Chemo-Enzymatic Synthesis of Poly(lactate-*co*-(3-hydroxybutyrate)) by a Lactate-Polymerizing Enzyme

Kenji Tajima,* Yasuharu Satoh, Toshifumi Satoh, Rumi Itoh, Xuerong Han, Seiichi Taguchi, Toyoji Kakuchi, and Masanobu Munekata

Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo 060-8628, Japan

Received November 17, 2008; Revised Manuscript Received January 25, 2009

ABSTRACT: In our previous paper, we synthesized poly-3-hydroxybutyrate [P(3HB)] by using the water-organic-solvent two-phase reaction system (TPRS), in which (*R*)-3-hydroxybutyrylCoA [(*R*)-3HBCoA] was continuously supplied to PHA synthase by the ester exchange reaction between CoA and thiophenol in thiophenyl (*R*)-3HB [(*R*)-3HBTP]. By applying TPRS to the screening, we found a lactate- (LA-) polymerizing enzyme from PHA synthases. The enzyme was an engineered PHA synthase, which stereoselectively copolymerized (*R*)-LA together with (*R*)-3HB. NMR and GPC revealed that the TPRS successfully synthesized poly(lactate-*co*-(3-hydroxybutyrate)) [P(LA-*co*-3HB)] using the LA-polymerizing enzyme as a catalyst. The molar ratios of LA in the copolymers were controllable in the range of 0 to 36 mol% by varying the ratio of (*R*)-LATP and (*R*)-3HBTP fed into the TPRS. The number-average molecular weight and the polydispersity of P(36 mol%-*co*-3HB) were 1.1×10^4 and 1.4, respectively. This is the first report on the chemo-enzymatic synthesis of P(LA-*co*-3HB) by a LA-polymerizing enzyme.

Introduction

From the standpoint of environmental preservation, biodegradable polymers have attracted much attention. Polylactate (PLA) is the most popular biodegradable plastic and has been widely studied all over the world. In general, PLA is prepared by the three processes below: (i) production of lactic acid by fermentation; (ii) purification of lactic acid and/or preparation of lactide; (iii) polycondensation of lactic acid or ring-opening polymerization of lactide. Lactic acid (LA) has two enantiomers, and the physical properties of PLAs are influenced by the ratio of L- [(*S*)-] and D- [(*R*)-] isomers in the polymers. Therefore, the optical purity of lactic acid as a starting material and stereocontrol during the polymerization reaction are significantly important. The major lactic acids in nature are L-type and racemic, and D-lactic acid is a minor product. The industrial fermentation method for L-lactic acid has already been established,[1−3] and several fermentation methods for D-lactic acid have been also reported.[4−6] Recently, the stereocomplex of PLLA and PDLA has drawn attention,[7,8] and the importance of D-lactic acid and PDLA production is increasing.

PLA is a very attractive material, as mentioned above, but its application is limited in part due to its brittle properties, such as poor elongation, slow crystallization rate, and so on. To improve the properties of PLA, several methods have been developed, such as copolymerization[9,10] and the formation of stereo complex.[7,8] Abe et al.[9] and Hanes et al.[10] reported the synthesis of copolymers by the ring-opening copolymerization of (*S*,*S*)-lactide with (*R*)-β-butyrolactone and by the ring-opening polymerization of (*S*,*S*)-lactide using polyhydroxyalkanoate (PHA) as a macroinitiator. The synthesized copolymers had better properties than those of PLA,[9,10] suggesting the usefulness of the copolymerization. In general, a highly purified lactide with high optical purity and a large amount of energy are needed for the chemical synthesis of PLA and LA-incorporated polymer by using ring-opening polymerization. Therefore, a more efficient process for producing polymers is widely sought. To develop an innovative process, research has been conducted in two steps: (I) screening of a lactate- (LA) polymerizing enzyme and analysis of a product synthesized by the selected enzyme and (II) construction of a microbial factory for large-scale production. The results of the latter research have been already reported.[11] Therefore; the results of the former research are described in this paper.

