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Hee-Jin Kang · Koichi Uegaki · Harumi Fukada
Kazuhiko Ishikawa

Improvement of the enzymatic activity of the hyperthermophilic cellulase from *Pyrococcus horikoshii*

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Abstract A hyperthermophilic β-1,4 endoglucanase (EGPh) from the hyperthermophilic archaeon *Pyrococcus horikoshii* exhibits a strong hydrolyzing activity toward crystalline cellulose. The characteristic features of EGPh are: (1) it appears to have disulfide bonds, which is rare among anaerobic hyperthermophilic archaeon proteins, and (2) it lacks a carbohydrate-binding domain, which is necessary for effective hydrolysis of cellulose. We first examined the relationship between the disulfide bonds and the catalytic activity by analyzing various cysteine mutations. The activities of the mutated enzymes toward carboxy methyl cellulose (CMC) increased without any loss in thermostability. Second, we prepared a fusion enzyme so that the thermostable chitin-binding domain of chitinase from *P. furiosus* was joined to the C-terminus of EGPh and its variants. These fusion enzymes showed stronger activities than did the wild-type EGPh toward both CMC and crystalline cellulose (Avicel).

Keywords Endoglucanase · Hyperthermophile · Site-directed mutagenesis · Active site · Chitinase · Disulfide bond

Introduction

Cellulases are some of the most important industrial enzymes. In particular, cellulases are already used in large quantities in the textile industry for the bio-polishing of cotton products, which is essential to remove excess fibers and to provide a soft texture. There are many additional potential uses of cellulases, e.g., for the treatment of biomass to produce sugars that can be converted into bioethanol. Cellulases can also be used to treat waste water (Bhat and Bhat 1997).

Various endoglucanases and their genes from eukaryotes and bacteria have been investigated. However, studies on archaeal endoglucanases have been limited. A few β-1,4 endoglucanases (endo-type cellulases) have been identified in the genome sequences of several hyperthermophilic archaea. A hyperthermostable endo-type cellulase (family 12) obtained from *Pyrococcus furiosus* showed no activity toward crystalline cellulose (Bauer et al. 1999). However, we have recently found that a hyperthermostable endo-type cellulase, EGPh, from *P. horikoshii* exhibited hydrolyzing activity toward crystalline cellulose (Kashima et al. 2005).

The amino-acid sequence of EGPh exhibits 43% homology with that of the endoglucanase (pdb:1ECE) from *Acidothermus cellulolyticus* (Ando et al. 2002; Sakon et al. 1996), suggesting their 3D structures are similar. We focused on the four conserved cysteine (Cys) residues (at positions 106, 159, 372, and 412) that form disulfide bonds in endoglucanase from *A. cellulolyticus*. The disulfide bonds in this enzyme have been thought to be essential for its thermostability (Matsumura et al. 1989). However, it has been reported that the disulfide bonds are not compatible with high temperatures of around 100°C (Volkin and Klibanov 1987). Thus, we constructed various Cys mutants to examine whether the four Cys residues in fact form disulfide bonds and if so, whether disulfide bond formation contributes to the hydrolyzing activity and/or hyperthermostability of EGPh. Here, we report the effects of disruption of the disulfide bonds by the Cys mutations on thermostability and hydrolyzing activity.

The amino acid sequence of EGPh also suggests that it lacks the cellulose binding domain (CBD). We expected that a fusion protein that was fused with a hyperther-

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H.-J. Kang · K. Uegaki · K. Ishikawa (✉)
National Institute of Advanced Industrial Science and Technology (AIST), 1-18-31, Midorigaoka, Ikeda
Osaka 563-8577, Japan
E-mail: kazu-ishikawa@aist.go.jp
Tel.: +81-72-7519526
Fax: +81-72-7519628

