Roles of Carbohydrate-Binding Module (CBM) of an Endo- β -1,4-Glucanase (Cel5L) from *Bacillus* sp. KD1014 in Thermostability and Small-Substrate Hydrolyzing Activity^S

Jae Pil Lee¹, Eun-Sun Shin², Min Yeol Cho², Kyung-Dong Lee³, and Hoon Kim^{1,2*}

¹Department of Pharmacy, and Research Institute of Life Pharmaceutical Sciences, Suncheon National University, Suncheon 57922, Republic of Korea

²Department of Agricultural Chemistry, Suncheon National University, Suncheon 57922, Republic of Korea

³Department of Oriental Medicine Materials, Dongshin University, Naju 58245, Republic of Korea

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*Corresponding author
Phone: +82-61-750-3751;
Fax: +82-61-750-3708;
E-mail: hoon@sunchon.ac.kr

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An endo- β -1,4-glucanase gene, *cel5L*, was cloned using the shot-gun method from *Bacillus* sp.. The gene, which contained a predicted signal peptide, encoded a protein of 496 amino acid residues, and the molecular mass of the mature Cel5L was estimated to be 51.8 kDa. Cel5L contained a catalytic domain of glycoside hydrolase (GH) family 5 and a carbohydrate-binding module family 3 (CBM_3). Chromatography using HiTrap Q and CHT-II resulted in the isolation of two truncated forms corresponding to 50 (Cel5L-p50) and 35 kDa (Cel5L-p35, CBM_3-deleted form). Both enzymes were optimally active at pH 4.5 and 55°C, but had different half-lives of 4.0 and 22.8 min, respectively, at 70°C. The relative activities of Cel5L-p50 and Cel5L-p35 for barley β -glucan were 377.0 and 246.7%, respectively, compared to those for carboxymethyl-cellulose. The affinity and hydrolysis rate of pNPC by Cel5L-p35 were 1.7 and 3.3 times higher, respectively, than those by Cel5L-p50. Additions of each to a commercial enzyme set increased saccharification of pretreated rice straw powder by 17.5 and 21.0%, respectively. These results suggest CBM_3 is significantly contributing to thermostability, and to affinity and substrate specificity for small substrates, and that these two enzymes could be used as additives to enhance enzymatic saccharification.

Keywords: *Bacillus* sp. KD1014, GH5 endo- β -1,4-glucanase, truncated enzymes, CBM, thermostability, saccharification

Introduction

Cellulosic biomasses are of particular interest as valuable raw materials for the production of cellulosic ethanol [1]. Complex polysaccharides can be hydrolyzed by cellulases such as endo- β -1,4-glucanase, exo- β -1,4-glucanase, and β -glucosidase. Among them, endo- β -1,4-glucanases, which are grouped into 13 glycoside hydrolase (GH) families; 5–9, 12, 44, 45, 48, 51, 74, 124, and 131 [2], play important roles in hydrolysis as they cleave internal glycosidic bonds in the chains [3]. Most of the enzymes grouped in GH families 5–9, 12, 44, 45, and 48 are cellulases acting on cellulose [4]. Of these, GH5 is the largest with more than 12,000 sequences

in the Carbohydrate-Active Enzyme (CAZy) database (<http://www.cazy.org>) that are divided into 54 subfamilies. Most GH5 enzymes exclusively display endocellulase activity, but a few are exocellulases [5]. Based on published data, more than 60% of GH proteins are single proteins without non-catalytic accessory domains, such as carbohydrate-binding modules (CBMs), and the rest were multidomain proteins with CBMs [6]. CBMs are classified into 83 families (<http://www.cazy.org>), and CBM2, 3, and 6 are targeting cellulose [6].

Improvements in saccharification processes have been studied intensively in the contexts of lignocellulosic biorefinery processes and the production of lignocellulosic

biofuels. Key process steps, such as the pretreatment of biomasses and enzymatic hydrolysis by enzyme cocktails and additives have been well investigated [1, 7]. In addition, the enhancement of the enzymatic saccharification of cellulose processes using synergistic proteins including CBMs, has also been studied [8].

