Thermostable Mutants of Glycoside Hydrolase Family 6 Cellobiohydrolase from the Basidiomycete Phanerochaete chrysosporium

(Received April 5, 2020; Accepted June 9, 2020)

Sora Yamaguchi,‡ Naoki Sunagawa,¹ Mikako Tachioka,¹,‡ Kiyohiko Igarashi,¹,³,⁴ and Masahiro Samejima¹,⁴

¹Department of Biomaterial Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo
(1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan)
²Deep-Sea Nanoscience Research Group, Research Center for Bioscience and Nanoscience,
Japan Agency for Marine-Earth Science and Technology
(Natsushima-cho, Yokosuka City, Kanagawa 237–0061, Japan)
³VTT Technical Research Centre of Finland
(P.O.Box 1000, Tietotie 2, Espoo FI-02044, Finland)
⁴Faculty of Engineering, Shinshu University
(4–17–1 Wakasato, Nagano City, Nagano 380–8553, Japan)

Abstract: Thermal inactivation of saccharifying enzymes is a crucial issue for the efficient utilization of cellulosic biomass as a renewable resource. Cellobiohydrolases (CBHs) are a kind of cellulase. In general, CBHs belonging to glycoside hydrolase (GH) family 6 (Cel6) act synergistically with CBHs of GH family 7 (Cel7) and other carbohydrate-active enzymes during the degradation of cellulosic biomass. However, while the catalytic rate of enzymes generally becomes faster at higher temperatures, Cel6 CBHs are inactivated at lower temperatures than Cel7 CBHs, and this represents a limiting factor for industrial utilization. In this study, we produced a series of mutants of the glycoside hydrolase family 6 cellobiohydrolase PcCel6A from the fungus Phanerochaete chrysosporium, and compared their thermal stability. Eight mutants from a random mutagenesis library and one rationally designed mutant were selected as candidate thermostable mutants and produced by heterologous expression in the yeast Pichia pastoris. Comparison of the hydrolytic activities at 50 and 60 °C indicated that the thermal stability of PcCel6A is influenced by the number and position of cysteine residues that are not involved in disulfide bonds.

Key words: cellobiohydrolase, random mutagenesis, protein engineering, glycoside hydrolase, enzymatic saccharification

INTRODUCTION

Cellulosic biomass is the most abundant carbon stock in nature, and its degradation to soluble sugars has the potential to replace fossil resources by providing an alternative raw material for fuels and chemicals. Further, as enzymatic saccharification of cellulosic biomass would not require strong acid or alkali, or intense heat, it should involve lower energy consumption than chemical or physical treatments. Cellobiohydrolases (CBHs) are a kind of cellulase and indispensable for the complete enzymatic hydrolysis of cellulose because they can degrade crystalline regions that are resistant to enzymatic hydrolysis by removing cellobiosyl units from the cellulose chain ends. Furthermore, the degradation of highly crystalline cellulose remains a key bottleneck for achieving efficient enzymatic saccharification.

CBHs that degrade crystalline cellulose are classified into either glycoside hydrolase family 6 (Cel6, EC.3.2.1.91) or 7 (Cel7, EC.3.2.1.176) in the Carbohydrate-Active EnZyme (CAZy) database (http://www.cazy.org/). Most Cel6 and Cel7 CBHs have a carbohydrate-binding module (CBM) and a catalytic domain (CD), which are connected by a flexible linker. Fungal Cel6 CBHs are important, because their hydrolytic activity is comparable to that of Cel7 CBHs, and synergistic hydrolysis occurs when crystalline cellulose is incubated with both Cel6 and Cel7 together. However, the thermal stability of Cel6 CBHs is generally lower than that of Cel7 CBHs. For example, the optimum temperatures of Cel6 and Cel7 CBHs from the thermophilic filamentous fungus Chaetomium thermophilum are 50 °C and 65 °C, respectively. This is a problem, because industrial-scale enzymatic saccharification is conducted at an elevated temperature to increase the hydrolysis rate. Therefore, increasing the thermal stability of Cel6 CBH should immediately lead to an increase in the efficiency of the commercial process.