H. Fukada
Graduate School of Life and Environmental Sciences,
Osaka Prefecture University, Sakai, Osaka 599-8531, Japan

mostable CBD would have increased hydrolyzing activity at high temperatures. However, at present, no hyperthermostable CBD is known. Instead of a CBD, we therefore attempted the utilization of the hyperthermostable chitin binding domain (ChBD1 and 2) of chitinase from *P. furiosus* (Nakamura et al. 2005; Oku et al. 2006). The structure of the ChBD2 (pdb:2CWR, 2CZN) is similar to the carbohydrate–binding module (CBM) 2 that is classified in the CAZy database (http://www.afmb.cnrs-mrs.fr/CAZY/) (Coutinho and Henrissat 1999). ChBD2 possesses the potential to bind not only chitin but also cellulose (in preparation). The study of the structure of ChBD1 is now in progress. As we previously reported, the activity of EGPh was increased for a fusion protein, EG*Pf*ChiCBM, which was constructed from EGPh and ChBD2 of chitinase from *P. furiosus* (Kashima et al. 2005). By the same analogy to this previous research, in this study we constructed a fusion protein comprising EGPh with Cys mutations, and ChBD1 and 2. Further, we report an improved hydrolyzing activity using this fusion protein with ChBD1 and 2.

Materials and methods

Construction and preparation of mutant and fusion proteins

Three mutations, C106/159/372/412A, C106/159A, and C372/412A (Cx/yA indicates that Cys x and Cys y were changed to Ala), were created by means of site-directed mutagenesis, which was performed by the PCR method (Mullis et al. 1986) using wild-type endoglucanase from *P. horikoshii* (EGPh, WT) as a template. The resulting mutated DNA fragments were digested with *Nde*I, *Kpn*I, and *Bam*HI, and then inserted into the corresponding site of the vector pET11a (Novagen, Madison, WI, USA). All vectors carrying the mutations were transformed into *Escherichia coli* XL-2 Blue (Stratagene, La Jolla, CA, USA).

We constructed a fusion protein (WT–ChBD1 and 2) using wild-type EGPh, and ChBD1 and ChBD2 of chitinase from *P. furiosus* (Chhabra and Kelly 2002; Oku et al. 2006). The sequences of ChBD1 (PF1234) and ChBD2 (PF1233) of *P. furiosus* are available at http://www.gib.genes.nig.ac.jp/single/index.php?Spid = PfurDSM3638. A plasmid containing EGPh was digested with *Bam*HI and *Eco*RI, and then ligated with the PCR fragment of the ChBD1 and 2 regions digested with the same restriction enzymes (Fig. 1).

The constructed plasmids were introduced into *E. coli* strain BL21(DE3)pLysS (for expression of Cys mutated enzymes) or Rosetta (DE3) (for expression of ChBD fusion enzymes). Each transformant was cultured in LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37°C until the OD600 reached 0.2, and then isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added (0.01 mM). Purification of the recombinant enzymes was carried out by the method reported previously (Kashima et al. 2005).

Determination of the number of free SH residues

Free SH residues of the enzymes were determined by the method of Ellman (1959). Briefly, for quantitative determination of free SH residues the proteins were dissolved in 0.1 M Tris HCl (pH 8.5) containing 6 M guanidine HCl (Gdn-HCl), and after incubation for 1 h at 85°C, 4 mM EDTA and 1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were added in solution with and without 10 mM DTT. Just before analysis, the residual DTT was removed with a PD-10 column (Sephadex G-25 M) (Pharmacia, Uppsala, Sweden). The absorbance of 4-nitrophenylthiolate at 412 nm was measured for 1 h at 37°C to determine the number of free SH residues, using an extinction coefficient of 13,600 M^{-1} cm^{-1} (Ellman 1958).

