Polymer Journal, Vol. 38, No. 7, pp. 737–741 (2006)

SHORT COMMUNICATIONS

Biosynthesis of Terpolythioesters with 3-Mercaptopropionate Unit

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(Received February 7, 2006; Accepted March 2, 2006; Published June 20, 2006)

KEY WORDS Biosynthesis / 3-Mercaptopropinic Acid / Terpolythioester / *Ralstonia eutropha* / [doi:10.1295/polymj.PJ2005228]

A wide variety of bacteria accumulate an optically active polymer of (R)-3-hydroxybutyric acid as an intracellular strage material of carbon and energy sources.[1,2] Poly[(R)-3-hydroxybutyrate] (P[3HB]) isolated from bacteria is a biodegradable and biocompatible thermoplastic with a melting temperature around 180 °C.[3,4] Bacterial P(3HB) has attracted industrial attention as an environmentally degradable plastic for a wide range of medical, marine, and agricultural applications.[5] However, there exist several deficiencies of bacterial P(3HB) as an engineering material for it's inherent properties of brittleness and thermal unstability above melting point.[6] To modify the properties of P(3HB), a series of copolymers such as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)],[4,7] poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)],[8] poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) [P(3HB-*co*-3HP)],[9,10] poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HH)],[11,12] and poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoate) [P(3HB-*co*-3HA)][13] have been produced by a variety of bacteria with respect to specific carbon sources. Further, a few terpolymers such as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) [P(3HB-*co*-3HV-*co*-4HB)],[14] poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxyvalerate) [P(3HB-*co*-3HV-*co*-4HV)][15] and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyheptanoate) [P(3HB-*co*-3HV-*co*-3HHp)][16] have been also produced. Recently, Steinbüchel *et al.* biosynthesized the coplymers consisting of 3HB and 3-mercaptoalkanoic acid units such as poly(3-hydroxybutyrate-*co*-3-mercaptopropionate) [P(3HB-*co*-3MP)][17] and poly(3-hydroxybutyrate-*co*-3-mercaptobutyrate) [P(3HB-*co*-3MB)][18] by *Ralstonia eutropha* H16 (DSM 428). In addition, P(3MP), poly(3-mercaptobutyrate) [P(3MB)] and poly(3-mercaptovalerate) [P(3MV)] homopolymers have been biosynthesized by engineered *Escherichia coli*.[19] These microbial homopolythioesters and copolythioesters containing sulfur in the backbone were designated as the representative of an eighth class of biopolymers, which were classified into nucleic acids, proteins, polysaccharides, polyhydroxyalkanoates, polyisoprenoid, lignin and polyphosphate, respectively.[17] However, the biosynthesis of the terpolythioesters containing 3-mercaptopropionate (3MP) unit have been yet not reported. In this study, we described at first the biosynthesis and properties of novel terpolythioesters, namely poly(3-hydroxybutyrate-*co*-3-mercaptopropionate-*co*-4-hydroxybutyrate) [P(3HB-*co*-3MP-*co*-4HB)] and poly(3-hydroxybutyrate-*co*-3-mercaptopropionate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3MP-*co*-3HV)], by a wild-type *Ralstonia eutropha* H16 (ATCC 17699), and found that the 3MP fraction in terpolythioester dramatically increased to as high as 71 mol % by using the mixed carbon sources cosisting of 3,3′-thiodipropionic acid (TDP) and γ-butyrolactone, compared with the value reported previously.[20] Microbial terpolythioesters are interesting materials because of their different chemical and physical properties compared with the corresponding copolyoxoesters and terpolyoxoesters. Generally, polythioethers are reported that the thermal stability is better than the polyoxyethers.[21] Therefore, the melting points or thermal decomposition for terpolythioesters may be expected to rise further than for thier oxygen analogues. The biodegradability of polyoxoesters, namely polyhydroxyalkanoates (PHA) are examined in detail, while little is known about that of the polythioesters. Hence, the study on the biodegradability of different polythioesters is desired.

