Bio-specific immobilization of enzymes on electrospun PHB nanofibers

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Abstract

Phasins are proteins found on the surface of natural polyhydroxyalkanoate (PHA) granules. Due to their high affinity for PHA, they can potentially be used as a fusion partner to immobilize other proteins. In this study, we investigated the immobilization of a lipase onto electrospun polyhydroxybutyrate nanofibers. Due to a superior surface area-to-volume ratio, PHB nanofibers retained much larger amounts of enzyme than conventional immobilization supports. More importantly, when used in combination with a phasin tag, the enzyme immobilized on PHB nanofibers exhibited markedly higher activity and reusability. Our approach combines the advantageous features of nanofibrous materials and the regio-specificity of biomolecular interactions for the efficient use of enzymes.

1. Introduction

Enzymes have many advantages over conventional chemical catalysts, including outstanding substrate specificity and more rapid catalytic turnover (Jaeger and Eggert, 2004). Since they operate under milder conditions and consume less energy, enzyme-based processes also provide an eco-friendly route for the production of chemicals (Houde et al., 2004). Enzyme immobilization is an attractive option to reduce production cost and develop novel biotransformation processes. While stable immobilization of enzymes can be achieved by covalent linkage of functional groups between enzymes and support materials, it often perturbs the native conformation of enzymes, leading to diminished enzyme activity (Homaei et al, 2013; Tischer and Wedekind, 1999; Zhou et al., 2015). By contrast, immobilization methods based on non-covalent sorption can be performed under milder conditions, allowing immobilized enzymes to better retain their activity. However, in general, non-covalently attached enzymes are easily displaced from supports, limiting their reusability. Furthermore, most current immobilization methods, including both covalent and non-covalent types, result in a random orientation of enzymes on the support surface, and only a fraction of immobilized enzymes are optimally oriented toward their substrates. This can also contribute to a decrease in enzyme activity after immobilization.

Polyhydroxyalkanoates (PHA) are a class of biodegradable polyesters produced by a number of bacterial species (Chodak, 2008). In the cytoplasm of bacterial cells, a few surface-associated proteins are present on the surface of PHA granules, including PHA synthases and phasins. Phasins are amphiphilic proteins located at the PHA-cytoplasm interface that form stable monolayers with a nearly ordered orientation on PHA granules (Dong et al. 2010; You et al. 2011; Prieto et al. 2016; Tarazona et al. 2019). From a biotechnological perspective, these proteins have a high affinity toward the polymeric surface, and they can be usefully employed for immobilization of target proteins by engineering genetic fusions. Indeed, a number of groups have demonstrated successful immobilization of proteins onto PHA supports (Moldes et al., 2004;

Peters et al., 2006; Seo et al., 2016; Wong et al., 2018; Yang et al., 2015) through fusion with PHA-binding proteins, including phasins. However, most of these studies focused on *in vivo* immobilization of enzymes onto PHA granules accumulated inside cells, with drawbacks including uncontrolled size distribution and limited surface area.

In the present study, we investigated the use of electrospun PHA nanofibers as an alternative support to immobilize phasin-fused recombinant enzymes. A lipase discovered via metagenomics analysis was immobilized onto electrospun polyhydroxybutyrate (PHB) nanofibers using phasin from *Aeromonas hydrophila* (PhaP) as an affinity fusion tag. Due to a high surface-area-to-volume ratio, the amount of enzyme that could be loaded onto PHB nanofibers was >100-fold greater than that which could be loaded onto PHB granules. Additionally, the immobilized lipase exhibited markedly higher stability and activity, allowing for repeated use without significant loss of activity. Our results demonstrate that PHB nanofibers can be used as a highly efficient and versatile support to immobilize enzymes when used in combination with a phasin tag.

