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Screening and characterization of a cellulase with endocellulase and exocellulase activity from yak rumen metagenome

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ABSTRACT

Cellulose saccharification is an important process in conversion from lignocellulosic biomass to biofuels and other chemicals, and requires concerted action of endocellulase, exocellulase and β-glucosidase. Thus, it is very interesting to discover and develop multifunctional cellulase in order to convert cellulose to glucose more efficiently. Here we report an endo/exocellulase Rucel5B with 336 amino acids cloned from yak rumen uncultured microorganism, and its recombinant expression in *Escherichia coli*. This cellulase possesses endo-β-1,4-glucanase activity of 220 U mg^{-1} against carboxymethylcellulose and exo-β-1,4-glucanase activity of 52.9 U mg^{-1} against 4-nitrophenyl-β-D-cellobioside, and is able to hydrolyze not only amorphous cellulose (carboxymethylcellulose, barley glucan, lichenan, phosphate acid swollen cellulose, etc.), but also crystalline cellulose (filter paper, avicel, etc.). The exo-type action mode of Rucel5B was confirmed by its release of cellobiose from cellooligosaccharides and crystalline cellulose, and its endo-type action mode was confirmed by a time-dependent decrease in the polymerization degree of hydrolysates when Rucel5B was incubated with soluble amorphous cellulose. Therefore, the enzymatic activities, the endo/exo-mode of action and the ability in saccharification of both amorphous and crystalline cellulose make Recul5B a very interesting candidate for efficient saccharification of cellulose.

1. Introduction

Cellulose, the linear polymer of glucose residues linked by β-D-(1-4)-glucopyranosidic bonds, is the main constitute of plants and is the most abundant renewable biomass on earth [1]. Cellulose can be converted to glucose through concerted action of three types of cellulases: endocellulase (endo-β-1,4-glucanases, EC 3.2.1.4), exocellulase (exo-β-1.4-cellobiohydrolase, EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [2,3]. The first step of cellulose hydrolysis, a critical one, is that the endocellulase randomly attacks the amorphous regions in cellulose chains to generate cellooligosaccharides and release free ends [4]. Then the exocellulase cleaves cellobiose from the free ends in both amorphous and crystalline regions through exo-mode of action. Exocellulase is very important for degradation of crystalline cellulose because exocellulase is the only enzyme that can degrade highly ordered crystalline regions [4]. Finally the released cellobiose is converted to glucose by β-glucosidase.

Cellulases are important industrial enzymes that are broadly utilized in bioethanol, textile, paper, detergent, forage, and beer-brewing industries [5,6]. In the conversion from cellulose biomass into bioethanol and other chemicals, saccharification of cellulose to glucose by cellulases is the key process [2,3]. Although a number of cellulases have been discovered from fungi and bacteria, it is still unable to meet the needs of industrial application because of certain limitations, such as stability, activity, sensitivity to byproducts [2,5]. So researchers are still making efforts to isolate novel cellulases, such as thermostable cellulases, halostable cellulases, high activity cellulases, and multifunctional cellulases [5–10]. Most cellulases were found to be monofunctional, however, there also exist bifunctional/multifunctional cellulases, which could be used as two/more kinds of monofunctional cellulases [5,11,12]. Several artificially constructed bifunctional/multifunctional cellulases have also been reported [13]. Hence, discovery and development of multifunctional cellulases are very useful for potential applications in efficient conversion from cellulose to glucose.

Microorganisms from various environments provide potential sources for novel cellulases. Traditional explore for novel cellulase is mainly based on the isolation and re-cultivation of

Abbreviations: GH, glycoside hydrolase; CMC, carboxymethylcellulose sodium; MuC, 4-methylumbelliferyl-β-D-cellobiopyranoside; PASC, phosphate acid swollen cellulose; pNPC, 4-nitrophenyl-β-D-cellobioside; pNPG, 4-nitrophenyl-β-D-glucopyranside; T_m, melting temperature; DP, degree of polymerization; TLC, thin-layer chromatography; HPAEC-PAD, high performance anion-exchange chromatography with pulsed amperometric detection.

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microorganisms, however, more than 90% of microorganisms resist laboratory cultivation [14,15]. Development of metagenomics provides a cultivation independent method to mine wide range of biocatalysts in unculturable microorganisms. Combining metagenomics method and high throughput functional screening, many novel cellulases-encoding genes have been cloned, such as endoglucanase Cel5A from soil metagenome, cellulase CelAM11 from abalone intestine [8,9,14,16–20].

