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Chapter 6

Protein Engineering of Enzymes Involved in Bioplastic Metabolism

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Environmental problems caused by petroleum-based plastics

The last half century has witnessed the development of synthetic plastics from petroleum resources, and more than 300 million tons of synthetic plastics are annually produced at present. The recently increased consumption of petroleum resources has led to environmental problems such as a depletion of the resources as well as a global warming due to a marked increase of atmospheric CO_2 level. In addition to these problems, wasted plastics used in short-term applications may cause the environmental damage to a wide variety of wild animals including terrestrial, aquatic animals and birds. Furthermore, it has been suggested that even the wasted plastics in the form of small particles potentially induce the alteration of pelagic ecosystems [1]. Therefore, the development of environmentally sound alternatives, such as bioplastics, to conventional petroleum-based plastics is urgently desired to sustain the environment [2-4].

1.2. Bioplastics

Bioplastics include biodegradable and bio-based plastics (Figure 1) [5, 6]. The former are produced from renewable or petroleum resources via biological or chemical processes, and degraded by enzymes and microorganisms in natural environment. The latter are synthesized from renewable resources via biological or chemical processes, and some of them show non-biodegradability although bio-based plastics are generally biodegradable. Poly(ε-caprolactone) (PCL), poly(ethylene succinate) (PES) and poly(butylene succinate) (PBS) are synthesized from petroleum resources via chemical processes, but they show an excellent biodegradability. Currently, cost-effective processes for the production of succinic acid and 1,4-butanediol, raw materials of PBS, from biomass resources are being developed.

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Meanwhile, poly(ethylene) (PE) and poly(propylene) (PP) are chemically synthesized from their monomers derived from biological sources, but they are not biodegradable. Poly(hydroxyalkanoate)s (PHAs) and poly(lactide) (PLA) show an excellent biodegradability, and are produced from renewable resources via biological and chemical processes, respectively. Thus, the bio-based bioplastics having biodegradability, such as PHAs and PLA, are the most favorable bioplastics to avoid the above-mentioned problems associated with the use of petrochemical-based synthetic plastics.

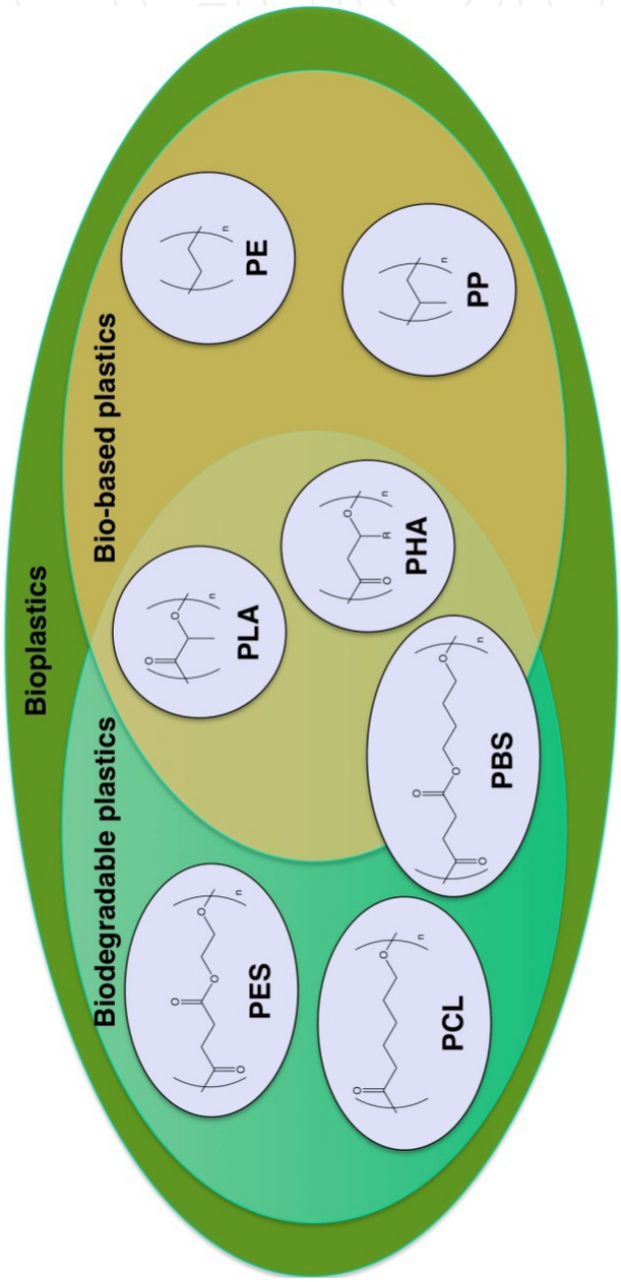

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Figure 1. Bioplastics comprised of biodegradable and bio-based plastics.

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1.2.1. PHAs

PHAs are the only bioplastics completely synthesized from renewable resources by a wide variety of microorganisms in soil, active sludge, marine and extreme environments [7, 8]. In the cells, PHAs form amorphous granules and is degraded by intracellular PHA depolymerases (i-PHA depolymerases) produced by the PHA-accumulating bacterium itself. In contrast, after PHAs are extracted from the cells, PHAs are converted to semicrystalline form and is degraded by extracellular PHA depolymerases (e-PHA depolymerases) secreted from microorganisms in natural environments, such as soil, active sludge, fresh water, and seawater [9, 10].

Many bacteria can synthesize various types of PHAs containing 3-, 4-, and 5-hydroxyalkanoate units, and over 150 different hydroxyalkanoates other than 3-hydroxybutyrate have been reported as constitutive units of PHAs [11]. PHAs consisting of short-chain hydroxyalkanoates (SCL-HAs; 3–5 carbon atoms) or medium-chain hydroxyalkanoates (MCL-HAs; 6–14 carbon atoms) have been detected. The former are thermoplastic in nature, whereas, the latter are elastomeric in nature. The physical and mechanical properties of PHAs can be regulated by varying monomer composition in order to gain properties comparable to petrochemical-based thermoplastics that have been used for various applications in industry, medicine, pharmacy, agriculture, and electronics [12]. Accordingly, PHAs have attracted industrial interest as bio-based, biodegradable, biocompatible, and versatile thermoplastics [13, 14].

1.2.2. PLA

PLA is representative bio-based plastics with good processability and transparency that are used in packaging, containers, stationary, etc. [15]. In addition, medical and agricultural uses of the material have been investigated because of their biocompatibility and biodegradability [16]. PLAs are produced from renewable biomass through a chemo-bioprocess consisting of fermentative production of lactic acid (LA) and chemical polymerization. LA is spontaneously polymerized by refluxing, but the molecular mass of yielded polymer tends to be low [17]. There are several methods for synthesizing high-molecular-mass PLAs: condensation, chain elongation, and ring-opening polymerization of cyclic lactides [15]. Currently, the major industrial method to produce PLAs is ring-opening polymerization which is catalyzed by heavy metal catalysts, typically tin [18, 19]. However, the trace residues of the heavy metal catalyst are unfavorable for certain applications, in particular, medical and food applications. Thus, replacement of the heavy metal catalyst with a safe and environmentally acceptable alternative is an important issue. For this purpose, enzymes are attractive targets because they are natural non-harmful catalysts that can drive the reactions under mild conditions. In addition, highly specific enzymatic reactions may be capable of synthesizing polymers with fine structure from crude materials, which would reduce the cost of preparing the starting substances. This could be an advantage over chemical polymerization of LA or lactides, since the chemoprocess requires extremely pure monomers (contamination of carbonic acids is known to inhibit polymerization), along with anhydrous and high temperature conditions to proceed.

In such a situation, LA-polymerizing enzyme (LPE) functioning in replacement of metal catalysts should enable the biosynthesis of PLA, even though it is enormously challenging both in terms of research and industrial implementation. The best solution could be the development of a PLA-producing microorganism introduced with LPE gene, but this has not been reported so far. In 2008, Taguchi et al. nonetheless successfully obtained encouraging results by developing a recombinant *Escherichia coli* strain allowing the synthesis of LA-based polyesters by introducing the gene encoding engineered PHA synthase with acquired LA-polymerizing activity [20, 21]. They thus achieved the one-step biosynthesis of a copolymer with 6 mol% of lactate and 94 mol% of 3-hydroxybutyrate units. This extremely important result represents a milestone towards the biological synthesis of PLA and confirms that the work is moving in the right direction, as mentioned in the section of 2.3. At present, the LA fraction in the copolyesters has been enriched up to 96 mol% [22], so the synthesis of homopolymers of LA represents a major goal. To that end, the current microbial cell factory ought to be improved with further evolved LA-polymerizing enzymes (LPE) and metabolic engineering-based optimization [23, 24]. Matsumura et al. likewise reported the lipase PC-catalyzed polymerization of cyclic diester-D,L-lactide at a temperature of 80-130 °C to yield PLA with molecular masses of up to 12,600 [25].

