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Biodegradation of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) by a novel P3/4HB depolymerase purified from *Agrobacterium* sp. DSGZ

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ABSTRACT: A poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P3/4HB)-degrading strain, *Agrobacterium* sp. DSGZ, was isolated from sewage by poly(3-hydroxybutyrate) (PHB) mineral agar plates. A novel P3/4HB depolymerase with a molecular weight of 34 kDa was purified through a novel single-step affinity chromatography method from the culture supernatant of the strain by using P3/4HB powder as a substrate. The purified depolymerase showed optimum activity at pH 7.0 and 50°C, and was stable at the pH range of 6.0 to 9.0 and temperature below 50°C. Enzyme activity was strongly inhibited by phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), hydrophobic reagents, and some metal ions. The depolymerase degraded poly(3-hydroxybutyrate) (PHB), poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHBV), P3/4HB, and polycaprolactone (PCL), instead of polylactic acid (PLA) or poly(butylene succinate) (PBS). Meanwhile, the depolymerase showed high hydrolytic activity against short-chain length esters, such as butyrate acid ester and caprylic acid ester. The main degradation products of the depolymerase were identified as hydroxybutyrate monomers and dimers, and the monomers were identified as 3-hydroxybutyrate (3HB) monomers and 4-hydroxybutyrate (4HB) monomers. The preparation procedure, crystallinity, and 4HB composition of the P3/4HB copolymer showed evident effect on degradation behavior, and change in crystallinity was the main factor affecting degradation. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 42805.

KEYWORDS: amorphous; biodegradable; blends; degradation; films

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INTRODUCTION

Polyhydroxyalkanoates (PHAs) are natural biodegradable polyesters that are synthesized and accumulated intracellularly during unbalanced growth by different kinds of bacteria.[1] PHAs have generated increased research interest in recent years because of their biodegradability, biocompatibility, and excellent mechanical properties compared with traditional nondegradable thermoplastics, such as polypropylene and polyethylene. Poly(3-hydroxybutyrate) (PHB) is the most extensively studied type in the PHA family and has been used successfully in the medical and packing fields as a renewable and biodegradable plastic. In published reports, the degradation of PHB in natural environment has been studied. Numerous types of microorganisms were selected, and a series of PHB depolymerase secreted by these organisms has been purified and characterized.[2–7] Enzymolysis by PHB depolymerase has been considered to be the root cause of PHB degradation. PHB depolymerase preferentially hydrolyzes the amorphous region of PHB with a disordered structure and subsequently degrades its crystalline region.[8,9]

More than 90 different hydroxyalkanoic acids have been found to comprise different PHBs, among which 4HB is more advantageous than the rest because of its ability to adjust the material properties of the PHB copolymer.[10] Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P3/4HB), which is composed of 3HB and 4HB, is synthesized in microorganisms by using a special production culture. Compared with other PHBs, P3/4HB exhibits better biocompatibility, thermal stability, and toughness for applications in agriculture and medical field.[11] At present, numerous studies have been carried out to improve material properties by changing the percentage of 4HB composition. However, only a few studies on its biodegradation have been reported, and P3/4HB depolymerases have rarely been purified and characterized.[12–15] Therefore, the mechanism by which the introduction of 4HB in PHB would change the biodegradability of the polymer remains unclear. Therefore, the mechanism of P3/4HB biodegradation by P3/4HB depolymerase requires in-depth study.

In the present study, P3/4HB-degrading bacteria, *Agrobacterium* sp. DSGZ, was isolated, and a novel P3/4HB depolymerase from the strain was purified by a new, simple, single-step affinity chromatography method. The purified enzyme was characterized, and the degradation mechanism of P3/4HB was explored.

Table I. Properties of P3/4HB Powder with Different Monomer Compositions

Sample	4HB content (mol %)	M_n	M_w	Polydispersity index
A	0	3.22×10^5	4.15×10^5	1.29
B	6.5	3.44×10^5	4.02×10^5	1.17
C	10.0	3.26×10^5	3.98×10^5	1.22
D	12.0	3.06×10^5	4.25×10^5	1.39
E	23.5	3.08×10^5	4.07×10^5	1.32

MATERIALS AND METHODS

Chemicals

P3/4HB powder was provided by Green Bio Co. (Tianjin, China). The molar ratios of 4HB, weight-average molecular weight, number-average molecular weight, and polydispersity index were determined by nuclear magnetic resonance (NMR Bruker AV400, Switzerland) spectroscopy and gel permeation chromatography (GPC) analysis and are listed in Table I. Unless otherwise stated, P3/4HB films were prepared by thermoforming method.[16] All chemicals used were of analytical grade.

Strain Isolation, Cultivation, and Assay of P3/4HB Degradation

The fermentation medium contained 0.15 g of P3/4HB, 0.5 g of $MgSO_4$ $7H_2O$, 1 g of NH_4Cl, 0.005 g of $CaCl_2$ $2H_2O$, 4.54 g of KH_2PO_4, and 11.94 g of Na_2HPO_4 $12H_2O$ per liter.[17] A strain was isolated from activated sludge samples, and P3/4HB-degrading activity was determined by hydrolysis transparent zone test. P3/4HB films (1.0 cm × 1.0 cm × 0.1 cm) were added into the fermentation medium to take the place of P3/4HB powder, and *Agrobacterium* sp. DSGZ was added to the medium at 37°C and 150 r/m. The surfaces of degraded P3/4HB films were observed with a scanning electron microscope.