Several GH5 endo- β -1,4-glucanases cloned from *Bacillus* sp. have been characterized [9–13]. In the present study, cloning of an endo- β -1,4-glucanase, belonging to GH5 with a CBM_3, from *Bacillus* sp. KD1014 isolated from soil was conducted. Two truncated forms of the enzyme were purified, and CBM_3 roles in substrate specificity, thermostability, and carbohydrate binding ability were investigated. Finally, their abilities to enhance saccharification were analyzed using pretreated biomasses.

Materials and Methods

Chemicals and Enzymes

Lichenan (Icelandic moss) and commercial enzymes (Novozym 188 and Celluclast 1.5L) were obtained from Megazyme (Wicklow, Ireland) and Novozyme (Bagsvaerd, Denmark), respectively. Substrates such as carboxymethyl-cellulose (CMC) and *p*-nitrophenyl- β -D-cellobioside (*p*NPC), and other chemicals were from Sigma-Aldrich (USA).

Cloning of a CMCase Gene and Analysis of the Gene

For shot-gun cloning, genomic DNA was isolated from *Bacillus* sp. KD1014, which was previously isolated from soil [14]. Cloning of a gene for a CMC-degrading enzyme was conducted as previously described [15], except for using LBCT (LB containing 0.5% CMC and 0.008% trypan-blue) agar plates for tooth-picked transformants instead of the step for Congo red staining. The conserved regions were analyzed using BLAST at NCBI (<http://www.ncbi.nlm.nih.gov>). Putative signal peptide was predicted by SignalP 4.1 in CBS (<http://www.cbs.dtu.dk/services/SignalP/>) [16]. The molecular mass and multiple alignment of the encoded protein were analyzed using DNA/MAN (Lynnon Biosoft, version 4.11, Canada).

Purification of the Enzyme

The enzyme was isolated from the selected clone as previously described [15] with slight modification. Briefly, the crude extract was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) and passed through a HiTrap Q column (5 ml, GE Healthcare, Sweden) using a linear NaCl gradient (0–1.0 M). Active fractions were further purified using a CHT-II column (5 ml, Bio-Rad, USA) using a sodium phosphate buffer gradient (10–500 mM, pH 6.8). Protein concentrations were determined using the Bradford method [17], and the protein was analyzed by SDS-PAGE using 11% polyacrylamide gels [18].

Enzyme Assay

Enzyme activity was analyzed using 0.5% CMC in 50 mM sodium citrate buffer (pH 5.5), as described previously [19], but at 50°C. The amount of reducing sugar released was determined using the dinitrosalicylic acid (DNS) method [20]. To investigate the substrate specificity of Cel5L, activities were determined using 0.5% barley β -glucan, birchwood xylan, lichenan or laminarin. Its hydrolyzing activities for filter paper (1 cm $\times$ 1 cm strip) and Avicel (0.5%) were observed after 2 h at 50°C. Hydrolyzing activities for *p*NPG and *p*NPC were assayed using 2.0 mM of each substrate at 50°C. One unit of hydrolyzing activity was defined as the amount of enzyme liberating 1 μ mol of reducing sugar or *p*NP per min.

Activity Staining

Enzyme activity was observed by activity staining after SDS-PAGE, as described previously [15] with some modifications. Briefly, proteins in crude extract or fractions were separated in 11.0% SDS-polyacrylamide gels containing 0.2% CMC. Gels were then washed with 10% isopropanol for 30 min and then cleaned three times with the assay buffer (pH 5.5) for 30 min, incubated at 50°C for 0.5–1 h, stained with 0.1% Congo-red, and washed with 1 M NaCl.

Characterization of Enzymes

Optimum temperature for the enzymes was determined by measuring enzyme activities at designated temperatures and thermostability was analyzed after pre-incubation of the enzymes [21]. Optimum pH levels for enzyme activity were determined using 50 mM sodium citrate (pH 3.5–6.0) buffer. The influences of various cations on enzyme activity were determined as described previously [21].

Binding with Insoluble Avicel

Binding abilities of enzymes to carbohydrate polymer were analyzed as described previously with slight modification [22]. Briefly, the purified enzymes were added to 1.5% Avicel solution in 0.3 ml of the assay buffer (pH 5.5), mixed with shaking for 40 min on ice and centrifuged at 15,000 $\times$ g for 10 min to remove enzymes bound to Avicel. Amounts of unbound enzymes were determined by measuring cellulase activity in supernatants.

