Structural insight into molecular mechanism of poly (ethylene terephthalate) degradation

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Plastics, including poly(ethylene terephthalate) (PET), possess many desirable characteristics and thus are widely used in daily life. However, non-biodegradability, once thought to be an advantage offered by plastics, is causing major environmental problem. Recently, a PET-degrading bacterium, *Ideonella sakaiensis*, was identified and suggested for possible use in degradation and/or recycling of PET. However, the molecular mechanism of PET degradation is not known. Here we report the crystal structure of *I. sakaiensis* PETase (IsPETase) at 1.5 Å resolution. IsPETase has a Ser–His–Asp catalytic triad at its active site and contains an optimal substrate binding site to accommodate four monohydroxyethyl terephthalate (MHET) moieties of PET. Based on structural and site-directed mutagenesis experiments, the detailed process of PET degradation into MHET, terephthalic acid, and ethylene glycol is suggested. Moreover, other PETase candidates potentially having high PET-degrading activities are suggested based on phylogenetic tree analysis of 69 PETase-like proteins.
Plastics are essential materials in our lives due to their desirable properties, such as lightness, durability, low price, easy processibility into many different forms, and non-degradability. However, non-degradability, which had been considered to be a great advantage of employing plastics, has been reconsidered as a major cause of environmental problems, in particular due to the accumulation of waste plastics in landfill and ocean. Plastics production has continuously increased and about 320 million tons of plastics were produced globally in 2015. Because most plastics are resistant to biodegradation and require a long time to degrade, the amount of plastic wastes to be accumulated is expected to reach 33 billion tons by 2050. Therefore, much effort has been exerted to reduce plastic wastes. To remove plastic wastes and recycle plastic-based materials, several chemical degradation methods such as glycolsysis, methanolysis, hydrolysis, aminolysis and ammonolysis have been developed. However, these methods generally require high temperature and often generate additional environmental pollutants. Alternatively, biocatalytic degradation might be applied as an ecofriendly method. Microbes can degrade plastics with ester bonds via enzymatic hydrolysis through colonization onto the surfaces of materials. The degree of biodegradability of plastics depends on their chemical and physical properties.

Poly(ethylene terephthalate) (PET) is an extensively and widely used polyester and is also resistant to biodegradation. According to a report by the US National Park Service, PET bottles require approximately 450 years to be decomposed. PET comprises terephthalamide (TPA) and ethylene glycol (EG), which are polymerized through ester linkage. Various bacterial hydrolases, such as cutinases, lipases, carboxylesterases, and esterases, have been shown to degrade PET, although to different extents. Among the PET-degrading enzymes, such as cutinases, lipases, carboxylesterases, and esterases, have been shown to degrade PET, although to different extents. Among the PET-degrading enzymes identified to date, TJH and TJH BTA-2 from Thermobifida fusca DSM43793, TJCut1 and TJCut2 from T. fusca KW3, LC cutinase from the metagenome in plant compost, cutinase from Saccharomonomas viridis AHK190, HIC from Thermomyces insolens, and lipase B from Candida antarctica have been shown to possess relatively higher degradability. However, the degradation activities are still too low for industrial applications.

To enhance enzymatic activity, several strategies have been adopted. Through site-directed mutagenesis of the active site, cutinases exhibit higher hydrolysis activity. Moreover, the introduction of Ca2+ or Mg2+ ions to esterases improves the thermal stability of the enzymes, leading to enhanced PET degradability. Recently, a dual enzyme system consisting of TJCut2 from T. fusca KW3 and LC cutinase or lipase from C. antarctica and cutinase from Humicola insolens was found to have synergistic effects. Despite these attempts, the PET degradation activity still remains low.

Recently, a new bacterial species, Ideonella sakaiensis, which can use PET as a carbon source, was isolated. The PETase of I. sakaiensis (IsPETase) can degrade PET at a moderate temperature (30 °C) and has relatively higher activity than other PET-degrading enzymes, such as cutinases and lipases. In addition, IsPETase showed higher specificity for PET. The superior capability of IsPETase for PET degradation has been receiving much attention. However, the detailed enzyme mechanism has not been elucidated, hampering further studies. Here we report the crystal structure and key structural features of IsPETase. While we determined the crystal structure (Protein Data Bank accession code, 5XJH) and this manuscript was under revision, another group independently determined the crystal structure of IsPETase (Protein Data Bank accession code, 5XG0). In this study, based on structural and biochemical studies of IsPETase, we propose the detailed molecular mechanism of IsPETase, so far the most efficient and more specific PET degrading enzyme, compared with other PET-degrading enzymes. In addition, we constructed the IsPETase variant with enhanced PET-degrading activity by structural-based protein engineering.

