Molecular weight-dependent degradation of D-lactate-containing polyesters by polyhydroxyalkanoate depolymerases from *Variovorax* sp. C34 and *Alcaligenes faecalis* T1

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Abstract

Polyhydroxyalkanoate depolymerase derived from *Variovorax* sp. C34 ($PhaZ_{Vs}$) was identified as the first enzyme that is capable of degrading isotactic P[67 mol% (*R*)-lactate(LA)-*co*-(*R*)-3-hydroxybutyrate(3HB)] [P(D-LA-*co*-D-3HB)]. This study aimed at analyzing the monomer sequence specificity of $PhaZ_{Vs}$ for hydrolyzing P(LA-*co*-3HB) in comparison with a P(3HB) depolymerase from *Alcaligenes faecalis* T1 ($PhaZ_{Af}$) that did not degrade the same copolymer. Degradation of P(LA-*co*-3HB) by action of $PhaZ_{Vs}$ generated dimers, 3HB-3HB, 3HB-LA, LA-3HB and LA-LA, and the monomers, suggesting that $PhaZ_{Vs}$ cleaved the linkages between LA and 3HB units and between LA units. To provide a direct evidence for the hydrolysis of these sequences, the synthetic methyl trimers, 3HB-3HB-3HB, LA-LA-3HB, LA-3HB-LA and 3HB-LA-LA, were treated with the PhaZs. Unexpectedly, not only $PhaZ_{Vs}$ but also $PhaZ_{Af}$ hydrolyzed all of these substrates, namely $PhaZ_{Af}$ also cleaved LA-LA linkage. Considering the fact that both PhaZs did not degrade PDLA homopolymer, the cleavage capability of LA-LA linkage by PhaZs was supposed to depend on the length of the LA-clustering sequence in the polymer chain. To test this hypothesis, PDLA oligomers (6 to 40 mer) were subjected to the degradation assay by PhaZs, and the result revealed that there was an inverse relationship between molecular weight of the substrates and their hydrolysis efficiency. Moreover, $PhaZ_{Vs}$ exhibited the degrading activity toward significantly longer PDLA oligomers compared to $PhaZ_{Af}$. Therefore, the cleaving capability of PhaZs used here toward the D-LA-based polymers containing the LA-clustering sequence was strongly associated with the substrate length, rather than the monomer sequence specificity of the enzyme.

Keywords: biobased polyester, polylactic acid, biodegradation, biodegradable material, size-dependent hydrolysis, monomer sequence specificity

Introduction

Biodegradable aliphatic polyesters, such as poly(lactic acid) (PLA) and poly(3-hydroxybutyrate) [P(3HB)], are well-studied polymer materials that could contribute to reduce the pollution from non-degradable plastics (Jendrossek and Handrick 2002; Tokiwa and Calabia 2006). During the biodegradation process, these polymers are hydrolyzed into water-soluble monomers and/or oligomers by their respective degrading enzymes, which are secreted by a variety of microorganisms in the environment. Although PLA and P(3HB) are similar to each other in the monomeric structure, they are known to be degraded by different classes of enzymes. For example, P[(*S*)-lactic acid] (PLLA) is degraded by certain proteases, lipases and cutinase-like protein (Shinozaki et al. 2013a), whereas poly[(*R*)-3-hydroxybutyrate] [P(D-3HB)] is degraded by P(3HB) depolymerases (Jendrossek et al. 1996; Tokiwa and Calabia 2006; Madhavan Nampoothiri et al. 2010; Shinozaki et al. 2013b). In contrast to the numerous studies on the degradation of these polymers, little is known regarding the degradation

of P[(*R*)-lactic acid] (PDLA). It has been proposed that PDLA was degraded by a cutinase like enzyme (Kawai et al. 2011).

In our previous studies, we developed engineered microbial systems for the production of a new type of lactate (LA)-based copolymer P[(*R*)-LA-*co*-(*R*)-3HB] (Taguchi et al. 2008; Matsumoto and Taguchi 2010; Shozui et al. 2011; Matsumoto and Taguchi 2013; Nduko et al. 2013a). Considering the substrate specificity of polyester-degrading enzymes, the degradation of the copolymer, a hybrid of PDLA and P(3HB), was an interesting research target. We previously performed the screening for isolating the LA-enriched P(LA-*co*-3HB)-degrading strains in the soil, and found a Gram-negative bacterium *Variovorax* sp. C34 as a degrader (AHU Culture Collection accession No. AHU1997) (Sun et al. 2014). A depolymerase excreted by the bacterium effectively degraded P(67 mol% LA-*co*-3HB), whereas the well-characterized P(3HB) depolymerase from *Alcaligenes faecalis* T1 (accession No.P12625.1) ($PhaZ_{Af}$) did not degrade this copolymer. This difference stimulated our interest in the mechanism underlying the enzymatic degradation of P(LA-*co*-3HB) by the isolated depolymerase.

Therefore, this study aimed at analyzing the mechanism for the different capacity of the depolymerases from *Variovorax* sp. C34 and *A. faecalis* T1 for degrading

P(LA-*co*-3HB). Because P(3HB) depolymerases hydrolyze the ester bond between 3HB units, a key question was the capability of the depolymerases to cleave the linkages between 3HB and LA units and/or between LA units. To answer this question, the substrate specificities of the depolymerases were evaluated using both polymeric and oligomeric substrates, and the molecular species of degradation products were determined to locate the cleavage sites. As a result, P(3HB) depolymerases were, for the first time, found to cleave the LA-containing sequences including LA-LA. In addition, the capability of cleaving the LA-LA linkage was strongly associated with the length of the substrates.

