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Amorphous Poly(3-hydroxybutyrate) Nanoparticles Prepared with Recombinant Phasins and PHB Depolymerase

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ABSTRACT: Phasin protein (PhaP) is known to anchor into the matrix of phospholipid surrounding polyhydroxyalkanoic acid (PHA) inclusion bodies formed in bacterial cells and regulate the size of the granules, as well as the number of PHA granules. To investigate the effect of phasin on the formation of artificial poly(3-hydroxybutyrate) (P(3HB)) granules *in vitro*, $(His)_6$-tagged or GST-fusion recombinant phasin was prepared and utilized for the artificial granule preparation. In addition, a P(3HB) depolymerase was coloaded with the recombinant phasin to prepare self-degradable phasin-coated P(3HB) granules. A water/chloroform two-phase emulsion technique was used, in which the emulsification was carried out by sonication, and the chloroform in the emulsion was removed by stirring-aided evaporation at room temperature or 65°C. Slower chloroform removal at room temperature produced better spherically shaped P(3HB) nanogranules, which were uniformly sized (~100 to 200 nm in diameter). The self-degradability of P(3HB) depolymerase-loaded P(3HB) nanogranules was investigated. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 41074.

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INTRODUCTION

Phasin (PhaP) is an amphiphilic protein responsible for the regulation of the size of polyhydroxyalkanoic acid (PHA) granules, and their number density in bacterial cells.[1–3] The existence of phasin localized in the interfacial region between cytosolic water and a hydrophobic PHA inclusion body was reported first by the Steinbüchel group.[3] The autoregulated repressor PhaR is known to regulate *phaP* expression[1]; however, the mechanism of the involvement of the phasin protein in the self-assembled formation of PHA granules in the cells is unknown.

The primary sequence structure of phasin is quite different among the genus. PhaP, found in short-chain-length PHA producing bacteria, has seven isotypes, which have 38–70% homology to one another.[4] Among the isotypes, only one PhaP (PhaP1 in *Ralstonia eutropha*) is expressed in the major fraction and localized on the surface of PHA granules. On the contrary, PhaF, found in medium-chain-length PHA producing *Pseudomonas spp.* has no isotype and high homology among the same genus.[2] *Pseudomonas* PhaF phasin has enriched repeated A (Alanine), K (Lysine), and P (Proline) amino acids in the C-terminal. Phasin molecules have a size between 16 and 23 kDa, and a hydrophobic amino acid-rich region responsible for binding to a PHA polymer. The phasin in which the hydrophobic region is removed does not bind to a PHA granule surface. Phasin proteins are thermally stable up to 121°C for 20 min.[5] They are also stable upon exposure to acid and alkali as well as organic solvents such as C1–C8 alkanol, acetone, acetonitrile, and chloroform.[5]

Because of the biocompatibility and *in vivo* degradability of polyhydroxybutyrate (PHB) in animals, the use of PHB in drug delivery has been evaluated in a number of studies.[6–11] However, the *in vivo* degradation was too slow, but nevertheless PHB was completely sorbed after 24–30 months.[9] Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) was used to prepare the PHA nanoparticles (NPs) loaded with 5-fluorouracil, an anticancer drug, as a drug delivery carrier.[8] 5-Fluorouracil[7] or gentamicin sulfate[6] was also loaded into poly(3-hydroxybutyrate-*co*−3-hydroxyvalerate)

microspheres (20–300 μm in diameter) or wafers, respectively, for its sustained release. Various methods for preparing PHA nanoparticles have been reported.[12–15] Interestingly, tumor-specific hybrid PHB NPs were prepared using the coupled enzymatic process with oil-in-water (O/W) emulsion methods as surface functionalized nanocarriers.[15] NPs of amphiphilic triblock copolymers based on poly(3-hydroxybutyrate) (P(3HB)) and poly(ethylene glycol) as drug carriers were also prepared by a precipitation/solvent evaporation technique without any surfactants[13] with their sizes ranging between ~20 and 150 nm. *In vitro* enzymatic polymerization of (R)-3-hydroxybutyryl coenzyme A by a PHA synthase in the presence of BSA and a detergent resulted in clusters composed of 100–250 nm granules.[14] Stable poly(3-hydroxyoctanoate) latex was prepared from *Pseudomonas oleovorans* using hypochlorite treatment.[12] Thus, depending on the condition of the emulsification, various types of PHA nanoparticles varying in size from 10 to 1000 nm can be prepared for effective drug delivery devices.[16]

If PHA is used as a long-term drug delivery matrix,[17–19] the PHA should be dissolved in an organic solvent to load the drug in a solvent evaporation method. However, if the drug is a polypeptide, the situation may be quite complicated because of its instability during the process.[20] Since phasin specifically binds to the surface of PHA granules, the fusion of the polypeptide to phasin might be a solution in the protein loading without activity loss if the fused polypeptide is stable during the emulsification process. Therefore, in this study, we tried to prepare artificial P(3HB) granules using a modified two-phase emulsion technique[21–23] assisted with recombinant phasin proteins. In addition, self-degradable P(3HB) granules were prepared by loading a P(3HB) depolymerase into them.