1.3. Toward an enhanced sustainable production

Three main issues have hindered widespread use of PHAs: (1) the high production cost compared to petroleum-based polymers with similar properties; (2) the inability to produce high-performance PHAs in substantial amounts; and (3) the difficulty in controlling the life cycle of PHAs, i.e., the control of their biodegradability and their effective chemical recycling.

To solve the former two issues, we have focused on the genetic engineering of PHAs metabolism, which will lead to the cost-effective biological production of PHAs and the improvement of their properties, such as molecular mass and monomer composition. In particular, protein engineering of PHA synthase can improve both PHA production efficiency and the properties of the generated polymer because PHA synthase plays a central role in PHA biosynthesis [26]. Here we would like to highlight the current special topic on the biosynthesis of new PHA polymers incorporating unusual monomer units such as LA by PHA synthase engineering. Further, gene cloning and expression in plants has created new possibilities of using photosynthesis to convert atmospheric CO_2 directly into PHA, in hopes of reducing production cost in the future.

In addition, to solve the latter issue, we have also focused on the engineering of PHB depolymerases. PHB is the most common form of PHAs. In natural environment, the microbial and enzymatic degradation of PHB is an important first step in the PHB recycling process. However, PHB degradation depends on the surrounding conditions and proceeds on the order of a few months in anaerobic sewage or a few years in seawater [13]. Such PHB degradation process is undesirable from the standpoint of the efficient use of biomass resources. To overcome this issue, chemical recycling using spent PHB materials as recyclable monomer-concentrated resources is rapidly gaining importance due to its high degradation rate [27]. In addition, as chemical recycling is cost-efficient and has low CO_2 emissions, it has great potential

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as a low-cost and environmentally compatible process. PHB monomerization, the first step in chemical recycling, is currently carried out via a thermal decomposition process. However, this chemical recycling method presents some drawbacks, such as racemization of the decomposed products, high reaction temperature, and contamination with residual metal catalysts [28-31]. As one of the solutions, the development of alternative PHB monomerization methods that use such enzymes as PHB depolymerases is highly awaited because those methods do not produce undesirable byproducts, have high enantio- and regioselectivities, and can be performed at moderate temperatures [32, 33]. Moreover, as the efficient use of biocatalysts requires suitable enzymes with high activity and stability under process conditions, the desired substrate selectivity, and high enantioselectivity, the improvement of PHB depolymerases is expected to result in the construction of an effective PHB chemical recycling system. In this chapter we will also provide some case studies on protein engineering of PHB depolymerase based on domain structure-based and random mutagenesis approaches.

2. Protein engineering of PHA synthases

2.1. Biochemical properties and engineering concepts of PHA synthases

PHA synthases catalyze the polymerization reaction of hydroxyalkanoate (HA) to polymer PHA. The monomer substrates of PHA synthase are mainly 3HA-CoAs with various side-chain lengths, and only *R*-enantiomer HA-CoAs are accepted for polymerization by synthase [34]. Over 60 different PHA synthases have been classified into four types based on their substrate specificities and subunit compositions of enzymes (Table 1) [35].

Type	Subunit(s)	Representative species	Substrate specificity
I	PhaC	*Ralstonia eutropha*	C3 - C5
		Aeromonas caviae	C3 – C7
II	PhaC	*Pseudomonas aeruginosa*	C6 – C14
		Pseudomonas sp 61-3	C3 – C12
III	PhaC - PhaE	*Allochromatium vinosum*	C3 – C5
IV	PhaC - PhaR	*Bacillus megaterium*	

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Table 1. The four classes of PHA synthases

Type I and type II PHA synthases consist of single subunits (PhaC). Type I PHA synthases, represented by *Ralstonia eutropha* enzyme, mainly polymerize SCL-monomers (C3–C5), whereas type II PHA synthases, represented by *Pseudomonas oleovorans* enzyme, polymerize MCL-monomers (C6–C20). Type III PHA synthases, represented by *Allochromatium vinosum* enzyme, consist of two hetero-subunits (PhaC and PhaE). PhaC subunits of type III synthase

are smaller than those of type I and II synthases, but possess catalytic residues. Like the type I synthases, these PHA synthases prefer to polymerize SCL-monomers (C3–C5). Type IV PHA synthases, represented by *Bacillus megaterium*, are similar to the type III PHA synthases with respect to possessing two subunits. However, unlike the PhaE of type III PHA synthases, a smaller protein designated as PhaR is required for full activity expression of type IV PhaC.

The lack of a suitable structural model for any PHA synthase has limited attempts to improve the activity and to alter the substrate specificity of these enzymes in "irrational" manners, such as random mutagenesis and gene shuffling [36, 37]. Generally, natural diversity provides us with attractive starting materials for artificial evolution as it represents functionalized sequence spaces to some extent. A tremendous population (over 60 species) of randomly screened PHA producing bacteria suggests that attractive prototype enzymes for molecular breeding would exist. Among them, enzyme evolution approach has been applied to the following type I and type II PHA synthases derived from some bacteria.

2.2. Activity improvement and substrate specificity alteration of PHA synthases

2.2.1. Application to type I PHA synthases

A pioneering study that established methods for protein engineering PHA synthase initiated in 2001 using the best-studied enzyme, the *R. eutropha* PHA synthase [38]. *In vitro* evolutionary program was firstly constructed by coupling an error-prone PCR-mediated point mutagenesis with the plate-based high-throughput screening method to generate mutants with acquired beneficial functions [38]. A mutant library of the *R. eutropha* PHA synthase gene was prepared by colony formation of transformant cells of *Escherichia coli*. It should be noted to meet a good correlation between the change in PHB accumulation resulting from the introduction of mutations into the *R. eutropha* PHA synthase gene and the change in the enzymatic activity of the mutants. To gain the mutants with increased activity, multi-step mutations, including an activity loss and an intragenic suppression-type activity reversion were attempted [39]. The mutant enzymes were once identified by primary mutation analysis, a secondary round of mutation was used to evolve these enzymes to proteins with better characteristics than the wild-type enzyme. As a result, through this intragenic suppression-type mutagenesis, an increased specific activity towards 3HB-CoA by 2.4-fold compared to the wild-type enzyme was acquired by a mutation of Phe420Ser (F420S) in a type I PHA synthase [39].

As a next case, screened beneficial mutation, Gly4Asp (G4D), exhibited higher levels of protein accumulation and PHB production compared to the recombinant *E. coli* strain harboring the wild-type PHA synthase [40]. As for intragenic suppression-type mutagenesis, second-site reversion is dependent or independent of primary mutation in the activity. Secondary mutations of F420S and G4D are the latter cases, being independent of primary mutation. Subsequently, site-specific saturation mutagenesis was also performed on the codon encoding the G4 residue of the *R. eutropha* PHA synthase and many substitutions resulted in much higher PHB content as well as higher molecular masses of the polymers [41].

Aeromonas caviae (*punctata*) PHA synthase is unique among type I PHA synthases since it can synthesize not only PHB homopolymer but also random copolyesters of 3HB and 3-hydrox-

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yhexanoate (3HHx). Kichise et al. performed the first successful *in vitro* molecular evolution experiments on PHA synthase from *A. caviae* by targeting to the limited region of the enzyme [42]. Two evolvants exhibited increased activity towards 3HB-CoA of 56% and 21%, respectively, compared to the wild-type enzyme by *in vitro* assays. These mutations led to enhanced accumulation (up to 6.5-fold higher than the wild-type enzyme) of P(3HB-*co*-3HHx) and increases in the 3HHx molar fraction (16-18 mol% compared to 10 mol% of the wild-type PHA synthase) in recombinant *E. coli* strains grown on dodecanoate. As an extended study, a combination of these two beneficial mutations (N149S/D171G) synergistically altered enzymatic properties, leading to synthesis of PHA copolymers with enhanced 3HA fraction and increased molecular mass from in the recombinant *R. eutropha* [43]. In a separate study, *A. caviae* PHA synthase was engineered *in vivo* using the mutator strain *E. coli* which has a 5,000-fold higher mutation rate than wild-type *E. coli*, and mutants were again screened for enhanced PHB accumulation in recombinant *E. coli* [44]. Also, mutants synthesized PHAs with increased molecular mass, but in contrast to the previous study, the 3HHx fraction was only slightly different from wild-type composition.

Junction site for interconnection of heterogeneous enzymes based on the predicted secondary structures allowed chimeragenesis of the PHA synthase from *R. eutroph*a with the partner PHA synthase from *A. caviae*. Successfully obtained chimera-mutant exhibited improved activity increase and expanded substrate specificity compared to the original enzymes [45]. As for PHA synthases, directed evolution studies have thus progressed through advancements from random approach to much more systematic approaches such as chimera-genesis, recombination and shuffling.