Materials and methods

Cloning and expression of the P(LA-*co*-3HB) depolymerase gene

The extracellular P(LA-*co*-3HB) depolymerase from *Variovorax* sp. C34 (accession No. AHU1997) was purified as previously described (Sun et al. 2014). The N-terminal amino acid sequence of this depolymerase was determined by Edman degradation using a Procise 491cLC system (Applied Biosystems) in the Instrumental Analysis Division of the Equipment Management Center at Hokkaido University.

Based on the determined N-terminal sequence of the P(LA-*co*-3HB) depolymerase from *Variovorax* sp. C34, homologous hypothetical P(3HB) depolymerases from *V. paradoxus* EPS (accession No. CP002417.1) and *V. paradoxus* S110 (accession No. CP001635.1) were selected from the GenBank database. A pair of primers (1f: 5′-CACGGCCGATTCGTCAG-3′, 6r: 5′-CCGGGTCTCAAGCATCG-3′) was designed based on the conserved sequences in the 5' and 3'-untranslated region (UTRs) of the hypothetical P(3HB) depolymerase genes to amplify the *Variovorax* sp. C34 depolymerase gene from chromosomal DNA.

For heterologous expression of the *Variovorax* sp. C34 depolymerase gene in *E. coli*, the gene was amplified by PCR using a pair of primers (FSigPhaZV: 5′-GAGACACATATGCCAGCGATGAAGTGGAAC-3′, RePhaZV: 5′-CATTGCGGATCCGAAAGACGGAAAAGGAAAAAGGCGT-3′) containing *Nde*I and *Bam*HI restriction sites (underlined), respectively. The resultant DNA fragment was digested with *Nde*I and *Bam*HI, and ligated into the same sites of the pET3a vector (Novagen, Madison, USA) to form pET3a-phaZ$_{Vs}$. *E. coli* BL21(DE3) was used as a host strain for expression of *PhaZ*$_{Vs}$.

Recombinant *E. coli* BL21(DE3) harboring pET3a-PhaZ_{Vs} was grown in M9 minimal medium supplemented with 100 μg/ml ampicillin at 37 °C, and 0.1 mM IPTG (final concentration) was added when the OD_{600} was 0.6. The cells were further cultivated at 30 °C for 12 h. Then, the cells were removed by centrifugation at 10,000 × *g* for 15 min at 4 °C, and the resulting culture supernatant was used for enzyme purification as described previously (Sun et al. 2014).

The P(3HB) depolymerase PhaZ_{Af} (accession No. P12625.1) was prepared as described previously (Sun et al. 2014).

Nucleotide accession number

The P(LA-*co*-3HB) depolymerase gene sequence of *Variovorax* sp. C34 has been deposited in DDBJ under accession number of LC011525.

Polymeric substrates

P(3HB) (M_n 28.3×10^4) was kindly provided by the Mitsubishi Gas Chemical Company (Tokyo, Japan). P(67 mol% LA-*co*-3HB) (M_n 1.3×10^4) was synthesized as previously described (Nduko et al. 2013b). PDLA (M_n 3.8×10^4) was kindly provided by Dr. Tadahisa Iwata (The University of Tokyo). PLLA (M_n 1.0×10^4) was purchased from

TAKI CHEMICAL (Hyogo, Japan).

Preparation of trimer methyl esters

The trimer methyl esters, 3HB-3HB-3HB-methyl, 3HB-LA-LA-methyl, LA-3HB-LA-methyl and LA-LA-3HB-methyl were prepared by multi-step coupling reactions. The 3HB and LA monomeric derivatives protected with a silyl-group at the hydroxyl end and with a benzyl ester group at the carboxylic end were prepared according to previously published methods (Tabata and Abe 2014). The silyl-protected monomeric derivative was combined with an equimolar amount of the benzyl-protected derivative in the presence of an equivalent amount of N,N′-dicyclohexylcarbodiimide (DCC) and 0.5 equivalents of N,N-dimethyl-4-aminopyridine (DMAP). The reaction mixture was allowed to stir at room temperature for 24 h under nitrogen and then filtered to remove the generated dicyclohexylurea. The filtrate was concentrated *in vacuo* and chromatographed (silica, 5-10% ethyl acetate in hexanes). The obtained di-protected dimer was hydrogenated to deprotect the benzyl group using the flow reactor of an EYELA CCR-1000G system (Tokyo Rikakikai, Japan) with 10% palladium on silica and an Air-tech NM-H-100. The silyl-protected dimeric derivative obtained from this procedure was reacted with the methyl ester monomeric derivative in

143 the same manner used for the dimeric reaction to yield the silyl-protected trimer methyl
144 esters. Equivalents (1.5-2.0) of tetra-n-butylammonium fluoride (TBAF) (1.0 M in
145 tetrahydrofuran, THF) buffered by 3 equivalents of acetic acid were added to the
146 silyl-protected trimer methyl esters in dry THF. The reaction mixture was stirred for 2
147 days and then poured into brine. The product was extracted using diethyl ether, dried
148 with $MgSO_4$, and concentrated *in vacuo*. The concentrate was chromatographed (silica,
149 20% ethyl acetate in hexanes) to yield a clear liquid. The structures of the trimers were
150 confirmed by ^1H NMR (Figure S1). The synthesis of LA-LA-LA-methyl using the
151 aforementioned method was unsuccessful (data not shown).

152 **Preparation of LA oligomer methyl esters**

153 LA oligomer was prepared by modified methanolysis based on a previous described
154 method (Arai et al. 2002b). In brief, PDLA (10 mg) was dissolved in 1 ml of chloroform
155 and combined with 1 ml 15% H_2SO_4/85% methanol. The reaction mixture in a 10-ml
156 glass tube with a screw cap was incubated at 100 °C for 10 min. After reaction, the
157 mixture was cooled down to room temperature and neutralized by adding 1 ml solution
158 containing 0.65 M NaOH and 0.9 M NaCl, and 500 μl 0.25 M Na_2HPO_4. The sample
159 was vortexed vigorously, and chloroform phase was transferred to a new tube, and dried

up. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the oligomer was performed using a Microflex (Bruker Daltonics) using positive ionization mode. An accelerating voltage was 19 kV. For preparing sample, the resulting oligomer was dissolved in 1 ml tetrahydrofuran (THF), and 10 µl aliquot was combined with 22 µl THF solution containing 1 mg/ml sodium trifluoroacetate and 40 mg/ml trans-2-[3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene] (DCTB) as a matrix.