We first attempted to find a LA-polymerizing enzyme. Although several systems for in vitro PHA synthesis have been reported to date,[12−15] we used the water-organic solvent two-phase reaction system (TPRS)[15] to screen a LA-polymerizing enzyme. Since PHA is insoluble in water, the ability to polymerize LA was detected by the formation of a precipitate. PHA synthases were chosen as candidates of LA-polymerizing enzymes based on the structural similarities of LA and hydroxyalkanoate (HA) which is a monomer of PHA. PHA synthase is a key enzyme for PHA biosynthesis, and about 50 PHA synthase (PhaC) genes have been cloned from various microorganisms.[16] PHA synthases have been divided into four classes based on their primary structures and substrate specificities.[17,18] We have obtained a new type of PHA synthase from *Bacillus* sp.,[19,20] and the synthase has been categorized into class IV based on differences in molecular weight, gene structure, kinetics, and so on.[18] In this study, we examined representative PHA synthases belonging to the individual classes and three engineered PHA synthases. Each PHA synthase was introduced into the TPRS, including thiophenyl (*R*)-lactate [(*R*)-LATP] and thiophenyl (*R*)-3-hydroxylbutyrate [(*R*)-3HBTP] as substrate precursors. In the TPRS, hydroxyalkanoylCoA (HACoA) is produced by the ester exchange reaction between CoA and thiophenyl alkanoate (HATP), and the HACoA is sequentially polymerized by PHA synthase. From the results of in vitro synthesis, a PHA synthase was selected as a LA-polymerizing enzyme, and the stereoselectivity of the enzyme was analyzed by using (*R*)-LATP or (*S*)-LATP as a substrate precursor. Then the structure and molecular weight of the product were analyzed by NMR and GPC, respectively. Finally, we attempted to rigorously control molar ratios of LA in P(LA-*co*-3HB).

We found a LA-polymerizing enzyme from PHA synthases by applying the TPRS to the screening of the enzyme. By using this enzyme, P(LA-*co*-3HB) was chemo-enzymatically synthe-

* Corresponding author. Telephone: +81 11 706-7816. Fax: +81 11 706-7816. E-mail: ktajima@eng.hokudai.ac.jp.

10.1021/ma802579g CCC: $40.75
Published on Web 02/27/2009

sized. In addition, the molar ratios of LA in the copolymers were controllable in the range of 0 to 36 mol% by varying the ratio of (*R*)-LATP and (*R*)-3HBTP fed into the TPRS. This is the first report on the chemo-enzymatic synthesis of P(LA-*co*-3HB).

Experimental Section

Materials. Hexane, chloroform, acetonitrile, thiophenol, methanol, ethanol, methyl lactate, and coenzymeA (CoA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). (*R*)-3-hydroxybutyric acid was a product of Sigma-Aldrich Japan (Tokyo, Japan). All other chemicals were of reagent grade or better.

Measurements. The ^{1}H NMR spectra of the polymers were obtained using a Bruker MSL400 spectrometer (400 MHz) at a 90° pulse with a 4 ms, 3,000 Hz spectral width and a 4 s repetition rate. The COSY spectrum was recorded in deuterated chloroform ($CDCl_3$) at 25 °C using a JEOL JNM-A400II instrument (400 MHz) and the chemical shifts reported in ppm with tetramethylsilane (TMS) as the internal reference.

The molecular weights of the obtained polymers were determined by gel-permeation chromatography (GPC) using tandem TSKgel Super HZM-H columns (6.0 mm i.d. × 150 mm; TOSOH, Tokyo) using chloroform as an eluate, and the calibration was performed using polystyrene samples as standards.

Differential scanning calorimetory data were recorded in the temperatures ranging from −50 to +220 °C on a Brucker AXS DSC3100 under nitrogen flow rate of 100 mL/min. The products were encapsulated in aluminum pans and heated from −50 to +220 at 10 °C·min^{-1} (first heating scan). The melt samples were then quenched until −50 at −40 °C·min^{-1}. They were heated from −50 to 220 at 10 °C·min^{-1} (second heating scan). The grass-transition temperature (T_g) was taken as midpoint on the heat capacity change. The melting temperature (T_m) was determined from positions of the endothermic peaks. The T_g and T_m of P(3HB) or P(17 mol%-*co*-3HB) were −2 and +155 °C or −1 and +147 °C.

Synthesis of thiophenyl esters. The synthesis of thiophenyl (TP) esters was performed according to the method described by Yuan et al.[21] Each methyl (*R*)-lactate or methyl (*S*)-lactate was used as a starting material in the synthesis of (*R*)-LATP or (*S*)-LATP. To purify the products, flash column chromatography was carried out on a silica gel 60 (230−400 mesh, Merck, Germany) using hexane containing 5% ethyl acetate as eluate. Thin-layer chromatography (TLC) was also performed on silica gel plates with a fluorescent indicator (0.25 mm, Merck). Compounds were detected by ultraviolet light.

