Purification of an Extracellular \( \text{d}(-)\)-3-Hydroxybutyrate Oligomer Hydrolase from Pseudomonas sp. Strain A1 and Cloning and Sequencing of Its Gene

KAIZHANG, MARISHIRAKI, AND TERUMISAITO*

Department of Biological Sciences, Faculty of Science, Kanagawa University, Hiratsuka, Japan

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An extracellular \( \text{d}(-)\)-3-hydroxybutyrate oligomer hydrolase was purified from a poly(3-hydroxybutyrate)-degrading bacterium, Pseudomonas sp. strain A1. The purified enzyme hydrolyzed the \( \text{d}(-)\)-3-hydroxybutyrate dimer and trimer at similar rates. The enzyme activity was inhibited by a low concentration of diisopropylfluorophosphate. The molecular weight of the hydrolase was estimated to be about 70,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A 10-kbp DNA fragment of A1 was detected by hybridization with the gene (2 kbp) of an extracellular poly(3-hydroxybutyrate) depolymerase from Alcaligenes faecalis. Subsequent subcloning showed that a Smal-KpnI fragment (2.8 kbp) was responsible for expression of the hydrolase in Escherichia coli and in an in vitro transcription-translation system. The expressed protein detected by immunostaining had the same molecular weight as the purified enzyme from A1. The protein band detected in the in vitro transcription-translation system had a molecular size of 72 kDa. The nucleotide sequence of the Smal-KpnI fragment was determined, and one open reading frame (2,112 nucleotides) was found. It specifies a protein with a deduced molecular weight of 72,876 (704 amino acids). In this sequence, the consensus sequence of serine-dependent hydrolysis, G-X-S-X-G, did not exist.
Enzyme purification. A 3HB-oligomer hydrolase was isolated from the PHB-degrading bacterium *Pseudomonas* sp. strain A1, which also secretes a PHB depolymerase. During

DNA sequence analysis. Nucleotide sequences were determined by the dideoxy nucleotide chain termination method (20) with single-strand templates purified using the Auto Read sequencing kit (Pharmacia Biotech, Tokyo, Japan). A series of ordered deletions was made on the M13mp19 vector by using the Kilo-Sequence deletion kit (Takara Shuzo Co., Kyoto, Japan). Sequence information was analyzed with GENETYX-MAC software (Software Development Co., Tokyo, Japan).

*In vitro* transcription-translation. The *E. coli* S30-based coupled transcription-translation system was performed according to the technical bulletin of the producer (Promega). Briefly, the purified plasmid DNA was mixed with amino acids containing [U-14C]leucine (11 GBq/mmol, 18.5 kBq; Amersham) and S30 extract. The mixture was incubated at 37°C for 1 h. After the reaction had been completed, a 5-μl aliquot was removed, dried, treated with sodium dodecyl sulfate (SDS), and applied on an 12.5% SDS-polyacrylamide gel. After electrophoresis, the gel was soaked in 10% acetic acid and 30% methanol for 1 h and then in an enhancer reagent (ENLIGHTNING; Dupont/NEN Research) for 30 min. The treated gel was dried, and the radioactivity on the gel was detected by using an overnight exposure to X-ray film (Kodak X-Omat AR).

Preparation of antibody. The rabbits used were 3 months old, male, and about 1.8 kg. After 2 weeks of growing in good condition, 1 ml of the purified enzyme (0.02 mg) and 1 ml of Freund's complete adjuvant (Wako Pure Chemical Industries, Tokyo, Japan) were mixed and injected in the center of the back by subcutaneous injection. The same amount of protein was added in a helix vein 2 weeks after the first injection. Finally, some blood was obtained by puncturing an aorta. The blood was placed at room temperature for 1 h and centrifuged, and the supernatant was preserved in −20°C.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (13). Protein was stained with Coomassie brilliant blue R-280. Electrophoretic analysis was according to the method of Towbin et al. (27).

Protein measurement and amino acid sequence analysis. Protein was measured by the method of Lowry et al. (14). The amino acid sequences of peptides were determined by using a sequenator (model 4774; Applied Biosystems, Inc., Foster City, Calif.).

Chemicals. Chemicals were obtained as follows: butyl-Toyopearl, Tosho (Tokyo, Japan); TEAE-cellulose, Serva Feinbiochemica. Dimeric and trimeric esters of D(-)-3-hydroxybutyric acid were prepared as described previously (25). Purified PHB granules for the assay of PHB depolymerase were prepared as described by Smibert and Krieg (24). PHB powder for bacterial cultivation was donated by Zeneca. Molecular weight markers were purchased from Pharmacia (LMW electrophoresis calibration kit), Gibco BRL (prestained protein molecular weight standards), and Amersham (14C methylated protein mixture, high-molecular-weight range).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D85373.

