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Co-expression of Two Polyhydroxyalkanoate Synthase Subunits from *Synechocystis* sp. PCC 6803 by Cell-Free Synthesis and Their Specific Activity for Polymerization of 3-Hydroxybutyryl-Coenzyme A

Keiji Numata,*[,†] Yoko Motoda,[†] Satoru Watanabe,[‡] Takashi Osanai,[§] and Takanori Kigawa[‡]

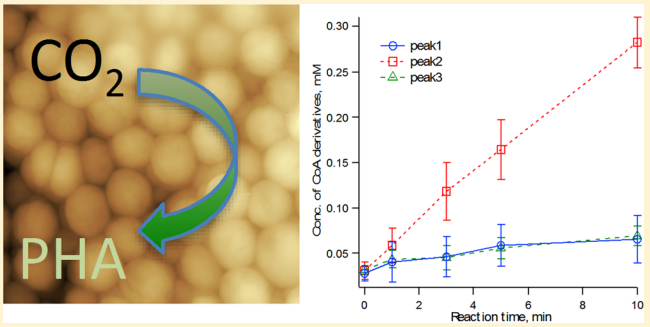
[†]Enzyme Research Team, RIKEN Center for Sustainable Resource Science, 2-1, Hirosawa, Wako-shi, Saitama 351-0198, Japan

[‡]Laboratory for Biomolecular Structure and Dynamics, RIKEN Quantitative Biology Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan

[§]Metabolic Systems Research Team, RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan

Supporting Information

ABSTRACT: *Synechocystis* sp. PCC 6803 is one of the most studied cyanobacteria for polyhydroxyalkanoate (PHA) synthesis, and its PHA synthase is known to consist of two subunits, namely, PhaC and PhaE. This report is the first to show the specific activity and related biochemical properties of PHA synthase from cyanobacteria. We have cloned and prepared a complex of PhaC and PhaE (PhaCE) from *Synechocystis* sp. PCC 6803 by the co-expression of PhaC and PhaE using a cell-free synthesis system. The specific activity of PhaCE was comparable to that of the class I PHA synthases, indicating that the low PHA productivity of cyanobacteria is not due to the activity of PHA synthase but may be caused by the other metabolic reactions related to PHA synthesis. The positive Hill coefficient of PhaCE as well as the size exclusion chromatography data indicates that dimeric PhaCE is a major active form that polymerizes 3-hydroxybutyryl-coenzyme A.



Polyhydroxyalkanoate (PHA) is a biological polyester synthesized for the intracellular storage of carbon and energy, which is needed for the survival of various microorganisms such as soil bacteria, cyanobacteria, etc.[1,2] PHA as a biopolymer is an attractive alternative to the petroleum-based plastics and thus helps in the reduction of carbon dioxide emissions. Efficient PHA production requires plant oil and/or sugars as a carbon source, which ultimately may still require multiple petroleum-based processes. Thus, an intuitive strategy for reducing carbon dioxide emissions would be to synthesize PHA using carbon dioxide as the sole carbon source and to employ phototrophic bacteria or cyanobacteria as the host bacteria. *Synechocystis* sp. PCC 6803 is one of the well-studied cyanobacteria for PHA synthesis.[3−5] Its PHA synthase is a class III synthase, which consists of two subunits, namely, PhaC and PhaE. Both PhaC and PhaE are ∼40 kDa and form a complex (PhaEC) during PHA polymerization.[6] Cyanobacteria, including *Synechocystis* sp. PCC 6803, synthesize poly[(*R*)-3-hydroxybutyrate] [P(3HB)] with relatively lower yields in comparison to those seen with soil bacteria such as *Ralstonia eutropha* H16 (*Cupriavidus necator* H16),[4,5] even though Miyake et al. reported relatively high yields of PHA with *Synechococcus* sp. MA19.[7] Therefore, the specific activity of PhaCE from cyanobacteria is considered relatively lower than that of major soil bacteria producing PHA.[8]

