The Journal of Microbiology (2010) Vol. 48, No. 3, pp. 393-398
DOI 10.1007/s12275-010-9361-0

Molecular Cloning, Purification, and Characterization of a Novel, Acidic, pH-Stable Endoglucanase from *Martelella mediterranea*

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(Received November 23, 2009 / Accepted March 3, 2010)

A novel gene encoding an endoglucanase designated Cel5D was cloned from a marine bacterium *Martelella mediterranea* by genomic library. The gene had a 1,113 bp opening reading frame encoding a 371-amino-acid protein with a molecular mass of 40,508 Da and containing a putative signal peptide (41 amino acids). Cel5D had low similarity (48-51% identity) with other known endoglucanases and consisted of one single catalytic domain, which belonged to the glycosyl hydrolase family 5. The maximum activity of Cel5D was observed at 60°C and pH 5.0. Cel5D displayed broad pH stability within the range of pH 3.0-11.0 and retained hydrolytic activity in the presence of a wide variety of metal ions and some chemical reagents. These characteristics suggest that the enzyme has considerable potential in industrial applications.

Keywords*: *M. mediterranea, endoglucanase, sodium carboxymethyl cellulase, pH-stable

Cellulose is the most abundant biological polymer (Schwarz, 2001). It is broken down by the synergistic action of three types of cellulase, which are endoglucanases (EC 3.1.2.4), exoglucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). The cellulases are often used in detergents, animal feed, the pulp, and paper industry, and even the food industry (Miettinen-Oinonen and Suominen, 2002). Recently, there has been considerable interest in the use of ethanol as an alternative energy source. Thus, a number of researchers have focused their attention on the bio-production of ethanol (Ohmiya *et al*., 1997; Sun and Cheng, 2002). However, the economic feasibility of bio-ethanol fuels depends on the development of enzymatic processes to hydrolyze the cellulosic polysaccharides into fermentable sugars (Lynd *et al*., 2002). Given this, the demand for cellulases is increasing rapidly, and has become the driving force for cellulase research (Bhat, 2000).

Endoglucanases catalyze the endohydrolysis of amorphous cellulose by random cleavage of β-1,4-glucoside bonds to yield long-chain oliosaccharides (Stutzenberger, 1991). This process is crucial for promoting the hydrolysis of β-glucosidase. Thus, endoglucanases yield significant improvements in modifying the properties of fibers and improving the strength of paper pulps compared with exoglucanases (Oyekola *et al*., 2007). Endoglucanases are also used in detergent products to brighten colors, soften fabrics, and remove soil particulates (Pang *et al*., 2009). The specificity of these applications suggests a need to identify a wide range of endoglucanases that have varying optimal pH and temperature values.

The majority of well-described enzymes have been isolated from fungi (Moriya *et al*., 2003). However, bacteria are an excellent source of cellulases, especially given the volume of bacteria in the ocean. In extreme conditions, microorganisms produce enzymes that enable the individual to adapt to a variety of environments (Fu *et al*., 2009). Endoglucanases that are active over a wide range of temperatures and pH are of particular interest as they can be used during industrial processes without incurring a cost for adjustments in temperature and pH. Moreover, thermostable endoglucanases may also serve as robust hydrolytic enzymes that can be integrated into industrial fermentation processes (Stutzenberger, 1991).

We isolated a novel endoglucanase gene from a marine bacterium *Martelella mediterranea* using an activity-based screening library. We then characterized the purified recombinant enzyme.

Materials and Methods

Bacterial strains and chemicals

The *M. mediterranea* donor strain was provided by the Third Institute of Oceanography (Xiamen, China) and was deposited in the China Center for Type Culture Collection (CCTCC) (accession number AB209166). The bacteria were cultivated in YED medium (0.5% yeast extract, 0.7% glucose, and 1.5% agar) (Rivas *et al*., 2005). *Escherichia coli* DH5α, BL21 (DE3) was purchased from TaKaRa (Japan), and recombinant *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin.

GST-Bind Purification kit was purchased from Novagen (Germany). The DNA Purification kit was purchased from Sangon (China) and the DNA restriction endonucleases were obtained from TaKaRa (Japan). All other reagents were purchased from Sigma (USA), unless otherwise specified.