The yak (*Bos grunniens*) is a kind of ruminant living on Qinghai-Tibetan Plateau in China. Their diet of pure grass pasture instead of grain (starch based) makes rumen a residence for highly active lignocellulose hydrolyases [21]. However, more than 85% of rumen microorganisms were uncultured and many novel enzymes present in difficult-to-culture microbes are not characterized yet [21], while metagenomic approach is suitable to resolve this problem. So in this study, we employed metagenome method and function-based screening to explore yak rumen cellulase resources, and isolated a cellulase named Recul5B, which exhibited both endo-β-1,4-glucanase and exo-β-1,4-glucanase activities. Besides, its enzymatic properties, and the endo-/exo-mode of action were fully investigated.

2. Materials and methods

2.1. Library construction and screening

The yak rumen samples were collected in Qinghai-Tibet Plateau, China, January, 2007. Metagenomic DNA was extracted as described previously [21]. Cosmid library was constructed using pWEB Cosmid Cloning Kit (Epicentre, Madison, USA) according to product manual and preserved in 96-well plates at −80 °C [22].

The library clones were incubated in LB medium containing 100 mg L^{-1} ampicillin at 37 °C for 16 h. After centrifugation, cell pellets were collected and resuspended in 30 μL of 50 mM sodium citrate (pH 6.0). The cells were lysed by freezing and thawing [23]. For endo-β-1,4-glucanase screening, the lysates were piped on agar plates containing 0.5% CMC, then the plates were incubated at 37 °C for 1 h, stained with 1% (w/v) Congo red for 20 min and destained with 1 M NaCl [24]. The positive clones would form a halo against a red background. For exo-β-1,4-glucanase screening, 10 μL of 0.1% (w/v) MuC (Sigma, St. Louis, USA) was added into the cell lysates and incubated at 37 °C for 1 h. The positive clones would emit blue florescence under UV light. Then the cosmid DNA of positive clone was extracted to construct subclone library in pGEM11z (Promega, Madison, USA) and also screened for endo-β-1,4-glucanase and exo-β-1,4-glucanase activities. The insert fragments of positive subclones were then separately sequenced.

The BLAST program at the NCBI was used for database searches [25]. Conserved domain analysis was performed by the online software SMART (http://smart.embl-heidelberg.de). Putative signal sequence was identified by SignalP 3.0 server (http://www.cbs.dtu.dk/services/). The phylogenetic tree was constructed with MEGA3 [26].

2.2. Expression and purification

The ORF of *Rucel5B* without signal peptide coding sequence was amplified by PCR with a sense primer (5′-ATAGAATTCCATCATCACCATCATCACGGCAACGGCTGGGTC-3′) and an antisense primer (5′-TGTAAGCTTACCCGCCTGTCCCTG-3′), and then cloned into pET21a (Novagen, Madison, USA). Recombinant Rucel5B was overexpressed in *Escherichia coli* BL21 (DE3) (Novagen, Madison, USA) according to pET System Manual, before the cells were suspended in lysis buffer (20 mM Tris–HCl, 50 mM NaCl, pH7.4) and lysed by sonication. Rucel5B was purified by Ni/NTA resin and analyzed by SDS-PAGE for size and purity. Protein concentration was determined by the BCA assay using bovine serum albumin as standard [27].

2.3. Enzymatic assay

Substrate CMC (Sigma, St. Louis, USA) was used for endoglucanase activity assay and pNPC (Sigma, St. Louis, USA) for exoglucanase activity assay. The standard assay condition for endoglucanase activity was to incubate 1 μg Rucel5B with 300 μL of 1% CMC in 50 mM sodium citrate (pH 6.5) at 60 °C for 2 min. The amount of reducing sugar released was measured with DNS reagent [28]. The standard assay condition for exoglucanase activity was to incubate 1 μg Rucel5B with 300 μL of 1 mM pNPC in 50 mM sodium citrate (pH 5.5) at 65 °C for 2 min. Then 100 μL 1 M Na_2CO_3 was added into the mixture to quench the reactions and visualize yellow color. The amount of *p*-nitrophenyl released was quantified by the absorbance at 405 nm. One unit (U) of endoglucanase or exoglucanase activity was defined as the amount of enzyme releasing 1 μmol reducing sugar from CMC or 1 μmol *p*-nitrophenyl from pNPC per minute. All the assays were performed in triplicate.

The pH optima for endoglucanase and exoglucanase activities were assayed at 60 °C for 2 min from pH 4.0 to 9.0 using appropriate buffers: sodium citrate (50 mM, pH 4.0–6.5), sodium phosphate (50 mM, pH 6.0–8.0), Tris–HCl (50 mM, pH 8.0–9.0). The temperature optima for endoglucanase and exoglucanase activities were determined at an interval of 5 °C from 25 °C to 80 °C in 50 mM sodium citrate, pH 6.0 for 2 min. The thermostability was determined by measuring residual endoglucanase activity under standard condition after treatment of the enzyme at 50, 55 and 60 °C for 10–60 min in the absence of substrates.

