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ENZYMOLOGY AND PROTEIN ENGINEERING

Poly-3-Hydroxybutyrate Functionalization with BioF-Tagged Recombinant Proteins

AQ: au Daniel Bello-Gil,[a*] Beatriz Maestro,[a*] Jennifer Fonseca,[a] Nina Dinjaski,[b*] M. Auxiliadora Prieto,[b] Jesús M. Sanz[a*]

[a]Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Elche, Spain

[b]Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CIB-CSIC), Madrid, Spain

ABSTRACT AQ:A Polyhydroxyalkanoates (PHAs) are biodegradable polyesters that accumulate in the cytoplasm of certain bacteria. One promising biotechnological application utilizes these biopolymers as supports for protein immobilization. Here, the PHA-binding domain of the *Pseudomonas putida* KT2440 PhaF phasin (BioF polypeptide) was investigated as an affinity tag for the *in vitro* functionalization of poly-3-hydroxybutyrate (PHB) particles with recombinant proteins, namely, full-length PhaF and two fusion proteins tagged to BioF (BioF–C-LytA and BioF–β-galactosidase, containing the choline-binding module C-LytA and the β-galactosidase enzyme, respectively). The protein-biopolyester interaction was strong and stable ~~in~~ a wide range of pHs and temperatures, and the bound protein was highly protected from self-degradation, while the binding strength could be modulated by coating with amphiphilic compounds. Finally, BioF–β-galactosidase displayed very stable enzymatic activity after several continuous activity-plus-washing cycles when immobilized in a minibioreactor. Our results demonstrate the potentialities of PHA and the BioF tag for the construction of novel bioactive materials.

IMPORTANCE Our results confirm the biotechnological potential of the BioF affinity tag as a versatile tool for functionalizing PHA supports with recombinant proteins, leading to novel bioactive materials. The wide substrate range of the BioF tag presumably enables protein immobilization *in vitro* of virtually all natural PHAs as well as blends, copolymers, or artificial chemically modified derivatives with novel ~~physical-chemical~~ properties. Moreover, the strength of protein adsorption may be easily modulated by varying the coating of the support, providing new perspectives for the engineering of bioactive materials that require a tight control of protein loading.

KEYWORDS PHB, phasins, affinity tag, protein immobilization, polyhydroxyalkanoates

The immobilization of peptides and proteins on polymer surfaces to develop bioactive supports represents a challenging goal in biotechnology and biomedicine (1, 2). As more biotechnologically relevant materials with new properties are described, novel methods for efficient protein functionalization are also required. Among the immobilization procedures, covalent methods ensure the strongest binding to the support, providing, in some cases, even more stability to the immobilized protein. However, the matrix is barely recoverable after enzyme inactivation, and these methods normally require the use of chemical-linking reagents that might perturb the native structure of the polypeptide (although interesting, alternative *in situ* systems have been recently described) (3). On the other hand, noncovalent methods are generally of a weaker intensity but have other important advantages, as they are normally ~~simple, gentle, and~~ do not require the chemical modification of the protein, thus diminishing

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Address correspondence to Jesús M. Sanz, jmsanz@umh.es.

* Present address: Daniel Bello-Gil, Institut d'Investigació Biomédica de Bellvitge (IDIBELL), Barcelona, Spain; Beatriz Maestro, Centro de Investigaciones Biológicas–CSIC, Madrid, Spain; Nina Dinjaski, Science & Technology Center, Tufts University, Massachusetts, USA; Jesús M. Sanz, Centro de Investigaciones Biológicas–CSIC, Madrid, Spain.

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the risk of denaturation. In addition, immobilization is reversible in most cases, enabling the straightforward regeneration of the support (4, 5).

Current trends in material research specifically focus on biocompatible and biodegradable, yet versatile and stable, supports. The polyhydroxyalkanoate (PHA) family possesses all these characteristics. PHAs are natural polyesters of 3-hydroxyalkanoic acids that accumulate intracellularly in several organisms as a reserve material. They possess a high variety of monomer compositions, providing them with different physicochemical properties and functionalities (6–9). Depending on the length of the carbon chain, PHAs are classified as (i) short-chain-length PHAs (~~[scl-PHAs]~~ 3 to 5 carbon atoms), (ii) medium-chain-length PHAs (~~[mcl-PHAs]~~ 6 to 14 carbon atoms), ~~and~~ (iii) long-chain-length PHAs (~~[lcl-PHAs]~~ more than 14 carbon atoms) (10). The scl-PHA poly-3-hydroxybutyrate (PHB) represents the most produced and commercially successful member of the PHA family (11, 12). The PHAs constitute, among natural polymers, the largest group of microbial polyesters that displays thermoplastic features (9, 13) (hence the commonly used term "bioplastics"), ~~and they~~ have been hailed as potential competitors of oil-derived plastics, not only because of their physical properties but also for their biocompatibility and biodegradability (8, 14, 15) and the high sustainability of their sources, as they naturally accumulate as a reserve material in several microbial (16) and engineered plant (17) species. The tunable structural features of these polyesters based on their variable compositions make them highly attractive for the development of next-generation biomaterials ~~susceptible~~ of being functionalized with peptides and proteins.

