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Original Article

Effects of residue 107 of the PET hydrolase Cut190 on its activity and thermal stability

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ABSTRACT

Structure-based protein engineering is a rational method to improve the catalytic activity and stability of enzymes. A cutinase from *Saccharomonospora viridis* AHK190, Cut190, hydrolyzes polyethylene terephthalate in the presence of Ca^{2+}. Our crystal structure analysis of the inactive S176A mutant of Cut190 S226P/R228S (Cut190*) showed that Thr107 changes its conformation largely upon binding of Ca^{2+} and the substrate to Cut190*, and this conformational change facilitates substrate binding. To analyze the role of Thr107, a series of Cut190* mutants, T107A, T107S, T107V, and T107M, were overexpressed in *Escherichia coli* and purified. The catalytic activities of the mutants were similar to that of Cut190*. Upon mutating to hydrophobic residues, such as Val and Met, the Michaelis-Menten constant increased, possibly due to increased hydrophobic interactions with not only the substrate but also the product. The secondary structure and thermal stability of Cut190*T107 mutants were also analyzed using circular dichroism. Upon mutation, the enzyme structure and stability were almost unchanged. In the presence of Ca^{2+}, unfolded protein states were largely perturbed, similar to what was observed in Cut190*.

KEYWORDS: Ca^{2+} binding, enzymatic activity, mutation, thermal stability.

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INTRODUCTION

A cutinase-type polyesterase (Cut190) cloned from a thermophilic isolate, *Saccharomonospora viridis* AHK190, has been shown to degrade polyethylene terephthalate (PET) [1]. Because PET has been widely used all over the world and waste PET has caused severe environmental problems, PET depolymerization *via* enzymatic catalysis during its chemical recycling, in which the monomer resulting from the depolymerization process can be used as new material, is one of the most useful solutions to achieve sustainable development goals (SDGs) [2, 3]. We have improved the activity and thermal stability of Cut190 by amino acid substitution to depolymerize PET, because depolymerization of the inner block of PET occurs above its glass transition temperature (T_g), which corresponds to approximately 70 °C [4, 5]. We showed that a mutant of Cut190, S226P/R228S, designated as Cut190*, has increased activity and stability [1]. Furthermore, we used Cut190* as a template for further mutational analyses [6-8].

The catalytic residue of Cut190 is Ser176, which forms a catalytic triad with His254 and Asp222. A unique feature of Cut190 is that its enzymatic activity and thermal stability are regulated by Ca^{2+} binding [1, 9]. Crystal structure analysis of the Cut190 S226P mutant first showed that Ca^{2+} binding changed the protein conformation from closed to open forms (Fig. 1) [10]. We further determined the crystal structures of Cut190* and its inactive mutant, Cut190*S176A, showing that Ca^{2+} binding

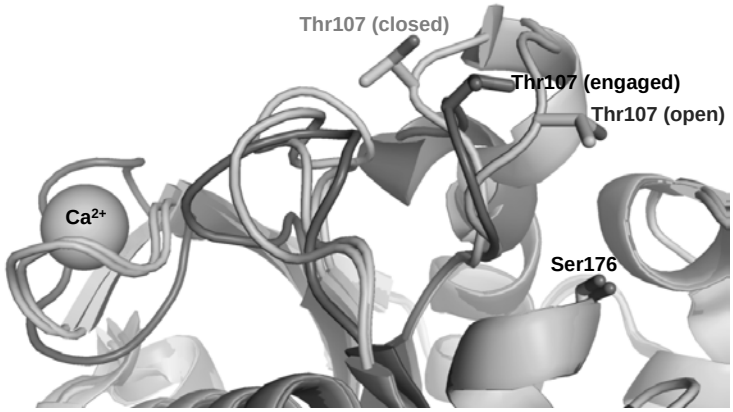

Fig. 1. Crystal structures of Cut190S226P and Cut190*S176A mutants. The Ca^{2+}-bound (open form) and -unbound (closed form) states of Cut190S226P are indicated by medium gray and light gray, together with the engaged form of Cut190*S176A indicated by dark gray. The side-chains of Thr107 and Ser176 are indicated by stick models, and Ca^{2+} is indicated by sphere. The figure was prepared using the PyMOL software (http://www.pymol.org/).