2.2.2. Application to type II PHA synthases

Contrasted with the type I PHA synthases, type II PHA synthases typically have substrate specificity towards MCL-3HA-CoA substrates but relatively poor substrate specificity towards SCL-3HA-CoA substrates like 3HB-CoA. An exception to this is the type II PHA synthase of *Pseudomonas* sp. 61-3 with significant substrate specificity towards the 3HB-CoA (Table 1). In the landmark study by Takase *et al*, the *in vitro* evolutionary technique was applied to the PhaC1 PHA synthase from *Pseudomonas* sp. 61-3 to increase the activity towards 3HB-CoA monomers [46]. Substitutions at two amino acid residues, Ser325 and Gln481 were found to dramatically effect the production of PHB homopolymer in recombinant *E. coli* with glucose as the carbon source. The codons for these amino acids were subjected to site-specific saturation mutagenesis and several individual substitutions were found that could dramatically increase the level of PHB production. These mutations were combined as double mutants to further increase the level of PHB production (340 - 400-fold higher than the wild-type enzyme) [46]. The changes in the *in vivo* produced P(3HB-*co*-3HA) copolymer molar compositions correlated well with the *in vitro* biochemical data of the substrate specificity and activity of the enzymes and represents one of the most well-rounded studies to date [47].

The findings obtained in these studies for the type II PHA synthase would be very useful for evaluating a similar evolution strategy to the other types of PHA synthases based on the amino acid sequence alignment of the PHA synthases. For example, position 481 in PhaC1 PHA

synthase from *Pseudomonas* sp. 61-3 was found to be one of the residues determining substrate specificity of the enzyme, as described above. Interestingly, the amino acid residues corresponding to the position of this enzyme are conserved within each type of PHA synthases; Ala for type I, Gln for type II, Gly for type III and Ser for type IV enzymes. Thus, the effects of mutating the highly conserved alanine (Ala510) of the *R. eutropha* PHA synthase (corresponding to the position 481 in *Pseudomonas* sp. 61-3 PhaC1) were analyzed via site-specific saturation mutagenesis. Mutations at Ala510 were found to affect the substrate specificity of the *R. eutropha* PHA synthase, allowing slightly higher 3HA incorporation compared to the wild-type PHA synthase in *R. eutropha* PHB^{-}4 (PHA negative mutant) [48].

The other two beneficial positions, Glu130 [49] and Ser477 [50], were also identified through the *in vitro* evolution screening. As illustrated in Figure 2 (A) and (B), "mutation scrambling" among four beneficial positions (130, 325, 477, 481) for activity increase, change in substrate specificity, and regulation of polymer molecular mass would further create new super-enzymes. Most recently, a possible mechanistic model for PHA polymerization has been proposed on the basis of the accumulated evolutionary studies [51]. Furthermore, the useful evolvants obtained through the systematic enzyme evolution have been supplied to other organisms including plants [52, 53]. The impacts of these reports prompted the other research groups to apply directed evolution to the individual PHA synthases of interests [54-56].

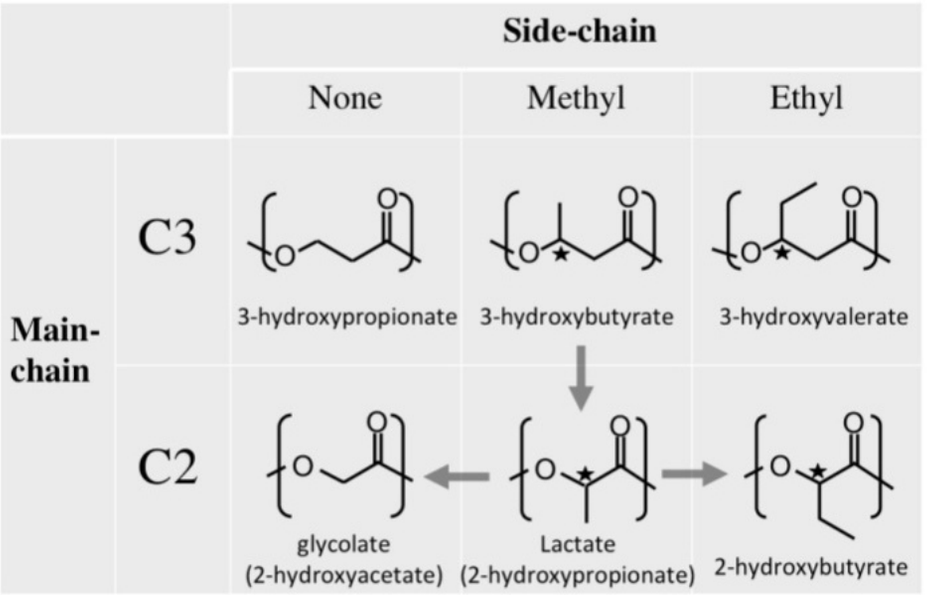

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Figure 2. Structural diversities in main-chain and side-chain of PHA back bone which can be recognized by natural and artificially evolved PHA synthases. Asterisks indicate the chiral center in monomer units of PHA.

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2.3. Engineering of lactate-polymerizing enzyme (LPE) from PHA synthases

The pioneering work on the exploration of LA-polymerizing activity by PHA synthases was reported by Valentin et al. [57]. In that attempt, the PLA biosynthesis was carried out by monitoring the activity of PHA synthases towards synthetic LA-CoAs (*R* and *S* enantiomers). Several PHA synthases were evaluated for LA-polymerizing activities and a class III PHA synthase from *Allochromatium vinosum* exhibited a weak CoA releasing activity [57]. In a similar report, Yuan et al. reported in detail the activity of *A. vinosum* PHA synthase towards (*R*)-LA-CoA [58]. Unfortunately, in either case, polymerization was not observed/was negligible, suggesting that PHA synthase could hydrolyze CoA ester to release CoA but not progress from there with polymerization to form a polymer.

In this context, Taguchi et al. formally reported the first prototype LPE in the year 2008 as a PHA synthase with an acquired LA-polymerizing activity through *in vitro* and *in vivo* experiments [20]. The first clue of LA-polymerizing activity was obtained through a water-organic solvent two-phase *in vitro* system [20, 59]. The activity towards LA-CoA was tested in the absence or presence of 3HB-CoA using representative PHA synthases belonging to the four classes of natural PHA synthases together with three engineered ($PhaC1_{Ps}$ mutants) from *Pseudomonas* sp. 61-3. The engineered PHA synthases were two single mutants[Ser325Thr (ST) and Gln481Lys (QK)] and one double mutant carrying the two mutations (STQK). The two mutants were selected based on their improved activity and/or broader substrate specificity [36, 37]. The natural synthases and mutants did not form a clear-polymer with LA-CoA alone but did with 3HB-CoA. However, when LA-CoA was supplied together with 3HB-CoA, one mutant, $PhaC1_{Ps}$(STQK) clearly exhibited a polymer-like precipitation. Subsequently, the analysis of the precipitant revealed that the precipitant consisted of 36 mol% of the LA unit. Therefore, this was the first report ever of a PHA synthase with ability to incorporate LA unit to form P(LA-*co*-3HB).

The finding that $PhaC1_{Ps}$(STQK) could polymerize LA was a demonstration of evolutionary engineering as a powerful tool for the generation of biocatalysts with desired properties. By demonstrating the *in vitro* activity of $PhaC1_{Ps}$(STQK) towards LA-CoA, it was presumed that heterologous expression of this LPE could result into an *in vivo* synthesis of LA-based polyesters thus creating microbial factories for LA-based polyesters synthesis.

In a subsequent study, based on the improved activity of a point mutation at position 420 (F420S) of a type I PHA synthase ($PhaC_{Re}$) from *R. eutropha* (Taguchi et al., 2002) [39], the same mutation was introduced into the ancestral LPE [$PhaC1_{Ps}$(STQK) from *Pseudomonas* sp. 61-3] to create a triple mutant of LPE with S325T and Q481K along with a new mutation, F392S which corresponds to F420S of $PhaC_{Re}$ [60]. When the new further engineered LPE [$PhaC1_{Ps}$ (STQKFS)] was expressed in *E. coli*, a copolymer with 45 mol% LA and polymer content of 62 wt% was synthesized in comparison with P(26 mol% LA-*co*-3HB) obtained with the prototype LPE, $PhaC1_{Ps}$(STQK) under aerobic culture conditions. Additionally, the cells harboring $PhaC1_{Ps}$ (STQKFS) synthesized P(LA-*co*-3HB) with 62 mol% LA with polymer content of 12 wt %. During the same study, saturation mutagenesis of LPE at the same site (position 392) yielded mutants that gave varying LA fractions in the copolymers however; F392S was superior to the other mutants in incorporating LA. This study demonstrated the effectiveness of enzyme

engineering of the LPE towards two directions; there was improved LA incorporation and polymer yield improvement for both aerobic and anaerobic culture conditions [60]. Furthermore, it will be interesting to note that copolymers incorporating 2-hydroxy acids (2HAs) such as 2-hydroxybutyrate [61] and glycolate (Matsumoto et al., 2011) [62] may lead to copolymers with novel properties (Figure 3). This expansion of PHA synthase to 2HAs-polymerizing enzymes has extensively prompted us to create further new enzymes with acquired activities toward new unusual monomer substrates, consequently create new polymers.