Degradation of polymeric substrates and analyses of the degradation products

P(3HB) and P(LA-*co*-3HB) were used as the substrates for the enzymatic degradation assay. The polymer emulsion was prepared as previously described (Sun et al. 2014). A 20 ml reaction mixture containing 0.08% (wt/vol) emulsified polymer and 1.2 U [activity toward P(3HB)] PhaZ in 25 mM ammonium acetate buffer (pH 7.0) was incubated at 30 ºC for 48 h. After degradation, the solubilized fraction and the remaining emulsion (water-insoluble fraction) were separated by centrifugation at 3,000 × *g* for 1 h at 4 °C, and dried *in vacuo*. The LA and 3HB units in each fraction were ethanolyzed and quantified using a gas chromatography-mass spectroscopy (GCMS-QP2010, Shimadzu) equipped with an InertCap 1MS capillary column (GL

Science Inc., Japan) as previously described (Arai et al. 2002a). The ^{1}H NMR spectra of the water-insoluble fraction in $CDCl_3$ were obtained using a JNM-ECS 400 spectrometer (JEOL RESONANCE, Tokyo, Japan) with tetramethylsilane used as an internal reference.

The enzyme in the solubilized fraction was removed by ultrafiltration using a Centricut 1 stage (KRB-U-10) (Kurabo, Japan). The resulting solution was subjected to size exclusion chromatography (SEC) using a HPLC equipped with a GF-210 HQ (Showa Denko, Japan) and a refractive index detector. Twenty five millimolar ammonium acetate (pH 7.0) was used as the mobile phase. The column was maintained at 60 °C and a flow rate of 1 ml/min. The eluted peaks were fractionated and the LA and 3HB content in the fractions were quantified by GC-MS as aforementioned. Each fraction was diluted in 25 mM ammonium acetate (pH 7.0) with 10% acetonitril to contain 10 ppm LA and 3HB total. The sample was subjected to electrospray ionization mass spectrometry (ESI-MS) analysis using a MicroTOF (Bruker Daltonics). The sample was injected directly by a syringe pump at room temperature at a flow rate of 180 μl/h. The ESI voltage was 4.5 kV in the negative mode and the drying temperature was 200 °C. Nitrogen was used as a nebulizer (1.6 bar) and drying gas (9.0 ml/min). All spectra were recorded in the range of 50-800 m/z.

Degradation of oligomeric substrates and analyses of the degradation products

Each trimer-methyl ester (6 mg) was dissolved in 15 μl of ethanol and combined with 150 μl of 250 mM ammonium acetate buffer (pH 7.0) and 1235 μl of ultrapure water. The enzyme solution (100 μl) containing 12 U [activity toward P(3HB)] of PhaZ was added to the reaction mixture and incubated at 30 °C with gentle shaking for 2.5 h. The resulting methyl esters were extracted with 1 ml of chloroform and subjected to GC analysis using a GC-2010 Plus system (Shimadzu, Kyoto, Japan) equipped with an InertCap 1 capillary column (GL Science Inc., Japan). The methanol in the reaction mixtures was determined using a GC equipped with ZB-WAX column (30 m × 0.25 mm i.d. × 0.25 μm, Phenomenex) and a temperature gradient as follows: 0 min, 40 °C; 0.5 min, 40 °C; 7.7 min, 220 °C; 12.5 min, 220 °C, at flow rate of 2.5 ml/min. The temperature of injector was 200 °C. The flame ionization detector (FID) was used at 250 °C.

For the degradation assay of LA oligomer, 1 mg LA oligomer methyl esters was combined with 100 μl of 250 mM ammonium acetate buffer (pH 7.0) and 875 μl of ultrapure water. The enzyme solution (25 μl) containing 3 U [activity toward P(3HB)] of PhaZ was added to the reaction mixture and incubated at 30 °C with gentle shaking

for 48 h. The water-insoluble fraction was extracted with 1 ml of chloroform and subjected to MALDI-TOF MS analysis using a Microflex as well as the case of PDLA oligomer described above. Solution of matrix containing 40 mg/ml DCTB and 1 mg/ml sodium trifluoroacetate in THF and 5 mg/ml oligomer were mixed in a volume ratio of 11:5. The spectra were recorded in the range of 0-3000 m/z. The amount of LA in the solubilized fraction was determined by GC-MS as aforementioned.

Results

Cloning and expression of a P(LA-*co*-3HB) depolymerase gene *phaZ*$_{Vs}$ from *Variovorax* sp. C34

For detailed enzymatic characterization, we cloned the gene encoding the depolymerase from *Variovorax* sp. C34. The N-terminal amino acid sequence of the depolymerase, AVPLPALGANPAEV, was homologous to those of hypothetical P(3HB) depolymerases from *V. paradoxus* EPS and *V. paradoxus* S110, which were closely-related strain of *Variovorax* sp. C34 based on the 16S rRNA seqnece (Sun et al. 2014). We noticed that the 5'- and 3'-UTRs of hypothetical P(3HB) depolymerase genes of *V. paradoxus* EPS and *V. paradoxus* S110 possessed highly conserved regions, respectively. Therefore, we attempted to amplify the depolymerase gene from

229 *Variovorax* sp. C34 using a pair of primer designed based on the conserved UTR
230 sequences. As the result, a 1.5 kb fragment was successfully amplified from *Variovorax*
231 sp. C34 chromosomal DNA.