and releasing can regulate enzyme function *via* closed, open, engaged, and ejecting forms [7, 8, 11]. Ca^{2+} binding changes the conformation of a substrate-binding loop, the β3-α2 loop, largely in an allosteric manner, which would contribute to the activity identified. We had expected that the mutation of Thr107 on the substrate-binding loop could change the enzyme property, because the conformation of Thr107 is largely changed in the crystal structures. Our crystal structures showed that Thr107 undergoes considerable movement; the distances between the βC of Thr107 and βC of Ser176 or Ala176 are 11.6 Å, 9.6 Å, and 7.2 Å in closed, open, and engaged forms, respectively (Fig. 1). In our previous study involving docking analysis with a PET analogue [12], Thr107, together with Phe106, contributed to substrate binding. The crystal structure of Cut190* S176A in complex with monoethyl succinate, corresponding to the pre-reaction state designated as the engaged form, revealed that conformational changes involving Thr107 resulted in the formation of a short tunnel near the active site, and the bound PET substrate might migrate through this tunnel [11]. In this study, we prepared a series of Cut190*T107 mutants, T107A, T107S, T107V, and T107M, and analyzed their enzymatic activities towards poly(butylene succinate-*co*-adipate) (PBSA). In addition, we analyzed the mutational effects on secondary structure and thermal stability using circular dichroism (CD).

MATERIALS AND METHODS

Proteins

The expression plasmids of Cut190*T107 mutants were constructed from that of Cut190* by site-directed mutagenesis. The proteins were overexpressed in *Escherichia coli* and purified as reported previously [11]. The harvested cells were resuspended in 20 mM Tris-HCl (pH 8.0) containing 300 mM NaCl and 10 mM imidazole and lysed by sonication at 4 ºC. After the cell debris was removed by centrifugation, the supernatant was loaded into a Ni-nitrilotriacetic acid column (Qiagen) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole, and eluted using 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl and 200 mM imidazole. Each purified protein was dialyzed in 10 mM Tris-HCl (pH 8.0). The protein concentration was spectrophotometrically determined using an absorption coefficient of 1.26 mg^{-1} cm^2 at 280 nm.

CD measurements

Secondary structure and thermal denaturation analyses were carried out using a Jasco J-725 spectropolarimeter as described previously [13]. The protein concentration was 0.04 mg mL^{-1} in 10 mM Tris-HCl (pH 8.0) in the absence or presence of $CaCl_2$, 0.25, 2.5, and 25 mM, respectively. Thermal denaturation curves were recorded in the temperature mode at 222 nm with a heating rate of 1.0 ºC min^{-1}.

Enzymatic activity measurements

The enzymatic activities were quantitatively measured by monitoring the decrease in the turbidity of a PBSA suspension at 600 nm, as described previously [1]. The reaction mixture included PBSA (to produce a final OD_{600} of approximately 1.0-1.5), 50 mM Tris (pH 8.2), 2.5 mM Ca^{2+}, and an appropriate quantity of Cut190* and its mutants in a total volume of 2 mL. The measurements were performed in duplicate.

