Enzyme-catalyzed Ring-opening Polymerization of β-Butyrolactone Using PHB Depolymerase

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Poly(3-hydroxybutyrate) [P(3HB)] was prepared by the ring-opening polymerization of (*R,S*)-*β*-butyrolactone (BL) using two types of PHB depolymerase with or without substrate-binding domains (SBD) as the catalyst. The SBD lacking PHB depolymerase exhibited better catalytic activities for the ring-opening polymerization of BL.

The syntheses of aliphatic polyesters, such as poly(3-hydroxybutyrate) [P(3HB)], have been extensively studied both by fermentation and chemical processes in the field of biodegradable materials science.[1,2] Recently, these reveal that in vitro enzymatic syntheses become an effective method to design and synthesize environmentally acceptable polymeric materials.[3] A variety of polymers have been synthesized by the ring-opening polymerization of 4-, 6-, 7-, 12-, 13- and 16-membered lactones using enzyme as a catalyst.[4-7] Among them, the enzyme-catalyzed polymerization of four-membered lactones will be a feasible way to prepare relatively low-molecular-weight poly(3-hydroxyalkanoate)s which may have attractive applications in the industrial field for the next generation.[8] We previously reported the lipase-catalyzed polymerization of *β*-butyrolactone (BL), preferential polymerization of (*R*)-BL, and the production of two structural isomers of linear and cyclic P(3HB).[9] It is known that bacterial P(3HB) is degraded by the PHB depolymerase, which is excreted as the extracellular enzyme, to produce monomeric and oligomeric 3-hydroxybutyrates. The enzyme consists of catalytic domain, putative linker region and SBD as observed in other enzymes which degrade water-insoluble substrates, such as cellulase and chitinase (Figure 1). The active site is a serine residue in the pentapeptide Gly-X_1-Ser-X_2-Gly, namely lipase box, which is common for serine hydrolase. However, preparation of P(3HB) using PHB depolymerase as the reverse reaction of the enzyme has not yet been reported.

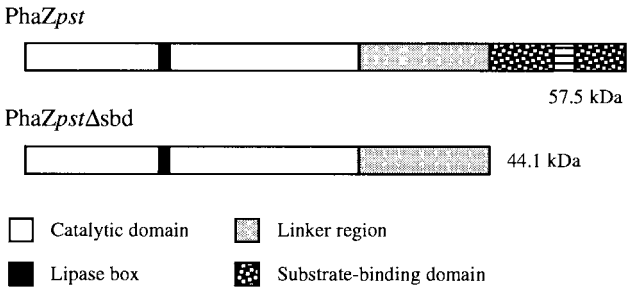
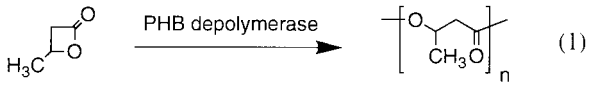


Figure 1. Domain structures of PhaZ*pst* and PhaZ*pst*Δsbd.

In this report, preparation of P(3HB) by the enzyme-catalyzed ring-opening polymerization of BL was studied using PHB depolymerase with respect to the catalytic activity for the polymerization and the effects of the SBD of the enzyme. The PHB depolymerase of *Pseudomonas stutzeri* YM1006 was purified,[10] and its gene was cloned and sequenced.[11] The PHB depolymerase (PhaZ*pst*) was purified to electrophoretic homogeneity from the culture of *P. stutzeri* YM1006 by hydrophobic column chromatography.[10] The PHB depolymerase lacking SBD (PhaZ*pst*Δsbd) was purified from the recombinant *Escherichia coli*.[12] The molecular weights were deduced to be 57.5 kDa and 44.1 kDa from consisted amino acids, respectively.[11]

The enzyme-catalyzed ring-opening polymerization of BL using PHB depolymerase was carried out as shown in Scheme 1. The general procedure is as follows: A mixture of enzyme[13] and BL was stirred in bulk under argon in a capped vial placed in a thermostated oil bath. After the reaction, the reaction mixture was dissolved in chloroform, and the insoluble enzyme was removed by filtration. The organic solvent was then evaporated under a slight reduced pressure to obtain the polymer. The molecular weight and the molecular weight dispersion of the polymer were measured by GPC.[14] The monomer conversion was determined by ^{1}H-NMR.[15] The polymer structure was analyzed by ^{1}H-NMR, ^{13}C-NMR, IR and elemental analysis.[16] Table 1 summarizes the results of the ring-opening polymerization of BL with and without enzyme. It was found that BL was readily polymerized by both the PhaZ*pst* and PhaZ*pst*Δsbd to obtain P(3HB) with a weight average molecular weight (M_w) greater than 3000 in a high monomer conversion. It was confirmed that both the M_w and the monomer conversion were significantly low in control experiment or without the enzyme, indicating that the PHB depolymerase actually promoted the polymerization of BL (entries 4, 9, 10 and 11 in Table 1). It was also found that the PhaZ*pst*Δsbd showed higher catalytic activities for the ring-opening polymerization of BL with respect to the M_w of the polymer. That is, lack of SBD of PHB depolymerase tended to promote the polymerization of BL to P(3HB). It is suggested that the SBD in PHB depolymerase was not necessary for the polymerization of BL. The SBD of PHB depolymerase may be responsible for the specific interaction of the producing P(3HB) polymer chain. Details of the polymerization mechanism with PHB depolymerase are now under study. We previously reported the lipase-catalyzed ring-opening poly-