EXPERIMENTAL

Manipulation of DNA and Plasmid Construction

Plasmid isolation, gel electrophoresis, transformation, PCR, and cloning for vector construction were performed through standard procedures.[24] The *phaP1* gene (coding the major protein among the four isotypes, *phaP1*, *phaP2*, *phaP3*, and *phaP4*[1] in *Ralstonia eutropha* H16 chromosomal DNA was PCR amplified using the primers: RephaP-Bam (5'-CATGGATCCATGATCCTCACCCCG-3'), which has a BamHIsite in the translation start codon region and RephaP-Eco (5'-GGAGAATTCTCAGGCAGCCGTCGT-3'), which has an EcoRIsite in the stop codon region. The amplified fragment was inserted into a pET-28a (Novagen, USA) vector for $(His)_6$-tagging expression and pGEX-linker2 (GE Healthcare Life Sciences) vector for glutathione S-transferase (GST, *Schistosoma japonicum*, protein size: 26 kDa) fusion expression generating pET-RephaP and pGEX-RephaP, respectively. The two constructed plasmids, pET-RephaP and pGEX- RephaP were introduced into *E. coli* BL21(DE3) by electroporation.[25] The two recombinants *E. coli* BL21(DE3) harboring pET-RephaP and pGEX-RephaP, respectively, were selected on an LB medium containing kanamycin and ampicillin, respectively, at 30°C. *E. coli* BL21(DE3) harboring pET-RephaP was inoculated to an LB medium containing kanamycin (30 μg/mL), cultivated for 3 h at 37°C, and then further cultivated in the presence of 0.1 m*M* IPTG for 3 h at 37°C and harvested. *E. coli* BL21(DE3) harboring pGEX-RephaP was inoculated to an LB medium containing ampicilin (50 μg/mL), cultivated for 3 h at 37°C, and then further cultivated in the presence of 0.1 m*M* IPTG for 12 h at 20°C and harvested.

Protein Purification

Both *R. eutropha* $(His)_6$-tagged PhaP and GST-fused PhaP were overexpressed as soluble proteins. $(His)_6$-tagged PhaP protein was purified by metal chelation chromatography loaded with Ni-NTA Chelating Agarose CL-6B (Peptron, Korea) and GST fusion protein by glutathione affinity chromatography loaded with Glutathione Sepharose™ 4 Fast Flow (GE Healthcare, USA) according to the protocol of the instructions.

Extracellular P(3HB) depolymerase was isolated from *Ralstonia pickettii* T1. *R. pickettii* T1 was inoculated to a P(3HB)-supplemental medium, cultivated for 24 h at 30°C, and then harvested. The enzyme was purified by hydrophobic affinity chromatography loaded with Toyopearl phenyl-650 M resin (Tosoh Bioscience, Japan) according to the protocol in the instructions. A 10 m*M* potassium phosphate buffer (pH 7.2) was loaded into the column for equilibration (l mL/min flow rate, 200 min), and the culture supernatant was then loaded into the column (l mL/min flow rate) and eluted by an ethanol gradient (0–40%), an eluted solution dialyzed against distilled water using a cellulose acetate membrane (Sigma, Seamless cellulose tubing, MW 12,000~14,000).

Preparation of Detergent or Protein-Assisted PHB Granules

Artificial amorphous P(3HB) granules were prepared according to the method of Horowitz and Sanders.[26] In the detergent treatment method, two types of detergent, sodium deoxycholate (SDC)[27] and cetyltrimethyl-ammonium bromide (CTAB), were used as anionic and cationic detergents, respectively. In the SDC method, P(3HB) homopolymer (MW = 611,200, determined by GPC) (synthesized in *R. eutropha* H16)[28] was dissolved in 1 mL of chloroform (5 (w/v)%), and an SDC solution of an appropriate concentration in 20 mL DW was added to the 1 ml P(3HB) chloroform solution (mixing ratio, 1 : 20). The two-layered solution was sonicated at 20 kHz for 1.5 min at room temperature to emulsify it. The emulsion solution was stirred at 65°C for 1 h and at room temperature for 4 h to remove the chloroform. The aggregated polymer particles were removed by filtration with a paper filter (pore size, 5 μm). The filtrate was dialyzed against 0.01 (w/v)% SDC for 24 h using a cellulose acetate membrane (MW cut-off 3,500, Membrane Filtration Product, USA). The granules isolated by centrifugation (1500 x *g*) were stored in a phosphate buffer (50 m*M* sodium phosphate, pH 8.0). The CTAB-treated granules, isolated by centrifugation from the filtrate, were kept in the same buffer without dialysis. All other granules prepared using $(His)_6$-tagged PhaP or GST-fusion PhaP were processed similarly.

In the modified method, 5 mL of an SDC solution in distilled water and/or $(His)_6$-tagged PhaP was added to 1 mL of the chloroform solution containing P(3HB) (3 (w/v)%). The two-layered solution was sonicated at 20 kHz (10%) for 3 min at room temperature. The emulsion solution was then centrifuged, and the isolated upper water-rich layer was stirred at room temperature for 30 min to 1 h to remove chloroform. The artificial

amorphous P(3HB) granules suspension was washed twice with 50 m*M* Tris-HCl (pH 8.0) and stored at 4°C for further studies. All other granules prepared using $(His)_6$-tagged PhaP and extracellular P(3HB) depolymerase were processed similarly.

Scanning Electron Microscopy (SEM)

The granule samples for visualization were fixed with a fresh solution of 2.5% glutaraldehyde (Electron Microscopy Sciences, USA) in a 0.2*M* sodium phosphate buffer (pH 7.2) for 3 h at 4°C, and the glutaraldehyde fixed samples were then washed twice with a phosphate buffer, followed by a second fixation with a fresh 1% osmium-tetra-oxide solution for 2 h at 4°C. The osmium-tetra-oxide fixed samples were dehydrated in a graded ethanol series (50, 60, 70, 80, 90, and 100% for 5 min each). Finally, the samples were coated with gold (fine coat sputter JFC-1100, JEOL) and observed using a field emission scanning electron microscope (FE-SEM) (Philips XL30 S FEG, Netherlands).