Saccharification of Pretreated Biomasses

Enzymatic hydrolyses of reed (*Phragmites communis*) and rice (*Oryza sativa*) straw powders were carried out as previously described [15], but with purified enzymes rather than crude extracts. Briefly, pretreated reed or rice straw powders were suspended at 6% in 5 ml of 50 mM sodium citrate buffer (pH 5.5) in 50 ml tubes. The concentrations of the enzymes used were 0.2 U/ml of Celluclast 1.5 L; 0.2 U/ml of Novozym 188; and 0.33 U/ml of Cel5L. Reactions were carried out for up to 72 h at 50°C with shaking at 150 rpm. Aliquots of the mixtures were withdrawn at designated times (0, 6, 12, 24, 48, and 72 h) and

centrifuged for 10 min at 12,000 ×g. Amounts of soluble sugar released were measured using the DNS method [20]. All experiments were performed in duplicate.

Results and Discussion

Cloning and Characterization of the CMCase Gene

One CMCase positive clone, ES 8-10, was selected on LBCT agar plates. In the insert DNA of 2.58 kb, an open reading frame (ORF) of 1,491 bp was identified as an endo-β-1,4-glucanase gene with high similarities to glycosyl hydrolase family 5 (GH5) enzymes and named *cel5L*. The gene was identified to encode a protein of 496 amino acid residues with a signal peptide of 29 amino acid residues. The molecular mass of mature Cel5L was calculated to be 51.8 kDa. A comparison with previously reported enzymes revealed a protein sequence with high similarity to CMCase of *B. subtilis* BSE616 (98%) (GenBank Accession No. D01057.1, BAA00859.1) [9], 97% similarity to the endoglucanase (Cel5A) of *B. subtilis* BME-15 (FJ172348, ACI15227) [23], 92% similarity to the endoglucanase of *B. subtilis* DLG (M16185, AAA22496.1) [24], and 62% similarity to the endoglucanase (Egl) of *Bacillus* sp. KSM-N252 (AB057671.1, BAB62295) [25]. Cel5L was found to be modular endoglucanase to contain a catalytic domain (residues 50–293), to belong to glycoside hydrolase family 5 (GH5), and to possess one CBM_3 domain (353–434th) at the C-terminal end of the catalytic domain (Fig. 1A).

Cel5L was found to belong to GH5 subfamily 2 (GH5-2), in which the endoglucanases of *B. subtilis* are grouped. Several residues are conserved in the GH5 catalytic domain of Cel5L, that is, catalytic residues Glu169 and Glu257, which act as proton donor and nucleophile, respectively, and Trp69, Arg92, His131, Asn168, Trp207, and Tyr231 residues [13, 23, 26, 27] (Fig. 1B). When other subfamilies of GH5, *i.e.*, GH5-1 endoglucanase (Cel5B) of *Thermobifida fusca* TM51 (AY298814, AAP56348) and GH5-4 bifunctional cellulase of *B. halodurans* TSLV1 (the same as BAB04322 cited by 28) were blasted together, Arg92, His131, Asn168, and Tyr231 and the two catalytic residues were found to be

strictly conserved (Fig. 1B). In addition, Val126, Pro170 in the NEP motif, Asn175, Asp221, Gly259 in the EWG motif, Phe270, and Trp291 residues were also found to be conserved in the catalytic domain.

The CBM of Cel5L was linked to the C-terminus of the GH5 catalytic domain, as is likely the case with most GH5 cellulases [29]. In some GH5 cellulases, two CBMs are linked to the catalytic domain, such as in Cel5A of *Bacillus* sp. 1139, which contains CBM17 and 28 in tandem [30].

Cel5L expressed in *E. coli* was predicted to have the same signal peptide as that in wild-type *Bacillus* sp. by SignalP 4.1 analysis, and based on this prediction, the molecular mass of mature Cel5L was 51.8 kDa.

The nucleotide sequence of *cel5L* has been deposited in the GenBank under the accession number MH923517.