**Results**

**Overall structure of IsPETase.** For structural determination of IsPETase, the signal peptide sequences (Met1-Ala33) were removed for the production of the core domain of the protein. The recombinant IsPETase protein had additional amino-acid residues at both N and C-termini (Met13-Met33 and Leu291–Gln312), due to the use of the pET15b vector. To elucidate the molecular mechanisms of IsPETase, its crystal structure was determined at 1.5 Å resolution (Fig. 1 and Supplementary Fig. 1). The structure reported here comprises residues Ser31–Gln292 visible in the electron density map. The refined structure was in good agreement with the X-ray crystallographic statistics for bond angles, bond lengths, and other geometric parameters (Supplementary Table 1). The asymmetric unit in the P2_12_1 space group contains one molecule of IsPETase, indicating that IsPETase exists as a monomer. The size-exclusion chromatography experiment also confirms that IsPETase functions as a monomer (Supplementary Fig. 2). The IsPETase structure belongs to the α/β hydrolase superfamily, and the central twisted β-sheet is formed by nine mixed β-strands (β1–β9) and surrounded by seven α-helices (α1–α7; Fig. 1b). As observed in other α/β hydrolase superfamily proteins such as lipases and esterases, IsPETase contained the conserved serine hydrolase Gly–x1–Ser–x2–Gly motif (Gly158–Trp159–Ser160–Met161–Gly162) located at the active site (Fig. 1a).

**Active site of IsPETase.** IsPETase has been shown to degrade PET into monomers such as bis(2-hydroxyethyl) terephthalate (BHET), mono(2-hydroxyethyl) terephthalate (MHET), and TPA (Supplementary Fig. 3). IsPETase also hydrolyzes BHET, which is a commercial monomer having similarity with the core structure of PET and has been widely used for studying PET. BHET is hydrolyzed by IsPETase to MHET with no further decomposition. In order to elucidate the substrate binding mode of IsPETase, we first attempted to determine its structure in complex with BHET. However, neither co-crystallization nor soaking of BHET into the IsPETase crystal was successful, potentially because we could not use high concentration of BHET in co-crystallization and soaking due to its low solubility. Alternatively, we speculated the substrate binding mode of the enzyme by covalent docking calculation using 2-hydroxyethyl-(mono-hydroxyethyl terephthalate), 2-HE(MHET)α, a four-MHET molecule mimicking PET (Supplementary Fig. 3). At the active site of IsPETase, three residues Ser160, His237, and Asp206 form a catalytic triad and Ser160 is postulated to function as a covalent nucleophile to the carbonyl carbon atom in the scissile ester bond, as in other carboxylesterases (Fig. 2a). Oxyanion of the tetrahydridal intermediate is stabilized by an oxyanion hole that consists of nitrogen atoms of Tyr87 and Met160 with distances of 2.90 and 2.83 Å, respectively (Fig. 2a). The substrate binding site is simulated to form a long, shallow L-shaped cleft on a flat surface with dimensions of approximately 25 and 29 Å (Fig. 2b–d). The surface of the substrate binding cleft is mainly hydrophobic and the length of the cleft is ~40 Å (Fig. 2b). Based on the scissile ester bond of 2-HE(MHET)α, the substrate binding site can be divided into two subsites, subsite I and subsite II, where one and three MHET moieties are bound, respectively (Fig.2b,e). For binding of the first MHET moiety in the subsite I, the benzene ring is positioned on a ravine between the two aromatic residues of Tyr87 and Trp185 (Fig. 2b, e). The π–π interactions between Trp185 and the benzene ring of the first MHET moiety with a
distance of ~3.6 Å seem to be a main contributor to the stabilization of the ligand (Fig. 2e). Met161 and Ile208 are also predicted to assist the binding of the first MHET by providing a hydrophobic surface at the bottom and the side of subsite I, respectively (Fig. 2e). Subsite II tends to form a longer and shallower cleft than subsite I and accommodates three MHET moieties (the second, third and fourth MHET moieties of 2-HE (MHET)₄) (Fig. 2b–d). Based on the binding of MHET, the subsite II is further divided into three parts, subsite IIa, IIb, and IIc (Fig. 2b, e). Subsite II is composed of residues including Thr88, Ala89, Trp159, Ile232, Asn233, Ser236, Ser238, Asn241, Asn244, Ser245, Asn246, and Arg280. Although the interaction between subsite II and three MHET moieties seems to be mainly mediated through hydrophobic interactions, carbonyl oxygen...
atoms in the fourth MHET moiety also form polar interactions with a main chain of Ser236 and a side chain of Asn246 in the subsite IIc (Fig. 2b, e). Arg280 is located at the end of subsite IIc and the residue seems to hinder the extension of the substrate binding site due to its positive charge and slightly protruding structure (Fig. 2b, c, e); this residue was further examined by protein engineering (see below).

In order to confirm the residues involved in enzymatic catalysis and substrate binding, site-directed mutagenesis experiments were conducted. First, three catalytic residues, Ser160, Asp206 and His237 forming a catalytic triad were identified. These residues were shown to be involved in the oxyanion hole and the catalytic triad (Fig. 2a).