In vitro specific activity of PhaC and the mechanisms of polymerization during PHA synthesis have been studied well for more than a decade using PhaC from *R. eutropha* H16 and *Allochromatium vinosum* as prototypes of the class I and class III PHA synthases.[9−18] The synthase is considered a member of the α,β-hydrolase superfamily with an active site nucleophile at the elbow of a strand−turn−helix structure, suggesting that two active sites (thiol groups of Cys) from each PhaC monomer could generate the required active site at their interface.[2,11] In our previous work, we have reported the in vitro specific activity of PhaC from *Aeromonas caviae*, with and without its substrate, to characterize the active intermediates of PhaC in the polymerization reaction.[19] We have synthesized PhaC using a cell-free protein expression system after successful cloning of a PhaC gene from *A. caviae*.[20,21] Although the earlier challenges in the expression and purification of PhaC using recombinant *Escherichia coli* were not efficient enough for in vitro assay, we successfully obtained in vitro specific activity of the PhaC from *A. caviae* using a cell-free protein expression system.[22]

PhaCE from *Synechocystis* sp. PCC 6803 has not been investigated for its in vitro activity because of difficulties in its

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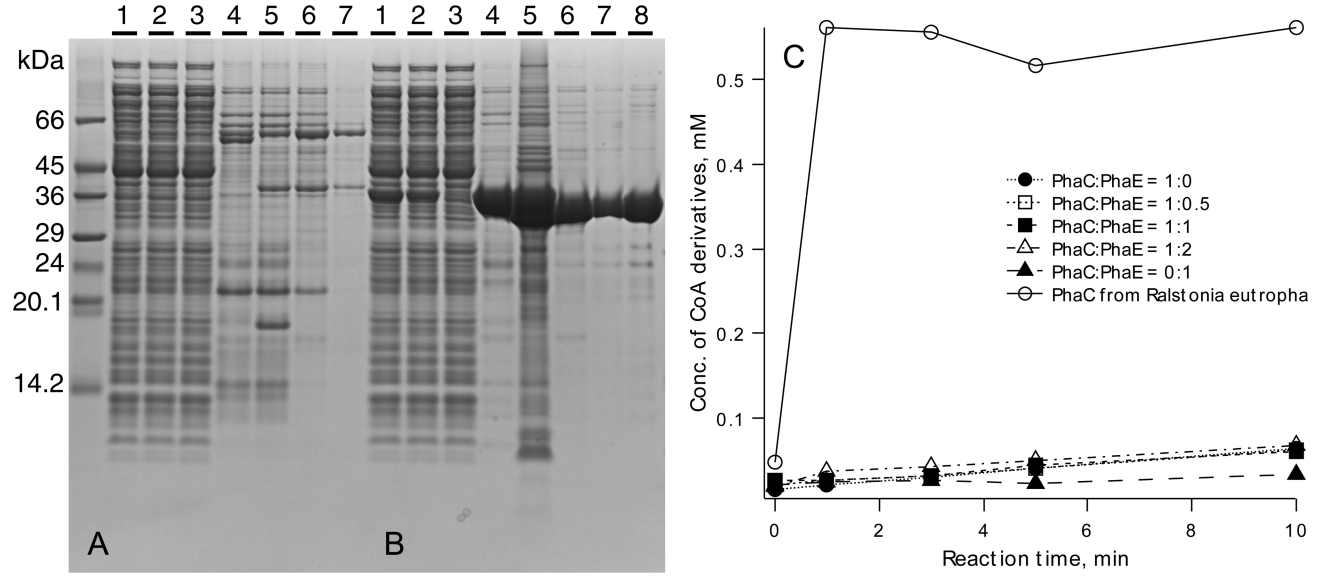

Figure 1. SDS−PAGE analysis of purified PhaC (A) and PhaE (B) from *Synechocystis* sp. PCC 6803 with an N11 (polyhistidine) tag: lane 1, total fraction; lane 2, supernatant; lane 3, flow-through of the first Ni-NTA system; lane 4, elution from the first Ni-NTA system; lane 5, TEV treatment to remove the tag; lane 6, elution after the second Ni-NTA system; lane 7, elution from the ion-exchange column; lane 8, elution from the gel filtration column. (C) Time course of release of CoA from 3HB-CoA catalyzed by a mixture of PhaC and PhaE (total concentration of 7.5 μM) with 1 mM 3HB-CoA. PhaC:PhaE molar ratios of 1:0, 1:0.5, 1:1, 1:2, and 0:1 were used. The data of PhaC from *R. eutropha* H16 were used as a positive control.

