1 **Enzymatic characterization of a depolymerase from the isolated**
2 **bacterium *Variovorax* sp. C34 that degrades poly(enriched**
3 **lactate-*co*-3-hydroxybutyrate)**

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Abstract

Poly[(*R*)-lactate-*co*-(*R*)-3-hydroxybutyrate] [P(LA-*co*-3HB)] is a biobased polyester with semitransparent and flexible properties produced in engineered bacteria carrying an LA-polymerizing enzyme. In this study, we attempted to isolate the P(enriched LA-*co*-3HB)-degrading bacteria from soil samples in order to identify enzymes with the capacity to degrade this new type of polymer. Among approximately 500 samples, the Gram-negative bacterium C34, which exhibited potent P(enriched LA-*co*-3HB)-degrading activity, was isolated based on the decrease in the turbidity of culture medium supplemented with emulsified P(67 mol% LA-*co*-3HB). Based on its 16S rDNA sequence, this isolated bacterium was identified as a member of *Variovorax* sp.. Next, we attempted to isolate and purify the depolymerase that contributes to the polymer degradation from the culture supernatant of strain C34. The purified enzyme had a molecular mass of 42 kDa and exhibited degradation activity towards the P(67 mol% LA-*co*-3HB) as well as 3HB homopolymer [P(3HB)], but not LA homopolymers (PDLA and PLLA). On the other hand, a well-characterized depolymerase of P(3HB) derived from *Alcaligenes faecalis* T1 did not degrade P(67 mol% LA-*co*-3HB), PDLA or PLLA. This result suggests that the newly isolated depolymerase differs from the P(3HB) depolymerase from *A. faecalis*.

Keywords: Lactate-based polymer; polyhydroxyalkanoate; enzyme; biodegradation

38 **1. Introduction**

39 At present, it is estimated that over 200 million tons of plastic are being produced
40 globally per year, and the production is still increasing [1]. These plastics are stable
41 and not readily degraded in the natural environment. Due to such lack of
42 biodegradability, this growing volume of disposed plastic has caused dangerous levels
43 of land and water pollution and attracted considerable attention as an issue requiring
44 environmental intervention. For these reasons, the development of biodegradable
45 plastic has attracted considerable research interest.

46 Polylactic acid (PLA) is one of the most widespread biodegradable plastics, used for
47 commodity purposes. The polymer is synthesized from fermentative (*S*)- and
48 (*R*)-lactic acid via chemical polymerization steps, and the stereochemistry of the
49 monomer greatly influences the enzymatic degradation of the polymers [2].

50 Poly[(*S*)-lactic acid] (PLLA) is degraded in the natural environment and compost [3].

51 Several microbes that degrade PLLA have been reported and the related degrading
52 enzymes, such as certain proteases and lipases, have been purified and characterized
53 [4-7]. In contrast, only one natural enzyme (cutinase like enzyme from *Cryptococcus*
54 *sp.* S-2) degrading poly[(*R*)-lactic acid] (PDLA) has been reported [2], suggesting that
55 PDLA is less biodegradable than PLLA.

56 Polyhydroxyalkanoates (PHAs) are bacterial polyesters and another major class of
57 biodegradable plastic. P(3HB) and its related polymers are highly biodegradable, and
58 are hydrolyzed by a variety of bacteria and fungi [8-12]. To date, many extracellular
59 P(3HB) depolymerases have been purified from different microorganisms and
60 characterized [13-16]. The characterized P(3HB) depolymerases have some common
61 properties such as molecular weight lower than 70 kDa, optimum pH at alkaline range
62 from 7.5 to 9.8 and good stability at a wide range of pH, and temperature [17]. During
63 the degradation process, P(3HB) depolymerases can hydrolyze P(3HB) into
64 water-soluble monomer and/or oligomers [17].

65 In our previous studies, we succeeded in producing P[(*R*)-lactate(LA)-*co*-(*R*)-3HB] in
66 recombinant *Escherichia coli* that expressed heterologous PHA biosynthetic enzymes.

