Recovery of active medium-chain-length-poly-3-hydroxyalkanoate polymerase from inactive inclusion bodies using ion-exchange resin

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A novel process for the purification of active medium-chain-length-polyhydroxyalkanoate (mcl-PHA) polymerase was developed. This process is based on solubilization and activation of inactive polymerase inclusion bodies by incubation with ion-exchange resin. The mcl-PHA polymerase 1 from *Pseudomonas oleovorans* was overproduced from the *Palk* promoter. Most of the polymerase produced was sequestered in the cytoplasm as an inactive form in insoluble aggregates. By incubating the protein aggregates with S-Sepharose ion-exchange resin in the presence of dithiothreitol and glycerol, the mcl-PHA polymerase could be

extracted in an active and soluble form with a final yield of about 5.2 mg/g of cell dry weight. The solubilized polymerase was able to catalyse the *in vitro* synthesis of mcl-PHA without any additional cell components, suggesting its potential application for production of biopolymer. The procedure used here may be of general value in solubilizing and activating purified inactive labile enzymes.

Key words: *in vitro* synthesis of polymer, protein inclusion bodies, *Pseudomonas*, recovery of enzyme activity.

INTRODUCTION

Poly-3-hydroxyalkanoates (PHAs), which are produced as intracellular storage material by many bacterial species, have attracted considerable attention on account of their potential applications as biodegradable plastics and as sources of valuable chiral synthons (for reviews, see [1,2]). The best known variants, for which the genetics and enzymology have been studied in detail, and which have been produced industrially, are the homopolymer poly-3-hydroxybutyric acid (PHB) and the copolymer PHB/V, which contains 3-hydroxyvaleric acid in addition to 3-hydroxybutyric acid (see review [2]). Since both of these monomers are short-chain hydroxyalkanoic acids, PHB and PHB/V are referred to as short-chain-length (scl) PHAs (note that 'short chain length' is a characteristic of the monomer; the polymer may well be a very long molecule, as is generally the case for PHB). Most other PHAs are referred to as medium-chain-length (mcl) PHAs, because the monomers are generally consisting of six or more carbon atoms [3].

There are several enzymes involved in the synthesis of PHAs. Among these enzymes, the PHA polymerases (these are also called PHA synthases) are the key enzymes, which incorporate 3-hydroxyacyl moieties into nascent PHA chains, using CoA-activated precursors (see review [2]). Since the polymerase encoding gene was first identified in *Ralstonia eutropha* [4], many PHA polymerases or the corresponding genes have been studied thus far (see review [2]). There has been some work on the enzymology of PHA polymerases. However, to date, only PHB polymerase has been purified and studied *in vitro* in detail [5,6]. Several groups have attempted to isolate mcl-PHA polymerases, but so far no success has been reported. Although mcl-PHA polymerases have proved difficult to isolate, it would be useful to have such enzymes available, both to study structural and functional characteristics, and also to use polymerases in the

synthesis of specific homo- and hetero-polymers *in vitro*. Thus there is a clear incentive to prepare active purified mcl-PHA polymerases.

Since very little PHA polymerase appears as free soluble protein in the wild-type *Pseudomonas* strains [7], we chose to overproduce this protein. In the present paper we describe the recovery of PHA polymerase activity from inclusion bodies which have been formed upon overexpression of the corresponding gene. The purified active polymerase allowed us to synthesize homopolymeric mcl-PHAs *in vitro*.

EXPERIMENTAL

Bacterial strains and plasmids

Pseudomonas putida GPp104 (PHA⁺) [8] was used in the present study. pAlkVSVG1 [pGEM-5Zf(+), *Palk*, VSV G tag, Ap^r] [9] and pVLT35 (*Ptac/lacI^q*, Mob⁺, RSF1010 *ori*, Sm^r/Sp^r) [10] were used to construct pET701 and pET702. pAlkVSVG1 contains the *Palk* promoter, which originates from the *P. oleovorans* alkane-degradation system and is known to allow efficient gene expression in *Pseudomonas* strains [9], and a sequence coding for 12 C-terminal amino acids of the vesicular-stomatitis-virus glycoprotein (VSV G), to which monoclonal antibodies are available. Plasmid pGec74 (pLAFR1, *alkST*, Tc^r) contains the regulatory gene for expression of the *Palk* promoter [11].