Circular dichroism (CD) spectra of Rucel5B were measured using JASCO J-715 CD spectropolarimeter (JASCO, Tokyo, Japan) from 35 °C to 90 °C at the rate of 1 °C min^{-1} over the wavelength range from 200 to 250 nm. The thermal denaturation melting curves were determined by Boltzman fitting of the ellipticity values from 35 °C to 90 °C at 222 nm, and the T_m was calculated according to the thermal melting curves.

Substrate specificity of Rucel5B was assayed using a serial of substrates. Specific activity against 1% (w/v) CMC, barley β-glucan (Megazyme, Bray, Ireland), lichenan (Megazyme, Bray, Ireland), laminarin (Sigma, St. Louis, USA), β-1,6-glucan (Sigma, St. Louis, USA), xylan from birchwood (Sigma, St. Louis, USA) and PASC [29] was assayed at 60 °C in 50 mM sodium citrate (pH 6.5) (CMC, lichenan, laminarin and birchwood xylan were prepared as colloidal suspension in water according to Megazyme's procedures). Activity against 12% (w/v) avicel PH101 (Sigma, St. Louis, USA) and 4% (w/v) Whatman NO. 1 filter paper (Sigma, St. Louis, USA, first smashed to about 1 mm × 1 mm and then prepared as suspension) was assayed in 50 mM sodium citrate (pH 6.0) at 37 °C for 8 h. Activity against 1 mM pNPG (Sigma, St. Louis, USA) and 1 mM pNPC was carried out at 65 °C in 50 mM sodium citrate (pH 5.5). One unit of activity against the above substrates was defined as the amount of enzyme releasing 1 μmol reducing sugar or 1 μmol *p*-nitrophenyl per minute using glucose or pNP as standard. Specific activity was defined as the units of enzyme per milligram of protein.

The values of Michaelis constant (K_m), turnover number (k_{cat}) and specificity constant (k_{cat}/K_m) on CMC, barley glucan, lichenan and pNPC were determined by the activities at 60 °C in 50 mM sodium citrate (pH 6.5) against 0.1–1% polysaccharides substrate, or the activities at 65 °C in 50 mM sodium citrate (pH 5.5) against 0.1–1 mM pNPC through double-reciprocal plot method [30].

Effects of metal ions on the activity of Rucel5B were determined against 1% (w/v) CMC or 1 mM pNPC under standard assay condition with metal ions at final concentrations of 5 mM. The inhibition or

Table 1
Substrate specificity and kinetic parameters of Rucel5B.

Substrate	Main linkage type	Specific activity ($U\,mg^{-1}$)	Relative activity (%)	K_m	k_{cat} (S^{-1})	k_{cat}/K_m
CMC	β-1,4-Glucan	220[a]	100	$13.5\,g\,L^{-1}$	163	$12.0\,L\,g^{-1}\,S^{-1}$
Barley glucan	β-1,3/1,4-Glucan	717	326	$2.0\,g\,L^{-1}$	478	$239\,L\,g^{-1}\,S^{-1}$
Lichenan	β-1,3/1,4-Glucan	693	315	$3.9\,g\,L^{-1}$	879	$227\,L\,g^{-1}\,S^{-1}$
Laminarin	β-1,3-Glucan	0	0	ND[b]	ND	ND
Glucan	β-1,6-Glucan	0	0	ND	ND	ND
Xylan from birchwood	β-1,4-Xylan	0	0	ND	ND	ND
pNPG	Aryl β-1,4-glucoside	0	0	ND	ND	ND
pNPC	Aryl β-1,4-glucoside	52.9	24.1	1.74 mM	69.6	$40.1\,mM^{-1}\,S^{-1}$
PASC	β-1,4-Glucan	23.5	10.7	ND	ND	ND
Avicel	β-1,4-Glucan	0.2	0.09	ND	ND	ND
Filter paper	β-1,4-Glucan	0.04	0.02	ND	ND	ND

[a] Specific activity against 1% (w/v) CMC, barley β-glucan, lichenan, laminarin, β-1,6-glucan, xylan and PASC was assayed at 60 °C in pH 6.5 citrate. Activity against 12% (w/v) avicel PH101 and 4% (w/v) Whatman NO. 1 filter paper was assayed at 37 °C in pH 6.0 citrate. Activity against 1 mM pNPG and 1 mM pNPC was carried out at 65 °C in pH 5.5 citrate. One unit of activity against the above substrates was defined as the amount of enzyme releasing 1 μmol reducing sugar or 1 μmol p-nitrophenyl per minute. Specific activity was defined as the units of enzyme per milligram of protein.
[b] Not detected.

activation effects on the activity were presented as relative activity to control which was measured with no addition of metal ions.