Degradation Kinetics of *Ralstonia pickettii* T1 Extracellular P(3HB) Depolymerase-Loaded P(3HB) Granules

The degradation of P(3HB) granules loaded with *Ralstonia pickettii* T1 extracellular P(3HB) depolymerase was monitored by measuring the decrease of optical density (O.D.) at 650 nm for the emulsion solution. The P(3HB) depolymerase-loaded P(3HB) granules were suspended in 1 mL of a 50 m*M* Tris-HCl buffer (pH 8.0) and their initial absorbance was 0.685 at 650 nm. The solution was incubated and shaken at 100 rpm at 37°C for the time intervals between measurements, and after transferring the solution to a UV cell cuvette, its O.D. was measured.[29] All experiments were performed in triplicate.

Emulsifying Properties of Recombinant Phasin

To observe an emulsifying property of phasin protein $(His)_6$-PhaP, 10 m*M* stearic acid was dissolved in 5 mL of octanol or chloroform. The organic solvent mixture was mixed with 1 mL of an aqueous phasin solution dissolved in a 10 m*M* sodium phosphate buffer (pH 7.2) and the mixture in a 10 mL test tube was inverted at 50 rpm at room temperature for 12 h.

Other Analytical Methods

The protein content was measured by the method of Lowry.[30] SDS-polyacrylamide gel electrophoresis was done by the procedure of Laemmli.[31]

RESULTS AND DISCUSSION

Purification and Characterization of Proteins

The recombinant proteins, $(His)_6$-PhaP and GST-fused PhaP, and extracellular P(3HB) depolymerase, were purified enough as shown by a single electrophoretic band [Figure 1(a)]. The size of *R. eutropha* PhaP and *Ralstonia pickettii* T1 extracellular P(3HB) depolymerase (PhaZ) deduced from their gene sequences is 19 kDa and 47 kDa, respectively. Thus, $(His)_6$-tagged *R. eutropha* PhaP, GST-fused *R. eutropha* PhaP, and *R. pickettii* PhaZ showed a reasonable size in comparison with their original sizes.

Phasin and phasin-like proteins are known to be stable against their exposure to organic solvents.[5] The proteins are localized on the surface of the PHA granules in the cells along with PHA granule surrounding lipids,[1–3] which means the hydrophobic/hydrophilic amphiphilic property of the proteins. A 12-h inversion of the organic solvent/water phase induced the formation of water-in-oil emulsion where phasin molecules migrated into the oil phase to emulsify the hydrophobic stearic acid in oil [Figure 1(b)]. The amphiphilic phasin showed better emulsifying effect in the chloroform phase than the octanol phase. This surfactant-like property clearly demonstrates the potential role of phasin in the formation of artificial PHA granules using a two-phase emulsifying technique.

Preparation and Characterization of Recombinant Phasin-Assisted P(3HB) Nanoparticles

Contrary to the emulsifying role of phasin transferred into the organic phase containing stearic acid [Figure 1(b)], in the emulsification of P(3HB), phasin remained in the water phase and emulsified the P(3HB) polymer in the chloroform phase [Figure 1(c)]. Thus, the emulsification process is considered to occur in the interfacial region between water and chloroform. However, the size and shape of P(3HB) nanoparticles may strongly depend on the processing condition such as a solvent removal method. In a conventional method, chloroform was removed by high temperature evaporation (e.g., at 65°C, the boiling point of chloroform). Hence, we initially employed the conventional method in P(3HB) granule preparations and then the room-temperature evaporation method for comparison.

We examined the effects of two different types of detergent, phasin concentration, and GST tagging of phasin under 65°C evaporation. In the presence of 0.05 w/v% detergent such as CTAB and SDC, compared with the native granules isolated from *R. eutropha* cells by a mild hypochlorite treatment, the two artificial granules were neither structurally unique nor uniform in size even though anionic SDC treated granules look better than cationic CTAB treated ones [Figure 2(a)]. The size of the SDC-treated granules showed a bimodal distribution: smaller ones in the range of approximately 100–200 nm, bigger ones in the range of approximately 400–500 nm. Phasin is known to regulate *in vivo* both the number of granules and their size in cells.[1–3] Therefore, we coadded 1 μg/mL or 5 μg/mL phasin to the above detergent treatment system [Figure 2(b)]. Compared with the phasin-free system, addition of 1 μg/mL phasin resulted in the formation of relatively well-defined spherical shape granules. A five-fold addition of phasin induced a maximum two-fold increase in diameter of the P(3HB) granules. This clearly demonstrates that the phasin affects the granule formation *in vitro* as well as *in vivo*.