EXPERIMENTAL

Culture Method

A wild-type *Ralstonia eutropha* H16 (formerly *Alcaligenes eutrophus*, ATCC 17699) was used in this study. The biosynthesis of terpolythioester with 3-mercaptopropionate (3MP) unit was carried out by a two-step batch cultivation. *R. eutroha* H16 cells were

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Table I. Production of terpolythioesters from thiodipropionic acid (TDP) and different substrates by *R. eutropha* for 48 h at 30 °C and pH 7.2[a]

No	Carbon source ($10\,gL^{-1}/5\,gL^{-1}$)	Cell dry weight (gL^{-1})	Polythioester[b] Content (wt %)	Composition[c] (mol %)			
				3HB	3MP	3HV	4HB
1	TDP/Propionic acid	3.08	15.7	61	7	32	0
2	TDP/Valeric acid	4.37	24.1	33	1	66	0
3	TDP/Levulinic acid	3.52	28.2	58	1	41	0
4	TDP/γ-Valerolactone	2.31	16.1	31	17	52	0
5	TDP/1,4-Butanediol	3.90	5.8	83	16	0	1
6	TDP/4-HBA[d]	3.05	10.6	59	14	0	27
7	TDP/γ-Butyrolactone[e]	3.06	13.4	72	6	0	22
8	TDP/γ-Butyrolactone	3.42	15.2	36	59	0	5
9	TDP/ε-Caprolactone	4.08	24.5	53	26	0	21

[a]When lactones were used as cosubstrates of TDP, the cultivation was carried out in media that lactones were added after TDP solution was ajusted to pH 7.2. [b]Polythioester content in cell dry weight. [c]Determined by ^{1}H NMR. [d]4-HBA denotes sodium 4-hydroxybutyrate. [e]The cultivation was carried out in media that TDP was added to γ-butyrolactone solution treated with equimolar NaOH and then was adjusted to pH 7.2.

first grown in the nutrient-rich medium containing yeast extract, polypeptone, fish extract, and $(NH_4)_2SO_4$ under aerobic conditions at 30 °C for 24 h. The cells were harvested by centrifugation, and transferred into a nitrogen-free mineral medium (100 ml) containing thiodipropionic acid (TDP) and different substrates that supplied 4HB unit or 3HV unit, respectively. When lactones were used as co-substrates of TDP, they were added after the mineral medium containing TDP was adjusted to pH 7.2. The mineral medium contained 0.265 g of KH_2PO_4, 0.380 g of $Na_2HPO_4 \cdot 12H_2O$, 0.02 g of $MgSO_4$ and 0.1 ml of microelement solution. The microelement solution contained 9.8 g of $FeCl_3$, 7.8 g of $CaCl_2$, 0.156 g of $CuSO_4 \cdot 5H_2O$, 0.119 g of $CoCl_2$, 0.118 g of $NiCl_2 \cdot 6H_2O$, and 0.104 g of $CrCl_3 \cdot 6H_2O$ (per liter of 0.1NHCl). The cells were aerobically cultivated in this medium for prescribed time at 30 °C and 120 rpm, harvested by centrifugation, and finally lyophilized. Polythioesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with *n*-hexane. The analysis of structure and composition of polythioesters was carried out with the infrared absorption spectrum (IR) and the nuclear magnetic resonance (NMR).

RESULTS AND DISCUSSION

The coplymer of 3-hydroxybutyrate and 3-mercaptopropionate [P(3MP-*co*-3HB)] was reported recently to be produced by cultivating cells of *Ralstonia eutropha* strain H16 (DSM 428) in mineral salts medium containing 3-mercaptopropionic acid or 3,3′-thiodipropionic acid (TDP) plus fructose or gluconic acid as carbon sources.[17] In this communication, we report that a wild-type *Ralstonia eutropha* strain H16 (ATCC 17699) produces novel terpolymers, poly(3-hydroxybutyrate-*co*-3-mercaptopropionate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3MP-*co*-3HV)] and poly(3-hydroxybutyrate-*co*-3-mercaptopropionate-*co*-4-hydroxybutyrate) [P(3HB-*co*-3MP-*co*-4HB)] from TDP plus 3HV unit- or 4HB unit-supplying substrates, respectively. Table I shows the accumulation of terpolythioesters. The compositions and chemical structures of the terpolythioesters were determined from ^{1}H NMR, ^{13}C NMR and IR spectroscopies. When *R. eutropha* strain was cultivated on $10\,gL^{-1}$ of TDP plus $5\,gL^{-1}$ of propionic acid as 3HV unit-supplying substrate in nitrogen-limited mineral salt medium at pH 7.2 for 48 h, 7 mol % of 3MP unit was detected in the accumulated polyesters together with 3HB and 3HV units. However, the 3MP unit was little introduced in polymers, when cultivated for 48 h on TDP plus valeric acid or levulinic acid as 3HV unit-supplying substrate. On the other hand, a terpolythioester P(3HB-*co*-3MP-*co*-3HV) with up to 17 mol % 3MP unit was produced when this strain cultivated on TDP plus γ-valerolactone as 3HV unit-supplying substrate. It seems that these results are because valeric acid and levulinic acid are more effective substurates for the 3HV unit containing polyester synthesis in *R. eutropha* than propionic acid or γ-valerolactone. That is, the valeric acid or levulinic acid as cosubstrate of TDP is more predominantly utilized than TDP for the polyester accumulation in *R. eutropha*, resulting in P(3HB-*co*-3HV) copolymer with minor 3MP unit. Next, the microbial synthesis of P(3HB-*co*-3MP-*co*-4HB) terpolythioesters was investigated by using sodium 4-hydroxybutyrate, 1,4-butanediol, γ-butyrolactone, and ε-caprolactone that are 4HB unit-supplying carbon sources as cosubsturate of TDP. As shown in Table I, when *R. eutropha* strain was cultivated on medium

