SHORT COMMUNICATIONS

Enzymatic Hydrolysis of Thioester Linkages in Bacterial Poly(3-hydroxybutyrate-co-3-mercaptopropionate)s by Poly(3-hydroxybutyrate) Depolymerase Isolated from *Ralstonia pickettii* T1

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One of major approaches to modify the bacterial poly(3-hydroxybutyrate) (PHB) while reserving its biodegradability has involved the copolymerization of its monomer with some hydroxyalkanoates (HA) via bacterial fermentation,¹ comprising 3-, 4-, 5- and 6-hydroxyalkanoates with various length of the main-chain or various substituents at different positions, such as 3-hydroxypropionate (3HP),^{2–5} 3-hydroxyvalerate (3HV)^{6,7} and so forth.

Recently, Steinbüchel's group^{8–14} has succeeded to isolate a novel class of biopolymers from the poly(hydroxyalkanoate) (PHA)-accumulating bacterium, comprising one kind of completely different backbone constituents, that is, thioesters, such as poly(3-mercaptopropionate), poly(3-mercaptoputyrate), poly(3-mercaptopvalerate), poly(3-hydroxybutyrate-co-3-mercaptopropionate) [P(3HB-co-3MP)] and poly(3-hydroxybutyrate-co-3-mercaptoputyrate). Physical properties, such as solubility, crystallinity and thermal stability, of the biodegradable polymers are modified with the introduction of thioester linkages.^{8,15}

However, few research have been devoted to the enzymatic hydrolysis of thioester linkages in P(3HB-co-3MP) except for the work most recently published.¹⁶ It is found that the PHA depolymerase isolated from *Schlegella thermodepolymerans* is specific for the oxoester linkages and that thioester linkages can not be cleaved by the enzyme. PHA depolymerases are known to be quite specific to the chemical structure and physical state of polyesters. It seems necessary to investigate further the enzymatic hydro-

lyzability of the thioester linkages with considering the possible substrate specificity of depolymerase. PHA depolymerase isolated from *Ralstonia pickettii* T1 (PhaZRpi) has been well purified and characterized, and thus is one ideal enzyme to study the degradation of P(3HB-co-3MP)s.

In this communication, the enzymatic degradability of thioester linkages in the comonomer compositionally well-fractionated P(3HB-co-3MP)s by PHA depolymerase PhaZRpi is evidenced by analyzing degradation products. It is the first observation of the degradation of thioester linkages by PHA depolymerase. Moreover, in comparison with P(3HB-co-3HP)s, the comonomer compositional dependence of the enzymatic degradability will be established.

EXPERIMENTAL

Materials and Preparation of Samples

P(3HB)¹⁷ and P(3HB-co-3MP)s^{10–13,15} were synthesized by using *Ralstonia eutropha* H16. Films for the enzymatic degradation were compression-molded at 195 °C for 5 min under a pressure of 5 MPa, using Toyoseiki Mini Test Press-10, and then quenched to 30 °C for crystallization of at least 4 weeks. The enzyme was isolated from *Ralstonia pickettii* T1.¹⁹

Analytical Procedures

Wide-angle X-ray diffraction (WAXD) measurements were carried out on a Rigaku RINT-2000 (Rigaku Co., Tokyo, Japan), working at 40 kV and

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200 mA, with Ni-filtered $\text{CuK}\alpha$ radiation ($\lambda = 0.15418 \text{ nm}$). The degree of crystallinity was estimated according to the method developed by Vonk.¹⁸

Enzymatic degradation rates were measured by weight-loss measurement, as described below.⁴ At 37°C , films with initial mass of about 10 mg and initial dimension of $10 \times 10 \times 0.1 \text{ mm}$ were incubated into 1.0 mL of 0.1 M phosphate buffer ($\text{pH} = 7.5$) with $1.0 \mu\text{g}$ of extracellular PHB depolymerase isolated from PhaZRpi.¹⁹ Films were removed after reacting for a period of time, washed with distilled water and dried to constant weight in vacuum before the weight measurement. The average weight loss rate for three films was reported as the result of one sample. Control tests in the buffer solution free from enzyme, showed no detectable weight losses over the time scale of the experiment.

After incubation of 20 h, water-soluble products were characterized by ^1H NMR (JEOL GSX-270 MHz NMR spectrometer). A small amount of sodium 2,2-dimethyl-2-silapentane-5-sulfate (DSS) was added in as the internal reference of proton chemical shift. HPLC analysis were performed on a SHIMADZU HPLC Organic Acid Analysis System, equipped with two Shim-pack SPR-H ($250 \text{ mm} \times 7.8 \text{ mm}$) columns, at 45°C . The mobile phase was 4 mM p-toluenesulfonic acid aqueous solution with a flow rate of 0.8 mL/min.