Enzyme assay

The hydrolytic activities of the enzymes toward 0.5% high-viscosity carboxy methyl cellulose (CMC) (Wako Pure Chemicals) and 0.5% Avicel (Merck, Darmstadt, Germany) were determined from the amounts of reducing sugars released, using the modified Somogi-Nelson method (Hiromi et al. 1963) at 85°C in 100 mM acetate buffer (pH 5.6). Avicel was prepared by swelling in 85% phosphoric acid, and then by removing the phosphoric acid by filtration and centrifugation according to the method of Wood (1988). One unit of activity is defined as 1 μmol of glucose equivalent released per min. Hydrolytic activity toward *p*-nitrophenyl cellobiose (G2-PNP) and *p*-nitrophenyl cellotetraoside (G4-PNP) (Sigma, St Louis, MO, USA) was measured spectrophotometrically at 420 nm from the absorbance of *p*-nitrophenol (PNP) liberated from G2-PNP and G4-PNP at 50°C in 100 mM acetate buffer (pH 5.6). The K_m and k_{cat} were determined using the non-linear least squares method (Wilkinson 1961).

The optimal temperatures and pH of the enzymes were examined using CMC as the substrate. The pH and buffers used were: pH 4–6 (50 mM acetate buffer) and pH 7–8 (50 mM phosphate buffer).

Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were carried out using a nanoDSCII instrument (Calorimetry Sciences Corp., UT, USA) with platinum tubing cells with a volume of 0.3 ml. Proteins were dialyzed against 50 mM phosphate buffer, pH 7.0. The dialysate buffer was used as a reference solution for the DSC scanning. Samples containing 1.0 mg/ml of protein were heated at 1°C/min from 25 to 125°C.

Results

Preparation of recombinant enzymes

In order to examine the effects of replacing Cys with Ala on the conformational stability and catalytic activity of EGPh, we constructed three mutated enzymes (C106/159/372/412A, C106/159A, and C372/412A). The replacement of Cys with Ala is the most conservative natural replacement for cystine (Klink et al. 2000). Each of the ChBDs of *P. furiosus* can bind crystal chitin and their structure is similar to that of the CBDs. These ChBDs exhibited some affinity for cellulose (Uegaki et al. in preparation). We anticipated that the fusion of EGPh with ChBD1 and 2 would increase the binding affinity for cellulose as well as the apparent activity toward cellulose. New recombinant fusion enzymes were constructed; WT–ChBD2, in which 11 amino acid residues at the N-terminus of ChBD2 were removed, and WT–ChBD1 (Fig. 1). Based on the findings that the mutated forms of EGPh exhibited improved enzymatic activity after replacement of the Cys residues and also after fusion with ChBD, we constructed mutated fusion proteins, C372/412A–ChBD2 and C106/159/372/412A–ChBD2, in which some Cys residues in EGPh–ChBD2 were replaced with Ala.

Determination of the number of SH residues in the enzymes

EGPh was not unfolded even in the presence of 6 M Gdn-HCl at room temperature, judging from CD spectra (data not shown), and in this unfolded condition no free SH was observed using DTNB. To determine whether this result really reflected the existence of disulfide bonds or merely indicated that the thiol groups were inside the protein core, it was necessary to incubate the EGPh in 6 M Gdn-HCl at 85°C for 1 h. After the heat-treatment, EGPh was unfolded (judging from CD spectra), and the presence of 1.9 mol of SH per mol of EGPh protein was determined, while in the presence of DTT, 4.7 mol of SH was detected per mole of EGPh. These results suggest that there is one disulfide bond and two free Cys residues in recombinant EGPh. To identify the position of the disulfide bond, we analyzed Cys-mutated enzymes. After the heat-treatment in the presence of 6 M Gdn-HCl to unfold the protein, no free SH residues in the C372/412A mutated structures were detected (no DTT added), while 2.1 mol of SH were detected per mole of C106/159A mutated structure (no DTT added). Therefore, these results strongly suggested that EGPh has one disulfide bond between C106 and C159.