RESULTS

Enzyme purification. A 3HB-oligomer hydrolase was isolated from the PHB-degrading bacterium *Pseudomonas* sp. strain A1, which also secretes a PHB depolymerase. During

FIG. 2. (A) Elution profile of 3HB-oligomer hydrolase from a butyl-Toyopearl column. The supernatant from culture was applied to a butyl-Toyopearl column (2.5 by 6 cm). Fractions 41 to 53 (220 ml) were pooled. ●, 3HB-oligomer hydrolase activity; ○, PHB depolymerase activity; □, A280. (B) Elution profile of 3HB-oligomer hydrolase from a TEAE-cellulose column. The concentrated enzyme solution from the butyl-Toyopearl step was applied to a TEAE-cellulose column (0.75 by 15 cm). Fractions 13 to 20 (31 ml) were pooled. ●, 3HB-oligomer hydrolase activity; ○, A280.
growth, A1 produced 3HB-oligomer hydrolase and PHB depolymerase, secreting them into the culture medium in 2 to 3 days (Fig. 1). The enzyme was purified by butyl-Toyopearl and TEAE-cellulose column chromatography. With a butyl-Toyopearl column, 3HB-oligomer hydrolase was separated from PHB depolymerase which was eluted with a buffer containing 10% ethanol (Fig. 2A). The fractions with 3HB-oligomer hydrolase activity were pooled. The pooled fraction was turbid probably due to a substance produced during PHB digestion. Most of turbidity was removed by centrifugation at 15,000 × g for 10 min. The resulting fraction was further purified on a TEAE-cellulose column, and the enzyme activity was eluted at about 0.1 M NaCl (Fig. 2B) with 17% yield (Table 1). The purified enzyme showed apparent homogeneity on SDS-polyacrylamide gel electrophoresis (Fig. 3A). The purified preparation (0.2 to 0.9 mg/ml) was stable at −20°C for at least 6 months.

Enzyme properties. The molecular mass of the purified enzyme was estimated to be about 70,000 Da by SDS-polyacrylamide gel electrophoresis (Fig. 3A). K_m and V_max values for the d(-)-3-hydroxybutyrate dimer were 0.18 mM and 25 μmol/min/mg, respectively (Table 2). K_m and V_max values for the trimer were almost the same as those for the dimer. The purified enzyme hydrolyzed methyl-d(-)-3-hydroxybutyrate at a much lower efficiency (Table 2). The purified enzyme hydrolyzed p-nitrophenyl acetate and p-nitrophenyl butyrate at the rate of 0.94 and 0.27 μmol/min/mg, respectively, but not p-nitrophenyl octanoate. Lipase activity with olive oil as a substrate in the purified preparation was under the limit of detection (<0.1 μmol/min/mg). The purified preparation did not hydrolyze PHB. Diisopropylfluorophosphate (DFP) (13 μM) inhibited enzyme activity completely. Phenylmethylsulfonyl fluoride (5 mM), Triton X-100 (1%, vol/vol), and dithiothreitol (10 mM), which are inhibitors of PHB depolymerases (26), did not affect hydrolase activity. The purified enzyme showed maximum activity at pH 7 to 8.5.

Cloning of the A1 3HB-oligomer hydrolase gene. A1 genomic DNA was digested completely with HindIII. The resulting fragments were separated on a 1% agarose gel and transferred onto a nylon membrane. The DNA fixed on the nylon membrane was hybridized with 32P-labeled 2.8-kbp fragment DNA prepared from the PHB depolymerase gene of A. faecalis T1. The DNA corresponding to the positive signal that was about 10 kbp was extracted from the agarose gels, ligated to HindIII-digested charomid 9,36, and introduced into E. coli DH5 by transfection. From about 20,000 ampicillin-resistant recombinant colonies, 11 positive colonies were selected by colony hybridization. Among positive colonies, a colony having the strongest 3HB-oligomer hydrolase activity was selected for further analyses. This colony was found to have about 10 kbp of foreign DNA. Two EcoRI fragments, 1.0 and 2.5 kbp, and an EcoRI-HindIII fragment, 6.0 kbp, were isolated and ligated into pUC19 with pUC carrying the 2.8-kbp fragment was used.

