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Characterization of a thermolabile poly(3-hydroxybutyrate) depolymerase from the marine bacterium *Shewanella* sp. JKCM-AJ-6,1α

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ABSTRACT

A DNA fragment carrying the gene encoding poly(3-hydroxybutyrate) [P(3HB)] depolymerase was cloned from the genomic DNA of the marine bacterium *Shewanella* sp. strain JKCM-AJ-6,1α (wild-type strain) from seawater. The gene was 2049 bp and encoded 683-amino acids protein with a molecular mass of 70,382 Da. A sequence homology revealed that the deduced protein contains a signal peptide, catalytic domain (CD), fibronectin type III linker domain (LD), and two substrate-binding domains (SBDs). Meanwhile, a P(3HB) depolymerase purified from wild-type strain (wPhaZ$_{She}$) had a smaller molecular mass (47 kDa) because it lacked the SBDs, compared with calculated one based on the sequence. wPhaZ$_{She}$ was unstable above 15 °C and had relatively high enzymatic activity in the presence of 0.5 M NaCl, demonstrating that the enzyme is thermolabile and halotolerant. In addition, there was no decrease in the activity by self-inhibition. In contrast, a recombinant form of the enzyme (rbPhaZ$_{She}$) produced in *Escherichia coli* BL21 had a stronger binding affinity for P(3HB) and exhibited self-inhibition. These results demonstrate that a truncated form P(3HB) depolymerase lacking SBDs (wPhaZ$_{She}$) has an advantage for P(3HB) degradation in marine environments with relatively high NaCl concentrations owing to a less self-inhibitory effect.

1. Introduction

General-purpose plastic products are dumped into the ocean at an alarming rate of 6.5 million tons each year [1]. Plastic waste is extremely stable and does not degrade in marine environments, causing damage to marine ecosystems [2–4]. For example, discarded fishing nets and lines—referred to as ghost nets—can entangle and kill marine organisms, including fish and other commercial species, through choking and by reducing feeding efficiency [5,6], resulting in economic losses for the fishing industry. In addition, plastic debris or bags were ingested by marine organisms such as turtles [7,8] or seabirds [9,10], which weakens and may kill these organisms. Rubbish can also cause serious damage to boats when it becomes entangled in propellers or blocks water intake valves [11]; in Asia-Pacific rim, it is estimated that such damage to marine industries costs over 1 billion USD per year [12–14]. Biodegradable plastics have attracted considerable attention as a potential solution to these problems.

Aliphatic polyesters are among the most promising biodegradable material. In particular, poly[(R)-3-hydroxybutyrate] [P(3HB)], which is synthesized by some bacteria [15,16], has properties that are similar to those of conventional plastics [15] but has excellent biodegradability. Moreover, P(3HB) and its copolymers are thermoplastics that are available in the form of fibers [17,18] and can therefore be used to make fishing nets and lines. To date, several P(3HB)-producing bacteria including *Vibrio*, *Colwellia*, *Moritella*, and *Shewanella* spp. have been isolated from marine environments [19–22], suggesting that P(3HB) originates from this milieu. P(3HB) and its copolymers degrade well in seawater [23,24], as compared to other biodegradable polymers such as poly(ethylene succinate) (PESu), poly(butylene succinate) (PBSu), and poly(butylene adipate) [25]. Therefore, P(3HB) has potential applications in a marine environment.

Various P(3HB)-degrading microorganisms have been isolated from natural sources [26–32], and the extracellular P(3HB) depolymerases they produce have been characterized [30,32–39]. P(3HB) depolymerases share the following biochemical and structural properties: (1) molecular mass of approximately 40–60 kDa;

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Abbreviations

a.a.	amino acid
AP	alkaline phosphatase
b.p.	base pair
CD	catalytic domain
LD	linker domain
DIG	digoxigenin
SBD	substrate-binding domain
DFP	diisopropyl fluorophosphates
DAN	diazo acetyl-DL-norleucine methyl ester
PMSF	phenylmethylsulfonyl fluoride
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
SDS	sodium dodecyl sulfate
GDNA	genomic DNA
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
E. coli	*Escherichia coli*
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
P(3HB)	poly(3-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-18mol%3-hydroxyvalerate)
PBSu	poly(butylene succinate)
PCL	poly(ε-caprolactone)
PESu	poly(ethylene succinate)
PLA	poly(lactic acid)
PMSF	phenylmethylsulfonyl fluoride
SSC	saline sodium citrate.

(2) a catalytic domain (CD), linker domain (LD), and substrate-binding domain (SBD), (3) an isoelectric point in the range of 7.5–9.8; (4) no binding to anion exchange carriers in a neutral buffer solution, but strong binding to hydrophobic carriers; (5) stability over a wide range of pH values, temperatures, and ionic strengths; and (6) inactivation by typical serine hydrolase inhibitors such as diisopropyl fluorophosphate (DFP) or phenylmethylsulfonyl fluoride (PMSF) [40,41].

