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Cel8H, a Novel Endoglucanase from the Halophilic Bacterium *Halomonas* sp. S66-4: Molecular Cloning, Heterogonous Expression, and Biochemical Characterization

Xiaoluo Huang[1], Zongze Shao[2], Yuzhi Hong[3], Ling Lin[1], Chanjuan Li[1], Fei Huang[1], Hui Wang[1], and Ziduo Liu[1]*

[1]*State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, P. R. China*
[2]*Key Laboratory of Marine Biogenetic Resources, The Third Institute of Oceanography, State of Oceanic Administration, Xiamen 361005, P. R. China*
[3]*College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, P. R. China*

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A recombinant *Escherichia coli* clone expressing an endoglucanase was identified from a genomic library of the halophilic bacterium *Halomonas* sp. S66-4, and the enzyme was designated Cel8H. The *cel*8H gene consisted of 1,053 bp and encoded 350 amino acids sharing the highest identity of 48% to other known endoglucanases. The protein was expressed in *E. coli* BL21 (DE3) and purified to homogeneity. The purified recombinant enzyme had an optimal activity of 4.9 U/mg at pH 5 and 45°C toward the substrate carboxymethylcellulose. It exhibited extraordinary properties which differed from endoglucanases reported previously at the point of high salt tolerance above 5 M, simultaneously with high pH stability at pH 4-12 and high temperature stability at 40-60°C. Various substrate tests indicated that the enzyme hydrolyzes β-1,4-glucosidic bonds specifically.

***Keywords*:** endoglucanase, salt tolerance, pH stability, temperature stability, *Halomonas* sp.

Endoglucanase, also called endocellulase, catalyses the cleavage of the internal glucoside linkage of a cellulose polysaccharide chain, and is involved in the physiological processes of carbon metabolism in many fungi, bacteria and archaea (Lynd *et al*., 2002) and also the cellulose biosynthesis in some bacteria such as *Agrobacterium tumefaciens* and *Acetobacter xylinum* (Matthysse *et al*., 1995; Yasutake *et al*., 2006). Endoglucanase works with exocellulase and glucosidase to degrade cellulose biomass in natural environment, and has many commercial applications, such as in the detergent, fabric, or bioenergy industries (Endo *et al*., 2001; Wang *et al*., 2009). Till now, numerous endoglucanase genes have been cloned from the moderate microorganisms and some extremophiles such as thermophiles and alkaliphiles (Lynd *et al*., 2002; Wang *et al*., 2009). Most of these enzymes are also purified and their biochemical characterizations or crystal structures are identified. Nevertheless, there is very limited information about endoglucanases from the halophilic extremophiles. Only recently, a salt-activated endoglucanase from a halotolerant, alkaliphilic bacterium *Bacillus agaradhaerens* (Hirasawa *et al*., 2006) and a cellulase with endoglucanase-like activity from a halophilic bacterium *Salinivibrio* sp. strain NTU-05 (Wang *et al*., 2009) were purified and characterized. Apart from the industrial applications, characterization of genes encoding endoglucanases from the halophilic species will be invaluable in clarifying their regulatory mechanisms and physiological functions. Study of these enzymes will also provide very useful information for elucidating the structure-function relationship of proteins with the optimal activity at high salt concentrations.

Halomonas spp. are halophiles that could tolerate high salt concentrations up to 20%-35% NaCl (Dobson *et al*., 1993). The group contains a large number of halophilic species, isolated from different terrestrial and aquatic environments with, in most cases, high salt concentrations (Garcia *et al*., 2004). We reported here the cloning and sequencing of a novel endoglucanase gene from a halophilic bacterium *Halomonas* sp. S66-4. The gene was expressed in *E. coli* BL21 (DE3) and the recombinant enzyme was purified to homogeneity and characterized. The amino acid compositions, charges, and theoretical isoelectric points of endoglucanases from halophilic and non-halophilic microorganisms were also compared.

Materials and Methods

Bacterial strains and plasmids

Halomonas sp. S66-4 was supplied by the Marine Culture Collection of China (MCCC 1A0S66-4). It could grow in media containing 2% to 25% NaCl with the optimal growth at 10% NaCl and shared 99.7% of its 16S rDNA sequence (EU670604) similarity to a halophilic bacterium *Halomonas salaria* M27^T (Kim *et al*., 2007). *E. coli* DH10B was used for construction of the genomic library in the pUC18 vector. *E. coli* BL21 (DE3) was used as a host for gene expression.