preparation using the recombinant *Escherichia coli* system, even though the PHA productivity of *Synechocystis* sp. PCC 6803 using carbon dioxide as a sole carbon source is a critical element to convert from carbon dioxide to Bioplastic. Also, the sequence of the PHA synthase from *Synechocystis* sp. PCC 6803 shows striking similarities to those of PhaC and PhaE from anoxygenic purple sulfur bacteria.[3] This fact makes PhaCE from *Synechocystis* sp. PCC 6803 a model PHA synthase of cyanobacteria and phototrophic bacteria. In this study, we have successfully synthesized PhaCE using co-expression of PhaC and PhaE by a cell-free protein expression system. We also confirmed that the specific activity of PhaCE is comparable to that of PhaC from *R. eutropha* H16 and *A. caviae* and is sufficiently high for efficient PHA production. The positive Hill coefficient of PhaCE and the size exclusion chromatography data indicate that dimeric PhaCE is a major active form that polymerizes 3-hydroxybutyryl-CoA (3HB-CoA).

MATERIALS AND METHODS

Cloning of PhaC and PhaE. Genomic DNA of *Synechocystis* sp. PCC 6803 was isolated by a standard procedure.[23] PhaC and PhaE were cloned by a two-step polymerase chain reaction (PCR), according to previous reports.[19,24] The forward (FW) and reverse (RV) primer sequences for the first PCR are listed in Table S1 of the Supporting Information. The first PCR program was identical to that of our previous report.[19] The second PCR was conducted according to previous reports,[19,24] for N11-SUMO-tag (N11-SUMO).[25,26] The N11 tag is a modified version of the natural polyhistidine tag.[24] It is a tandem tag of N11 and a small ubiquitin-modifying protein (SUMO).[25,26] A tobacco etch virus (TEV) protease recognition sequence was inserted to cleave the target PhaC and PhaE from the partner protein in N11 tag constructs.[27] Table S2 of the Supporting Information lists the tags used in this study. After the 10th cycle, the annealing temperature was changed to 64 °C and the duration of the extension was prolonged for 5 s per cycle. The last step was incubation at 72 °C for 7 min. The resultant product was immediately cooled to 10 °C. Its concentration was determined with a PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, CA).

Cell-Free Synthesis of PHA Synthase. The dialysis-mode cell-free protein synthesis method was used in this study, according to the literature.[19−21,28] A cell-free giant-scale dialysis using a dialysis membrane with a molecular weight cutoff (MWCO) of 15 kDa (Pierce, Rockford, IL) was performed using the reported reaction condition.[19] In this study, 4.0% polyethylene glycol (average molecular weight of 8000 Da) was added to the reaction mixture. The internal solution was dialyzed in a dialysis tube (Spectra/Por 7, MWCO of 15 kDa, Spectrum) against the external solution at 23 °C for 16 h while being shaken.[28]

Purification of PHA Synthase. Nine milliliters of the internal solution with the tagged protein was purified by AKTA Express (GE Healthcare, Little Chalfont, U.K.), using a literature procedure.[19] The buffers used for the purification were from our previous study.[19] Briefly, the protein solution was purified with HisTrap [5 mL, nickel-nitrilotriacetic acid (Ni-NTA) column, GE Healthcare]. To remove the tags, TEV (N11 tag) or SUMO protease (N11-SUMO tag) was added to the eluted fraction of the protein at a final concentration of 10 μg/mL.[24] The protein in the flow-through fraction was purified with HiPrep 26/10 desalting and HiTrapQ HP columns (1 mL, GE Healthcare) and further loaded onto HiLoad 16/60 Superdex 75 (GE Healthcare) to concentrate the purified protein. The yield of the purified protein was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Bovine serum albumin was used as the protein standard. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed using 15 to 20% precast Tris-HCl gels (DRC Co Ltd., Kyoto, Japan). The gel was stained with Coomassie brilliant blue.

Specific Activity Assay of PHA Synthase. The specific activities of PhaC and PhaCE were determined by the method