232 The DNA sequence analysis revealed that the fragment contained an ORF encoding a
233 protein of 484 amino acid residues bearing the distinct structural modules, putative
234 signal peptide, catalytic domain, fibronectin type III linkage and type I substrate binding
235 domain. This deduced amino acid sequence had homology to the P(3HB)
236 depolymerases from *Delftia acidovorans* (71% identity, accession No. BAA19791.1)
237 (Kasuya et al. 1997), *Acidovorax* sp. AT4 (69%, accession No. BAA35137.1)
238 (Kobayashi et al. 1999), *Comamonas* sp. (46%, accession No. AAA87070.1)
239 (Jendrossek et al. 1995), and *Comamonas testosteroni* (46%, accession No.
240 BAA22882.1) (Kasuya et al. 1994), which were classified as catalytic domain type II
241 denatured extracellular short-chain-length polyhydroxyalkanoates (dPHAscl)
242 depolymerase (Knoll et al. 2009). In addition, the conserved arrangement of the
243 catalytic amino acid residues (Ser46, Asp133, His166, and His255) among catalytic
244 domain type II extracellular dPHAscl depolymerase (Jendrossek and Handrick 2002)
245 was also found in this deduced amino acid sequence. Therefore, it was concluded that
246 the cloned gene encoded a catalytic domain type II extracellular dPHAscl depolymerase,

247 which was designated as PhaZ$_{Vs}$.

248 To investigate the substrate specificity of PhaZ$_{Vs}$, the *phaZ*$_{Vs}$ gene was expressed in *E.*
249 *coli* using the T7 promoter system. The purified recombinant PhaZ$_{Vs}$ was recovered
250 with a 37% yield and a specific activity of 68.3 U/mg protein. The molecular mass of
251 this enzyme was estimated to be 43 kDa by SDS-PAGE, which was the same size as the
252 native PhaZ$_{Vs}$ from *Variovorax* sp. C34 (Fig. 1).

253 **Identification of the molecular species of P(3HB) and P(LA-*co*-3HB) degradation**
254 **products generated using PhaZ$_{Vs}$**

255 The recombinant PhaZ$_{Vs}$ was subjected to the P(3HB) degradation assay. PhaZ$_{Vs}$
256 completely degraded P(3HB) emulsion into the water-soluble degradation product,
257 which contained monomer and oligomers (Fig. 2d). The degraded products fractionated
258 from SEC was subjected to ESI-MS analysis, revealing that the fraction was 3HB-3HB
259 (m/z = 189) homodimer (Fig. 3a). The small amount of 3HB (m/z = 103) monomer was
260 also detected in the fraction. The 3HB oligomers longer than dimer were not detected,
261 indicating that the trimer is the minimum substrate that can be recognized by PhaZ$_{Vs}$.

262 The degradation products of P(67 mol% LA-*co*-3HB) generated by action of PhaZ$_{Vs}$

were analyzed using the same method. The resultant solution of copolymer emulsion treated with $PhaZ_{Vs}$ remained slightly turbid. The water-soluble fraction of the degradation product was estimated to be 96.5 wt% of the copolymer used, and was composed of 69 mol% LA and 31 mol% 3HB (Table 1), which is nearly identical to the monomer composition of the original copolymer. The water-soluble fraction contained oligomers and monomers (Fig. 2e), which were determined to be LA-LA (m/z = 161) and 3HB-3HB (m/z = 189) homodimers, LA-3HB and/or 3HB-LA (m/z = 175) heterodimers, and LA (m/z = 89) and 3HB (m/z = 103) monomers (Fig. 3b). No trimers or longer oligomers were detected. This result indicated that ester bonds between the LA and 3HB units were hydrolyzed by $PhaZ_{Vs}$. Moreover, $PhaZ_{Vs}$ should also cleave LA-LA linkage because of the absence of LA-LA-LA trimer in the degradation product. No degradation of P(LA-*co*-3HB) was observed without $PhaZ_{Vs}$ (data not shown).

It should be noted that a small amount of copolymer emulsion remained after the degradation reaction, which was determined based on turbidity decrease, reached its plateau. The water-insoluble but chloroform-soluble fraction was composed of nearly entirely LA units together with trace amounts of 3HB units (Table 1). ^{1}H NMR resonances of this sample were identical to those of the PDLA homopolymer (Fig. 4). In addition, a small quartet at δ 4.19, which was close to the resonance of the methine

proton of methyl lactate (δ 4.28, data not shown), was ascribed to the CH group of the LA unit at the hydroxyl terminus. Based on the ratio between the peak of the terminal unit and that of the polymeric unit, the number-averaged molecular weight of the PDLA-like fraction was estimated to be approximately 2,900.

Hydrolysis of the chemically synthesized trimers containing LA and 3HB units by PhaZs

The aforementioned results demonstrated that $PhaZ_{Vs}$ hydrolyzed ester bonds in the all dyad sequences, 3HB-3HB, LA-3HB, 3HB-LA and LA-LA. We initially thought that the capacity to cleave LA-containing sequences should distinguish $PhaZ_{Vs}$ and $PhaZ_{Af}$ in terms of the ability to degrade P(67 mol% LA-*co*-3HB). To test this hypothesis, four synthetic trimer methyl esters, 3HB-3HB-3HB-methyl, 3HB-LA-LA-methyl, LA-3HB-LA-methyl and LA-LA-3HB-methyl, were used as the degradation substrates for analyzing the hydrolysis of LA/3HB containing sequences. The treatment of the substrates with $PhaZ_{Vs}$ resulted in the generation of the corresponding methyl esters (Table 2), indicating that this enzyme hydrolyzed ester bonds in 3HB-3HB, 3HB-LA, LA-3HB and LA-LA dyads. For comparison, $PhaZ_{Af}$ from *A. faecalis* T1 was subjected to the same experiment. Surprisingly, $PhaZ_{Af}$ also exhibited hydrolyzing activity toward