Site-directed mutagenesis experiments were conducted to further examine the role of Arg280 at the end of subsite IIc. The results showed that the negative charge of Arg280 hindered the extension of the substrate binding site, which was confirmed by protein engineering experiments (see below).

Fig. 2 Active site of IsPETase. a Catalytic triad and the docking model of the reaction intermediate of 2-HE(MHET)₄ in IsPETase. The three residues of Ser160, Asp206 and His237 forming a catalytic triad are shown as cyan-colored sticks and labeled appropriately. The distances of the interaction involved in the oxyanion hole and the catalytic triad are also shown. b Substrate binding site of IsPETase. The IsPETase structure is presented as an electrostatic potential surface model. The 2-HE(MHET)₄ docking model is shown as an orange-colored stick, and the cleavage site is highlighted as a red box. Subsites I, IIa, IIb, and IIc of the substrate binding site are indicated with red, green, cyan, and magenta-colored dotted circles, respectively. Arg280 residue at the end of subsite IIc is also indicated. c, d Side views of the substrate binding mode of IsPETase in b. e Residues involved in the active site of IsPETase. The IsPETase structure is presented as a cartoon diagram with a gray color. Residues involved in binding of 2-HE(MHET)₄ are shown as a line model, and those constituting subsite I and subsite II are distinguished with colors of magenta and light-blue, respectively, and the ester bond that is cleaved by the enzyme is indicated with a star mark. The 2-HE(MHET)₄ docking model is shown as an orange-colored stick. The hydrogen bonds formed between the residues and the substrate are shown as red-colored lines.
and His237, were replaced with Ala, and hydrolytic activities were measured using BHET as a substrate. Three variants, IsPETaseS160A, IsPETaseD206A, and IsPETaseW185A, showed almost complete loss of the activity (Fig. 3a), indicating that these residues are involved in catalysis. Next, Tyr87, Trp185, Met161, and Ile208 residues, which constitute subsite I, were replaced with Ala. Two mutants, IsPETaseY87A and IsPETaseW185A, showed only 5% BHET hydrolytic activity compared with IsPETaseWT (Fig. 3a). This result indicates that abolishment of the π–π interactions of these residues with the benzene ring of the first MHET moiety severely decreases stabilization of the first MHET moiety. The IsPETaseW185A and IsPETaseS238A variants exhibited 52% and 46% activity, respectively, compared with IsPETaseWT (Fig. 3a). It indicates that these residues also contribute to the constitution of subsite I, although these residues are not as crucial as Tyr87 and Trp185 residues. We also replaced residues of Trp159, Ser238, and Asn241, which constitute subsite II, with Ala. IsPETaseW159A, and IsPETaseN241A showed only 8% and 18% BHET hydrolytic activities compared with IsPETaseWT (Fig. 3a), suggesting that these residues are crucial in the constitution of subsite II. However, IsPETaseS238A showed a similar level of BHET hydrolytic activity compared with IsPETaseWT (Fig. 3a). The result suggests that replacing of Ser238 with Ala does not seem to affect BHET hydrolytic activity of the enzyme. Next, the PETase activities of the above variants were measured using PET film as a substrate. Variants of the catalytic triad with Ala showed almost complete loss of enzyme activities, and those involved in constitution of the substrate binding site exhibited decreased PETase activities compared with IsPETaseWT (Fig. 3b).

As we described above, Arg280 at the end of subsite IIC is a polar residue and shows protruding shape, which seems to hinder stable binding of PET substrate beyond the fourth moiety (Fig. 2b, c, e). Based on these results, we predicted that the substitution of Arg280 into a small hydrophobic residue might allow more stable binding of longer substrate, subsequently leading to an increase in PETase activity. We replaced Arg280 with Ala, and measured both the BHET hydrolytic and the PETase activities. IsPETaseR280A showed similar activity on BHET hydrolysis compared with IsPETaseWT (Fig. 3a), and the result can be explained by the fact that Arg280 is located distal from the catalytic site and thus does not directly participate in substrate binding when BHET is used as a substrate. As hypothesized, IsPETaseR280A showed increased PETase activity by 22.4% in 18 h and 32.4% in 36 h, compared with IsPETaseWT, when PET film was used as a substrate (Fig. 3b). To investigate whether the replacement of Arg280 with Ala indeed changed the conformation of substrate binding site (subsite IIC) allowing longer substrate binding, we determined the structure of IsPETaseR280A at a 1.36 Å resolution (Supplementary Table 1). As expected, compared with IsPETaseWT, IsPETaseR280A showed an extended subsite IIC by providing hydrophobic and non-protruding cleft (Fig. 3c). It is interesting that the replacement of Arg280, located distal from the catalytic site with a distance of 22.8 Å, with Ala enhanced the enzymatic activity. This result could not be obtained without reliable docking calculation, which identified unique substrate binding characteristics of IsPETase.