Numerous studies have attempted to improve the thermal
stability of Cel6 CBHs by applying two major strategies, i.e., random mutagenesis and rational design. Random mutagenesis is generally employed when information about the target enzyme is limited. On the other hand, in the process of rational design, key amino acid(s) to be changed are firstly identified based on the enzyme structure and the interaction between enzyme and substrate, and then the designed mutants are prepared and characterized.\(^1\)

The methylotrophic yeast *Pichia pastoris* is a suitable host for the expression of fungal Cel6 CBHs because it performs post-translational modifications found in the eu-karyote and it can secrete the fungal proteins in up to gram quantities per liter of culture.\(^2\) For example, production of Cel6 CBH from the wood-decaying fungus *Phanerochaete chryosporium* (PcCel6A) in *P. pastoris* using a Mini jar- fermentor is as much as 4.6 g/L at 160 h of cultivation.\(^3\)

Therefore, random mutagenesis combined with expression in *P. pastoris* has been employed to improve the catalytic efficiency and thermal stability of fungal Cel6 CBHs.\(^4\)\(^5\) However, the contribution of each individual mutation to the activity of mutants with multiple mutations has not been investigated, e.g., by preparing mutants with each single mutation, although this information would be useful for rationally enhancing the activity even further.

Regarding the rational design of thermostable Cel6 CBHs, free cysteine (cysteine residues that do not form a disulfide bond) has been a target of substitution.\(^6\)\(^7\) These studies analyzed the thermal stability of the mutants by measuring the incubation temperature at which the enzyme loses 50 % of its activity, the residual activity, and the half-life. However, these methods do not provide information about the hydrolytic activity during incubation with substrates at elevated temperatures, though this ability is critical for achieving more efficient saccharification of cellulose biomass.

In the present study, therefore, we heterologously expressed in *P. pastoris* a series of mutants of fungal PcCel6A with substitutions based on either random mutagenesis\(^8\) or rational design.\(^9\) We compared the activities of these mutants by incubating them with amorphous phosphoric acid-swollen cellulose or crystalline cellulose III\(_\alpha\) at different temperatures. Based on the results, we discuss the critical features for increased thermal stability of PcCel6A.

**MATERIALS AND METHODS**

**Materials.** DNA polymerases PrimeSTAR Max (TaKaRa Bio Inc., Shiga, Japan) and KOD-Plus (Ver.2; Toyobo Co., Ltd, Osaka, Japan) were used to amplify mutated DNA. One Shot\textsuperscript{®} TOP10 Chemically Competent *Escherichia coli* (Thermo Fisher Scientific Inc., MA, USA) was used to amplify the plasmid. *Pmel* (New England Biolabs, MA, USA) was used to linearize the amplified plasmid for the transformation of *P. pastoris* strain KM71H (Thermo Fisher Scientific). *P. pastoris* containing wild-type PcCel6A gene was produced according to the reported method.\(^10\) Colonies containing WT or mutant PcCel6A genes were grown in 1 % yeast, 2 % peptone (YP) medium with 2 % glycerol at 30 °C. Then, expression was induced in YP medium in a conical tube at 26.5 °C by the addition of methanol (1 % (v/v), final concentration) every other day. Aliquots of 80 μL of yeast culture were sampled for 3 days after the induction of the enzyme expression and centrifuged at 4 °C with 10,000 × G for 10 min. The third-day culture was centrifuged at 4 °C with 3,000 × G for 5 min and centrifuged again at 15,000 × G for 10 min at 4 °C. The supernatants were filtered with a 0.20 μm filter (Acrodisc\textsuperscript{®} Syringe Filters with PVDF Membrane; Pall Corporation, NY, USA). The protein concentration of the supernatants and filtrate was determined using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-rad Laboratories, CA, USA); the absorbance was measured at 595 nm with a Thermo Scientific Multiskan\textsuperscript{®} GO (Thermo Fisher Scientific). The expression level of enzymes was evaluated by SDS-PAGE using 12 % polyacrylamide gel after deglycosylation with endoglycosidase H and α-mannosidase at 37 °C for 1 h. A picture of the gel was taken with a CanoScan (Canon Inc., Tokyo, Japan) at 50 % exposure time. The image was modified to change the shape of the lanes from trapezoidal to rectangular by using GIMP (Ver. 2.10.14.0, https://www.gimp.org) and converted to a 32-bit gray scale with ImageJ (Ver.1.52a, https://imagej.nih.gov/ij/). The amount of PcCel6A was estimated by comparing the peak area calculated by measuring the color intensity of bands between 50 and 75 kDa and those of a protein marker (Bio-rad Laboratories, Inc.).