Preparation of PHA synthases. We selected PHA synthases from *Ralstonia eutropha* (*R. eutropha* = *Cupriavidus necator*), *Pseudomonas* sp. 61-3, *Synechosystis* sp. PCC6803 and *Bacillus* sp. INT005,[19,20] and three engineered PHA synthases from *Pseudomonas* sp. 61-3[22,23] as candidates to synthesize P(LA-*co*-3HB). The PHA synthases from *R. eutropha*, *Pseudomonas* sp. 61-3, *Synechocystis* sp. PCC6803, and *Bacillus* sp. INT005 were expressed as CRe, CPs, CSs, and CBs, respectively. All PHA synthases were prepared using a His-tag system according to the method described in a previous paper.[14] The PHA synthase gene from each bacterium was amplified by PCR using specific primers. Each PCR product was purified, digested with appropriate restriction enzymes, and inserted into pQE30 (Qiagen, Hilden, Germany), which expressed a His-tagged fusion protein. Recombinant *Escherichia coli* BL21(DE3) harboring both pREP-4 and pQEREC, pQEPSC(WT), pQEPSC(ST), pQEPSC(QK), pQEPSC(STQK), pQESSC, or pQEBSC were used to produce CRe, CPs(WT), CPs(ST), CPs(QK), CPs(STQK), CSs, or CBs, respectively. The engineered PHA synthases were two single mutants (Ser325Thr (ST) and Gln481Lys (QK)) and a double mutant carrying these mutations (STQK).[22,23] PHA synthases were purified and assayed by the reported protocols,[14] and (*R*,*S*)-3-hydroxybutyrylCoA [(*R*,*S*)-3HBCoA] was used as a substrate for the assay.

Screening of a LA-Polymerizing Enzyme. TPRS has been used for the bioconversion of substrates with low solubility in water.[24,25]

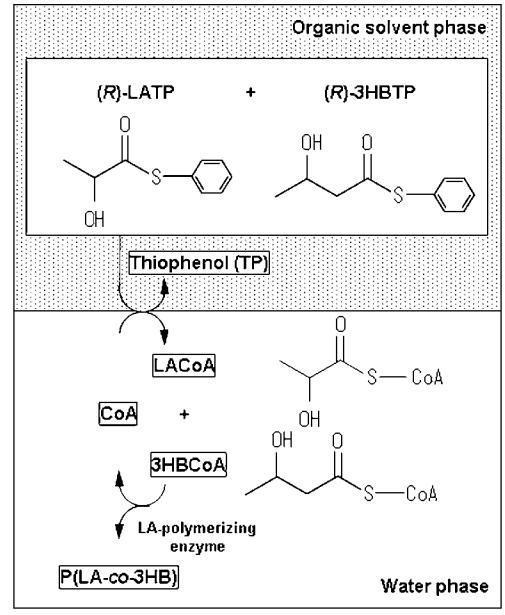

Figure 1. Chemo-enzymatic synthesis of P(LA-*co*-3HB) using TPRS.

In this system, substrate precursors and enzymes are dissolved in the organic solvent phase and the water phase, respectively. We applied the TPRS[15] to the screening of an LA-polymerizing enzyme. The reaction system is shown in Figure 1. A water phase (5 mL) contains 100 mM sodium phosphate (pH 7.5) and 1.0 mM CoA. (*R*)-LATP (25 μmol) and (*R*)-3HBTP (25 μmol) were dissolved in 5 mL of hexane. The water phase was poured into a test tube with a screw cap, and then hexane containing the monomer precursors was added into the tube. Each purified PHA synthase was added into a reaction mixture to start the reaction. Then, 200 μg of enzyme for CRe, CSs, or CBs and 2.5 mg for CPs, CPs(ST), CPs(QK), or CPs(STQK) were used for the reactions. The polymerization reaction was carried out at 30 °C in static for 72 h. The polymerizing activity of PHA synthase was judged by the generation of the polymer-like precipitation and the formation of thiophenol (TP) with the progress of the reaction.

