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Cloning and Analysis of Medium-Chain-Length Poly(3-Hydroxyalkanoate) Depolymerase Gene of *Pseudomonas luteola* M13-4

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Abstract The gene encoding the extracellular medium-chain-length poly(3-hydroxyalkanoate) (MCL-PHA) depolymerase of *Pseudomonas luteola* M13-4, $phaZ_{Plu}$, was cloned and analyzed. It was found to be 849 bp, with a deduced protein of 282 amino acids, and was revealed to have a typical leader peptide at its N terminus. The amino acid sequence of $PhaZ_{Plu}$ revealed relatively low identity (69 to 72%) with those of other *Pseudomonas* MCL-PHA depolymerases. In comparison with the amino acid sequences of all available MCL-PHA depolymerases, the depolymerase was found to consist of three domains in sequential order; signal peptide, an N-terminal substrate binding domain, and a catalytic domain, indicating that $PhaZ_{Plu}$ belongs to the type IV depolymerases family. The enzyme also contained Asn as an oxyanion hole amino acid.

Key words: Molecular analysis, MCL-PHA depolymerase gene, *Pseudomonas luteola* M13-4

Poly(3-hydroxyalkanoates) (PHAs) are synthesized by many bacteria, and accumulate as discrete granules at levels as high as 90% of the cell dry weight. Medium-chain-length PHAs (MCL-PHAs) are composed of C_6 to C_{16} 3-hydroxy alkanoates, and are synthesized from fatty acids or other aliphatic carbon sources, with the composition of the resulting PHA depending on the growth substrates used [2, 5, 14, 17, 18]. MCL-PHAs have a much lower level of crystallinity and are more elastic than short-chain-length PHAs (SCL-PHAs) [4, 19]. These MCL-PHAs potentially have a different range of applications from those of SCL-PHAs.

Extracellular PHA depolymerases are divided into two groups, SCL-PHA depolymerases and MCL-PHA depolymerases, which differ in respect to the substrate specificity for SCL-PHAs or MCL-PHAs. The great majority of PHA-degrading microorganisms are known to produce only one type of PHA depolymerase that acts upon either the SCL-PHAs or MCL-PHAs [6]. In contrast to SCL-PHA degraders and their depolymerases, which have been extensively investigated, microorganisms producing extracellular MCL-PHA depolymerases are known to be relatively uncommon, and only a limited number of reports have demonstrated the biochemical characteristics of MCL-PHA depolymerases [9–13, 25, 26]. Moreover, only three of the nucleotide sequences of MCL-PHA depolymerases have been deposited in the GenBank database to date [9, 25]. Thus, the molecular biological properties of MCL-PHA depolymerases remain to be properly elucidated.

Recently, we reported on the biochemical properties of a novel MCL-PHA depolymerase from the marine isolate *Pseudomonas luteola* M13-4 [22]. The enzyme has the unique property to hydrolyze not only various types of MCL-PHAs but also SCL-PHA consisting of 3-hydroxybutyrate and 3-hydroxyvalerate. Since all MCL-PHA depolymerases analyzed thus far degrade MCL-PHAs but are inactive toward SCL-PHAs, we investigated the mechanism of the broad substrate specificity of the enzyme through the elucidation of its primary structure. In this paper, the cloning and sequence analysis of MCL-PHA depolymerase from the *P. luteola* M13-4, and a comparison of its molecular structure with those of other MCL-PHA depolymerases are described.

Materials and Methods

Organism, Culture Conditions, and Enzyme Purification

P. luteola M13-4 isolated from a seawater sample was grown in a basal salt medium, supplemented with 0.2% poly(3-hydroxyoctanoate) (PHO) as the sole carbon and energy source, as described previously [22]. The culture was incubated at 30°C on a shaking incubator at 180 rpm

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for 3 days. The purification of enzyme was performed as described previously [22].

Amino Acid Sequencing

The purified enzyme was electroblotted directly from an SDS-PAGE gel to a polyvinylidene difluoride membrane (Sequi-Blot PVDF; Bio-Rad, U.S.A.), and the N-terminal amino acid sequence determined at the Korea Basic Science Institute using an Applied Biosystems Procise 491 automatic sequencer (Applied Biosystems Inc., U.S.A.).