67 In particular, an engineered PHA synthase (PhaC_{1ps}STQK) that gained
68 lactate-polymerizing activity played a central role in the system [18]. P(LA-*co*-3HB)
69 exhibited distinctly different properties from PLA and P(3HB) homopolymers in that
70 the copolymers were flexible and stretchy compared to the more rigid homopolymers.

71 In addition, recent progress in the metabolic engineering P(LA-*co*-3HB) production
72 has improved the productivity of the copolymer, which is now even higher than that
73 of P(3HB) [19]. These developments suggest the potential of the copolymers to be
74 utilized in a wide range of applications. However, to date, the microbial degradation

75 of the copolymer has not been reportedly investigated.

76 In this study, we attempted to isolate the P(enriched LA-*co*-3HB)-degrading bacteria
77 from soil samples in order to identify enzyme(s) with the capacity to degrade this new
78 type of polymer. We chose a copolymer with relatively high LA fraction (67 mol%) as
79 the target polymer. As a result, we successfully isolated a P(LA-*co*-3HB)-degrading
80 bacterium and purified a depolymerase involved in the degradation process.

81 **2. Materials and methods**

82 **2.1. Polymers**

83 P(3HB) (M_n 28.3×10^4) was kindly provided by the Mitsubishi Gas Chemical
84 Company (Tokyo, Japan). P(LA-*co*-3HB) (M_n 1.3×10^4) with a 67 mol% LA fraction
85 was biosynthesized as described previously [20], Chemically synthesized PDLA (M_n
86 3.8×10^4) was kindly provided by the group of Prof. Tadahisa Iwata (The University of
87 Tokyo). PLLA (M_n 1.0×10^4) was purchased from TAKI CHEMICAL (Hyogo, Japan).

88 **2.2. Polymer emulsion preparation**

89 The polymer emulsion was prepared by a sonication method [21]. Twenty milligrams
90 of each polymer were dissolved in 1 mL dichloromethane. The polymer solution was

91 combined with 20 mL of water and emulsified by sonication (40W) on ice for 30 sec
92 repeated 3 times. The dichloromethane was evaporated at 50 °C for 5 h. One gram of
93 the emulsified polymer was added to 1 L of a modified yeast extract medium
94 (YE-medium) containing 1000 mg (NH₄)₂SO₄, 250 mg yeast extract, 200 mg
95 MgSO₄·7H₂O, 100 mg NaCl, 20 mg CaCl₂·2H₂O, 10 mg FeSO₄·7H₂O and 0.8 mg
96 MnSO₄·5H₂O in 10 mM KH₂/K₂HPO₄ (pH7.0).

97 2.3. Isolation of P(LA-*co*-3HB)-degrading microbes

98 The soil samples were taken at summer season in 5 cm deep from the earth surface of
99 the Hokkaido University campus, Sapporo, Japan. For the enrichment culture,
100 approximately 1 g of each soil sample was suspended in 10 mL of sterilized water.
101 Then 10 µL aliquot of the suspension were inoculated into 5 mL liquid YE-medium
102 supplemented with 0.1% (wt/vol) emulsion of P(LA-*co*-3HB) or P(3HB) and
103 cultivated at 30 °C for 1 week with shaking. The decrease of the turbidity of the
104 medium was monitored visually. Culture medium exhibiting the cell growth was
105 inoculated into fresh medium. This step was repeated three times. Samples exhibiting
106 an efficient decrease in turbidity were chosen and subjected to halo formation assay.

107 To detect the P(LA-*co*-3HB)-degrading activity in the culture media, the supernatants
108 of selected cultures were spotted on agar plates containing P(LA-*co*-3HB) emulsion

109 and incubated for 1 week at 30 °C. For colony isolation, the selected sample was
110 inoculated onto a plate containing YE-medium with 0.1% (wt/vol) P(LA-*co*-3HB)
111 emulsion. The cells that grew on the plate and displayed a clear halo around the
112 colonies were chosen and stored in 30% glycerol at -80 °C.

113 2.4. Assay of P(LA-*co*-3HB)-degrading enzyme

114 For halo formation, the cells were grown on the YE-medium plates containing the
115 P(3HB), P(LA-*co*-3HB), PDLA and PLLA emulsion, respectively, at 30 °C for 120 h.
116 The halo formation was visually determined.