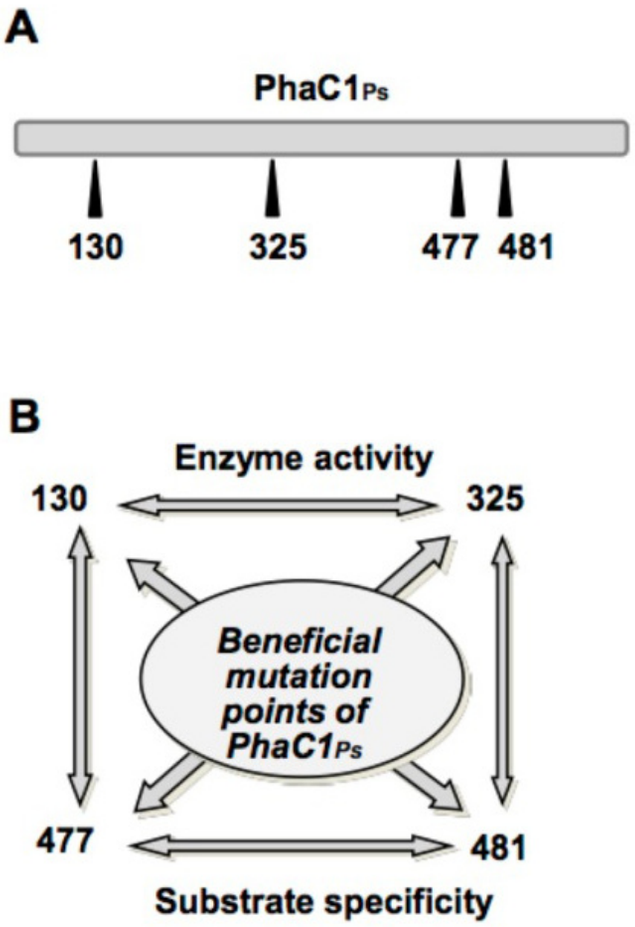

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Figure 3. Functional mapping of beneficial positions (A) and relationships among the residues related to enzymatic activity and substrate specificity of PHA synthase from *Pseudomonas* sp. 61-3 (B).

Regarding the reports on LPE, the following several studies have been published [63, 64]. Currently, the best-studied PHA synthase from *R. eutropha* has been successfully engineered to LPE by only single mutations at beneficial position corresponding to the position 481 of *Pseudomonas* sp. 61-3 PHA synthase [65]. This implies the functional compatibility between PHA members also for acquiring LPE activity. In the prospect, advanced types of LPE will be supplied by artificial evolution of the prototype LPE as well as exploration of

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natural PHA synthases with potentially possessing new substrate specificities such as LA-polymerizing activity.

3. Protein engineering of PHB depolymerase

3.1. Biochemical and genetic properties of PHB depolymerases

A number of PHA depolymerases have been purified from diverse PHA-degrading microorganisms and characterized [9, 10, 12]. As described earlier, depending on the substrates and localization of PHA depolymerases, PHA depolymerases are grouped generally into four families: PHA depolymerases degrading the native intracellular granules (i-PHA_{MCL} depolymerases and i-PHA_{SCL} (i-PHB) depolymerases) and PHA depolymerases degrading the denatured extracellular PHA granules (e-PHA_{MCL} depolymerases and e-PHA_{SCL} (e-PHB) depolymerases). To date, the genes of about 30 PHA depolymerases with experimentally verified PHA depolymerase activity have been identified. On the basis of their sequence similarity, the PHA Depolymerase Engineering Database has been established as a tool for systematic analysis of PHA depolymerase family [66].

Among the PHA depolymerases, multi-domain e-PHB depolymerases have been extensively examined [9]. The multi-domain e-PHB depolymerases generally have a domain structure consisting of a catalytic domain (CD) at N-terminus, a substrate-binding domain (SBD) at C-terminus, and a linker region connecting the two domains, while e-PHB depolymerases from *Penicillium funiculosum* ($PhaZ_{Pfu}$) and PhaZ7 from *Paucimonas lemoignei* ($PhaZ7_{Ple}$) have emerged as two exceptions (single-domain e-PHB depolymerases) [9, 67-69]. Genetic analysis also shows that e-PHB depolymerases contain a lipase box pentapeptide [Gly-X_1-Ser-X_2-Gly] as an active residue, indicating that these enzymes are one of the serine hydrolases. As an example, the domain structure of e-PHB depolymerase from *Ralstonia pickettii* T1 ($PhaZ_{RpiT1}$) is illustrated in Figure 4(A). Such domain structure has been found in many biopolymer-degrading enzymes, such as cellulase, xylanase, and chitinase, which are capable of hydrolyzing water-insoluble polysaccharides [70-73]. The enzymatic degradation of PHB by the multi-domain e-PHB depolymerases is considered to proceed via a two-step reaction at the solid-liquid interface, as shown in Figure 4(B). The e-PHB depolymerase approaches and adheres to the PHB surface via SBD, followed by hydrolysis of the polymer chain by CD. Accordingly, it is considered that elucidation of the mechanisms of enzyme adsorption and enzymatic hydrolysis will contribute to the development of new PHB polymer materials with the desired environmental stability and biodegradability as well as the development of improved e-PHB depolymerases that can be used to effectively recycle PHB materials.

From a biological viewpoint, the structure-function relationship of maluti-domain e-PHB depolymerases has been studied extensively, and several mutants were designed to analyze the function of each domain, in particular, SBD. Using a truncated multi-domain e-PHB depolymerases, Behrends et al., Nojiri and Saito, and our group revealed that the C-terminal domain is essential for PHB-specific binding [74-76]. Further, Nojiri and Saito genetically prepared many mutants of $PhaZ_{RpiT1}$ in various forms such as inversions, chimeras, and fusion

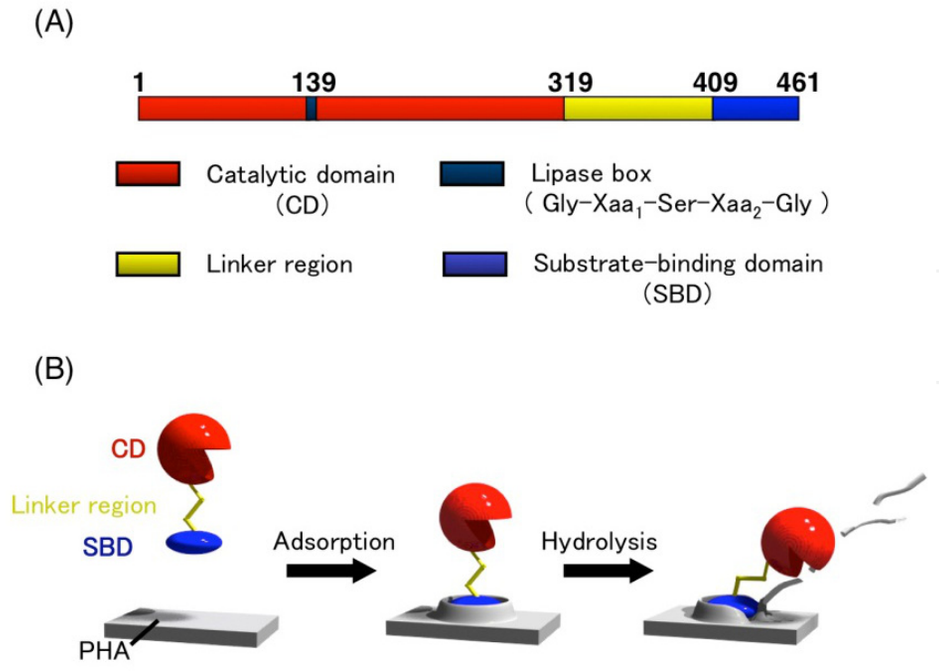

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Figure 4. A) Domain structure of e-PHB depolymerase from *Ralstonia pickettii* T1 (PhaZ*RpiT1*). (B) Schematic illustration of the enzymatic degradation of PHA by e-PHB depolymerase.

to extra linker domains, and demonstrated that its SBD organization also influences the PHB degradation but not water-soluble substrates. Doi and co-workers prepared fusion proteins of SBDs of several PHB depolymerases with glutathione-S-transferase [77-81], and demonstrated specific interactions based on molecular recognition between SBD and polyester surface.

3.2. Effects of chemical and solid-state structures and surface properties of PHAs on enzymatic degradation

Chemical structures of PHAs have influence on their enzymatic hydrolysis by multi-domain e-PHB depolymerases. Various types of PHAs including racemic PHA [82-89] and 3HA oligomers [90, 91], PHAs with different main- and side-chain lengths (Kasuya et al., 1997) [77], and random copolymers of (*R*)-3HB with various hydroxyalkanoate units [92-95] have been synthesized to examine their enzymatic degradation by a variety of e-PHB depolymerases. For instance, Abe et al. proposed a schematic model of the enzymatic cleavage of the PHA chain by $PhaZ_{RpiT1}$ (Figure 5), in which its active site can recognize at least three neighboring monomer units with a certain degree of difference in main-chain length [93]. Besides the chemical structure, the solid-state structure and surface properties of PHAs also influence the enzymatic hydrolysis. For example, the amorphous regions in PHA materials are preferentially hydrolyzed, followed by the hydrolysis of crystalline regions as a rate-limiting step in the enzymatic degradation process [96, 97]. Further, the enzymatic degradation rate of PHA materials decreases with increasing crystallinity, crystal size, and regularity of the chain

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packing state. In addition, Abe and co-workers demonstrated using proteinase K that the change in the surface properties of PLA film induced by end-capping with alkyl ester groups (carbon numbers 12 to 14) leads to a decrease in their enzymatic degradation rates [98, 99].

