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Molecular Characterization of the Poly(3-hydroxybutyrate) Depolymerase Gene from *Penicillium funiculosum*

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Summary: A cDNA encoding *Penicillium funiculosum* P(3HB) depolymerase ($PhaZ_{Pfu}$) was cloned from a cDNA library. This cDNA contained a 1,020-bp open reading frame (ORF) that encoded 339 amino acids. Edman degradation of $PhaZ_{Pfu}$ indicated that 20 amino acids from the N terminus function as a signal peptide. Homology analysis revealed that $PhaZ_{Pfu}$ lacks linker and substrate-binding domains, both of which are observed in bacterial P(3HB) depolymerases. This may account for a weak binding affinity of $PhaZ_{Pfu}$ to the P(3HB) surface.

Keywords: degradation; enzymes; P(3HB) depolymerase; Penicillium funiculosum; $PhaZ_{Pfu}$; substrate-binding domain

Introduction

Poly(3-hydroxybutyrate) (P(3HB)) is accumulated in bacterial cells as a carbon storage materials, and it is one of the most promising candidates for developing biodegradable thermoplastics. A wide variety of P(3HB)-degrading microorganisms belonging to domains Prokaryote and Eukaryote have been isolated from various environments.[1,2,4,6–8] Sang et al. proposed that fungi comprise the predominant group of P(3HB) degraders in the soil environment due to their faster colony growth rate than that of bacteria.[4] However, to date, only a few studies have been reported on fungal degraders and their P(3HB) depolymerases as compared to those on bacteria. In our previous study, we focused on the P(3HB)-degrading fungus *Penicillium funiculosum* IFO 6354; we purified its P(3HB) depolymerase ($PhaZ_{Pfu}$) and characterized the biochemical properties of this enzyme.[1] $PhaZ_{Pfu}$ possesses several unique properties distinct from those observed in bacterial P(3HB) depolymerases. $PhaZ_{Pfu}$ showed a low binding affinity to solid substrates, whereas bacterial enzymes specifically adsorbed onto the solid polyester surface due to the presence of a substrate-binding domain (SBD). Further, the X-ray single-crystal structure analysis revealed that $PhaZ_{Pfu}$ is composed of a single domain.[2] In order to gain a better understanding of the relationship between the structure and function of the enzyme, we cloned and sequenced the gene encoding $PhaZ_{Pfu}$ ($phaZ_{Pfu}$).

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Materials and Methods

Nucleotide Manipulation

Total RNA and genomic DNA were prepared from the fungal mycelia by the phenol-chloroform method. The nucleotide sequence was determined by the dideoxy chain-termination method using a DSQ

1000 sequencer (Shimadzu co., Kyoto). Database searches were performed using the BLAST program on the NCBI website. The DNA and amino acid sequences were aligned using the ClustalW program on the DDBJ website.

Cloning of the Genomic DNA Region Encoding $phaZ_{Pfu}$

Based on the N-terminal amino acid sequence (TALPAFNVNPNSVSVSGLSS GGYMAAQLGV) determined by Edman degradation, we designed primer 1 (5′-AT GGACC(A/G/C/T)GC(A/G/C/T)TT(T/C) AA(T/C)GT(A/G/C/T)AA(T/C)CC(A/G/ C/T)AA(T/C)-3′) and primer 2 (5′-GG(A/ G/C/T) GG(A/G/C/T)TA(T/C)ATGGC (A/G/C/T) CA(G/A)AAGCTTGG-3′). A part of the DNA fragment encoding the N-terminal region of $PhaZ_{Pfu}$ was obtained by PCR amplification.