Characteristics of the recombinant enzymes

Activity toward G2-PNP

The kinetic parameters of K_m and k_{cat} determined using G2-PNP are shown in Table 1. G2-PNP and G4-PNP are water-soluble substrate analogues useful for characterizing the activity center of cellulase (Percival Zhang et al. 2006). The K_m, k_{cat}, and k_{cat}/K_m values for G2-PNP of the Cys-mutated enzymes and fusion enzymes were similar to those of WT. This result indicates that

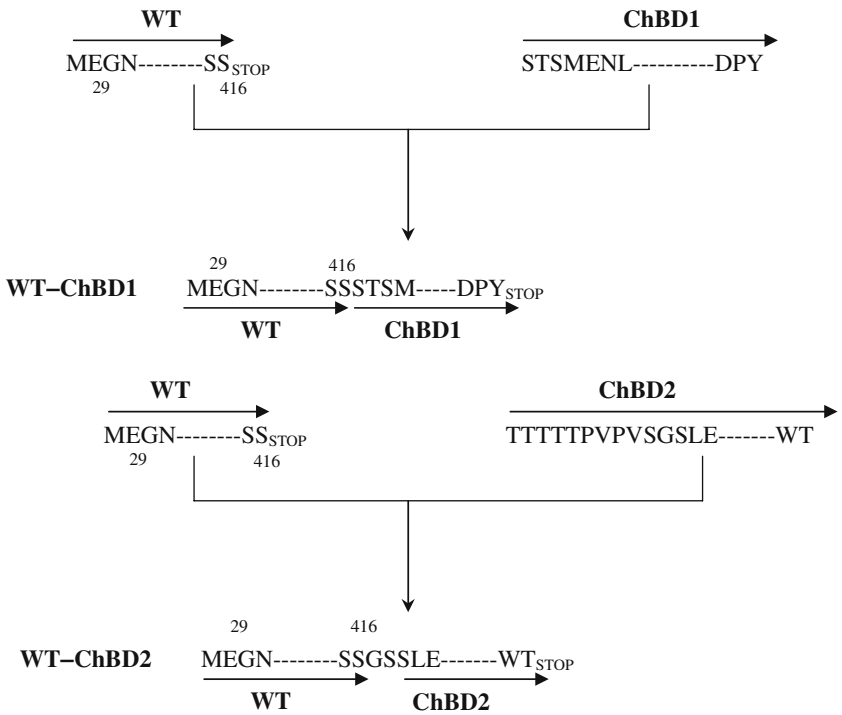

Fig. 1 Construction of fusion proteins WT–ChBD1 and WT–ChBD2 using the endoglucanase from *P. horikoshii* (WT) and the chitin binding domain (ChBD) of chitinase from *P. furiosus*. Eleven amino residues have been removed from the N-terminus of ChBD2 (Oku and Ishikawa 2006)

Table 1 Enzymatic parameters of the recombinant enzymes with G2-PNP

Enzyme	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m
WT	0.95 ± 0.33	0.41 ± 0.05	0.43 ± 0.15
C106/159/372/412A	0.78 ± 0.06	0.47 ± 0.01	0.60 ± 0.04
C106/159A	0.46 ± 0.16	0.24 ± 0.02	0.54 ± 0.18
C372/412A	0.35 ± 0.02	0.43 ± 0.01	1.23 ± 0.07
WT–ChBD1	0.97 ± 0.31	0.43 ± 0.03	0.44 ± 0.14
WT–ChBD2	0.98 ± 0.21	0.69 ± 0.03	0.70 ± 0.15
C372/412A–ChBD2	0.89 ± 0.09	0.58 ± 0.04	0.65 ± 0.07
C106/159/372/412A–ChBD2	0.42 ± 0.15	0.51 ± 0.04	1.21 ± 0.44

