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Screening and Characterization of a Cellulase Gene from the Gut Microflora of Abalone Using Metagenomic Library

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A metagenomic fosmid library was constructed using genomic DNA isolated from abalone intestine. Screening of a library of 3,840 clones revealed a 36 kb insert of a cellulase positive clone (pAM11E10). A shotgun clone library was constructed using the positive clone (pAM11E10) and further screening of 3,840 shotgun clones with an approximately 5 kb insert size using a Congo red overlay revealed only one cellulase positive clone (pAM11L9). The pAM11L9 consisted of a 5,293-bp DNA sequence and three open reading frames (ORFs). Among the three ORFs, cellulase activity was only shown in the recombinant protein (CelAM11) coded by ORF3, which showed 100% identity with outer membrane protein A from *Vibrio alginolyticus* 12G01, but no significant sequence homology to known cellulases. The expressed protein (CelAM11) has a molecular weight of approximately 37 kDa and the highest CMC hydrolysis activity was observed at pH 7.0 and 37°C. The carboxymethyl cellulase activity was determined by zymogram active staining and different degraded product profiles for CelAM11 were obtained when cellotetraose and cellopentaose were used as the substrates, while no substrate hydrolysis was observed on oligosaccharides such as cellobiose and cellotriose.

***Keywords*:** abalone, cellulase, metagenomic library, gut microflora

Cellulase has been isolated in plants, molds, fungi, bacteria, protista, and herbivorous invertebrates such as arthropods, nematodes, and mollusks, where the majority of cellulose degradation is carried out by symbiotic microbes in their ecological enviroments (Suzuki *et al*., 2003). Recent studies also have proven that prokaryotes may play an important role in the horizontal cellulase gene transfer to commensals such as termites, nematodes, and sea squirts (Angus and Mark, 2005). Mid-gut gland of marine animals possess glucan degrading genes for two molluscan glucanases, β-1,4-endoglucanase (glycoside hydrolase family 45) and cellulase (glycoside hydrolase family 9), from the blue mussel *Mytilus edulis* and the abalone *Haliotis discus hannai*, respectively (Tachibana *et al*., 2005). These finding raised the possibility of the presence of several types of β-glucanases, which may be produced by symbiotic microbes in their digestive tracts of marine invertebrates.

In recent years, fosmid cloning system has been commonly used widely for the identification of novel genes from organisms that are not easily cultured, the analysis of genomic sequencing and DNA fingerprinting. This screening system, which uses fosmid vector and insert size of 40 kb directly isolated from genomic DNA, has found out a wide range of genes such as those encoding chitinase, dehydrogenease, oxidoreductase, amylase, esterase, endoglucanase, and cyclodextrinase (Ammiraju *et al*., 2005; Suenaga *et al*., 2007).

Here, we identified and characterized a cellulase gene that was highly active toward carboxymethyl cellulose using a metagenomic fosmid library constructed from the gut microflora of abalone.

Materials and Methods

Metagenomic library construction

Abalone samples were obtained from the South Sea near Yeosu in the Republic of Korea during February, 2009. DNA of gut microflora of abalone was prepared by directed DNA extraction and purification as previously described (Kim *et al*., 2007). A metagenomic fosmid library from these samples was then constructed by an adjusted protocol as described previously (Yun *et al*., 2005).

Library screening and sequence analysis

The metagenomic fosmid library was screened for carboxymethyl cellulolytic activity using a Congo red overlay method (Teather and Wood, 1982). The DNA sequences of the positive clones were then determined using an AB1 2720 Thermocycler (Applied Biosystems) and an ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, version 3.1) in accordance with the manufacturer's instructions. The BLAST program at the National Center for Biotechnology Information (NCBI) was used for database searches and sequence comparisons. Amino acid sequences were aligned using

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Table 1. PCR primers used in this study

ORF	Primer	Sequence (restriction enzyme sites are underlined)
1	AM 1229F	5'-TGGGGAGCTCATGACACATCATTCATATTGGTTC-3'
	AM 2626R	5'-GCCGGATCCGTCAAGCTTATCCGCTTTGC-3'
2	AM 2743F	5'-TGGGGAGCTCATGAAAGTATTCAGCAACTTCGAT-3'
	AM 3864R	5'-GCCGGATCCAATTTTATCCGCTACGGTTAACG-3'
3	AM 4275F	5'-TGGGGAGCTCATGCTGCCGCGGTGTG-3'
	AM 5291R	5'-GCCGGATCCTTCAGTTGTTTGGTATTCTTCAAACGC-3'

the CLUSTALW software package (MEGA 4.0).