Native PHA granules are subcellular structures formed by a polymeric core surrounded by granule-associated proteins (GAPs) (18). Microbial PHA preparations usually contain adsorbed phospholipids as well (19), although the actual occurrence of this coating *in vivo* has been recently questioned and might represent a purification artifact from the cell extracts (20–22). Phasins, the main component of GAPs, have a relevant role in the PHA intracellular metabolism, participating in events~~, including~~ the control of the size, number, and localization of granules in the cell (23, 24). The high affinity of phasins for PHA suggests the application of such polypeptides as affinity tags for the immobilization of proteins on polyester granules. Despite its potential, only a few examples of phasin-mediated immobilization of recombinant proteins have been described to date (25–31). In particular, Prieto and coworkers (32) developed the BioF tag derived from the PHA-binding domain of the PhaF phasin from *Pseudomonas putida* KT2440, a protein that is involved in the intracellular stabilization and localization of the PHA granule as well as in its equal distribution between daughter cells upon cell division, due to the presence of a DNA-binding domain (33, 34). Several fusion proteins containing the BioF sequence were expressed and found to be associated *in vivo* with native mcl-PHA granules in *P. putida* without compromising its functionality (32). The fusion proteins could be recovered from the granules by a mild treatment with detergents. In a step toward effective biotechnological applications, native granules ~~were produced~~ containing a variant of the Cry1Ab toxin with insecticidal properties (35), demonstrating that the functionalization of PHA polyesters with BioF-tagged proteins constitutes a promising field for designing bioactive polymers of interest. However, several aspects of these *in vivo* systems need be addressed for their successful application, such as the control of granule size and the amount of immobilized protein, as well as the overall conditions of the environment regulating its binding (pH, temperature, etc.).

To date, most studies have dealt with the interaction between a particular PHA and the cognate phasin with which it naturally interacts *in vivo*, but the peculiar organization of phasins as predominantly amphipathic α-helical proteins that recognize the biopolymer through relatively nonspecific interactions (23) enables speculation on the possibility of a wider PHA substrate range for this family of polypeptides. It is also evident that a single versatile tag able to recognize different types of PHAs would imply an important biotechnological capacity. Therefore, we decided to characterize the binding of several BioF fusions to scl-PHA particles such as PHB. Although several

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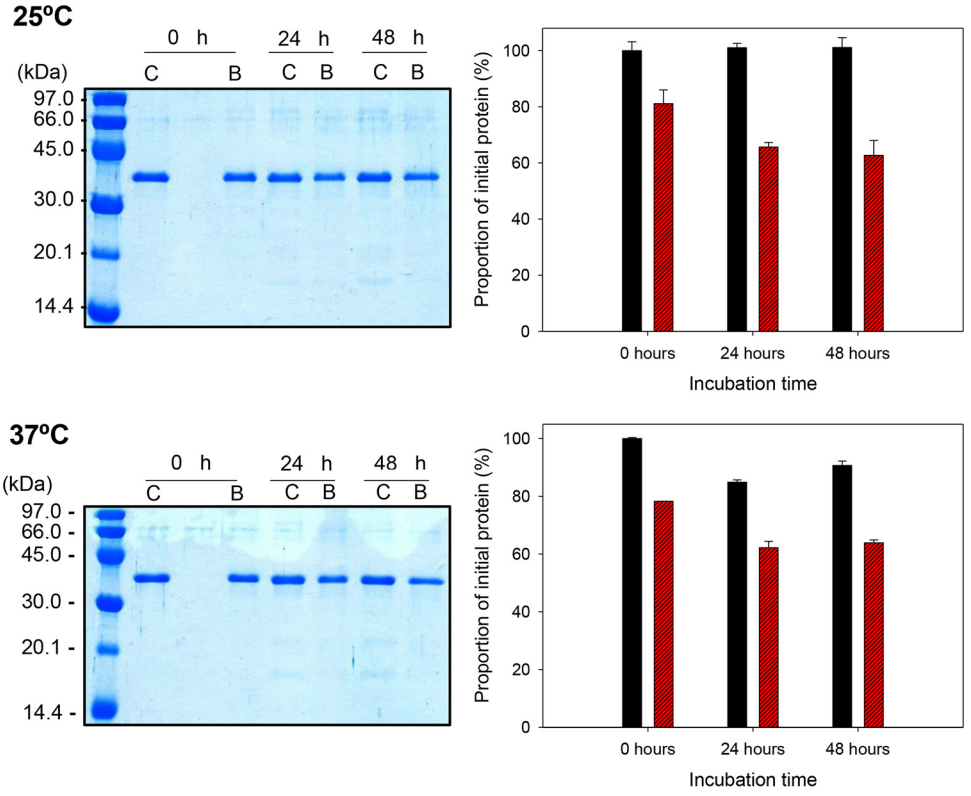

FIG 1 (Left) Stability of PhaF binding to PHB at 25 and 37°C analyzed by SDS-PAGE. C, control of soluble protein; B, bound protein eluted from PHB with 1% ~~SDS~~ (wt/vol) after heating at 90°C for 10 min. (Right) Results of gel densitometry, shown as the percentages of the initial applied protein. Black solid bars indicate soluble control protein, whereas red hashed bars represent the particle-bound protein.

phasins naturally bound to PHB inclusions have been described (23, 24), our approach considered the aim of expanding the applications of the BioF tag (a naturally mcl-PHA-binding polypeptide) rather than finding a strong tag strictly recognizing a particular PHA. Furthermore, we have assessed the functional performance of a BioF–β-galactosidase hybrid protein immobilized on PHB as a proof of concept for the development of PHA-based enzymatic bioreactors. We believe that our results confirm that BioF-based PHA functionalization procedures possess an evident potential in biomedicine and bioprocess technology.

