Lipase-Catalyzed Reaction of Molecularly Pure Linear and Cyclic Poly(3-hydroxybutanoate)s: Evidence of Cyclic Polymer Formation

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Molecularly pure linear and cyclic poly(3-hydroxybutanoate) [P(3HB)] polymer species were subjected to hydrolysis, transesterification and intramolecular esterification by lipase to produce two series of linear and cyclic polymer structures having higher and lower molecular weights. It was found that a cyclic polymer was produced by the lipase-catalyzed reaction of the polymer chain.

Poly(3-hydroxybutanoate) [P(3HB)] is one of the most promising candidates for the biodegradable commodity plastics which can be produced both microbially[1] and chemically.[2] The *in vitro* synthesis of P(3HB) using *(R)*-3-hydroxybutyryl-CoA with PHB synthase or polymerase has recently been reported.[3] The *in vitro* synthesis using an enzyme is independent of the metabolism, and therefore, one may be able to design and synthesize functional polymers. It has been revealed that lipase acted as an effective catalyst for the polymerization of lactones, hydroxy acids, and diols with diacids to produce the corresponding polyesters.[4,5] We previously reported that the enzyme-catalyzed polymerization of β-butyrolactone yielded both linear and cyclic P(3HB)s.[6] It is reported that the enzyme-catalyzed polymerization of β-lactone involved enzyme-activated monomers in a living polymerization mechanism; however, the molecular weight of the resulting polymer became constant within several thousands and no linear relationship between the molecular weight and monomer conversion was established in the region of higher than 50% monomer conversion.[6] These phenomena may indicate that the extensive hydrolysis, transesterification and intramolecular esterification reactions related to the polymer chain occurred in the lipase-catalyzed polymerization system. However, the lipase-catalyzed reaction of the P(3HB) polymer chain has not yet been reported. Detailed studies are now needed.

In the present study, in order to analyze these reactions related to the polymer chain by lipase, molecularly pure P(3HB)s having linear and cyclic structures were subjected to an enzymatic reaction. The molecularly pure P(3HB) was isolated from P(3HB) obtained by the lipase-catalyzed ring-opening polymerization of *(R,S)*-β-butyrolactone.[7]

Fractionation was carried out by preparative supercritical fluid chromatography (SFC).[8] The molecular weight was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).[9] It was found that P(3HB) could be fractionated according to the polymer structures as well as by the molecular weights. Figure 1 shows the typical SFC profiles of P(3HB) having M_n = 1620 and M_w/M_n = 1.8[10] obtained by the lipase-catalyzed ring-opening polymerization of *(R,S)*-β-butyrolactone.[7] These peaks could be isolated by repeated fractionation using SFC according to the polymerization degree, and the linear and cyclic structures by gradient elution using ethanol with/without acetic acid as the modifier. The fractionated polymer structure was identified by both ^{1}H NMR[11] and MALDI-TOF MS.

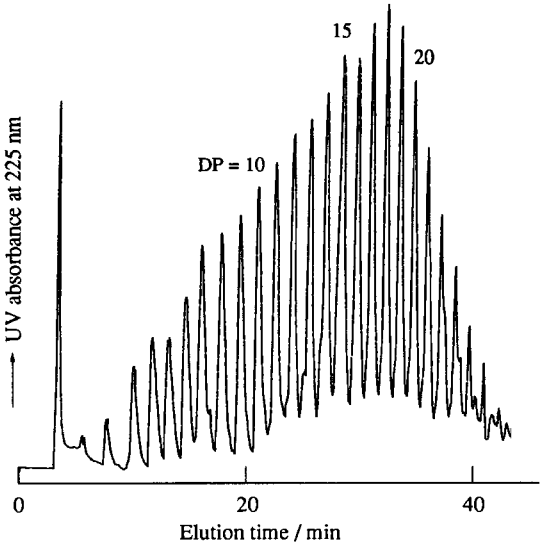

Figure 1. SFC profiles of poly(3-hydroxybutanoate) having M_n = 1620 and M_w/M_n = 1.8 obtained by the lipase-catalyzed polymerization of β-butyrolactone. The peak at 3 min is due to chloroform used as solvent for sample solution.