Recombinant DNA techniques

Isolation and analysis of plasmid DNA were carried out as described by Sambrook et al. [12]. Two oligonucleotide primers with the following sequences: AGCGTCGTACATGTCTAAC-

Abbreviations: DTT, dithiothreitol; DCPK, dicyclopropyl ketone; VSV G, vesicular-stomatitis-virus glycoprotein; PHB, poly-3-hydroxybutyric acid; PHB/V, co-polymer containing 3-hydroxyvaleric acid in addition to 3-hydroxybutyric acid; PHA, poly-3-hydroxyalkanoate; mcl- and scl-PHA, medium- and short-chain-length PHA; LB, Luria-Bertani; GC, gas chromatography; cdw, cell dry weight.

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AAGAAC and ACGCTCGTGAACGTAGGTGCC were designed and synthesized (Microsynth G.m.b.H., Balgach, Switzerland) to perform site-directed mutagenesis (exchanged nucleotides are underlined) and to amplify the *phaC1*-containing fragment.

pET701 was constructed as follows. For integration of appropriate restriction sites, a site-directed mutagenesis was performed which resulted in a change of three base-pairs in *phaC1*, but the amino acid sequence of the PHA polymerase was not changed. Instead of introducing a *NcoI* site at the start codon of *phaC1*, an *AflIII* site (which produces cohesive ends compatible with the *NcoI* site) was generated because this led to fewer base-pair changes and because the *phaC1* gene does not contain an internal *AflIII* site, while it does contain several *NcoI* sites. The 1.7 kb PCR fragment was digested with *AflIII*, isolated and ligated into *NcoI*/*EcoRV*-digested pAlkVSVG1, resulting in the 5 kb plasmid pET701.

pAlkVSVG1 is derived from the ColE1-derived pGEM-5Zf(+) (Promega) and cannot replicate in *Pseudomonas*. pET702 was constructed from pVLT35, which contains a RSF1010 replicon and replicates both in *Escherichia coli* and in *Pseudomonas*. In order to remove the *tac* promoter, pVLT35 was digested with *ApaI* and *PstI*, producing an 8.3 kb fragment. Since there are several *PstI* sites inside *phaC1*, pET701 was digested with *ApaI* and *NsiI*, generating cohesive ends compatible with *PstI*. The 2 kb *Palk-phaC1-tag* containing fragment was then ligated to the *ApaI*/*PstI* sites of pVLT35, resulting in the 10.3 kb plasmid pET702.

Media, growth conditions and cell disruption

Luria–Bertani (LB) medium was used as rich medium [12]. For testing the expression system for PHA polymerase production, *Pseudomonas* recombinants were grown on E2 minimal medium [13]. As carbon source, 15 mM sodium octanoate was added. All recombinants were precultured in LB medium at 30 °C with appropriate antibiotics (tetracycline, 12.5 µg/ml; streptomycin, 100 µg/ml), then transferred into minimal medium in a 1:100 dilution.

To induce the *Palk* promoter, pGEc74 was always co-transformed with *Palk* expression plasmids, and dicyclopropyl ketone (DCPK) was added to 0.03% (v/v) in the early exponential phase. Cell growth was monitored by measuring D_{450} [14].

Pseudomonas cells were harvested and washed once with Tris/HCl (50 mM, pH 8). The pellets were resuspended in the same buffer to a D_{450} of 40–50. Cells were disrupted by three passages through a French pressure cell at 75.8 MPa (11 000 lbf/in²) and 4 °C.

Protein analysis

Proteins were separated by gel electrophoresis as described by Laemmli [15]. The amount of PHA polymerase relative to total protein was determined by densitometric scanning (Molecular Dynamics). Total protein was assayed with a Lowry-based method (Bio-Rad detergent compatible assay) in the presence of SDS with BSA as the standard. Immunoblotting of polymerase was carried out as previously described [9].

N-terminal sequencing

Approx. 10 µg of protein was applied on a protein gel, followed by transfer on to a PVDF membrane (Immobilon-PSQ; Millipore Corp., Bedford, MA, U.S.A.) [12]. N-terminal sequencing of the appropriate band was carried out by the service laboratory in Molekularbiologie, ETH Hönggerberg, Switzerland.