these substrates and generated monomer-methyl esters (Table 2). In particular, the generation of LA-methyl from 3HB-LA-LA-methyl indicated that $PhaZ_{Af}$ did hydrolyze ester bond in LA-LA dyad, although the reaction rate of $PhaZ_{Af}$ was slower than that of $PhaZ_{Vs}$ (Fig. 5). It was contrasted with the result that $PhaZ_{Af}$ was shown to not degrade P(67 mol% LA-*co*-3HB) (Sun et al. 2014). Taken these facts together, it was suggested that the hydrolysis of LA-clustering sequence by PhaZ depended on the molecular weight of the substrate. No degradation of these trimers was observed without PhaZs (data not shown).

Hydrolysis of LA oligomers by PhaZs

In order to evaluate the effect of the substrate molecular weight on LA-LA hydrolysis by action of PhaZs, the D-LA oligomers of approximately 6-40 mers (Fig. 6a) were used for PhaZ assay. MALDI-TOF-MS analysis of the degradation products indicated that $PhaZ_{Af}$ fully degraded D-LA oligomers of 8 mer and shorter. However, the enzyme only partially degraded the oligomers of 9-31 mers, and the degradation efficiency of the oligomers was inversely correlated with their molecular weight (Fig. 6b). In contrast, $PhaZ_{Vs}$ exhibited significantly higher degradation activity toward oligomers of 31 mer and shorter compared to $PhaZ_{Af}$ (Fig. 6c). These results clearly demonstrated that the

capacity to degrade long D-LA oligomer was a major determinant distinguishing $PhaZ_{Vs}$ and $PhaZ_{Af}$.

Discussion

$PhaZ_{Vs}$ was identified as the first depolymerase that degrades P(67 mol% LA-*co*-3HB) (Sun et al. 2014). On the other hand, a well-studied P(3HB) depolymerase $PhaZ_{Af}$ was found to not degrade this copolymer. This phenomenon drew our interest in analysis of the cleaved sites in P(LA-*co*-3HB) by action of $PhaZ_{Vs}$ in comparison with $PhaZ_{Af}$. We initially expected that $PhaZ_{Vs}$ and $PhaZ_{Af}$ could be distinguished based on the substrate specificity toward LA-containing sequences. However, a surprising result was that $PhaZ_{Af}$ also had a capacity to cleave the LA-containing oligomers. This result indicated that the difference in the capacity of $PhaZ_{Vs}$ and $PhaZ_{Af}$ in degrading P(LA-*co*-3HB) was not determined by recognition of the monomer sequence, rather, it was caused by the dependency of PhaZ activity on the length of LA-clustering sequence (Fig. 6).

To date, PhaZs have been typically characterized using high-molecular-weight homopolymers and/or low-molecular-weight model substrates, such as *p*-nitrophenylbutyrate (Sznajder and Jendrossek 2014). The PhaZ assay using the copolymer described in this study, however, led to the finding of the hidden PhaZ

activity toward PDLA oligomer. This result suggests that PhaZs, which exhibited no activity toward high-molecular-weight substrates, might degrade oligomer substrates. Therefore, the size of substrate should be recognized as an importance factor for characterization of PhaZs.

The degradation of P(67 mol% LA-*co*-3HB) by $PhaZ_{Vs}$ remained the small amount of non-solubilized fraction, which was identified to be PDLA oligomers of 40 mer in average. This result agreed with the finding that $PhaZ_{Vs}$ degraded PDLA oligomer of 34 mer and shorter. This substrate length-dependency of PhaZ for hydrolysis of D-LA oligomer should be a critical factor affecting the biodegradability of P(LA-*co*-3HB), because the D-LA clustering region contained in the copolymer may last longer period in the environment. In our previous screening, a number of P(67 mol% LA-*co*-3HB)-degrading bacteria were isolated from the soil, suggesting the widespread distribution of the PDLA oligomer-degrading bacteria in the soil. The character of these bacteria and the fate of the PDLA oligomer in the natural environment are important targets of the next stage of the study.

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Figure captions

Fig. 1 SDS-PAGE of recombinant $PhaZ_{Vs}$ expressed in *E. coli*. Lanes: 1, 1.8 μg of $PhaZ_{Vs}$ purified from culture supernatant of *Variovorax* sp. C34; 2, size marker; 3, 10 μg of the dialysate after ammonium sulfate precipitation; 3, 1.2 μg of $PhaZ_{Vs}$ purified from the culture supernatant of recombinant *E. coli*.

Fig. 2 Size exclusion chromatography of water-soluble degradation products of polymers generated by action of $PhaZ_{Vs}$. (a): 1 mg/ml 3HB standard; (b): 1 mg/ml LA standard; (c): 0.06 U/ml $PhaZ_{Vs}$ solution; (d): 0.8 mg/ml P(3HB) treated with $PhaZ_{Vs}$; (e): 0.8 mg/ml P(67 mol% LA-*co*-3HB) treated with $PhaZ_{Vs}$. The peak eluted at 11 min was ammonium acetate.

Fig. 3 ESI-MS analysis of water-soluble oligomer fractions. Samples were fractionated from SEC analysis shown in Fig. 2. (a) oligomer fraction derived from P(3HB). (b)

oligomer fraction derived from P(LA-*co*-3HB). The calculated m/z of $[M-H]^-$ ions from the expected species are 89 (LA), 103 (3HB), 161 (LA-LA), 189 (3HB-3HB), and 175 (LA-3HB and/or 3HB-LA), respectively.