A high expression of phasin *in vivo* is known to decrease the size of the granules and increase their number. To understand the *in vitro* effect of phasin itself on the granule formation, a concentration-dependent experiment was carried out against the two types of phasins, $(His)_6$-PhaP, and GST-PhaP [Figure 2(c)]. High-temperature evaporation method was employed in the granule preparation. An increase in the phasin concentration rather increased the size of the P(3HB) granules contrary to the *in vivo* formation. For both types of phasins, at a low protein concentration, the size distribution was quite broad, but at a higher concentration, the granules of two different groups of

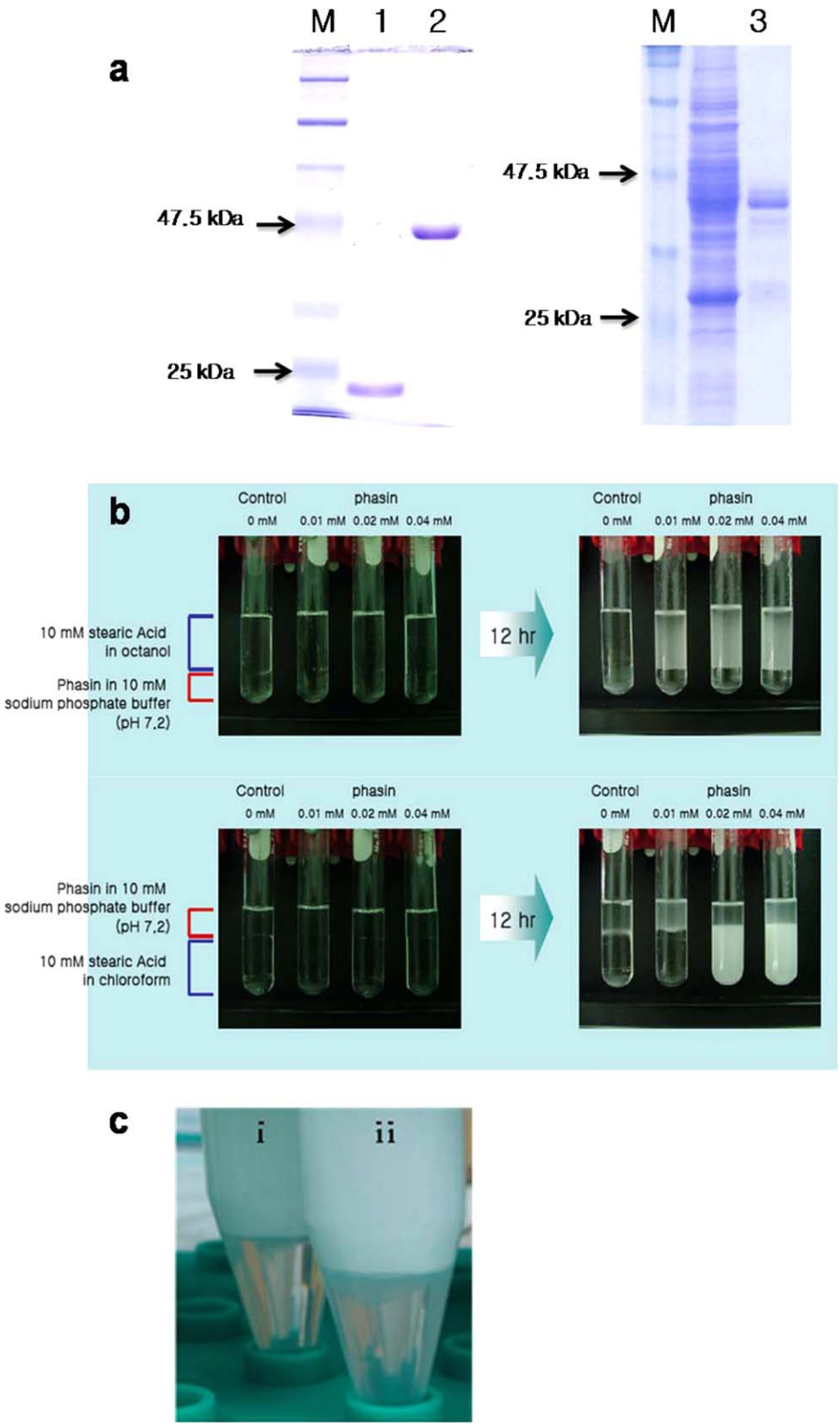

Figure 1. (a) SDS-PAGE of purified phasin proteins and extracellular P(3HB) depolymerase. Lane M, Molecular standard maker; Lane 1, $(His)_6$-tagged *Ralstonia eutropha* phasin (19 kD); Lane 2, *Ralstonia pickettii* T1 P(3HB) depolymerase (47 kD); Lane 3, GST-fused *Ralstonia eutropha* phasin (45 kD).; (b) Emulsifying property of $(His)_6$-tagged phasin. Phasin concentration was 200 μg/mL; (c) Emulsification phase layers of P(3HB) in chloroform (lower) and SDC in water (upper). (i) P(3HB) granules with 0.05% sodium deoxycholate, (ii) P(3HB) granules with 0.05% sodium deoxycholate and 2.5 μg/mL depolymerase. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