![Image](https://example.com/image1.png)

**Figure 3.** (A) SDS-polyacrylamide gel electrophoresis of the purified 3HB-oligomer hydrolase. The purified 3HB-oligomer hydrolase (1 μg) (right) and molecular weight markers (left) were subjected to electrophoresis on 10% acrylamide gels. Proteins were stained with Coomassie brilliant blue R-250. (B) In vitro transcription-translation of the 2.8-kbp DNA fragment from A1 cloned in pUC19. The products of DNA cloned in pUC19 were analyzed by the E. coli S30 coupled translation system. 14C-labeled translation products were analyzed on an SDS-polyacrylamide gel (12.5%) and then detected by using an overnight exposure to X-ray film. Lanes: 1, 4C-labeled molecular size marker; 2, pUC19 (4 μg); 3, pUC19 containing the 2.8-kbp fragment (Smal-KpnI, 3.3 μg); 4, purified 3HB-oligomer hydrolase (1 μg). The bands at around 14 and 30 kDa were α peptide of β-galactosidase and β-lactamase expressed by the pUC vector, respectively. Bands in lanes 1 to 3 were detected by autoradiography, and the purified protein in lane 4 was stained with Coomassie brilliant blue R-250. (C) Western blotting and immunostaining of the expressed 3HB-oligomer hydrolase in E. coli. Lanes: 1, purified 3HB-oligomer hydrolase from A1 (0.1 μg); 2, crude extract of E. coli with a pUC19 containing the 2.8-kbp Smal-KpnI fragment (1.5 μg).

**Table 1.** Purification of 3HB-oligomer hydrolase from *Pseudomonas* sp. strain A1

<table>
<thead>
<tr>
<th>Step</th>
<th>Amt of protein (mg)</th>
<th>Activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>100</td>
<td>720</td>
<td>7.2</td>
<td>100</td>
</tr>
<tr>
<td>Butyl-Toyopearl</td>
<td>14</td>
<td>180</td>
<td>13</td>
<td>25</td>
</tr>
<tr>
<td>TEAE-cellulose</td>
<td>5.4</td>
<td>120</td>
<td>22</td>
<td>17</td>
</tr>
</tbody>
</table>

* Enzyme was purified from a 1.6-liter culture as described in the text.

**Table 2.** Kinetic properties of 3HB-oligomer hydrolases from *Pseudomonas* sp. strain A1 and *E. coli*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_m (mM)</th>
<th>V_max (U/mg)</th>
<th>V_max/K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.18</td>
<td>25</td>
<td>138</td>
</tr>
<tr>
<td>Dimer</td>
<td>0.17</td>
<td>30</td>
<td>176</td>
</tr>
<tr>
<td>methyl-d(-)-3-Hydroxybutyrate</td>
<td>100</td>
<td>32</td>
<td>0.32</td>
</tr>
</tbody>
</table>

* The crude extract from *E. coli* with pUC carrying the 2.8-kbp fragment was used.
with pUC19. Only the cells with pUC19 that contained the 6.0-kbp fragment showed strong 3HB-oligomer hydrolase activity.

**Subcloning and in vitro expression of the hydrolase gene.** Figure 4 shows a restriction map of the 6-kbp EcoRI-HindIII fragment. According to this map, Smal-HindIII (4 kbp), SphI-HindIII (3 kbp), and Smal-KpnI (2.8 kbp) fragments were obtained. Among these fragments, Smal-HindIII and Smal-KpnI fragments contained the region responsible for expression of the hydrolase activity. The 2.8-kbp Smal-KpnI fragment expressed in vitro an about 72-kDa protein band that is similar in size to the purified hydrolase (Fig. 3B).

**Properties of the expressed 3HB-oligomer hydrolase in E. coli.** The crude extracts from E. coli with pUC19 carrying the 2.8-kbp fragment (Smal-KpnI) were analyzed by immunodetection on nitrocellulose membranes after SDS-polyacrylamide gel electrophoresis.
The 3HB-oligomer hydrolase from A. faecalis sp. strain A1 shows similar properties to that of Alcaligenes faecalis T1 (23): the molecular size is 70 kDa in the A1 enzyme and 74 kDa in the T1 enzyme, the pH optimum is 7 to 8.5 in the A1 enzyme and 8.5 in the T1 enzyme, and both enzymes are inhibited similarly with a low concentration of DFP. The nucleotide sequence of the 3HB-oligomer hydrolase gene was analyzed with the 2.8-kbp fragment. Within the 2.8-kbp sequence, one open reading frame (2,112 nucleotides) was found (Fig. 5, ORF1). It specifies a protein with a deduced molecular weight of 72,876 (704 amino acids). The initiation codon is preceded by a putative Shine-Dalgarno sequence. Since 3HB-oligomer hydrolase is secreted from cells, a signal sequence probably exists at the N terminus. We examined the N-terminal amino acid sequence of the purified protein twice but failed. The 3HB-oligomer hydrolase gene in A1 has about 47 and 20% identity to the PHB depolymerase gene in A. faecalis in nucleotide and amino acid sequences, respectively. Downstream of the 3HB-oligomer hydrolase in the 6-kbp fragment, we found another open reading frame. In this open reading frame (Fig. 5, ORF2) the sequence corresponding to the N-terminal amino acid sequence of the PHB depolymerase purified from A1 (A-V-T-A-G-P-G-A-W-S-S-Q-Q-T-W-A-A-D-S-V-) (22) was found. Therefore, it is highly possible that the 3HB-oligomer hydrolase gene and PHB depolymerase gene are arranged in tandem.