(1)

Table 1. Polymerization of (*R,S*)-β-butyrolactone using PHB depolymerase from *Pseudomonas stutzeri* YM1006

Entry[a]	Enzyme[b]	Wt %[c]	Temp / °C	Time / d	Conversion / %	$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$
1	PhaZ*pst*	5	60	3	17	730	1.2
2	PhaZ*pst*	3	80	3	25	1170	1.3
3	PhaZ*pst*	5	80	3	95	1740	1.4
4	t-PhaZ*pst*	5	80	3	16	1080	1.1
5	PhaZ*pst*Δsbd	5	60	3	21	1470	1.3
6	PhaZ*pst*Δsbd	1	80	3	29	1460	1.5
7	PhaZ*pst*Δsbd	3	80	3	51	1920	1.3
8	PhaZ*pst*Δsbd	5	80	3	97	3420	1.4
9	t-PhaZ*pst*Δsbd	5	80	3	12	1020	1.3
10	---	---	60	3	2	600	1.2
11	---	---	80	3	5	840	1.1

[a]Entries 4 and 9 : Control experiments. Entries 10 and 11 : blank tests. [b]The enzymes t-PhaZ*pst* and t-PhaZ*pst*Δsbd are thermally inactivated PHB depolymerase activity at 100 °C before the reaction. PhaZ*pst*: PHB depolymerase from *P.stutzeri* YM1006, PhaZ*pst*Δsbd: PHB depolymerase lacking SBD. [c]Weight % of enzyme to BL.

merization of BL. It is difficult to simply compare the polymerization results because of the difference in thier purity. However, the rate of polymerization of BL by PhaZ*pst*Δsbd was faster than that using porcine pancreatic lipase (PPL) or *Candida cylindracea* lipase under the same reaction conditions of 3-d polymerization at 80 °C in bulk.[9]

In conclusion, it has been found that PHB depolymerase could catalyze the polymerization of BL to produce P(3HB). The SBD moiety of PHB depolymerase was not essential for the enzyme-catalyzed polymerization of BL. Polymerization of BL was facilitated by removing the SBD of PHB depolymerase. This is the first report on the in vitro enzyme-catalyzed synthesis of P(3HB) using the PHB depolymerase.

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13 Enzymes: Activities of PhaZ*pst* and PhaZ*pst*Δsbd are 65 U/mg protein and 24 U/mg protein, respectively.
14 The number-average molecular weight (M_n), weight-average molecular weight (M_w) and molecular weight dispersion (M_w/M_n) were measured by a gel permeation chromatography (GPC) using GPC columns (Shodex K-803 + K-8006 + K-800D, Showa Denko Co., Ltd., Tokyo, Japan) with refractive index detector. Chloroform was used as the eluent. The GPC system was calibrated with polystyrene standards.
15 ^{1}H-NMR spectra were recorded with a JOEL Model GSX-270 (270 MHz) spetrometer (JOEL Ltd., Tokyo, Japan). The monomer conversion of BL to P(3HB) was determined by comparison of the ^{1}H-NMR spectral integration intensities for the δ = 1.58 ppm peak corrsponding to the methyl protons of monomeric BL with the corresponding methyl protons of P(3HB) at δ = 1.25 ppm.
16 The spectral data and elemental analysis of P(3HB) (entry 3 in Table 1) are shown to be representative. IR (KBr): 2986 (CH_2), 1734, 1186 (ester C=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$): δ = 1.3 (d, 3H), 2.6 (m, 2H), 5.3 (m, 1H). ^{13}C-NMR (67.5 MHz, $CDCl_3$): δ = 19.8 (CH_3), 40.8 (CH_2), 67.6 (CH), 169.3 (ester C=O). Anal. Calcd for $(C_4H_6O_2)_n$: C, 55.81; H, 7.02%. Found: C, 55.76; H, 6.90%.