Fig. 4 ^{1}H NMR analysis of the water-insoluble fraction in the degradation products of P(LA-*co*-3HB) by action of $PhaZ_{Vs}$. (a) Original polymer, (b) the water-insoluble fraction after $PhaZ_{Vs}$ treatment, (c) PDLA.

Fig. 5 Time profile of degradation of 3HB-LA-LA-methyl by action of PhaZs. (a) $PhaZ_{Vs}$, (b) $PhaZ_{Af}$. 3HB-LA-LA-methyl (white square); LA-methyl (black diamond); methanol (black triangle). Data is an average of three trials.

Fig. 6 MALDI-TOF MS analysis of the water-insoluble degradation products of PDLA oligomers by action of PhaZs. (a) PDLA oligomer without PhaZ treatment (control); (b) degradation product of sample (a) by action of $PhaZ_{Af}$ (black). The original sample (a) was merged (red); (c) degradation product of sample (a) by action of $PhaZ_{Vs}$ (black). The original sample (a) was merged (red).

Table 1. The yield and composition of the P(67 mol% LA-*co*-3HB) degradation product generated by $PhaZ_{Vs}$

	Major component	Yield (wt%)			Composition (mol%)	
		LA	3HB	Total	LA	3HB
Water-soluble fraction	Monomers	27.8±0.5	19.4±1.4	47.2±1.6	30.5±0.5	17.8±1.3
	Dimers	35.4±0.7	13.9±0.4	49.3±1.1	38.8±0.8	12.8±1.0
	Total	63.2±1.1	33.3±1.5	96.5±2.4	69.4±1.2	30.6±1.4
Water-insoluble fraction	Oligomers	2.3±0.5	Trace	2.3±0.5	100	Trace

The yield and molar composition of each fraction were determined by GC-MS. The monomers and oligomers were separated using SEC. The molecular weights of the molecular species in the oligomer fraction were determined using ESI-MS.

Table 2. Degradation products of synthetic trimers by $PhaZ_{Vs}$ and $PhaZ_{Af}$[a]

Depolymerase	Substrates[b]	Hydrolysis product (mol%)		
		3HB-methyl	LA-methyl	Methanol
$PhaZ_{Vs}$	3HB-3HB-3HB-methyl	91.3±2.7		8.5±1.4
	3HB-LA-LA-methyl		54.2±2.2	21.4±2.3
	LA-3HB-LA-methyl		35.8±4.1	4.6±1.7
	LA-LA-3HB-methyl	9.9±2.4		73.9±1.4
$PhaZ_{Af}$	3HB-3HB-3HB-methyl	89.7±2.4		7.4±1.2
	3HB-LA-LA-methyl		51.8±2.2	17.4±2.6
	LA-3HB-LA-methyl		31.4±3.1	10.3±2.3
	LA-LA-3HB-methyl	10.6±1.2		72.1±4.4

[a]The presence of methyl esters and methanol were determined using GC. [b]After enzymatic reaction, the substrates were fully consumed.