Chemo-Enzymatic Synthesis of P(LA-*co*-3HB) by Using TPRS. The chemo-enzymatic synthesis of P(LA-*co*-3HB) was performed by the same method with the screening of a LA-polymerizing enzyme. After polymerization reaction, a product was accumulated at the bottom (aqueous phase) of a test tube. After the hexane was removed, 5 mL of chloroform was poured into the reaction tube. The chloroform and aqueous phases were mixed by a vortex mixer, then the tube was incubated for 3 h at 70 °C to solve the product into the chloroform, completely. After the cooling to room temperature, the chloroform (lower) phase was filtered through a 0.2 μm poly(tetrafluoroethylene) (PTFE) membrane to remove insoluble materials such as a denatured enzyme, then the filtrate was concentrated into about 1 mL by using an evaporator. Ten milliliters of methanol was added to the solution, which was then stored at 4 °C for 16 h to precipitate a polymer. The obtained precipitate was collected using a 0.2 μm PTFE membrane, and the dried powder was used for the analysis.

Results and Discussion

Ester Exchange Reaction between Thiophenol and CoA. The ester exchange reaction between the thiophenyl group and CoA is necessary to form substrates. It has been reported that the ester exchange progresses under weak alkaline conditions[26] and the optimal pH of PHA synthase is around 7.0.[27] From these facts, pH 7.5 was selected as the pH value of the reaction buffer. To confirm complete progression of the ester exchange reaction under these conditions, (*R*)-LATP in aceto-

Table 1. Control of Molar Ratios of LA and 3HB in Polymers

run	molar ratio in a reaction mixture [(*R*)-LATP/(*R*)-3HBTP]	molar ratio in a polymer[a] [(*R*)-LA/(*R*)-3HB]	yield (%)	$M_n \times 10^{-4}$[b]	M_w/M_n
1	0/100	0/100	53.4	2.2	2.5
2	25/75	17/83	31.5	1.1	1.6
3	50/50	36/64	17.7	1.1	1.4
4	75/25	n.d.	7.9	n.d.[d]	n.d.
5	90/10	n.d.	trace	n.d.	n.d.
6	100/0	n.d.		n.d.	n.d.
7	50[c]/50	0/100	12.6	0.78	1.6

[a] Determined by NMR. [b] Determined by GPC. [c] (*S*)-LATP was used as a substrate precursor. [d] Key: n.d., not determined.

nitrile was added to the 100 mM sodium phosphate buffer (pH 7.5) containing CoA. The mixture was incubated at 30 °C with stirring.[26,28] After the reaction, 1 M H_3PO_4 (0.13 mL) was added to the solution to stop the reaction, and the mixture was then analyzed by HPLC. A peak corresponding to CoA decreased as the reaction progressed, and a peak corresponding to (*R*)-LACoA appeared (Figure S1). These results confirmed that the ester exchange progressed and that (*R*)-LATP was converted into (*R*)-LACoA. The ester exchange rate of (*R*)-LATP was lower than that of (*R*)-3HBTP.

Screening of a LA-Polymerizing Enzyme and Its Stereoselectivity. In the screening of a LA-polymerizing enzyme, an engineered PHA synthase, CPs(STQK),[22,23] from *Pseudomonas* sp. 61-3 obviously produced a white precipitate (Figure S2). To confirm the progress of the reaction, the organic solvent phase was analyzed by TLC. The TP esters decreased when TP increased (Figure S3), suggesting that the reaction successfully progressed. From these results, we selected CPs(STQK) as a LA-polymerizing enzyme.

In general, enzymes have substrate specificities, and PHA synthase recognizes (*R*)-hydroxyalkanoylCoAs as substrates. In LA, there are L- (*S*-form) and D- (*R*-form) isomers. Therefore, (*S*)-LATP was synthesized as well as (*R*)-LATP to confirm the stereoselectivity of the LA-polymerizing enzyme, CPs(STQK), toward LACoA. (*S*)-LATP (25 μmol) and (*R*)-3HBTP (25 μmol) were dissolved in 5 mL hexane, and the hexane solution was poured into 5 mL aqueous phase. The polymerization reaction was started by the addition of the LA-polymerizing enzyme to the water phase. The polymerization reaction was carried out at 30 °C in static for 72 h. In the ^{1}H NMR spectrum of the product obtained from the reaction mixture, only peaks derived from P(3HB) were observed (Figure S4b). This suggests that the LA-polymerizing enzyme could not incorporate (*S*)-LA into the polymer. The yield and the molecular weight of the product obtained using (*S*)-LATP were lower than those using (*R*)-LATP. Since (*S*)-LATP can be converted into (*S*)-LACoA as well as can (*R*)-LATP, the decreases in the yield and molecular weight (run 7 in Table 1) could be due to the decrease of available CoA molecules. Other reasons could be the binding of (*S*)-LACoA onto the catalytic site and the inhibition of polymer elongation.