DNA isolation and manipulation

DNA extraction, plasmid DNA preparation, and DNA digestion were carried out according to established protocols (Sambrook and Russell,

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2001).

Genomic library construction and screening

The genomic DNA from *M. mediterranea* was partially digested with *Sau*3AI. Fragments ranging from 4 to 9 kb were harvested by agarose gel electrophoresis, eluted from agarose, and cloned into the *Bam*HI site of a dephosphorized pUC18 plasmid. The ligation mixture was transformed into *E. coli* DH5α using heat shock process. The positive clones formed white colonies when grown on LB plates supplemented with ampicillin and 5-bromo-4-chloro-3-indolyl-D-Galactopyranoside (X-Gal). The clone with CMC-hydrolyzing-activity formed a clear halo following the Congo red staining method (Wood *et al*., 1988). The CMCase clones were selected for further analysis.

Gene analysis

DNA sequencing was performed by the Beijing Genomics Institute (BGI). The DNA and protein sequences were aligned using BlastN and BlastP, respectively (http://www.ncbi.nlm.nih.gov/Blast/). Multiple sequence alignments were conducted by CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/). The signal peptide in the deduced amino acid sequence was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). A protein functional analysis was performed by InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/).

Gene cloning

The coding sequence, excluding the predicted signal peptide, was amplified by PCR using two primers: 2928F (5′-ACTGGATCCGCA CAGAACTGTGAAATGAC, containing a *Bam*HI site) and 2928R (3′-ATTCTCGAGTCAGTTGCCGTCGCCGTTGC, containing an *Xho*I site).

The PCR product was gel purified, digested with *Bam*HI and *Xho*I, and ligated into the corresponding sites of the vector pGEX-6p-1. The recombinant plasmid pGEX-*cel*5D was then transformed into *E. coli* DL21 (DE3). Cellulase-producing recombinants were identified by the Congo red method (Wood *et al*., 1988). The nucleotide sequences of these recombinants were determined by DNA sequencing.

Gene expression and purification of the recombinant protein

A positive transformant was picked, containing glutathione-S-transferase (GST)-tagged pGEX-*cel*5D, from a single colony. The transformant was grown overnight at 37°C in the LB medium, supplemented with 100 μg/ml ampicillin. The culture was then incubated in fresh LB medium (1:100 dilution) containing ampicillin to an OD_{600} of 0.6. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM. The cells were incubated at 18°C for 8 h and harvested by centrifugation. Following centrifugation, the cells were washed, resuspended in phosphate buffer (140.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4), and disrupted by French press (Lin *et al*., 2009). The mixture was then centrifuged at 10,000×g for 30 min at 4°C and the supernatant was collected. The GST-tagged endoglucanase was purified through a GST-Bind Purification kit (Pharmacia), following the manufacturer's instructions. The GST tag was removed by digestion using a PreScission Protease solution (10 U/μl, Sweden, Pharmacia) and the purified protein was eluted with 1 ml phosphate buffer. The purity was confirmed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) analysis on 12% polyacrylamide gels.

Enzyme assay

The cellulase activity was quantified by measuring the quantity of reducing sugars (as glucose equivalents) following reaction with 3′,5′-dinitrosalicylic acid (DNS) (Miller, 1959). One unit (U) of enzyme activity was defined as the amount of protein required to release an amount of reducing sugars equivalent to 1 μmol of glucose per min (using glucose as the standard).

Cel5D activity was measured in a total volume of 100 μl containing 90 μl of 1% CMC dissolved in McIlvaine buffer (0.2 M Na_2HPO_4/0.1 M citric acid, pH 5.0) and 10 μl of the appropriately diluted enzyme solution. The reaction mixture was incubated at 60°C for 30 min. The reaction was terminated by adding an equivalent volume of DNS reagent, as described previously (Miller, 1959), and the mixture was treated in a boiling water bath for 10 min. We then added 200 μl distilled water and quantified color development by measuring the absorbance of the solution at 540 nm. The protein concentration was measured by the Bradford assay, with BSA as standard (Bischoff *et al*., 2006). Cel5D activities with other substrates (avicel, barley glucan, oat-spelt xylan, birchwood xylan, chitin, starch, locust bean gum, and laminarin) were assayed under the same reaction conditions by replacing CMC with other substrates (1% initial concentration).