RESULTS

***In vitro* interaction of the PhaF phasin with lipid/GAP-free PHB particles.** The binding capability of the BioF polypeptide to mcl-PHA granules has been widely demonstrated *in vivo* (32, 35, 36). Nevertheless, native PHA granules are surface coated by several GAPs and probably by a lipid layer that might interfere with the BioF binding *in vivo*. Therefore, in a first approach, we tested the ability of the entire PhaF phasin to be immobilized *in vitro* on commercially available PHB particles devoid of any protein or lipid coating. Figure 1 shows that, in sodium phosphate (PNa) buffer at 25°C or 37°C, PhaF could be clearly adsorbed on PHB, indicating that the substrate range of the BioF tag also includes scl-PHAs and that no other granule-associated components are needed for efficient binding.

The stability of this PhaF-PHB interaction was evaluated ~~both at 25 and at~~ 37°C. After 48 h of incubation, approximately 65% of the initially added PhaF (representing 75% of the initially bound protein) remained stably attached to the polyester at both temperatures without significant hints of protein degradation (Fig. 1). Furthermore, incubation at pHs other than 7.0 did not appear to have a direct influence on immobilization, as the protein remained similarly adsorbed to the particles in all cases (see Fig. S3 in the

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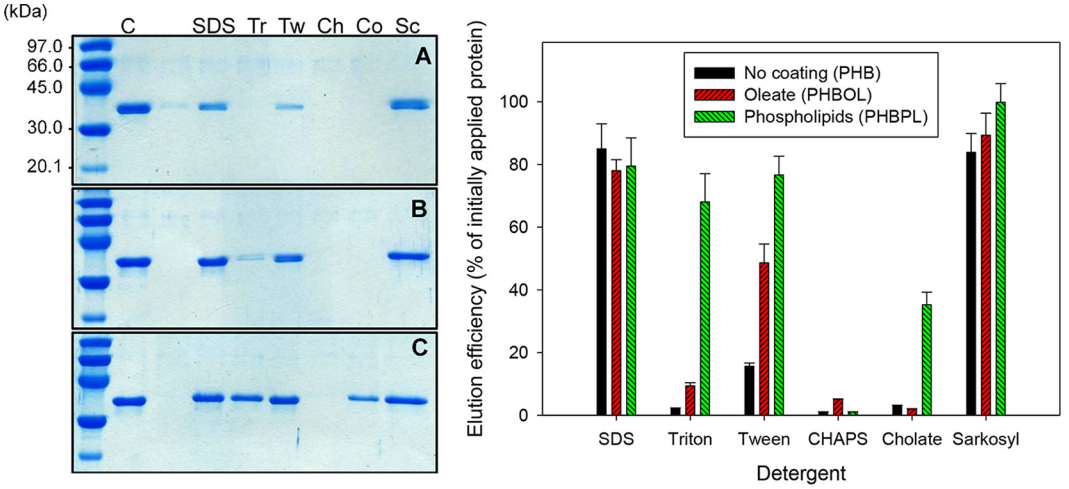

FIG 2 Elution of PhaF from different preparations of PHB upon incubation with detergents and the dependence on granule coating. CN, control of soluble protein; SDS, 1% (wt/vol) SDS; Tr, 1% (vol/vol) Triton X-100; Tw, 1% (wt/vol) Tween 20; Ch, 3% (wt/vol) CHAPS; Co, 3% (wt/vol) sodium cholate; Sc, 3% (wt/vol) sarcosyl. (A) Naked PHB; (B) PHB coated with sodium oleate (PHBOL); (C) PHB coated with phosphatidylglycerol and phosphatidylcholine (PHBPL); (D) quantification of elution efficiency by gel densitometry.

supplemental material). This result supports the hypothesis that ionic forces are minor contributors to PHA recognition (34). To test the strength of the hydrophobic interactions, protein desorption was induced by incubations with several detergents at 25°C (Fig. 2A; see also Fig. S4). PhaF was eluted from the polymer particles with the greatest efficiency by SDS and sarcosyl, and only partially (18%) with Tween 20 (Fig. 2A). However, other detergents such as Triton X-100, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and sodium cholate were unable to desorb the protein at the concentrations tested (Fig. 2A).

The affinity of PhaF to PHB was also quantified by a Langmuir analysis (Fig. 3). The binding isotherm acquired at 25°C reveals a maximum loading capacity of 20 ± 2 μg PhaF/mg PHB and a dissociation constant of 0.5 ± 0.1 mg/ml (18.3 μM) (Table 1). These values are similar to those from other PHA-binding proteins (37).

PHB coating interferes with PhaF binding. The mainly hydrophobic nature of the phasin-PHA binding interaction makes it interesting to evaluate the effect of covering the polyester with amphipathic compounds on the stability of PhaF adsorption. First, commercially available PHB particles were coated with oleic acid (PHBOL particles), a common component in artificial PHA preparations (38). Remarkably, the oleate cover

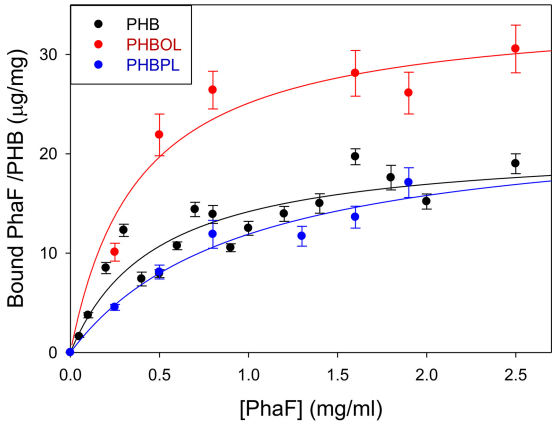

FIG 3 Analysis of the saturation capacity of PHB preparations by Langmuir isotherm analysis. Each point in the plot represents the mean from three independent experiments.