Gene Isolation and Sequence Analysis

The genomic DNA of *P. luteola* M13-4 was isolated according to the method of Ausubel *et al.* [1]. On the basis of the sequences of the N-terminus and a tryptic peptide (boxed peptides in Fig. 1), oligonucleotide primer mixtures were designed for amplification experiments. PCR was carried out in 50-μl reaction mixtures, containing 50 ng of total DNA, deoxynucleoside triphosphate (2.5 mM each), *Taq* DNA polymerase buffer (10 mM Tris-HCl, pH 9.0, 40 mM KCl, and 1.5 mM $MgCl_2$), 10 pmol of each primer (Forward, 5' GCN TCN CCN TGN ACN GAR 3'; and Reverse, 5' CTN GTR TTR TAN CCN CC 3'), and 2.5 U of *Taq* DNA polymerase (Promega, U.S.A.). This mixture was heated to 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final elongation step of 8 min at 72°C. The PCR product (469 bp) was cloned into a pGEM T-easy vector, according to manufacturer specifications (Promega, U.S.A.), and both strands sequenced. Using the primers designed on the basis of the sequence of the PCR product (Table 1), the sequences of the unknown regions were determined using DNA walking ACP (Annealing Control Primer)-PCR technology, with a DNA Walking *SpeedUp* kit, according to the manufacturer's manual (Seegene, USA). The full sequence for the PHO depolymerase of *P. luteola* M13-4 was submitted to GenBank, under the accession number AY781279.