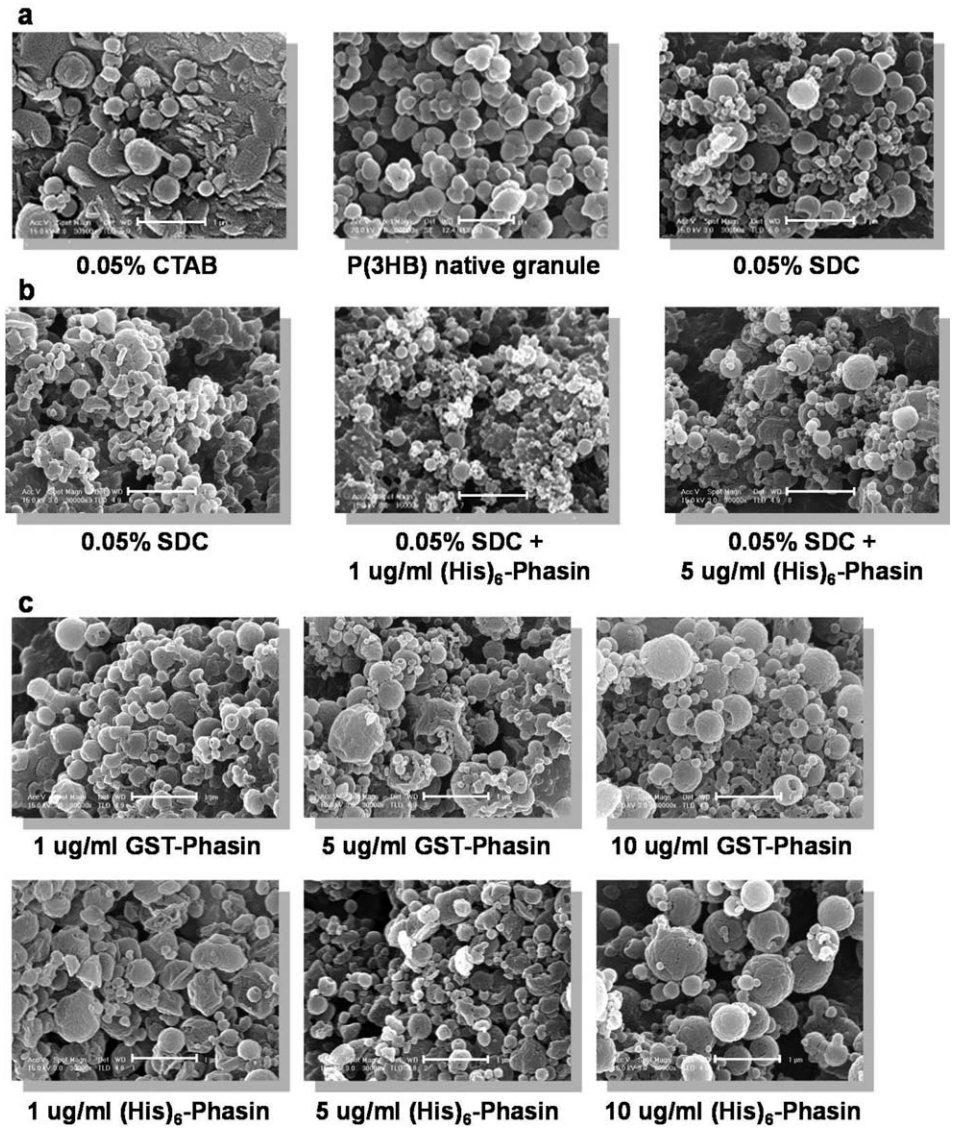

Figure 2. (a) Effect of the type of detergent on the shape of P(3HB) artificial granules. CTAB (cationic detergent hexadecyl trimethyl-ammonium bromide) and SDC(anionic detergent sodium deoxycholate) were used. The chloroform in the water-rich phase was removed by heating the emulsion at 65°C. The native granules were isolated from *R. eutropha* H16 cells grown with fructose by a mild hypochlorite treatment.; (b) Effect of $(His)_6$-phasin concentration on the shape of P(3HB) artificial granules prepared in the presence of 0.05% SDC via 65°C chloroform removal.; (c) Electron micrographs of artificial P(3HB) granules prepared with varying concentrations of two types of phasin, $(His)_6$-phasin and GST-phasin via 65°C chloroform removal. The preparation was carried out under detergent-free condition to see the *in vitro* effect of phasin itself on the granule formation. Scale bar, 1 μm.

size[32] (specifically, for GST-PhaP, 100–200 and 400–500 nm) were predominant. This bimodal size distribution may indicate the presence of two different types of nucleation origins (e.g., in oil-rich and water-rich phase). At the phasin concentration of 10 μg/mL, GST-PhaP-treated granules exhibited a more uniform size distribution, better-shaped, and more smooth surface morphology than $(His)_6$-PhaP treated ones. However, $(His)_6$-PhaP treatment resulted in an approximately three times bigger but more roughly organized granules. The longer polar GST chain of GST-PhaP was considered to play a role in its better controlled-shaping.

Compared with the granules prepared using the 65°C evaporation method [Figure 2(a–c)], the room-temperature evaporation method resulted in granules that are structurally unique and uniform in size (Figure 3). The size of the SDC-only treated granules was in the range of 100–200 nm in diameter [Figure 3(a)]. Totally, 2.5 μg/mL or 25 μg/mL of $(His)_6$-phasin was coadded to the SDC treatment system. Compared with the phasin-free system, the addition of 2.5 μg/mL $(His)_6$-phasin resulted in the formation of relatively well-defined spherically shaped granules [Figure 3(b)]. A 10-fold addition of $(His)_6$-phasin (25 μg/mL) induced even better spherically shaped granules [Figure 3(c)]. A high expression of phasin *in vivo* is known to decrease the size of the granules and increase their number. However, in this *in vitro* case, the concentration of the phasin protein did not seem to have a noticeable effect on the granule