2.4. Determination of the mode of action

To determine the mode of action of Rucel5B, 1% cellobiose, 0.1% MuC, 1% cellotetraose (Sigma, St. Louis, USA), 12% avicel and 4% filter paper were separately incubated with Rucel5B at 50 °C for 8 h, while 1% CMC, 1% barley glucan and 1% lichenan were separately incubated with Rucel5B at 50 °C for indicated time. The hydrolysates were analyzed by TLC or HPAEC-PAD. For TLC analysis, 2–10 μL samples were spotted onto a Silica gel 60 F_{254} TLC plate (Merck, Darmstadt, Germany) and developed with butanol/ethanol/H_2O (5:3:2, v/v/v) for 1.5 h, then visualized by spraying the plates with a 10% solution of sulfuric acid in methanol. For HPAEC-PAD analysis, 1 μL of cellotetraose hydrolysates was diluted in 3 mL H_2O and analyzed on a Dionex system (Dionex, Sunnyvale, USA) equipped with a CarboPac PA-1 column (0.4 cm × 25 cm; Dionex, Sunnyvale, USA). The elution ($1\,mL\,min^{-1}$) involved a linear gradient of 0–250 mM sodium acetate in 100 mM NaOH for 30 min.

3. Results

3.1. Cloning and bioinformatics analysis of Rucel5B

The rumen metagenomic library was screened for endo-β-1,4-glucanase and exoglucanase activities using CMC and pNPC as substrate, respectively. Out of 4000 library clones, one positive clone exhibited both endoglucanase and exoglucanase activities. After construction of subclone library and screening followed by sequencing analysis, an ORF encoding a protein of 336 amino acids with a predicted 24-residues signal peptide was obtained and designated as *Rucel5B*.

BLAST analysis showed that Rucel5B shares the highest 83% identity in the amino acid sequence with a cellulase DC9-2 (ACA61152) from buffalo rumen uncultured bacterium [9], followed by celludextrinase (ADB80111), cellulases (ACA61168) and endoglucanase (ADB80108) also from buffalo rumen uncultured bacterium, with identity of 82.1%, 82.1% and 81.8%, respectively. Phylogenetic analysis showed that Rucel5B is clustered with GH family 5 cellulases from uncultured buffalo rumen bacteria (Fig. 1a), suggesting that Rucel5B and its closely related cellulases may be from a new group of unculturable bacteria that reside specifically in rumen. The conserved domain analysis indicated that Rucel5B contains a domain from residue 35 to 310 which belongs to GH family 5 (pfam: PF00151). The nucleotide sequence of Rucel5B has been deposited in GenBank with the accession number GQ849224.

3.2. Expression and purification of Rucel5B

Recombinant Rucel5B was heterogenously expressed in *E. coli* BL21 (DE3), and purified from the supernatants of cell lysate by Ni/NTA resin. SDS-PAGE analysis revealed that the purified Rucel5B was a single band with an apparent molecular weight of about 40 kDa, which was consistent with the theoretical value of 38.4 kDa (Fig. 1b).

3.3. Enzymatic properties of Rucel5B

The optimal pH and optimal temperature of Rucel5B were assayed against substrates CMC and pNPC. Its optimal pH was 6.5 against CMC and 5.5–6.5 against pNPC (Fig. 2a). Its optimal temperature was 60 °C against CMC and 65 °C against pNPC (Fig. 2b). The thermostability analysis showed that Rucel5B was stable after treatment at 50 °C or 55 °C for 1 h (Fig. 2c). The thermostability of Rucel5B was further evaluated by thermal denaturation melting curve obtained by CD spectra from 35 °C to 80 °C at 222 nm (Fig. 2d), from which the T_m was calculated as 60 °C.

The substrate specificity of Rucel5B was measured against various substrates. Rucel5B was active towards CMC, barley glucan, lichenan, pNPC, PASC, avicel and Whatman NO.1 filter paper, but not active towards laminarin, β-1,6-glucan and birchwood xylan (Table 1). The linkage type of these substrates (Table 1) indicated that Rucel5B could hydrolyze β-1,4 glucosidic linkage, but not β-1,3 glucosidic linkage, β-1,6 glucosidic linkage or β-1,4 xylosidic linkage. The substrate type revealed that Rucel5B was not only active towards amorphous cellulose (CMC, barley glucan, lichenan, PASC), but also towards crystalline cellulose (avicel and filter paper).