Genomic library construction and screening

Genomic DNA of *Halomonas* sp. S66-4 was partially digested with *Eco*RI and DNA fragments with sizes ranging from 2-7 kb were purified and ligated into *Eco*RI-digested and CIAP-treated pUC18 vector. The ligation products were transformed into *E. coli* DH10B

* For correspondence. E-mail: lzd308@yahoo.cn; Tel: +86-27-8728-1429; Fax: +86-27-8728-0670

and then the transformants were spread on plates containing 1 mM isopropyl-D-thiogalactopyranoside (IPTG) and 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for white-blue screening, and incubated overnight at 37°C. For identification of the endoglucanase-producing *E. coli* transformants, all the white colonies were picked up and transferred to carboxymethylcellulose (CMC) plates (0.001% $MgSO_4$, 0.005% KH_2PO_4, 0.001% $CaCl_2$, 0.6% NaCl, 0.2% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.1% yeast extract, 0.5% CMC, and 1.5% agar), then incubated at 37°C for 24 h, and the plates were visualized using a Congo red staining method (Teather and Wood, 1982).

Sequence analysis

Nucleotide sequencing was accomplished by the Sangon Company (Shanghai, China). The open reading frame (ORF) and the promoter region in the obtained DNA fragments were predicted using FGENSB and BPROM (http://linux1.softberry.com/berry.phtml). The possible signal peptide was found using SIGNALP (http://www.expasy.org). Amino acid and nucleotide sequence alignment were analyzed using BLASTP and BLASTN (http://www.ncbi.nlm.nih.gov/). The multiple sequence alignment was carried out using CLUSTALW program in BIOEDIT. Other sequence analyses were performed using DNASTAR.

Cloning the *cel*8H gene

The *cel*8H coding region from the putative signal peptide cleavage site to a region downstream of the stop codon was amplified using the primers F-*Bam*HI (5-GTGGGATCCAATTTCGCTGTCGGCGATC) and R-*Eco*RI (GTGGAATTCCTATCCGGCGCTCAGGCT) with the following conditions: 94°C, 4 min; 94°C, 30 sec; 55°C, 30 sec; 72°C, 90 sec for 30 cycles then 72°C, 10 min; 4°C, 10 min. Products were then digested by *Bam*HI and *Eco*RI, and purified by agarose gel electrophoresis, then ligated into similarly treated pGEX-6p-1 vectors and transformed into electro-super-competent cells of *E. coli* BL21 (DE3). A number of clones were sequenced and a clone with the correct sequence, harboring the plasmid pGEX-6p-1-ns-*cel*8H, was used for further protein expression and characterization experiments.

Protein expression and purification

The *E. coli* BL21 (DE3) clone, harboring the plasmid pGEX-6P-1-ns-*cel*8H, was grown overnight at 37°C in Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin. The culture was then inoculated into fresh LB medium (1:100 dilution) containing 100 μg/ml ampicillin and grown at 37°C to an OD_{600} of 0.6. IPTG was then added to a final concentration of 0.5 mM. After induction at 28°C for 12 h, cells were harvested and resuspended in 50 ml PBS buffer (0.8% NaCl, 0.02% KCl, 0.142% Na_2HPO_4, 0.027% KH_2PO_4; pH 7.4), then disrupted through a French cell press at 20,000 psi cell pressure. The cell lysate was centrifuged at 13,000×g for 30 min at 4°C and the supernatant was collected. The protein was then purified using glutathione *S*-transferase (GST) Gene Fusion System (GE Healthcare, Sweden) as described (Cao *et al.*, 2008). The GST-Cel8H fusion protein from *E. coli* cell-free extracts was bonded to glutathione affinity resins, and then recombinant Cel8H was released from the bound GST protein by PreScission protease. The quantification of the protein was determined using the Bradford reagent (Sigma, USA) with bovine serum albumin (BSA) as a standard (Bradford, 1976) and the purity of the extracted proteins was analyzed by sodium dodecyl sulfate-denatured polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of enzyme activity

Standard enzyme assay was carried out according to the method described below. Enzyme (1 μg) was mixed with 200 μl CMC [1.0% (w/v) in phosphate-citrate buffer, pH 5]. After incubation at 45°C for 1 h, dinitrosalicylic acid reagent (200 μl) was added, and the mixture was heated in a boiling water bath for 5 min. The absorbance was measured at 540 nm. One unit of enzyme activity was defined as the quantity of enzyme capable of releasing 1 μmol of glucose equivalent per min (glucose as standard).