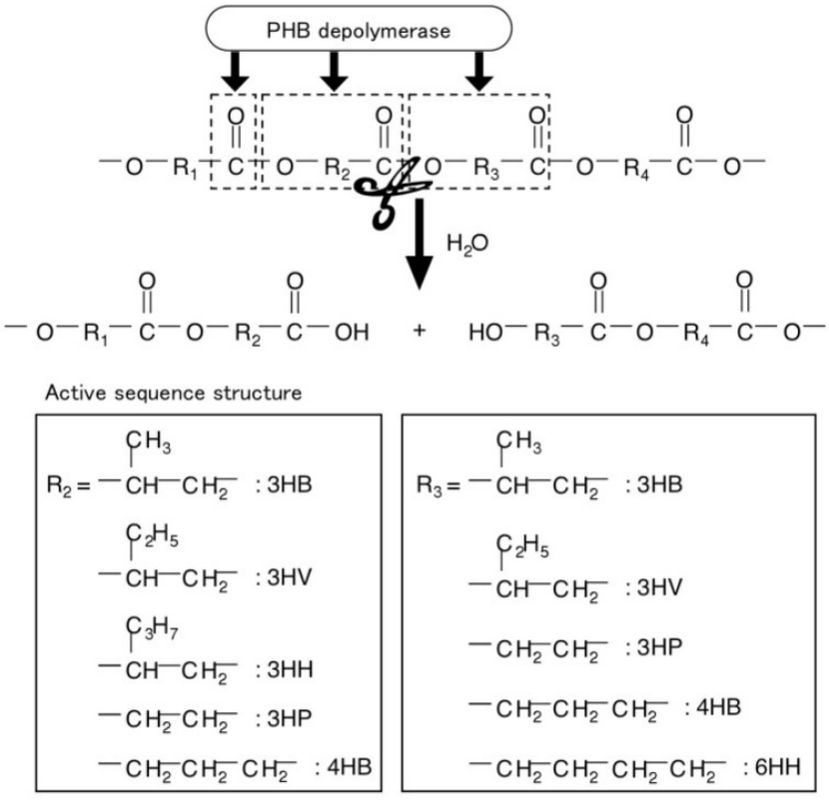

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Figure 5. Schematic model of enzymatic cleavage of an ester bond in various sequences by PHB depolymerase.

To investigate the influence of the chemical structure or surface properties of polymer on enzymatic adsorption at nano-level sensitivity, several studies using quartz crystal microbalance (QCM) and atomic force microscopy (AFM) have been performed. Yamashita et al. investigated the $PhaZ_{RpiT1}$ adsorption to the film surface of several polymers including polyethylene, polystyrene and PHA using the QCM technique, and found that the enzyme showed adsorption specificity for PHA [100-102]. In addition, AFM analysis of $PhaZ_{RpiT1}$ on polyester surface has revealed that small ridges are formed around the enzyme molecule due to movement of some polyester chains at the adsorption area, suggesting that a strong chemical interaction exists between the enzyme and the polyester chains [102, 103]. Furthermore, AFM analysis of interaction between PHB single crystal and a hydrolytic-activity-disrupted $PhaZ_{RpiT1}$ mutant has demonstrated that its SBD disturbs the molecular packing of PHB polymer chains, resulting in fragmentation of the PHB single crystal [104]. Taking these findings into consideration, the specific adsorption of PHB depolymerase to the PHB surface probably involves both the adsorption of the enzyme to the surface and the non-hydrolytic

disruption of the substrate to promote PHB degradation. Recently, we have developed the AFM technique by using an AFM tip modified with SBD protein to evaluate the interaction between the SBD molecule and the PHB surface at the molecular level. Through this, it has been shown that the adsorption force of one SBD molecule to the PHB surface is approximately 100 pN [105, 106].

3.3. Analysis of polymer binding ability of e-PHB depolymerase using directed evolution technique

The structural aspects of an enzyme generally provide crucial information about the interaction between the enzyme and its ligand. Some researchers have reported the tertiary structures of polymer-degrading enzymes, such as glycoside hydrolases and single-domain e-PHB depolymerases, and proposed an interaction model between the enzymes and the polymer surfaces [68, 107-109]. However, because of the paucity of information about the 3D structures of multi-domain e-PHB depolymerases, there are few insights into which and how amino acid residues in their SBD are involved in the enzyme adsorption to PHB surface.

Directed evolution is a useful and powerful tool to explore, manipulate, and optimize the properties of an enzyme as no information on the tertiary structure of the enzyme is required and new and unexpected beneficial mutations can be discovered [110-112]. Random mutagenesis via error-prone PCR (epPCR) and DNA recombination are widely used approaches to generate a large mutant pool and screen for the desired characteristics [113, 114]. Using those approaches, many enzymes with improved substrate specificity, catalytic activity, thermostability, or solubility were obtained [115]. Further, analysis of the effects of mutations could also provide useful information for the improvement of enzyme function.

To improve e-PHB depolymerases, it is important to understand the mechanisms underlying its adsorption and hydrolysis, such as which and how amino acid residues participate in the catalytic process. To clarify this issue, we have investigated the interaction between $PhaZ_{RpiT1}$ and PHB surface by a combination of PCR random mutagenesis targeted to only SBD and an *in vivo* screening system as shown in Figure 6(A) [116]. In the analysis of recombinants showing low PHB-degrading activity, Ser410, Tyr412, Val415, Tyr428, Ser432, Leu441, Tyr443, Ser445, Ala448, Tyr455, and Val457 were replaced with other residues having hydropathy indices opposite to theirs at high frequency (Figure 6(B)). The results suggested that $PhaZ_{RpiT1}$ adsorbs to the PHB surface not only via the formation of hydrogen bonds between hydroxyl groups of Ser at these positions of the enzyme and carbonyl groups in the PHB polymer, but also via the hydrophobic interaction between hydrophobic residues at above-mentioned positions and methyl groups in the PHB polymer.

Nevertheless, because only little knowledge was obtained on the biochemistry and kinetics of the purified mutant enzymes, the roles of these amino acids (Ser410, Tyr412, Val415, Tyr428, Ser432, Leu441, Tyr443, Ser445, Ala448, Tyr455, and Val457) and their contributions to the enzymatic activity remain poorly understood, resulting in little information to develop e-PHB depolymerases. Among these positions, Leu441, Tyr443, and Ser445 were predicted to form a β-sheet structure to orient in the same direction as shown in Figure 6(B). As polymer-degrading enzymes generally align their amino acid residues in a plane to interact with polymer surfaces,