The analysis of specific activity showed that the endo-β-1,4-glucanase activity of Rucel5B against 1% CMC was $220\,U\,mg^{-1}$, and the exo-β-1,4-glucanase activity against 1 mM pNPC was $52.9\,U\,mg^{-1}$. Meanwhile, Rucel5B exhibited $717\,U\,mg^{-1}$ activity against 1% barley glucan and $693\,U\,mg^{-1}$ activity against 1% lichenan. Rucel5B showed higher activity against barley glucan and lichenan than that against CMC, which was consistent with several endoglucanases reported in previous papers and may be due to different solubility of substrates [8,9]. Kinetic parameters of Rucel5B for CMC, barley glucan, lichenan and pNPC were determined separately under standard conditions (Table 1). The K_m and k_{cat} were $13.5\,g\,L^{-1}$, $163\,S^{-1}$ against CMC, $2.0\,g\,L^{-1}$, $478\,S^{-1}$ against barley glucan, $3.9\,g\,L^{-1}$, $879\,S^{-1}$ against lichenan and 1.74 mM, $69.6\,S^{-1}$ against pNPC.

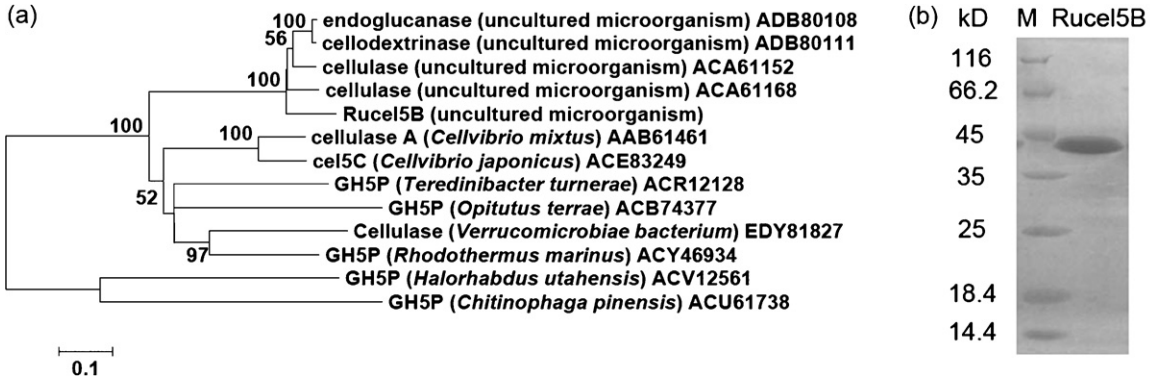

Fig. 1. Phylogenetic analysis of Rucel5B and SDS-PAGE analysis of purified Rucel5B. (a) Phylogenetic tree of Rucel5B. GH5P: glycoside hydrolyase family 5 protein. (b) SDS-PAGE analysis of purified recombinant Rucel5B. *Lane M*: protein molecular weight markers.

Table 2
Effects of metal ions on the activity of Rucel5B.

Chemical	Relative activity (%)	
	Endoglucanase	Exoglucanase
Control	100	100
$MgCl_2$	114	91.1
$CaCl_2$	89.3	91.2
$CoCl_2$	129	103
$NiSO_4$	115	94.2
$CuCl_2$	49.3	102
$ZnCl_2$	104	87.6

Effects of metal ions on the activity of Rucel5B were measured in the presence of 5 mM metal irons (Table 2). The addition of 5 mM Mg^{2+}, Ni^{2+} and Co^{2+} could enhance the endoglucanase activity by 15–30%, the addition of 5 mM Ca^{2+} and Cu^{2+} inhibited the endoglucanase activity by about 10% and 50%, respectively, while the addition of 5 mM Zn^{2+} had no statistical difference on the activity (Table 2). The exoglucanase activity was reduced by 5–10% in the presence of 5 mM Mg^{2+}, Ca^{2+}, Ni^{2+} and Zn^{2+}, while no statistical difference was detected in the presence of 5 mM Co^{2+} and Cu^{2+} (Table 2).

3.4. Mode of action of Rucel5B

To determine Rucel5B's mode of action, different cellooligosaccharides and cellulose substrates were used and the hydrolysates were subjected to chromatography analysis. The TLC results in Fig. 3a showed that Rucel5B could not hydrolyze cellobiose (DP 2), but could degrade MuC to release cellobiose. To analyze the hydrolysates of cellotetraose (DP4), more sensitive HPAEC-PAD was used. As shown in Fig. 3b, Rucel5B hydrolyzed cellotetraose