Purification of Two Truncated Enzymes

Cel5L was purified from the transformant using HiTrap Q and CHT-II chromatography. The amount of enzyme bound by the High-Q column was much less than that bound by the HiTrap Q column (data not shown). When proteins in extract were separated using the HiTrap Q column, one activity peak was observed (Fig. S1), and when pooled active fractions were further purified using the CHT-II column, two partially resolved activity peaks were observed (Fig. S1). To check the enzyme species, CMCase activities were analyzed using a crude extract of the transformant by activity staining after SDS-PAGE and renaturation methods. Two active bands were observed, and these proteins were named P1 and P2, respectively (Fig. 2A). Enzyme activity of P1 was greater than that of P2. The active bands observed represented truncated forms produced by internal proteolytic cleavages, as has been reported for other heterologous gene expressions [15, 31–34]. Fractions eluted from the CHT-II column revealed P2 was eluted before P1, which corresponded to molecular masses of 35 and 50 kDa, respectively. Both forms were pooled and named Cel5L-35 and Cel5L-50, respectively (Fig. 2B). The two forms were nearly homogeneous, though Cel5L-35 appeared along with minor bands. The average

Table 1. Purification of two truncated endo-β-1,4-glucanases in the transformant cloned from *Bacillus* sp. KD1014.

Procedure	Enzyme	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Supernatant		5.0	17.8	212.7	11.9	1.0	100
Hi-Trap chromatography		7.5	0.82	54.7	67.0	5.6	25.7
CHT-II chromatography	Cel5L-p50	2.0	0.10	11.6	110.5	9.3	5.4
	Cel5L-p35	3.5	0.14	11.8	86.2	7.2	5.5

Fig. 1. Putative conserved domains of Cel5L (A) and multiple alignment of the amino acid sequences of Cel5L and other endo- β -1,4-glucanases (B).

Asterisks represent catalytic residues. Closed and open red circles represent conserved amino acid residues in GH5-2 members and in GH5-1 and GH5-4 blasted together, respectively. Closed triangles represent conserved residues. The inverted long triangle indicates the tentative cleavage site of a signal peptide in Cel5L as determined by SignalP4.1. Conserved motifs are represented as orange boxes. CBM_3, carbohydrate binding module_3.

Fig. 2. Staining of enzyme activities after SDS-PAGE and renaturation of fractions eluted by CHT-II chromatography (A), and SDS-PAGE of the purified truncated enzymes (B). (A): C represents crude extract. Numbers at the tops of figures represent fraction numbers. (B): M, molecular weight markers; C, crude extract of the clone ES 8-10; 1, active fractions pooled from HiTrap chromatography; 2 and 3, active fractions corresponding to Cel5L-p35 and Cel5L-p50, respectively, obtained by CHT-II chromatography. For activity staining, the gel was made by adding 0.5% CMC, whereas the SDS-PAGE gel was made without CMC to enable accurate molecular mass determination.

specific activities of Cel5L-p50 and Cel5L-p35 were 110.5 and 86.2 U/mg protein, respectively (Table 1). Based on estimated molecular masses, it was predicted Cel5L-50 and Cel5L-35 were composed of approximately 455 and 318 amino acid residues, respectively, *i.e.* 30–484th and 30–347th residues, respectively. Considering the size of the CBM_3 domain (353–434th), Cel5L-50 contained complete CBM_3, whereas Cel5L-p35 contained only a catalytic domain without entire CBM_3, presumably due to internal proteolytic cleavage, as has been reported for other endoglucanases in *E. coli* [15, 19, 35]. Actually, as the purification progressed, an extra minor band corresponding to a smaller molecular mass was observed by second column chromatography in activity stained gel (Figs. 2A and 2B). These truncation products have also been observed for other modular or multimodular enzymes [15, 31–34].