Cloning of cDNA Encoding $phaZ_{Pfu}$

Based on the DNA sequence encoding the N-terminal amino acid sequence of $PhaZ_{Pfu}$ that was determined by sequencing the gDNA fragment, we designed primer PF-N2 (5′-CTGATCTAGAGGTACCG GATCCACGGC CCTACCTGCCTTCA ATGT-3′). Next, a part of cDNA was cloned using a 3′-Full RACE Core Set (TaKaRa BIO INC, Kyoto, Japan) with PF-N2 as the primer and total RNA of *P. funiculosum* as the template, according to the manufacturer's instructions. After the resultant fragment was labeled by the DIG-HIGH prime kit (Roche Diagnostics K.K., Tokyo, Japan), it was used as a probe for screening the positive clones obtained from the *P. funiculosum* cDNA library. Positive clones were isolated from the cDNA library by plaque hybridization with the probe. According to the manufacturer's instructions, one of the positive clones (pTriplExPFDP) was subcloned into the plasmid pTriplExPFDP by the *in vivo* excision method. Next, the insert of pTriplExPFDP was amplified by PCR. The PCR mixture contained a thermopol buffer, deoxynucleoside triphosphate, 20 pmol of each primer, 5′ pTriplEx sequencing primer (5′-CTCCGAGATCTGGACGAGC-3′) and 3′ pTriplEx sequencing primer (5′-TAATACGACTCACTATAGGG-3′), 10 ng of pTrriplExPFDP as the template, and 1 U of Deep Vent DNA polymerase (New England BioLabs. Inc., Beverly, MA, USA). A DNA thermal cycler (MJ Japan, Tokyo, Japan) was used for amplification of the gene under the following conditions: denaturation at 95 °C for 20 s, 25 cycles of denaturation at 94 °C for 5 s, and annealing and extension at 68 °C for 5 min. By using T4 ligase, the amplified DNA was ligated to pUC18 that was digested by the restriction endonuclease *Sma*I, thereby yielding the recombinant plasmid pUCPFDP.

The nucleotide sequence of $phaZ_{Pfu}$ was deposited in the DDBJ nucleotide sequence data base under accession number AB281621.

Results and Discussion

Cloning and Northern Analysis of the cDNA of $phaZ_{Pfu}$

A 72-bp DNA fragment encoding the N-terminal region of $PhaZ_{Pfu}$ was obtained by PCR using a set of degenerate primers that were designed based on the N-terminal amino acid sequence determined by Edman degradation. Southern blotting analysis with the DIG-labeled PCR product for the genomic DNA fragment of *P. funiculosum* digested with various restriction enzymes revealed that a 3.5k-bp *Eco*RI fragment contained $phaZ_{Pfu}$. A cDNA encoding the enzyme was obtained by the 3′ RACE method using a primer that was designed based on the determined N-terminal amino acid sequence and the total RNA of *P. funiculosum* was used as the template.

P. funiculosum produced a large amount of $PhaZ_{Pfu}$ when it was cultivated on a medium containing P(3HB) as the sole carbon source. On the other hand, the production of $PhaZ_{Pfu}$ was not observed in cultures grown on a carbon-containing media that were supplemented with sugars or organic acids such as succinate. Here, we

examined whether the regulation of the gene expression occurs on transcription by northern hybridization. A 1.2-kbp RNA isolated from the P(3HB)-grown cells hybridized to the probe designed based on the *phaZ*$_{Pfu}$ cDNA. This sizes of the RNA and cDNA were similar. When considered together, these findings suggest