The K_m and k_{cat} values of the purified recombinant Cel8H were determined by measuring the enzymatic activity with various concentrations of CMC as the substrate. The concentrations ranged from 0.5 mg/ml to 15 mg/ml. The data was plotted according to the Lineweaver-Burk method.

To study the hydrolyzed products of CMC by the recombinant Cel8H, products generated from reaction with the substrate CMC were separated 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. The plate was visualized by spraying with a 9:1 (v/v) mixture of methanol and sulfuric acid containing 0.2% methanolic orcinol.

To investigate the substrate specificity of the enzyme, the activity was determined under the same conditions by replacing CMC with each of the following substrates: 0.5% barley glucan (Sigma), 0.5% laminarin (Sigma), 1% avicel (Sigma), 1% filter paper (Whatman, Maidstone, UK), 1% birch wood xylan (Sigma), 1% mannan (Sigma).

To determine the optimum pH, enzyme activity assays were carried out at pH 3-12 under standard conditions except for pH. The pH stability was determined by incubating the enzyme at 4°C for 24 h at each pH followed by measuring the activity under standard conditions at pH 5. Buffers used were phosphate-citrate buffer (pH 3-8) and glycine-NaOH buffer (pH 8-12). The final pH values of the reaction solution were determined after addition of the enzyme and the substrate. The optimum temperature of activity was measured at 20°C to 70°C under standard conditions except for temperature. For the temperature stability study, the enzyme was incubated at 40°C, 50°C, 60°C, and 70°C at pH 5 for 24 h, and the residual activity was measured under the standard conditions at 45°C.

To investigate the effects of salt concentrations on the enzyme activity, 0.2 to 5 M NaCl were added to the enzyme activity reaction solution and used for the enzyme assay. To study the effects of chemical reagents on the enzyme activity, a certain concentration of metal ions, surfactants, and chelating agents were added to the enzyme assay solution and the residual activity was measured.

Nucleotide sequence accession number

The nucleotide sequence of the endoglucanase gene (*cel*8H) from *Halomonas* sp. S66-4 was submitted to the GenBank database under the accession number of FJ458448.

Results

Cloning and nucleotide sequence analysis of the *cel*8H gene

The genomic library of *Halomonas* sp. S66-4 in *E. coli* DH10B containing a total of 10,000 clones was constructed using the pUC18 vector and screened for endoglucanase activity on CMC agar plates. A colony with obvious halo-generating ability was isolated and identified by sequencing, and a 3.3 kb DNA fragment was found to contain in the recombinant clone.

Genes in the 3.3 kb DNA fragment were predicted using FGENSB in the softberry website. The result indicated that a complete endoglucanase ORF was located in the DNA region

Table 1. Amino acid compositions, charges, and theoretical isoelectric points of endoglucanases from halophilic and non-halophilic microorganisms

Microorganisms	Enzyme	Acidic residues (%)	Basic residues (%)	Net charge	p*I*
Halomonas sp. S66-4	endoglucanase	12.89	8.81	-12.22	4.69
Cellulomonas uda	endoglucanase	8.63	11.90	11.70	9.82
Pectobacterium chrysanthemi	endoglucanase	9.06	8.74	-0.42	6.76
Gluconacetobacter xylinus	endoglucanase	8.48	8.19	-0.28	6.87
Clostridium thermocellum	endoglucanase	10.56	8.99	-5.91	5.79
Bacillus agaradhaerens	endoglucanase	15.75	5.25	-40.52	4.04
Salinivibrio sp. strain NTU-05	cellulase[a]	14.50	12.3	-5.224	5.54

[a] this cellulase has endoglucanase-like activity

from 1,536 bp to 2,588 bp, and the gene was designated *cel*8H. The ORF contained 1,053 bp that started with an ATG codon and terminated in a TGA codon and the overall G+C content was 61.6%. Upstream of the ORF, there was a region relatively close to the bacteria promoter containing the -10 (TTTTATT) and -35 (TTGCCA) regions, located at 38 bp and 66 bp from the start codon. No significant homology was found between *cel*8H and other gene sequences in the GenBank database by BlastN analysis.

Amino acid sequence analysis and comparison

Analyzing the protein with SIGNALP software, a possible signal peptide of 32 amino acids was found located at the N terminal of the enzyme, and thus the resulting mature protein polypeptide was calculated to be 318 amino acids in length with a total molecular mass of 36 kDa.