2. Materials and Methods

2.1. Preparation of recombinant proteins

Genes encoding superfolder green fluorescent protein (sfGFP) and LipM7, a lipase recently discovered in our laboratory via metagenomics analysis (Table S1, to be published elsewhere) were cloned into the plasmid pET21a (Novagen, Wisconsin, USA) between the *Nde* I and *Xho* I restriction enzyme sites to generate pET21a-sfGFP and pET21a-LipM7, respectively. For construction of plasmids encoding N-terminal phasin fusion proteins (PhaP-sfGFP and PhaP-LipM7), phasin gene *phaP*from *Aeromonas hydrophila* (accession number, UniprotKB-O32470) was synthesized by GenScript (Nanjing, China) and inserted between the*Hind* III and *Xho* I sites of pET21a-sfGFP or pET21a-LipM7. To prepare recombinant proteins fused with phasin at the C-terminus (sfGFP-PhaP and LipM7-PhaP), sfGFP and LipM7 genes were first cloned into the pET21a vector between the *Nde* I and *Hind* III sites, followed by insertion of the phasin sequence between the *Hind* III and *Xho* I sites (see Table S2 for primers used for plasmid construction).

Recombinant proteins were prepared from cultures of *Escherichia coli* strain BL21 (DE3) (Novagen, Darmstadt, Germany) transformed with corresponding plasmids. Cells were grown at 37°C in a 2 L baffled flask containing 400 mL of Luria-Bertani medium supplemented with 50 μg/mL of ampicillin. When the absorbance at 600 nm (OD_{600}) reached 0.5 to 0.6, the culture broth was supplemented with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubated at 20°C for a further 20 h. Cells were harvested by centrifugation (6,800 × g , 20 min) and lysed by sonication in 10 mL of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 10 mM imidazole, and 1 mg/mL lysozyme. The supernatant of the centrifuged lysate (6,800 × g , 20 min) was loaded onto a column containing 2 mL Ni-NTA agarose resin (Qiagen, Hilden, Germany). After extensive washing with 20 mM imidazole in 50 mM sodium phosphate buffer, resin-bound proteins were eluted with 250 mM imidazole solution (1 mM imidazole for sfGFP-PhaP and PhaP-sfGFP). The eluate was dialyzed against 100 mL of 20 mM sodium phosphate buffer (pH 7.4). After adjusting to a protein concentration of 0.5 mg/mL, the protein solutions were stored at 4°C prior to use.

The purity of proteins was analyzed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The sizes of resolved proteins were estimated using PM2700 Protein Markers (SMOBIO, Hsinchu, Taiwan). Protein concentration was determined by the Bradford assay following the manufacturer's protocol (BioRad, Hercules, CA, USA). Fluorescence of sfGFP, sfGFP-PhaP, and PhaP-sfGFP was measured using a VICTOR X2 plate reader (Perkin Elmer, Waltham, MA, USA) set at wavelengths of 485 and 535 nm for excitation and emission, respectively. The enzymatic activity of LipM7 lipase was determined by a colorimetric method using p -nitrophenyl decanoate (pNPD) as a substrate, as described previously (Choi et al., 2013).

2.2. Fabrication of PHB nanofibers by electrospinning

PHB granules were dissolved in a 7:3 mixture of 2,2,2-trifluoroethanol and chloroform to 10% (w/v). The polymeric solution was transferred into a syringe and delivered to the needle at a flow rate of 0.5 mL/h.

A constant voltage of 10 kV was applied to induce fiber formation. For preparation of nanofibers in two-dimensional (2D) sheet form, electrospun fibers were collected on aluminum foil connected to the ground. Three-dimensional (3D) nanofibrous sponges were prepared by collecting nanofibers in a coagulation bath filled with a 6:4 mixture of t-butanol and water. Prepared PHB nanofibers were dried in vacuum for 24 h, and stored in a desiccator prior to use.