Substrate Specificities of the Truncated Enzymes

The relative activities of Cel5L-p50 and Cel5L-p35 for barley β-glucan (β-1,3-1,4-glucan) were 377.0 and 246.7%, respectively, as compared with CMC (β-1,4-glucan) (Table 2). These higher activities for barley β-glucan than CMC resembled those of GH5-2 endoglucanases [29,36] and a fungal GH5-5 cellulase GtCel5 of *Gloeophyllum trabeum* [2]. However, the two enzymes showed low but moderate hydrolytic activities, 35.0 and 32.7%, respectively, for lichenan (another type of β-1,3-1,4-glucan with a different ratio of β-1,3 to β-1,4). This result was similar to that of BICel5B of *B. licheniformis* [37], but differed from those of

BsCel5A of *B. subtilis* 168 and the GtCel5 of *G. trabeum*, which had higher lichenase activity than CMCase activity [2, 11], and the Cel5B of *T. fusca* with undetectable lichenases activity [38]. Both Cel5L-p50 and Cel5L-p35 showed low activities for laminarin (β-1,3-1,6-glucan), however, the activity of Cel5L-p35 was 2.8 times higher than that of Cel5L-p50 (71.6% and 25.2%, respectively). No or negligible activities were observed toward birchwood xylan (β-1,4-xylose), Avicel, or filter paper (Table 2), as has been reported for Cel5A of *B. subtilis* BME-15 and the GtCel5 [2,23], and the BsCel5A with undetectable Avicelase activity [29], but not for GH5 cellulase of *B. amyloliquefaciens* DL-3, which has significant Avicelase activity [10].

Table 2. Substrate specificities of Cel5L-p50 and Cel5L-p35.

Substrate	Relative activity (%)	
	Cel5L-p50	Cel5L-p35
CMC	100	100
Barley β-glucan	377.0 ± 0.63	246.7 ± 1.97
Lichenan	35.0 ± 0.65	32.7 ± 0.25
Laminarin	25.2 ± 0.73	71.6 ± 0.57
Xylan	ND	ND
Avicel	ND	6.9 ± 0.74
Filter	0.85 ± 0.18	1.4 ± 0.35
pNPC	58.9 ± 0.38	191.9 ± 1.04
pNPG	ND	1.5 ± 0.59

ND, not detectable

Fig. 3. Effects of pH (A) and temperature (B) on the enzyme activities of Cel5L-p50 and Cel5L-p35 and on their thermostabilities after preincubation at 60 and 70°C (C).

Enzyme activities were measured using 50 mM Na-citrate buffer.

Interestingly, Cel5L-p50 and Cel5L-p35 hydrolyzed a small substrate of *p*NPC with activities of 58.9 and 191.9%, respectively, indicating the hydrolysis of *p*NPC by Cel5L-p35 was 3.3 times higher than that by Cel5L-p50. Substrate specificities of the enzymes showed Cel5L was able to efficiently hydrolyze β -1,4 linkages of mixed-linked β -1,3-1,4-glucans such as barley β -glucan, but with strict β -1,3 to β -1,4 ratio sensitivity, which may have been due to the higher ratio of β -1,4 to β -1,3 linkages in β -glucans than in lichenans [39].

Enzymatic Properties of the Truncated Enzymes

Cel5L-p50 and Cel5L-p35 showed the characteristics of acidic enzymes with maximal CMCase activity at pH 4.5 (Fig. 3A), similar to an acidic cellulase Cel-1 of buffalo rumen metagenome [40], though Cel5L-p35 was more resistant to pH changes than Cel5L-p50. Regarding optimum temperature, Cel5L-p50 and Cel5L-p35 both exhibited maximal CMCase activities at 55°C and showed similar activity/temperature characteristics (Fig. 3B). At 70°C, the those of Cel5L-p50 and Cel5L-p35 were about half activities of maximal activities, 46.2 and 48.8%, respectively. When

compared with other GH5-CBM_3 cellulases, the optimum temperature of Cel5L-p50 and Cel5L-p35 was similar to that of Cel5A of *B. subtilis* BME-15 and the cellulase of *B. amyloliquefaciens* DL-3 (both 50°C). However, optimum pH was similar to that of Cel5A (pH 5.0), but quite different from that of the cellulase of *B. amyloliquefaciens* DL-3 (pH 7.0) [10, 23].

Cel5L-p50 and Cel5L-p35 retained 50.0 and 85.0% of their activities after 30 min of preincubation of each enzyme at 60°C (Fig. 3C). The half-lives of Cel5L-p50 and Cel5L-p35 activities were 4.0 and 22.8 min at 70°C, which were comparable to the half-life of Cel5B of *T. fusca* (~ 8 min) [38]. Truncation of CBM_3 by complete deletion from Cel5L increased thermal stability, as was observed for the endoglucanase of *B. subtilis* JA18 after deletion of CBM_3 [41]. During storage at 4°C, Cel5L-p50 proved to be less stable than Cel5L-p35 (data not shown).