**PET degradation mechanism by IsPETase.** Based on the structural observations and biochemical studies described above, we propose the following PET degradation process. To start PET degradation, the PETase secreted from the bacterium would first bind to the PET surface using its flat hydrophobic surface that has a substrate binding cleft (Fig. 2b–d). The PET degradation process can be divided into two steps, nick generation step and terminal digestion step. In the nick generation step, four MHET moieties are bound to each subsite (one MHET moiety to subsite I and three MHET moieties to subsite II) and the scissile ester bond seems to be positioned between subsite I and II near the catalytic Ser160 residue (Fig. 4a). Then, the cleavage of one ester bond causes the formation of a nick in PET, resulting in generation of two PET chains with different terminals: TPA-terminal released from subsite I and HE-terminal released from subsite II (Fig. 4a).

In the terminal digestion step, two PET chains having the HE- and the TPA-termini are digested into MHET monomers in somewhat different manners. For digestion of PET having the HE-terminal (HEP), the terminal MHET and the next three MHET moieties bind to subsite I and subsite II, respectively, and breakage of the ester bond results in the production of one MHET monomer and HEPET–(HEPET)_{n-1} (Fig. 4b). Subsequent digestion of HEPET–(HEPET)_{n-1} is expected to occur in a manner similar to that of the first cleavage process. Digestion of PET having the TPA-terminal (TPAPET) is also expected to occur through positioning of the terminal TPA and the next three MHET moieties at subsite I and subsite II, respectively (Fig. 4b). Cleavage of the ester bond seems to produce one TPA molecule and HEPET–(HEPET)_{n-1}, and this HEPET–(HEPET)_{n-1} undergoes subsequent cleavage as observed in the HEPET degradation process (Fig. 4b). Alternatively, HEPET and TPAPET can also be digested though binding of PET polymer chains and the enzyme in the reverse direction, although this type of digestion might be less efficient than the above digestion. In this case, one or two MHET moieties, instead of three MHET moieties, can bind to subsite II (Fig. 4b). These bindings can produce a variety of PET monomers and dimers such as 2-HE (MHET), (MHET)_{2}, MHET and BHET, which can be finally digested to MHET, TPA and EG (Fig. 4b). Continuous digestions of HEPET and TPAPET proceed in a combinatory manner, as described above, resulting in accumulation of four molecules, including MHET, TPA, BHET, and EG (Fig. 4b). BHET can be further degraded into MHET and EG, and finally, three molecules, MHET, TPA, and EG, accumulate due to PET degradation (Fig. 4b). In addition, it is worth to note that degradation of PET film by IsPETase accumulates significant amount of TPA (Fig. 2b), although IsPETase cannot hydrolyze MHET to TPA and EG. Based on the PET degradation process we propose here, it can be also concluded that accumulation of TPA from PET film degradation is mainly derived from terminal digestion step of TPAPET.

**Structural comparison with other PET degrading enzymes.** Structural comparison using the DALI server showed that the structure of IsPETase is quite similar to those of cutinases from *T. fusca* KW3 (TfCut2, PDB code 4CG1, Z-score 42.4), *S. viridis* (SvCut, PDB code 4WFJ, Z-score 42.3), and *Thermobifida alba* (TaCut, PDB code 3VIS, Z-score 42.1). These structural homologs have been identified to have a PET-degrading activity and share ~50% amino-acid identity with IsPETase. In order to provide a structural basis for why IsPETase shows much higher PETase activity than these other PET-degrading enzymes, we compared the structure of IsPETase with other three PET-degrading enzymes. As residues constituting the substrate binding site are almost conserved within these enzymes, the structure of IsPETase was compared with that of TfCut2, a representative cutinase studied for PET degradation. Three residues constituting the Ser–His–Asp catalytic triad are located at the same positions in TfCut2 (Fig. 5a), indicating that these enzymes catalyze PET degradation through the same catalytic mechanism. The residues constituting subsite I are also identical in both IsPETase and TfCut2. It suggests that the
binding mode of the first MHET moiety to subsite I is similar in both enzymes (Fig. 5a). However, significant structural differences were observed in the conformation of subsite II. In TfCut2, His169 and Phe249 residues are located at the corresponding positions of Trp159 and Ser238 in subsite II of IsPETase, respectively (Fig. 5a). To verify whether the residues, Trp159 and Ser238, play a crucial role in the high PET-degrading activity of IsPETase, these two residues were replaced with His and Phe,

![Graph](image)