Activity toward G2-PNP was measured at 50°C. The increase in absorbance at 420 nm due the production of *p*-nitrophenol was measured. The concentration of *p*-nitrophenol was calculated by comparison with the standard curve for a *p*-nitrophenol solution in 100 mM acetate buffer (pH 5.6). The k_{cat} values were determined from the initial velocity measured using the non-linear least squares method (Wilkinson 1961)

the active center of EGPh is not influenced by replacement of Cys with Ala and by the addition of ChBD2. The hydrolytic activities toward G4-PNP (10 mM) for releasing PNP were determined to be 0.029, 0.017, and 0.005 mM min^{-1} mg^{-1} for WT, C106/159/372/412A, and WT–ChBD2, respectively. However, for G4-PNP, the cleavage site is not just at one position and some products play a role in inhibition of this enzyme (data not shown). Therefore, we were not able to determine the values of K_m and k_{cat} for G4-PNP.

Activity toward CMC

The activity toward the soluble cellulosic substrate CMC, measured by the Somogy-Nelson method, is shown in Fig. 2. The activity was increased for all of the recombinant enzymes. The activities of C106/159/372/412A, C106/159A, and C372/412A were increased by approximately 2.1-, 1.7-, and 1.6-fold, respectively. The replacement of Cys 106 and 159 with Ala, and of Cys 372 and 412 with Ala, both enhanced the activity considerably. However, the activity increment was largest when the four Cys were replaced with Ala. The activities of the fusion proteins WT–ChBD1 and WT–ChBD2 were increased by 1.2-, and 1.9-fold, respectively, as compared to that of WT. Based on the above results, we further constructed mutated fusion proteins C372/412A–ChBD2 and C106/159/372/412A–ChBD2. As shown in Fig. 2, the activities of C372/412A–ChBD2 and C106/159/372/412A–ChBD2 were increased relative to WT by about 1.7- and 2.3-fold, respectively. The 2.3-fold increase is the highest among the results obtained here. The synergistic effect on the hydrolytic activity was not observed by Cys replacement with Ala and fusion with ChBD.

The optimal temperatures and pH for the activity were examined using CMC. The optimal temperature for most of the recombinant enzymes was determined to be 95°C, however, that of WT–ChBD2 and C106/159/372/412A was decreased to 90°C. The optimal pH was between 5.5 and 6.0 for most of the enzymes, which was the same as that of WT. However, the optimal pH range for WT–ChBD2 was between pH 5 and 7, slightly wider than that of WT.

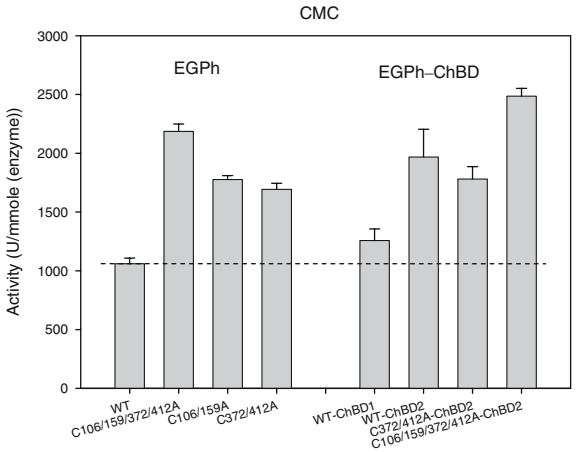

Fig. 2 Activities of the wild-type and recombinant enzymes toward CMC. Activities were measured at 85°C in 100 mM acetate buffer (pH 5.6), using 0.5% CMC as the substrate. The activity per 1 mmol of the enzyme was determined

Activity toward Avicel

Figure 3 shows the activities toward Avicel, an insoluble microcrystalline cellulose substrate. The activities of the Cys-mutated enzymes were found to be decreased as compared with that of WT. However, the activities of the fusion enzymes WT–ChBD1 and WT–ChBD2 increased, WT–ChBD2 exhibiting the highest activity; about twofold of that of WT. This is an impressive result showing the effects of introducing the chitin-binding domains. On the other hand, the activities of the mu-