**Activity measurements of crude enzymes.** Culture supernatants of WT or mutant PcCel6A (10 μL) were incubated with 100 μg of PASC or cellulose III, prepared as described previously\(^12\)\(^13\) in 100 mM sodium acetate buffer (pH 5.0) with a total volume of 200 μL using 96-well plates at 50 or 60 °C with shaking at 1,000 rpm. After two hours of
were heated at 98 °C for 3 min and filtered again. The filtrate was incubated at 60 °C for 48 h and Gly421 lies within the loop near the active site. G421A was chosen to test the effect of removing Gly421. C240S/C393S were selected by rational design (see the text). The overall structure of PcCel6A was created by superposing the structure of the catalytic domain taken from PDB ID 5XCY, 16 and the structure of the carbohydrate-binding module predicted using Protein Homology/analogy Recognition Engine V 2.0 (Phyre2, http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) 17 on the overall structure of TrCel6A modelled previously.15 The points of mutations were introduced into images of these domains with the PyMOL Molecular Graphics System, Version 1.0.0.0 Schrödinger, LLC.

#### RESULTS AND DISCUSSION

Improving the thermal stability of fungal Cel6 CBHs is critical to increase the saccharification rate of cellulosic biomass, because these enzymes are the least stable among the cocktail of cellulosytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

The total protein concentration expressed by transformed *P. pastoris* increased with cultivation time after the induction of protein expression by adding methanol to the culture, as shown in Fig. 2. The production levels of W267C and G421D were higher than that of WT. The content of WT and Cys393 in the CD should not form a disulfide bond because they are distant from other cysteine residues. The double mutant C240S/C393S was rationally designed as a candidate thermostable mutant by substituting the two cysteine residues with serine.

**Expression amount of PcCel6A mutants.**

The total protein concentration expressed by transformed *P. pastoris* increased with cultivation time after the induction of protein expression by adding methanol to the culture, as shown in Fig. 2. The production levels of W267C and G421D were higher than that of WT. The content of WT and Cys393 in the CD should not form a disulfide bond because they are distant from other cysteine residues. The double mutant C240S/C393S was rationally designed as a candidate thermostable mutant by substituting the two cysteine residues with serine.

*Fig. 1. Residues of PcCel6A selected for mutagenesis.***

Mutations C25Y, A103T, A105D, M257I, W267C, G346D, and G421D were selected on the basis of a random mutagenesis experiment (see the text). G421A was chosen to test the effect of removing Gly421. C240S/C393S were selected by rational design (see the text). The overall structure of PcCel6A was created by superposing the structure of the catalytic domain taken from PDB ID 5XCY, 16 and the structure of the carbohydrate-binding module predicted using Protein Homology/analogy Recognition Engine V 2.0 (Phyre2, http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) 17 on the overall structure of TrCel6A modelled previously.15 The points of mutations were introduced into images of these domains with the PyMOL Molecular Graphics System, Version 1.0.0.0 Schrödinger, LLC.

Expression amount of PcCel6A mutants.

The total protein concentration expressed by transformed *P. pastoris* increased with cultivation time after the induction of protein expression by adding methanol to the culture, as shown in Fig. 2. The production levels of W267C and G421D were higher than that of WT. The content of WT and Cys393 in the CD should not form a disulfide bond because they are distant from other cysteine residues. The double mutant C240S/C393S was rationally designed as a candidate thermostable mutant by substituting the two cysteine residues with serine.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.

#### Preparation of PcCel6A mutants.

Amino acid residues that were expected to influence the thermostability of PcCel6A were chosen based on reported experimental results of random mutagenesis14 or rational design, 15 as shown in Fig. 1. Eight single mutations with different characteristics were chosen from the mutant library generated by introducing random mutations into the cocktail of cellulolytic enzymes. The current study provides insights into the factors that determine the thermal stability of PcCel6A.
should have a negligible impact on our findings, because the effects of glycosylation of the CD of Cel6 CBH from *Trichoderma reesei* (TrCel6A) on the enzyme structure and interactions with a ligand were minimal.

Thermal properties of PcCel6A mutants.