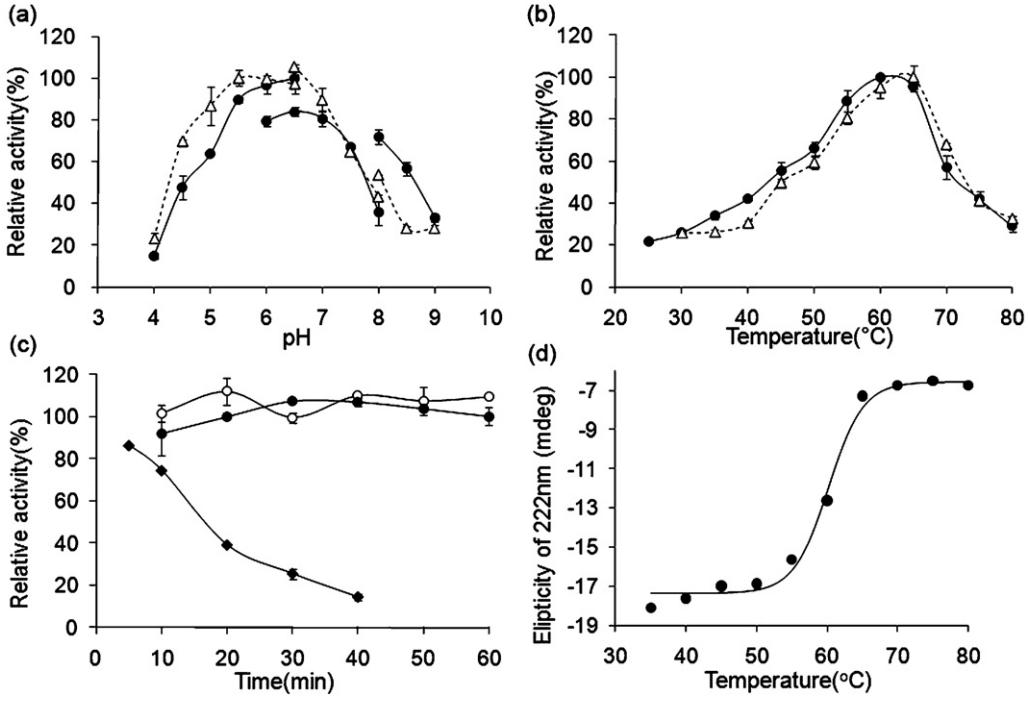

Fig. 2. Enzymatic properties of recombinant Rucel5B. (a) Optimal pH of Rucel5B against CMC (•) and pNPC (Δ). Rucel5B was incubated with 1% (w/v) CMC or 1 mM pNPC at a pH range from 4.0 to 9.0 at 60 °C for 2 min. (b) Optimal temperature of Rucel5B against CMC (●) and pNPC (Δ). Rucel5B was incubated with 1% (w/v) CMC or 1 mM pNPC in sodium citrate (pH 6.0) at an interval of 5 °C from 25 °C to 80 °C for 2 min. (c) Thermostability of Rucel5B. Rucel5B was preincubated at 50 °C (○), 55 °C (●) and 60 °C (♦) for 10–60 min in the absence of substrate. The residual activity of endoglucanase was assayed with CMC as substrate at the standard condition. (d) Thermal denaturation melting curve of Rucel5B. Circular dichroism spectra of Rucel5B (●) were collected from 35 °C to 90 °C at the rate of 1 °C min^{-1} over the wavelength range from 200 to 250 nm, the ellipticity values at 222 nm were analyzed by Boltzman fitting. All the results represent the mean ± SD of three independent experiments performed in triplicate.