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1   GTCAAAACCTTGCTCGAAAATGGCTACGCGGTGATTGCACCTAGCGCACTTCACAGAAGT  60
61  ATGCCAAGCGAGGGGGGGGGGGGGTCATCATCATCCTTAGCCTCACCGCCATCGCTGGC  120
    M  P  S  E  G  G  G  G  V  I  I  L  S  L  T  A  I  A  G
121 ACAGCCCCGGCTCTCGCTGCCTCACCCTGTACCGAGCGCGCAAAATCACTACTGTTGCCA 180
    T  A  P  A  L  A  A  S  P  C  T  E  R  A  K  S  L  L  L  P
181 GGCAAGATCAGCTGCAGCTACAGATCTACCTGGATACCTTCAAGCAGTATCTCCAATCGC 240
    G  K  I  S  C  S  Y  R  S  T  W  I  P  S  S  S  I  S  N  R
241 AAGGTGATCTATCAGTTGCCTTCAGGCACGGCCCCGACCGGCGGCTGGCCGGTAGTGCTG 300
    K  V  I  Y  Q  L  P  S  G  T  A  P  T  G  G  W  P  V  V  L
301 ATTTATCAGGGGTCATTTTTCCCGTTGAACAATTTCACCTACTACACAACAGACCCGTTT 360
    I  Y  Q  G  S  F  F  P  L  N  N  F  T  Y  Y  T  T  D  P  F
361 GGCGGTTACTACGAAGGGCGCGTAGTCAAAACCTTGCTCGAAAATGGCTACGCGGTGATT 420
    G  G  Y  Y  E  G  R  V  V  K  T  L  L  E  N  G  Y  A  V  I
421 GCACCTAGCGCACCAGCAGACCTGTTCTGGCATACCAACCTTCCAGCGCTGGCCAGCAAC 480
    A  P  S  A  P  A  D  L  F  W  H  T  N  L  P  A  L  A  S  N
481 TACGAACTGAGCACCGACTACACCTTTCTGACCAACGTGTTTCAGGCAATCGATGACGGC 540
    Y  E  L  S  T  D  Y  T  F  L  T  N  V  F  Q  A  I  D  D  G
541 GACTTCGGCCCGCTCAACGGCAACCGAAAGTACGCTACCGGCATCTCCAGCGGCGGCTAC 600
    D  F  G  P  L  N  G  N  R  K  Y  A  T  G  I  S  S  G  G  Y
601 AACACCAGCCGGATGGCCGTGTCCTTCCCGGGGGAGTTCAAGGCTCTAGCAGTTCAATCC 660
    N  T  S  R  M  A  V  S  F  P  G  E  F  K  A  L  A  V  Q  S
661 GGGTCTTACGCCACCTGCAGCGGGCCAGTGTGCTCCGTGCCGGATGAACTGCCAGCAGAC 720
    G  S  Y  A  T  C  S  G  P  V  C  S  V  P  D  E  L  P  A  D
721 CACCCGCCAACCTACTTCCTCCATGGCTTTGTTGATCTAACGGTGCCGTGGTGGAGCATG 780
    H  P  P  T  Y  F  L  H  G  F  V  D  L  T  V  P  W  W  S  M
781 GACATGTACTACGACCGACTGCTGTACCAGAACATCCCAACCGGACGCTACACCAAAACC 840
    D  M  Y  Y  D  R  L  L  Y  Q  N  I  P  T  G  R  Y  T  K  T
841 ACAGGGGGCCATGAGTGGTTTCCCGCGTCAGCCGCCAAAGTACTGGCCTGGTTCAACGCA 900
    T  G  G  H  E  W  F  P  A  S  A  A  K  V  L  A  W  F  N  A
901 TACCCTTAGCAGGCCTCAAGCCCGGGCGGCCGCACACACGGCAACCCGGGCCGGCCTGGC 960
    Y  P  *
```

Fig. 1. The nucleotide sequence of the MCL-PHA depolymerase gene from *P. luteola* M13-4 with the deduced amino acid sequence.

The putative ribosome-binding site is in boldface. The signal peptide is marked in black, with the binding positions of the sense or antisense primers used for PCR boxed. The broken line indicates the sequence determined by the Edman degradation method, and the double underlined sequences are the trypsin-derived amino acid sequences, as determined by mass spectrometry. A possible terminator sequence is indicated by the asterisk.

RESULTS

Characterization of the MCL-PHA Depolymerase Gene (*phaZ*$_{Plu}$)

The nucleotide sequence obtained (960 bp) was determined for both strands. An open reading frame of 849 bp was identified, which encoded the MCL-PHA depolymerase structural gene, *phaZ*$_{Plu}$. The assumed coding sequence started with an ATG initiation codon at position 61, which was preceded by a potential ribosome-binding site (5'-AGAAG-3'), and extended to a TAG stop codon at position

Table 1. Oligonucleotides used in the DNA walking ACP-PCR experiments.

Oligonucleotide	Sequence	Annealing temperature
C-PHO-1	5'-GGGTCATTTTTCCCGTTGAA-3'	55 °C
N-PHO-1	5'-CAGAACAGGTCTGCTGGTGC-3'	
C-PHO-2	5'-ATGGCTACGCGGTGATTGCA-3'	59 °C
N-PHO-2	5'-TAACCGCCAAACGGGTCTGT-3'	
C-PHO-3	5'-GCATACCAACCTTCCAGCGC-3'	60 °C
N-PHO-3	5'-ATCAGCACTACCGGCCAGCC-3'	

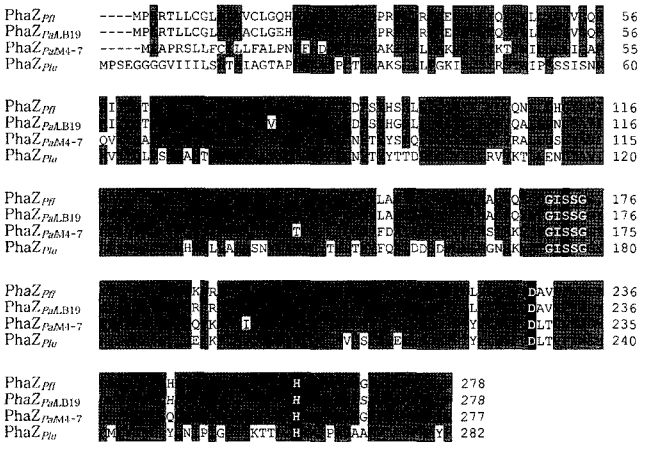

Fig. 2. Amino acid alignment of the MCL-PHA depolymerase of *P. luteola* M13-4, compared with those of other MCL-PHA depolymerases.
Identical amino acids are indicated by shading. The lipase box is indicated in white letters and the catalytic amino acids (Ser, Asp, and His) by the black box with white letters.

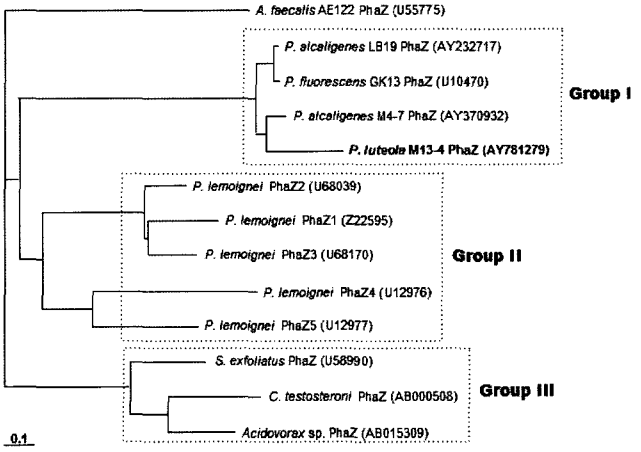