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TABLE 1 Binding properties of PHB preparations for PhaF[a]

Material	q_{max} (μg protein/mg PHB)	K_d (mg/ml)
PHB	20 ± 2	0.5 ± 0.1
PHBOL	35 ± 3	0.4 ± 0.1
PHBPL	24 ± 4	1.0 ± 0.4

[a]Calculated by Langmuir analysis (see Materials and Methods for the equation and Fig. 3).

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somehow increases the affinity for PhaF, while significantly augmenting its maximum binding capacity (Table 1 and Fig. 3). This may be explained in terms of a specific stabilizing interaction between oleate molecules and PhaF that has been described before (34). Nevertheless, and for the same reason, the PhaF-loaded PHBOL particles were more sensitive to competing detergents whose structures mimic that of oleate (i.e., long hydrocarbon chains), namely, Triton X-100 and especially Tween 20 (Fig. 2B; see also Fig. S4). Finally, with the aim of mimicking the lipid layer inherently found in usual PHA preparations, PHB granules were coated with a 1:1 (wt/wt) lipid mixture of phosphatidylcholine and phosphatidylglycerol (PHBPL particles). In this case, the affinity for PhaF was severely diminished (Table 1 and Fig. 3), and the protein could be eluted with an appreciable yield by all tested detergents except CHAPS (Fig. 2C). To check for any possible destabilizing effect on the PHB particle caused by the phospholipid layer, we evaluated the aspect, stability, and degree of crystallinity of both ~~lipid treated- and~~ nontreated PHB materials by differential scanning calorimetry (DSC) and scanning electron microscopy (SEM). Figure 4 and Table 2 show that the size and morphology of PHB granules did not appreciably change upon coating. Data in Table 2 also indicate that lipid coating ~~only produced~~ a moderate decrease in crystallinity (65% to 44%), even after the lyophilization step, while thermal stability and particle size were also largely unaffected. These results demonstrate that granule coatings by amphiphilic compounds affect the BioF-PHB interaction to ~~a variable extent~~ without substantially affecting particle integrity.

F4/T2

Immobilization of BioF-tagged hybrid proteins on PHB. The results shown so far indicate that the PhaF phasin is likely to bind to different types of PHAs such as PHB. This adds a novel functionality for the BioF polypeptide to serve as a versatile affinity tag for anchoring recombinant proteins of interest to a wide range of polyhydroxyalkanoates. To further evaluate the biotechnological potential of this result, we analyzed the *in vitro* binding of the BioF–C-LytA protein (formerly named FLyt), a fusion of BioF

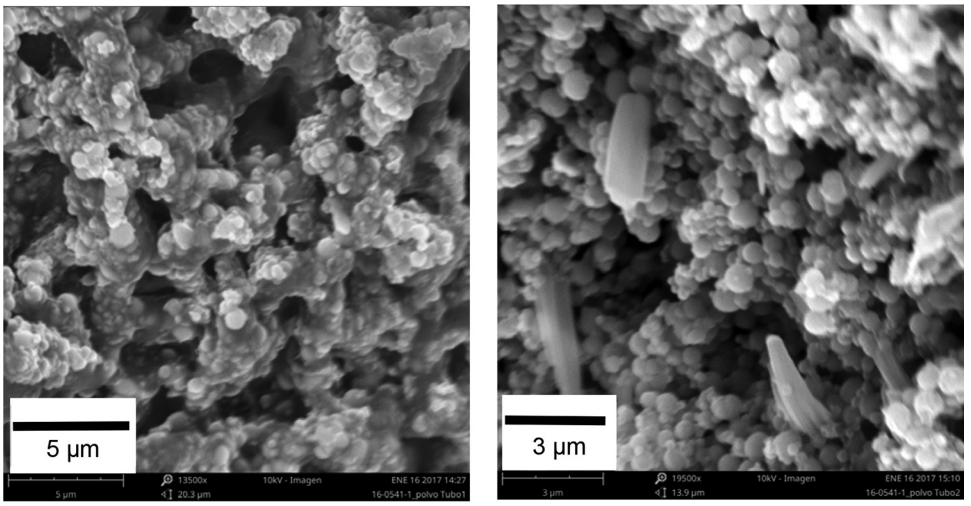

FIG 4 SEM micrographs of PHB and PHBPL preparations.

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TABLE 2 Calorimetric characterization of PHB preparations

Material	T_m[a] (°C)	ΔH_m[b] (J/g)	Crystallinity[c] (%)	Mass loss upon heating (%)	Particle size[d] (μm)
PHB	173.3 ± 0.9	95 ± 3 ~~J/g~~	65	20.0	0.5–1
PHBPL	173.0 ± 0.9	70 ± 20 ~~J/g~~	44	25.5	0.3–0.9

[a]T_m, melting temperature.
[b]ΔH_m, melting enthalpy.
[c]Calculated according to reference 53.
[d]By SEM.