P3/4HB-Degrading Enzyme Assay

Approximately 0.4% (w/v) P3/4HB powder was emulsified with 0.07% (w/v) sodium dodecyl sulfate (SDS) in 20 m*M* phosphate buffer (pH 7.5). About 1 mL of diluted enzyme solution and 3 mL of P3/4HB emulsion were then mixed at 50°C for 1 h. The decrease in turbidity of P3/4HB emulsions was measured at a wavelength of 650 nm by using a UV spectrophotometer. One unit (U) of P3/4HB depolymerase activity was defined as the amount of enzyme required to decrease absorbance by 0.001 at 650 nm per min.[18]

Purification of P3/4HB Depolymerase

One liter of P3/4HB medium culture with the bacterial strain was fermented at 37°C for 36 h, and the fermented supernatant was obtained by centrifugation at 12,000 *g* and 4°C for 20 min. The P3/4HB powder at a final concentration (0.05%, w/v) was added to the supernatant. After incubation at 8°C to 10°C for 6 h to 8 h, the mixture was filtered through Whatman No. 1 filter paper. P3/4HB depolymerase adsorbed on the P3/4HB powder was eluted with 100 mL of phosphate buffer (20 m*M*, pH 7.5) containing 0.1% (v/v) Triton X-100. The elution mixture was magnetically stirred for 1 h at room temperature 28 ± 2°C. The eluted component was lyophilized and stored at −20°C.[15]

Protein Measurement

Proteins were quantified by Bradford method by using bovine serum albumin as the standard protein. The molecular weight of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by using standard low-molecular weight markers (Takara).

Biochemical Characterization of P3/4HB Depolymerase

The optimal temperature and pH for the enzymatic reaction were determined by testing the activity of the purified enzyme at a temperature range of 30°C to 70°C and pH range of 4.0 to 10.0 (sodium citrate buffer for pH 4.0–6.0, potassium phosphate buffer for pH 6.0–8.0, tris-HCL buffer for pH 8.0–9.0, and glycine–NaOH buffer for pH 9.0–10.0). To determine the thermostability and pH stability of the P3/4HB depolymerase, purified enzymes were stored at different temperatures from 10°C to 70°C for 12 h or in different buffers (pH 4.0 to pH 10.0) at 4°C for 24 h. Residual activity was assayed under standard conditions.

To determine the effects of metal ions on enzyme activity, a series of metal ions (Cu^{2+}, Na^+, Mn^{2+}, Co^{2+}, Ca^{2+}, Fe^{2+}, and Mg^{2+}) with final concentrations of 1 or 10 m*M* were added to the purified enzyme solutions, followed by incubation at 4°C for 2 h. Residual activity was assayed under standard conditions.

To determine the effects of chemicals on the enzyme, various chemical reagents at final concentrations of 1% (v/v) and 10% (v/v) were added to the purified enzyme solutions, followed by incubation at 4°C for 2 h. Residual activity was assayed under standard conditions.

Substrate Specificity

To assay polymer-degrading activity, PHB, poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHBV), polycaprolactone (PCL), poly(butylene succinate) (PBS), and PLA were emulsified as substrates, and activity was determined after the P3/4HB-degrading enzyme assay. The activity of the purified enzyme on *p*-NP acyl-esters, including acetate (C2), butyrate (C4), caproate (C6), caprylate (C8), laurate (C12), and palmitate (C16), was measured by monitoring the production of *p*-nitrophenyl from esters.[19] Kinetic assays were carried out under optimal temperature and pH conditions with *p*-NPC (1 mg mL^{-1} to 10 mg mL^{-1}) as the substrate. The values of K_m, V_{max}, and K_{cat} were calculated from a Lineweaver–Bark plot.[20,21]

Analysis of P3/4HB Hydrolysis Products

A reaction mixture with 1 mL of diluted purified enzyme and 3 mL of P3/4HB emulsion was incubated at 50°C for 12 h. The degradation products formed during the enzyme reaction were analyzed by tandem quadrupole mass spectrometry (Quattro Premier XE) with the following instrument parameters: capillary voltage, 3.0 kV; cone voltage, 20 V; source temperature, 110°C; cone gas flow, N_2, 80 L/h; desolvation temperature, 380°C; desolvation gas flow, N_2, 60 L/h; and vacuum degree, 1.9×10^{-4} Pa.[22]

A P3/4HB film (4HB, 10.0 mol %) was degraded by P3/4HB depolymerase at 50°C for 24 and 72 h, and the degradation products were lyophilized and analyzed by NMR spectrometry (Bruker AV400, Switzerland) at 400 MHz with a spectrum of

32 K data points. An inactivated enzyme powder was used as the blank control.

Catalytic Kinetic Analysis of the Depolymerase on P3/4HB

P3/4HB (4HB: 6.5, 12.0, and 23.5 mol %) powder was emulsified with 0.06 g of SDS in 20 m*M* phosphate buffer (pH 7.5). Each P3/4HB emulsion substrate was diluted to different concentrations (1 mg/mL to 5 mg/mL) and mixed with the purified enzyme. Enzymatic activity was assayed under standard conditions, and the values of K_m and V_{max} were calculated from a Lineweaver–Bark plot.[20,21]

Degradation of Various P3/4HB Copolymers by the Purified Enzyme

P3/4HB (4HB, 12.0 mol %) films with different crystallinities were prepared by non-isothermal crystallization method.[23] After measurement by differential scanning calorimetry (DSC) analysis, the crystallinities of P3/4HB films were calculated as 25.21%, 33.19%, 37.12%, and 40.72%, respectively. These P3/4HB films were degraded by the purified enzyme, and weight loss was detected to investigate the effect of crystallinity on P3/4HB degradation.