Hydrolytic activity in the culture supernatant of WT and mutant enzymes was tested using PASC and cellulose III as substrates. As shown in Fig. 4A, specific activities of PASC hydrolysis by A103T, M257I, and C240S/C393S were significantly improved, compared with WT, at 60 °C. However, when crystalline cellulose III was used as a substrate, thermal inactivation of A103T, M257I, and WT apparently occurred at 60 °C, though the C240S/C393S double mutant remained active. Ala103 has an α-helix at the surface of the enzyme. It was reported that many advantageous mutations of *Pc* Cel6A based on the sequences of thermophilic fungal Cel6 CBHs are located on the surface of the enzyme. Since A103T involves the substitution of compact Ala with bulky Thr, we speculate that its stability might be increased as a result of filling a cavity on the surface of the enzyme that would otherwise be accessible to the solvent. In a previous study, M257I showed an increase of 1.2 °C in the temperature required to reduce the initial
Hydrolytic activities towards PASC and cellulose III at 50 °C (A) and 60 °C (B), and specific activities at 50 °C (C) and 60 °C (D). The reaction conditions were the same as for Fig. 4. N = 3. Error bars represent ± 1 standard deviation.

Fig. 5. Comparison of the hydrolytic activities at 50 and 60 °C towards PASC and cellulose III at 50 °C (A) and 60 °C (B), and specific activities at 50 °C (C) and 60 °C (D). The reaction conditions were the same as for Fig. 4. N = 3. Error bars represent ± 1 standard deviation.

Fig. 6. Comparison of the hydrolytic activities at 50 and 60 °C towards PASC (A) and cellulose III (B), and specific activities towards PASC (C) and cellulose III (D). The reaction conditions were the same as for Fig. 4. N = 3. Error bars represent ± 1 standard deviation.
activity by 50% within 120 min. This might be due to the substitution with a more hydrophobic amino acid, Ile, because Met257 is surrounded by hydrophobic side chains of amino acids in the α-helix. If we compare the amino acid sequences of the characterized fungal Cel6 CBHs, branched chain amino acids such as Leu that are more hydrophobic than Met appear frequently at the position corresponding to Met257 in PcCel6A. Thus, it is plausible that these hydrophobic residues improve the stability of the enzyme, since they are highly conserved among fungal Cel6 CBHs.

Comparison of the hydrolytic activities towards amorphous (PASC) versus crystalline cellulose (cellulose III) is helpful to judge the origin of activity changes. When the activity is plotted by putting the glucose yield from PASC versus crystalline cellulose (cellulose III) on the horizontal axis and that from cellulose III on the vertical axis, we typically see second-degree polynomial curves, as we previously reported. The plot should deviate from the curve if the relative activity, i.e., amorphous vs. crystalline, is changed by mutation. As shown in Fig. 5, the activity of C25Y and W267C are located in the CBM and at the entrance to the catalytic tunnel, respectively. Since Cys25 forms a disulfide bond with Met257 in PcCel6A, it is plausible that these residues improve the stability of the enzyme, since they are highly conserved among fungal Cel6 CBHs.

Fig. 7. Location of the N- and C-terminal loops (A) and the close-up views of Cys240 (B) and Cys393 (C).

The structure of the catalytic domain of PcCel6A WT was taken from PDB ID 5XCY and modified with the PyMOL Molecular Graphics System. N- and C-terminal loops consist of amino acid residues 174-178 (green) and 390-425 (cyan), respectively. Free cysteine Cys240 and Cys393 are colored orange and shown by sticks. Side chains of the residues around 8 Å from Cys240 and Cys393 are shown by lines. Residues that might be interacting with Cys240 and Cys393 directly or indirectly (Gln187, Asn362, Cys361 and Cys408) are also represented by sticks.

When the hydrolytic activity in the reaction at 50 °C is plotted on the horizontal axis against that at 60 °C on the vertical axis, we typically see second-degree polynomial curves, as we previously reported. The plot should deviate from the curve if the relative activity, i.e., amorphous vs. crystalline, is changed by mutation. As shown in Fig. 5, the activity of C25Y and W267C are located in the CBM and at the entrance to the catalytic tunnel, respectively. Since Cys25 forms a disulfide bond with Met257 in PcCel6A, it is plausible that these residues improve the stability of the enzyme, since they are highly conserved among fungal Cel6 CBHs.