The fractionated P(3HB) was subjected to an enzymatic reaction and the reaction products were analyzed by MALDI-TOF MS with respect to the molecular weight dispersion. That is, the fractionated P(3HB) was dissolved in diisopropyl ether (1% solution), and then lipase from *Candida antarctica*[12] was added and stirred at 60 °C for 48 h. After the reaction, the reaction mixture was filtered through a Celite pad with suction, and the diisopropyl ether was evaporated to obtain the reaction mixture. This was analyzed by the MALDI-TOF MS. Typical results for the fractionated cyclic type P(3HB)s with the degree of polymerization (DP) of 11 and 12 are shown in Figure 2. In order to get a sufficient amount of fractionated polymer for the enzymatic reaction and analyses, two fractions of DP=11 and 12 were combined for the reaction in this experiment (Figure 2a).

It was found that the isolated polymer fraction subjected to the lipase-catalyzed reaction produced two series of polymers with linear and cyclic structures having the higher and lower molecular weights. As shown in Figure 2c, the 11 and 12-mer of 3-hydroxybutanoate with cyclic structures subjected to the enzymatic reaction produced both linear and cyclic P(3HB)s composed of about 8-mer to 25-mer of 3-hydroxybutanoate after a 48-h incubation at 60 °C in diisopropyl ether solution. It was also found that the fractionated uniform linear P(3HB) was

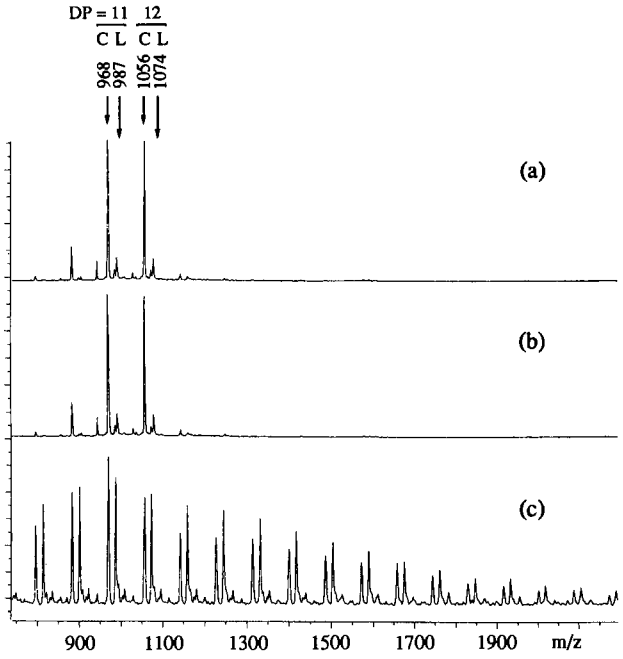

Figure 2. MALDI-TOF MS spectra of fractionated P(3HB)s with DP = 11 and 12 [M+Na$^+$]. (a) initial cyclic P(3HB); (b) incubation for 48 h at 60 °C without enzyme as blank test; (c) reaction with lipase from *Candida antarctica* (Novozym 525) for 48 h at 60 °C. C: cyclic form, L: linear form.

subjected to the enzymatic reaction with lipase to produce two series of linear and cyclic structures having the higher and lower molecular weights. Similar results were obtained in toluene instead of diisopropyl ether. Under these conditions without lipase, no significant change was observed, suggesting that the lipase actually catalyzed the reaction (Figure 2b).

It is indicated that the cyclic P(3HB) was first cleaved by lipase to produce the acyl-enzyme intermediate which was further reacted with other P(3HB) polymer fractions (transesterification) or water (hydrolysis) to produce the higher or lower molecular weight linear fractions of P(3HB). In addition, the acyl-enzyme intermediate might react with the hydroxy terminal group of the same polymer chain in a back-biting way to form the cyclic P(3HB) (intramolecular esterification). Cyclization was more facilitated when lipase was used instead of chemical catalyst, such as alkali-metal alkoxides. This might be a characteristic feature for the enzymatic reaction. Therefore, both cyclic and linear P(3HB)s with the higher and lower molecular weight P(3HB) were produced from both the cyclic and linear P(3HB)s by lipase. These proposed enzymatic reaction mechanisms are summarized in Figure 3. It is reported that the lipase-catalyzed ring-opening polymerization of β-butyrolactone proceeded in a living way[4]; however, due to the extensive hydrolysis, transesterification and intramolecular esterification reaction, the resulting P(3HB) polymer contained a relatively wide molecular weight dispersion with linear and cyclic structures. Details of this reaction are now under study.