P3/4HB films prepared by thermo-forming and solvent-forming methods were used to detect the effect of preparation method on P3/4HB degradation. P3/4HB films (1.0 cm × 1.0 cm × 0.1 cm) were degraded in a solution containing P3/4HB depolymerase (0.2 mg/mL, pH 7.5) at 50°C and 130 r/min. Purified depolymerase was replaced every 24 h. The weight loss of copolymer films was measured to reveal the effect of preparation method on P3/4HB degradation.

P3/4HB copolymer films with different 4HB concentrations (0, 6.5, 10.0, 12.0, and 23.5 mol %) (1.0 cm × 1.0 cm × 0.1 cm) were applied to test the effect of the number of 4HB units on P3/4HB degradation. P3/4HB films were prepared by conventional solvent casting techniques from chloroform solutions of polymer using glass Petri dished as casting surfaces, and the solution-cast films were aged for 3 weeks to reach equilibrium crystallinity prior to analysis. Film weight loss was measured to characterize P3/4HB film degradation.

RESULTS AND DISCUSSION

Isolation and Identification of the P3/4HB-Degrading Strain

A P3/4HB-degrading strain forming a clear zone on P3/4HB-emulsified agar medium was isolated from activated sludge. The nucleotide sequence of the 16S rRNA of the strain revealed 99% homology with *Agrobacterium tumefaciens* stain A2P3 (EU221409.1). The morphological, physiological, and biochemical properties of the strain also showed that the isolate belonged to *Agrobacterium* sp. Hence, the strain was named *Agrobacterium* sp. DSGZ. The strain could grow in a basic medium with P3/4HB substrate as the sole carbon source.

After the degradation of P3/4HB films by *Agrobacterium* sp. DSGZ, films became thinner, and the holes on P3/4HB films grew larger and deeper (Figure 1). These results indicated that the biodegradation of P3/4HB films by the strain began from the surface and extended to the interior of the polymer step by step. After 7 d of degradation, the P3/4HB film lost its integrated appearance, and weight loss reached 80%. The strain was confirmed to secrete extracellular depolymerase, which was responsible for degradation.

Purification of Extracellular P3/4HB Depolymerase

An extracellular P3/4HB depolymerase from the supernatant of *Agrobacterium* sp. DSGZ was purified to homogeneity by affinity chromatography. The enzyme was purified by 2.76-fold with a recovery of 9.2% as summarized in Table II. The image in Figure 2 illustrates that the molecular weight of the purified enzyme was 34 kDa, as analyzed by using SDS-PAGE.

In this article, a simple, one-step, P3/4HB substrate binding affinity chromatography method was applied. This method was more convenient and efficient than previous approaches.[4–7,11] A recovery yield of 9.2% and 2.76-fold purification proved that purification was successful. This article was the first to report the purification of P3/4HB depolymerase by using single-step affinity chromatography with P3/4HB powder. The method was feasible for the purification of proteins that strongly interact with the substrate. In the past few years, many PHB depolymerases were purified and characterized. Large molecular weights (about 50–70 kDa) of the depolymerase were observed because of multiple domains, including catalytic, linker, and substrate-binding domains.[24] The purified P3/4HB depolymerase from *Agrobacterium* sp. DSGZ with a molecular weight of 34 kDa, was smaller than many PHB depolymerases. Adsorption experiments of the enzyme onto typical polymers were conducted, and results showed that the purified enzyme could adsorb to PHAs specifically rather than to PLA and PCL. Thus, the depolymerase was speculated to contain a small, specific substrate-binding domain or a certain region that replaced the substrate-binding domain, thereby performing the special adsorption function between enzyme and substrate.

Biochemical Characterizations of the Purified Enzyme

Effects of Temperature and pH. The P3/4HB-degrading activity of the purified enzyme was assayed within the temperature range of 30°C to 70°C, and the maximum activity was observed at 50°C [Figure 3(a)]. The purified enzyme could be assayed stably at temperature levels below 50°C for 2 h. Enzyme activity decreased by nearly 70% when assayed at 70°C for 2 h [Figure 3(b)]. P3/4HB degradation by the purified enzyme was assayed under standard conditions and change in pH from 4.0 to 10.0. As shown in Figure 3(c), the optimum pH of P3/4HB depolymerase for P3/4HB degradation was 7.0. The purified enzyme was stable at the pH range of 5.0 to 10.0, but showed a nearly 40% decrease in activity at pH 10.0 [Figure 3(d)].

Effects of Metal Ions and Chemicals. Metal ions and chemicals were frequently used to probe the response of enzyme catalytic sites to inhibition or stimulation. Tables III and IV showed the effects of various metal ions and chemical reagents on the activity of the purified enzyme. Most metal ions exerted no significant effect at 1 m*M*. However, Mn^{2+} and Co^{2+} remarkably inhibited enzyme activity at this concentration.

The purified enzyme was sensitive to the assayed chemicals, and evident activity loss was measured even at low concentrations (1% (v/v) and 1 m*M*). Ethylenediaminetetraacetic acid (EDTA)