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with the C-LytA choline-binding module (32), to synthetic PHB granules. Only the binding of BioF–C-LytA to native mcl-PHA granules *in vivo* had been previously studied (32). As shown in Fig. 5, purified BioF–C-LytA was able to bind to PHB at 37°C with an affinity comparable to that of the PhaF phasin (77% of applied protein, see Fig. 1) and also independently of pH and ionic strength (data not shown). The stability against detergents followed a ~~similar trend as~~ that for PhaF, with sarcosyl and SDS being the most efficient eluting agents (see Fig. S5). Unexpectedly, the residual elution capability of Tween 20 for PhaF was not detected in the case of BioF–C-LytA. Nevertheless, this effect might be ascribed to secondary interactions taking place between the polymer and the relatively hydrophobic aromatic-rich C-LytA moiety (39) that is likely to strengthen the binding. On the other hand, in contrast to the PhaF case (Fig. 2), CHAPS was able to elute a small but detectable amount of BioF–C-LytA protein (Fig. S5). This is probably due to the fact that the quaternary ammonium group in the detergent (Fig. S4) may act as a specific choline-like ligand of the C-LytA moiety (40) and helps release the protein from the polymer. Importantly, while BioF–C-LytA is unstable in solution and shows evident signs of autodegradation at 37°C, a clear protective effect provided by the PHB matrix is evident when the protein is immobilized on the polymer (Fig. 5).

Construction of PHB-based enzymatic minibioreactors. Once the capability and functionality of the BioF tag as an immobilization system of fusion proteins to commercial preparations of PHB were confirmed, we evaluated the possibility of using this platform for the construction of enzyme bioreactors. As a proof of concept, we assessed the performance of the hybrid protein BioF–β-galactosidase (formerly named FLac) in a PHB-based minibioreactor, where the BioF polypeptide is fused to the ~~E.~~ *coli* β-galactosidase protein (32). Even though the BioF–β-galactosidase enzyme could only be purified to approximately 35% (Fig. S2), it still represents a suitable system, since the majority of industrial applications involving immobilized enzymes do not require a greater extent of protein purity (41). The partially purified BioF–β-galactosidase prep-

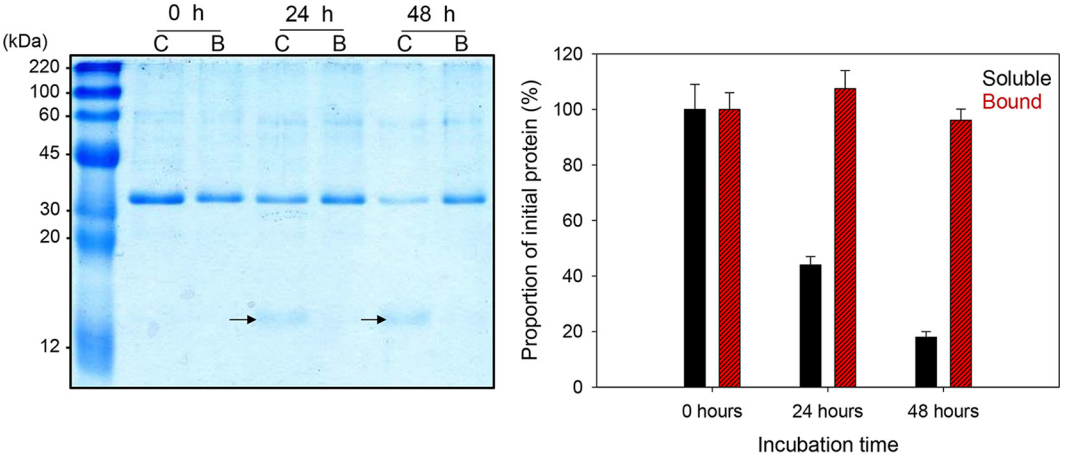

FIG 5 (Left) Analysis by SDS-PAGE of the stability of BioF–C-LytA binding to PHB at 37°C. C, control of soluble protein; B, bound protein eluted from PHB with 1% ~~SDS~~ (wt/vol) after heating at 90°C for 10 min. Black arrows indicate degradation fragments of BioF–C-LytA in solution. (Right) Results of gel densitometry, shown as percentages of initial applied protein.

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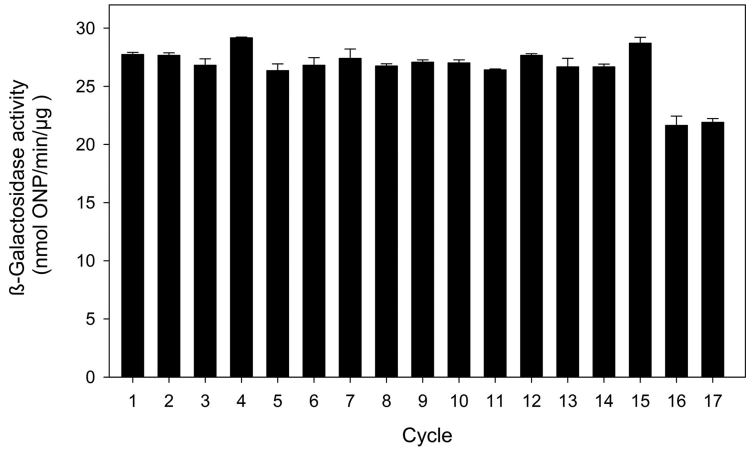

FIG 6 Activity of PHB-immobilized BioF–β-galactosidase during 16 continuous cycles. Time between cycles was 20 ~~min except cycles 15 and 16 that~~ were carried out after 48 and 96 h, respectively. Each experiment represents the mean from six determinations.

aration was adsorbed on PHB, and the enzymatic activity was assayed in 14 reaction/washing cycles (10 min each), followed by two other cycles assayed after 48 and 96 h (Fig. 6). After such incubations, the enzymatic activity was still conserved at 75% ± 7% with respect to the initial activity, suggesting that the enzyme kept its stability under these conditions and that protein desorption from the matrix was not relevant.