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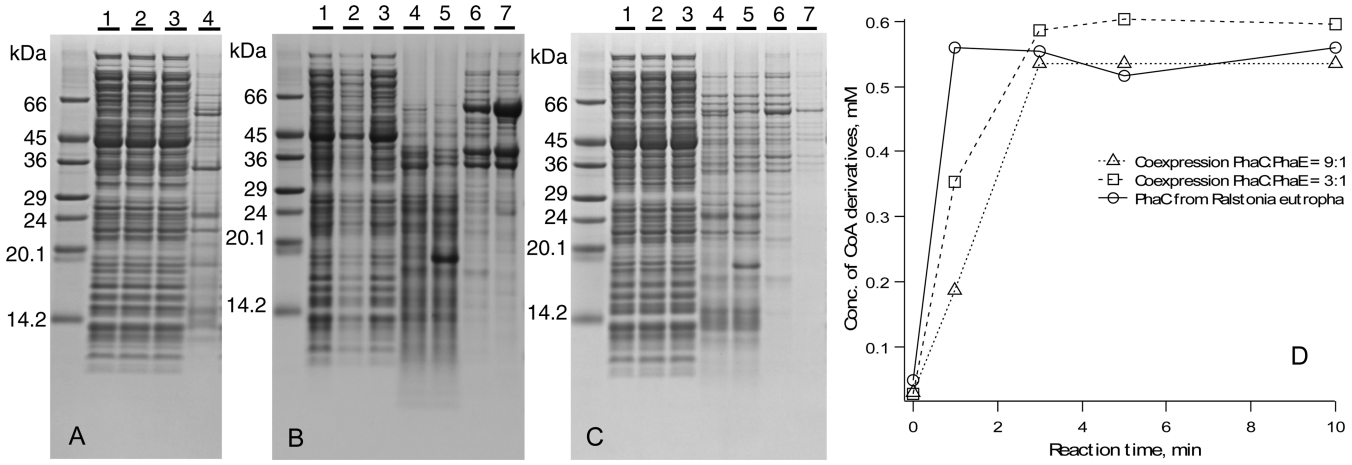

Figure 2. SDS−PAGE analysis of purified PhaCE from *Synechocystis* sp. PCC 6803 with a polyhistidine tag. (A) Co-expression of PhaC and PhaE with a 1:1 PhaC:PhaE DNA template molar ratio. (B) Co-expression with a 3:1 PhaC:PhaE template molar ratio. (C) Co-expression with a 9:1 PhaC:PhaE template molar ratio. In each gel: lane 1, total fraction; lane 2, supernatant; lane 3, flow-through of the first Ni-NTA system; lane 4, elution from the first Ni-NTA system; lane 5, SUMO protease treatment to remove the tag; lane 6, elution after the second Ni-NTA system; lane 7, elution from the ion-exchange column. (D) Time course of release of CoA from 3HB-CoA catalyzed by co-expressed PhaCE (total concentration of 7.5 μM) with 1 mM 3HB-CoA. Template ratios for the co-expression between PhaC and PhaE are 9:1 and 3:1. The data of PhaC from *R. eutropha* H16 were used as a positive control.

of Gerngross et al.[18] In this study, the modified method, which was reported previously,[19] was used. Briefly, coenzyme A released during the PHA synthase-catalyzed reaction can be measured using Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[29] The assay mixture (360 μL) contained 100 mM sodium phosphate (pH 7.5), 0.12 mM Triton X-100, an appropriate amount of 3HB-CoA (35, 70, 350, 700, 1000, 1400, or 2100 μM), and variable amounts of purified PhaC or PhaCE (0.75, 7.5, 10, or 15 μM). Reactions were initiated by the addition of PHA synthase. Dithionitrobenzoic acid (DTNB) was added to the mixture, and then the absorbance at 405 nm was mesaured. One unit was defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate/min. The specific activity was determined from the CoA release ranging from 0 to 1 min of the reaction, because of the absence of an obvious lag phase in the reaction. The Hill coefficients and the microscopic dissociation constant were calculated from the sigmoidal curve fitting of these data, according to a previous report.[19]

Size Exclusion Chromatography. Ten microliters of the reaction mixture contained PhaC and PhaE, 3HB-CoA, and 100 mM sodium phosphate (pH 7.5). It was incubated for 15 min at 25 °C and then loaded onto a GE Superdex200 column (GE Healthcare Life Sciences, Buckinghamshire, U.K.; separation range of 10−600 kDa, exclusion limit of 1300 kDa) equilibrated with 20 mM NaP_i (pH 7.0) containing 200 mM Na_2SO_4 at 25 °C. The reaction mixture containing PhaC and PhaE was eluted with the same buffer at a flow rate of 1 mL/min at 25 °C. The molecular weight was determined on the basis of a calibration curve prepared using the following molecular weight standards: thyroglobulin (669 kDa, 19.1 min), apoferritin (443 kDa, 22.5 min), catalase (240 kDa, 25.3 min), alcohol dehydrogenase (150 kDa, 25.8 min), and bovine serum albumin (BSA, 66 kDa, 27.9 min). The molecular weights, the elution times of the standards, and a calibration curve based on the relationship between the molecular weights and the elution time of the standards are shown in Figure S1 of the Supporting Information.