### DISCUSSION

The 3HB-oligomer hydrolase from Pseudomonas sp. strain A1 shows similar properties to that of Alcaligenes faecalis T1 (23); the molecular size is 70 kDa in the A1 enzyme and 74 kDa in the T1 enzyme, the pH optimum is 7 to 8.5 in the A1 enzyme and 8.5 in the T1 enzyme, and both enzymes are inhibited similarly with a low concentration of DFP.

We cloned the structural gene for the extracellular 3HB-oligomer hydrolase from Pseudomonas sp. strain A1 into a vector plasmid, pUC19, confirmed expression of the gene in E. coli, and sequenced the gene for this hydrolase. It is possible that the promoter sequence derived from A1 is involved in expression of the 3HB-oligomer hydrolase gene in E. coli, since the level of expression did not change in the presence of IPTG (data not shown). The open reading frame assigned for the hydrolase gene has 2,112 nucleotides corresponding to the deduced amino acid sequence of 704 amino acids that add up to a molecular weight of 72,876. The in vitro product migrated slightly slower than the purified enzyme in SDS-polyacrylamide gel electrophoresis and gave a molecular size of about 72 kDa (Fig. 3B). The expressed protein in E. coli detected by immunostaining had a molecular size of about 70 kDa (Fig. 3C).

These results indicate that the open reading frame represents the structural gene for the hydrolase. The possibility that the PHB depolymerase gene from A. faecalis used as a probe hybridized to the 3HB-oligomer hydrolase gene in A1 seems low due to relatively low homology. In the DNA fragment cloned, it was found that the 3HB-oligomer hydrolase gene and PHB depolymerase gene are arranged in tandem, although sequencing of the latter has not been completed (Fig. 5). Since the deduced amino acid sequence corresponding to the N-terminal amino acid sequence of the mature PHB depolymerase of A1 (22) was almost identical to the N-terminal amino acid sequence of mature PHB depolymerase from A. faecalis (A-T-A-G-P-G-A-W-S-S-Q-Q-T-W-A-A-D-S-V-) (19), we assume that the PHB depolymerase gene in A. faecalis used as a probe probably hybridized to a portion of the PHB depolymerase gene in the 10-kbp fragment from A1.

Since the enzyme activity in the purified hydrolase was inhibited by a low concentration of DFP, we suppose that an active serine residue is involved in the catalytic mechanism of this hydrolase. Inspection of the sequence of the structural gene failed to find the presence of the conserved serine-containing pentapeptide, Gly-X-Ser-X-Gly, that plays an essential role in catalysis by lipase, protease, and esterase (1). PHA depolymerases were found to have such a conserved pentapeptide (10). Oligomer hydrolase and PHA depolymerase are similar in catalytic properties as observed for hydrolysis of ester bonds of d-(-)-3-hydroxybutyric acid. It should be noted, however, that Ala-Thr-Ser-Gly (residues 176 to 180) and Ala-Ile-Ser-Thr-Gly (residues 187 to 191) are found in the deduced amino acid sequence (Fig. 5). In the lipase of Bacillus subtilis, the sequence of the conserved pentapeptide was reported to be Ala-His-Ser-Met-Gly (5). Furthermore, the conserved sequence Gly-X-Ser-X-Ala was found in subtilisins from various microorganisms (1). We cannot determine which sequence present in the A1 hydrolase plays a role in the catalytic function of this enzyme. Recently, a new family of lipolytic enzymes was suggested (28). In this group of enzymes including lipase/acyltransferase, lipase, thioesterase/protease, and aryler esterase from various origins, active-site serine is the first in the sequence Gly-Asp-Ser-Leu-Ser (Phe). However, we cannot find such a sequence in the 3HB-oligomer hydrolase gene from A1. Although extracellular 3HB-oligomer hydrolase has a similar catalytic function to PHB depolymerase, these two hydrolases are quite different in molecular weight (oligomer hydrolase, ~70,000; PHB depolymerase, 45,000 to 50,000 [22]) and amino acid sequence including the sequence around the active-site serine.

The physiological role of extracellular 3HB-oligomer hydrolase is not fully clear. This type of enzyme was found only in A. faecalis T1 (23), Pseudomonas sp. strain A1 in this study, and Pseudomonas pickettii K1 (unpublished data), as a member of the extracellular PHB depolymerase system. PHB depolymerases purified from these bacteria show relatively low activity for hydrolysis of the d-(-)-3-hydroxybutyrate dimer compared to their PHB depolymerization activity (22). It is possible that these bacteria secrete 3HB-oligomer hydrolase besides PHB depolymerase to compensate the relatively low hydrolysis activity of the PHB depolymerase for the dimer.

### ACKNOWLEDGMENT

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### REFERENCES