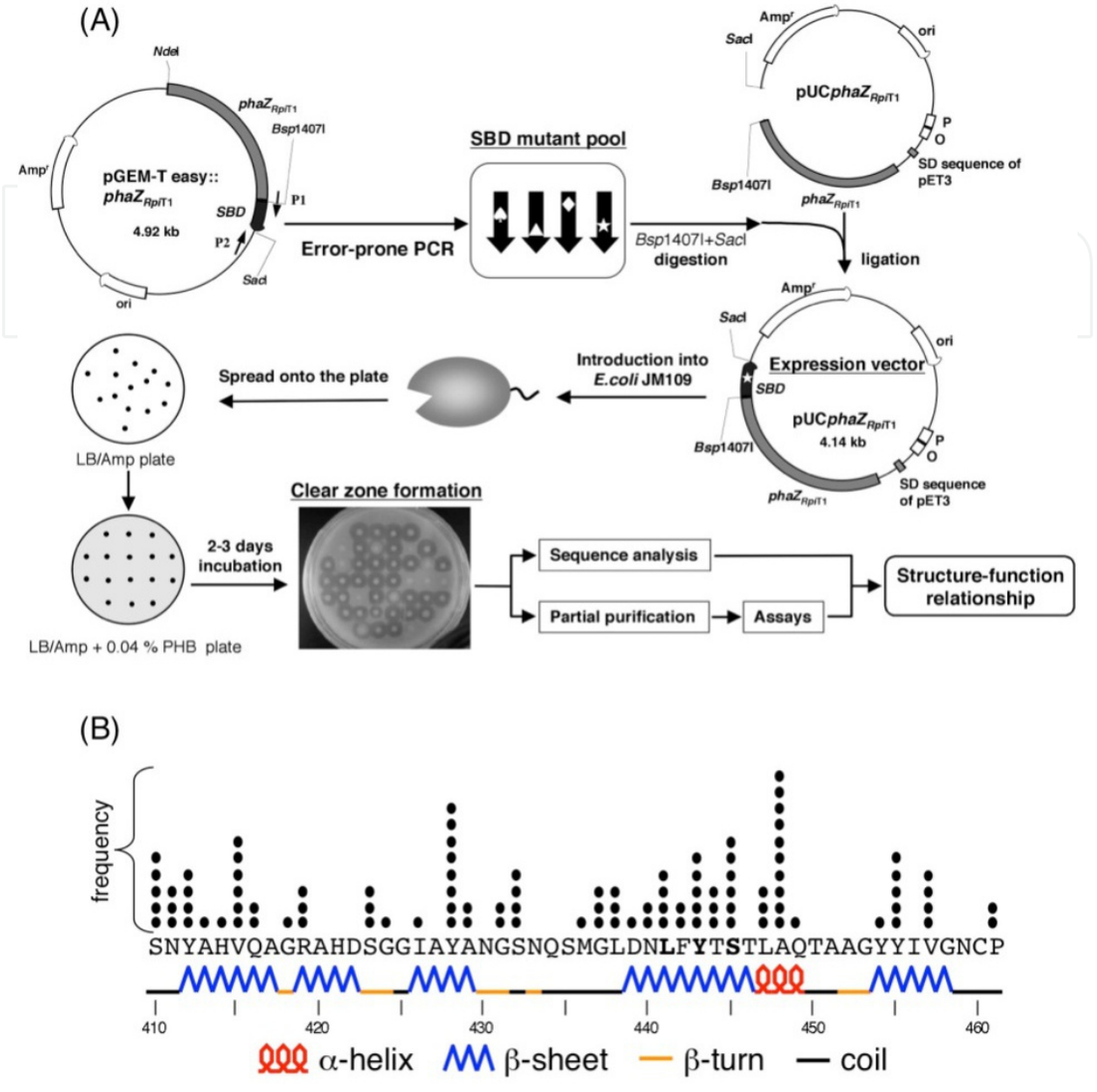

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Figure 6. A) *In vivo* assay system for assessment of mutational effects of the substrate-binding domain of $PhaZ_{RpiT1}$ on PHB degradation. Schematic flow diagram of the system is illustrated. This system is composed of PCR-mediated random mutagenesis in the substrate-binding domain region of $PhaZ_{RpiT1}$ gene, preparation of mutant library, primary plate assay of PHB degradation (clear-zone formation), nucleotide sequencing and PHB degrading and adsorbing assays of partially purified mutant enzymes. (B) Positions and frequencies of PCR-mediated single mutations in the region coding for SBD of $PhaZ_{RpiT1}$, together with its predicted secondary structure.

these three residues in $PhaZ_{RpiT1}$ may interact directly with the PHB surface. Since the hydropathy indices of such mutations as L441H (replacement of Leu441 with His), Y443H (replacement of Tyr443 with His), and S445C (replacement of Ser445 with Cys) dramatically changed among the mutations at these positions, their PHB-binding and -degrading properties were examined in detail [117]. Functional analyses of the purified L441H, Y443H, and S445C enzymes indicated that these mutations had no influence on their structures and their ability

to cleave the ester bond, while their PHB-degrading activity differed from that of the wild type. Kinetic analysis of PHB degradation by the mutants suggested that the hydrophobic residues at these positions are important for the enzyme adsorption to the PHB surface, and may more effectively disrupt the PHB surface to enhance the hydrolysis of PHB polymer chains than the wild-type enzyme. Further, surface plasmon resonance (SPR) analysis revealed that these substitutions mentioned above altered the association phase rather than the dissociation phase in the enzyme adsorption to the polymer surface.

Recently, Hisano et al. determined the crystal structure of $PhaZ_{Pfu}$ and proposed that hydrophobic residues, including Tyr, Leu, Ile, and Val, contribute to adsorption to the PHB surface, and that hydrophilic residues (Ser and Asn) located around the mouth of the enzyme crevice may also contribute to the affinity of the enzyme for PHB [68]. Jendrossek group determined $PhaZ7_{Ple}$ crystal structure and demonstrated that the enzyme was enriched in hydrophobic amino acids including eight tyrosine residues [108]. All tyrosine residues (Tyr103, Tyr105, Tyr172, Tyr173, Tyr189, Tyr190, Tyr203, and Tyr204), which are located at the surface of $PhaZ7_{Ple}$ but are far from the active site (Ser136), were changed to alanine or serine and the substitution effects were examined [118]. It turned out that mutation of Tyr105, Tyr189 or Tyr190 resulted in reduced PHB-degrading activity and in occurrence of a lag phase of the depolymerase reaction, indicating that these residues are possibly involved in the enzyme adsorption. Similar results have been obtained for the e-PHA_{MCL} depolymerase of *Pseudomonas fluorescens* GK13 by Jendrossek et al. [119]. They reported that several hydrophobic amino acids (Leu15, Val20, Ile26, Phe50, Phe63, Tyr143 and Val198) were identified to be involved in interaction between the enzyme and poly(3-hydroxyoctanoate) substrate surface. This finding was supported with the recent study by Ihssen et al. (2009) [120].

3.4. Improvement in SBD function of $PhaZ_{RpiT1}$

The above-mentioned findings imply that PHB binding ability of $PhaZ_{RpiT1}$ can be improved by substituting a hydrophilic residue with a hydrophobic one at the positions of 441, 443 and 445. Tyr at position 443 was targeted for substitution with a more highly hydrophobic amino acid residue because its hydrophobicity shows medium to high degree compared to those of general naturally occurring amino acid residues [121].

Table 2 shows the hydrophobicity, the potential for β-sheet formation, and the volume of 20 common amino acid residues [122-124]. In this table, the properties of the original amino acid residue are colored blue and the desirable characteristics of the amino acid residues are colored orange, respectively. In the design of a mutant enzyme with an amino acid substitution at this position, the following factors were taken into consideration: (1) to achieve higher hydrophobicity than the original residue, (2) to retain the β-sheet structure, and (3) to change as little as possible the volume of the amino acid residue after the substitution. As a result, the substitution of Tyr443 with Phe (Y443F) was considered to be appropriate. Analysis of the purified Y443F enzyme indicated that the mutation had no influence on the structure and the ester bond cleavage activity, while this mutant had higher PHB degradation activity than the wild type. Thus, this finding supports our previous assumption and indicates the importance of highly hydrophobic residues at these positions for PHB degradation.

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amino acid residue	hydrophobicity[a]	Pb[b]	volume[c]
Ile	4.5	1.60h	100.1
Val	4.2	1.70h	83.9
Leu	3.8	1.30h	100.1
Phe	2.8	1.38h	113.9
Cys	2.5	1.19h	65.1
Met	1.9	1.05h	97.7
Ala	1.8	0.83i	53.2
Gly	-0.4	0.75b	36.1
Thr	-0.7	1.19h	69.7
Ser	-0.8	0.75b	53.4
Trp	-0.9	1.37h	136.7
Tyr(wild type)	-1.3	1.47h	116.2
Pro	-1.6	0.55b	73.6
His	-3.2	0.87i	91.9
Asn	-3.5	0.89i	70.6
Gln	-3.5	1.10h	86.3
Asp	-3.5	0.54b	66.7
Glu	-3.5	0.37b	83.0
Lys	-3.9	0.74b	101.1
Arg	-4.5	0.93i	104.1

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[a] J. Kyte and R. F. Doolittle, 1982 [122].

[b] P. Y. Chou and G. D. Fasman, 1978 [123]. Pb: potential for β-sheet formation ; h: former ; i: indifferent ; b: breaker.

[c] A. A. Zamyatnin, 1972 [124].

Table 2. Hydrophobicity, potential for β-sheet formation, and volume of amino acid residues

3.5. Cell surface display system for protein engineering of $PhaZ_{RpiT1}$

Cell surface display is a valuable technique for the expression of peptides or proteins on the surface of bacteria and yeasts by fusion with the appropriate anchoring motifs [125]. Therefore, the cell surface display of functional and useful peptides and proteins, such as enzymes, receptors, and antigens, has become an increasingly used strategy in various applications, including whole-cell biocatalysts and bioabsorbents, live vaccine development, antibody production, and peptide library screening. In addition, this method is very useful for enzyme

library screening because the displayed protein is accessible to the external environment and thus, is able to interact with substrates easily, allowing the screening of large libraries [126].

A variety of surface anchoring motifs, including outer membrane proteins, lipoproteins, autotransporters, subunits of surface appendages, and S-layer proteins, have been employed to achieve the display systems [125, 127, 128]. We used the OprI anchoring motif for the functional display of $PhaZ_{RpiT1}$ on *Escherichia coli* cell surface [129]. The displayed enzyme retained its intrinsic characteristics, that is, hydrolytic activity for *p*-nitrophenyl butyrate (pNPC4) and the ability to adsorb to and degrade PHB, indicating that the engineered *E. coli* can be used in the form of a whole-cell biocatalyst by overcoming the uptake limitation of such substrates as insoluble PHB. These findings also indicate that the whole-cell catalyst is a promising and suitable tool to screen for mutant $PhaZ_{RpiT1}$ with enhanced catalytic activity.