RESULTS

Activity of the Mixture of PhaC and PhaE. The expression levels of PhaC and PhaE determined by cell-free synthesis were evaluated using SDS−PAGE (Figure 1A,B), which reveals that the expression levels of PhaE were much higher than that of PhaC. Both PhaC and PhaE were expressed with N11 and SUMO tags for the purification process. After the SUMO treatment and the purification of PhaC and PhaE by multiple steps using Ni-NTA, ion-exchange, and gel filtration columns, purified PhaC and PhaE were concentrated to approximately 30 μM. Yields of purified PhaC and PhaE were approximately 7.8 and 61 mg, respectively, in a 1 L-scale reaction mixture. In lane 7 of Figure 1A, bands around 40 and 60 kDa correspond to monomeric and dimeric PhaC, respectively. Purified PhaC and PhaE were mixed in 100 mM sodium phosphate (pH 7.5) containing 0.12 mM Triton X-100 and characterized using a polymerization activity assay of 3HB-CoA (Figure 1C). The mixture of subunits PhaC and PhaE did not show significant activity compared to the activity of PhaC from *R. eutropha* H16, based on the release of CoA from the polymerization of 3HB-CoA. Five different PhaC:PhaE molar ratios (1:0, 1:0.5, 1:1, 1:2, and 0:1) were investigated. However, none of them showed any significant activity (Figure 1C).

Co-expression of PhaC and PhaE: PhaCE Formation. Co-expression of PhaC with the N11 tag and PhaE without any tag by cell-free synthesis was performed using three PhaC:PhaE template DNA molar ratios, namely, 1:1, 3:1, and 9:1. Co-expression using 1:1 PhaC:PhaE molar ratio templates did not efficiently yield PhaC and PhaE (Figure 2A). However, with 3:1 and 9:1 PhaC:PhaE template molar ratios, PhaC and PhaE were successfully co-expressed (Figure 2B,C), resulting in the yields of purified PhaCE of approximately 26 and 8.3 mg, respectively, in a 1 L-scale reaction mixture. In lane 7 of panels B and C of Figure 2, bands around 35, 40, and 60 kDa correspond to monomeric PhaE, monomeric PhaC, and dimeric PhaC, respectively. On the basis of the co-expression levels of PhaC and PhaE with a 3:1 PhaC:PhaE template molar ratio, the PhaC:PhaE molar ratio was approximately 1:1 (Figure 2B). The PhaE without any tag sequence was copurified with

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PhaC, indicating that PhaE formed a complex with PhaC during the co-expression. The polymerization activity of PhaCE was evaluated with 1 mM 3HB-CoA. PhaCE synthesized with a 3:1 PhaC:PhaE template molar ratio showed a slightly higher activity that is comparable to the activity of PhaC from *R. eutropha* H16 (Figure 2D) based on the release of CoA from the polymerization reaction. These result indicate that co-expression of PhaC and PhaE can provide PhaCE showing a true activity, whereas mixing of PhaC and PhaE does not induce formation of the PhaCE complex.

Effect of the Concentration of PhaCE and 3HB-CoA. The polymerization activity of PhaCE, which was synthesized at 3:1 PhaC:PhaE template DNA molar ratios, was studied as a function of its concentration based on the release of CoA from 3HB-CoA (Figure 3). The polymerization activity of PhaCE

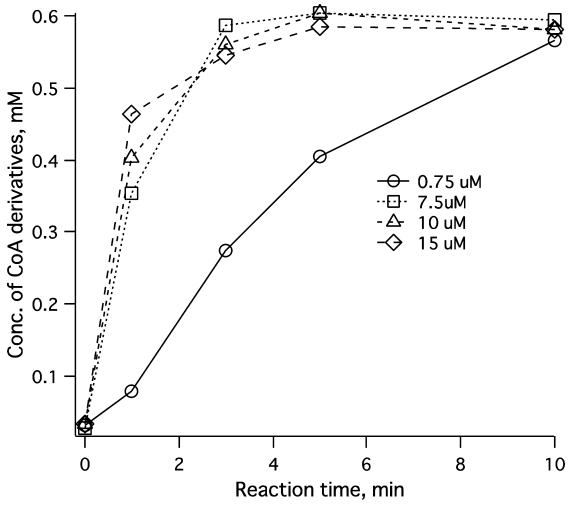

Figure 3. Time course of release of CoA from 3HB-CoA catalyzed by PhaCE at various concentrations ranging from 0.75 to 15 μM. The concentration of 3HB-CoA was 1 mM.

increased beyond 0.75 μM and then reached a plateau at 7.5, 10, and 15 μM. The activity was approximately 4 units/mg, comparable to the activity of PhaC from *R. eutropha* H16, suggesting that the activity of PhaCE is sufficiently high for efficient PHA production.