Fig. 3. Comparison of the amino acid sequences of different PHA depolymerases (dendrogram from Kimura distance matrix [15] after multiple alignment of mature proteins).
GenBank accession numbers are shown in parenthesis.

907. Analysis of the codon usage of $phaZ_{Plu}$ revealed an extreme bias for guanine and cytosine in the third codon position (69.6%), with cytosine as the preferred nucleotide (47.4%). The $phaZ_{Plu}$ coded for a protein of 282 amino acids (30,534 Da), and a putative signal peptidase cleavage site predicted between Ala-26 and Ala-27, resulting in a molecular mass of 28,171 Da for the mature protein. The high content of hydrophobic amino acids (43.4%) in the mature protein predicted a strong hydrophobic peptide. Additionally, the N-terminal amino acid sequences of the mature $PhaZ_{Plu}$ contained many hydrophobic amino acids, such as Phe, Val, and Leu, suggesting the substrate-binding domain was located in this region. The sequence contained the complete sequences of the three trypsin-derived peptide fragments of the MCL-PHA depolymerase, with a *p*I of the predicted mature protein of 6.08. The DNA sequence of MCL-PHA depolymerase and the predicted translation product are shown Fig. 1.

Comparison with Other MCL-PHAs Depolymerases

The nucleotide sequence identity of $phaZ_{Plu}$ compared with those of other MLC-PHA depolymerase genes ranged from 70% (with *P. fluorescens* GK13 PHO depolymerase $phaZ_{Pfl}$, and *P. alcaligenes* LB19 MCL-PHA depolymerase $phaZ_{PalLB19}$) to 73% (with MCL-PHA depolymerase of *P. alcaligenes* M4-7 $phaZ_{PalM4\text{-}7}$). The identity of the $PhaZ_{Plu}$ amino acid sequence compared with other MCL-PHA depolymerases varied between 69 ($PhaZ_{Pfl}$ and $PhaZ_{PalLB19}$) and 72% ($PhaZ_{PalM4\text{-}7}$) (Fig. 2). A comparison of the various PHA depolymerases, in terms of the DNA-deduced mature protein sequences, is shown in Fig. 3. The clustering obtained evidenced that the MCL-PHA depolymerase group was unrelated to SCL-PHA depolymerases, and the MCL-PHA depolymerase of *P. luteola* M13-4 was related to other MCL-PHA depolymerases.

Validation of the Primary Structure

Inspection of the primary sequence of $PhaZ_{Plu}$ revealed the presence of three domains (a signal peptide, a substrate binding domain, and a catalytic domain), as found with all other MCL-PHA depolymerases. The catalytic domain contained an identical lipase box pentapeptide sequence, G-X-S-X-G, which is characteristic of serine hydrolases, such as lipases, esterases, and all PHA depolymerases. $PhaZ_{Pfl}$ had a His as the oxyanion hole, but the oxyanion packets of $PhaZ_{Plu}$ replaced the His with an Asn (Table 2).

Table 2. Alignment of the catalytically active amino acids of MCL-PHA depolymerases.

Peptide	Pos. Oxyanion hole	Pos. Ser	Pos. Asp	Pos. His	Reference
$PhaZ_{Pfl}$	111 QNLLD**H**GYAVIA	172 LNAQRQYATGI**S**SGGYNT	228 FLHGFV**D**AVV	260 LGG**H**EWFAAS	[26]
$PhaZ_{PalLB19}$	111 QALLD**N**GYAVIA	172 LNAQRQYATGI**S**SGGYNT	228 FLHGFV**D**AVV	260 LGG**H**EWFAAS	[10]
$PhaZ_{PalM4\text{-}7}$	110 RALLD**S**GYAVIA	171 LNSQRKYATGI**S**SGGYNT	227 FLHGFV**D**LTV	259 LGG**H**EWFAAS	[9]
$PhaZ_{Plu}$	115 KTLLE**N**GYAVIA	176 LNGNRKYATGI**S**SGGYNT	232 FLHGFV**D**LTV	264 TGG**H**EWFPAS	This work

An alignment of the regions around the catalytic triad amino acids Ser, Asp, and His and around the putative oxyanion hole (indicated by boldfaced letters) is shown. The numbering refers to the DNA-deduced premature polypeptides.

DISCUSSION

The gene for the MCL-PHA depolymerase of *P. luteola* M13-4, $phaZ_{Plu}$, was sequenced with the deduced amino acid sequence. The $phaZ_{Plu}$ codes a 282 amino acid preprotein, which through usual processing produced a mature protein containing 256 amino acids (Figs. 1 and 2). The enzyme had only one N-terminal processing site (Ala-26 and Ala-27) and was different from that of the PHO depolymerase of *P. fluorescence* GK13 [25, 26] and the MCL-PHA depolymerase of *P. alcaligenes* LB19 [9, 10], which exhibit two N-terminals.