3.6. Protein engineering of CD region of $PhaZ_{RpiT1}$ using cell surface display system

In contrast to SBD, there is little knowledge on the CD of $PhaZ_{RpiT1}$, such that which and how amino acid residues in the CD contribute to the enzymatic activity remain poorly understood, and this has resulted in the lack of information for the improvement of the CD function of $PhaZ_{RpiT1}$. The CD of $PhaZ_{RpiT1}$ was targeted for the directed evolution, employing random mutagenesis and DNA recombination to enhance its ester bond cleavage ability (Figure 7) [130]. The mutant genes generated from these reactions were expressed as surface-displayed enzymes, and the mutant enzymes were screened through a high-throughput system using pNPC4, a water-soluble substrate. As a result, clones displaying mutant enzymes with a 4- to 8-fold increase in pNPC4 hydrolysis activity were obtained in comparison with those displaying the wild type. This result was roughly consistent with the results of pNPC4 hydrolysis using purified enzymes with the unfused and undisplayed forms, concluding that the current screening system is feasible and effective for the search of improved enzymes.

As the aliphatic part in pNPC4 is similar to the monomer unit in PHB polymer chain and pNPC4 is generally used as a model substrate, changes in pNPCn hydrolysis rates by the purified mutant enzymes as a function of the chain length of the aliphatic part in *p*-nitrophenyl esters (pNPCn, n=2 to 6) can provide the information regarding the substrate recognition of the enzyme. The results of pNPCn hydrolysis by the mutants demonstrated that the elevation on their pNPCn hydrolysis activity for each pNPCn substrate occurred. DNA sequencing showed that eight improved mutant enzymes contained N285D or N285Y mutations. As beneficial mutations are accumulated and deleterious mutations are simultaneously removed from the improved mutants through DNA recombination procedures [131], the N285D and N285Y mutations found here are probably beneficial for pNPCn hydrolysis. Kinetic studies revealed that the increase in catalytic efficiency for pNPCn hydrolysis by the mutant enzymes is attributed to the high V_{max} values.

As opposed to pNPCn hydrolysis by the N285D and N285Y mutant enzymes, their PHB degradation rates were slower than that of the wild-type enzyme, indicating that these mutations are unfavorable for PHB degradation. The kinetics of PHB degradation demonstrated that the N285D and N285Y mutations lowered the hydrolysis activity for the PHB

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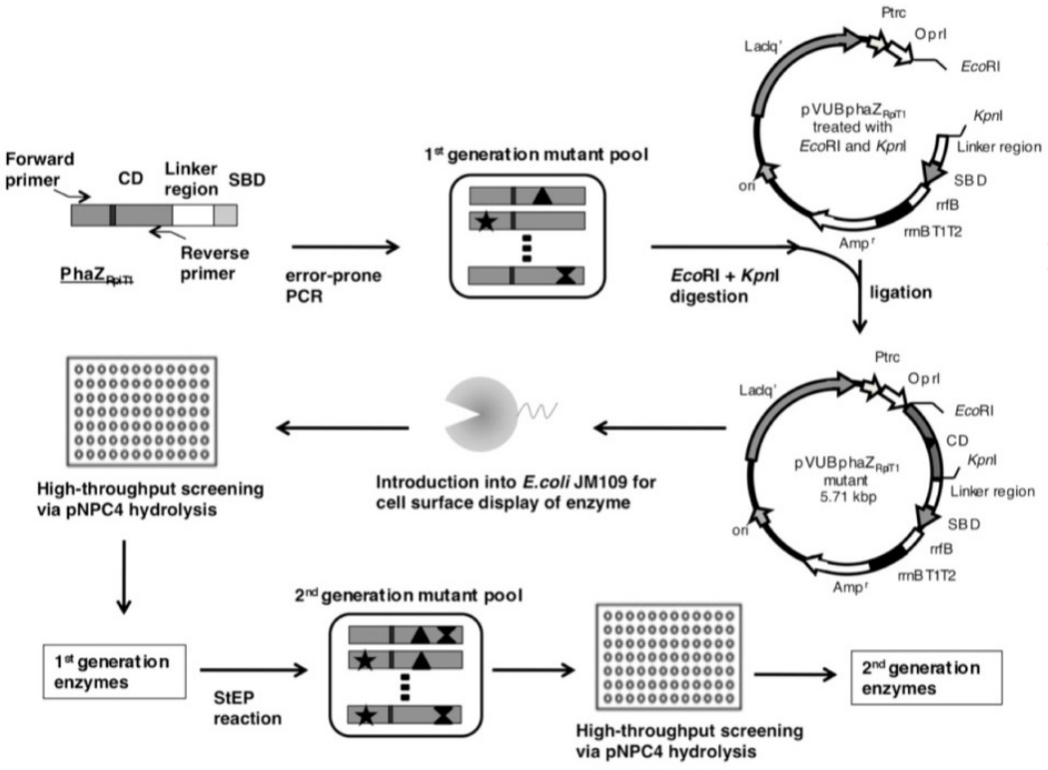

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Figure 7. Directed evolution targeted at the catalytic domain (CD) of $PhaZ_{RpiT1}$ using the *in vivo* screening system in the cell surface display system. A schematic diagram of the mutational effects analysis is illustrated. This system consists of random mutagenesis by error-prone PCR in the CD of $PhaZR_{piT1}$, cell surface display of enzyme, high-throughput microplate screening via *p*-nitrophenyl butyrate (pNPC4) hydrolysis, staggered extension process (StEP), and nucleotide sequencing.

polymer chain compared to the wild-type enzyme despite retention of the binding activity for the PHB polymer surface.

3.7. Proposed models of the active site in e-PHB depolymerases

The correct orientation of a PHB polymer chain to the active site is necessary to realize effective PHB degradation by e-PHB depolymerase. Hisano et al. have determined the crystal structures of $PhaZ_{Pfu}$-3HB trimer complex as well as $PhaZ_{Pfu}$ enzyme alone [68]. In the $PhaZ_{Pfu}$-3HB trimer complex, 3HB trimer binds to the crevice with its carbonyl terminus towards the catalytic residues (Figure 8(A)). From the structural insight gained from $PhaZ_{Pfu}$, they proposed the mechanism of action of $PhaZ_{Pfu}$. Figure 8(B) shows the location of the catalytic residues and the interaction between PHB polymer chain and the residues in the subsite of the active site of $PhaZ_{Pfu}$. In their model, Ser39 participates in the nucleophilic attack of the carbonyl carbon of a PHB chain, resulting in the formation of a covalent acyl-enzyme intermediate followed by the hydrolysis by an activated water molecule. The nucleophilicity of the hydroxyl group of Ser39 is enhanced by the His155-Asp121 hydrogen bonding system.

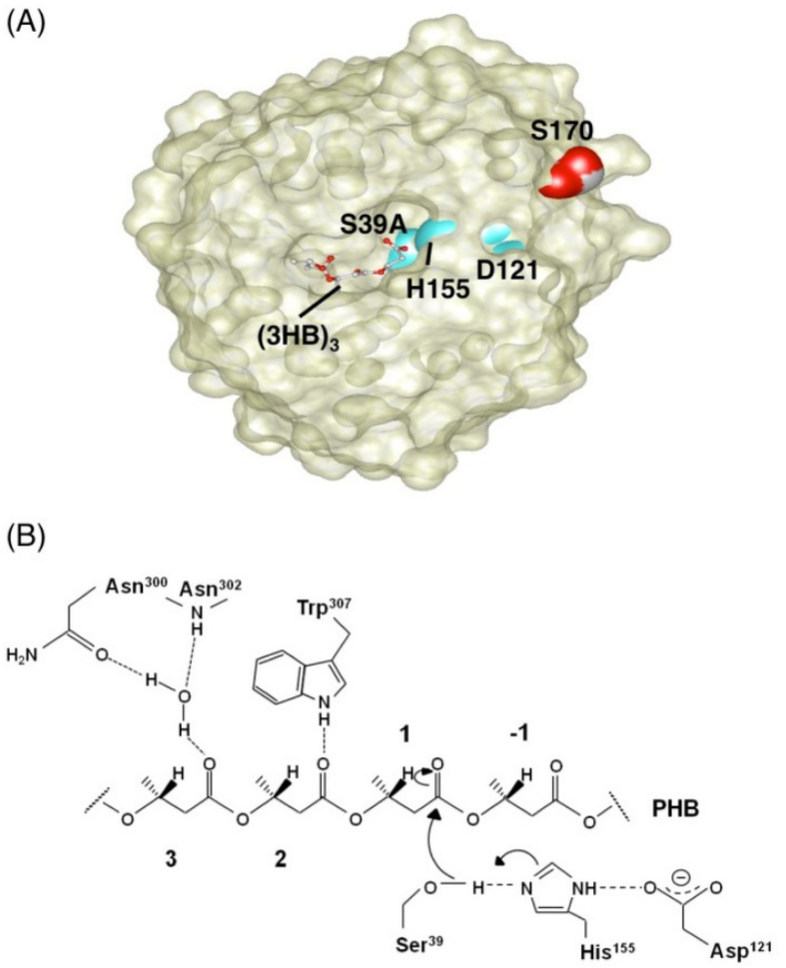

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Figure 8. A) Molecular surface representation of $PhaZ_{Pfu}$. 3HB trimer in the crevice is shown as a ball and stick model. The positions of catalytic triad residues (S39A, D121, and H155) (cyan), as well as residue S170 (color-coded according to molecular species) are indicated. (B) Proposed model of the active site in $PhaZ_{Pfu}$ by Hisano et al. (Hisano et al. 2006).