**Fig. 3** PETase activity of the variants. **a** Hydrolytic activities of IsPETase and its variants using BHET as a substrate. PETase activities of IsPETase and its variants were measured using BHET concentration of 200 mg L\(^{-1}\) and enzyme concentration of 50 nM. The amount of produced MHET was monitored by HPLC analysis. The PETase activities of the IsPETase variants were compared with that of the wild-type. **b** PETase activity of IsPETase proteins using the PET film as a substrate. PET film degradation activity of IsPETase proteins were measured using enzyme concentration of 200 nM. The amount of produced MHET and TPA was monitored by HPLC analysis. The PETase activities of the IsPETase variants were compared with that of the wild-type. The IsPETase\(^{R280A}\) variant showing an increased activity is highlighted with a star mark. **c** Electrostatic potential surface presentation of IsPETase\(^{R280A}\) structure. The 2-HE(MHET)\(_4\) molecule is labeled. The Arg280 residue in IsPETase\(^{WT}\) and the Arg280Ala (R280A) change in IsPETase\(^{R280A}\) are indicated with dotted circles. Error bars represent the s.d. values obtained in duplicate experiments.
Fig. 4 Schematic diagram of PET degradation process. a Nick generation step. The TPA and EG moieties of the PET polymer are presented with orange-colored hexagons and purple-colored lines, respectively. IsPETase is shown with a dark-gray color diagram. Subsite I and subsite II of IsPETase are shown as an orange and green-colored diagrams, and labeled as I and II, respectively. The catalytic Ser160 residue is shown as a red-colored rectangle. The PETn polymers with the TPA-terminal and with the HE-terminal are labeled. The TPA moieties of these PETn polymers are labeled by numbers 1, 2, 3, 4, and 5 from each terminal. b Terminal digestion step. Each enzymatic reactions in terminal digestion step is shown as a diagram. The cleaved products from the enzymatic reactions are indicated with black-colored arrows. The light green-colored dotted lines indicate the paths the PET polymer products go for the next reaction. Six PET-related compounds, 2-HE(MHET)2, (MHET)2, MHET, TPA, BHET, and EG, that are cleaved products from terminal digestion step, are shown. The final degradation product (MHET, TPA and EG) are labeled in bold.

respectively, the corresponding residues in TjCut2. The IsPETaseW159H and the IsPETaseS238F variants showed dramatically decreased hydrolytic activities from both uses of BHET and PET as a substrate (Fig. 3a, b). The differences in these residues seem to make subsite IIA of TjCut2 narrower and deeper than that of IsPETase, resulting in reduced accessibiliy of the second MHET moiety to its binding site in TjCut2 (Fig. 5b, c). Furthermore, more striking structural difference was observed in the connecting loop of β8-α6 (Figs. 1a and 5a). Compared with TjCut2, IsPETase has an extended loop in the region with three extra-residues (Asn244, Ser245, and Asn246; Figs. 1a and 5a). Interestingly, the unique conformation of the extended loop in IsPETase allows the formation of subsite IIb and IIc by constituting a continuous cleft from subsite IIA (Fig. 5b, c). On the other hand, the conformation of the region in TjCut2 prevents the formation of subsite IIb and IIc by blocking the formation of the continuous cleft (Fig. 5b,c).

In addition to structural differences in subsite II, existence of two disulfide bonds is another important structural feature of IsPETase. In other PET-degrading enzymes, one disulfide bond is observed near the C-terminal; the disulfide bond is formed between Cys281 and Cys299 in TjCut2, between Cys287 and Cys302 in SvCut, and between Cys276 and Cys294 in TaCut (Fig. 5d). The disulfide bond is also conserved in IsPETase and formed between Cys273 and Cys289 (Fig. 5d). Since the disulfide bond is located at the opposite side of the active site, it can be assumed that the disulfide bond has no direct effect on the enzyme activity, but rather influences the structural stability of the enzyme. Interestingly, IsPETase has an additional disulfide bond between Cys203 and Cys239 in the vicinity of the active site (Fig. 5e). However, all other PET-degrading enzymes have Ala residues at the corresponding positions (Fig. 5f). Since internal disulfide bonds tend to increase the thermal stability of proteins, another IsPETase variant without the additional disulfide bond was generated to investigate how the disulfide bond affects the thermal stability of IsPETase. The Tm values of IsPETaseW/T and IsPETaseC203A/C239A variant were 46.8 and 33.6 °C, respectively (Supplementary Fig. 4), suggesting that the additional disulfide bond plays an important role in the thermal stability of IsPETase.

As expected, the PETase activity of IsPETaseC203A/C239A was dramatically decreased compared with that of IsPETaseW/T (Fig. 3a, b). The Tm of TjCut2 was measured to be 67.9 °C, which is much higher than that of IsPETaseW/T. The reason with high Tm value of TjCut2 is expected as T. fusca is a thermophilic bacterium, and its high thermal stability is due to other structural features of the protein even without the additional disulfide bond.