In conclusion, it was confirmed that linear and cyclic P(3HB) polymer species were subjected to hydrolysis, transesterification and intramolecular esterification by lipase to produce two series of polymers of linear and cyclic structures having the higher and lower molecular weights. A cyclic polymer was produced by the lipase-catalyzed reaction of the polymer chain.

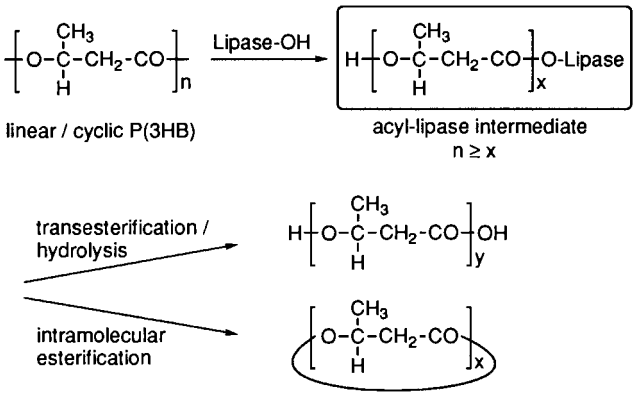

Figure 3. Proposed lipase-catalyzed reaction mechanism of linear and cyclic P(3HB) polymers.

Lipase from *Candida antarctica* (Novozym 525) was kindly supplied by Novo Nordisk Bioindustry Ltd. (Chiba, Japan). This work was supported by a Grant-in-Aid for Scientific Research on Priority Area, "Sustainable Biodegradable Plastics", No 11217212 (1999) from the Ministry of Education, Science, Sports and Culture (Japan).

References and Notes

1 a) E. A. Dawes and P. J. Senior, *Adv. Microb. Physiol.* **10**, 135 (1973). b) Y. Doi, "Microbial Polyesters", VCH Publishers, New York (1990). c) Y. Steinbuchel and H. E. Valentin, *FEMS Microbiol. Lett.* **128**, 219 (1995).
2 Y. Hori and T. Hagiwara, *Biolog. Macromol.*, **25**, 237 (1999).
3 L. Su, R. W. Lenz, Y. Takagi, S. Zhang, S. Goodwin, L. Zhong, and D. P. Martin, *Macromolecules*, **33**, 229 (2000).
4 S. Kobayashi, S. Shoda, and H. Uyama, *Adv. Polym. Sci.*, **121**, 1 (1995).
5 S. Matsumura, *Function & Materials (Kinouzairyou)*, **17**(**9**), 32 (1997).
6 S. Matsumura, Y. Suzuki, K. Tsukada, and K. Toshima, *Macromolecules*, **31**, 6444 (1998).
7 P(3HB) was prepared by the lipase-catalyzed ring-opening polymerization of *(R,S)*-β-butyrolactone in bulk using 20% *Candida rugosa* lipase at 60 °C for 48 h basically according to Ref. 6.
8 The preparative SFC was performed on a JASCO SCF-201 chromatograph equipped with a 10 mm i.d. x 250 mm column packed with silica gel (SFCpak SIL-5, JASCO) using super critical fluid of CO_2 as mobile phase, and ethanol with/without acetic acid as the modifier. The fluid pressure was controlled to 25 MPa and column temperature was 70 °C. Chromatograms were recorded using a UV detector operated at a wavelength of 225 nm.
9 MALDI-TOF mass spectrum was measured on a Brucker Protein TOF in reflection mode using 2,5-dihydroxybenzoic acid as the matrix.
10 The number-average molecular weight (M_n) and weight-average molecular weight (M_w) were measured by a size-exclusion chromatography (SEC) using SEC columns (Shodex K-803L + K-806L + K-800D, Showa Denko Co., Ltd., Tokyo) with a refractive index detector. Chloroform was used as the eluent. The SEC system was calibrated with a polystyrene standard.
11 The spectral data of *(R,S)*-P(3HB) having an M_n of 1620 are shown to be representative. IR(KBr): 2984 (CH_2), 1748, 1184 (ester C=O) cm^{-1}. ^{1}H-NMR (270 MHz: $CDCl_3$): δ = 1.3 (m, 3H), 2.6 (m, 2H), 5.3 (m, 1H).
12 Enzyme: *Candida antarctica* lipase (Novozym 525, 14100 LU/mL according to the supplier) was supplied by Novo Nordisk Bioindustry Ltd. (Chiba, Japan).