The amino acid distribution, net charge, and theoretical isoelectric point of Cel8H without the signal peptide were analyzed and compared with the endoglucanases of non-halophilic bacteria included in Table 1. The salt activated endoglucanase from a halotolerant, alkaliphilic bacterium *Bacillus agaradhaerens* (Hirasawa *et al*., 2006) and the partial amino acid sequence of a cellulase with endoglucanase-like activity from an isolated halophilic bacterium *Salinivibrio* sp. strain NTU-05 (Wang *et al*., 2009) were also included for comparison. To our knowledge, these are the only two endoglucanase amino acid sequences originating from halophilic microorganisms with experimental evidences. As shown in Table 1, the three endoglucanases from halophiles showed a relatively higher percentage of acidic residues, and lower net charge and theoretical isoelectric points.

Based on the amino acid alignments by BLASTP, the Cel8H was classified into glycosyl family 8. It showed the highest identity (48%) with a putative endoglucanase from *Azotobacter vinelandii* AVOP (ZP_00418773). It also had 39% identity with an endoglucanase from *Pectobacterium chrysanthemi* (Cho *et al*., 2002), 37% identity with the endoglucanase from *Gluconacetobacter xylinus* (Yasutake *et al*., 2006), 36% identity with the endoglucanase from *Cellulomonas uda* (Nakamura *et al*., 1986) and 25% identity with the endoglucanase from *Clostridium thermocellum* (Alzari *et al*., 1996). Multiple sequence alignment was

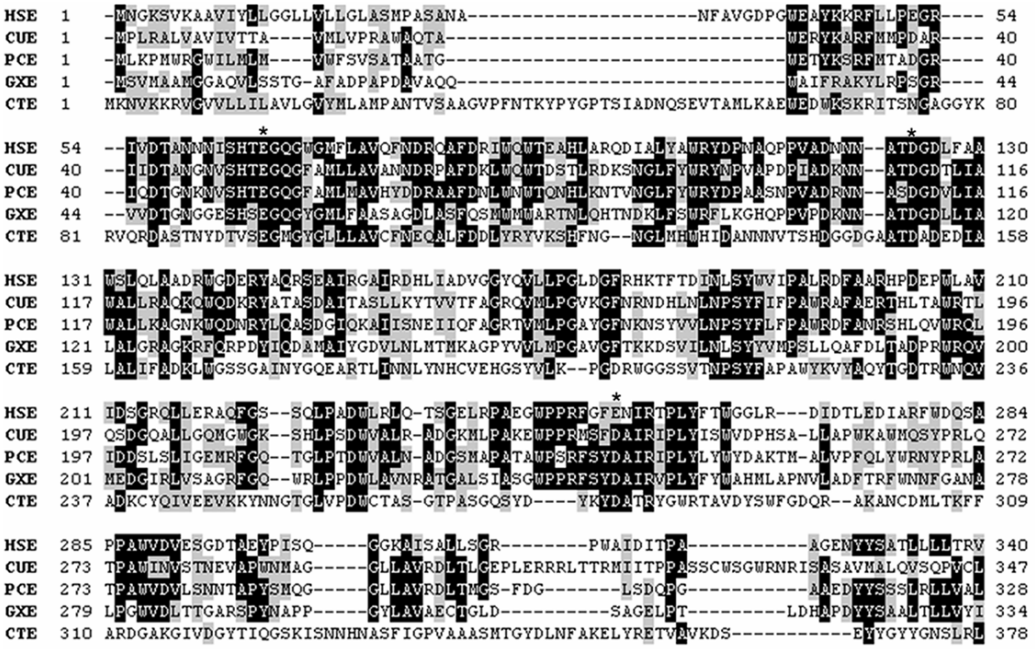

Fig. 1. Multiple sequence alignment between Cel8H and other family 8 endoglucanases. The multiple sequence alignment was performed using CLUSTAL W program in BIOEDIT. Shaded areas indicate regions of high amino acid similarity; black regions indicate regions of 100% similarity. The predicted catalytic residues are marked by asterisks (*). Abbreviations: HSE, the endoglucanase from *Halomonas* sp. S66-4 (this work); CUE, the endoglucanase from *Cellulomonas uda* (Nakamura *et al*., 1986); PCE, the endoglucanase from *Pectobacterium chrysanthemi* (Cho *et al*., 2002); GXE, the endoglucanase from *Gluconacetobacter xylinus* (Yasutake *et al*., 2006); CTE, the endoglucanase from *Clostridium thermocellum* (Alzari *et al*., 1996).