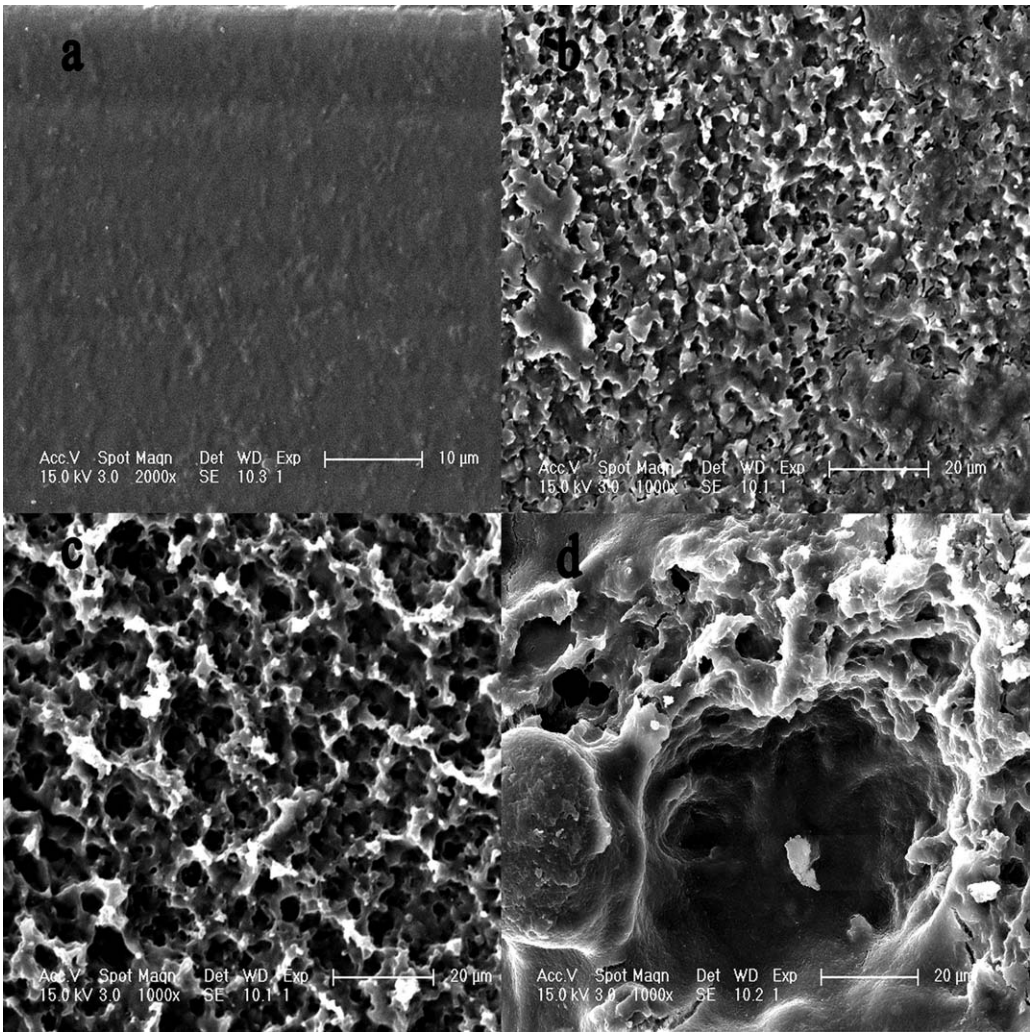


Figure 1. SEM images of the surfaces of P3/4HB films. Without strain (a); degradation by the strain for 1 day (b); degradation for 2 days (c); and degradation for 4 days (d).

inhibited the P3/4HB-degrading activity of the purified enzyme, and this finding suggested that the enzyme exhibited metal ion dependence at its active site. Phenylmethylsulfonyl fluoride (PMSF) partly inhibited its P3/4HB-degrading activity, which indicated that a serine residue was involved in the active side of the depolymerase.[25] Certain hydrophobic reagents, including Triton X-100, Tween-80, and glycerin, also markedly inhibited P3/4HB degradation by the purified enzyme. Such results suggested that the enzyme contains hydrophobic groups at its active site. However, low-concentration Triton X-100 (0.1%, v/v) revealed no obvious effect on the activity of the depolymerase, and more than 90% of enzyme activity remained. Therefore, the hydrophobic reagent with low concentration could be used as eluent to purify the P3/4HB depolymerase.

Substrate Specificity of the Purified Enzyme. Several representative polymers were applied as substrates to test the enzymatic activity of the purified P3/4HB depolymerase, and the results are shown in Table V. The enzyme showed degradation activity toward PHB and PHBV, in addition to P3/4HB, low activity against PCL, and nearly no activity toward PLA and PBS. This result indicated that the purified enzyme showed high specificity for PHAs, especially P3/4HB.

Table II. Purification of the Extracellular PHB Depolymerase from *Agrobacterium* sp. DSGZ

Purification steps	Total volume (mL)	Amount of total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold of purification	Recovery (%)
Crude culture supernatant	1000	937	2042	2.18	1	100
The elution solution	95	31.26	188	6.01	2.76	9.2

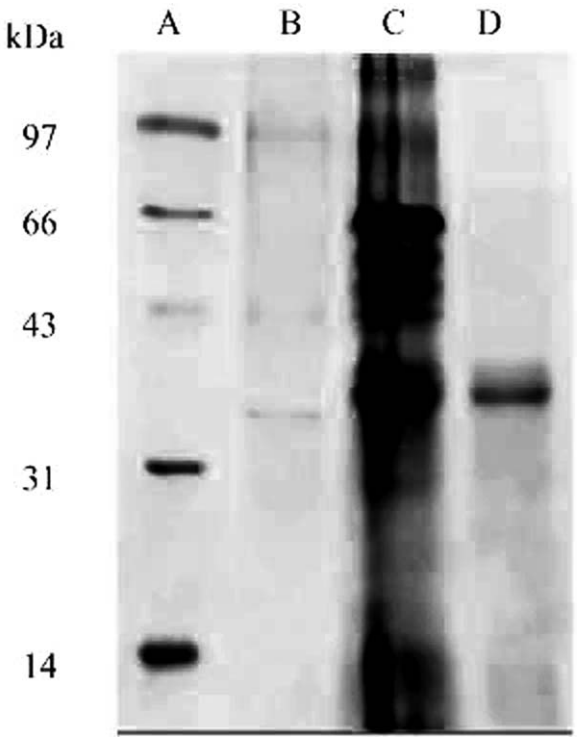


Figure 2. Commassie Blue Fast Stained 12% SDS-polyacrylamide gel electrophoresis with enzyme samples before and after purification. Lane A: protein marker; lane B: non-adsorbed; lane C: crude enzyme; and lane D: purified P3/4HB depolymerase.