These effects of free cysteine might explain the decline in the specific activity of A103T, M257I, and WT towards crystalline cellulose at 60 °C (Fig. 5D). As shown in Fig. 7, Gln187 and Cys408 that are supposed to be directly or indirectly interacting with Cys240 and Cys393, respectively, take double conformation. Therefore, thermal stabilization of C240S/C393S might be resulted from stabilizing these double conformational residues. Simulations suggest that processive CBHs are more likely to perform the rate-limiting step of dissociation from crystalline cellulose by backing up along the cellulose chain without opening the substrate-enclosing loops rather than by opening the loops.

The fact that the mobility of the C-terminal loop (amino acid 390-425) is calculated to be less than that of the N-terminal loop (amino acid 174-178) may be related to the existence of a disulfide bond (Cys361-Cys408) near Cys393. If Cys393 weakens this disulfide bond via Asn362 (and perhaps H2O), and the bond is consequently cleaved more readily, the C-terminal loop might not retain its immobile structure at higher temperature, which could interfere with dissociation of the enzyme from crystalline cellulose.
cellulose (cellulose III, in this experiment). Moreover, since the number of free cysteine residues is 0 in C240S/C393S, 3 in C25Y and W267C, and 2 in other mutants and WT, the hydrolytic activity towards amorphous cellulose at the higher temperature (Fig. 6A) might also be connected to the number of free cysteine residues in the enzyme.

CONCLUSION

In this work, we identified several mutants of Pc-Cel6A that show higher activities than WT at 60 °C. Our results indicate that the number and position of free cysteine residues are critical factors affecting the thermal stability of Pc-Cel6A. We are currently conducting X-ray crystal structure analysis to better understand the structural basis of the thermal stabilization by the specific substitutions without depending on structure modeling. Our findings should be helpful to increase the efficiency of industrial-scale enzymatic saccharification of cellulose.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

S.Y. is grateful for financial support from UTokyo Sustainable Agriculture Education Program during a Master’s course. The authors are grateful for Grants-in-Aid for Scientific Research (B) (15H04526, 18H02252 and 19H03013 to K.I.) from the Japan Society for the Promotion of Science (JSPS), a Grant-in-Aid for Innovative Areas from the Japanese Ministry of Education, Culture, Sports, and Technology (MEXT) (No. 18H05494 to K.I.). In addition, K.I. thanks Business Finland (BF, formerly the Finnish Funding Agency for Innovation (Tekes)) for support via the Finland Distinguished Professor (FiDiPro) Program “Advanced approaches for enzymatic biobased materials utilization and modification (BioAD)”.