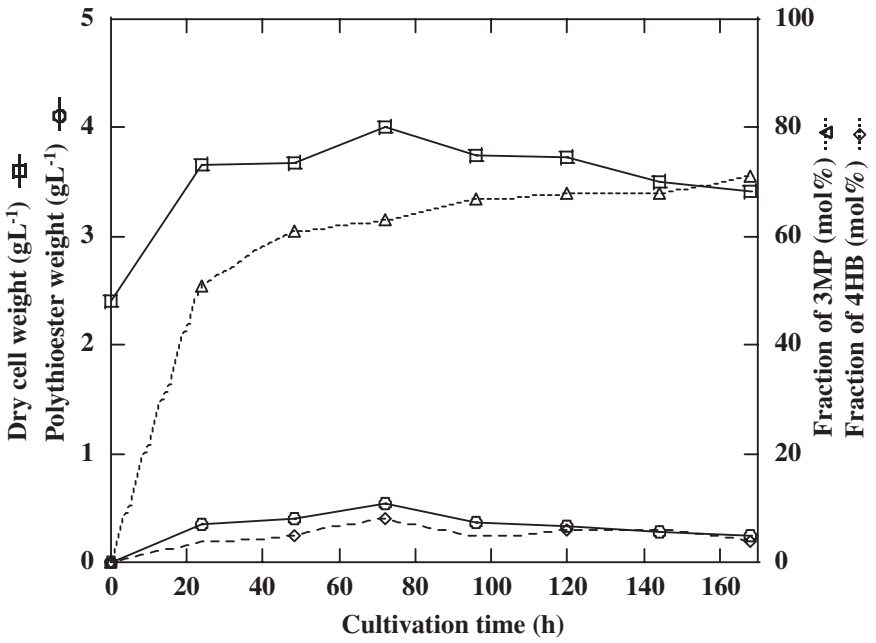

Figure 1. Time course of terpolythioester [P(3HB-*co*-3MP-*co*-4HB)] production by the batch cultivation of *R. eutropha* in the mineral medium containing 15 gL^{-1} of TDP and 5 gL^{-1} of γ-butyrolactone at pH 7.2.