117 P(LA-*co*-3HB)-degrading activity was determined by a turbidimetric method using
118 P(LA-*co*-3HB) emulsion. The 1.5 mL reaction mixture contained 1.2 mL of
119 P(LA-*co*-3HB) emulsion, 150 µL of 250 mM potassium phosphate buffer (pH 7.0)
120 and 150 µL enzyme solution. The reaction was initiated by adding the enzyme
121 solution and the turbidity was monitored at 650 nm using a spectrophotometer at
122 30 °C. One unit of the enzyme was defined as the amount of protein required to
123 decrease the OD at 0.1 per min.

124 2.5. Identification of microorganism

125 The genomic DNA of isolated microorganism was extracted by Wizard® Genomic

DNA purification kit (Promega). The 16S rDNA was amplified using a pair of primers, 5'-CAACGAGCGCAACCCT-3' and 5'-GGTTACCTTGTTACGACTT-3', as reported by [22]. A phylogenic tree was constructed by MEGA6 software based on the neighbor-joining method [23].

The isolated strain, identified as *Variovorax* sp. C34, was deposited to the AHU (Agriculture Hokkaido University) Culture Collection of Hokkaido University with a number of AHU1997.

2.6. Purification of the P(LA-co-3HB)-degrading enzyme

An Isolated bacterium was cultured in modified YE-medium supplemented with 0.1% P(3HB) emulsion and 0.1% yeast extract at 30 °C for 120 h on a rotary shaker at 120 rpm. All subsequent purification steps were performed at 4 °C or on ice. Cells were removed by centrifugation at $10,000 \times g$ for 15 min. Ammonium sulfate was added to the culture supernatant obtained at 40% saturation for the precipitated proteins. After being maintained at 4 °C over night, the precipitated proteins were harvested by centrifugation at $10,000 \times g$ for 10 min and dissolved in 0.1 mM potassium phosphate buffer (pH 7.0) with 0.1 mM CaCl₂. The crude enzyme solution was desalted by using a HitrapTM 5 mL desalting column (GE Healthcare) equilibrated with the same buffer.

Desalted crude enzyme solution was applied onto a sucrose density gradient isoelectric-focusing electrophoresis system [24]. The 1.5 – 0.5% of the broad pH range ampholyte (pH 3.5-11) (Pharmacia LKB) was stabilized with a sucrose density gradient (0 - 50%) in a cooling jacket-covered glass column. Isoelectric-focusing electrophoresis was performed at the constant voltage of 400 V for 48 h under 0 °C. The sample was 2 mL for each fraction. The active fractions (i.e. 8 mL in 4 fractions) were combined. To remove the ampholyte and sucrose, a HitrapTM desalting column was used, as described above. The purified enzyme was stored at -80 °C.

2.7. Enzymatic characterization of the P(LA-*co*-3HB)-degrading enzyme

The N-terminal amino acid sequence of the purified P(LA-*co*-3HB)-degrading enzyme was determined by Procise 491cLC (Applied Biosystems) in the Instrumental Analysis Division of the Equipment Management Center in Hokkaido University.

The effects of inorganic and organic compounds on the P(LA-*co*-3HB)-degrading enzyme activity were evaluated using 1 to 5 mM of NaCl, MgCl₂, CaCl₂, CuCl₂, ZnCl₂, MnCl₂, FeCl₃, CdCl₂, HgCl₂, AgNO₃, phenylmethanesulfonyl fluoride (PMSF), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), dithiothreitol (DTT), iodoacetic acid (IAA), sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (2-ME), respectively. P(LA-*co*-3HB)-degrading enzyme was incubated with each of the

chemicals on ice for 1 h. The reaction mixture was diluted with 3 volumes of 10 mM potassium phosphate buffer (pH 7.0), and then subjected to measurement of the enzymatic activity.

P(3HB), P(LA-*co*-3HB), PDLA and PLLA were used for the polymer degradation assay as the substrate. Enzymatic reaction was performed in a 5 mL reaction mixture containing 0.08 % (wt/vol) of each polymer, 25 mM potassium phosphate buffer (pH 7.0) and 0.3 U P(LA-*co*-3HB)-degrading enzyme. Molecular weight of polymer was estimated by gel permeation chromatography (GPC) equipped with tandem columns KF-805L and K-803L (Shodex, Japan). Polystyrene standards (M_n : 1.20×10^3 to 3.1×10^6) were used to calibrate.