F6

DISCUSSION

Polyhydroxyalkanoate-based technologies based on functionalization with proteins possess a high biotechnological potential that, nevertheless, has not yet found the expected industrial development and application. These limitations are particularly linked with the restrictions of the production systems. Therefore, to achieve a wide industrial application and performance comparable to ~~those~~ other commercially available polymers, improved PHA-based protein immobilization platforms need to be developed (42). Moreover, the need for high-added-value applications~~,~~ and particularly the construction of bioactive PHA~~,~~ supports ~~that~~ protein functionalization might boost the commercial importance of PHA. Nevertheless, the construction of bioactive PHA systems by protein immobilization has been investigated to a limited degree at the laboratory scale and is still a nascent technology that needs scaling up for industrial applications (43). Very efficient covalent methods for PHA functionalization-based translational fusions with ~~the~~ PhaC from *Ralstonia eutropha* have been described in detail by Rehm's group, leading to a variety of biotechnological and biomedical applications (3, 44, 45). However, in the case of noncovalent methods like those based in phasins, several key technical issues, such as control of PHA-protein association/dissociation and granule size, still remain unresolved.

Herein, we have evaluated the *in vitro* binding properties of the PHA-binding domain of the PhaF phasin from *P. putida* KT2440 (BioF tag) to scl-PHAs such as PHB particles devoid of any coating. The BioF-PHB interaction is very stable with respect to temperature (25 to 37°C) (Fig. 1), time (48 to 96 h) (Fig. 1, 5, and 6), pH (2 to 9) (Fig. S3 in the supplemental material), and detergent action (Fig. 2). Similar results were obtained with the fusion protein BioF–C-LytA (Fig. 5; see also Fig. S5), demonstrating that binding to PHB is mostly ascribed to the BioF sequence independently of the fused polypeptide. Furthermore, the binding of the fusion protein to PHA may strongly protect the adsorbed protein from degradation, as revealed for BioF–C-LytA (Fig. 5). Our results indicate that the BioF tag is especially suited to provide a robust and stable, yet straightforward, protein immobilization platform not only to its natural cognate mcl-PHA substrate but also to commercially available PHB and, probably, to many other PHA types. To date, only limited examples of phasin binding to pure noncoated PHA

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preparations have been reported (30, 46). However, more studies of this kind are necessary, since several applications, especially in the biomedical field, require homogeneous materials free of endotoxins or other incompatible components.

The molecular nature of the binding between BioF and mcl-PHAs is likely to be mainly based on hydrophobic, nonspecific interactions, according to the predicted secondary structure of the PhaF phasin (34). This structural model predicts that the BioF sequence folds into a long, tetrameric amphipathic α-helix that recognizes the surface of the PHA granule through the nonpolar face of the helix. As expected for a mainly hydrophobic interaction, the BioF-PHB binding is susceptible to detergent treatment. The efficiency of the detergent treatment strongly depends on the detergent type. The agents with the highest eluting performances are SDS and sarcosyl (Fig. 2; see also Fig. S5), which is in agreement with similar *in vivo* results when mcl-PHAs have been used as the immobilization substrate (32). Therefore, these should be the compounds of choice to eventually regenerate the PHB support in any biotechnological process that requires a new loading with fresh protein. Moreover, SDS and sarcosyl are also the detergents with the longest linear hydrocarbon moieties (Fig. S4), which are likely to better intercalate within the presumably elongated BioF-PHA interface (34). While Tween 20 also has a long linear hydrophobic chain, its polar moiety is sizeable, probably hampering the interaction with the hydrophobic side of the helix (Fig. S4). Consequently, this detergent displays an intermediate eluting behavior that is enhanced when the protein-granule interaction is weakened upon granule coating (Fig. 2). Finally, Triton X-100 and sodium cholate possess the shortest linear chains and subsequently show the poorest elution properties. CHAPS represents a peculiar case, because its structure is devoid of linear chains and, accordingly, does not elute PhaF from the PHB granule (Fig. 2). However, the binding of the BioF–C-LytA chimera is somehow susceptible to this detergent both *in vitro* (Fig. S5) and *in vivo* (32), probably because CHAPS behaves as a ligand of the C-LytA moiety and helps elute the protein by weakening a secondary non-BioF-related interaction. This indicates that the polypeptide fused to the BioF tag may also contribute, positively or negatively, to strength of the binding to PHA.