Chemo-Enzymatic Synthesis of P(LA-*co*-3HB) Using TPRS. To confirm that the precipitate was a LA-incorporated polyester, the purified sample was analyzed by GPC and NMR. The number-average molecular weight of the polymer was 1.1 $\times$ 10^4 (run 3), which was smaller than that of P(3HB) obtained by the same method. An unimodal peak with the polydispersity of 1.4 was observed in the GPC chart (Figure S5).

The ^{1}H NMR spectra of (a) a mixture of PLA and P(3HB) and (b) the product (run 3) obtained from the reaction mixture are shown in Figure 2. In the ^{1}H NMR spectrum of a mixture (Figure 2a), the simple mixed peaks of P(3HB) and PLA were observed, while very complex peaks, which were obviously different from those of the mixture of the PLA and P(3HB),

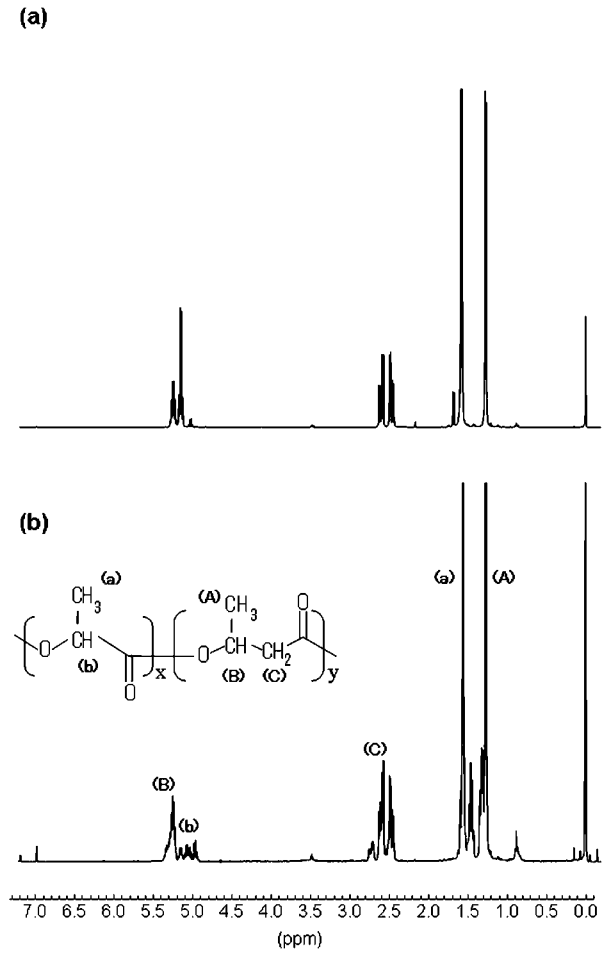

Figure 2. ^{1}H NMR spectra of (a) a mixture of PLA and P(3HB) and (b) P(36 mol% LA-*co*-3HB) (run 3) obtained from the reaction mixture containing (*R*)-LATP and (*R*)-3HBTP as substrate precursors and CPs(STQK) as a catalyst, respectively.

were observed in the ^{1}H NMR spectrum of the product (Figures 2b, S6, and S7). This suggests that the product was a copolymer composed of LA and 3HB units and was not a simple mixture or block copolymer of PLA and P(3HB). To determine the assignments for the ^{1}H-peaks, the 2D NMR (COSY) chemical shift correlation was measured (Figure 3). The peaks at around 2.6 ppm appear to be the methylene protons in 3HB units, and the assignment of the methine proton and methyl protons in 3HB units can be determined by the correlation between methine or methyl protons and methylene protons. The peaks at around 5.1 ppm appear to be the methine protons in LA units, and the assignment of the methyl protons in LA units can be determined by the correlation between methine and methyl protons. These results suggested that the product was the copolymer composed of LA and 3HB.