Phylogenetic tree analysis. Having understood the reasons for the much higher PETase activity of IsPETase compared with other known PET-degrading enzymes as described above, we became interested in comparatively analyzing all possible 69 PETase-like enzymes from phylogenetically diverse organisms. For this, a maximum-likelihood phylogenetic tree was constructed (Fig. 6a and Supplementary Fig. 5). PETase-like enzymes can be classified into two types, type I and type II. Fifty-seven enzymes, including TjCut2, belong to type I, and the remaining twelve enzymes including IsPETase belong to type II. Type II PET-degrading enzymes can be further classified into two subtypes, type IIA and type IIb. Among 12 type II enzymes, eight enzymes belong to type IIA and four enzymes including IsPETase belong to type IIb. In all 69 proteins, three residues constituting the catalytic triad, such as Ser, His, and Asp, are conserved (Fig. 6b), indicating that these enzymes have the same catalytic mechanism. The residues constituting subsite I are also highly conserved in all proteins, suggesting that the binding mode of the first MHET moiety to subsite I is quite similar among these proteins (Fig. 6b). However, there are major differences in key residues comprising subsite II and in the presence of additional disulfide bond depending on the type of enzyme. In type I PET-degrading enzymes, there is no additional disulfide bond and the extended loop found in IsPETase. Moreover, all of these enzymes possess His and Phe/Tyr residues at the corresponding positions of Trp159 and Ser238 in IsPETase, respectively (Fig. 6b). These structural features suggest that type I PET-degrading enzymes have much lower PET-degrading activity compared with...
Fig. 5 Structural comparison of PET-degrading enzymes.  

- **a** Superposition of the structures of IsPETase and TfCut2. Both structures of IsPETase and TfCut2 are shown as gray-colored cartoon model. The 2-HE(MHET)$_4$ docking model of IsPETase is shown as an orange-colored stick. Residues constituting subsite I and subsite II of IsPETase and TfCut2 are shown as a line models with cyan and magenta colors, respectively, and labeled. The unique residues observed in IsPETase, Trp159 and Ser238, are indicated with star marks. The extended loop of IsPETase distinguishable from TfCut2 is shown in light-blue color. 

- **b, c** Structural difference on subsite II of IsPETase (b) and TfCut2 (c). The structures of IsPETase and TfCut2 enzymes are presented as surface models with a gray color. The 2-HE(MHET)$_4$ docking model of IsPETase is shown as an orange-colored stick. The Ser328 and Trp159 residues in subsite II and extended loop of IsPETase corresponding phenylalanine and histidine residues and extended loop in other PET-degrading enzymes are distinguished and labeled, respectively.

- **d** Disulfide bond found in PET-degrading enzymes. The disulfide bond found in each all four PET-degrading enzymes is shown as a stick model, respectively and the residues forming the disulfide bond are labeled. 

- **e, f** Additional disulfide bond found in IsPETase. The IsPETase structure is presented as a stick model and the omit electron densities (magenta mesh) of the residues constituting the additional disulfide bond in IsPETase are contoured at 2.0 $\sigma$ (e). The additional disulfide bond region in IsPETase is compared with the corresponding regions in other PET-degrading enzymes (f). The residues forming the additional disulfide bond in IsPETase and those located at the corresponding positions in other PET-degrading enzymes are shown as a stick model and labeled appropriately.
IsPETase. Unlike type I enzymes, all type II PET-degrading enzymes have additional disulfide bonds and the extended loop (Fig. 6b). However, substantial differences were observed in residues constituting subsite II and the extended loop depending on type IIA and type IIB (Fig. 6b). Although residues constituting subsite II and the extended loop are conserved among type IIB enzymes, type IIA enzymes have a Phe or Tyr residue at the position of Ser238 in IsPETase (Fig. 6b). Because the IsPETase $^{S238F}$ variant exhibited much lower PET-degrading activity compared with IsPETase WT, the type IIA proteins are predicted to have lower PET-degrading activities compared with IsPETase. Furthermore, type IIA enzymes have highly variable residues at the extended loop (Fig. 6b), implying that environment around subsite IIB and IIC of type IIA enzymes might be quite different.
from that of type IIb enzymes. Based on the same reasoning, the other enzymes of type IIb are predicted to have PET-degrading activities similar to that of IsPETase. In addition to IsPETase, enzymes originating from bacteria, such as Acidovorax delafieldii, [Polyangium] brachysporum DSM 7029, and Burkholderiales bacterium RICFSPLOWO2_02_FULL_57_36, belong to type IIb. Interestingly, all four bacteria of type IIb enzymes belong to the order Burkholderiales, suggesting that these four bacteria seem to have similarly evolved.

**Discussion**

Until recently, enzymes using PET as a natural substrate have not been identified, and PET degradation studies have mainly been performed using cutinase and lipase family enzymes. However, degradation of PET with these enzymes was not effective due to their low affinities to PET, which leads low PET degrading activity. Recently, PETase from I. sakaiensis was reported to have much higher PET degradation efficiency than those enzymes previously examined. In this work, we determined the crystal structures and reported the structural features conferring high PET-degrading activity on IsPETase based on the docking calculations. While this paper of ours was under revision process, Han et al. also reported the crystal structure of IsPETase and its catalytic mechanism. Since they failed to obtain complex structure of IsPETase with various ligands, they ended up using inactive variants of IsPETase instead of wild-type IsPETase and succeeded in making complex structures of IsPETase variant (Ser131Ala and Arg103Gly) with two ligand, 1-(2-hydroxyethyl) 4-methyl terephthalate (HEMT) and p-nitrophenol (pNP), respectively.