The effects of mono- and divalent cations on Cel5L-p50 and Cel5L-p35 were not significant, with the exceptions of Mn^{2+} , Co^{2+} , Cu^{2+} , and Ba^{2+} (Table 3). Cel5L-p50 and Cel5L-p35 were inhibited by 40.8 and 50.0%, respectively, by Mn^{2+} at 5 mM, but the degree of this inhibition was not

Table 3. Effects of cations on the activities of Cel5L-p50 and Cel5L-p35.

Cation	Concentration (mM)	Relative activity (%)	
		Cel5L-p50	Cel5L-p35
Control		100	100
NaCl	2	46.1 ± 1.38	98.6 ± 0.62
	5	123.1 ± 0.61	116.1 ± 0.44
KCl	2	47.9 ± 1.10	90.9 ± 1.00
	5	124.8 ± 0.25	116.9 ± 0.44
MgCl ₂	2	58.0 ± 0.55	98.5 ± 0.69
	5	119.3 ± 0.86	112.2 ± 0.15
MnCl ₂	0.01	88.9 ± 1.07	88.9 ± 0.24
	0.02	73.4 ± 1.25	81.8 ± 1.43
	0.1	41.2 ± 0.15	48.2 ± 0.29
	0.5	50.7 ± 0.30	50.9 ± 0.59
	1	53.2 ± 0.45	49.6 ± 0.15
	2	42.6 ± 0.67	56.4 ± 0.80
	5	50.0 ± 1.34	59.2 ± 1.44
CaCl ₂	2	66.0 ± 1.38	96.8 ± 0.39
	5	100.9 ± 0.49	105.4 ± 0.15
CuCl ₂	2	160.9 ± 1.38	122.0 ± 0.62
	5	166.6 ± 0.74	142.6 ± 0.65
CoCl ₂	2	48.1 ± 0.83	61.6 ± 0.77
	5	49.4 ± 0.49	60.1 ± 0.07
BaCl ₂	2	79.2 ± 0.83	86.0 ± 0.46
	5	125.8 ± 0.98	112.9 ± 0.29
FeCl ₂	2	76.7 ± 1.10	83.7 ± 0.69
	5	97.4 ± 0.25	98.8 ± 0.51
ZnCl ₂	2	73.5 ± 0.55	87.9 ± 0.77
	5	106.0 ± 0.61	104.0 ± 1.02

concentration-dependent and exhibited a saturation pattern at 0.01–5.0 mM. The enzymes were inhibited by 33.3 and 39.2%, respectively, by Co²⁺ at 2 mM, and by 42.6 and 48.28%, respectively, at 5 mM. However, both enzymes were stimulated to 161.0 and 132.6, respectively, by Cu²⁺ at 2 mM, and increased to 215.3 and 176.7%, respectively, by Cu²⁺ at 5 mM. Finally, Cel5L-p50 activity was increased to 135.2% by Ba²⁺ at 5 mM. The inhibitions of Cel5L-p50 and Cel5L-p35 by Mn²⁺ and Co²⁺ were similar to those of the cellulase of *B. amyloliquefaciens* DL-3 [10], but unlike those of GH5 endoglucanase EG5C of *Paenibacillus* sp. IHB B 3084 [42], and the bifunctional GH5 cellulase of *B. halodurans* TSLV1 [28], which were enhanced by these ions. Activations of both enzymes by Cu²⁺ were similar to those of EG5C and the bifunctional cellulase [28,42]. Comparisons of cation

Table 4. Kinetic parameters of Cel5L-p50 and Cel5L-p35 for CMC and *p*NPC.

Enzyme	Substrate	K _m [*]	V _{max} (U/mg protein)
Cel5L-p50	CMC	0.77 ± 0.094	143.3 ± 8.2
	<i>p</i> NPC	0.33 ± 0.006	52.2 ± 0.7
Cel5L-p35	CMC	0.80 ± 0.094	164.6 ± 10.8
	<i>p</i> NPC	0.19 ± 0.02	180.0 ± 16.1

*CMC, %; *p*NPC, mM.

effects suggest the presence of CBM_3 had an insignificant impact on the effects of cations.