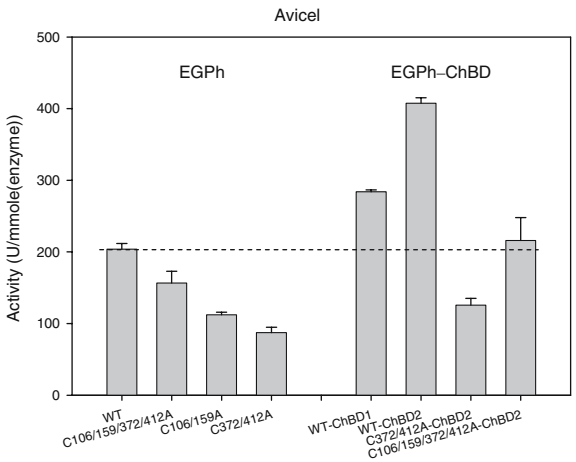

Fig. 3 Activities of the wild-type and recombinant enzymes toward Avicel, representing crystalline cellulose. Activities were measured at 85°C in 100 mM acetate buffer (pH 5.6), using Avicel. The activity per 1 mmol the enzyme was determined

tated fusion proteins C372/412A–ChBD2 and C106/159/372/412A–ChBD2 did not show remarkable increases.

Thermostability of the enzymes

Figure 4 shows a DSC thermogram for the recombinant enzymes at pH 7.0. The peak temperature (T_m) values for C106/159/372/412A, C106/159A, and C372/412A decreased to 95.0–96.5°C from 100.1°C for WT. The small difference in T_m showed that the thermostability of the recombinant enzymes was not substantially altered by the replacement of Cys with Ala. Even if Cys residues were removed, the hyperthermostability, as judged by DSC, was not greatly altered. As distinct from the case of mesophilic enzymes, however, the effect of the disulfide bond is not always significant on the thermostability (Kanaya et al. 1991; Wakarchuk et al. 1994). A factor other than the disulfide bond must be involved in the thermostability of these hyperthermophilic enzymes.

The enthalpy of heat denaturation ΔH of WT, C106/159/372/412A, C106/159A, and C372/412A was 23, 18, 23, and 17 J/g, respectively. The ΔH of proteins with mutations on Cys 372 and 412 was therefore lower than ΔH of WT or C106/159A proteins. The removal of the disulfide bond (C106/159A protein) induces only a small decrease in the denaturation temperature but does not affect ΔH. Cys 372 and 412 are not involved in a disulfide bond, but these results suggest that they are critical in maintaining the hyperthermostability of the enzyme.

Discussion

By amino acid sequence comparisons between EGPh and endoglucanase from *A. cellulolyticus*, and inference from the structure of the *A. cellulolyticus* endoglucanase

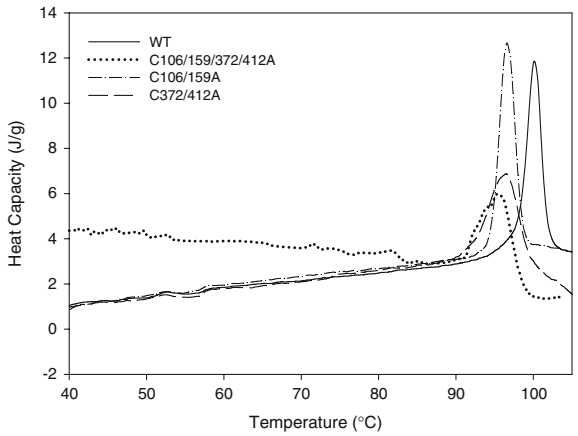

Fig. 4 Differential scanning calorimetry of the recombinant enzymes. The protein concentration of enzymes was 1 mg/ml in 50 mM sodium phosphate, pH 7.0. The T_m values of WT, C106/159/372/412A, C106/159A, and C372/412A were 100.1, 95.0, 96.5, and 96.5°C, respectively. The ΔH of WT, C106/159/372/412A, C106/159A, and C372/412A were 23, 18, 23, and 17 J/g, respectively