3. Results and discussion

3.1. Isolation and identification of bacterial strain C34

A total of 500 soil samples collected from different locations such as farms, compost dumps, dumpsters, sport fields and building sites in Hokkaido University were applied to the enrichment liquid culture. Among them, 216 samples decreased the turbidity of media containing P(LA-*co*-3HB) and P(3HB), 93 samples decreased the turbidity of P(3HB)-containing medium but not P(LA-*co*-3HB), and 191 samples

178 exhibited no decrease in the turbidity of either polymer. This result suggests that the
179 microorganisms isolated in this study can be grouped into two groups, that can
180 degrade P(3HB) only, and that degrade P(LA-co-3HB) as well as P(3HB). In addition,
181 P(LA-co-3HB)-degrading microorganisms were common inhabitants of the soil of
182 these sampling sites.

183 Out of 216 samples, 23 samples exhibited a considerable turbidity decrease of media
184 containing P(LA-co-3HB). Thus, the supernatants of the 23 cultures were applied to
185 the agar plates containing P(LA-co-3HB) emulsion for degradation activity assay.

186 After 7 days incubation, 8 samples exhibited the halo formation, indicating the
187 presence of P(LA-co-3HB)-degrading enzyme in the supernatants. Of these, sample
188 No. 34 (taken from the location of 43°04'38.1"N, 141°20'03.8"E), which generated
189 the largest halo, was selected for microorganism isolation after streaking it on the
190 same plate. Among the colonies grown on the plates, the bacterium C34, which grew
191 well and generated a clear halo, was chosen as a candidate P(LA-co-3HB)-degrading
192 enzyme producer. The isolated strain C34 also generated a halo on the P(3HB) plates,
193 but not the PDLA-containing plates. For comparison, recombinant *E. coli* expressing
194 the gene encoding the P(3HB) depolymerase (PhaZ_{Af}) from *A. faecalis* T1 was grown
195 on the same plates. As the result, PhaZ_{Af} only degraded P(3HB), but not
196 P(LA-co-3HB), suggesting the isolated C34 strain secretes degrading enzyme(s) with

a different substrate specificity than PhaZ_{AF} (Fig.1).

The isolated strain C34 was a Gram-negative and rod-shaped bacterium (data not shown). The sequence of a 340 bp 16S rDNA fragment amplified from C34 chromosomal DNA had 99 % identity with that of *Variovorax* sp. HI-I4 (accession number DQ205307.1), indicating that the bacterium C34 belongs to the genus of *Variovorax* sp. Therefore, the isolated bacterium was identified as *Variovorax* sp. C34. Fig. 2 shows a neighbor joining phylogenetic tree based on the 16S rDNA sequence of *Variovorax* sp. C34. *Variovorax* sp. C34 is located close to the extracellular P(3HB) depolymerase secreting bacteria, such as *Acidovorax* sp. [25], *Comamonas* sp. [26] *Comamonas testosteroni* [27] and *Delftia acidovorans* [28], which belong to the family of *Comamonadaceae*.

3.2. Purification of the P(LA-co-3HB)-degrading enzyme

During the course of this study, we found that the addition of P(3HB) emulsion was effective for increasing the production of the P(LA-co-3HB)-degrading enzyme. Therefore, the following cultivation of the *Variovorax* sp. C34 was carried out by supplementation of P(3HB) emulsion as an enzyme inducer. The purification procedures are summarized in Table 1. By means of the purification steps, a P(LA-co-3HB) depolymerase was purified to a final yield of 25% with a specific

215 activity of 51.9 U mg⁻¹ from 9 L culture supernatants. Upon density
216 isoelectric-focusing electrophoresis, the fraction exhibiting the highest
217 P(LA-*co*-3HB)-degrading activity was eluted at pH 9.0, which was thought to be the
218 pI of this enzyme. The purified protein migrated as a single band on SDS-PAGE and
219 its molecular mass was calculated to be approximately 42 kDa (Fig. 3).