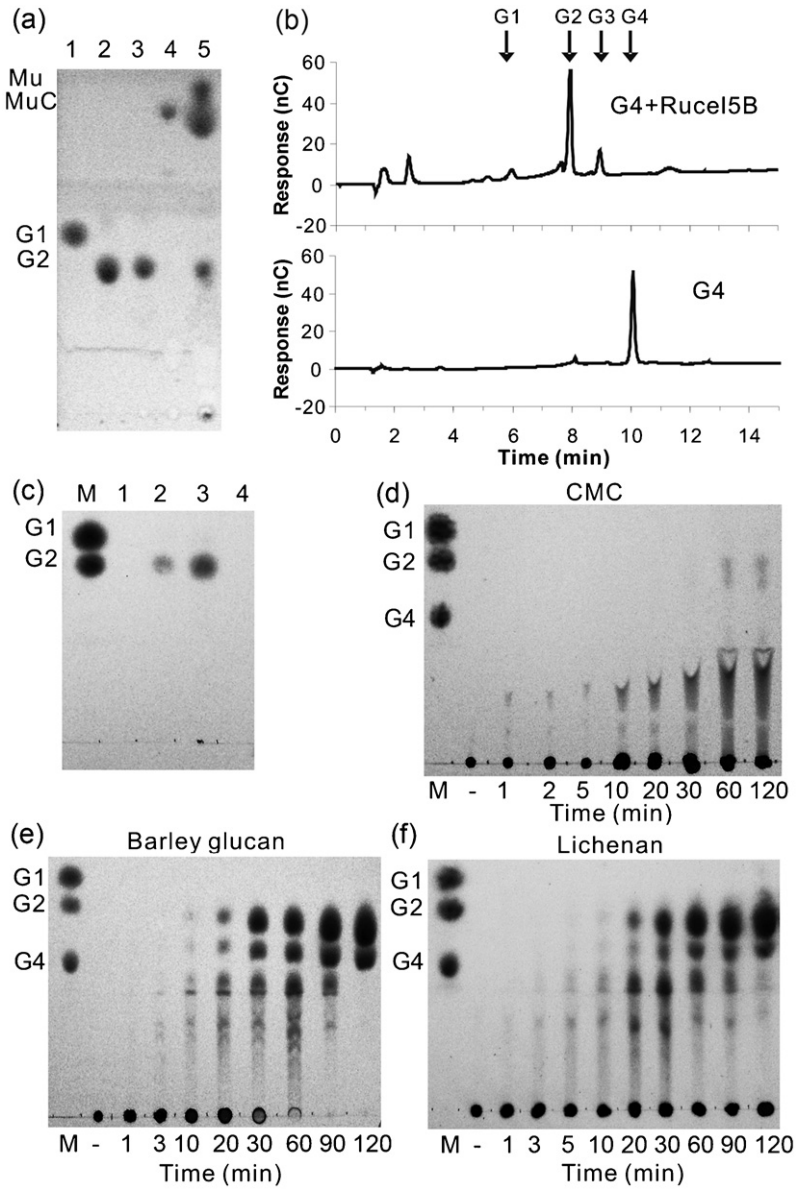

Fig. 3. Determination of the mode of action of Rucel5B. (a) TLC analysis of cellobiose and MuC hydrolysates by Rucel5B. 1% cellobiose or 0.1% MuC were separately incubated with Rucel5B at 50 °C for 8 h. *Lane* 1, glucose (G1) marker; *Lane* 2, cellobiose (G2) marker; *Lane* 3, cellobiose + Rucel5B; *Lane* 4, MuC marker; *Lane* 5, MuC + Rucel5B. (b) HPAEC analysis of cellotetraose (G4) hydrolysates by Rucel5B. 1% cellotetraose was incubated with Rucel5B at 50 °C for 8 h. (c) TLC analysis of filter paper and avicel hydrolysates by Rucel5B. 12% avicel or 4% Whatman NO.1 filter paper were separately incubated with Rucel5B at 50 °C for 8 h. *Lane* 1, filter paper negative control; *Lane* 2, filter paper + Rucel5B; *Lane* 3, avicel + Rucel5B; *Lane* 4: avicel negative control. (d) Time course degradation of CMC by Rucel5B through TLC analysis. 1% CMC in sodium citrate (50 mM, pH 6.0) was incubated with Rucel5B at 50 °C for indicated time. (e) Time course degradation of barley glucan by Rucel5B through TLC analysis. 1% barley glucan in sodium citrate (50 mM, pH 6.0) was incubated with Rucel5B at 50 °C for indicated time. (f) Time course degradation of lichenan by Rucel5B through TLC analysis. 1% lichenan in sodium citrate (50 mM, pH 6.0) was incubated with Rucel5B at 50 °C for indicated time.

to release cellobiose as the dominant product, while cellotriose and glucose were minor products. Considering that dominant cellobiose released from celloligosaccharides or cellulose (polysaccharides) is the feature of most reported exocellulase [2,5,31], the results that Rucel5B can cut cellobiose from cellooligosaccharides suggest Rucel5B's exo-mode of action. In the degradation of crystalline cellulose (avicel and filter paper) by Rucel5B, cellobiose was also detected as exclusive product (Fig. 3c), which confirmed its exo-mode of action. It has been reported that there are two classes of exocellulases each working from different ends of cellulose, whether Rucel5B's exo-action proceeds from the reducing or non-reducing end requires future studies using radiolabeled or chemically reduced cellooligosaccharide substrates [32,33].

On the other hand, the time-dependent degradation of soluble amorphous cellulose CMC indicated that Rucel5B hydrolyze CMC through an endo-mode of action. At the beginning of the reaction, only high molecular mass products were released and the polymerization degree of hydrolysates was decreased obviously as the reaction proceeded, while the released cellobiose was observed after 60 min of incubation when high DP cellooligosaccharides were further hydrolyzed to low DP cellooligosaccharides (Fig. 3d). The degradation of soluble amorphous cellulose barley glucan and lichenan by Rucel5B also supported the endo-mode of action (Fig. 3e and f). The formation of high DP oligosaccharides could be seen after 1 min of incubation, and the oligosaccharides were gradually accumulated within 30 min and further degraded to low DP products after 60 min. In the first 10 min of the reaction,

the DP of the products was rapidly decreased before cellobiose was released, which were consistent with the characterization of other endoglucanase [8].