2.3. Immobilization of proteins

PHB nanofibers (5 mg), 150 mg of PHB granules (Goodfellow, Huntingdon, UK), 25 mg of Duolite A568 (Novozymes, Kalundborg, Denmark), or 25 mg of Sipernat D17 (Degussa, Frankfurt, Germany) were suspended 1 mL of protein solutions (0.2-0.3 mg) and incubated overnight at 4degC or for 1 h at 30degC under constant shaking. These supports were recovered by a brief centrifugation step and washed three times with 50 mM TRIS-HCl buffer (pH 8.0). The amount of protein adsorbed to the support was calculated by measuring the protein concentration in the buffer solution sampled before and after the immobilization procedures.

2.4. Characterization of lipase immobilization on PHB supports

Different concentrations of LipM7-PhaP dissolved in 10 mM sodium phosphate (pH 7.4) were immobilized on PHB nanofibers and PHB granules (average diameter: 2.5 μm). The amount of LipM7-PhaP immobilized on the PHB support was estimated by quantifying unbound proteins. The maximum loading capacity ($q_{\max}$) and the dissociation constant (k_{d}) were determined by applying the protein concentration to the Langmuir isotherm model.

2.5. Analysis of transesterification reactions by immobilized LipM7-PhaP

PHB nanofibers containing immobilized LipM7-PhaP (100 mg) were added to a 10 mL solution of 1 M octanoic acid and 1 M MeOH. The reaction mixture was incubated at 30°C under constant orbital shaking at 250 rpm. Samples (100 μL) were withdrawn every 12 h until the reaction reached equilibrium. Withdrawn samples were mixed with 900 μL of chloroform containing n-hexane as an internal standard. Synthesized methyl octanoate, and residual octanoic acid and methanol were analyzed by gas chromatography on an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm, J&W Scientific; Agilent, Santa Clara, CA) equipped with a flame ionization detector (FID). Helium was used as the carrier gas at a flow rate of 1.3 mL/min. The temperatures of the injector and detector were set at 250°C and 340°C, respectively. Starting from 100°C, the oven temperature was sequentially raised to 260°C (15°C/min) and 315°C (5°C/min), and held at this temperature for 15 min. Samples (1 μL) were injected in the split mode.

3. Results and Discussion

3.1. Use of PhaP as a fusion partner for immobilization of enzymes

Due to its high affinity for PHB (Zhao et al., 2016) and relatively small size compared to phasins from other bacterial species (Table S3), we investigated the use of PhaP from *Aeromonas hydrophila* as an affinity tag to immobilize proteins on PHB surfaces. In the initial experiments, we examined whether fusion with PhaP affected the expression of target proteins. When PhaP-sfGFP and sfGFP-PhaP were expressed in *E. coli* strain BL21(DE3), both fusion proteins were produced at similar levels to those of the parental sfGFP (Figure 1A, upper panel). Similarly, expression of LipM7 lipase was not significantly affected by N- or C-terminal fusion with phasin (Figure 1B, upper panel), indicating that PhaP could be fused upstream or downstream of target proteins without affecting the efficiency of gene expression.

The location of the PhaP fusion could significantly affect the biological activity of target proteins. In the case of sfGFP, the fusion protein with C-terminal PhaP displayed ˜33% higher fluorescence than the control sfGFP (Figure 1A, lower panel). It has been reported that the functionality of a fluorescence protein can be enhanced when fused with a foreign protein sequence (Solovyov et al., 2011, Huang et al., 2016). However, the enzymatic activity of LipM7 exhibited greater dependence on the location of phasin fusion. Although LipM7-PhaP was not significantly different from wild-type LipM7 in terms of its ability to hydrolyze pNPD, PhaP-LipM7 exhibited <25% the activity of LipM7 (Figure 1B, lower panel). Although the structure of this

lipase has not been determined, this result suggests that the N-terminus of this enzyme plays an important role in catalytic activity, in accordance with previous results showing that lipase activity can be disrupted when fused to N-terminal fusion partners (Gustavsson et al., 2001; Singh et al., 2018; Malunavicius et al., 2018).