Biochemical characterization

The hydrolytic specific activities of Cel5D with different substrates were evaluated by replacing 1% CMC with a range of substrates, identified above (Table 1). The relative activity with each substrate was then calculated in comparison with CMC.

To investigate the activities of Cel5D with various oligosaccharides and CMC, the hydrolysis products obtained from substrates were analyzed by thin-layer chromatography (TLC) on a silica gel 60 F254 plate (0.20-0.25 mm) with n-butanol-acetic acid-water (3:2:2, v/v) as the running solvent. Then, the plate was visualized by spraying with a 9:1 (v/v) mixture of methanol and sulfuric acid containing 0.2% methanolic orcinol, as described previously (Fu *et al*., 2009).

The optimum pH for the purified enzyme activity was determined at 60°C and pHs, ranging from 2.0 to 9.5. The optimum temperature for the purified enzyme activity was determined over the range of 10-80°C at pH 5.0. We estimated the pH stability by pre-incubating the purified enzyme solution with a range of buffers (pH 2.0-11.0) at 4°C for 24 h. The thermostability of the purified enzyme was measured by pre-incubating the enzyme at a range of temperatures (10-80°C) for 1 h without substrate. The residual enzyme activity was measured in each instance under standard conditions (60°C, pH 5.0). The following buffers was used: McIlvaine buffer (0.2 M Na_2HPO_4/0.1 M citric acid) between pH 2.0-8.0 and 0.1 M glycine-NaOH between pH 8.0-11.0.

Table 1. Hydrolytic specific activities of purified recombinant Cel5D on various substrates

Substrate	Specific activity (U/mg)
CMC	1.44±0.04[a]
Avicel	< 0.01
Barley glucan	3.12±0.15
Oat-spelt xylan	< 0.01
Birchwood xylan	< 0.01
Chitin	< 0.01
Starch	< 0.01
Locust bean gum	< 0.01
Laminarin	< 0.01

[a] Standard deviations of specific activity

Table 2. Effect of metal ions and chemical reagents on the activity of purified recombinant Cel5D

Metal ions and chemical reagents	Concentration (mM)	Relative activity (%)
Control	1	100
K^+	1	115±0.6
Na^+	1	117±0.4
NH_4^+	1	109±0.8
Mg^{2+}	1	113±0.5
Ca^{2+}	1	99±0.3
Cu^{2+}	1	120±0.5
Zn^{2+}	1	137±1.0
Mn^{2+}	1	135±1.2
Fe^{2+}	1	121±0.7
Hg^{2+}	1	96±0.8
Co^{2+}	1	115±0.6
Pb^{2+}	1	97±0.4
EDTA	1%	105±0.8
Dithiothreitol	1%	119±0.5
β-Mercaptoethanol	1%	106±0.4
SDS	1%	3.2±1.2

[a] Standard deviations of specific activity

The influence of several metal ions and chemical reagents (outlined in Table 2) on recombinant enzyme activity was evaluated by reaction mixtures containing a 1 mM solution of each of the metal ions or chemical reagents at 60°C, pH 5.0.

The K_m, V_{max}, and K_{cat} values for the purified enzyme were determined using 0.25-40 mg/ml CMC and barley glucan as the substrates in McIlvaine buffer (pH 5.0) at 60°C. The data were plotted according to the Lineweaver-Burk method.

Nucleotide sequence accession number

The nucleotide sequence of *cel*5D from *M. mediterranea* was submitted to GenBank (accession number 1242045).

Results

Gene cloning and sequence analysis

6800 clones were screened in an *M. mediterranea* genomic DNA library for that hydrolyzed CMC. Based on the screening we selected one clone (D2928) that possessed halo-producing activity. The plasmid DNA insert from D2928 was 3 kb, including a 1,113 bp open reading frame (ORF) which had an overall G+C content of 62.98%. The ORF encoded a 371-residue polypeptide including a putative signal peptide of 41 residues.