Enzyme overexpression and purification

The putative cellulase genes were amplified from the CMC positive clones using sense primers with a *Sac*I site and antisense primers with a *Bam*HI site (Table 1). The amplified DNA was then ligated into *Sac*I and *Bam*HI double digested pCold II (TaKaRa), after which the construct was transformed into *E. coli* BL21 (DE3) cells. The transformed cells were grown in 500 ml of LB broth at 37°C until an optical density at 600 nm of 0.5 was reached, at which point the transformed cells were allowed to stand at 15°C for 30 min. Next, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the flasks were then further incubated at 15°C for 24 h to induce the expression of recombinant proteins. The recombinant proteins were then concentrated against 50 mM Tris buffer (pH 7) at 4°C using an Amicon concentrator (10 kDa, cut-off filter) prior to purification. To purify the 6·His:CelAM11, 10 ml of crude cell lysates were loaded onto an iminodiacetic acid (IDA) MiniExcellose affinity column (Bioprogen, Korea) and then washed three times with 10 ml of equilibration buffer (50 mM phosphate, 0.5 N NaCl, pH 7.0). The recombinant proteins were subsequently eluted with 5 ml of 0.5 M imidazole in the same buffer (50 mM phosphate, 0.5 N NaCl, pH 7.0).

Determination of enzymatic activity

The optimal enzyme temperature and pH were estimated at temperatures and pHs ranging from 30°C to 40°C and 6.0 to 9.0, respectively, using a Nelson-Somogyi assay (Green *et al*., 1989). A standard curve was prepared using glucose at concentrations ranging from 31.5 μg/ml to 1,000 μg/ml based on measurement of the absorbance at 525 nm using a spectrophotometer (Mecasys Co., Ltd., Korea) (Green *et al*., 1989). Inhibition and enhancement of the cellulase activity was determined by a Nelson-Somogyi assay in the presence of metal ions and nicotinamide adenine dinucleotide (NAD^+). To assess whether the combination of metal ions (X) and NAD^+ (Y) is synergistic, additive or antagonistic, the fractional fold activity (FFA) index was calculated as follows: (A+B) / 4, where A=(fold activity of combination X+Y) / (fold activity of chemical X alone), B=(fold activity of combination X+Y) / (fold activity of chemical Y alone). The combination acts synergistically when a FFA index of two chemical components is more than 1; additively, FFA=1; antagonistically, FFA<1.

Detection of cellulase activity on gels

Purified recombinant enzyme CelAM11 was treated with sample buffer without 2-mercaptoethanol and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels according to the method described by Laemmli (1970). After SDS-PAGE, the proteins in the gel were renatured by three times, 30-min incubations in renaturation buffer (50 mM Tris-HCl buffer; pH 7.0, 10 mg/ml of casein, 2 mM EDTA, 0.01% NaN_3) containing 25% methanol. The polyacrylamide gel was then washed with 100 mM Tris-HCl buffer (pH 7.2) and overlaid onto a 2% agarose gel containing 1 mg/ml of CMC, 10 mg/ml of NaCl, and 100 mM Tris-HCl buffer (pH 7.2). The gel was then incubated at 30°C for 20 h, followed by flooding with an excess of 0.1% aqueous Congo red for 20 min, after which it was washed with excess 1 M NaCl solution.