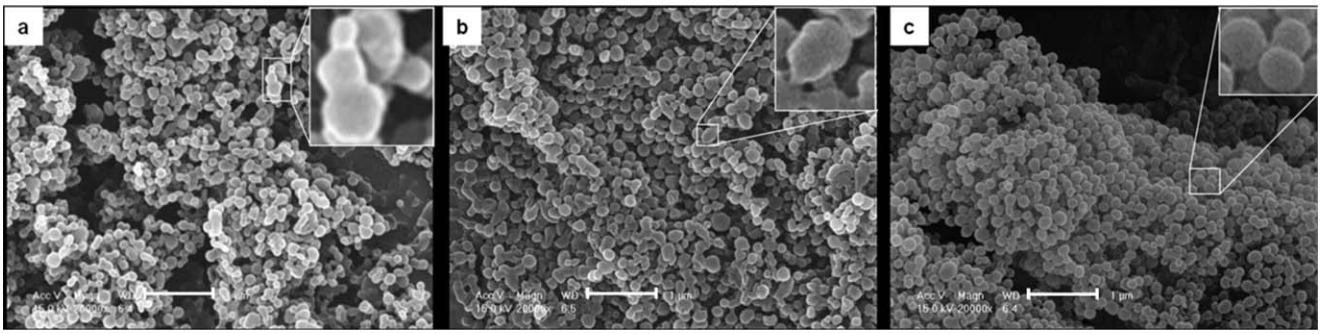

Figure 3. Artificial amorphous P(3HB) granule prepared using different $(His)_6$-phasin concentration ((a) 0 μg/mL, (b) 2.5 μg/mL, (c) 25 μg/mL). The emulsification was carried out with 0.05 (w/v)% SDC. The separated water-rich emulsion phase was slowly stirred to remove chloroform at room temperature. Phasin treatment resulted in the formation of relatively well-defined spherical shape granules. Scale bar 1 μm.

size. In an organic solvent/water emulsion system, the phasin exhibited a concentration dependence on the granule formation totally different from shown in the *in vivo* cells. This must be due to the different environmental factors (organic solvent, temperature, ionic strength, pH, mechanical agitation, etc.) as well as to the organic solvent-induced conformational change of the phasin molecules at the organic solvent/water interface, resulting in a totally different interfacial energy of the phasin. Therefore, to simulate the *in vivo* procedure of P(3HB) granule formation, a new and totally different protocol must be developed. However, compared with the blender agitated emulsion technique producing 20–300 μm spheres,[7] the technique (room temperature method) developed in this study is considered to be very useful to produce 100–200 nm spheres, 10^3 times smaller in size than the mechanical agitation.

Preparation and Degradation Kinetics Analysis of Extracellular P(3HB) Depolymerase Added P(3HB) Nanoparticles

For the design of degradation controlled P(3HB) granules, the formation of P(3HB) granules in the presence of extracellular P(3HB) depolymerase was investigated. As shown in Figure 4, the shapes of the granules prepared in the presence of phasin (25 μg/mL) and extracellular P(3HB) depolymerase (2.5 μg/mL) [Figure 4(b)] were well defined, and their size increased up to ~500 nm, which is ~5 times larger than the enzyme-free

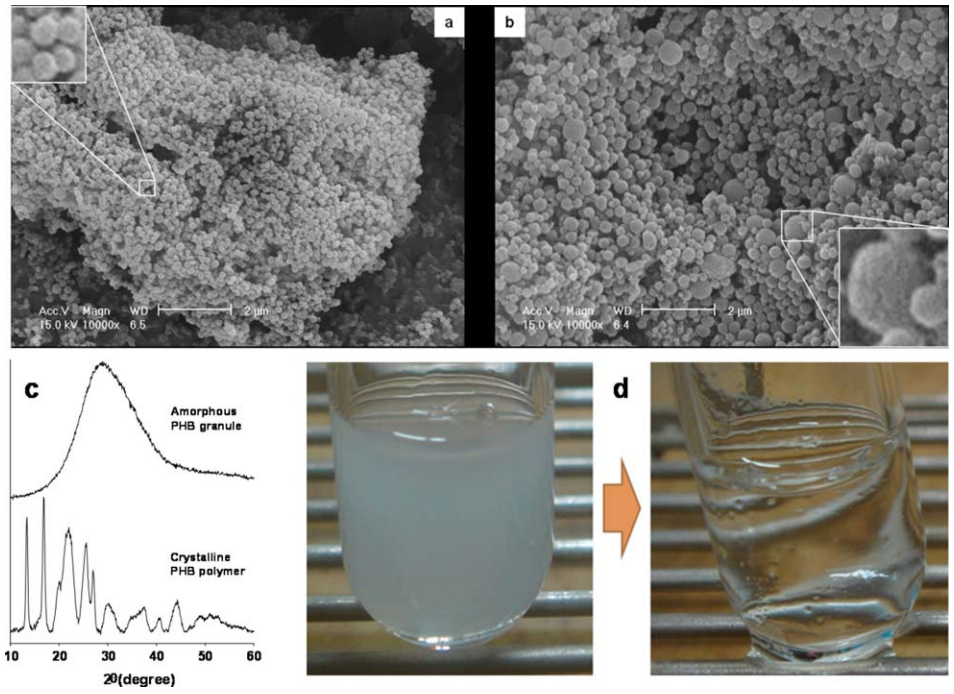

Figure 4. Preparation of self-degradable artificial amorphous P(3HB) granules via room temperature chloroform removal process. (a) extracellular P(3HB) depolymerase free (0.05% SDC + 25 μg/mL phasin) system; (b), treated with extracellular P(3HB) depolymerase (2.5 μg/mL) (0.05% SDC + 25 μg/mL phasin). The addition of the enzyme resulted in several times bigger (up to ~500 nm in diameter) granules and wider size distribution. Scale bar 2 μm.; (c) X-ray diffractogram and (d) Self-degradation test at 37°C for the amorphous PHB granule prepared with the assistance of phasin and extracellular P(3HB) depolymerase. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