220 3.3. Polymer degradation by the purified P(LA-*co*-3HB) depolymerase

221 The time course of the degradation by P(LA-*co*-3HB) depolymerase was determined
222 using the various polymer emulsions, i.e. P(LA-*co*-3HB), P(3HB), PLLA and PDLA
223 (Fig. 4). The purified depolymerase degraded both P(LA-*co*-3HB) and P(3HB), but
224 not PLLA or PDLA, which is consistent with the results of the halo formation test on
225 the agar plates, as described above (Fig.1). After the reaction, the P(LA-*co*-3HB)
226 degradant was subjected to GPC analysis, but no peak was observed in the measurable
227 range of molecular weight (10³ to 10⁶) (data not shown). Thus, the most part of
228 P(LA-*co*-3HB) was thought to be degraded into low molecular weight segments
229 (<10³). We are currently analyzing the structures of the degradation products of
230 P(LA-*co*-3HB) that should provide important information to reveal which linkages
231 between LA and 3HB units in the copolymer were hydrolyzed by the enzyme.
232 The degradation of P(LA-*co*-3HB) by P(LA-*co*-3HB) depolymerase was faster than

that of P(3HB). This result could be interpreted to mean that the crystallinity of a random copolymer is lower than that of a homopolymer [29], containing more amorphous regions, and is thus more rapidly degraded than the crystallized region [30, 31]. On the other hand, it was also found that PhaZ_{Af} hydrolyzed P(3HB), but not P(LA-*co*-3HB) (Fig. 1). This phenomenon suggested that the presence of the LA units in the polymer chain significantly inhibited the hydrolysis of the polymer by PhaZ_{Af}, even with the lowered crystallinity of the polymer. The mechanism for the low activity of PhaZ_{Af} toward P(LA-*co*-3HB) is also an interesting target for future studies.

3.4. Effects of pH and temperature on the enzymatic activity

The optimum pH of the purified P(LA-*co*-3HB) depolymerase was found to be 8.0 (Fig. 5 a), which is similar to that of the P(3HB) depolymerase from *Paucimonas lemoignei* [17], *Caldimonas manganoxydans* [32] and *Aspergillus fumigatus* [33]. The pH stability of the enzyme was determined by measuring enzymatic activity at various pHs ranging from 2.0 to 12.0. The enzyme retained more than 90% activity over a range of pH 6.0-12.0 (Fig. 5 b) after incubation on ice for 1 h. The effect of temperature on the enzymatic activity was determined to be between 10 and 70 °C at pH 7.0. The optimal temperature of this enzyme was 55 °C (Fig. 6 a). After incubation

in a temperature range of 10-70 °C for 1 h, the residual activities of the enzyme were measured. The enzymatic activity was stably maintained at temperatures below 40 °C (Fig. 6 b). The thermal properties of P(LA-*co*-3HB) depolymerase are similar to those of the P(3HB) depolymerase from *A. faecalis* T1 [34].

3.5. Effect of metal ions and organic inhibitors on the enzymatic activity

The sensitivity of the enzyme towards various inhibitors was evaluated (Table. 2). The enzymatic activity of the P(LA-*co*-3HB) depolymerase decreased to 41% and 12% by 1 h incubation in 5 mM of Hg²⁺ and Ag⁺ respectively. However, the P(LA-*co*-3HB) depolymerase was insensitive to the chemical reagents (see Materials and Methods) tested such as PMSF, EDC, DTT, iodoacetic acid, SDS and 2-ME. It has been known that some P(3HB) depolymerases are sensitive to these inhibitors, while others are not [17, 26, 35]. Thus, this result does not exclude the possibility that the newly isolated enzyme is an esterase having serine residue as an active center. The primary structure of the enzyme will be needed to clarify this problem.

4. Conclusion

A P(LA-*co*-3HB)-degrading bacterium *Variovorax* sp. C34 was isolated from soil using P(enriched LA-*co*-3HB) emulsion. A 42 kDa depolymerase, which was purified

from the culture medium of the bacterium, was found to contribute to the degradation of P(LA-co-3HB). The newly isolated depolymerase differed from PhaZ_{Af} in the hydrolysis capacity toward (*R*)-LA-containing polymer.