consisting of TDP and 1,4-butanediol, the 3MP unit was introduced up to 16 mol % but the 4HB unit was little introduced in polymer, and the content of polythioester in dried cells was below 6 wt %. When *R. eutropha* was cultivated on medium consisting of TDP and sodium 4-hydroxybutyrate, P(3HB-*co*-3MP-*co*-4HB) terpolythioesters were produced at 10.6 wt % of dried cell weight but the molar fractions of 3MP unit were up to 14 mol %. In contrast, when lactones (5 gL^{-1}) such as γ-butyrolactone and ε-caprolactone were supplemented as cosubstrate of TDP (10 gL^{-1}), the 3MP fractions up to 59 mol % and 4HB fractions up to 21 mol % were introduced to terpolymers for 48 h, respectively. The yield of terpolythioester was relatively high up to 25 wt % of dried cell weight. Further, P(3HB-*co*-3MP-*co*-4HB) terpolythioester with up to 71 mol % 3MP fraction was biosynthesized, when *R. eutropha* strain was cultivated on 15 gL^{-1} of TDP plus 5 gL^{-1} of γ-butyrolactone for 168 h, as shown in Figure 1. This 3MP molar fraction (71 mol %) is the highest value in copolyesters containing 3MP unit produced by a wild type *R. eutropha* until now. It is worth noting that among the co-substrates of TDP tested, γ-butyrolactone raises the molar fraction of 3MP in copolymer. When *R. eutropha* was cultivated in mineral media that γ-butyrolactone was added to TDP solution adjusted to pH 7.2 with NaOH, the terpolythioesters with high molar fractions of 3MP were produced, but 4HB molar fractions were below 10 mol % (No. 8 in Table I and Fig. 1). In contrast, when cultivated in media that TDP was added to γ-butyrolactone solution treated with equimolar NaOH for 1 h, and then adjusted to pH 7.2, *R. eutropha* produced the terpolythioesters with up to 22 mol % 4HB unit and minor 3MP fractions (below 6 mol %). The 4HB fraction obtained by the latter method was almost similar value to the case which was cultivated in media consisting of sodium 4-hydroxybutyrate and sodium thiodipropionate at pH 7.2 (No. 6 and No. 7 in Table I). These results suggest that γ-butyrolactone is assimilated slower than sodium 4-hydroxybutyrate or sodium thiodipropionate in *R. eutropha*. Hence, it resuts in the production of terpolythioesters with high fraction of 3MP and minor 4HB when cultivated in media containing sodium salt of TDP and γ-butyrolactone. The structure and composition of terpolythioesters produced in a wild type *R. eutropha* were analyzed by IR and NMR spectroscopies. In the IR specta (date not shown) of terpolythioesters, two strong absorption bands were observed at 1735 cm^{-1} due to the stretching of the oxoester carbonyl group, and at 1681 cm^{-1} due to that of the thioester carbonyl group. Figure 2 shows the 67.5 MHz ^{13}C NMR spectrum of typical terpolythioester, P(26 mol % 3HB-*co*-68 mol % 3MP-*co*-6 mol % 4HB), together with the chemical shift assignment for each carbon resonance. The oxoester carbonyl resonances at 169–173 ppm were resolved into peaks six or above, while the thioester carbonyl resonances at 195–199 ppm were resolved into five peaks. These peaks may arise from the different diad and triad sequences of connecting the 3HB, 3MP, and 4HB units, by referring oxoester carbonyl resonances of 3HB-4HB and 4HB-3HB diad sequences in P(3HB-*co*-4HB) copolyesters[8] and