Determination of PHA

To determine the polyester content and composition, samples were treated with chloroform and H₂SO₄/methanol (85:15, v/v) [16] and analyzed using a CP-Sil 5 CB column (Chrompack) on a gas chromatograph (Fisons) [16]. Gas-chromatography–MS (GC–MS) analyses were performed on a Fisons MD800 mass spectrometer, using electron impact at 70 eV [17].

Synthesis and purification of polymerase substrate 3-hydroxyoctanoyl-CoA

CoA was coupled to 3-hydroxyoctanoic acid (Sigma) by using acyl-CoA synthetase (Sigma) as described previously [18]. The product, 3-hydroxyoctanoyl-CoA, was purified over an ODS-2 reverse phase column by HPLC [18], dried using a SpeedVac apparatus, and then dissolved in 50 mM Tris/HCl, pH 8.

Isolation of PHA polymerase inclusion bodies

Inclusion bodies were isolated as described previously [19] with the following modifications: to separate PHA granules which contain active PHA polymerase from the polymerase inclusion bodies, the cell lysates were layered on to a 20% (w/v) sucrose solution and ultracentrifuged at 110 000 *g* for 2.5 h [20]. After this centrifugation, PHA granules remained on the top of the sucrose solution and the polymerase inclusion bodies were precipitated into the pellet fraction. The pellet fraction was then resuspended in washing buffer (0.5% Triton X-100/1 mM EDTA) and centrifuged at 10 000 *g* for 10 min to separate membrane proteins from inclusion bodies [19]. The pelleted inclusion bodies were washed at least three times with 50 mM Tris, pH 8.0, and finally resuspended in the same buffer to 1 mg/ml.

Solubilization of PHA polymerase

Solubilization or extraction of polymerase from the inclusion bodies was achieved by mixing 0.5 ml of buffer A [50 mM Tris/HCl (pH 7.5)/50 mM NaCl/1 mM EDTA/1 mM dithiothreitol (DTT)/1 mM PMSF/10% (v/v) glycerol], containing about 20 µg of polymerase inclusion bodies, with an equal bed volume of Q- or S-Sepharose Fast Flow, equilibrated in buffer A, and shaking the suspensions gently at 4 °C for 2–3 h. As controls, either inclusion bodies, Q-Sepharose or S-Sepharose were/was omitted from the mixture. The suspensions were then centrifuged at 10 000 *g* for 10 min to remove the resins. The pelleted resins were further resuspended in buffer A and 0.5 M NaCl, and were shaken at 4 °C for 2 h to elute adsorbed polymerase. The suspensions were centrifuged again at 10 000 *g* for 10 min. The supernatants obtained after the two centrifugations were collected and used for polymerase studies.

In vitro polymerase activity assay

The polymerase activity was followed by monitoring the depletion of substrate 3-hydroxyoctanoyl-CoA by HPLC as described previously [20]. One unit is defined as 1 µmol of substrate depletion/min.

In vitro synthesis of polymeric mcl-PHA

The *in vitro* formation of mcl-PHA was followed by detecting the chloroform-extractable product. A typical reaction mixture contained the following components, in a final volume of 450 µl: 0.5 mM 3-hydroxyoctanoyl-CoA, 50 mM Tris, pH 8, and the

soluble polymerase (0.5–1 μg). Assay mixtures were incubated at 30 °C, samples (60 μl) were taken at different time points, and the reactions were stopped by the addition of 140 μl of 1% trichloroacetic acid. The solution was then extracted with 500 μl of chloroform, which contained 0.01 mg/ml methyl benzoate. A 400 μl sample of the chloroform phase was transferred to a Pyrex tube, mixed with 600 μl of chloroform (containing 0.01 mg/ml methyl benzoate) and 1 ml of H_2SO_4 /methanol (85:15, v/v). The mixture was then analysed for the presence and composition of PHA as described previously [16].