The substrate-binding mode in complex with HEMT or pNP in the first TPA binding site, corresponding to the first MHET moiety in this study, agrees with what we described in this study. Based on the complex structure, they focused on the wobbling tryptophan and serine located near the active site. Because we also observed multi-occupancy of Trp156 (Trp185 in our study), this suggested mechanism indicated by the reduced activity of S185H variant is interesting.

On the other hand, we performed docking calculation using a longer substrate, 2-HE(MHET)4 and showed a substrate of four PETase instead of wild-type IsPETase and succeeded in making complex structures of IsPETase variant (Ser131Ala and Arg103Gly) with two ligand, 1-(2-hydroxyethyl) 4-methyl terephthalate (HEMT) and p-nitrophenol (pNP), respectively.

The substrate-binding mode in complex with HEMT or pNP in the first TPA binding site, corresponding to the first MHET moiety in this study, agrees with what we described in this study. Based on the complex structure, they focused on the wobbling tryptophan and serine located near the active site. Because we also observed multi-occupancy of Trp156 (Trp185 in our study), this suggested mechanism indicated by the reduced activity of S185H variant is interesting.

The crystal structure of this variant (Arg280Ala) was solved as well, which showed much higher PET-degrading activity. Then, the crystal structure of this variant (Arg280Ala) was solved as well, which showed that the structure was altered to better accommodate PET substrate as we hypothesized. This is an important finding as the structure-based engineering of a residue (Arg280), which is based on the structure of cutinase from Thermobifida alba (TcCut, PDB code 3VIS, 50% sequence identity) as a search model. The model building was performed using the WinCoot program and the refinement was performed with REFMACS. The data statistics are summarized in Supplementary Table 1. X-ray diffraction data of IsPETase crystal were collected at 100 K at Beamline 6D at the Pohang Accelerator Laboratory (Pohang, Korea). The data were then indexed, integrated, and scaled using the HKL2000 software suite. The IsPETase crystals belonged to the space group P212121, with unit cell parameters of a = 43.48 Å, b = 50.40 Å, and c = 129.49 Å. With one molecule of IsPETase per asymmetric unit, the Matthews coefficient was 2.64 Å³ Da⁻¹, which corresponds to a solvent content of 53.38%. The structure of IsPETase was determined by molecular replacement with the CCP4 version of MOLREP using the structure of cutinase from Thermobifida alba (TcCut, PDB code 3VIS, 50% sequence identity) as a search model. The model building was performed using the WinCoot program and the refinement was performed with REFMACS. The data statistics are summarized in Supplementary Table 1. The refined models of IsPETase and IsPETase have been deposited in the Protein Data Bank with PDB code 5XJH and 5YNS, respectively.

**Molecular docking calculations** Molecular docking of the tetrahedral intermediate from 2-HE(MHET)₄ to IsPETase structures was carried out by mixed approaches of flexible and covalent docking using AutoDock and AutoDock Vina. The ligand molecule of IsPETase was prepared with Vina and ProDock and nonpolar H atoms were merged onto both the ligands and the targets using AutoDockTools prior to performing the docking. For the generation of pdbqt files of both rigid and flexible receptor, flexible residues (Trp78, Trp159, Ser160, Met161, Trp185, His237, Ser238, and Asn241) were selected, and the bonds were flexible and covalent docking using AutoDock and AutoDock Vina.
in the side chain of each residues were allowed to rotate. The grid box was centered at \(-3.249, \mu 25.239\) and \(-29.093\) with sizes of 90.7, 74.7, and 122.7 Å, respectively. Prior to monodock docking, non-covalent docking calculations using AutoDock Vina was performed, and nine output poses were generated with their calculated free energy of binding from its own scoring function. The best docking model with the lowest binding energy (\(-7.1 \text{kcal mol}^{-1}\)) was selected, and the conformation of the model was used as an evaluation standard for the following calculation. Furthermore, the induced conformation of the flexible residues in the best model was applied to the receptor for covalent docking. Then, the covalent docking using AutoDock was conducted according to the previous report\(^{11}\). A total of 200 docking poses were evaluated based on the proper distances of the oxyamin hole, and the best pose with the binding energy of \(-10.27 \text{kcal mol}^{-1}\) (from the semi-empirical free energy force field of AutoDock) was selected by similarity to the non-covalent docking result. The docking pose was finally minimized using OPLS3 force field\(^{42}\) in the Schrödinger suite.

**PETase in vitro assay using bis-hydroxyethyl terephthalate.** To compare the activity of the variants of IsIPetase, bis-hydroxyethyl terephthalate (BHET) was chosen as a substrate for enzyme assay. The BHET stock solution was prepared by dissolving 2.5 g of BHET in dimethyl sulfoxide. The assay protocols were based on the previously reported paper\(^{17}\). The enzyme assay was performed in buffer solution (80 mM Na2HPO4 –HCl, 40 mM NaCl) at pH 7.0 with 200 mg L\(^{-1}\) of BHET. The enzyme reaction was started by the addition of 30 nM enzyme and was kept at 30 °C for 30 min. Then, the reaction was terminated by heating at 85 °C for 15 min. The samples were centrifuged at 13,200 r.p.m. for 10 min, and the supernatant was applied to LC analysis.