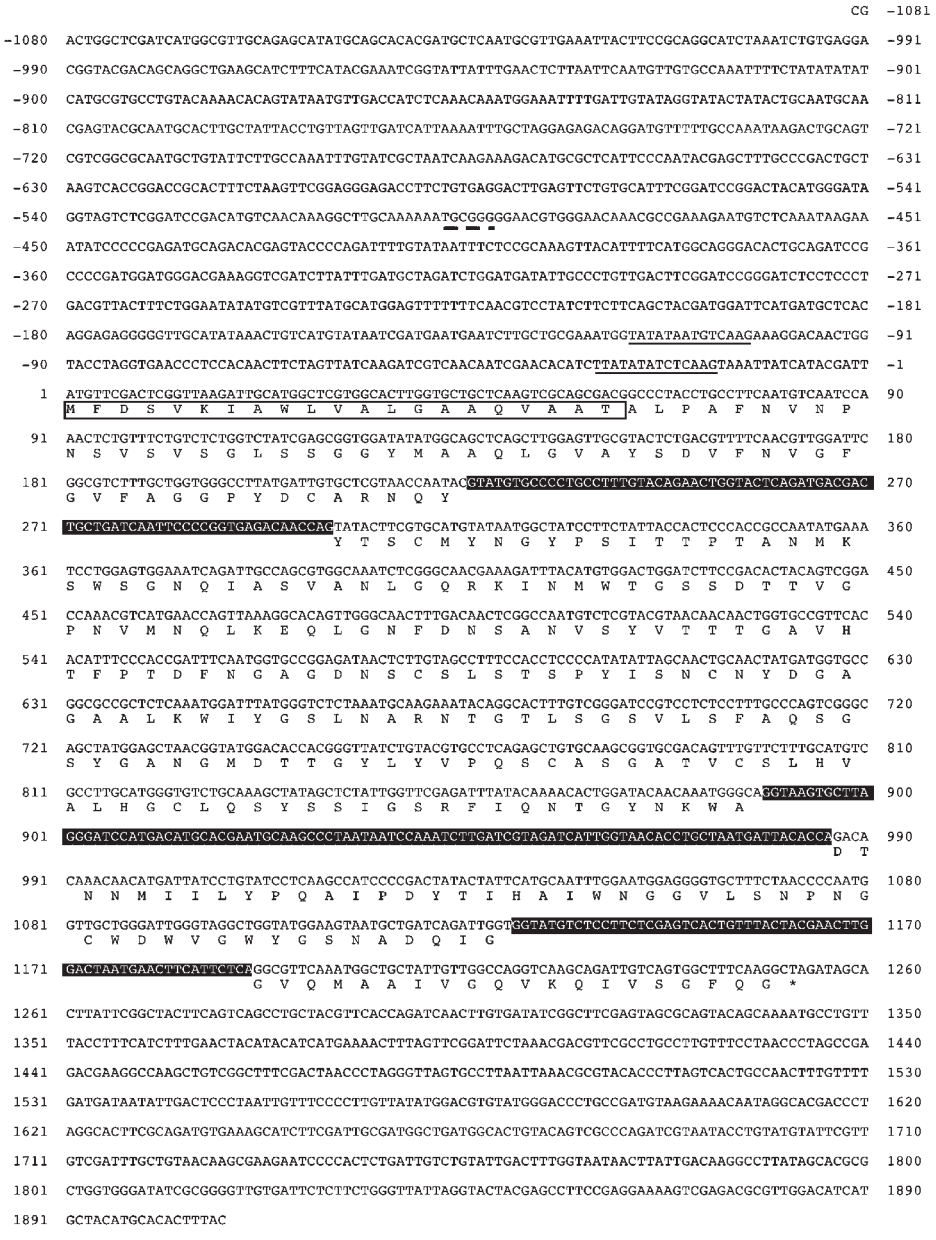

Figure 1.

Genomic DNA sequence of *phaZ*$_{Pfu}$ and the upstream and downstream regions. The broken line indicates a possible CreA-binding site. Solid lines indicate a possible TATA box. Thick black solid lines indicate introns. The boxed 20 amino acid residues indicate a possible signal peptide. The deduced amino acid sequence is shown below the DNA sequence.