Kinetic analysis of the purified enzyme on *p*-nitrophenyl acylesters (C2–C16) was carried out to test the catalytic characteristics of the enzyme on different chain lengths of substrates. The values of K_m, V_{max}, and K_{cat} were calculated from the Lineweaver–Bark curve, and results are listed in Table VI. The values of K_{cat}/K_m of C4 (228.53 s^{-1} mM^{-1}) and C8 (152.34 s^{-1} mM^{-1}) were significantly larger than those of other substrates (C2: 57.01 s^{-1} mM^{-1}, C12: 18.71 s^{-1} mM^{-1}, and C16: 28.74 s^{-1} mM^{-1}). These findings indicated that the enzyme showed higher enzymatic activity if the substrate was composed of C4 and C8. In the current research, *p*-NPC substrates with different chain lengths were used to determine the property of enzymes for the degradation of ester. Esterases hydrolyze short-chain fatty acids preferentially, whereas lipases give preference to long-chain fatty acids. In our research, the P3/4HB depolymerase enzyme showed high affinity for substrates comprising *p*-NPC (C≤10), thereby indicating that the enzyme is an esterase instead of a lipase.[20,21]

Analysis of the Degradation Products of P3/4HB by the Purified Enzyme

Mass spectrometry (MS) Analysis of P3/4HB Hydrolysis Products. After the incubation of P3/4HB emulsions with the purified enzyme for 1 h at 50°C, the water-soluble products were analyzed by a mass spectrometer. The hydroxybutyric acid (HB) monomer and HB–HB dimers, rather than trimers or other oligomers, were detected in the products (Figure 4). The spectrum of the control with inactive enzyme revealed no peaks

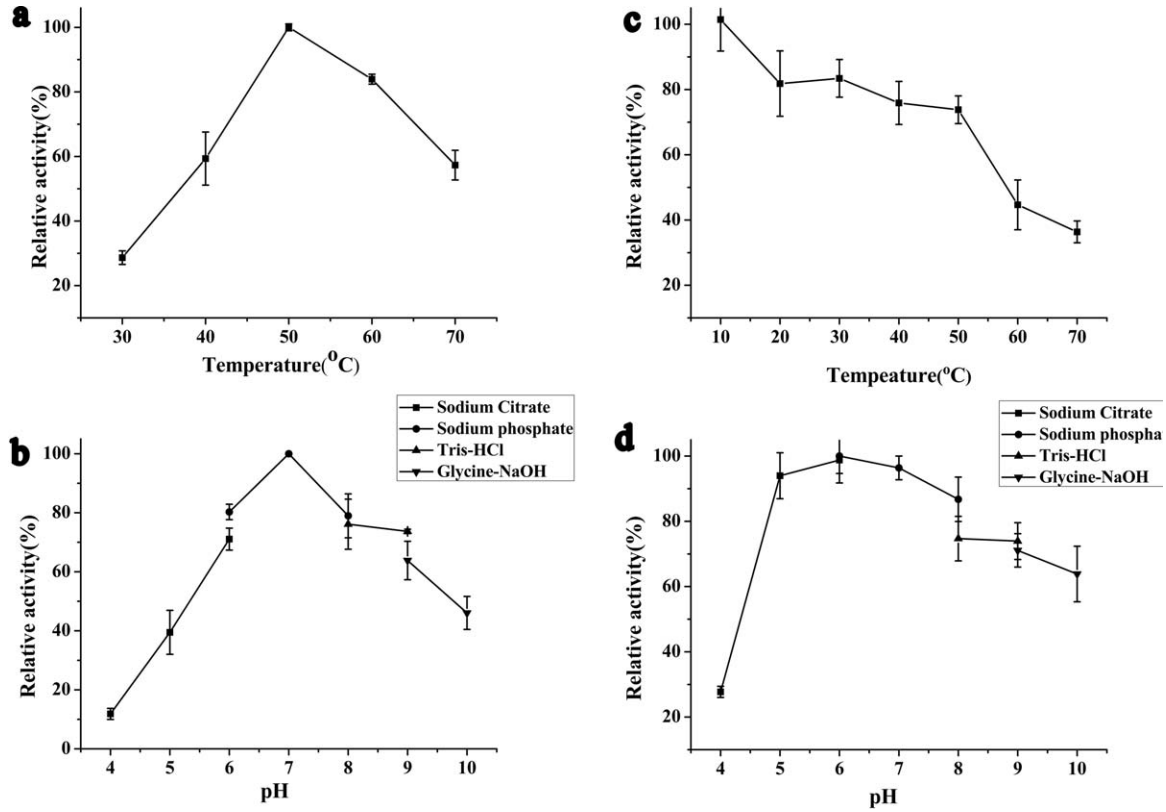


Figure 3. Effects of pH and temperature on the P3/4HB-degrading activity of the purified enzyme. Optimal temperature (a) and optimal pH (c); effect of the thermal stability of P3/4HB-degrading enzyme (b); and stability of the purified enzyme at different pH (d).