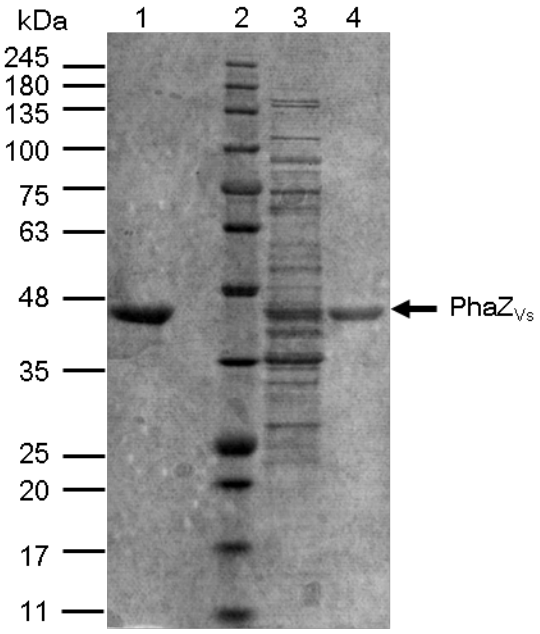


Figure 1

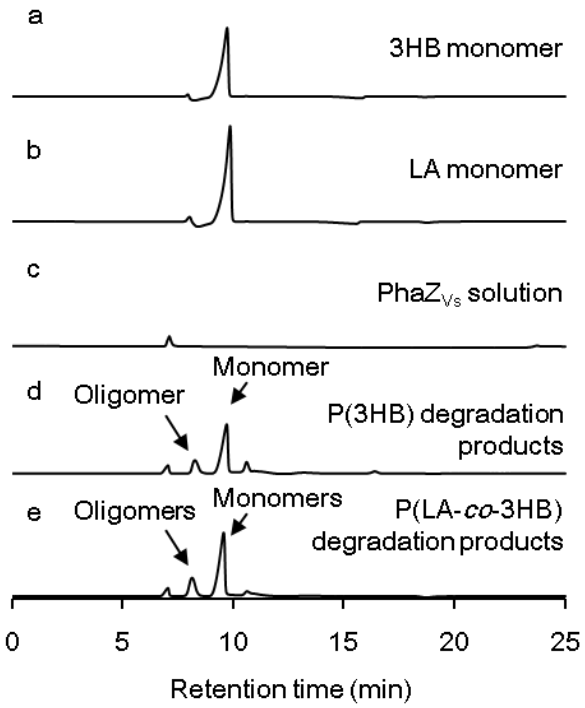
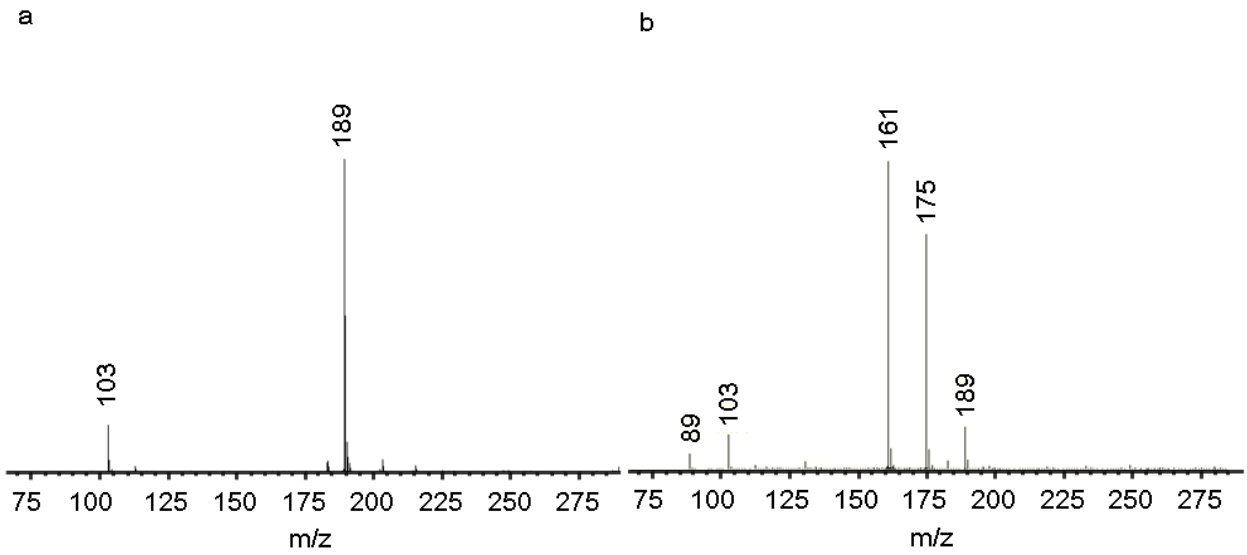

a
3HB monomer
b
LA monomer
c
PhaZVs solution
Monomer
d
Oligomer
P(3HB) degradation products
Monomers
e
Oligomers
P(LA-co-3HB) degradation products
0
5
10
15
20
25
Retention time (min)

Figure 2


a
189
103
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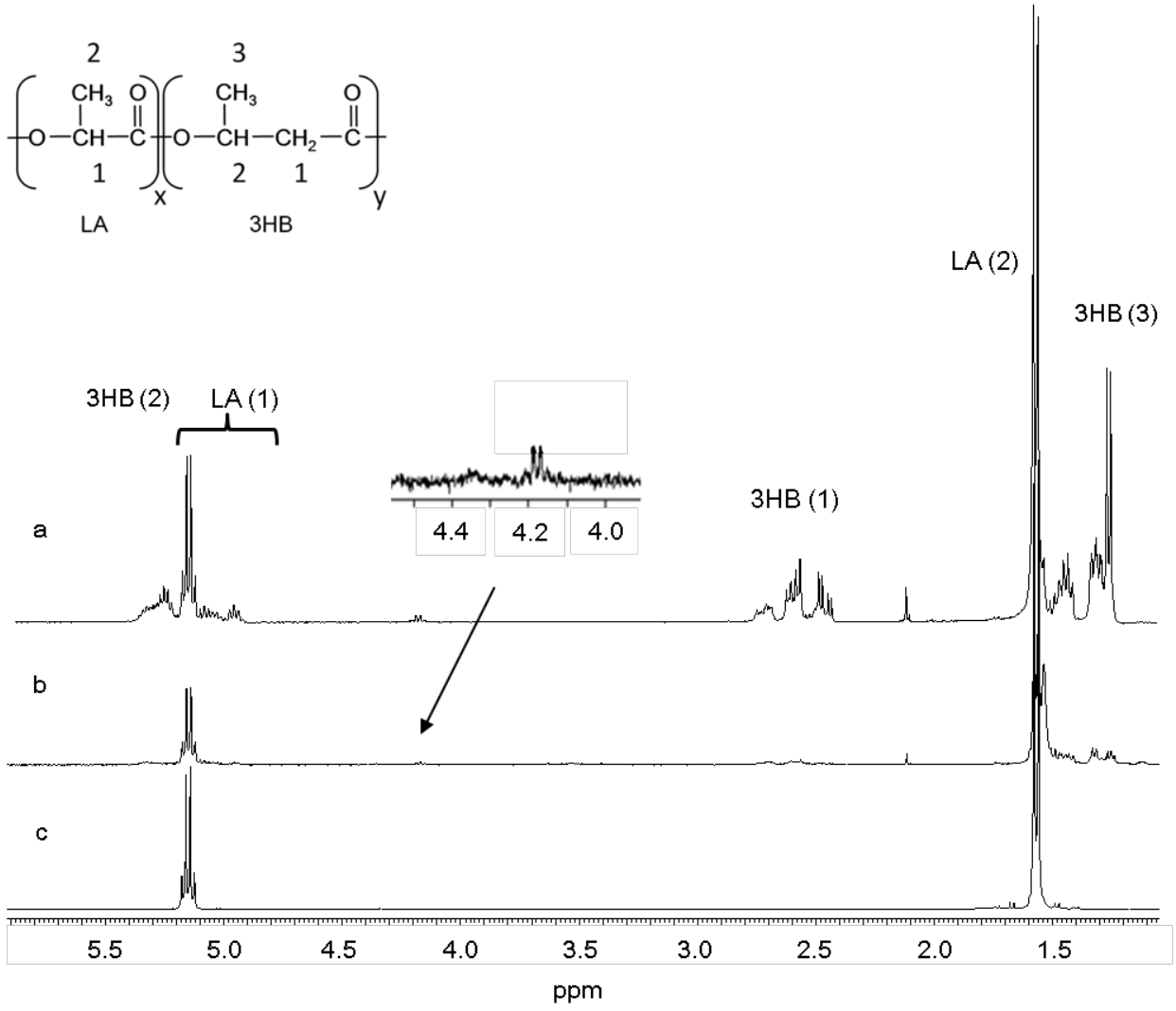
m/z
b
161
175
189
89
103
75 100 125 150 175 200 225 250 275
m/z