The deduced amino acid sequence of the ORF was aligned with available protein sequences from the GenBank and SWISSPROT databases (Fig. 1). The sequence encoding one single catalytic domain shared a low level of similarity with endo-1,4-β-glucanase from the glycoside hydrolase family 5 (GH5). The overall sequence of *cel*5D shared 51, 48, and 49% identity with putative endoglucanases from *Rhizobium etli* CIAT 652 (or *Rhizobium etli* CIAT 894), *Sinorhizobium*

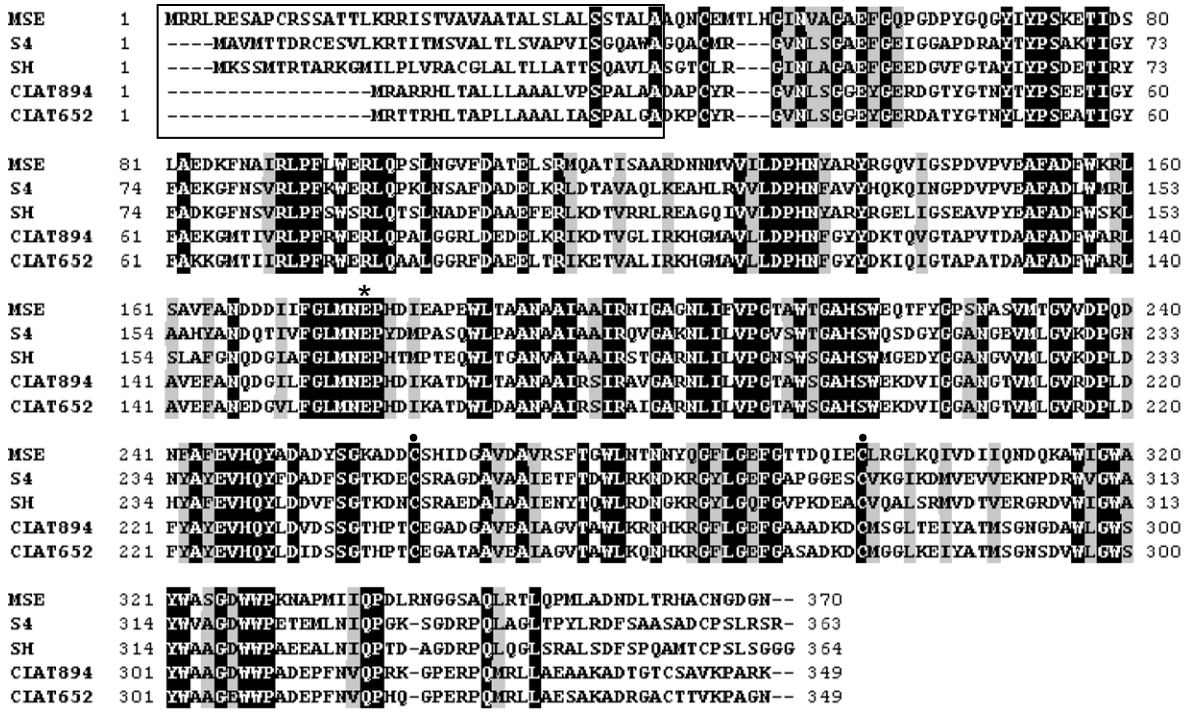

Fig. 1. Homology of Cel5D with the catalytic domains of other cellulases. Shaded areas indicate regions of 100% similarity; black areas indicate regions of 100% identity. The putative signal peptide sequence is contained within the box. The star indicates the catalytic residue and the dots represent the conserved cysteines. The GH5 endoglucanases S4, SH, CIAT894, and CIAT652 were isolated from *Agrobacterium vitis* S4 (gi 222106954), *Sinorhizobium meliloti* (gi 12005274), *Rhizobium etli* CIAT 894 (gi 218680146), and *Rhizobium etli* CIAT 652 (gi 190889902).

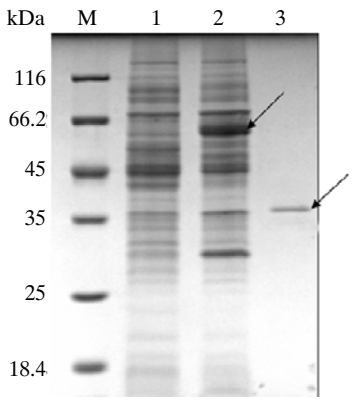

Fig. 2. 12% SDS-PAGE analysis of purified Cel5D stained by Coomassie blue. Lanes: M, protein molecular weight marker; 1, crude extract of control *E. coli*; 2, crude extract of *E. coli* harboring the expression plasmid with the *cel*5D gene. The molecular mass of GST-Cel5D is marked by the arrow. Lane 3: purified Cel5D. The molecular mass of Cel5D is marked by the arrow. The recombinant protein was purified using a GST-Bind purification kit.

meliloti, and *Agrobacterium vitis* S4, respectively, at the protein level.