**PETase in vitro assay using PET film.** IsIPetase assays were performed as previously reported\(^{32}\) with slight modifications described below. To analyze the degradation rate of PET by PETases, commercial PET film (UBIGEO, Korea) was used as the substrate for enzyme assay. The PET film was prepared in a circular form with 6 mm diameter. The PET film was soaked in 300 μL of pH 9.0 glycine-NaOH buffer with 200 nM of enzyme. The reaction mixture was incubated at 30 °C for 18 and 36 h. The enzyme reaction was terminated by heating at 85 °C for 15 min. Then, the samples were centrifuged at 13,200 r.p.m. for 10 min, and the supernatant was analyzed by LC. After the enzyme reaction, the film was washed with 1% SDS and 20% ethanol in distilled water.

**Melting temperature (Tm) measurement.** Thermal stability of IsIPetase\(^{WT}\), IsIPetase\(^{R280A/C299A}\) and TjCut2 proteins was determined by measuring melting curves at both pH 7.0 and pH 9.0 with the Protein thermal shift dye (Applied Biosystems) in a StepOnePlus Real-Time PCR (Thermo Fisher Scientific) according to manufacturer’s instructions. Briefly, 1 μg of protein was mixed with 1× protein thermal shift dye (Applied Biosystems) in 20 μl and signal changes reflecting protein denaturation were monitored by increasing temperature from 25 to 90 °C. Melting temperatures were determined from the first derivative curve.

**Phylogenetic tree analysis.** Iterative searching for PETase-like proteins was done using the side chain of each residues were allowed to rotate. The grid box was centered at \(-3.249, \mu 25.239\) and \(-29.093\) with sizes of 90.7, 74.7, and 122.7 Å, respectively. Prior to monodock docking, non-covalent docking calculations using AutoDock Vina was performed, and nine output poses were generated with their calculated free energy of binding from its own scoring function. The best docking model with the lowest binding energy (\(-7.1 \text{kcal mol}^{-1}\)) was selected, and the conformation of the model was used as an evaluation standard for the following calculation. Furthermore, the induced conformation of the flexible residues in the best model was applied to the receptor for covalent docking. Then, the covalent docking using AutoDock was conducted according to the previous report\(^{11}\). A total of 200 docking poses were evaluated based on the proper distances of the oxyamin hole, and the best pose with the binding energy of \(-10.27 \text{kcal mol}^{-1}\) (from the semi-empirical free energy force field of AutoDock) was selected by similarity to the non-covalent docking result. The docking pose was finally minimized using OPLS3 force field\(^{42}\) in the Schrödinger suite.

**PETase in vitro assay using bis-hydroxyethyl terephthalate.** To compare the activity of the variants of IsIPetase, bis-hydroxyethyl terephthalate (BHET) was chosen as a substrate for enzyme assay. The BHET stock solution was prepared by dissolving 2.5 g of BHET in dimethyl sulfoxide. The assay protocols were based on the previously reported paper\(^{17}\). The enzyme assay was performed in buffer solution (80 mM Na2HPO4 –HCl, 40 mM NaCl) at pH 7.0 with 200 mg L\(^{-1}\) of BHET. The enzyme reaction was started by the addition of 30 nM enzyme and was kept at 30 °C for 30 min. Then, the reaction was terminated by heating at 85 °C for 15 min. The samples were centrifuged at 13,200 r.p.m. for 10 min, and the supernatant was applied to LC analysis.

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Received: 1 June 2017 Accepted: 2 January 2018
Published online: 26 January 2018
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**Acknowledgements**

We thank Dr. Oh-Hyun Kwon, the Chairman of Samsung Advanced Institute of Technology for his insight and advice on the need for developing strategies for efficient degradation and recycling of plastics. This work was supported by the Technology Development Program to Solve Climate Changes on Systems Metabolic Engineering for Biorefineries from the Ministry of Science and ICT (MSIT) through the National Research Foundation (NRF) of Korea (NRF-2012M1A2A2026556 and NRF-2012M1A2A2026557). Experiments at PLS-II 6D beamline were supported in part by UCRF, MSIT and POSTECH.

**Author contributions**

S.Y.L. and K.-J.K. conceived the project. S.J., I.J.C., H.S., and K.-J.K. designed research. S.J., I.J.C., and H.S. performed research. T.J.S. performed X-ray crystallographic experiment. H.F.S., H.-Y.S., and S.Y.C. analyzed the data. S.J., I.J.C., H.S., and S.Y.L. wrote the paper.

**Additional information**

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-02881-1.

**Competing interests:** The authors declare no competing financial interests.

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