Table III. Effects of Metal Ions on the Activity of P3/4HB Depolymerase

Metal ion	Concentration	Relative activity (%)
None		100
$CuCl_2$	1 mM	95.8±1.9
	10 mM	87.2±1.7
NaCl	1 mM	107.1±2.9
	10 mM	105.5±3.0
$MnCl_2$	1 mM	45.3±2.1
	10 mM	6.2±1.7
$CoCl_2$	1 mM	47.5±4.2
	10 mM	0
$CaCl_2$	1 mM	87.8
	10 mM	61.7±3.6
$FeSO_4$	1 mM	103.9±0.5
	10 mM	69.3±0.5
NaN_3	1 mM	82.8±1.5
	10 mM	79.7±0.7
$MgSO_4$	1 mM	92.7±0.3
	10 mM	56.5±1.1

corresponding to HB monomers or dimers. These results indicated that the purified P3/4HB depolymerase mainly hydrolyzed P3/4HB via an exo-wise action to release one or two HB units at a time.

^{1}H NMR Analysis of P3/4HB Hydrolysis Products. The ^{1}H NMR spectra of degradation products generated by the purified enzyme after 24 and 72 h of enzymatic degradation are shown in Figure. 5. The selected regions in the ^{1}H NMR spectra of both investigated samples corresponded to the occurrence of signals ascribed to protons ($\delta = 3.54$ ppm) of the 3-hydroxybutyrate monomer and those ascribed to protons ($\delta = 1.33$, 4.17 ppm) of the 4-hydroxybutyrate (4HB) monomer. However, the special

Table IV. Effects of Chemicals on the P3/4HB Depolymerase

Chemical	Concentrate	Relative activity (%)
None		100
Methanol	1% (v/v)	76.9
	10% (v/v)	51.9±5.7
Ethanol	1% (v/v)	82.7±1.9
	10% (v/v)	36.5±1.9
	0.1% (v/v)	91.2±1.3
Triton X-100	1% (v/v)	27.7±3.8
	10% (v/v)	0
Tween 80	1% (v/v)	0
	10% (v/v)	0
PDMF	1 mM	65.4±3.8
	10 mM	46.1±3.8
EDTA	1 mM	65.4±3.8
	10 mM	19.2
Glycerin	1% (v/v)	30.8±3.8
	10% (v/v)	0

Table V. Substrate Specificity of the P3/4HB Depolymerase From *Agrobacterium* sp. DSGZ

Substrate	Specificity activity (U/mg)
P3/4HB	6.22
PHB	2.95
PHBV	4.12
PCL	0.33
PLA	Not detectable
PBS	Not detectable

Table VI. Kinetic Parameters of Degradation of *p*-Nitrophenyl Acyl Esters by the Purified Enzyme

Substrate (*p*-Np esters)	*K*m (m*M*)	*K*cat (s^{-1})	*K*cat/*K*m (s^{-1} mM^{-1})
C2	0.550	31.38	57.01
C4	0.886	202.41	228.53
C6	0.986	101.91	103.40
C8	0.778	118.55	152.34
C12	6.945	129.97	18.71
C16	1.829	52.67	28.74

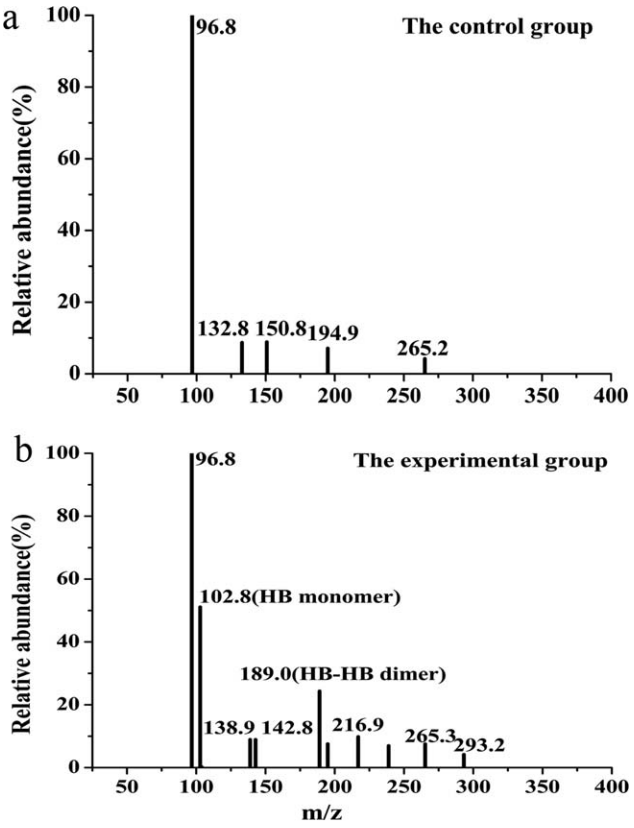


Figure 4. MS of degradation products of P3/4HB depolymerase. After degradation by the inactivation of enzyme (a) and after degradation by the purified enzyme (b).