RESULTS AND DISCUSSION

Overproduction of PHA polymerase

Previously we have found that, in the wild-type *P. oleovorans* or *P. putida* strains, PHA polymerase mainly appears as the PHA-granule-attached form [7,18]. We have tried to isolate PHA polymerase from PHA granules with various extraction procedures. However, extraction of the PHA polymerase from PHA granules invariably resulted in activity loss of polymerase (G. de Roo and B. Witholt, unpublished work). We do not yet understand why PHA polymerase is inactivated by its removal from granules. It is possible that the polymerase is active only when bound to a hydrophobic surface, normally provided by the granule; perhaps there is a requirement for an initiator of polymerization, or perhaps the protein denatures on extraction from PHA granules. None of these explanations is particularly convincing, since PHB polymerase has been purified and is functional in aqueous buffers [5,21]. We therefore decided to overproduce the PHA polymerase and then recover the enzyme activity by refolding.

In our first attempts to reach high-level production of PHA polymerase, the corresponding encoding gene *phaC1* was cloned under the control of *Plac* and *Ptac* promoters. Expression of these constructs, however, resulted in less than 0.1% PHA polymerase of total cell protein in recombinant strains (results not shown). We therefore constructed the plasmid pET702 in which *phaC1* expression is under the control of the *Palk* promoter [9], and *phaC1* is tagged with a sequence coding for 12 C-terminal amino acids of VSV G [9], to which monoclonal antibodies are available. The construct was tested in the PHA polymerase-negative *P. putida* mutant GPp104. In the recombinant harbouring pET702 in combination with pGec74 containing the *alkS* regulatory gene for *Palk* expression, induction led to the appearance of an extra band at about 62 kDa (Figure 1A, lane 3 versus lane 2). Western blotting using anti-(VSV G) antibodies showed that the PHA polymerase was absent in uninduced cells (lane 9) and clearly present in induced cells (lane 10), indicating that *phaC1* expression is tightly regulated by the *Palk* promoter. We typically obtained PHA polymerase levels of about 15–25% of the total protein in *Pseudomonas*.

The VSV G-tagged polymerase was also tested for its ability to restore PHA accumulation in *P. putida* GPp104. Although the host is not capable of accumulating PHA [8], GPp104 carrying pET702 and pGec74 produced about 30% (w/w) PHA per cell dry weight (cdw). Without induction, only trace amounts of PHA (0.5% PHA/cdw) were detected, demonstrating that the tagged PHA polymerase synthesized from pET702 is functional in *Pseudomonas*.

Purification of PHA polymerase inclusion bodies

When cells containing PHA polymerase were lysed, most of the polymerase precipitated into the pellet fraction after low-speed centrifugation (Figure 1A, lane 5 versus lane 4), suggesting that

Figure 1 Expression, purification and solubilization of *phaC1*-VSV G

The *Pseudomonas* recombinant was grown in minimal medium E2 with octanoate as carbon sources at 30 °C. DCPK (0.03%) was added in the early exponential phase, and incubation continued for 8 h. Culture samples were analysed by SDS/12% -PAGE. (A) Overproduction of PHA polymerase. Lanes: 1, protein size markers (kDa); 2, uninduced sample; 3, induced sample; 4 and 5, soluble and insoluble fractions after the cell lysate was centrifuged at 3000 g for 10 min; 6 and 7, soluble supernatant and insoluble pellet fractions of the whole cell lysate after centrifugation in 20% (w/v) sucrose at 110 000 g for 2.5 h; 8, purified PHA polymerase inclusion bodies; 9 and 10, Western blotting of samples from lanes 2 and 3 respectively. (B) Solubilization of polymerase from inclusion bodies. Each sample corresponds to 4 μg of inclusion bodies. Lanes: 1, protein size markers (kDa); 2, solubilized polymerase after inclusion bodies were incubated with S-Sepharose; 3, soluble fraction after S-Sepharose was incubated in buffer A; 4, solubilized polymerase after inclusion bodies were incubated in buffer without S-Sepharose.

the enzyme might be present in inclusion bodies or insoluble aggregates. These inclusion bodies were separated from the PHA granule-attached polymerase and the majority of other proteins in the cell lysate by centrifugation through 20% (w/v) sucrose at 110 000 g for 2.5 h. After this centrifugation, the polymerase accounted for more than 50% of total pellet protein (Figure 1A, lane 7; Table 1). N-terminal sequencing confirmed the identity of the polymerase band. We further purified the pellet fraction by washing it with Triton X-100 to remove contaminating proteins. On the basis of SDS/PAGE, the final preparation contained more than 98% pure polymerase (Figure 1A, lane 8; Table 1). The final yield of the purified PHA polymerase inclusion bodies was estimated to be 55.8 mg/g of cdw (Table 1). However, in these inclusion bodies no PHA polymerase activity was detected (Table 1).