From the comparison among ^{1}H NMR spectra of PLA, P(3HB) and P(LA-*co*-3HB), it was suggested that the peaks at ca. 5.26 and 5.16 ppm were methine protons in 3HB* of 3HB-3HB*-3HB and LA* of LA-LA*-LA, respectively (Figure 4). The peaks corresponding to LA-LA*-LA were scarcely observed in the ^{1}H NMR spectrum of P(17 mol% LA-*co*-3HB) [Figure 4b, run 2], suggesting that almost LA units were randomly incorporated in the copolymer (Figure 4). From the result of 2D-NMR analysis, the peaks between ca. 4.95 and 5.13 ppm observed in the ^{1}H NMR spectrum of P(LA-*co*-3HB) were confirmed to be a methine proton in LA unit. The peaks at ca. 4.97 ppm existed apart from those of LA-LA*-LA; therefore, those could be methine proton in LA* of 3HB-LA*-3HB. While, the intensities of the peaks at ca. 5.06 ppm were increased with

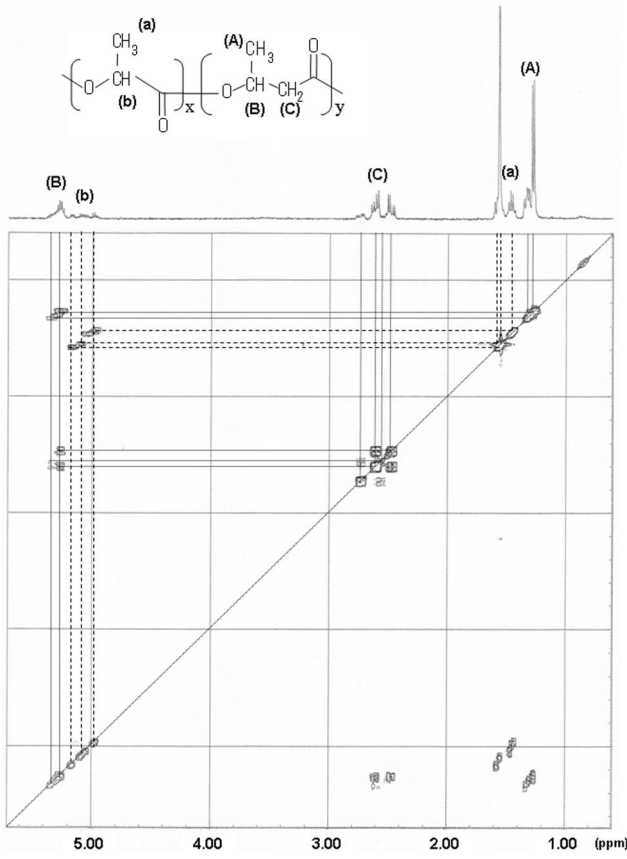

Figure 3. $^{1}H-^{1}H$ chemical shift correlation NMR spectrum (COSY) of P(36 mol % LA-*co*-3HB) (run 3) obtained from the reaction mixture containing (*R*)-LATP and (*R*)-3HBTP as substrate precursors and CPs(STQK) as a catalyst, respectively.

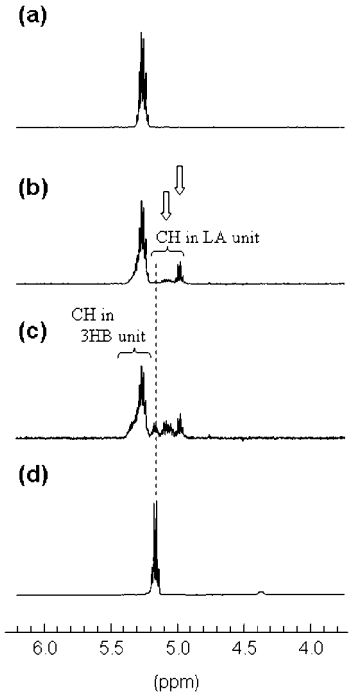

Figure 4. ^{1}H NMR spectra of (a) P(3HB) (run 1), (b) P(17 mol% LA-*co*-3HB) (run 2), (c) P(36 mol% LA-*co*-3HB) (run 3), and (d) PLA.

the increment of LA ratio in P(LA-*co*-3HB) (Figure 4, parts b and c); hence, the peaks could correspond to the methine protons in LA* of LA-LA*-3HB and 3HB-LA*-LA.

The molar ratios of LA unit in the products, which were calculated from the integral area in the ^{1}H NMR spectra, were 17 mol% and 36 mol%, respectively (runs 2 and 3). The obtained polymer was soluble in chloroform but insoluble in methanol, ethanol, acetone, and hexane.

From the results of GPC and NMR analyses, we concluded that the obtained product was P(LA-*co*-3HB) with a random sequence.