The amino acid sequence similarity among $PhaZ_{Pfl}$, $PhaZ_{Pal/LB19}$, and $PhaZ_{Pal/M4\text{-}7}$ ranged from 87 to 97%, suggesting the possibility of horizontal transfer of the MCL-PHA depolymerase gene in the *Pseudomonas* strains [9]. The amino acid sequence of $PhaZ_{Plu}$ revealed 69 to 72% identity with those of other *Pseudomonas* MCL-PHA depolymerases (Fig. 2). From these results, it was suggested that the $phaZ_{Plu}$ might evolve separately from other *Pseudomonas* MCL-PHA depolymerase genes. A comparison of the different PHA depolymerases, in terms of their mature protein sequences, is shown in the dendrogram of Fig. 3. The clustering gave evidence of *Alcaligenes faecalis* AE122 PhaZ being unrelated to other PhaZs. The other PhaZs can be placed into three main groups; (i) MCL-PHA depolymerases, (ii) SCL-PHA depolymerases of *P. lemoignei*, and (iii) other SCL-PHA depolymerases. The PhaZ of *P. luteola* M13-4 is related to those of typical MCL-PHA depolymerases (group I). Comparison of the primary amino acid sequences of all available MCL-PHA depolymerases revealed that depolymerases generally consist of three domains, in the sequential order of a signal peptide, an N-terminal substrate binding domain, and a catalytic domain. The catalytic domain contains a lipase box, G-X_1-S-X_2-G. Typical lipases have mostly occupied X_1, with His or Tyr, but variable occupation at X_2. However, in all the extracellular PHA depolymerases analyzed, including $PhaZ_{Plu}$, the X_1 is a Leu or Ile, and the X_2 an Ala or Ser. The Ser-176 residue of the lipase box has been shown to be essential for activity in all the PHA depolymerases analyzed so far [27, 29]. Asp-232 and His-264, together with Ser-176, are known to form the catalytic triad, including an oxyanion hole in the active enzyme [24, 25]. From these results, the $PhaZ_{Plu}$ can be classified as a type IV depolymerase, according to the conclusion offered by Kingbeil *et al.* [16]. The sequential order of the catalytic amino acids of all type IV depolymerases is the oxyanion hole, and a lipase box Ser, Asp, and His, which share relatively high amino acid homologies to each other, with the exception of the oxyanion hole. The oxyanion hole amino acid, which is found in extracellular PHA depolymerases, is known to participate in the transient state stabilization of the hydrolysis reaction by allowing the formation of a hydrogen bond to the negatively charged oxygen atom of the active site Ser [6]. The oxyanion hole amino acid of all reported PHB depolymerases [3, 7, 8, 16, 19, 24, 28, 30], and that of the PHO depolymerase of *P. fluorescence* GK13, is a His [25], but that of the MCL-PHA depolymerase of *P. luteola* M13-4 and *P. alcaligenes* LB19 is replaced by an Asn (Table 2). The Nσ2 of Asn has been described as being in a good location for hydrogen bond donation to the oxyanion [23].

The MCL-PHA depolymerase of *P. luteola* M13-4 exhibits significant substrate specificity toward SCL-PHA copolyesters consisting of 3-hydroxybutyrate and 3-hydroxyvalerate in addition to MCL-PHAs [22]. In contrast, other MCL-PHA depolymerases are specific for MCL-PHAs and do not hydrolyze SCL-PHAs. Therefore, the apparent differences in the substrate specificity of these enzymes can be explained by the differences in the amino acid sequence of the mature proteins influencing the three-dimensional structure. Further experiments including *in vitro* evolution of $PhaZ_{Plu}$ and characterization of resulting mutants will be necessary to determine the effect of amino acid substitutions on the substrate specificity of the enzyme.

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