2, 3 days cultured *Pseudomonas* sp.; lane 3, 4 days cultured *Pseudomonas* sp.; lane 4, 4.16 h cultured *E. coli* HB101[pPE5]. Roman numerals indicate the esterase forms.

**Identification of the esterase produced by recombinant *E. coli* with *Pseudomonas* sp. KWI-56 esterase**

Agarose gel electrophoresis of the samples of the PCR products showed that both PCR products generated a single product of the same mobility (data not shown). These findings led to the conclusion that the cloned esterase gene originated from the chromosome of *Pseudomonas* sp. KWI-56. Figure 6 shows nondenaturing PAGE of the cell extracts of *Pseudomonas* sp. and of the recombinant *E. coli* HB101[pPE5], which were activity stained. It turned out that the *Pseudomonas* strain produced six intracellular esterases (I-VI), I and II being the main fractions, and that the transformed *E. coli* synthesized esterase V. More direct evidence for the above conclusion was obtained by sequencing N-terminal amino acids of the purified esterase. The enzyme was found to have the terminal sequence of Met-Ile-Phe-His-Asn-Gly-Asn-Val, which agreed with that deduced from the DNA sequence.²³

**References**

Carboxylesterase from *Pseudomonas* sp.

755