Control of Molar Ratios of LA and 3HB in Polymers. In general, the physical properties of PHA vary with the monomer compositions in the polymers. It is easy to control the monomer composition in PHA in vitro; that is one advantage of in vitro synthesis. To confirm the possibility of control in the monomer composition, (*R*)-LATP and (*R*)-3HBTP were added to the reaction mixture at various molar ratios (LA/3HB: 0/100, 25/75, 50/50, 75/25, 90/10, 100/0) in the same way as mentioned in the Experimantal Section. The monomer compositions and molecular weights of PHAs synthesized at various LA/3HB ratios are summarized in Table 1. The yields of the polymers were decreased with the increase in the ratio of (*R*)-LATP, e.g., 2.3 mg for 0/100 of LA/3HB (run 1), 1.3 mg for 25/75 of LA/3HB (run 2), 1.0 mg for 50/50 of LA/3HB (run 3), and 0.3 mg for 75/25 of LA/3HB (run 4). In the experiment using only (*R*)-LATP (run 5), LA-homopolymer, PLA, could not be synthesized. The molar ratios of LA in the product increased with the increase of the (*R*)-LATP concentration in the reaction mixture, suggesting that it is possible to control the monomer ratios in the polymers. The molar ratios of LA in the polymers were slightly lower than those in the corresponding reaction mixtures, meaning that this could be due to the low ester exchange rate of LATP. The yields and molecular weights of the products decreased when the concentration of (*R*)-LATP increased; this could be also due to the low ester exchange rate of LATP.

The elongation of a polymer chain needs the immobilization and activation of a hydroxyl group and the nucleophilic attack of the activated hydroxyl group to the carbonyl carbon in an elongating polymer. Since the position of the carbonyl carbon in the elongating polymer is not changed, the position of the hydroxyl group in the donor molecule is important to the progress of a reaction. The reaction rate for a nucleophilic attack of LA could be lower than that of 3HB, because the molecular length of LA is shorter than that of 3HB. This could cause the decreases in the yields and molecular weights of P(LA-*co*-3HB) with the increases of their LA ratios (Table 1, runs 2, 3, 4, 5, and 6).

Conclusion

We found a lactate- (LA-) polymerizing enzyme from PHA synthases by using the TPRS as a screening method. The selected enzyme was an engineered PHA synthase, which stereoselectively copolymerized (*R*)-LA together with (*R*)-3HB. The molar ratios of LA in P(LA-*co*-3HB) were controllable in the range of 0 to 36 mol% by varying the ratio of (*R*)-LATP and (*R*)-3HBTP fed into the TPRS. The number-average molecular weight (M_n) and the polydispersity (M_w/M_n) of P(36 mol% LA-*co*-3HB) were 1.1×10^4 and 1.4, respectively. This is the first report on the in vitro synthesis of P(LA-*co*-3HB) by a LA-polymerizing enzyme.

Very recently, we reported on the in vivo production of P(LA-*co*-3HB) using the LA-polymerizing enzyme screened by the TPRS.[11] In that paper, a microbial factory was constructed, which made it possible to produce P(LA-*co*-3HB) in one step. That is, we succeeded in constructing a new methodology to develop novel PHAs. Since the TPRS can supply substrates with various structures to PHA synthase, it is possible to find a combination of a substrate and enzymes that can recognize and polymerize the substrate. This successful example suggests the

usefulness of our system in the development of a new type of PHA. By using this methodology, it will be possible to produce novel biopolyesters with multifunctions.