(Sakon et al. 1996), we surmised that two disulfide bonds would be formed in EGPh by Cys 106 and Cys 159, and by Cys 372 and Cys 412. However, we identified only one disulfide bond between Cys 106 and Cys 159. This result suggests that the tertiary structure of EGPh would be somewhat different from that of endoglucanase from *A. cellulolyticus*, even though they share a high sequence homology. It is worth noting that *P. horikoshii* grows under extensively anaerobic conditions. We wonder whether the disulfide bond is actually formed under these anaerobic conditions, and whether it is really required for stability and activity. As shown in Fig. 4, the Cys mutations did not change the thermal stability, and this indicated that the disulfide bond is not involved in thermal stability. Compared with WT, the activity of the Cys-mutated proteins toward CMC increased, whereas the K_m and k_{cat} determined using G2-PNP were almost the same as those of WT. The activity toward Avicel was not influenced by the mutations. These results indicate that the Cys residues as well as the disulfide bond do not participate in the active center. The binding affinity toward the crystalline cellulose does not seem to be improved by the mutations. However, the mutation of Cys residues to Ala was effective in increasing activity toward a soluble polymer substrate (CMC). This indicates that the substrate binding cleft around the active site may change into a more flexible conformation, to form a suitable E–S complex for the soluble polymer substrate.

From the results for the fusion enzymes, the addition of ChBD should cause an increase in the hydrolytic activity toward cellulosic substrates. An endoglucanase (TmCel74) from *Thermotoga maritima* was reported to lack a carbohydrate-binding module (CBM) (Chhabra and Kelly 2002). The hydrolytic activity of TmCel74 was increased by adding CBM of chitinase from *P. furious*. This is consistent with our finding of the effects of adding ChBD on the activity toward crystalline cellulose (Avicel). The binding affinity of EGPh for the polymer substrate seems to be amplified by the presence of ChBD. It has also been reported that the hyperthemophilic chitinase from *P. furiosus* exhibits improved activities when chitin-binding domains (ChBD1 and ChBD2) are added to the catalytic domains (AD1 and AD2) (Oku and Ishikawa 2006). In addition, the activity of EGPh was increased by adding ChBD2 (Kashima et al. 2005). Recently, the structure of ChBD2 was resolved by NMR and X-ray crystallography (Nakamura et al. 2005; in preparation), and structural analysis of ChBD1 is progressing. From the structural analysis of ChBD2, the N-terminus region (11 amino acid residues) of ChBD2 was found to be a flexible region. This seems to be related to a vulnerable region where proteases may attack the fusion enzyme. The new fusion enzyme we report here, which lacks these 11 amino acid residues, was found to exhibit sufficient stability in long storage.

On adding ChBD1 or ChBD 2, the k_{cat} of EGPh for G2-PNP and the activities toward CMC and Avicel were found to increase, however, the effect of ChBD2 was much higher than that of ChBD1. This showed that the

effect of ChBD on the activity increase was different according to the type of ChBD. Also, it indicates that the construction of a fusion protein using ChBD from another thermophilic endoglucanase represents a possible strategy for obtaining higher activity toward crystalline cellulose substrate.

In this study, we have examined two effects in EGPh: Cys replacement with Ala, and fusion with ChBD. The results obtained here suggest possible techniques for increasing the activities of cellulases lacking the carbohydrate-binding domain: (i) elimination of Cys residues or disulfide bonds, especially for an increase in activity toward soluble polymer substrates, and (ii) addition of a carbohydrate-binding domain, which can even be obtained from chitinase. We found that the removal of Cys did not substantially affect the hyperthermostability of the enzyme. We hope that the techniques presented here will be useful for improving EGPh and for extending its applicability.

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