RESULTS AND DISCUSSION

Normally, the bacterially synthesized copolyesters show extremely broad comonomer compositional distributions, *i.e.*, they are really blends of copolyesters with very different comonomer composition.^{3,7,20,21} To clarify exclusively the comonomer compositional dependent physical properties and biodegradation behavior, biosynthesized P(3HB-*co*-3MP)s with 18–28 mol % 3MP unit were well fractionated by the solvent-nonsolvent technique into a series of P(3HB-*co*-3MP) fractions with different 3MP unit content and much narrow comonomer compositional distribution.¹⁵ Totally, eight fractions with 3MP unit content varying from 2.9 to 61.2 mol % were selected as samples for this research. The molecular characteristics of fractionated P(3HB-*co*-3MP)s and the bacterial P(3HB) were tabulated in Table I.

In Figure 1, the crystallinity of fractionated P(3HB-*co*-3MP)s is plotted as a function of the 3MP unit content. The data of fractionated P(3HB-*co*-3HP)s⁴ are also included for comparison. Both show similar dependence on the commoner unit content. With increasing the 3MP/3HP unit content, the crystallinity of P(3HB-*co*-3MP) and P(3HB-*co*-3HP) decreases in the 3HB-rich region while increases in the 3HB-poor

Table I. Characteristics of the bacterial P(3HB) and the well-fractionated bacterial P(3HB-*co*-3MP) samples

Sample code	3MP ^a /mol %	M_n^b / $\times 10^{-5}$	M_w/M_n	Crystallinity ^c /%
P(3HB)	0	6.5	1.7	60
P(3HB- <i>co</i> -2.9 mol %3MP)	2.9	4.0	2.1	49
P(3HB- <i>co</i> -7.0 mol %3MP)	7.0	7.2	1.6	44
P(3HB- <i>co</i> -8.3 mol %3MP)	8.3	7.1	1.6	43
P(3HB- <i>co</i> -8.5 mol %3MP)	8.5	4.7	1.9	41
P(3HB- <i>co</i> -11.0 mol %3MP)	11.0	3.1	2.0	44
P(3HB- <i>co</i> -30.7 mol %3MP)	30.7	2.0	2.8	33
P(3HB- <i>co</i> -51.4 mol %3MP)	51.4	1.5	2.6	13
P(3HB- <i>co</i> -61.2 mol %3MP)	61.2	0.9	2.1	16

^aDetermined by ^1H NMR in chloroform solution. ^bMeasured by GPC. ^cEstimated by WAXD.

Figure 1. The degree of crystallinity estimated from WAXD patterns as a function of 3MP/3HP unit content for P(3HB), P(3HP), fractionated P(3HB-*co*-3MP)s, and fractionated P(3HB-*co*-3HP)s.²⁰

region, as both the 3HP and 3MP unit can not cocrystallize with the 3HB unit.

In Figure 2 are shown enzymatic degradation rates of fractionated P(3HB-*co*-3HP)s and P(3HB-*co*-3MP)s. Normally, the enzymatic degradability is quite dependent on the crystallinity of PHAs,⁴ as the ester bonds in crystalline phase are much more difficult to be cleaved by active sites of P(3HB) depolymerase than those in amorphous phase.²² Therefore, the enzymatic degradation rate of P(3HB-*co*-3MP)s first increases with the incorporation of 3MP unit into the backbone. With further increasing the 3MP unit content, however, another crucial process, *i.e.*, adsorption of enzyme on the surface of polyesters, should be taken into consideration, with noting only crystalline phase can provide a binding support to the enzyme.^{23,24} It is expected that the amount of the enzyme adsorbed on substrate surface will decrease with the crystallinity.

Figure 2. Enzymatic degradation rates at 37 °C estimated from weight loss rates as a function of 3MP/3HP unit content for P(3HB), P(3HP), fractionated P(3HB-co-3MP)s, and fractionated P(3HB-co-3HP)s.²⁰

Thus, the delicate balance between the crystalline phase specific adsorption and the amorphous phase preferential degradation leads to produce of a maximum degradation rate ($1.65 \text{ mg} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$) for P(3HB-co-3MP)s with 11 mol %3MP. Similarly, a maximum degradation rate ($2.5 \text{ mg} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$) can be observed for P(3HB-co-3HP)s with 24.1 mol %3HP.⁴

When the comonomer unit (3MP or 3HP) content increase to around 60 mol %, both P(3HB-co-3MP) and P(3HB-co-3HP) are hardly biodegraded. The poor adsorption of the enzyme to the fully amorphous surface of P(3HB-co-60 mol %3HP) should be responsible for its nondegradability.⁴ However, the crystalline phase specific adsorption can not fully explain the almost nondegradability of P(3HB-co-51.4 mol %3MP) and P(3HB-co-61.2 mol %3MP), as they still reserve 13 and 16% crystallinity, respectively. In addition, the P(3HB-co-3MP) with 3MP unit content in the 15–60 mol % region is found to be degraded much slower than their oxoester-counterpart independent of their similarity in the crystallinity. From the results shown above, it is suggested that the thioester linkages are more hardly degraded by enzymes than the oxoester ones.