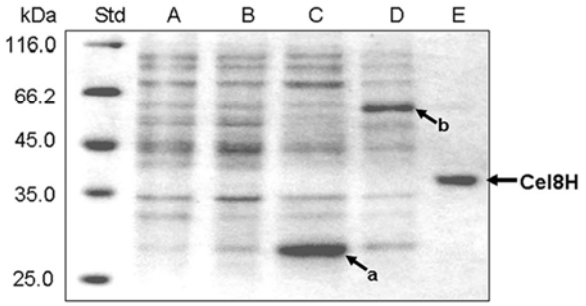

Fig. 2. SDS-PAGE analysis of the purified endoglucanase (Cel8H) from *Halomonas* sp. S66-4. Std: the standard protein marker; A, un-induced cell lysate of *E. coli* BL21 (DE3) harboring pGEX-6p-1; B, un-induced cell lysate of *E. coli* BL21 (DE3) harboring pGEX-6p-1-ns-*cel*8H; C, IPTG-induced cell lysate of *E. coli* BL21 (DE3) harboring pGEX-6p-1; arrow "a" indicate induced GST band; D, IPTG-induced cell lysate of *E. coli* BL21 (DE3) harboring pGEX-6p-1-ns-cel8H, arrow "b" indicate GST-Cel8H band; E, purified Cel8H.

conducted using sequences which had experimental evidences (Fig. 1). From this alignment it was obvious that Cel8H had many conserved amino acid residues, although the enzyme showed a low identity with other endoglucanases.

Expression and purification of the Cel8H

Due to the low level of expression of Cel8H in the primary clone in the DH10B, the gene region corresponding to the mature enzyme was introduced into the pGEX-6p-1 vector, yielding the vector pGEX-6p-1-ns-*cel*8H and expressed in the *E. coli* BL21 (DE3). As shown in Fig. 2, after induction, cell lysate of *E. coli* harboring pGEX-6p-1-ns-*cel*8H showed a thick band at approximately 62 kDa (Lane D), consistent with the predicted molecular mass of Cel8H (36 kDa) with a GST tag (26 kDa). This confirms that GST-Cel8H fusion enzyme was expressed efficiently. The purified Cel8H gave a band similar to the expected molecular mass (36 kDa) on SDS-PAGE (Lane E). After measuring the protein amount using the Bradford method, the concentration of the purified enzyme was determined to be 0.3 mg/ml.

Characterization of the recombinant Cel8H

The highest activity of the Cel8H enzyme with the CMC substrate was 4.9 U/mg. The K_m and K_{cat} of the enzyme with the CMC substrate were 37.5 mg/ml and 4.8×10^3 min^{-1}. The main hydrolyzed products of the CMC substrate were cellotriose and cellotetraose, when separated by TLC (Fig. 3). The enzyme showed an activity of 1.9 U/mg to the substrate barley glucan while it showed no activity to the substrates laminarin, avicel, filter paper, birch wood xylan, and mannan.

The pH and temperature dependence of the activity of Cel8H is shown in Fig. 4. The enzyme retained considerable activity between pH 4.0-9.0 with a maximum at pH 5.0. It was also very stable over a wide range of pH, maintaining over 90% activity after incubation at 4°C for 24 h in the pH range of 4-12. The temperature optimum of the enzyme was 45°C and over 60% activity was also found at 30°C and 60°C. The enzyme showed good temperature stability, retaining over 90% activity after incubation at 40°C for 24 h, and more than 60% activity after incubation at 50°C for 24 h or at 60°C for 12 h.

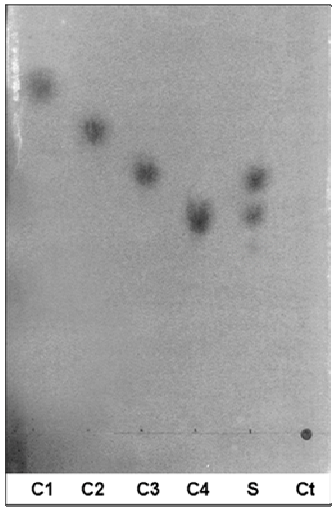

Fig. 3. Thin-layer chromatographic analysis of hydrolyzed products of CMC by the recombinant Cel8H. C1, glucose; C2, cellobiose; C3, cellotriose; C4, cellotetraose; S, CMC treated with the recombinant Cel8H; Ct, CMC.