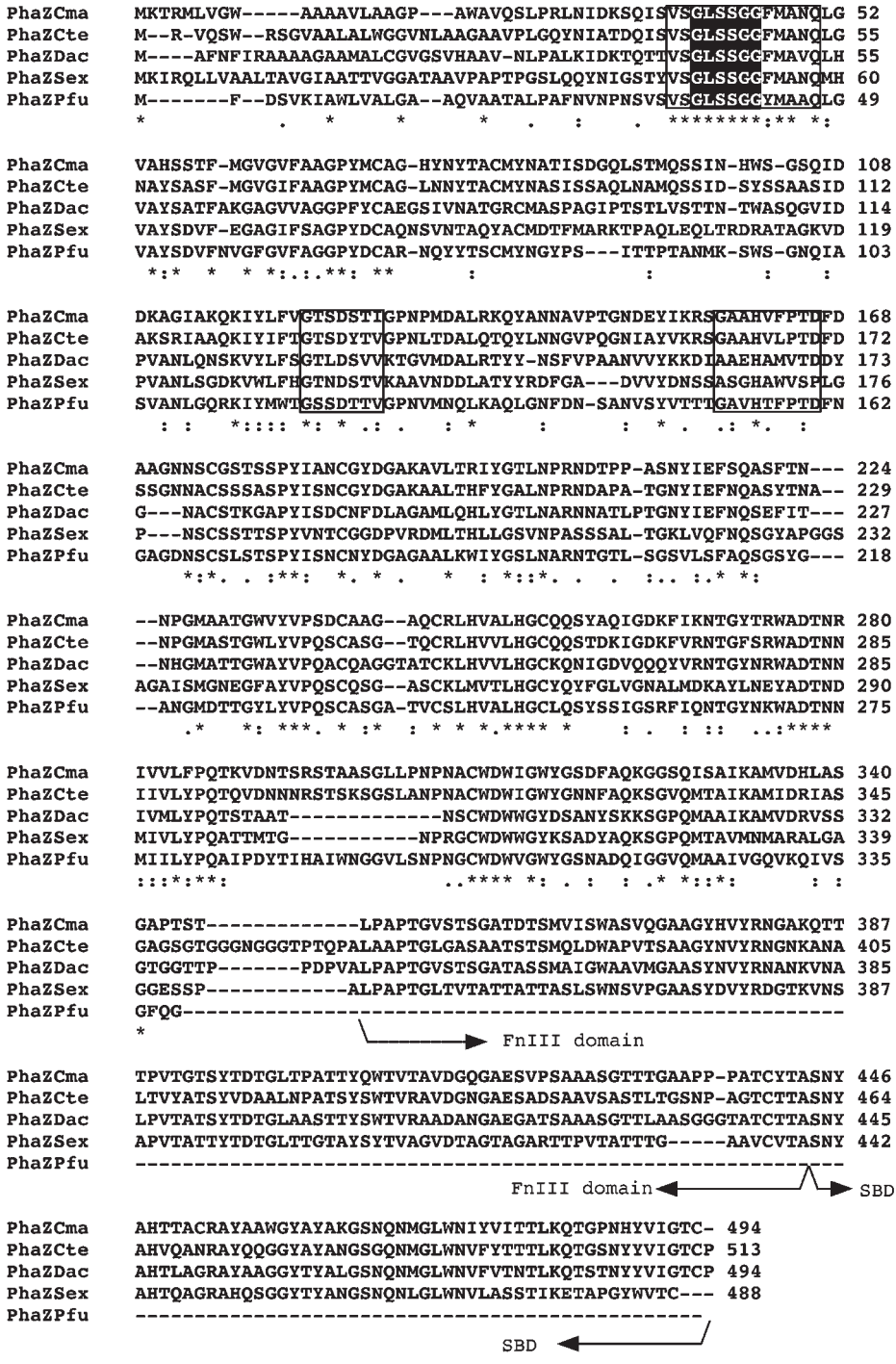

Figure 2.
Alignment of type II P(3HB) depolymerases from various microorganisms. The boxed regions indicate consensus sequences that form a catalytic triad (Ser-Asp-His).

that the degradation products from P(3HB) are involved in PhaZ$_{Pfu}$ production at the transcriptional level.

Nucleotide Sequence and Deduced Amino Acid Sequences

The genomic DNA sequence of *phaZ$_{Pfu}$* and the deduced amino acid sequence of the gene product are shown in Figure 1. The cDNA contains an open reading frame (ORF) of 1,020 bp beginning from the start codon. This ORF encodes 339 amino acids. Three introns interrupt the coding sequence in the cDNA sequence. The G+C content of the ORF was 48.5% and that at the third position of codons was 47.9%. The amino acid residues from 21 to 50 of the deduced gene product were identical to the N-terminal amino acid sequence determined for the mature P(3HB) depolymerase secreted by *P. funiculosum*. This suggests that the 20-amino acid polypeptide at the N terminus of the deduced protein encoded in the cDNA is a signal peptide. Based on the amino acid sequence, the molecular mass of mature protein was deduced to be 33,524 Da; this was in good agreement with the value estimated by SDS-polyacrylamide gel electrophoresis (33kDa). Upstream of the cDNA, 2 possible TATA boxes existed. A homologous region of CreA-binding site[3] was detected 507-502bp upstream of the translation start codon, suggesting that the gene is controlled by catabolite repression. This supports the result of northern hybridization.

Mature PhaZ$_{Pfu}$ comprised a polypeptide of 319 amino acids containing conserved amino acid residues of a catalytic triad (Ser-39, Asp-121, and His-155) and an oxyanion hole (His-248), which functions as an active center in many known serine hydrolases, and a lipase box (Gly-Leu-Ser39-Ser-Gly), which functions as an active center (Ser-39) in the vicinity of N terminus. Mature PhaZ$_{Pfu}$ exhibited significant homologies to the catalytic domain (CD)s of TypeII P(3HB) depolymerases, and the identity levels were as follows: *Caldimonas manganoxidans* P(3HB) depolymerase PhaZ$_{Cma}$, 49%;[5], *Comamonas* sp. P(3HB) depolymerase PhaZ$_{Csp}$, 48%;[6]*Comamonas testosteroni* P(3HB) depolymerase PhaZ$_{Cte}$, 48%;[9]*Delfitia acidovorans* P(3HB) depolymerase PhaZ$_{Dac}$, 39%;[7] and *Streptomyces exfoliatus* P(3HB) depolymerase PhaZ$_{Sex}$, 37%.[8]

On the other hand, PhaZ$_{Pfu}$ lacked linker domain (LD), namely, a fibronectin type III-like domain as well as a substrate-binding domain (SBD) that are observed in bacterial P(3HB) depolymerases (Figure 2). This single domain structure of PhaZ$_{Pfu}$ might be responsible for the considerably lower binding affinity to the P(3HB) surface than those of bacterial enzymes.[1]

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