In order to evaluate enzymatic hydrolyzability of thioester linkages, the water-soluble degradation product were analyzed by ^1H NMR and HPLC measurements. In Figure 3 are shown HPLC chromatograms of the buffer, 3-mercaptopropionic acid and the water-soluble degradation products from P(3HB-co-11.0 mol %3MP), P(3HB-co-30.7 mol %3MP) and P(3HB-co-61.2 mol %3MP) after 20 h incubation. Clearly, four well separated elution peaks can be

Figure 3. HPLC elution curves of water-soluble products after 20 h incubation of P(3HB-co-11.0 mol %3MP), P(3HB-co-30.7 mol %3MP) and P(3HB-co-61.2 mol %3MP) and those of 3-mercaptopropionic acid and the buffer.

observed, corresponding to four water-soluble degradation products of P(3HB-co-3MP)s. The assignments of HPLC chromatograms were carried out with the assistance of 1D ^1H NMR (*e.g.*, Figure 4) and 2D ^1H – ^1H COSY NMR analysis (data not shown). The assignment of 3MP elution peaks can be further confirmed by the HPLC elution time of 3-mercaptopropionic acid. The elution times of water-soluble products increase in the order of $3\text{HB} < 3\text{HB}-3\text{HB} < 3\text{MP} < 3\text{HB}-3\text{MP}$. In Figure 4 are shown the 1D ^1H NMR spectra of water-soluble degradation products of P(3HB-co-61.2 mol %3MP). Clearly, three kinds of products, that is, 3HB, 3MP and 3HB–3MP, can be detected, and the amount of 3MP monomer is comparable to that of the 3HB–3MP dimer. In addition, it is noted that no 3MP and 3MP–3HB dimer can be detected in the water-soluble degradation product.

Both HPLC and ^1H NMR measurements detected quite a bit of 3MP monomer in the water-soluble degradation product. Totally, four kinds of ester linkages, *i.e.*, 3HB–3HB, 3HB–3MP, 3MP–3HB and 3MP–3MP, distribute along the backbone of P(3HB-co-3MP). Among them, 3HB–3MP and 3MP–3MP are thioester linkages. With ascending the origin of 3MP monomer during biodegradation, it is clear that the produce of 3MP monomer should experience the cleavage of thioester linkage at least once. In addition, no 3MP–3MP dimer can be detected in the water-soluble degradation products of all samples. The sequence distribution of the comonomers 3HB and 3MP in P(3HB-co-3MP) is not random and does not obey Bernoullian statistics, as 3MP-oligomers consisting of up to 16 3MP units were detected in the degra-

Figure 4. ^1H NMR spectra of water-soluble products after 20 h incubation of P(3HB-co-61.2 mol %3MP).
*DSS

dation product.¹⁶ The cleavage of the 3MP–3MP linkage will certainly produce some detectable amount of 3MP dimer. Inversely, the absence of 3MP dimers indicates that 3MP–3MP linkages can not be hydrolyzed by the enzyme, which is well accordant with the previous report.¹⁶ Therefore the cleavage of 3HB–3MP thioester linkage is the most plausible pathway to produce the 3MP monomer. In addition, the absence of 3MP–3HB dimer possibly indicates that the cleavage most likely occurs at the 3MP–3HB oxoester linkage itself but not at the left adjacent thioester linkage.

In conclusion, both the enzymatic hydrolysis rate of P(3HB-co-3MP) and that of P(3HB-co-3HP) show strong dependence on the comonomer unit content, as the crystallinity of copolyesters varies with the comonomer unit content. In comparison with P(3HB-co-3HP)s, the biodegradation of P(3HB-co-3MP)s is also quite related to the poor biodegradability of thioester linkages. It is of interest to note that it may be 3HB–3MP thioester linkages but not 3MP–3MP ones that can be hydrolyzed by PHB depolymerase. The selective enzymatic hydrolysis of thioester linkages may be related to the substrate specificity of PHB depolymerase, which will be explored in our future work.

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