Figure 3


2
3
CH3
O
CH3
O
O—CH—C—O—CH—CH2—C
1
2
1
x
y
LA
3HB
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3HB (3)
3HB (2)
LA (1)
3HB (1)
4.4
4.2
4.0
a
b
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3.0
2.5
2.0
1.5
ppm

Figure 4

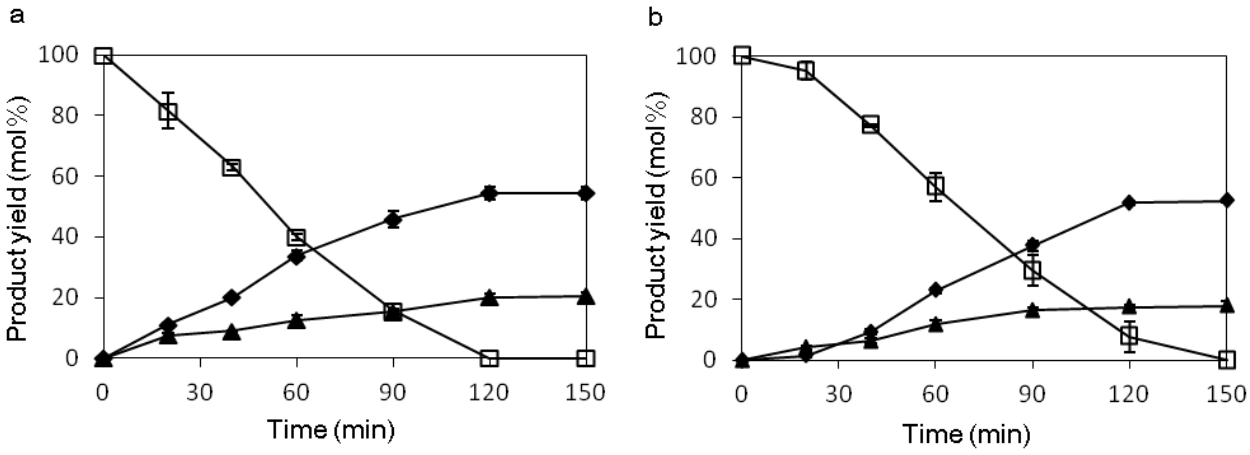

Figure 5

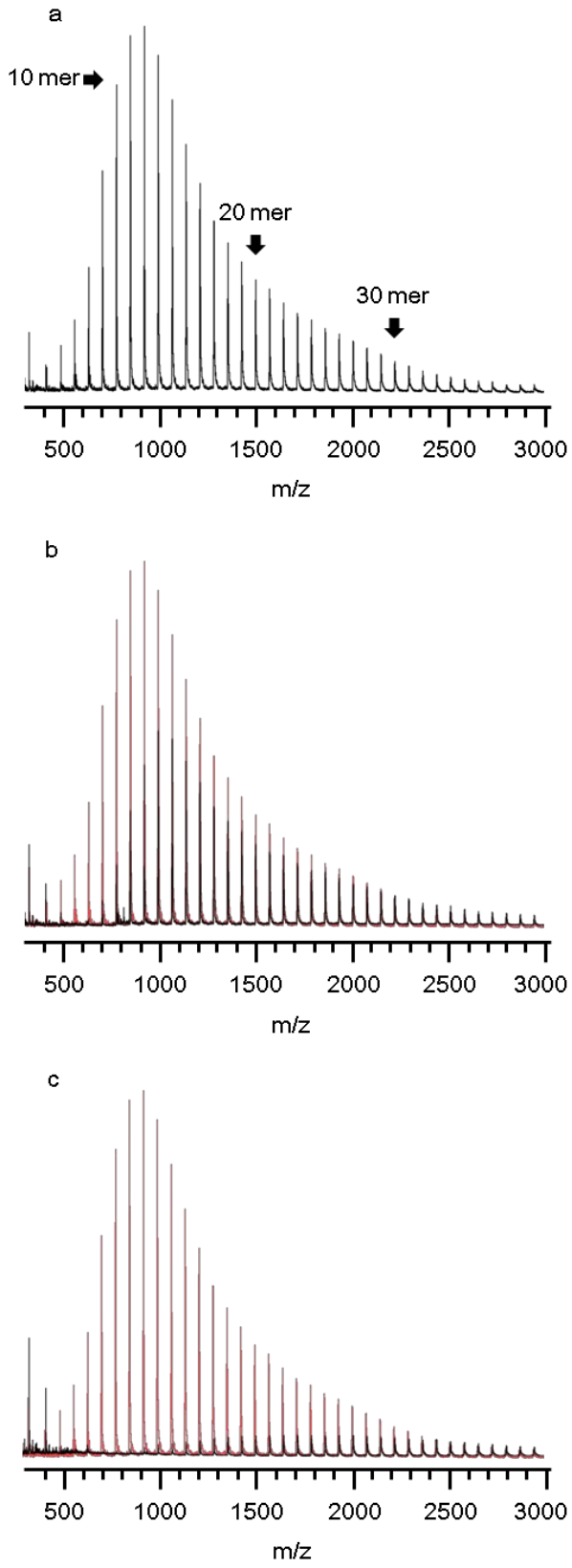
a
10 mer
20 mer
30 mer
500
1000
1500
2000
2500
3000
m/z
b
500
1000
1500
2000
2500
3000
m/z
c
500
1000
1500
2000
2500
3000
m/z

Figure 6