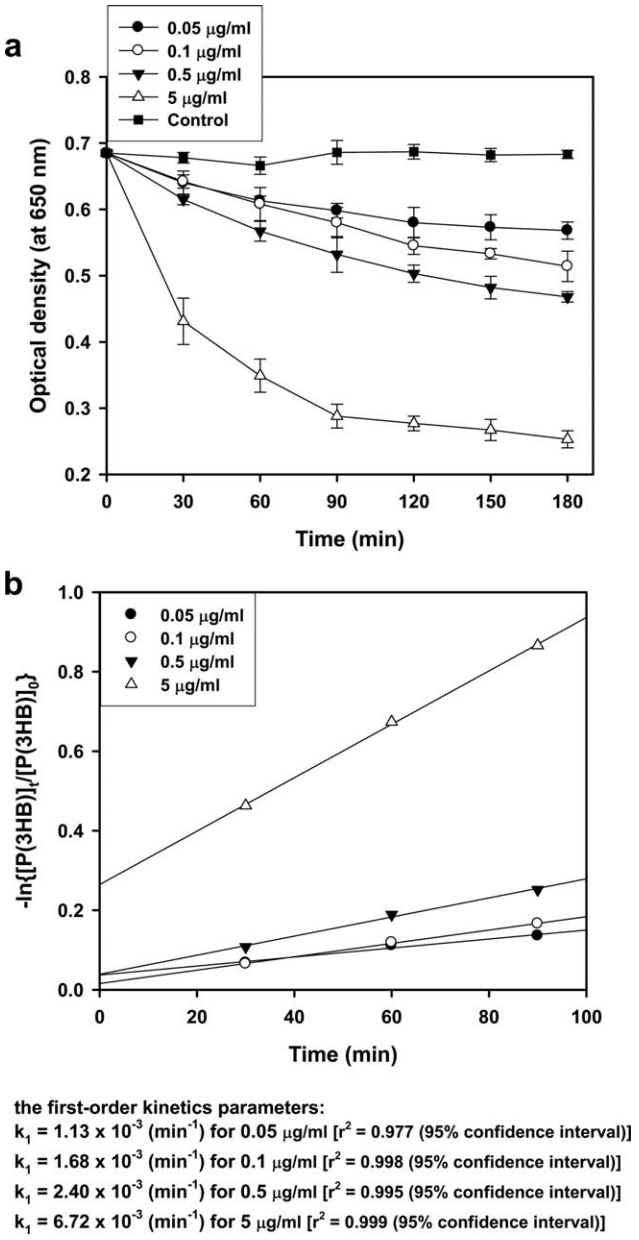

Figure 5. Self-degradation analysis of amorphous P(3HB) granule processed with various concentrations of extracellular P(3HB) depolymerase. The self-degradation of the enzyme treated granules was followed a first-order kinetics analysis. The degradation reaction was carried out at 37°C. (a) Optical density profiles during degradation reaction; (b) Determination of first-order degradation rate constants for the enzyme-loaded P(3HB) granules. All experiments were performed in triplicate.

[Figure 4(a)] preparations. The size distribution of the enzyme-treated granules is much wider than the enzyme-free controls. The amorphous nature of the enzyme-treated granules suspended in water was confirmed by XRD analysis [Figure 4(c)]. The extracellular P(3HB) depolymerase-free, phasin-only treated P(3HB) nanoparticles were not degraded. However, the enzyme added P(3HB) granules, despite the exposure of the enzyme to the harsh conditions during the process (with the presence of chloroform), were degraded and the emulsions turned into transparent solutions [Figure 4(d)]. Thus, a further detailed degradation analysis of amorphous P(3HB) granules, which were prepared using 0.05% SDC, 25 μg/mL phasin protein, and 0.05∼5.0 μg/mL extracellular P(3HB) depolymerase, was carried out by monitoring the change in optical density at 650 nm at 37°C using a spectrophotometer [Figure 5(a)]. The major degradation products of the enzyme reaction were known to be 3-hydroxybutyric acid (3HB) and 3HB dimer, which was confirmed by HPLC analysis (data not shown). The loading of a higher amount of enzyme induced a faster degradation. First-order degradation kinetics was assumed.[33,34] The calculated k_1 values of amorphous P(3HB) granules processed with 0.05, 0.1, 0.5, 5 μg/mL extracellular P(3HB) depolymerase are 1.13×10^{-3} (min^{-1}), 1.68×10^{-3} (min^{-1}), 2.40×10^{-3} (min^{-1}), 6.72×10^{-3} (min^{-1}), respectively [Figure 5(b)]. Thus, the addition of the enzyme and phasin in the P(3HB) granule processing might be helpful for designing amorphous P(3HB) nanoparticles carrying any functional molecules and releasing them in a timely manner by controlling the degradation kinetics.[13,16]

CONCLUSIONS

Room-temperature chloroform removal produced more uniformly sized nanoparticles probably because of a slower diffusive migration of the chloroform molecules from the inner core of the particles to their surface, and eventually minimizing the chance of particle–particle association between chloroform induced sticky surfaces. Phasin is stable with organic solvents, also having a high thermal stability, and thus it can be utilized as an emulsifying agent of PHA in an organic solvent/water immiscible medium. Thus, amphiphilic phasin can be used as a biosurfactant, specifically for the manufacture of various PHA nanoparticles. In addition, the artificial P(3HB) nanoparticles prepared using phasin and extracellular P(3HB) depolymerase can be used to make functional nanoparticles with auto-degradability. In these types of nanoparticles, the release of loaded functional molecules can be controlled by adjusting the degradation kinetics of the polymer nanoparticles. Thus, these nanoparticles can be applied in the drug delivery system for the controlled release of drugs.