Marine enzymes differ from other enzymes in their biochemical properties, exhibiting high salt tolerance, hyperthermostability, barophilicity, or cold adaptivity [42]. Many marine enzymes including proteases, carbohydrases, and peroxidases have been widely studied, but there have been few studies on P(3HB) depolymerases from marine environments [43–47]. Marine P(3HB) depolymerases have unique properties such as two SBDs and a higher molecular mass (>60 kDa). Little is known about how marine P(3HB) depolymerases degrade P(3HB) in the ocean.

We isolated the P(3HB)-degrading marine bacterium *Shewanella* sp. strain JKCM-AJ-6,1α from coastal seawater around the Yaizu port in Suruga Bay, Japan [48]. This bacterium forms a large clear zone at low temperatures and can grow in NaCl concentrations ranging from 0 to 0.8 M. It also secretes a large amount of P(3HB) depolymerase ($PhaZ_{She}$) into the growth medium in the presence of P(3HB) or (R)-3-hydroxybutyric acid. To clarify the degradation mechanism of P(3HB) in the marine environment, we cloned and sequenced the $phaZ_{She}$ gene. We also investigated the relationship between $PhaZ_{She}$ structure and function using wild-type and recombinant forms of the enzymes.

2. Materials and methods

2.1. Chemicals

P(3HB) was a gift from Mitsubishi Gas Chem. Co. (Tokyo, Japan). Poly(3-hydroxybutyrate-*co*-18mol%3-hydroxyvalerate) [P(3HB-*co*-3HV)] was provided by Akzo Nobel (formerly ICI; Tokyo, Japan). Poly(lactic acid) (PLA) and poly(ε-caprolactone) (PCL) were gifts from Shimadzu Co. (Kyoto, Japan) and Daicel Chemical Industries Co. (Osaka, Japan), respectively. PESu was a gift from Nippon Shokubai Co. (Tokyo, Japan). PBSu was a gift from Showa High Polymer (Tokyo, Japan). Poly(60mol%butylene adipate-*co*-40 mol% butylene terephthalate) was a gift from BASF AG (Ludwigshafen, Germany). These polymers were purified by dissolving in methylene chloride or chloroform and reprecipitating with methanol. The films were prepared by the solvent-cast technique from chloroform solutions of polyesters, using Petri dishes as casting surfaces. To prepare melt-crystallized film, solvent-casting films were inserted between two Teflon sheets and were compression-molded on a Toyoseki Mini Test Press by heating at 200 °C for 1 min. After melting, the film was maintained at 85 °C and isothermally crystallized for 3 days.

2.2. Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cells were grown in Luria-Bertani (LB) medium [pH 7.0; components (g L^{-1}): tryptone peptone, 10; yeast extract, 5; NaCl, 5] supplemented with ampicillin (50 μg/mL) at 37 °C. *E. coli* DH5α and BL21 (DE3) were used as host strains for vector transformation and protein expression, respectively. Strain JKCM-AJ-6,1α was cultured at 30 °C in LB medium. Mineral medium [pH 7.0; components (g L^{-1}): KH_2PO_4, 4.6; $NaHPO_4{\cdot}12H_2O$, 11.6; $MgSO_4{\cdot}7H_2O$, 0.5; NH_4Cl, 1.0; $FeCl_3{\cdot}6H_2O$, 0.1; yeast extract, 0.5] and M9 medium [pH 7.0; components (g L^{-1}): KH_2PO_4, 3.0; $NaHPO_4{\cdot}12H_2O$, 6.0; NH_4Cl, 1.0; 1 mM $MgSO_4$; 10 mg Vitamin B1; 0.1 mM $CaCl_2$] were used for the production of enzymes. In the case of solid media, 1.5% agar was added to the medium.

2.3. Cloning and sequencing

Genomic (g) DNA was isolated from strain JKCM-AJ-6,1α by a standard procedure [49] and digested with *Eco*RI, *Bam*HI, *Hind*III, and *Pst*I. The fragment containing $phaZ_{She}$ was identified by Southern blotting. After hybridization with a digoxigenin (DIG)-labeled 412-mer nucleotide encoding part of JKCM-AJ-6,1α P(3HB) depolymerase, the fragment was amplified by PCR with primers she03F (5′-GTNCCNGAYGCNATGAAYAA-3′) and she08R (5′-GCRTTYTGYTGRTTRTARCA-3′), which were designed based on the P(3HB) depolymerases (accession nos. ABI41661, ABI40356, EGM70854, and EGM76427) of strain a closely related species. The blots were prehybridized for 1 h, then hybridized for 16 h at 37 °C and washed twice at 25 °C for 15 min in 2 × saline sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS), then twice at 65 °C for 15 min in 0.1 × SSC and 0.1% SDS. The reaction was visualized with anti-DIG-alkaline phosphatase (AP) conjugate (Enzo Life Sciences, Farmingdale, NY, USA). AP activity was detected with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich, St. Louis, MO, USA). Target gDNA fragments identified by Southern blotting were ligated into the pUC18 vector digested with *Eco*RI, which was then transformed into *E. coli* DH5α. Strains containing the plasmid with the gene of interest were identified by the formation of a clear zone on M9 agar plates containing 0.2% P(3HB),