The BioF-PHB interaction is affected if the polyester is coated *in vitro* with amphiphilic compounds such as oleic acid and, especially, phospholipid layers (Fig. 2 and 3; Table 1). This dependence on granule coating has also been observed for the binding of the PhaP phasin from *Ralstonia eutropha* to polyhydroxyoctanoate surfaces, which was prevented by the presence of a phospholipid cover in the granule (46). Given its elongated shape, BioF is likely to be attached to the PHA polymer in parallel with its surface (34) but not deeply penetrating into the granule, as this would certainly impose an excessive desolvation energy penalty to the polar residues in the hydrophilic face of the helix. Hence, it is not surprising that any coating of the matrix should affect the direct association between the affinity tag and the polyester by decreasing the amount of available PHB surface. Far from constituting a drawback, this dependence on coating represents a useful characteristic to rationally modulate the strength of BioF-tagged protein adsorption and may find its application in processes requiring a fine tuning of the interactions, such as the controlled release of therapeutic proteins and peptides (47, 48) or the straightforward regeneration of enzymatic reactors after enzyme inactivation.

To date, the main applications of protein-functionalized PHAs have been aimed ~~for~~ biomedical purposes, such as diagnosis, vaccines, drug delivery, and cell imaging (42). However, the development of PHA-based enzymatic reactors has been analyzed to a very limited degree. Therefore, in this work, we have studied the enzymatic activity of the chimeric protein BioF–β-galactosidase immobilized on PHB particles as a proof of concept for the construction of these systems. Our procedure led to systems showing stable galactosidase activity during several continuous cycles of reaction/washing as well as an extended enzyme performance (at least 96 h) (Fig. 6). These results confirm the strong interaction between the BioF tag and PHB particles and pave the way for the construction of sustainable reusable PHA supports designed for enzymatic biotransformations.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and general growth conditions. Liquid cultures of *Escherichia coli* were grown at 37°C in Luria-Bertani (LB) broth (49) supplemented with kanamycin (0.05 mg/ml) or streptomycin (0.05 mg/ml), depending on the plasmid resistance, in an orbital shaker (200 rpm; INNOVA 4000). Bacterial growth was followed by turbidimetry at 600 nm using an Evolution 201 spectrophotometer (Thermo Scientific).

Overproduction and purification of recombinant proteins. As a general procedure, overexpression was carried out in every case in freshly transformed cells with the corresponding expression vector according to $CaCl_2$ transformation protocols (49). The purity of protein preparations was checked by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) (50) using the Mini-Protean systems from Bio-Rad. Gels were stained with EZBlue (Sigma-Aldrich). The concentration of pure proteins was assessed by absorption spectroscopy at 280 nm (Evolution 201; Thermo Scientific) with theoretical molar extinction coefficients of 19,071 $M^{-1} \cdot cm^{-1}$ (PhaF) and 109,320 $M^{-1} \cdot cm^{-1}$ (BioF–C-LytA) using the online software ProtParam from the Expasy toolbox (http://web.expasy.org/protparam). Samples containing partially purified BioF–β-galactosidase were quantified by using the protein assay dye reagent concentrate from Bio-Rad and bovine serum albumin (Sigma-Aldrich) as the standard. Enzyme fractions were stored at −20°C until further use.

PhaF protein was overexpressed in *E. coli* BL21(DE3) cells harboring the pETPhaF plasmid and purified by hydrophobic interaction chromatography on butyl-Sepharose (GE, Healthcare) as previously described (33).

For the purification of the Bio–C-LytA protein, *E. coli* CC118 cells harboring plasmid pNFA2 (32) were grown at 37°C and 200 rpm until the culture reached an optical density at 600 nm (OD_{600}) of 0.6. Then, *lyt* gene overexpression was induced for 14 h at 30°C by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation at 4°C (10,000 × *g*), resuspended in 20 mM sodium phosphate (PNa) buffer (pH 7.0) plus 100 mM NaCl (50 ml per liter of culture), disrupted by sonication (Branson 250), and centrifuged again for 10 min at 4°C (10,000 × *g*). Insoluble inclusion bodies containing denatured BioF–C-LytA were redissolved in 8 M urea (25 ml per liter of culture), followed by centrifugation for 10 min at 4°C (10,000 × *g*). Simultaneous refolding and purification of BioF–C-LytA was accomplished in one step by directly loading the supernatant onto a Sephadex G-100 gel filtration column (50 cm by 1 cm; Bio-Rad), employing PNa buffer plus 100 mM NaCl as the mobile phase. Fractions with more than 90% purity, as assessed by SDS-PAGE, were stored at 4°C (Fig. S1 in the supplemental material).

The purification of the BioF-β-galactosidase fusion was accomplished by using *E. coli* CC118 cells harboring plasmid pNFL2 (32) grown and processed in a similar way to that for the BioF–C-LytA protein (see above), except that BioF-β-galactosidase protein was found predominantly in the soluble fraction after sonication. Ammonium sulfate was slowly added at 4°C with stirring to a final concentration of 1.7 M, and the solution was then centrifuged at 4°C (10,000 × *g*) and the supernatant was applied to a butyl Sepharose 4 fast flow column (GE Healthcare) (10 cm by 1 cm) previously equilibrated with PNa buffer containing 1.7 M ammonium sulfate. The column was thoroughly washed with PNa buffer plus 0.75 M ammonium sulfate, and then BioF–β-galactosidase was finally eluted with PNa buffer. The result of the partial purification is shown in Fig. S2, and the amount of BioF–β-galactosidase with respect to the total protein was estimated at 35% ± 3% upon gel densitometry using ImageJ (51). Attempts to further purify the protein were unsuccessful in our hands.