For $PhaZ_{RpiT1}$, Bachmann and Seebach proposed that this enzyme has four subsites (2, 1, -1, and -2) in its active site, in which three of the subsites must be occupied by (*R*)-3-hydroxybutyrate (3HB) units for cleavage to occur at the center of the active site [90]. Homology modeling of $PhaZ_{RpiT1}$ using the SWISS-MODEL program based on the crystal structure of $PhaZ_{Pfu}$ (PDB accession no. 2d81A) was performed to speculate the possible localization of Asn285 in the active site. Figure 9(A) shows the homology modeling structure of $PhaZ_{RpiT1}$, in which the modeled residue range was positioned from 124 to 294. The residue Asn285 (color-coded according to molecular species) of $PhaZ_{RpiT1}$ is located at the mouth of the crevice and also located immediately above His273, which corresponds to His155 in subsite -1 of $PhaZ_{Pfu}$. However, Asn285 was positioned as if to cover the subsite -1 and to inhibit the substrate access. Taking the homology modeling results and the aforementioned information on the cleavage mechanism into consideration, we propose a simple schematic model for $PhaZ_{RpiT1}$, as shown in Figure 9(B). In

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this model, Ser139 participates in the nucleophilic attack of the carbonyl carbon of a PHB chain, and its nucleophilicity is enhanced by the His273-Asp121 hydrogen bonding system. The residue Asn285 is positioned relatively close to His273 located in subsite -1 as if to cover the subsite. The location of Asn285 in the subsite probably leads to the regulation of the recognition of substrate molecules, such as pNPCn and PHB polymer chain, possibly indirectly via conformational change. A similar situation has been described in lipases and $PhaZ7_{Ple}$ where activation via conformational change is required to uncover the active site [108, 109].

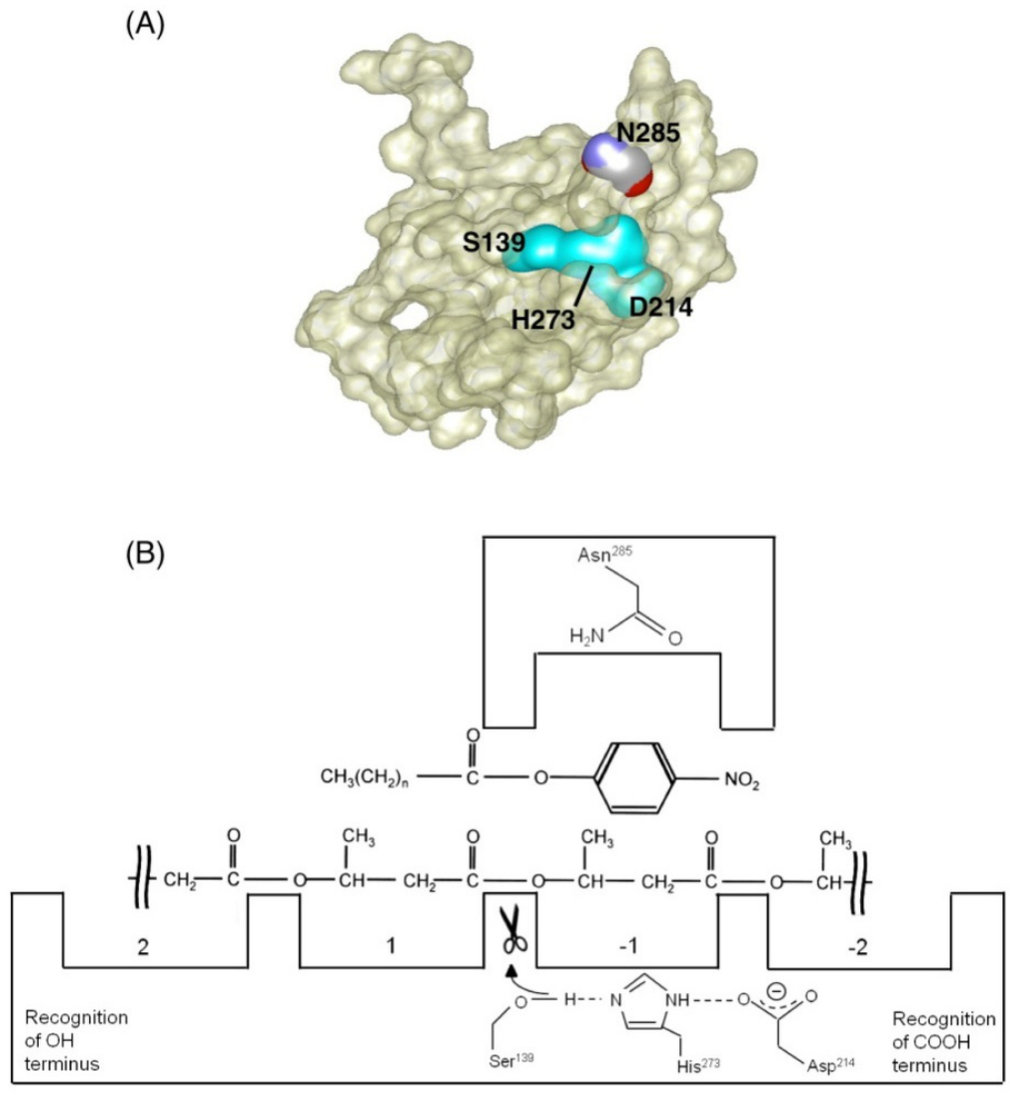

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Figure 9. A) Molecular surface representation of the homology model of $PhaZ_{RpiT1}$. The positions of catalytic triad residues (S139, D214, and H273) (cyan), as well as residue N285 (color-coded according to molecular species) are indicated. (B) Newly proposed schematic model of the active site in the CD of $PhaZ_{RpiT1}$.

4. Conclusion

This review describes the development of PHA synthases to synthesize the wide variety of custom-made bioplastics as well as PHB depolymerase with higher activity for PHB adsorption or pNPCn hydrolysis.

Bioplastics present a multitude of benefits as substitutes for conventional petroleum-based plastics. Among them, PHAs are one of the desirable alternatives to petrochemical-derived polymers because PHAs are produced directly from renewable resources completely by biological process and can be renewed over a relatively-short time. However, three main issues have hindered widespread use: the high production cost compared to petroleum-based polymers with similar properties; the inability to produce high-performance PHA polymers in substantial amounts; and the difficulty in controlling the life cycle of PHA polymers, i.e., the control of their biodegradability and their efficient recycling. Thus, with the development in recombinant DNA technology and high-throughput screening techniques, protein engineering methods and applications on the improvement of processes of bioplastic production as well as bioplastic degradation are becoming increasingly important and widespread.

The enzyme modification by protein engineering is an increasingly important scientific field. The well-known methods of rational design and directed evolution as well as new techniques including computational design, catalytic antibodies and mRNA display will be crucial for de novo design of enzymes. With recent advances in recombinant DNA technology tools including omics technologies and high-throughput screening facilities, improved methods for protein engineering will be available for easy modification or improvement of more enzymes for further specific applications.

Against such backgrounds, directed evolution of enzymes involved in PHA biosynthesis as well as metabolic engineering approaches of bacterial hosts will become the driving force to establish bioprocesses for the controlled production of PHAs with desired monomer compositions. In addition, systems-level analysis of metabolic, signaling, and regulatory networks is also making it possible to comprehensively understand global biological processes taking place in PHA-accumulating strains. The resultant knowledge will provide new targets and strategies for the improvement of PHA production, including tailor-made PHAs with desired monomer compositions and molecular masses.

Furthermore, from the viewpoint of preserving the ecosystem, bioplastics are most beneficial when they can be actually biodegraded. In order to achieve it, it is vital to elucidate the biodegradation mechanism of bioplastics and engineer their depolymerases. By contrast to PHA synthases, there have been very few protein engineering studies of PHA depolymerases using directed evolutionally methods, resulting in the less information about the improvement of PHA biodegradability as well as PHA depolymerases so far. In addition, as one of the recent trends in green polymer chemistry, *in vitro* bioplastic synthesis using isolated bioplastic-degrading enzymes has been developed because of the close relationship between the substrate specificities of the enzymes for polymer degradation and polymer synthesis. *In vitro* enzymatic polymerization offers many advantages, including easier control of polymer structure and

monomer reactivity than conventional chemical methods. Accordingly, novel bioplastic-degrading enzymes evolved by protein engineering are expected to become useful biocatalysts for the bioplastic production in the future.

Here, we present the recent approaches of protein engineering with potential for a total recycle system of bioplastics via combination of biological production with biological degradation. In the future, custom-made prominent enzymes generated via evolutionary engineering will be utilized extensively to create high-performance bioplastics from renewable resources in various organisms and applied to effective and eco-friendly chemical recycling of bioplastics.

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