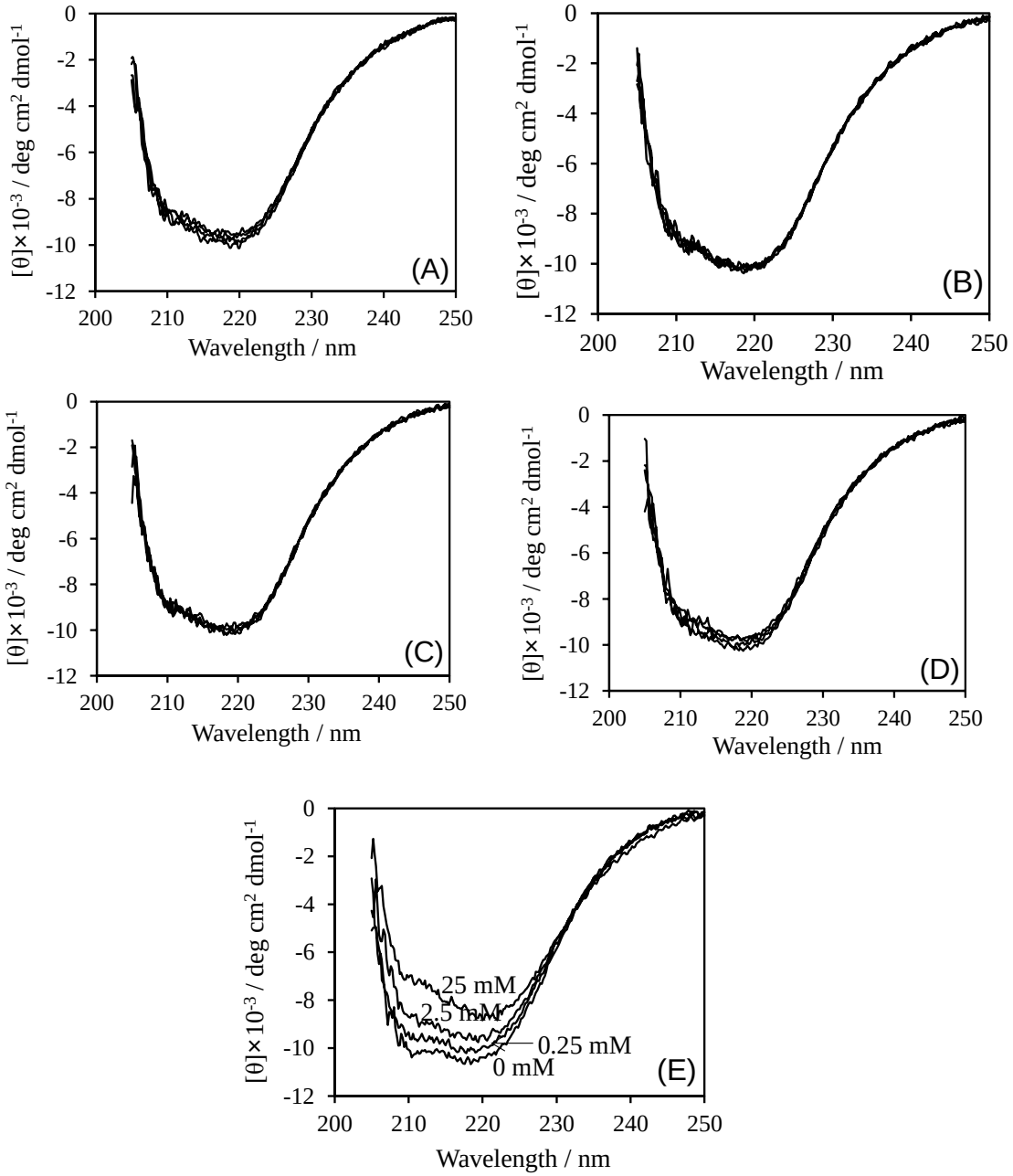

Fig. 2. Far-UV CD spectra of Cut190* (A), Cut190*T107A (B), Cut190*T107S (C), Cut190*T107V (D), and Cut190*T107M (E) in the absence or presence of Ca^{2+} (0.25, 2.5, 25 mM) at 20 °C. The spectra of Cut190* (A), Cut190*T107A (B), Cut190*T107S (C), Cut190*T107V (D) are almost superimposed.

RESULTS AND DISCUSSION

The secondary structures of Cut190*T107 mutants in the absence or presence of Ca^{2+} were analyzed using CD (Fig. 2). Upon mutation, the secondary structures were almost unchanged. The far-UV CD spectra of T107M in the presence of Ca^{2+} changed with increasing Ca^{2+} concentrations, probably due to partial aggregation induced by Ca^{2+} binding. In contrast, the secondary structures of other Cut190*T107 mutants were unchanged, even in the presence of 25 mM Ca^{2+}. The effects of Ca^{2+} on thermal stability were examined by monitoring CD values at 222 nm, while increasing the temperature (Fig. 3). It should be noted that the θ values after the transition in the absence of Ca^{2+} were different from those in the presence of Ca^{2+}, indicating that