Acknowledgment. We thank Dr. Teruoki Tago and Dr. Ken'ichiro Matsumoto, Hokkaido University, for their useful comments on this paper. We also thank Mr. Eiji Yamada, Hokkaido University, for his technical support in NMR measurements. This work was supported by the Industrial Technology Research Grant Program in 2003 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan and the Global COE Program (Project No. B01: Catalysis as the Basis for Innovation in Materials Science) and a Grant-in-Aid for Scientific Research (No. 18760587) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Supporting Information Available: Figures showing HPLC chart of LACoA, photograph of P(LA-*co*-3HB) synthesized by TPRS, result of TLC analysis of an organic solvent phase after reaction, ^{1}H NMR spectra of products obtained from reaction mixtures containing only (*R*)-3HBTP or both (*S*)-LATP and (*R*)-3HBTP, GPC chart of P(LA-*co*-3HB), and enlarged ^{1}H NMR spectra of P(LA-*co*-3HB). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Yu, R.-C.; Hang, Y. D. *Biotechnol. Lett.* **1989**, *11*, 597–600.
(2) Miura, S.; Arimura, T.; Hoshino, M.; Kojima, M.; Dwiarti, L.; Okabe, M. *J. Biosci. Bioeng.* **2003**, *96*, 65–69.
(3) Saitoh, S.; Ishida, N.; Onishi, T.; Tokuhiro, K.; Nagamori, E.; Kakimoto, K.; Takahashi, H. *Appl. Environ. Microbiol.* **2005**, *71*, 2789–2792.
(4) Fukushima, K.; Sogo, S.; Miura, S.; Kimura, Y. *Macromol. Biosci.* **2004**, *4*, 1021–1027.
(5) Ishida, N.; Suzuki, T.; Tokuhiro, K.; Nagamori, E.; Onishi, T.; Saitoh, S.; Kitamoto, K.; Takahashi, H. *J. Biosci. Bioeng.* **2006**, *101*, 172–177.
(6) Okino, S.; Suda, M.; Fujikura, K.; Inui, M.; Yukawa, H. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 449–454 .
(7) Tsuji, H.; Hyon, S.-H.; Ikada, Y. *Macromolecules* **1991**, *24*, 5651–5656.
(8) Tsuji, H. *Macromol. Biosci.* **2005**, *5*, 569–597.
(9) Abe, H.; Doi, Y.; Hori, Y.; Hagiwara, T. *Polymer* **1997**, *39*, 59–67.
(10) Haynes, D.; Abayasinghe, N. K.; Harrison, G. M.; Burg, K. J.; Smith, D. W., Jr. *Biomacromolecules* **2007**, *8*, 1131–1137.
(11) Taguchi, S.; Yamada, M.; Matsumoto, K.; Tajima, K.; Satoh, Y.; Munekata, M.; Ohno, K.; Kohda, K.; Shimamura, T.; Kambe, H.; Obata, S. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 17323–17327.
(12) Jossek, R.; Steinbüchel, A. *FEMS Microbiol. Lett.* **1998**, *168*, 319–324.
(13) Liu, S.-J.; Steinbüchel, A. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 545–552.
(14) Satoh, Y.; Tajima, K.; Tannai, H.; Munekata, M. *J. Biosci. Bioeng.* **2003**, *95*, 335–341.
(15) Tajima, K.; Satoh, Y.; Nakazawa, K.; Tannai, H.; Erata, T.; Munekata, M.; Kamachi, M.; Lenz, R. W. *Macromolecules* **2004**, *37*, 4544–4546.
(16) Rehm, B. H.; Steinbüchel, A. *Int. J. Biol. Macromol.* **1999**, *25*, 3–19.
(17) Steinbüchel, A.; Hustede, E.; Liebergesell, M.; Pieper, U.; Timm, A.; Valentin, H. *FEMS Microbiol. Rev.* **1992**, *9*, 217–230.
(18) Rehm, B. H. A. *Biochem. J.* **2003**, *376*, 15–33.
(19) Tajima, K.; Igari, T.; Nishimura, D.; Nakamura, M.; Satoh, Y.; Munekata, M. *J. Biosci. Bioeng.* **2003**, *95*, 77–81.
(20) Satoh, Y.; Minamoto, N.; Tajima, K.; Munekata, M. *J. Biosci. Bioeng.* **2002**, *94*, 343–350.
(21) Yuan, W.; Jia, Y.; Tian, J.; Snell, K. D.; Müh, U.; Sinskey, A. J.; Lambalot, R. H.; Walsh, C. T.; Stubbe, J. *Arch. Biochem, Biophys.* **2001**, *394*, 87–98.
(22) Takase, K.; Matsumoto, K.; Taguchi, S.; Doi, Y. *Biomacromolecules* **2004**, *5*, 480–485.
(23) Taguchi, S.; Doi, Y. *Macromol. Biosci.* **2004**, *4*, 146–156.
(24) Antonini, E.; Carrea, G.; Cremonesi, P. *Enzyme Microb. Technol.* **1981**, *3*, 291–296.
(25) Aono, R.; Doukyu, N.; Kobayashi, H.; Nakajima, H.; Horikoshi, K. *Appl. Environ. Microbiol.* **1994**, *60*, 2518–2523.
(26) Zhang, S.; Kamachi, M.; Takagi, Y.; Lenz, R. W.; Goodwin, S. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 131–136.
(27) Song, J. J.; Zhang, S.; Lenz, R. W.; Goodwin, S. *Biomacromolecules* **2000**, *1*, 433–439.
(28) Zhang, S.; Takagi, Y.; Lenz, R. W.; Goodwin, S. *Biomacromolecules* **2000**, *1*, 244–251.

MA802579G