REFERENCES

1) S.P. Voutilainen, T. Puranen, M. Siika-Aho, A. Lappalainen, M. Alapuranen, J. Kallio, S. Hooman, L. Viikri, J. Vehmaanperä, and A. Koivula: Cloning, expression, and characterization of novel thermostable family 7 cellobiohydrolases. Biotechnol. Bioeng., 101, 515–528 (2008).
2) V. Lombard, H. Golacconda Ramulu, E. Drula, P.M. Coutinho, and B. Henriques: The carbohydrate-active enzymes database (CAzY) in 2013. Nucleic Acids Res., 42, 490–495 (2014).
3) K. Igarashi, T. Uchihashi, A. Koivula, M. Wada, S. Kimura, T. Okamoto, M. Penttilä, T. Ando, and M. Samejima: Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface. Science, 333, 1279–1282 (2011).
4) I. Wu and F.H. Arnold: Engineered thermostable fungal Cel6A and Cel7A cellobiohydrolases hydrolyze cellulose efficiently at elevated temperatures. Biotechnol. Bioeng., 110, 1874–1883 (2013).
5) X.J. Wang, Y.J. Peng, L.Q. Zhang, A.N. Li, and D.C. Li: Directed evolution and structural prediction of cellobiohydrolase II from the thermophilic fungus Chaetomium thermophilum. Appl. Microbiol. Biotechnol., 95, 1469–1478 (2012).
6) Y.P. Zhang, M.E. Himmel, and J.R. Mielczan: Outlook for cellulase improvement: Screening and selection strategies. Biotechnol. Adv., 24, 452–481 (2006).
7) S. Macauley-Patrick, M.L. Fazenda, B. McNeil, and L.M. Harvey: Heterologous protein production using the Pichia pastoris expression system. Yeast, 22, 249–270 (2005).
8) K. Igarashi, M. Maruyama, A. Nakamura, T. Ishida, M. Wada, and M. Samejima: Degradation of crystalline cellulos by Phanerochaete chrysosporium cellobiohydrolase II (Cel6A) heterologously expressed in methylotrophic yeast Pichia pastoris. J. Appl. Glycosci., 59, 105–110 (2012).
9) M. Tachioka, N. Sugimoto, A. Nakamura, N. Sunagawa, T. I, T. Uchiyama, K. Igarashi, and M. Samejima: Development of simple random mutagenesis protocol for the protein expression system in Pichia pastoris. Biotechnol. Biofuels, 9, 1–10 (2016).
10) P. Heinzelman, C.D. Snow, M.A. Smith, X. Yu, A. Kannan, K. Boulware, A. Villalobos, S. Govindarajun, J. Minshull, and F.H. Arnold: SCHEMA recombination of a fungal cel lulase uncovers a single mutation that contributes markedly to stability. J. Biol. Chem., 284, 26229–26233 (2009).
11) I. Wu, T. Heel, and F.H. Arnold: Role of cysteine residues in thermal inactivation of fungal Cel6A cellobiohydrolases. BBA - Proteins Proteomics, 1834, 1539–1544 (2013).
12) A. Nakamura, H. Watanabe, T. I, T. Uchihashi, M. Wada, T. Ando, K. Igarashi, and M. Samejima: Trade-off between processivity and hydrolytic velocity of cellobio hydrolases at the surface of crystalline cellulose. J. Am. Chem. Soc., 136, 4584–4592 (2014).
13) M. Wada, H. Chanzy, Y. Nishiyama, and P. Langan: Cellulose III, crystal structure and hydrogen bonding by synchrotron X-ray and neutron fiber diffraction. Macromolecules, 37, 8548–8555 (2004).
14) M. Tachioka, A. Nakamura, T. I, K. Igarashi, and M. Samejima: Crystal structure of a family 6 cellobiohydrolase from the basidiomycete Phanerochaete chrysosporium. Acta Crystallogr. Sect. F Struct. Biol. Commun., 73, 398–403 (2017).
15) Y. Ito, A. Ikeuchi, and C. Imamura: Advanced evolutionary molecular engineering to produce thermostable cellulase by using a small but efficient library. Protein Eng. Des. Sel., 26, 73–79 (2013).
16) C. Steentoft, Y. Vakhrushev, H.J. Joshi, Y. Kong, M.B. Vester-christensen, K.T. Schjoldager, K. Lavrsen, S. Dabelsteen, N.B. Pedersen, L. Marcos-silva, R. Gupta, E.P. Bennett, U. Mandel, S. Brunak, H.H. Wandall, S.B. Levy, and H. Clausen: Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. EMBO J., 32, 1478–1488 (2013).
17) J.G. Duman, R.G. Miele, H. Liang, D.K. Grel, K.L. Sim, F.J. Castellino, and R.K. Borth, O-Mannosylation of Pichia pastoris cellular and recombinant proteins. Biotechnol. Appl. Biochem., 28, 39–45 (1998).
18) C.M. Payne, Y.J. Bomble, C.B. Taylor, C. McCabe, M.E. Himmel, M.F. Crowley, and G.T. Beckham: Multiple functions of aromatic-carbohydrate interactions in a processive cellulase examined with molecular simulation. *J. Biol. Chem.*, **286**, 41028–41035 (2011).

19) A. Koivula, T. Kinnari, V. Harjunpää, L. Ruohonen, A. Teleman, T. Drakenberg, J. Rouvinen, T.A. Jones, and T.T. Teeri: Tryptophan 272: An essential determinant of crystalline cellulose degradation by *Trichoderma reesei* cellobiohydrolase Cel6A. *FEBS Lett.*, **429**, 341–346 (1998).

20) V. Gupta and K.S. Carroll: Sulfenic acid chemistry, detection and cellular lifetime. *Biochim. Biophys. Acta - Gen. Subj.*, **1840**, 847–875 (2014).

21) J.V. Vermaas, R. Kont, G.T. Beckham, M.F. Crowley, M. Gudmundsson, M. Sandgren, J. Ståhlberg, P. Väljamäe, and B. C. Knott: The dissociation mechanism of processive cellulases. *Proc. Natl. Acad. Sci. USA*, **116**, 23061–23067 (2019).

22) L.A. Kelley, S. Mezulis, C.A.M. Yates, M.A.N. Wass, and M.J.E. Sternberg: The Phyre2 web portal protein modeling, prediction and analysis. *Nat. Protoc.*, **10**, 845–858 (2015).