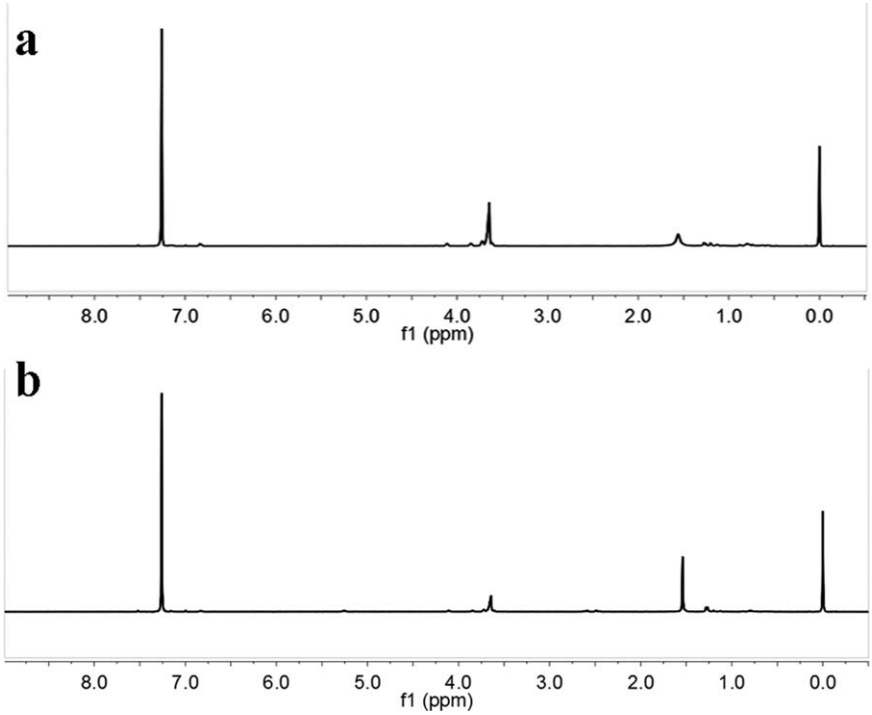
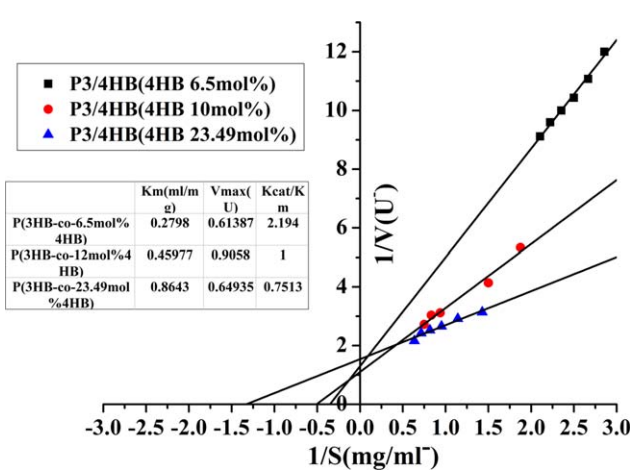


Figure 5. ^{1}H NMR spectra of degradation products by the purified enzyme. ^{1}H NMR spectra of the degradation products after 24h of degradation (a) and ^{1}H NMR spectra of the degradation products after 72h of degradation (b).

signals of 3HB and 4HB were not found in the blank control group.[26,27] According to the obtained peak areas, 3HB monomer production occurs to a greater extent than 4HB monomer production over 24 h of degradation. However, as degradation proceeded for 72 h, the 4HB monomer yield increased significantly compared with 3HB yield.

Catalytic Kinetic Analysis of the Depolymerase on P3/4HB

Catalytic kinetic analysis of the depolymerase on P3/4HB molecular chain with different 4HB ratio was carried out by emulsified P3/4HB without crystallinity. The K_m, V_{max}, and K_{cat} of the depolymerase on various P3/4HB copolymers were calculated from the Lineweaver–Burk curves (Figure 6). Emulsified P3/4HB copolymers showed similar degradation speeds despite the change in 4HB content from 6.5 mol % to 23.5 mol %, thereby suggesting that the 4HB content of P3/4HB exerted no significant effect on the cutting of the molecular chain without crystallinity. Hence, we concluded that the degradation difference of P3/4HB with different 4HB contents may depend on the property of the copolymer rather than on molecular chain enzymolysis.



	Km(ml/mg)	Vmax(U)	Kcat/Km
P(3HB-co-6.5mol% 4HB)	0.2798	0.61387	2.194
P(3HB-co-12mol%4HB)	0.45977	0.9058	1
P(3HB-co-23.49mol %4HB)	0.8643	0.64935	0.7513

Figure 6. Lineweaver-Burk curves of degradation of different P3/4HB copolymers by the purified enzyme. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

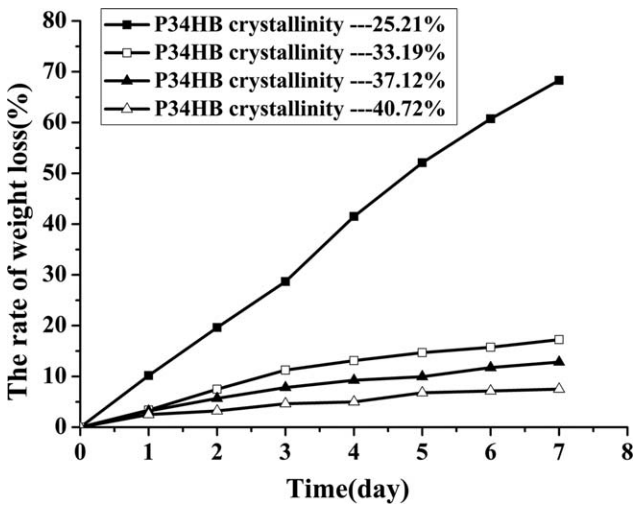


Figure 7. The weight loss of P3/4HB (4HB, 12.0 mol %) copolymers with different crystallinity.

