Biosynthesis of a Novel Microbial Tetracopolyester Containing 4-Hydroxyhexanoate by \textit{Rhodococcus ruber}

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\textbf{Abstract:} Novel poly (3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) [P(3HB-co-3HV-co-3HHx)] was synthesized from hexanoate by \textit{Rhodococcus ruber}. The polyhydroxyalkanoate (PHA) synthase of \textit{R. ruber} can accept thioesters of 3- and 4-hydroxyalkanoates with carbon atoms from four to six. The PHA synthase can also incorporate 4-hydroxybutyrate (4HB) and 4-hydroxyhexanoate (4HHx) monomer into PHA from structurally related substrates. \textit{R. ruber} could accumulate an unusual PHA consisting of 3HB, 3HV, 3HHx, and 4HHx if the cells were cultured on a mineral salts medium containing 4HHx as a sole carbon source. The structure of polymer was confirmed by gas chromatography-mass spectrometry (GC-MS).

\textbf{Keywords:} GC-MS, 4-hydroxyhexanoate, polyhydroxyalkanoate, \textit{Rhodococcus ruber}

\section*{Introduction}

The use of biodegradable polymers has been proposed as a solution to environmental problems, and polyhydroxyalkanoate (PHA) is considered as one of such biodegradable polymers with desirable properties [1-4]. Following the discovery of poly-3-hydroxybutyrate [P(3HB)], various copolymers of 3-hydroxyalkanoates with chain length ranging from three to sixteen carbon atoms and with various functional side chains have been reported [5-11].

The technical properties and biodegradability of PHA mainly depend on the structure and composition of polymer. The degradation rate of poly (3HB-co-4-hydroxybutyrate (4HB)) which was first identified as a biopolyester containing a monomer constituent other than 3-hydroxyalkanoates is faster than that of P(3HB) homopolymer, since ester linkage between 4HB monomer units is sterically more accessible for extracellular depolymerase [12,13]. Recently, the identification of 4-hydroxyhexanoate (4HHx) as a new constituent of PHA synthesized by the recombinant \textit{Pseudomonas putida} possessing the hybrid plasmid encoding the PHA biosynthetic genes of a photosynthetic sulfur purple bacterium, \textit{Thiocapsa pfennigii}, has been reported [14]. So far, no wild type bacteria have been described in detail that synthesize a copolyester containing 4HHx as a new monomer constituent. This paper describes the incorporation of 4HHx monomer in a novel tetracopolyester by a naturally occurring bacterium, \textit{Rhodococcus ruber}, and biosynthesis of this biopolyester from structurally related carbon substrates.

\section*{Experimental}

\textbf{Culture Condition}

\textit{N-limited one-stage culture:} bacteria were cultivated aerobically at 30°C or other given temperature in a mineral salts medium with the ammonium chloride concentration of 0.05 or 0.005% (w/v) to allow extensive accumulation of PHA and with carbon substrate concentration of 0.5% (v/v) and others as indicated in the references [15]. After 72 h of cultivation on the mineral salts medium, cells were harvested by centrifugation, washed with 0.9% (w/v) sodium chloride buffer (pH 7.0), lyophilized, and analyzed for content and composition of PHA. \textit{N-free two-stage production:} Nutrient broth (NB)-grown cells were harvested by centrifugation, washed with 100 mM potassium phosphate buffer (pH 7.0), and transferred to same volume of N-free mineral salts.
medium containing a carbon source. After aerobically incubated at 30°C for 48 hr, cells were harvested, lyophilized and analyzed for PHA.

**Fermentation**

N-free two-stage production was carried out in a 2 liter fermenter (B. E. Marubishi, Japan). The temperature and pH were controlled at 30°C and 7.0, respectively. The dissolved oxygen was automatically controlled at 50% (of air saturation) by regulating air flow rate and stirrer speed.

**Analytical Procedure**

Cell mass concentration was determined by measuring optical density at 600 nm using UV spectrometer (Shimadzu UV-2101PC) and intrapolating with the calibration curve.

The polymer content of lyophilized cells and the composition of PHA were determined by GC (HP5890 series II) equipped with a HP-1 capillary column (25 m, 0.32 mm) and a flame ionization detector. A 10 mg of sample was subjected to methanolysis in the presence of methanol/sulphuric acid (85:15 (v/v)) at 100°C for 2 hr. A 2 mL of the organic phase was analyzed after split injection (split ratio 1:40). The temperatures of the injector and detector were 200°C and 220°C, respectively. A temperature program was used for efficient separation of peaks (90°C for 5 min, temperature increase of 8°C /min, 150°C for 25 min). Standard curves were constructed with synthesized methyl ester of known-concentration or internal standard.