The effects of NaCl concentrations on the enzyme activity are shown in Fig. 5. Nearly constant activity was found in the presence of 1-3 M NaCl. The activity of the enzyme decreased at higher salt concentrations. At 5 M NaCl, about 40% activity remained.

The relative activity of the recombinant Cel8H in the presence of different metal ions or chemical reagents is shown in Table 2. The activity of the enzyme was strongly inhibited by Hg^{2+}, SDS, and Cu^{2+}, and slightly inhibited by Pb^{2+} and EDTA. Fe^{2+} enhanced the activity of the enzyme significantly. Other metal ions and chemical reagents showed nearly no effect on the activity of the enzyme.

Table 2. Effects of metal ions, surfactants, and chelating agents on the activity of Cel8H

Effectors	Concentration	Relative activity (%)
Control		100
Metal ions		
Mg^{2+}	1 mM	101±0.7[a]
Ca^{2+}	1 mM	92±0.8
Mn^{2+}	1 mM	102±1.2
Fe^{2+}	1 mM	158±0.5
Co^{2+}	1 mM	97±0.3
Cu^{2+}	1 mM	45±0.4
Zn^{2+}	1 mM	97±0.6
Hg^{2+}	1 mM	2±1.5
Pb^{2+}	1 mM	76±0.8
Surfactants		
SDS	5% (w/v)	16±0.9
Triton X-100	1% (v/v)	90±0.6
Chelating agent		
EDTA	1 mM	78±0.3

[a] Relative activity±SD.