3.2. Effect of PhaP location on PHB-binding activity

For the proposed approach to be a valid option for enzyme immobilization, the PHB-binding activity of phasin fusion proteins should be carefully considered alongside the enzymatic activity of the target enzyme. Because the hydrophobic region of its C-terminal end needs to be exposed for PhaP to interact with PHA (Zhao et al. 2016), we presumed that it would be more favorable to place a fusion partner in in front of phasin. This presumption was confirmed by observation of PHB nanofibers under fluorescence microscopy after incubation with sfGFP-PhaP or PhaP-sfGFP. As expected, when PHB nanofibers were incubated with the same molar amount of fusion proteins, much greater fluorescence was observed from those incubated with sfGFP-PhaP (Figure 2). By comparison, binding of PhaP-sfGFP to PHB nanofibers was only marginally higher than the control sfGFP.

In accordance with this result, the amount of LipM7-PhaP on PHB nanofibers was almost twice that of PhaP-LipM7 (Figure 3A). Furthermore, time-course analysis revealed an exceptionally stable maintenance of LipM7-PhaP on PHB nanofibers. As shown in Figure 3B, upon storage in buffer solution, >70% of immobilized LipM7-PhaP remained on PHB nanofibers after 28 days. By comparison, wild-type LipM7 adsorbed onto PHB nanofibers was almost completely released into buffer within a week. These results clearly indicate that C-terminal fusion of PhaP is desirable for maintaining the enzymatic activity of LipM7 and the binding activity of PhaP. LipM7-PhaP was therefore used in subsequent experiments.

3.3. Characterization of LipM7-PhaP binding to PHB nanofibers

A 0.2 mg sample of LipM7-PhaP was added to 1 mL of immobilization buffer (50 mM TRIS-HCl, pH 8.0) containing 5 mg of PHB nanofibers, and the amount of protein in buffer solution was measured periodically to estimate the quantity of fiber-bound proteins. As shown in Figure 4A, LipM7-PhaP rapidly partitioned onto the PHB nanofibers, and 50% saturation was reached in 15 min. The binding of LipM7-PhaP reached a plateau after 70 min.

To test the selectivity of LipM7-PhaP binding to PHB nanofibers, PHB nanofibers were incubated with a clarified lysate of *E. coli*cells expressing LipM7-PhaP. After extensive washing, proteins bound to PHB nanofibers were eluted with 10% SDS solution. The results in Figure 4B demonstrate that PHB nanofibers specifically interact with LipM7-PhaP. While the relative abundance of LipM7 in SDS-eluted proteins was comparable to that in the cell lysate (lanes A and B of the LipM7 group), LipM7-PhaP was eluted in high purity. This indicates that most of the *E. coli* proteins were outcompeted by LipM7-PhaP, and were removed during the washing step. The maximum loading capacity ($q_{\max}$) and the dissociation constant (k_{d}) determined by Langmuir isotherm model analysis were 12.5 mg per gram of PHB nanofibers and 0.92 μM, respectively (Table 1 and Figure S1). Although thek_{d} value for electrospun nanofibers was 5-fold higher than for the granular supports, the enlarged surface area of the nanofibers allowed them to anchor 120-fold more enzyme.

3.4. Enzymatic activity of lipases immobilized on PHB nanofibers

The advantages of phasin-mediated enzyme immobilization on PHB nanofibers was highlighted by comparing with conventional immobilization methods. Compared with Duolite A568 and Sipernat D17, the same weight of PHB nanofibers could retain 2- to 3-fold more enzyme (Figure 5A). Interestingly, differences in enzymatic activity between different immobilization supports were pronounced for immobilized LipM7-PhaP. PHB nanofibers loaded with LipM7-PhaP exhibited 3- to 10-fold higher lipase activity than Duolite A568 and Sipernat D17 (Figure 5B), indicating that the enzyme immobilized on PHB nanofibers possessed higher specific enzymatic activity than enzyme immobilized on conventional supports. This was attributed to the oriented immobilization of enzymes onto the PHB surface.