Table 1 Purification of PHA polymerase from *P. putida* GPP104[pET702, pGEc74]*

Purification step	Total protein (mg)	PHA polymerase (% of total protein)†	Polymerase activity (units)§	Specific activity (units/mg)§
Pellet fraction†	13.00	50	—	—
Inclusion bodies†	5.58	98	—	—
Solubilized polymerase†	0.52	100	2.29	4.40

* PHA polymerase was purified from 100 mg of cells (dry weight); the data presented in this Table are the averages from two independent experiments.

† The pellet fraction was obtained by centrifugation of cell lysate in 20% (w/v) sucrose at 110 000 *g* for 2.5 h; the inclusion bodies were obtained by washing the pellet fraction with Triton X-100 to remove contaminating proteins; soluble polymerase was obtained by incubating polymerase inclusion bodies with S-Sepharose as described in the Experimental section.

‡ Polymerase levels were estimated by densitometric scanning of SDS/PAGE gels.

§ The polymerase activity was determined with 3-hydroxyoctanoyl-CoA by substrate depletion as described previously [20].

Solubilization and activity recovery of PHA polymerase with ion exchange resin

To recover polymerase activity from the inclusion bodies, we first tried conventional methods in which inclusion bodies were dissolved in 8 M urea, followed by rapid dilution in several renaturation buffers [22]. However, no polymerase activity was obtained (results not shown). Previously it was reported that some protein inclusion bodies could be solubilized and activated by interacting with ion-exchange resins [23], and we therefore tested this approach with S- and Q-Sepharose Fast Flow. We were unsuccessful with Q-Sepharose, but we found that S-Sepharose partially solubilized the polymerase (Figure 1B, lane 2). About 10% polymerase was recovered from the inclusion bodies, leading to a final yield of the soluble enzyme of 5.2 mg/g cdw (Table 1). The solubilized polymerase exhibited a maximum specific activity of 4.40 units/mg with 3-hydroxyoctanoyl-CoA in the presence of 1 mg/ml BSA (Table 1). In all of our experiments, recovery of activity occurred only by exposure of polymerase inclusion bodies to S-Sepharose in buffer, but not by exposure to the buffer alone.

Factors affecting solubilization of polymerase and its activity

We have tested different conditions which might influence the recovery of polymerase from inclusion bodies. We noted that omission of DTT in buffer A led to essentially complete loss of the polymerase activity, omission of glycerol gave an approx. 6-fold reduced enzyme activity, omission of PMSF resulted in degradation of polymerase. Furthermore it was found that the activity of polymerase solubilized from the inclusion bodies was

time-dependent. Activity started to decrease after 3 h incubation in buffer A, and, after 8 h, no polymerase activity could be detected.

Different storage conditions were also investigated for retention of activity. We found that the soluble polymerase lost its activity completely by storage at -20°C for 24 h in buffer A, even in the presence of DTT and glycerol, indicating that the soluble polymerase is rather unstable, as has also been found for solubilized PHB polymerase [5,21]. The reason for this is not clear at present. It is possible that there are component(s) which are necessary for stabilizing the polymerase and are abundant in the cells but are missing in purified soluble polymerase. Further optimization for recovery efficiency and improvement for polymerase stability by using different detergents are under investigation.

Various additives were tested on the basis of what we have recently reported for PHA-granule-attached polymerase [20] (Table 2). We found that the soluble polymerase was completely inactivated by the addition of 0.5% (v/v) Triton X-100. A positive effect on polymerase activity was observed after the addition of BSA (1 mg/ml) or phospholipids (1 mg/ml), leading to about 1.5-fold higher activity compared with the control (Table 2). This is similar to what was reported for PHA-granule-attached polymerase [20]. Addition of NaCl (0.5 M) or EDTA (5 mM) did not lead to significant effects on polymerase activity.