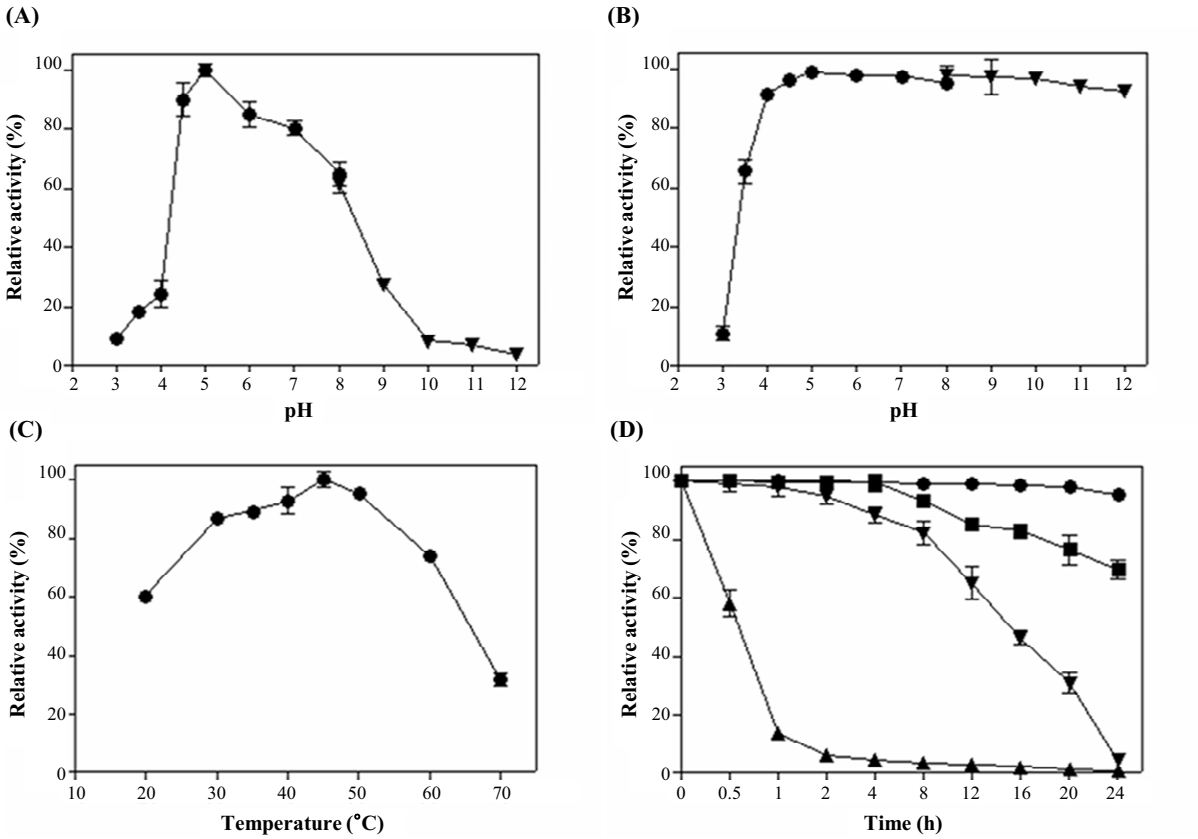

Fig. 4. Effects of pH and temperature on enzyme activity and stability. (A) The optimum pH. The enzyme activity was measured at the indicated pH at 45°C for 1 h. Buffers used were phosphate-citrate (pH 3-8, "●") and glycine-NaOH (pH 8-12, "▼"). (B) The pH stability. The enzyme stability was determined by incubating the enzymes at different pH at 4°C for 24 h, and the residual activities were measured at pH 5. (C) The optimum temperature. The enzyme activity was measured at temperatures ranging from 20°C to 70°C. (D) The temperature stability. Temperature stability was carried out by incubating the enzyme at 40°C (●), 50°C (■), 60°C (▼), and 70°C (▲) at pH 5 for 24 h, and the residual activity was measured under the standard conditions at 45°C. Error bars represent the standard deviation.

Discussion

Cel8H was the most active against the substrate CMC (polymerized by β-1,4-glucosidic bonds) and had relatively lower activity against barley glucan (polymerized by β-1,4 and β-1,3 glucosidic bonds). It was not active against laminarin (polymerized by β-1,6 and β-1,3 glucosidic bonds). These activities are similar to those of the glucanases from *Rhodothermus marinus* (Halldorsdottir *et al*., 1998), *Rhizopus oryzae* (Murashima *et al.*, 2002), *Bacillus sphaericus* JS1 (Singh *et al.*, 2004), and *Bacillus* sp. AC-1 (Li *et al*., 2006), which hydrolyzed the β-1,4-glucosidic bonds specifically. The enzyme showed no activity to insoluble avicel and filter paper. The main hydrolyzed products of Cel8H to the substrate CMC were cellotriose and cellotetraose. These activities are similar to those of some endo-type glucanases that hydrolyzed the internal bond of soluble cellulose polysaccharide randomly (Halldorsdottir *et al*., 1998; Akita *et al*., 2005). Thus, Cel8H could be defined as endo-β-1,4-endoglucanase.

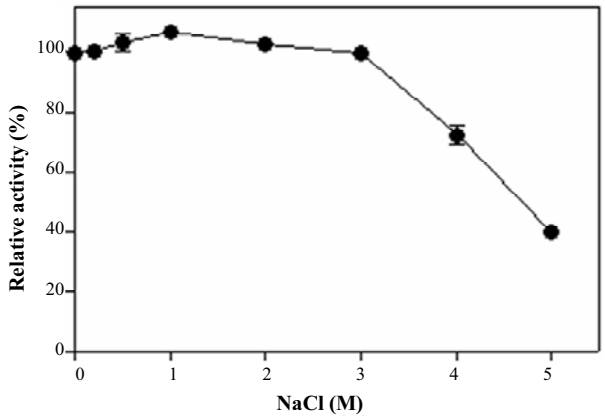

Fig. 5. Effects of salt concentrations on the enzyme activity. Different concentrations of NaCl were added into the enzyme assay solution and the relative activity was measured. Error bars represent the standard deviation.

On the basis of sequence comparison, Cel8H was assigned to glycosyl hydrolase family 8. Glycosyl hydrolases family 8 is a group of converting enzyme that cuts the β-1,4-glucosidic bonds between the running sugar residues (Alzari *et al*., 1996). The catalytic mechanism of the CelA, an endoglucanase from *Clostridium thermocellum*, revealed that a conserved Glu residue (Glu95) was required as the proton donor and two Asp residues (Asp152, Asp278) were the catalytic bases (Alzari *et al*., 1996). Correspondingly, similar amino acid

residues in Cel8H were Glu67, Asp124, and Glu254. Since Glu67 and Asp124 were strictly conserved in glycosyl hydrolase family 8, the functions of these two residues are likely to be the same with those in CelA. However, Glu254 replaced Asp278 in CelA and other endoglucanases originating from family 8 (Fig. 1). Although Glu was proven to be the catalytic base in family 5, 10, 11 (http://www.cazy.org) cellulases, it has never been reported in family 8. Therefore, a new catalytic mechanism may exist in Cel8H.