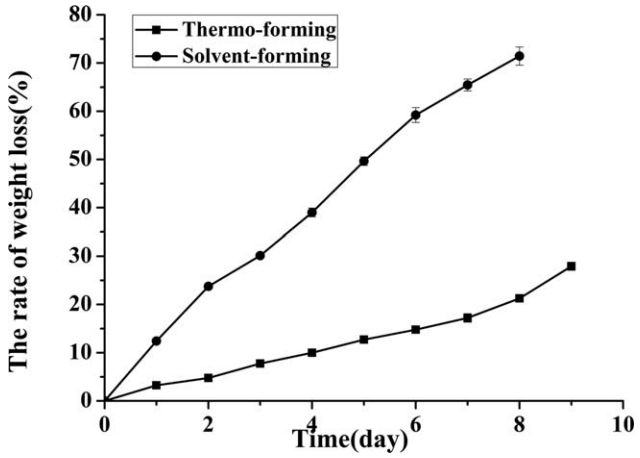


Figure 8. The weight loss of P3/4HB (4HB, 12.0 mol %) copolymer films prepared by thermo-forming and solvent-forming.

Influence of Material Properties on Degradation Characteristics

Crystallinity. P3/4HB samples with different crystallinities were degraded by depolymerase at different speeds for degradation (Figure 7). As crystallinity increased from 25.21% to 40.72%, the degradation rate of P3/4HB (4HB, 12.0 mol %) films decreased by 90% (Figure 7). We can conclude that P3/4HB films with low crystallinity are more likely to be degraded than films with high crystallinity, and the result was consistent with those of previous reports.[14,28,29]

Preparation Process. The effect of preparation process, such as thermo-forming and solvent-forming, was investigated by measuring the weight loss of copolymer films. As shown in Figure 8, the preparation method exerted a remarkable influence on the degradation of P3/4HB by the purified enzyme. The P3/4HB film prepared by solvent-forming was more easily degraded than that produced by thermo-forming. Through further research, we found that the most evident difference between thermo-formed and solvent-formed films was their crystallinity. P3/4HB films prepared by solvent-forming possessed low crystallinity and exhibited a relatively loose structure compared with films prepared by thermo-forming method. Therefore, solvent-formed P3/4HB films were hydrolyzed by depolymerase more easily.

Ratio of 4HB Monomers in P3/4HB Copolymer. Enzymatic rate increased with increasing 4HB composition, and the maximum rate was achieved at 10 mol % 4HB. Decreases in P3/4HB degradation rate were observed when the 4HB ratio in the copolymer film was increased to 12.0 and 23.5 mol % (Figure 9). The effect of 4HB ratio on copolymer degradation was not linear. A 4HB content of 10 mol % appeared to be the point of inflection. The degradation rate of P3/4HB decreased when the 4HB content was lower or higher than 10.0 mol %. Further studies were conducted to explain this effect. The properties of P3/4HB copolymers with different 4HB contents were detected, and the degree of crystallinity was observed to vary significantly among samples. Interestingly, the change in degradation rate was consistent with the change in crystallinity, thereby suggesting that the degradation of P3/4HB with different 4HB contents may be affected by the crystallinity of the copolymer. In previous reports, PHB possessed the most regular structure among all known PHAs, which are known to have fairly high degrees of crystallinity.[30] When other units were introduced to PHB, the residual structural regularity was damaged, and a decrease in the crystallinity of the material may be observed.[31–34]

CONCLUSIONS

In the present article, an extracellular P3/4HB depolymerase has been purified from *Agrobacterium* sp. DSGZ by a novel affinity chromatography method. Furthermore, the biochemical properties, including molecular weight, optimum pH and temperature, stability, and chemical inhibition, of the purified P3/4HB depolymerase have been characterized. The purified enzyme exhibited an exo-type action by which the depolymerase cleaved the first or the second ester bond in the end chain of P3/4HB and released HB monomers and HB–HB dimers. Meanwhile, the effects of the physicochemical properties, including crystallinity, preparation method, and 4HB composition, of the P3/4HB copolymer itself on P3/4HB degradation were detected. The results indicated that crystallinity is a dominant factor that

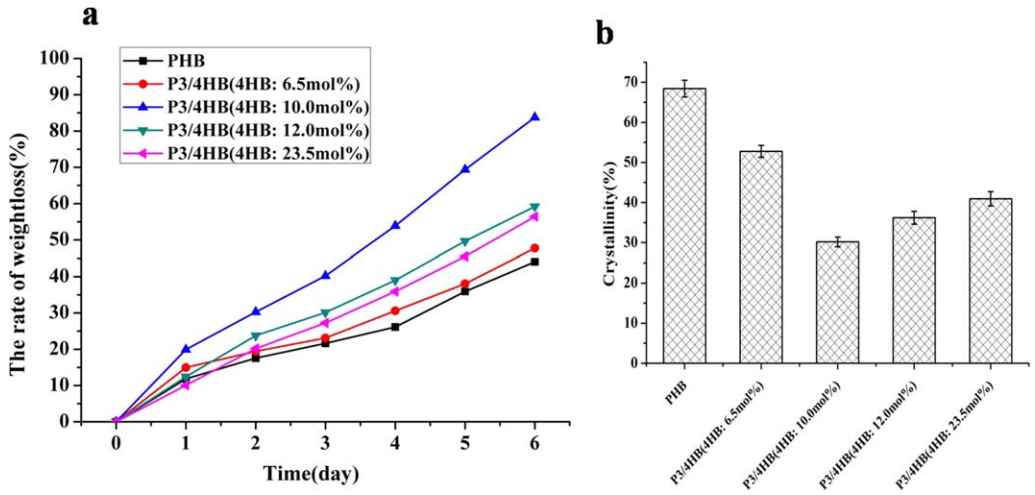


Figure 9. The weight loss of P3/4HB (4HB 0, 6.5, 10.0, 12.0, and 23.5 mol %) films by the purified enzyme (a) and different crystallinities of P3/4HB films (b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

affected P3/4HB biodegradation behavior. A study on the molecular structure and function of P3/4HB depolymerase is underway and aims to explore the catalytic degradation mechanism of the enzyme on the P3/4HB substrate.

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