4. Discussion

Bifunctional or multifunctional cellulase is one of the strategies evolved to improve the degradation of cellulosic biomass by resolving the contradiction between limited energy for enzyme synthesis and substrate variety [34]. The extra activity compared with monofunctional cellulase gives bifunctional/multifunctional cellulase advantage in application. However, most natural cellulases are monofucntional, and only a few bifunctional/multifunctional cellulases have been reported. For example, CelEdx16 from an anaerobic ruminal bacterium is an endo/exoglucanase with endoglucanase activity of 15.9 U mg^{-1} and exoglucanase activity of 0.036 U mg^{-1} [5]; Avicelase II from *Bacillus circulans* has endoglucanase activity of 14.3 U mg^{-1} and exo-cellobiohydrolase activity of 2.6 U mg^{-1}[12]; Endoglucanase S from *Streptomyces* sp. LX also exhibits both endoglucanase and exoglucanase activities [11]. The characterization of the action mode during hydrolysis has confirmed that Rucel5B is also such an endo/exoglucanase, and provides a new member for bifunctional cellulases. Besides, the reference to the cellulase records in Braunschweig Enzyme Database (http://www.brenda-enzymes.info) and other published papers in recent years [5,7–9,12,31] showed that only a few endocellulases possess more than 200 U mg^{-1} activity against CMC, and exocellulases with more than 10 U mg^{-1} activity against pNPC were even less. So Rucel5B is a new bifunctional endo/exocellulase with relative high activity.

In function-based screening for cellulase, CMC is broadly used as substrate for endo-type cellulase, while MuC or pNPC is used as substrate for exo-type cellulase, because they are soluble derivatives of cellulose and have visible appearance for positive reaction [3]. However, these derivatives could only represent amorphous cellulose, while in industrial application, cellulases are more commonly used to attack insoluble cellulose which has both amorphous and crystalline regions. The crystalline structure of cellulose substrate is one of the major reasons that cellulosic materials are difficult to be degraded [2]. Avicel and Whatman NO. 1 filter paper are two kinds of crystalline cellulose substrate with similar crystallinity index to pretreated natural cellulose [2]. Rucel5B reported here was active towards not only amorphous cellulose, but also crystalline cellulose such as avicel and filter paper (Table 1), indicating its cellulolytic ability and potential in application.

Cellulase C67-1 (ACA61137) is also a bifunctional GH5 enzyme cloned from rumen uncultured bacterium, however, it exhibited quite different substrate specificity and thermostability from Rucel5B [9]. C67-1 is a bifunctional endoglucanase/xylanase with endoglucanase activity of 73 U mg^{-1} and xylanase activity of 14.4 U mg^{-1}, but has no exoglucanase activity. The temperature optimum for C67-1 is 45 °C, and it would completely lose its activity when incubated above 50 °C for 1 h, while Rucel5B showed optimal temperature at 60–65 °C and is stable at 55 °C for 1 h. So the discovery of Rucel5B provides new data for analysis of the relationship between enzyme structure and function.

It is interesting that the average temperature of rumen environment is only 39 °C, while the rumen sourced Rucel5B exhibits a much higher temperature optimum and T_m than that. However, similar cases have also been reported, e.g., an endoglucanase from ruminal bacterium *Ruminococcus albus* has a temperature optimum of 55 °C [35], and another endoglucanase with a temperature optimum of 55 °C was cloned from buffalo rumen metagenome [36]. Since thermostability is an important property for the biotechnological and industrial applications of commercial enzymes, thermostable enzymes have been studied intensively [7,10,23,37]. The relatively high optimal temperature and thermostability of Rucel5B make it useful for further structure/function-based enzyme engineering and design to improve thermostability and enzymatic efficiency through experimental and computational approaches, such as those in Refs. [38–40].

During the hydrolysis of various cellulose substrates, it seems that Rucel5B would dominantly display endo-mode of action at the beginning of soluble amorphous celluloses hydrolysis (Fig. 3d–f), and dominantly display exo-mode of action in crystalline cellulose hydrolysis (Fig. 3c). It may be because that in the beginning of amorphous cellulose hydrolysis there are numerous random attacked sites for endocellulase, but the free ends for exocellulase are much less, while in crystalline cellulose hydrolysis there are few amorphous regions for endocellulase, but the free ends are still available to exocellulase.

5. Conclusion

In conclusion, an endo/exoglucanase Rucel5B was identified from yak rumen uncultured microorganism. Rucel5B can degrade not only soluble amorphous cellulose, such as CMC, barley glucan and lichenan, through endo-mode of action, but also crystaline cellulose, such as avicel and filter paper, through exo-mode of action. Accordingly, the enzymatic activities, the endo/exo-mode of action and the ability in saccharification of both amorphous and crystalline cellulose make Recul5B a very interesting candidate for efficient saccharification of lignocellulosic biomass.

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