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REFERENCES

1. Pötter, M.; Steinbüchel, A. *Biomacromolecules* **2005**, *6*, 552.
2. Prieto, M.; Bühler, B.; Jung, K.; Witholt, B.; Kessler, B. *J. Bacteriol.* **1999**, *181*, 858.
3. Steinbüchel, A.; Aerts, K.; Babel, W.; Follner, C.; Liebergesell, M.; Madkour, M. H.; Mayer, F.; Pieper-Fürst, U.; Pries, A.; Valentin, H. E.; Wieczorek, R. *Can. J. Microbiol.* **1995**, *41*, 94.
4. Pfeiffer, D.; Jendrossek, D. *J. Bacteriol.* **2012**, *194*, 5909.

5. Handrick, R.; Technow, U.; Reichart, T.; Reinhardt, S.; Sander, T.; Jendrossek, D. *FEMS Microbiol. Lett.* **2004**, *230*, 265.
6. Khang, G.; Choi, H. S.; Rhee, J. M.; Yoon, S. C.; Cho, J. C.; Lee, H. B. *Kor. Polym. J.* **2000**, *8*, 253.
7. Khang, G.; Kim, S. W.; Cho, J. C.; Rhee, J. M.; Yoon, S. C.; Lee, H. B. *Biomed. Mater. Eng.* **2001**, *11*, 89.
8. Lu, X. Y.; Zhang, Y.; Wang, L. *J. Appl. Polym. Sci.* **2010**, *116*, 2944.
9. Martin, D. P.; Williams, S. F. *Biochem. Eng. J.* **2003**, *16*, 97.
10. Williams, S. F.; Martin, D. P.; Horowitz, D. M.; Peoples, O. P. *Int. J. Biol. Macromol.* **1999**, *25*, 111.
11. Xiong, Y. C.; Yao, Y. C.; Zhan, X. Y.; Chen, G. Q. *J. Biomater. Sci. Polym. Ed.* **2010**, *21*, 127.
12. Alain, D.; Eric, S. *Macromolecules* **1998**, *31*, 6426.
13. Chen, C.; Yu, C. H.; Cheng, Y. C.; Yu, P. H.; Cheung, M. K. *Biomaterials*, **2006**, *27*, 4804.
14. Hiraishi, T.; Kikkawa, Y.; Fujita, M.; Normi, Y.; Kanesato, M.; Tsuge, T.; Sudesh, K.; Maeda, M.; Doi, Y. *Biomacromolecules* **2005**, *6*, 2671.
15. Lee, J.; Jung, S. G.; Park, C. S.; Kim, H. Y.; Batt, C. A.; Kim, Y. R. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2941.
16. Soppimath, K. S.; Aminabhavi, T. M.; Kulkarni, A. R.; Rudzinski, W. E. *J. Control. Release* **2001**, *70*, 1.
17. Shah, M.; Choi, M. H.; Ullah, N.; Kim, M. O.; Yoon, S. C. *J. Nanosci. Nanotechnol.* **2011**, *11*, 5702.
18. Shah, M.; Naseer, M.; Choi, M. H.; Kim, M. O.; Yoon, S. C. *Int. J. Pharm.* **2010**, *400*, 165.
19. Shah, M.; Ullah, N.; Choi, M. H.; Kim, M. O.; Yoon, S. C. *Eur. J. Pharm. Biopharm.* **2012**, *80*, 518.
20. Zhu, G.; Mallery, S. R.; Schwendeman, S. P. *Nat. Biotechnol.* **2000**, *18*, 52.
21. Barnard, G. N.; Sanders, J. K. M. *J. Biol. Chem.* **1989**, *264*, 3286.
22. Bonthrone, K. M.; Clauss, J.; Horowitz, D. M.; Hunter, B. K.; Sanders, J. K. M. *FEMS Microbiol. Lett.* **1992**, *103*, 269.
23. Horowitz, D. M.; Sanders, J. K. M. *Can. J. Microbiol.* **1995**, *41*, 115.
24. Sambrook, J.; Russell, D. W. Molecular Cloning: A Laboratory Manual, 3rd ed.; Cold Spring Horbor Laboratory, Cold Spring Harbor: New York, **2001**.
25. O'Connell, K. P.; Gustafson, A. M.; Lehmann, M. D.; Thomashow, M. F. *Appl. Environ. Microbiol.* **2000**, *66*, 401.
26. Horowitz, D. M.; Sanders, J. K. M. *J. Am. Chem. Soc.* **1994**, *116*, 2695.
27. Kobayashi, T.; Shiraki, M.; Abe, T.; Sugiyama, A.; Saito, T. *J. Bacteriol.* **2003**, *185*, 3485.
28. Song, J. J.; Shin, Y. C.; Yoon, S. C. *J. Microbiol. Biotechnol.* **1993**, *3*, 123.
29. Hiraishi, T.; Ohura, T.; Ito, S.; Kasuya, K.; Doi, Y. *Biomacromolecules* **2000**, *1*, 320.
30. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
31. Laemmli, U. K. *Nature* **1970**, *227*, 680.
32. Musyanovych, A.; Rossmanith, R.; Tontsch, C.; Landfester, K. *Langmuir* **2007**, *23*, 5367.
33. Choi, M. H.; Rho, J. K.; Lee, H. J.; Song, J. J.; Yoon, S. C.; Lee, S. Y. *Biomacromolecules* **2003**, *4*, 424.
34. Yoon, S. C.; Choi, M. H. *J. Biol. Chem.* **1999**, *274*, 37800.