The protein concentration in crude extracts was determined by the method of Bradford. A 50 μL of appropriately diluted sample was mixed with 1 mL of Bradford solution (Serva Blue G250 70 mg/50 mL of 96% (v/v) ethanol; 100 ml 85% (w/v) phosphoric acid; final volume of 1 liter with DDW), after 10 min absorbance was measured at 595 nm and protein concentration was determined.

**Characterization of Polymer by GC-MS Analysis**

Intracellular PHA was isolated from lyophilized cells by extraction with chloroform in a Soxhlet apparatus. After overnight reflux, the chloroform solution was concentrated by using rotary evaporator. The polymer was precipitated from chloroform by addition to 10 volume of ethanol and the precipitate was separated from the solvents by filtration. The resultant polymer was washed with methanol and remaining solvents were removed by exposure of the polymer to air stream. The precipitation procedure was repeated if necessary.

The methyl esters of 4-hydroxyalkanoic acids were analyzed by a HP 5890 series II GC equipped with DB-WAX column (polar, 30 m, 0.32 μm diameter, 0.25 μm film thickness) and mass data were acquired and processed with Jeol JMS-A505WA mass spectrometer. A 1 μL of sample in organic phase was injected (split ratio 1 : 80) with helium as carrier gas, and the temperature of oven was programmed for separation of peaks (temperature increment of 15°C/min from 50°C up to 200°C). The temperature of detector was 250°C. Ionizing energy for MS operation was 70 eV. The structure of monomer was identified by analysis of characteristic peaks and comparison with standard mass spectra if available.

**Synthesis of 4-, 5- and 6-hydroxyalkanoate**

4-, 5- and 6-hydroxyalkanoates were obtained by alkaline saponification from the corresponding lactones. To chilled aqueous solutions of 20% (v/v) corresponding lactones, 10 N NaOH was added dropwise until the pH of the solution increased to 12. The solution was stirred for 1 hr and then neutralized by addition of 1 M HCl.

**Results and Discussion**

**Accumulation of PHA from Various Carbon Sources**

*Rhodococcus ruber* NCIMB 40126 can synthesize an P(3HB-co-3HV) containing primarily 3-hydroxyvalerate (3 HV) from structurally unrelated carbon substrates [8, 15]. In the beginning *R. ruber* was examined for its ability to accumulate copolyesters containing various hydroxyalkanoates in addition to 3HB and 3HV from structurally related carbon substrates (Table 1). When propionate, valerate and 4-hydroxyvalerate (4HV) were supplied as a sole carbon source, the accumulated polymer contained 3HV as the principal monomer as high as 99 mol%. In case of 4HB and 4HV, incorporation of 4HB and 4HV into the accumulated polymer occurred, and these results indicated that the PHA synthase of *R. ruber* has the activity toward 4-hydroxyacyl-CoA as a substrate. It was also demonstrated that a terpolyester of 3HB, 3HV, and 3-hydroxyhexanoate (3HHex) was accumulated from hexanoate by *R. ruber*, which means hydroxyacyl-CoA with six carbon number can be accepted as a substrate for the PHA synthase of *R. ruber*. From the above results, it was clearly expected that *R. ruber* could accumulate a copolyester containing 4HHex monomer from 4HHex. *R. ruber* was cultured on a mineral salts medium supplemented with 4HHex as a sole carbon source in N-limited one-stage fed-batch mode or N-free two-stage fed-batch mode. As shown in Table 1, 4HHex was detected as a new monomer constituent from the PHA synthesized by *R. ruber*. The structure of PHA containing 4HHex is shown in Figure 1. To confirm the incorporation of 4HHex as a new monomer constituent, methanolysis products of purified PHA were analyzed by
Table 1. Accumulation of PHA from Various Carbon Sources by *R. rubber*