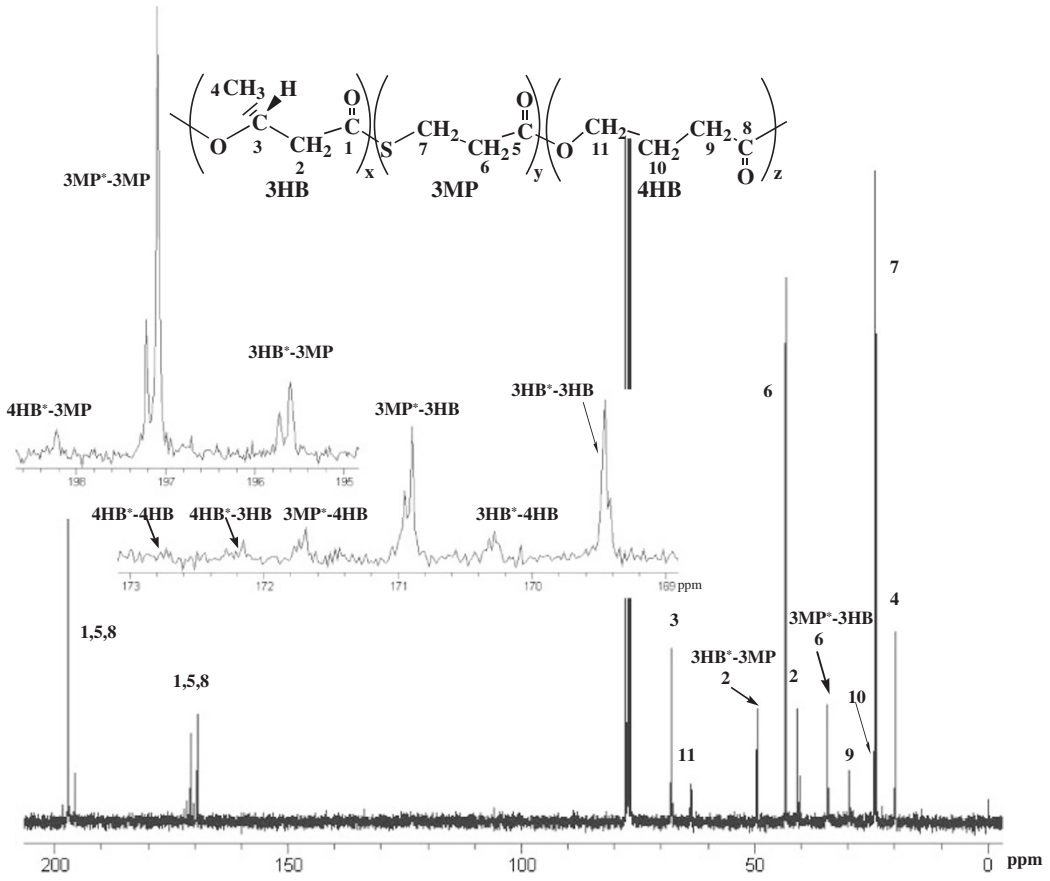

Figure 2. ^{13}C NMR spectrum of terpolythioester [P(26% 3HB-*co*-68% 3MP-*co*-6% 4HB)] in $CDCl_3$.

thioester carbonyl resonances of 3HB-3MP and 3MP-3HB diad sequences in P(3HB-*co*-3MP) copolythioesters,[17] respectively. Then, the nine peaks at 169.45, 170.28, 170.89, 171.44, 172.16, 172.45, 195.60, 197.09 and 198.22 ppm may be assigned to 3HB*-3HB, 3HB*-4HB, 3MP*-3HB, 3MP*-4HB, 4HB*-3HB, 4HB*-4HB, 3HB*-3MP, 3MP*-3MP and 4HB*-3MP diad sequences, respectively. The result of the relative peak areas of these nine fine resolved carbonyl carbon resonances may suggest the existence of the random sequence distribution of three monomeric units in P(3HB-*co*-3MP-*co*-4HB). The number average molecular weights (M_n) of P(3HB-*co*-3MP-*co*-3HV) with 17 mole % 3MP and 52 mole % 3HV units, and P(3HB-*co*-3MP-*co*-4HB) with 71 mol % 3MP and 4 mole % 4HB units were 1.6×10^5 and 3×10^4 respectively. M_ns of terpolythioesters with high 3MP fractions were slightly lower than these of P(3HB-*co*-3MP) copolymers with 26–54 mol % 3MP fractions reported by Lütke-Eversloh *et al.*[20] The thermal analysis of terpolythioesters by DSC showed that glass transition temperatures (T_g) of P(3HB-*co*-3MP-*co*-4HB) with *ca* 6 mol % 4HB units lowered from $-2.0\,^\circ C$ to $-12.4\,^\circ C$ as the fractions of 3MP unit increased 0 to 71 mol %, while melting temperatures (T_m) of those terpolymers appeared two around 130 °C and 167 °C with the increase of mole fractions of 3MP unit, and that T_g of P(3HB-*co*-3MP-*co*-4HB) with *ca* 35 mol % 3MP units lowered from $-3.1\,^\circ C$ to $-21.4\,^\circ C$ as the fractions of 4HB unit increased 0 to 22 mol %, while T_m of those terpolymers also lowered from 168 °C to 154 °C (date not shown). Enzymatic degradation of terpolythioester P(3HB-*co*-23 mol % 3MP-*co*-16 mol % 4HB), compared with copolymers P(3HB-*co*-23 mol % 3MP) and P(3HB-*co*-19 mol % 4HB) was carried out in a 0.1 M phosphate buffer solution (NaH_2PO_4/Na_2HPO_4, pH 7.4) containing porcine pancreas lipase at 37 °C. After the incubation, the polymer films were removed from the reaction solution, washed with distilled water, and dried to constant weight *in vacuo*. The weight loss of terpolythioester showed 26 wt % for 8 d, while those of copolymers with 3HB and 3MP or 4HB units were 0 wt % and 6 wt %, respectively. These results may suggest higher biodegradability of terpolythioesters with 3MP unit than corresponding copolymers. Further studies of biosynthesis and biodegadability of the terpolythioesters consisting of 3HB, 3MP and 4HB or 3HV units are in progress.

In conclusion, it has been demonstrated that the novel terpolythioesters, namely, poly(3-hydroxybutyrate-*co*-3-mercaptopropionate-*co*-4-hydroxybutyrate)

[P(3HB-*co*-3MP-*co*-4HB)] and poly(3-hydroxybutyrate-*co*-3-mercaptopropionate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3MP-*co*-3HV)] were biosynthesized by a wild-type *R. eutropha* H16 and found that the 3MP fraction in terpolythioesters dramatically increased to as high as 71 mol % by using the mixed carbon sources consisting of 3,3′-thiodipropionic acid (TDP) and lactones.

This work was supported by a Grant-in-Aid for Scientific Reserch (No. 16550133) from Japan Society for the Promotion of Science (JSPS).

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