Furthermore, nanofiber-immobilized enzyme could be used without substantial loss of activity over repeated reaction cycles. As shown in Figure 6, LipM7-PhaP immobilized on PHB nanofibers retained >74% of initial activity after 50 reaction cycles. By contrast, LipM7 adsorbed onto PHB nanofibers lost more than half of initial activity after only three reaction cycles.

Additionally, nanofiber-immobilized LipM7-PhaP was successfully used for a synthetic reaction. When employed for the transesterification of octanoic acid to produce methyl octanoate, ˜70% of octanoic acid was converted into methyl octanoate after 60 h (Figure S2).

4. Conclusions

Herein, we demonstrated that electrospun PHB nanofibers can be used as an efficient immobilization support for enzymatic processes. In addition to the high surface area-to-volume ratio, strong and specific interactions with phasins enabled selective immobilization of phasin-fused enzymes in a uniform orientation. The combined use of PHB nanofibers and phasin fusion tags cold contribute to the development of efficient enzymatic processes for the production of chemical materials. The bio-compatibility of PHB also makes it suitable for immobilization in biomedical applications.

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Figure Legends

Figure 1. Expression of PhaP-fused proteins in *E. coli*cells. (A) sfGFP, sfGFP-PhaP, and PhaP-sfGFP were overexpressed in the transformed *E. coli* strains. After lysis of the *E. coli*cells, expressed proteins were analyzed by SDS-PAGE (upper panel), or by the measurement of sfGFP fluorescence (lower panel). (B) LipM7, LipM7-PhaP, and PhaP-LipM7 overexpressed in the *E. coli* cells were analyzed by SDS-PAGE (upper panel), or by the measurement of lipase activity (lower panel). Lanes: M, molecular weight markers; C, lysates of *E. coli* cells without IPTG induction; T, lysates of IPTG-induced *E. coli* cells; S, soluble lysate fractions of the IPTG-induced *E. coli* cells.

Figure 2. Comparison of sfGFP immobilized on PHB nanofibers via N-terminal or C-terminal fusion of PhaP. sfGFP-PhaP, and PhaP-sfGFP were incubated in a buffer solution containing the PHB-nanofibers. After the incubation, PHB-nanofibers were recovered by centrifugation and washed with 50 mM Tris-HCl buffer (pH 8.0). sfGFP fluorescence on the surface of the nanofibers were observed under a fluorescence microscope (Eclipse 80i microscope, Nikon, Tokyo, Japan).

Figure 3. Immobilization of PhaP-fused LipM7 on PHB nanofibers.(A) The amounts of immobilized proteins on PHB-nanofibers. (B) Stability of immobilized enzymes on PHB nanofibers. Samples were withdrawn from the storage buffer at the indicated time points and protein concentrations were measured to determine the amounts of LipM7 remained on the PHB nanofibers. Open circles, LipM7; filled circles, LipM7-PhaP.

Figure 4. Characterization of LipM7-PhaP immobilization on the PHB nanofibers. (A) Kinetics of LipM7-PhaP immobilization on PHB-nanofibers. Samples were withdrawn during the incubation of purified LipM7-PhaP with PHB nanofibers, and measured for protein concentration to monitor the binding of LipM7-PhaP over time. (B) Selective binding of LipM7-PhaP on the PHB nanofibers. After overexpression of LipM7 or LipM7-PhaP, crude cell lysate was incubated with PHB nanofibers as described in Materials and Methods. After washing (lanes marked W), immobilized proteins were eluted with SDS solution (lanes marked E). M, molecular weight markers.

Figure 5. Comparison of LipM7-PhaP immobilized on conventional supports and PHB nanofibers. (A) Amounts of LipM7-PhaP immobilized on various supports of same weight. (B) Specific activity of LipM7-PhaP immobilized on various supports.

Figure 6. Repeated use of the PHB nanofiber-immobilized enzyme. Enzymatic activity of the immobilized LipM7 or LipM7-PhaP was measured over fifty reaction cycles. Open circles, LipM7; filled circles, LipM7-PhaP.