Enzyme expression and purification

The vector pGEX-6P-1 was used as it enhanced the dissolubility of the fusion protein without influencing the properties of the protein. The pGEX-*cel*5D was expressed in *E. coli* DL21 (DE3). The *E. coli* transformant containing pGEX-*cel*5D yielded a band at 62 kDa (Fig. 2, lane 2). This is consistent with the predicted combined molecular mass of Cel5D (36 kDa) with a GST tag (26 kDa). The purified Cel5D yielded a band at 36 KDa which agrees with the expected molecular mass (lane 3). Our results suggest that the expression and purification procedures were effective.

Characterization of the recombinant Cel5D

The specific activity of purified Cel5D towards CMC was 1.6 U/mg. The K_m, V_{max}, and K_{cat} values, using CMC as the substrate, were 28.4±0.1 mg/ml, 455±0.3 μmol/(min·mg) and 36.4/sec, respectively. When barley glucan was used as the substrate, the respective values were 8.8±0.1 mg/ml, 87.7±0.2 μmol/(min·mg), and 3.5/sec. Cel5D produced mainly cellobiose, cellotriose and cellotetraose from cellopentaose and CMC which were separated by thin-layer chromatography (Fig. 3). The hydrolytic specific activities of Cel5D with the different substrates is shown in Table 1.

The maximum enzyme activity towards CMC occurred at pH 5.0 (Fig. 4), suggesting the enzyme is acidic cellulase. The enzyme was stable over a wide pH range. Greater than 60% of maximum activity was retained after treatment at pH 3.0-11.0 for 24 h. The optimum temperature, at which the enzyme had the greatest activity, was 60°C at pH 5.0. The enzyme was stable ranging from 10 to 60°C, and retained more than 80% of the maximum activity after incubation for 1 h. Above 60°C, enzyme activity declined rapidly. Approximately 40% of the maximum value was retained at 70°C.

The metal ions and chemical reagents had no significant

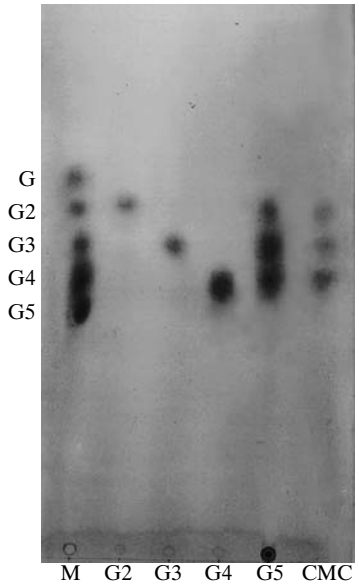

Fig. 3. TLC analysis of products from hydrolysis of oligosaccharide and CMC by Cel5D. M, marker; G, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose.

effect on enzyme activity (Table 2). On the other hand, SDS significantly inhibited enzyme activity. The relative activity was only 3.2% in the presence of SDS.

Discussion

Cellulose-producing microorganisms are commonly isolated from terrestrial environments. We identified a novel endoglucanase Cel5D from a cellulase-producing deep-sea bacterium *M. mediterranea*. This is the first description of an endoglucanase from *M. mediterranea*.

Our analysis of the protein structure and functional domains of Cel5D revealed that this enzyme contains a single catalytic domain belonging to GH5. In general, cellulases possess a cellulose-binding domain (CBD) which plays an important role during binding and facilitates the activity of the catalytic domain in the presence of insoluble, but not soluble, substrates (Linder and Teeri, 1997). And it is well established that the removal of the CBD has little influence on activity of cellulases towards soluble substrates (Gilkes *et al*., 1988). So, Cel5D can hydrolyze CMC (soluble cellulose) by the absence of a CBD. Cel5D possessed activity towards CMC (polymerized by β-1,4-linked glucose residues) and barley glucan (polymerized by β-1,3-1,4-linked glucose residues), but no activity towards laminarin (polymerized by β-1,3-1,6-linked glucose residues) or other substrates. Together, these data suggest that the hydrolytic activity of Cel5D is specific for β-1,4-glucosidic bonds. The exo-type glucanases hydrolyze cellulose from the free ends of cellulose chains, producing monosaccharides. Conversely, endo-type glucanases randomly cleave within the cellulose chain (Evans *et al*., 2000). Cel5D mediated CMC hydrolysis yielded cellobiose, cellotriose, and cellotetraose, suggesting that this enzyme is an endo-type glucanase. On the other hand, Cel5D can react with cellopentose instead of other smaller oligosaccharides to produce cellobiose, cellotriose,