The specific activity of PhaCE as a function of 3HB-CoA concentration (up to 2100 μM, i.e., in excess of PhaC, 0.75 μM) was characterized (Figures 4) and displayed sigmoidal kinetics. The cooperativity of PhaCE with 3HB-CoA was estimated by the Hill coefficient (n = 1.5 ± 0.2) and the microscopic dissociation constant (K_m = 478 ± 31 μM) based on the Hill plot. The Hill coefficient represents a degree of cooperativeness of PhaCE with 3HB-CoA. In case the Hill coefficient is >1, generally, the enzyme shows a positive cooperative binding with a substrate. Given the positive value of the Hill coefficient in this study, PhaCE seems to multimerize with 3HB-CoA.

Size Exclusion Chromatography of PhaCE. Multimerization of PhaCE was characterized quantitatively by size exclusion chromatography (Figure 5A). PhaCE at 0.75 μM without 3HB-CoA was examined. It revealed three major components corresponding to peak 1 (19.6 min), peak 2 (26.5 min), and peak 3 (30.4 min) on a chromatogram. SDS−PAGE analysis (Figure 5B) of each fraction from size exclusion chromatography indicated that peak 1 contained PhaC and

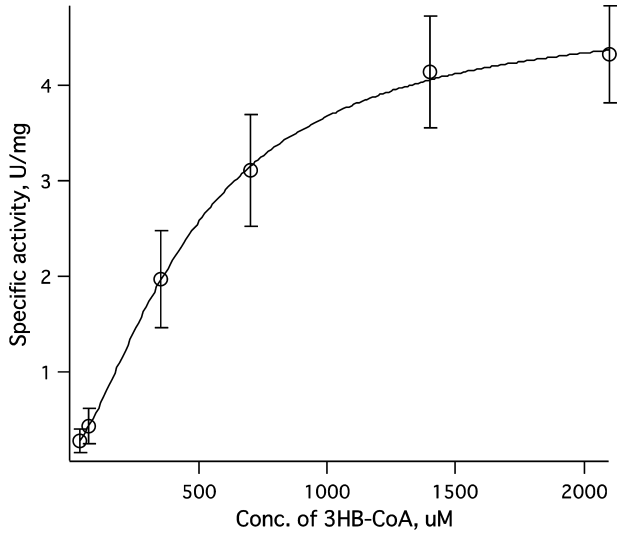

Figure 4. Specific activity of PhaCE as a function of 3HB-CoA concentration. The concentration of PhaCE was 0.75 μM. Sigmoidal curve fitting is represented by a solid line.

PhaE, peak 2 contained bands corresponding to PhaC and PhaE, while peak 3 contained lower-molecular weight impurities and a small amount of PhaC and PhaE. Only peak 2 contained two bands around 66 kDa, which correspond PhaC. According to the elution time of the marker proteins (Figure S1 of the Supporting Information), peak 2 with an elution time of 26.5 min, which corresponds to 142.7 kDa, could be assigned to dimeric PhaCE (PhaC, 43.0 kDa; PhaE, 36.2 kDa; PhaCE, 79.2 kDa). Peak 1 might be due to aggregation of PhaCE with impurities. Peak 3, based on their individual molecular weights, corresponded to monomeric PhaC and PhaE without any complex formation. The polymerization activity of each peak was characterized on the basis of CoA release (Figure 5C). Peak 2 (0.46 μM) demonstrated a significant activity similar to that of overall PhaCE shown in Figure 3. Thus, the dimeric PhaCE in peak 2 contributed mainly to the polymerization activity. Previous reports of the gel chromatography profiles of class I PHA synthase suggest that a substrate such as 3HB-CoA is needed to induce dimer formation or multimerization of PhaC.[17,19] Therefore, the mixture of PhaCE (7.5 μM) and 1 mM 3HB-CoA was characterized by size exclusion chromatography (Figure S3 of the Supporting Information), resulting in a broad peak at 19.6 min that implies the presence of the aggregates of PhaCE and 3HB-CoA over 600 kDa.