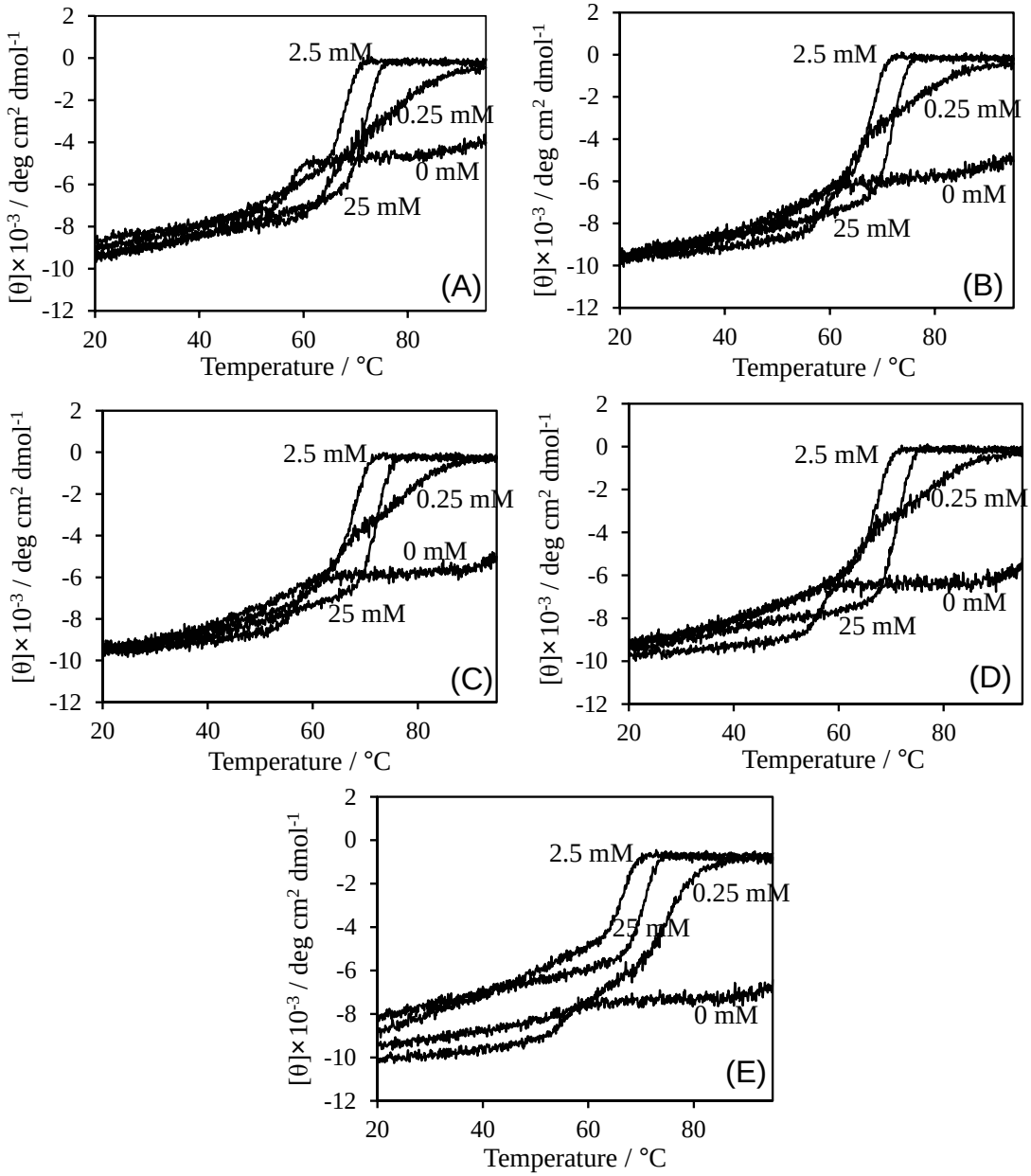

Fig. 3. Thermal denaturation curves of Cut190* (A), Cut190*T107A (B), Cut190*T107S (C), Cut190*T107V (D), and Cut190*T107M (E) in the absence or presence of Ca^{2+} (0.25, 2.5, 25 mM).