The salt tolerant cellulase is of great interest for treating agricultural waste and the bioremediation of cellulose materials at the conditions of low water (Wang *et al*., 2009). Cel8H, exhibiting high salt tolerance with keeping full enzyme activity under 3 M NaCl and retaining considerable activity even at 5 M NaCl, should provide a new biocatalyst for such biotechnological uses. Enzymes, which are insensitive to high salt concentrations, often show a higher acidic amino acid content, compared with the similar enzymes originating from non-halophilic bacteria (Lanyi, 1974; Fukuchi *et al*., 2003). The excess acidic amino acid residues are localized on the protein surface and contribute to bind large amounts of water molecules and metal ions to maintain the activity of the protein (Dym *et al*., 1995). In agreement with this, endoglucanases from halophiles *Halomonas* sp. S66-4, *Bacillus agaradhaerens* and *Salinivibrio* sp. strain NTU-05 were shown to be relatively acidic proteins. This fact could partially explain the halotolerance mechanism of the endoglucanases. However, the difference in the amount of acidic amino acids of the salt tolerant endoglucanase from *Halomonas* sp. S66-4 and endoglucanases from non-halophilic bacteria, especially the endoglucanase from *Clostridium thermocellum*, was not so obvious. This indicated that the salt tolerance mechanism of endoglucanase from halophilic bacteria may be very complicated and requires further investigation.

pH stability analysis indicated that Cel8H was stable at both acidic and alkaline condition, showing nearly full activity after incubation at pH 4-8 (phosphate-citrate buffer) and 8-12 (glycine-NaOH buffer) for 24 h. These are different from other endoglucanases, since they showed stability at a narrower pH range. For example, an alkaline endoglucanase from alkaliphilic *Bacillus* isolates showed good pH stability only in neutral and alkaline region (pH 6-11) (Endo *et al*., 2001) and an acidic endoglucanase from *Actinomyces* sp. 40 has good pH stability only in neutral and acidic region (pH 4-7) (Min *et al*., 1994). In industries which use endoglucanases, such as production of bioethanol, fabrics, and detergents, the pH conditions are often changeable (Gole *et al*., 2001). Thus, the high pH stability of Cel8H provides great potential for industrial applications. Moreover, the enzyme showed good temperature stability, with high retention of enzyme activity at 40℃ to 60℃ for several hours. As the optimum activity of the enzyme was at 45°C, Cel8H may be one of the most stable enzymes among the mesophilic endoglucanases. Though the cellulase from *Salinivibrio* sp. strain NTU-05 has a remarkable stability from 10°C to 20°C, the stability decreased sharply at higher temperatures (Wang *et al*., 2009). A cellulase with endoglucanase-like activity derived from metagenomes showed superior stability at 40°C, however the stability decreased sharply when the temperature reached above 50°C (Voget *et al*., 2006). Since temperature stability is a most important factor for enzyme being used in research or industry (Gole *et al*., 2001), high stability would make Cel8H a strong candidate for commercialization.

In the previous reports, Co^{2+} was shown to affect the activities of many endoglucanases prominently. For instance, the activities of endoglucanases, RCE1 and RCE2 from *Rhizopus oryzae* were inhibited remarkably by Co^{2+} (Murashima *et al*., 2002) and the activity of an endoglucanase from *Bacillus sphaericus* JS1 increased by 157% in the presence of 1 mM Co^{2+} (Singh *et al*., 2004). However, Co^{2+} showed no effects on the activity of the Cel8H. Fe^{2+} was reported to inhibit the activity of some endoglucanases, such as endoglucanases from *Bacillus* sp. AC-1 (Li *et al*., 2006) and *Daldinia eschscholzii* (Karnchanatat *et al*., 2008), and have no effects on other endoglucanases (Voget *et al*., 2006; Wang *et al*., 2009). Nevertheless, the activity of Cel8H was markedly enhanced by Fe^{2+}. Although mechanisms for such properties of Cel8H were obscure and need to be further investigated, these results may provide a very useful reference for studying on endoglucanases from halophiles.

To sum up, it is the first time to clone and sequence an endoglucanase gene (*cel*8H) from the genus *Halomonas* sp. The gene was expressed successfully in the non-halophilic host *E. coli* BL21 (DE3) and the recombinant enzyme was purified. The enzyme showed distinct properties with high salt tolerance, high pH and temperature stability simultaneously which would make it a good candidate for biotechnological uses. Since very limited information is available for endoglucanases from halophiles, the *cel*8H gene should prove to be very important for elucidating the physiological functions of such enzymes among the group of halophilic microorganisms.

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