■ DISCUSSION

Investigation of the biochemical properties of the PHA synthase from cyanobacteria, which can synthesize PHA directly from carbon dioxide via photosynthesis, was limited because of the low expression levels of recombinant PhaC and PhaE in *E. coli*. Class III PHA synthases have received less attention than class I PHA synthases like those from *R. eutropha* H16 and *A. caviae*. Stubbe and co-workers have studied the *Al. vinosum* enzyme as a prototype of the class III PHA synthase.[9−18] The Cys149Ser mutant of PhaC, which catalyzes polymer formation at a rate 1/2200 times that of wild-type PhaC, was used to investigate the role of Cys149 and covalent and noncovalent 3HB-CoA intermediates of PhaC from *Al. vinosum*.[16] These studies provide direct evidence of the

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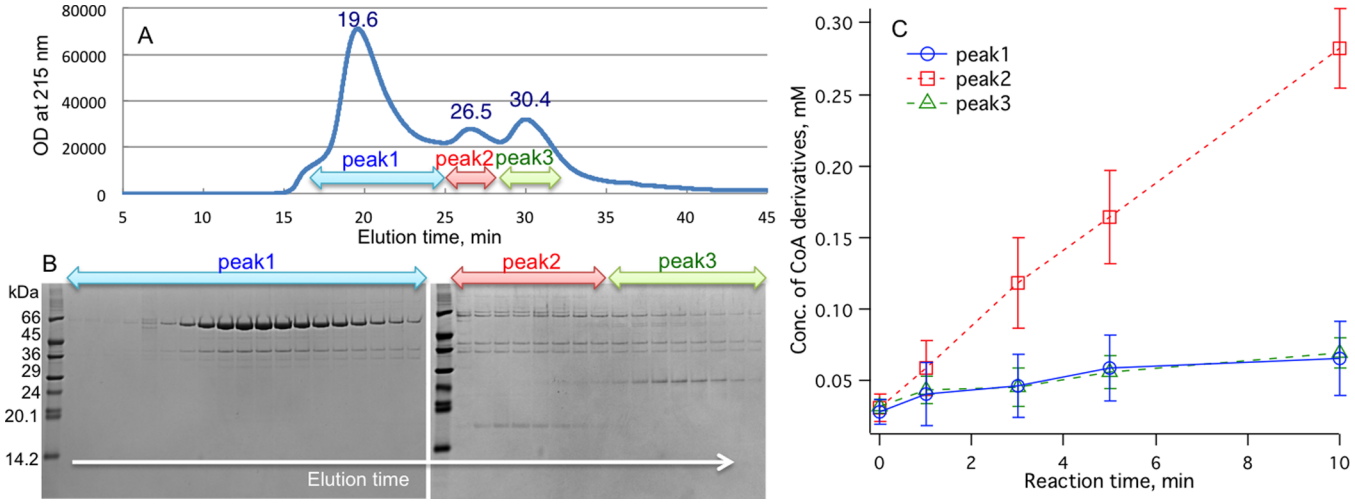

Figure 5. (A) Size exclusion chromatography profile of PhaCE, which was co-expressed using a 3:1 PhaC:PhaE template ratio. The concentration of PhaCE was 0.75 μM. Three peaks are labeled as peaks 1−3. (B) SDS−PAGE analysis of the PhaCE sample collected in each fraction. (C) Time course of release of CoA from 3HB-CoA catalyzed by PhaCE collected in peak 1, peak 2, and peak 3 fractions. The concentrations of PhaCE and 3HB-CoA were 0.75 μM and 1 mM, respectively.

importance of covalent and noncovalent catalysis in the polymerization reaction of synthase from *Al. vinosum*. In this study, we successfully cloned another class III PHA synthase (PhaC and PhaE) gene from *Synechocystis* sp. PCC 6803 and prepared the complex of PhaC and PhaE, namely PhaCE, by a cell-free protein co-expression system. Because of the difficulty of their expression and purification using recombinant *E. coli*, this is the first report of the in vitro specific activity of PhaCE from cyanobacteria such as *Synechocystis* sp. PCC 6803. We revealed that co-expression of PhaC and PhaE is needed to realize their in vitro specific activity for polymerization of 3HB-CoA. The specific activity of PhaCE was significantly higher than that of the mixture of PhaC and PhaE (Figures 1 and 2) and comparable to that of PhaC from *R. eutropha* H16, one of the most active PHA synthases (Figures 2 and 4). Therefore, we concluded that the low PHA productivity of cyanobacteria is not due to PHA synthase activity but might be caused by the other pathways related to PHA synthesis.