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Culture method</th>
<th>PHA content (% of DCW)</th>
<th>Composition (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3HB</td>
</tr>
<tr>
<td>Acetate (1.0% × 2)</td>
<td>A</td>
<td>10.1</td>
<td>23.1</td>
</tr>
<tr>
<td>Glucose (1.5% × 1)</td>
<td>A</td>
<td>15.9</td>
<td>27.4</td>
</tr>
<tr>
<td>Propionate (0.2% × 3)</td>
<td>A</td>
<td>21.2</td>
<td>1.2</td>
</tr>
<tr>
<td>3HB (0.25% × 1,3/buOH)</td>
<td>A</td>
<td>7.0</td>
<td>64.7</td>
</tr>
<tr>
<td>1,3/buOH (0.2% × 2)</td>
<td>B</td>
<td>12.8</td>
<td>56.1</td>
</tr>
<tr>
<td>4HB (0.25% × 2)</td>
<td>B</td>
<td>20.9</td>
<td>19.4</td>
</tr>
<tr>
<td>Valerate (0.25 × 3)</td>
<td>A</td>
<td>63.5</td>
<td>1.3</td>
</tr>
<tr>
<td>4HV (0.25% × 3)</td>
<td>A</td>
<td>43.3</td>
<td>0.7</td>
</tr>
<tr>
<td>5HV (0.25 × 2)</td>
<td>B</td>
<td>18.4</td>
<td>28.6</td>
</tr>
<tr>
<td>Hexanoate (0.25% × 3)</td>
<td>A</td>
<td>29.9</td>
<td>50.1</td>
</tr>
<tr>
<td>Octanoate (0.25% × 3)</td>
<td>A</td>
<td>10.6</td>
<td>73.5</td>
</tr>
<tr>
<td>4HHx (0.25% × 3)</td>
<td>A</td>
<td>35.7</td>
<td>57.7</td>
</tr>
<tr>
<td>4HHx (0.4% × 2)</td>
<td>B</td>
<td>38.2</td>
<td>39.4</td>
</tr>
</tbody>
</table>

Culture method A: Cells were cultured at 30°C for 72 h in a N-limited mineral salts medium containing given carbon substrates. Culture method B: Harvested cells which were grown on a NB medium for overnight were incubated at 30°C for 48 h in a N-free mineral salts medium containing given carbon substrates. Carbon substrates were successively added in portion as indicated in the Table. Abbreviation: 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHx, 3-hydroxyhexanate; 4HB, 4-hydroxybutyrate; 4HV, 4-hydroxyvalerate; 4HHx, 4-hydroxyhexanoate; 5HV, 5-hydroxyvalerate; 1,3/buOH, 1,3-butanediol; DCW, dry cell weight; Tr, trace amount.

Figure 1. Chemical structure of tetracopolyester synthesized by *R. rubber* from 4-hydroxyhexanoic acid as a sole carbon substrate.

GC-MS. Two GC peaks with the same retention times of methyl ester of 4HHx and γ-caprolactone, the characteristic byproduct which was spontaneously generated during the methanolysis of 4HHx, were detected (Data not shown) and their mass spectra were confirmed by comparison with those of authentic standards (Figure 2). The molecular ion-related mass fragments (M-1) at m/z 145 for methyl ester of 4HHx and at m/z 113 for γ-caprolactone clearly reflected their molecular weights.

Production of P(3HB-co-3HV-co-3HHx-co-4HHx)

In order to investigate the incorporation of 4HHx into PHA in more detail, *R. ruber* was cultured in a 2-liter fermentor with controlled pH and DO (7.0 and 50% of air saturation, respectively). *R. ruber* was grown aerobically at 30°C in a NB medium for overnight and then harvested by centrifugation. Washed cells were aseptically resuspended into a N-free mineral salts medium containing 1% (w/v) 4HHx and then incubated for 60 h. As shown in Figure 3, the mol% of each monomer changed during production stage. The mol% of 4HHx remained almost constant at low level. The mol% of 3HHx increased during production stage and reached maximum at the end of production stage, whereas the mol% of 3HB and 3HV were higher during early production stage and then decreased. It could be speculated that 4HHx was more easily converted to central metabolic intermediates for cell growth and maintenance via β-oxidation pathway and excess conversion of carbon source led to formation of 3HB and 3HV monomer during the initial production stage, which resulted in
accumulation of PHA with 3HB and 3HV at a much higher mol% [17]. Protein concentration did not significantly increase during production stage and cell mass increment was mainly due to extensive accumulation of PHA.

**Evaluation of Substrate Specificity and Proposed Biosynthetic Pathway**

The unusual carbon substrates like 5-hydroxyhexanoate (5HHx), 6-hydroxyhexanoate (6HHx), and 4-hydroxyoctanoate (4HO) were also provided as a sole carbon source to evaluate the possibility of incorporation of these monomers into polymer as new constituents. From 5HHx, a novel tetrapolyester containing 4HHx as a new constituent was accumulated, but the mol% of 5HHx was below one mol% of polymer (Table 2). When 6HHx was supplied, incorporation of 6HHx was not detected and tercopolyester of 3HB, 3HV, and 3HHx with 3HV as the principal monomer was synthesized by *R. ruber*. In case of 4HO, *R. ruber* accumulated a novel tercopolyester of 3HB, 3HV, 3HHx, and 3-hydroxyoctanoate (3HO).

The polymer production with cosubstrate was tested to increase the mol% of 4HHx and 5HHx based on the strategy that 4HHx and 5HHx will be more efficiently copolymerized into PHA if additional carbon sources will support cell growth and maintenance. Cosubstrate altered the composition of PHA, but there is no increment in mol% of 4HHx and 5HHx (Table 3).