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379 **Fig. 1** Halo formation obtained with the isolated C34 bacterium on YE-medium plates
 380 containing emulsion of P(3HB) (a), P(LA-co-3HB) (b), PDLA (c) and PLLA (d),
 381 respectively; recombinant *E. coli* expressing the phaZ_{Af} gene from *A. faecalis* T1 on
 382 YE-medium plates containing emulsion of P(3HB) (e), P(LA-co-3HB) (f), PDLA (g)
 383 and PLLA (h), respectively. The plates were incubated at 30 °C for 120 h

384 **Fig.2** Phylogenetic analysis of isolated bacterium C34 based on its 16s rDNA
 385 sequence. Phylogenetic analysis was performed using the MEGA5.2 software. Except
 386 for bacterium C34, the 16s rDNA sequences of aligned microorganisms were
 387 retrieved from GenBank (<http://www.ncbi.nlm.nih.gov>). The numbers at the nodes
 388 indicate the bootstrap percentages of 1,000 resamplings

389 **Fig.3** SDS-PAGE of P(LA-co-3HB)-degrading enzyme from *Varovorax* sp. C34.
 390 Lanes: 1, Molecular mass marker; 2, 15 µg protein of the dialysate after ammonium
 391 sulfate precipitation; 3, 4.5 µg of the purified P(LA-co-3HB)-degrading enzyme

392 **Fig.4** Enzymatic degradation of P(LA-co-3HB) (square), P(3HB) (diamond), PDLA
 393 (triangle) and PLLA (circle) emulsion by the P(LA-co-3HB)-degrading enzyme

Fig.5 Effect of pH on the (a) optimum and (b) stability of the purified P(LA-*co*-3HB)-degrading enzyme. The optimum pH for enzyme activity was determined at 30 °C in 25 mM potassium phosphate buffer (pH 6-8) (diamond) and boric acid/NaOH buffer (pH 8-10) (circle). For determination of pH stability, the enzyme was incubated with 10 mM of KCl/HCl (pH 2-4), citric acid/Na₂HPO₄ (pH 4-6), potassium phosphate (pH 6-8), boric acid/NaOH (pH 8-10) or KCl/NaOH (pH 10-12) on ice for 1 h and subjected to enzymatic assay at 30 °C in 25 mM potassium phosphate buffer (pH 7.0). The relative activity is expressed as a percentage of the maximum of the enzyme activity under the assay conditions using P(LA-*co*-3HB) as the substrate

Fig.6 Effect of temperature on the (a) optimum and (b) stability of the purified P(LA-*co*-3HB)-degrading enzyme. The temperature optimum for enzymatic activity was determined at temperatures ranging from 10 to 70 °C. For determination of thermal stability, the enzyme samples were incubated at 10 to 70 °C for 1 h and subjected to enzymatic assay at 30 °C. The relative activity is expressed as a percentage of the maximum of the enzyme activity under the assay conditions using P(LA-*co*-3HB) as the substrate

Table1. Purification of the P(LA-*co*-3HB)-degrading enzyme from *Variovorax* sp. C34

	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification fold
1. Culture supernatant	190	270	1.42	100	1
2. (NH ₄) ₂ SO ₄ precipitation	22	164	7.45	61	5
3. Isoelectric focusing	1.3	67.5	51.9	25	36

Table 2. Effect of metal ion and organic inhibitors on the enzymatic activity of the P(LA-*co*-3HB)-degrading enzyme

Reagent	Residue activity (%)	
	1 mM	5 mM
None		100
NaCl	100	100
MgCl ₂	100	93
CaCl ₂	110	100
CuCl ₂	100	86
AgNO ₃	100	12
ZnCl ₂	120	109
MnCl ₂	100	90
FeCl ₃	89	100
CdCl ₂	100	100
HgCl ₂	76	41
PMSF	100	109
EDC	100	100
DTT	100	100
IAA	100	100
SDS	100	100
2-ME	100	100

Fig. 1

Fig.2

Fig.3

Fig.4

Fig.5

a

b

Fig.6

a

b