The size exclusion chromatography data support the hypothesis that PhaCE seems to form dimers to exert polymerization activity (Figure 5), similar to that of the class I PHA synthase from *R. eutropha* and *A. caviae*.[17,19] Moreover, the positive Hill coefficient ($n > 1$) shows positive cooperativity of PhaCE with 3HB-CoA, which means that the presence of 3HB-CoA bound to PhaCE further enhances its affinity for dimerization (Figure 4). The microscopic dissociation constant, K_m, represents the equilibrium constant for dissociation of the enzyme from the substrate. Several K_m values for PHA synthases have been previously reported. The K_m of PHA synthase from *R. eutropha*, class I PHA synthase, for 3HB-CoA is reported to be 103 μM (final concentration of the enzyme not described),[30] whereas the K_m for granule-bound PHA synthase from *R. eutropha* with 3HB-CoA was 680 μM.[31] Also, in our previous report, the K_m of PhaC from *A. caviae* (7.5 μM) was 77 ± 5 μM.[19] Here we have found the K_m of PhaCE to be 478 ± 31 μM, indicating an affinity of PhaCE for 3HB-CoA lower than that of class I PHA synthases from *R. eutropha* and *A. caviae*. On the basis of the results for the Hill coefficient, K_m, and specific activity of PhaCE, those biochemical parameters are slightly different from those of but the dimerization behavior was similar to that of the class I PHA synthase. According to the similarity of PhaCE between *Synechocystis* sp. PCC 6803 and purple sulfur bacteria,[3] PHA synthase of purple sulfur bacteria could show biochemical properties similar to those of the PhaCE in this study.

The aggregation of PhaC and PhaE was detected by size exclusion chromatography (Figure 5A, peaks 1 and 3). It is thought that impurities in the PhaC and PhaE samples could induce aggregation. We performed multiple purification steps using Ni-NTA, ion-exchange, and gel filtration columns; however, the PhaCE sample still contained traces of impurities. In lane 7 of Figure 2B, faint bands corresponding to possible impurities were recognized in addition to PhaC and PhaE. The specific activity determined in this study might be lower than the actual activity because of the presence of impurities. Peak 2 in Figure 5A shows obvious polymerization activity in comparison to the other two peaks, suggesting that the dimeric PhaCE is the major active form to polymerize 3HB-CoA. This is also supported by the positive Hill coefficient of PhaCE.

■ CONCLUSION

This report is the first to show and discuss the specific activity of PHA synthase from cyanobacteria. We have cloned and prepared a PhaCE from *Synechocystis* sp. PCC 6803 by co-expression of PhaC and PhaE using a cell-free synthesis. The specific activity of PhaCE was comparable to those of the class I PHA synthases, indicating that the low PHA productivity of cyanobacteria is not caused by the activity or biochemical properties of PHA synthase but may be caused by the other metabolic reactions related to PHA synthesis. The positive Hill coefficient of PhaCE (n = 1.5 ± 0.2) as well as the size exclusion chromatography data indicates that the dimeric PhaCE is a major active form that polymerizes 3HB-CoA. These findings, using a cell-free co-expression system, illustrate utilization of cyanobacteria as an efficient PHA producer via photosynthesis.

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■ ASSOCIATED CONTENT

Supporting Information

Additional data (Tables S1 and S2 and Figures S1−S3). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan. Phone: +81-48-467-9525. Fax: +81-48-462-4664. E-mail: keiji.numata@riken.jp.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PHA, polyhydroxyalkanoate; PhaC and PhaE, PHA synthase subunits; PhaCE, complex of PhaC and PhaE; 3HB-CoA, 3-hydroxybutyryl-coenzyme A; PCR, polymerase chain reaction; FW, forward; RV, reverse; dNTP, deoxyribonucleotide triphosphate; N11, natural polyhistidine tag; SUMO, small ubiquitin-modifying protein; TEV, tobacco etch virus; EDTA, ethylenediaminetetraacetic acid; MWCO, molecular weight cutoff; Ni-NTA, nickel-nitrilotriacetic acid; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); *n*, Hill coefficient; K_m, microscopic dissociation constant.

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