From the above results, it can be concluded that the PHA synthase of *R. ruber* possesses the substrate specificity toward 3-, 4-, and 5-hydroxyacyl-CoA with carbon number from four to eight but possesses only low activity for 4- and 5-hydroxyhexanoyl-CoA as a substrate. It could be also inferred that *R. ruber* has the efficient metabolic regulation system for converting 4HHx and 5HHx to 3-hydroxyacyl-CoA and/or central metabolic intermediates.

Although the biosynthetic pathway for 4HHx in *R. ruber* has not been investigated at enzymatic level, a reasonable assumption would be that 4-hydroxyhexanoate is converted to 4-hydroxyhexanoyl-CoA thioester by acyl transferase, followed by esterification with growing polymer chain by the PHA synthase of *R. ruber* (Figure 4) [18]. The novel PHA containing 4HHx and 5HHx with rather low mol% was synthesized by *R. ruber*. Metabolic alteration for increment in mol% of 4HHx and
Table 3. Accumulation of PHA from Various Cosubstrates by R. ruber

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Culture method</th>
<th>PHA content (％ of DCW)</th>
<th>3HB</th>
<th>3HV</th>
<th>3HHx</th>
<th>4HHx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexanoate(0.2%)+4HHx(0.2%)</td>
<td>A</td>
<td>48.3</td>
<td>50.6</td>
<td>19.7</td>
<td>27.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Hexanoate(0.2%)+4HHx(0.2%)</td>
<td>B</td>
<td>39.1</td>
<td>52.7</td>
<td>10.7</td>
<td>34.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Hexanoate(0.2%)→4HHx(0.4%)</td>
<td>C</td>
<td>48.3</td>
<td>50.6</td>
<td>19.7</td>
<td>28.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Hexanoate(0.3%)+4HHx(0.2%)</td>
<td>A</td>
<td>53.6</td>
<td>56.0</td>
<td>23.3</td>
<td>19.9</td>
<td>1.8</td>
</tr>
<tr>
<td>Hexanoate(0.3%)+4HHx(0.2%)</td>
<td>B</td>
<td>42.8</td>
<td>49.9</td>
<td>26.9</td>
<td>21.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Hexanoate(0.5%)+4HHx(0.4%)</td>
<td>C</td>
<td>35.7</td>
<td>51.3</td>
<td>29.9</td>
<td>18.1</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Culture method A: Cells were cultured at 30℃ for 72 h in a N-limited mineral salts medium containing given carbon substrate. Two carbon substrates were successively added in portion by three times. Culture method B: Harvested cells which were grown on a NB medium for overnight were incubated at 30℃ for 48 h in a N-free mineral salts medium containing given carbon substrate. Two carbon substrates were successively added in portion by three times. Culture method C: Cells were cultured at 30℃ in a N-limited mineral salts medium for 24 h with the first carbon substrate, and then the second carbon substrate was sequentially added. The cells were further incubated for 48 h. Abbreviation: 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHx, 3-hydroxyhexanoate; 4HHx, 4-hydroxyhexanoate, 4H0, DCW, dry cell weight.

Figure 4. Proposed biosynthetic route for PHA containing 4-hydroxyhexanoate. (1, 3-ketohiolase; 2, reductase; 3, PHA synthase; 4, 4-hydroxyacyl-SCoA dehydrase; 5, hydratase; 6, unknown enzyme).

5HHx needs to be investigated.

Conclusion

*Rhodococcus ruber* could synthesize poly (3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) [P(3HB-co-3HV-co-3HHx)] from hexanoate and P(3HB-co-4-hydroxybutyrate (4HB)-co-3HV) from 4HB, which means that the PHA synthase of *R. ruber* can accept thioesters of 3- and 4-hydroxyalkanoates with carbon atoms from four to six. The novel polyhydroxyalkanoate (PHA) consisting of 3HB, 3HV, 3HHx, and 4-hydroxyhexanoate (4HHx) was synthesized by *R. ruber* if the cells were cultured on a mineral salts medium containing 4HHx as a sole carbon source, and the structure of polymer was confirmed by gas chromatography-mass spectrometry (GC-MS). Other unusual carbon substrates like 5-hydroxyhexanoate (5HHx), 6-hydroxyhexanoate (6HHx), and 4-hydroxyoctanate (4HO) were also provided as a sole carbon source for the biosynthesis of novel PHA containing new monomers whose structures are related to those of given carbon substrates. Incorporation of detectable amount of those monomers did occur only for 5HHx, but the mol% of 5HHx was below one mol% of polymer.

References

(2000).