In vitro synthesis of homopolymeric mcl-PHA by the soluble polymerase

3-Hydroxyoctanoyl-CoA was incubated with the solubilized polymerase for various time periods. The reactions were stopped by the addition of 1% trichloroacetic acid. The polymeric

Table 2 Effect of various additives on PHA polymerase activity*

Additive	Concentration	PHA polymerase activity (units/mg of polymerase)	Percentage of activity
None†	—	2.47	100
Triton X-100	0.5%, v/v	≤ 0.01	0
BSA	1 mg/ml	4.40	180
phospholipids	1 mg/ml	3.71	150
NaCl	0.5 M	2.45	100
EDTA	5 mM	2.44	100

* The activity of soluble PHA polymerase in buffer A was taken as 100; the effect of additives on polymerase activity is shown relative to this activity.

† A typical reaction mixture (100 μl) contained: 0.4–0.6 mM 3-hydroxyoctanoyl-CoA, 50 mM Tris/HCl, pH 8, and 0.5–1 μg of soluble polymerase.

Figure 2 *In vitro* formation of chloroform-extractable mcl-PHA by purified polymerase

Purified soluble polymerase (1 μ g) was exposed to substrate 3-hydroxyoctanoyl-CoA. Samples were taken at different time points. The formation of chloroform-extractable product was analysed by GC and GC-MS as described in the Experimental section. $\blacklozenge$, soluble fraction recovered from inclusion bodies after incubation with S-Sepharose; $\triangle$, $\square$ and $\circ$, Controls ($\square$, control 1, no polymerase was added in the assay mixture; $\triangle$, control 2, no substrate was added in the assay mixture; $\circ$, control 3, soluble fraction recovered from inclusion bodies after incubation with buffer alone without S-Sepharose).

product was extracted into chloroform, and analysed by GC and GC-MS as described in the Experimental section. This procedure allowed us to detect only monomers derived from the chloroform-extractable PHA. It does not detect unchanged substrate (3-hydroxyoctanoyl-CoA) or 3-hydroxyoctanoic acid which might be formed in the assay mixture, since this substrate and the free acid remained in the water phase under our assay conditions. This was evidenced by using radioactively labelled monomers (results not shown).

No chloroform-extractable material was detected when substrate or polymerase was omitted from the reaction mixture (Figure 2). Chloroform-extractable material was also absent when inclusion bodies were used (Figure 2). PHA formation was only observed when substrate and the PHA polymerase solubilized from inclusion bodies were added to the assay mixture. From these data we can conclude that the formation of the chloroform-extractable material did not require other components except the polymerase and its substrate. The formation of PHA followed a sigmoidal curve, suggesting that there was an initial activation step before PHA synthesis could be observed. Ultimately, incubation of 1 μ g of soluble polymerase led to the incorporation of 15 nmol of 3-hydroxyoctanoic acid into chloroform-extractable PHA (Figure 2). 3-Hydroxyoctanoate accounted for 99.9% of this PHA and the PHA could therefore be regarded as a homopolymer. However, the exact physical state of the polymer produced (such as molecular mass, granule size, substrate specificity etc.) remains to be experimentally determined.

The finding that synthesis of polymer can occur in the presence of substrate and polymerase without any other additives is in accord with the previous findings of Gerngross and co-workers, who showed that PHB can be synthesized *in vitro* without additional factors except substrate and enzyme [6]. The kinetics of mcl-PHA production are also reminiscent of the kinetics seen with PHB polymerase, namely an initial low rate which

accelerates as the synthesis proceeds. Further work has to be done to understand the nature of this activation during polymer synthesis. A likely possibility is that the nascent polymer chain improves the catalytic characteristics of the polymerase, perhaps by binding to the sites through which the enzyme binds to native PHA granules.

In the present study we have established an efficient process to obtain soluble, active mcl-PHA polymerase which had previously proved difficult to isolate in an active form. Ion-exchange resin was applied to recover the activity of inactive PHA polymerase produced as inclusion bodies. We anticipate that the procedure applied here may be of potential use in solubilizing and activating other inactive enzymes.

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