Kinetics of the Truncated Enzymes

The Lineweaver-Burk plots of Cel5L-p50 and Cel5L-p35 were linear (Fig. S2). K_m values for CMC were similar, 0.77 and 0.80%, respectively, and V_{max} values were 143.3 and 164.6 U/mg protein, respectively. K_m values for *p*NPC were 0.33 and 0.19 mM, and V_{max} values were 52.2 and 180.0 U/mg protein, respectively (Table 4). These results showed that differences between substrate affinities or specific activities of the two enzymes for polymeric substrates such as CMC were not significant. Nonetheless, Cel5L-p50 had 1.7 times lower substrate affinity and 3.4 times less specific activity than Cel5L-p35 for the monomeric substrate *p*NPC. We consider differences stemmed from the deletion of the CBM of Cel5L, which is composed of 80 amino acid residues from residues 353 to 434. On the other hand, the CBM-truncated endoglucanase of *B. subtilis* JA18 was found to have greater V_{max} and K_m values (1.78- and 1.52-fold increase, respectively) for CMC than the CBM-containing endoglucanase [41].

Carbohydrate Binding Properties of the Truncated Enzymes

To analyze the binding capacity of CBM_3 in Cel5L-p50, residual enzyme activities were measured after binding to Avicel using purified Cel5L-p50 (containing CBM_3) and Cel5L-p35 (containing no CBM_3). Residual activity of Cel5L-p50 after binding to Avicel was significantly decreased by 70%, whereas that of Cel5L-p35 increased to 140% (Table S1). Although the reason for this observed increase in Cel5L-p35 residual activity is not clear, these results suggest deletion of CBM_3 abolished binding ability to microcrystalline cellulose.

Enzymatic Saccharification of Pretreated Biomasses

The production of reducing sugar was investigated using pretreated reed and rice straw powders by adding purified Cel5L-p50 or Cel5L-p35 to a set of the commercial enzymes

Fig. 4. Saccharification of pretreated reed (A) or rice straw (B) powders by purified Cel5L-p50 and Cel5L-p35.

(Celluclast 1.5L + Novozym 188). Productions of reducing sugar from pretreated reed or rice straw powders reached a maximum after 48–72 h (Fig. 4). The addition of Cel5L-p50 increased the saccharification values for reed and rice straw powders by 7.2% and 17.5%, respectively (Fig. 4). When Cel5L-p35 was added to the commercial enzyme set, the saccharification values of the reed and rice straw powders were increased by 4.1% and 21.0%, respectively (Fig. 4). Substrate specificities for pure β -1,4-glucan (CMC) and mixed-linked β -1,3-1,4-glucans (barley β -glucan or lichenan) and for small substrates probably contributed to observed differences between reed and rice straw powders. It has been previously reported saccharification efficiencies were increased by using pretreated materials [43], an optimized crude enzyme cocktail [44], and an ionic liquid-tolerant cocktail for an ionic liquid-pretreated biomass [45]. In addition, Cel5L-p50 or Cel5L-p35 could be used as additives to improve the saccharification of lignocellulosic substrates, because they both enhance degree of saccharification of pretreated biomasses, regardless of CBM_3 deletion.

In conclusion, an endo- β -1,4-glucanase gene of *Bacillus* sp. KD1014 was cloned and its encoded products characterized. The endo- β -1,4-glucanase (Cel5L) belonged to GH5 and contained a CBM_3 module. Two truncated enzymes, that is, CBM_3-containing Cel5L-p50 and CBM_3-deleted Cel5L-p35, were purified from the transformant. Examination of the efficacies of these two truncated enzymes showed deletion of CBM_3 increased the thermostability, and affinity and reactivity for a small substrate (pNPC), but dramatically decreased binding affinity to Avicel. Notably, Cel5L-p35 enhanced saccharification of pretreated rice straw powder by 21.0% when added to a commercial enzyme preparation. These results suggest the two

truncated forms might be potentially useful additives for the saccharification of lignocellulosic biomasses.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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