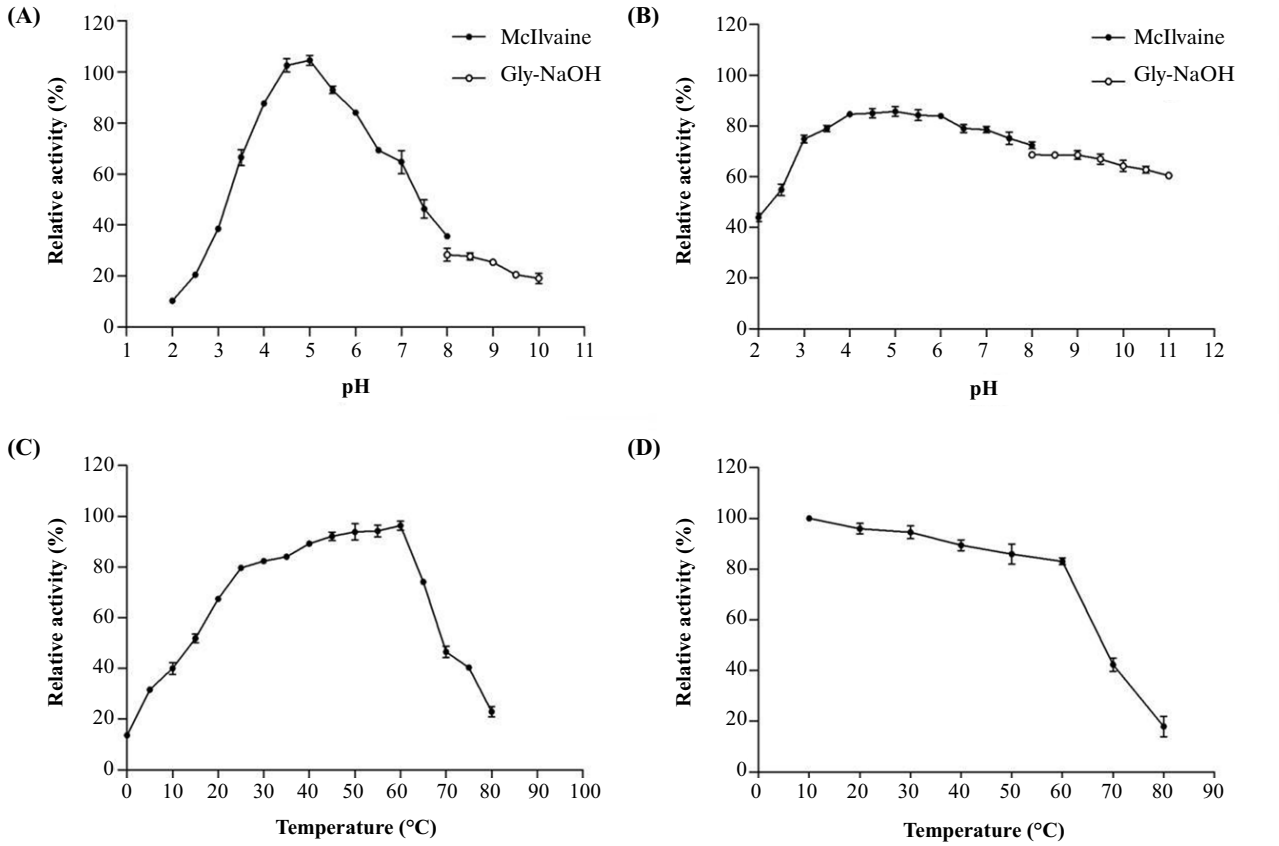

Fig. 4. Characterization of the purified recombinant Cel5D. (A) Effect of pH on Cel5D activity. The assay was performed at 50°C in buffers ranging from pH 2.0-9.5. (B) pH stability of Cel5D activity. The enzyme was incubated at 4°C for 24 h in buffers ranging from pH 2.0-11.0. The activity was then measured in McIlvaine buffer (pH 5.0) at 60°C. (C) Effect of temperature on Cel5D activity, assayed in McIlvaine buffer (pH 5.0). (D) Thermostability of recombinant Cel5D. The enzyme was pre-incubated at temperatures ranging from 10-70°C in McIlvaine buffer (pH 5.0). Residual activity was measured at 60°C.

and cellotetraose, suggesting that cellopentose is the minimal unit that can be recognized by Cel5D.

A number of family 5 endoglucanases share similar pH optima (pH 6.0-10.0), including CelB1 from *Bacillus* sp. strain N186-1 (pH 7.0) (Sanchez-Torres *et al*., 1996), Egl-252 from *Bacillus* sp. strain KSM-N252 (pH 10.0) (Endo *et al*., 2001), and endoglucanases from *Paenibacillus* spp. (pH 6.0-8.5) (Akinori *et al*., 2007). In contrast, Cel5D is an acidic cellulase that functions most optimally at pH 5.0. Duan *et al*. (2009) also identified an acidic cellulase, C67-1, that had a pH optima of 4.5 and was stable within the range of pH 3.5-10.5. Interestingly, Cel5D was stable after treatment with buffers that ranged in pH from 3.0 to 11.0 for 24 h, a wider range than for C67-1. Many industrial process involving cellulases rely on the use of extreme pH conditions to reduce contamination by other bacteria. Thus, these processes often require acidophilic enzymes to guarantee the degradation of fiber particles. In most instances, adjusting the process to a neutral pH is uneconomical and inefficient in a production setting (Turner *et al*., 2007). Therefore, enzymes that are pH-stable over a wide range yield considerable cost saving.

Cel5D was comparably stable at a range of temperatures when compared with other GH5 endoglucanases. The purified enzyme exhibited maximum activity at 60°C and was functional within the range of 25-60°C. Moreover, Cel5D retained more than 80% of the maximum activity when assayed at 10-60°C. In contrast, CelA, from *Cellvibrio mixtus*, was unstable following