the former solution contains soluble unfolded proteins while the latter contains the insoluble unfolded proteins. By increasing the temperature above 90 °C in the absence of Ca^{2+}, soluble unfolded proteins would change to the insoluble form. In the presence of 0.25 mM Ca^{2+}, two transitions were observed. The first transition around 60-70 °C would correspond to the thermal unfolding of the soluble form, and the second would correspond to the further transition to the insoluble form, probably the aggregated form. These results indicated that Ca^{2+} changes not only the conformation of native state but also that of denatured state in a Ca^{2+} concentration-dependent manner. As reported previously [9, 13], Ca^{2+} binding would alter protein conformations into less fluctuated forms, which would correlate to a different form in the denatured state. Assuming a simple two-state transition for experiments in the absence or presence of Ca^{2+} (2.5 mM and 25 mM), the apparent melting temperature (T_m) values were determined using a fitting procedure (Table 1). The temperature at which thermal transition commenced in the CD experiments also supported the notion that the thermal stability of Cut190*T107 mutants increased with elevating Ca^{2+} concentrations, similar to what was observed with Cut190* (Fig. 3).

Table 1. Thermal stabilities of Cut190*T107 mutants, T_m (ºC), in the absence or presence of Ca^{2+} analyzed using CD.

Enzyme	Ca^{2+} / mM		
	0	2.5	25
Cut190* [a]	55.9	68.4	72.3
Cut190*	57.2	67.3	71.7
Cut190*T107A	58.4	67.6	71.8
Cut190*T107S	56.6	67.5	71.2
Cut190*T107V	56.1	66.8	71.2
Cut190*T107M	54.8	66.5	70.4

[a]Data were taken from Oda *et al*., 2018 [6].

The enzymatic activity of the Cut190*T107 mutants towards PBSA in the presence of 2.5 mM Ca^{2+} was analyzed, and the kinetic parameters are summarized in Table 2. Both the Michaelis-Menten constant (K_m) and catalytic rate constant (k_{cat}) increased or decreased upon the mutation, resulting in similar k_{cat}/K_m values. Upon mutating to hydrophobic residues, such as Val and Met, K_m values increased, indicating that hydrophobic interactions with not only the substrate but also the product were elevated.

We have investigated structure-activity relationships involving Cut190 and generated mutants with improved activity and stability [1, 6-8, 12]. In this study, we focused on amino acid residue 107, which is involved in substrate binding. We had expected that mutation to a hydrophobic residue would increase the substrate-binding affinity, resulting in decreased K_m. However, K_m values for Cut190*T107V and Cut190*T107M were increased (Table 2), probably due to augmented product-binding affinity. This is in good agreement with previous results wherein the mutation of Phe106 to Ala decreased the K_m [12]. In the present study, mutants with increased K_m exhibited elevated k_{cat}, resulting in catalytic activity comparable to that of Cut190*. We recently succeeded in generating Cut190* mutants with increased thermal stability, which could efficiently hydrolyze PET at 70 °C, T_g of PET [8]. To improve the catalytic activity

Table 2. Enzyme kinetic parameters of Cut190* and its T107 mutants for the reaction of PBSA degradation.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Cut190* [a]	0.089	27	308
Cut190*T107A [b]	0.036	8.7	239
Cut190*T107S	0.083	25	302
Cut190*T107V	0.122	30	246
Cut190*T107M	0.172	60	348

[a]Data were taken from Oda *et al*., 2018 [6].
[b]Data were taken from Kawabata *et al*., 2017 [12].

and thermal stability of enzymes *via* protein engineering, various mutants will be needed and their functional evaluation will have to be performed.

CONCLUSION

The secondary structure of Cut190*T107 mutants, T107A, T107S, T107V, and T107M, were similar to that of Cut190*. The thermal stability increased in a Ca^{2+} concentration-dependent manner. The unfolded state in the presence of Ca^{2+} was different from that in the absence of Ca^{2+}, possibly due to solubility. Upon the mutation of Thr107 of Cut190* to hydrophobic residues such as Val and Met, both K_m and k_{cat} values increased, resulting in k_{cat}/K_m values similar to that of Cut190*. The results indicated that hydrophobic interactions with not only the substrate but also the product were elevated.

CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interest, financial or otherwise.

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