Preparation and characterization of coated PHB particles. Artificial oleate-coated PHB granules (PHBOL) were prepared by the oil-in-water emulsion method as previously described (38). Artificial phospholipid-coated PHB granules (PHBPL) were prepared according to a previously described procedure (52) with minor modifications. First, one volume of 5% PHB (wt/vol) (Sigma-Aldrich) solution in chloroform was emulsified with 20 volumes of an aqueous solution of 2% sodium cholate (wt/vol) (Sigma-Aldrich) by ultrasonication (20 kHz, 1 to 3 min). Then, chloroform was removed from the emulsion under vacuum conditions (40°C for 60 min), and the artificial granules coated with sodium cholate were harvested by centrifugation (10,000 × *g* for 10 min) and washed twice with equal volumes of distilled water. Granules were resuspended with equal volumes of PNa buffer containing L-α-phosphatidyl-D-L-glycerol (5 mg/ml; Sigma-Aldrich) and L-α-phosphatidylcholine (5 mg/ml; Sigma-Aldrich) from egg yolk previously emulsified by sonication in an aqueous solution of sodium cholate 2% (wt/vol). The resulting mixture was extensively dialyzed in 20 mM PNa buffer (pH 7.0) containing 25 g/liter Amberlite XAD-2 (Sigma-Aldrich) to remove sodium cholate. Finally, artificial phospholipid-coated PHB granules were concentrated by lyophilizing to dryness (Telstar Cryodos).

The characterization of the PHB material was achieved by thermal analysis. Samples of approximately 5 to 10 mg were placed in an aluminum capsule and subjected to differential scanning calorimetry (DSC) on a Pyris Diamond DSC calorimeter (PerkinElmer, Waltham, MA). The samples were heated under a nitrogen atmosphere from 20°C to 200°C at a rate of 20°C/min. The degree of crystallinity was estimated from the experimental melting enthalpies (ΔH_m) assuming a value of 146 J/g for a 100% crystalline PHB (53). PHB particles were also analyzed by scanning electron microscopy (SEM) in a Phenom-World ProX microscope operating at 10 kV. Samples were placed in the microscope plate and sputter coated with gold to ensure electrical conductivity. Both SEM and DSC analyses were carried out in the Plastics Technology Centre AIMPLAS (Valencia, Spain).

Protein binding to PHB particles. As a general methodology, 5 mg from different preparations of PHB (PHB, PHBPL, or PHBOL) was incubated for 15 min with 20 to 30 μg of a BioF-containing protein (PhaF or BioF–C-LytA) in a final volume of 100 μl PNa buffer at temperatures of 25 or 37°C. After mild mixing on a digital rotary mixer (Ovan), the unbound protein fraction was separated from the polyester

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by centrifugation (10 min at 10,000 × *g*). PHB beads were washed five times with 500 μl PNa buffer plus 0.1 M NaCl and finally resuspended in 100 μl of PNa buffer. To check the strength of the protein-PHB interaction, the resuspension buffer contained the corresponding detergents: ~~1% SDS (wt/vol), 3% sarcosyl (wt/vol), 1% Tween 20 (wt/vol), 1% Triton X-100 (vol/vol), 3% sodium cholate (wt/vol), and 3% CHAPS (wt/vol)~~ (Sigma-Aldrich). After 15 min of incubation at 25°C, samples were centrifuged and the eluted protein fractions were analyzed by UV spectroscopy and SDS-PAGE.

To determine the affinity of the protein-PHB interaction and the maximum protein binding capacity by Langmuir analysis (54, 55), 10 mg of PHB was incubated with increasing concentrations of PhaF (0 to 2.5 mg/ml) in PNa buffer (500 μl). After mild mixing (30 min at 25°C) on a digital rotary mixer (Ovan), the unbound protein fraction was recovered from the polyester by centrifugation (10 min at 10,000 × *g*). The amount of PhaF bound to PHB (μg PhaF/mg PHB) was calculated by subtracting the unbound protein from the initial added protein. Data were fitted to the equation $q = (q_{max}C)/(K_d + C)$ (54, 55), where q is the amount of protein bound on PHB (μg protein/mg PHB), q_{max} is the maximum adsorption capacity of PHB granules (μg protein/mg PHB), C is the concentration of protein added (mg/ml), and K_d is the dissociation constant (mg/ml).

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***β*-Galactosidase enzyme activity.** The β-galactosidase activity of the immobilized hybrid BioF–β-galactosidase protein was assayed spectrophotometrically according to Miller (56). BioF–β-galactosidase (approximately 2.5 μg, as estimated by gel densitometry [see above]) was first immobilized on 0.5 mg of PHB as described above for PhaF. After extensive washing with PNa buffer, the resin was incubated for 10 min at 25°C with 5 mM *o*-nitro-phenyl-β-D-galactopyranoside (ONPG) on a digital rotary mixer (Ovan). We previously checked that the enzymatic reaction was linear within these time limits (data not shown). The sample was then briefly centrifuged (10,000 × *g*), the supernatant was removed, and the reaction was stopped with 0.12 M Na_2CO_3. Enzymatic activity was assessed following the formation of *o*-nitrophenol at 420 nm (ε_{420} = 4,500 $M^{-1} \cdot cm^{-1}$), after subtracting a blank that was ~~equally~~ processed but without the enzyme added. The remaining PHB support was extensively washed with PNa buffer to remove remaining traces of ONPG and *o*-nitrophenol and subjected to new enzymatic cycles by the addition of fresh 5 mM ONPG.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02595-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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