incubation at temperatures above 55°C for 15 min (Fontes *et al*., 1997). Similarly, Ba-EGA, from *Bacillus* sp. AC-1, retained greater than 80% of its activity following incubation at 50°C, but less than 60% following incubation at 60°C for 1 h (Li *et al*., 2006). MI-ENG1 retained about 80% of its activity between 45°C and 60°C, but the enzyme was inactivated above 65°C (Bera-Maillet *et al*., 2000).

Understanding the effect of metal ions and reagents on enzyme activity is important as many industrial applications require their addition at various stages of the process. None of the metal ions we tested, or EDTA, had a significant effect on Cel5D activity, suggesting that the reaction is not dependent on divalent cations. This contrasts with most other cellulases that are influenced by one or several metal ions (Voget *et al*., 2006; Feng *et al*., 2007). Moreover, the activity of Cel5D was not affected by dithiothreitol (DTT) or β-mercaptoethanol, which are known to inhibit other related enzymes. Thus, Cel5D may be used in bioconversion processes that include a variety of treatments such as metal ion or chemical reagent pretreatment, prior to, or simultaneously with, enzyme treatments.

In summary, we identified a novel endoglucanase from the deep-sea bacterium *M. mediterranea*. This endoglucanase possesses a number of useful traits, including a broad pH-tolerance range, thermal stability, and independence from the effects of metal ions and chemical reagents. These traits make the enzyme ideally suited for applications that rely on the saccharification process, including the production of bioethanol, fabrics, food, and animal feed. Our data provide further information on the diversity and function of cellulases, and highlight the potential use for *M. mediterranea* in the design of industrial processes involving cellulose breakdown.

Acknowledgements

We would like to thank Dr. Qifa Zhang for valuable advice. This study was supported by grants from the National Natural Science Foundation of China (30770021 and 30570057), the 111 project (B07041), and the National Infrastructure of Natural Resources for Science and Technology Program of China (No. 2005DKA21209).

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