Analysis of reaction products by thin-layer chromatography

Cellooligosaccharides (cellobiose, cellotriose, cellotetraose, and cellopentaose) were purchased from Sigma-Aldrich. To determine if CelAM11 had a cellulase activity, 10 mM cellooligosaccharides (cellobiose, cellotriose, cellotetraose, and cellopentaose) were digested with 25 μl of CelAM11 (2.29 mg/ml) in 50 mM Tris buffer (pH 7)

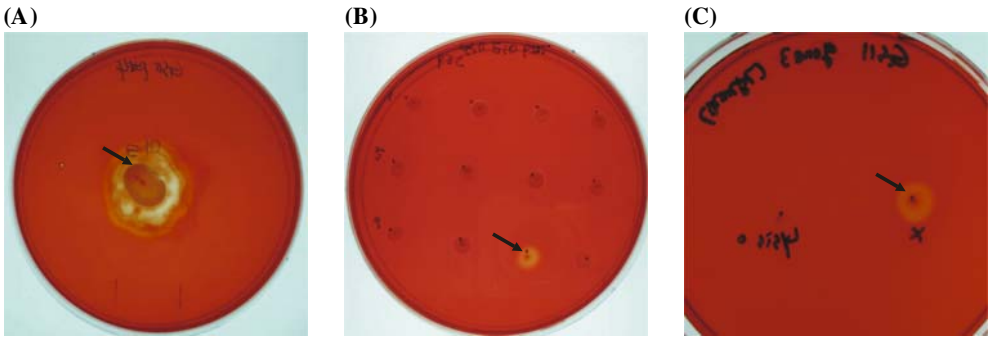

Fig. 1. Plate screening for CMC-hydrolyzing activity using a Congo red assay. CMC-hydrolyzing activities of clones, pAM11E10 (A), from metagenomic library clone, pAM11L9 (B) from shotgun library and clone, CelAM11 of ORF3 expressed protein (C). Arrows indicate carboxymethyl cellulase-positive colonies in the Congo red assay.

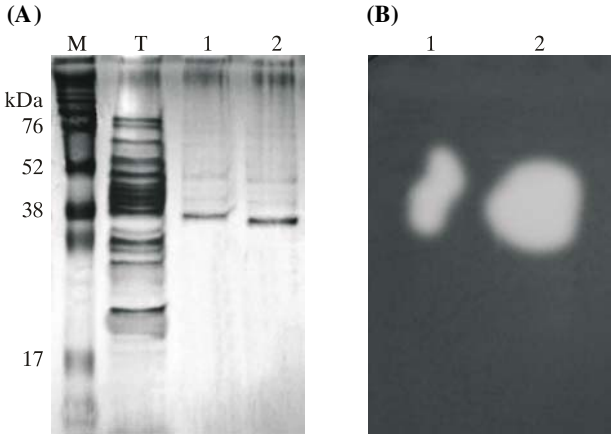

Fig. 2. Nondenaturing SDS-PAGE analysis and zymogram activity staining of purified recombinant CelAM11 protein. The left panel (A) shows a polyacrylamide gel stained with Coomassie Brilliant Blue R-250 after SDS-PAGE without 2-mercaptoethanol. Lanes: M, molecular weight marker proteins; T, cell lysate; 1, eluates in 0.1 M imidazole solution; 2, eluates in 0.5 M imidazole solution. The right panel (B) shows the results of each zymogram activity staining of purified recombinant protein (CelAM11) (lane 1) and cellulase obtained from *Aspergillus* sp. (lane 2) in agarose gel containing carboxymethyl cellulose.

at 37°C. Subsequently, the reaction products were developed in a mixture of 1-propanol, nitromethane and water (5:3:2, v/v/v) for 2 h, after which the hydrolysis products were separated by thin-layer chromatography (TLC) on a silica gel plate (Analtech, USA). Following separation, the sugars were visualized by spraying the plates with a mixture of 1 ml phosphoric acid and 10 ml stock solution (1 g diphenylamine, 1 ml aniline, 100 ml acetone) (Voget *et al.*, 2006).

Nucleotide sequence accession number

The accession number of CelAM11 gene in GenBank was HM235800.

Results and Discussion

Screening of a cellulolytic enzyme gene

A fosmid library screening for clones expressing cellulase activity on CMC identified a 36 kbp insert of cellulase positive clone (pAM11E10, Fig. 1A) from 3,840 clones screened of a total of 90,000 clones constructed. To identify the open reading frames (ORFs) responsible for cellulase activity in pAM11E10, further screening of subclones (average 5 kb insert size) constructed by a shotgun clone library using Congo red overlay identified only one cellulase positive clone, pAM11L9 (Fig. 1B). The pAM11L9 clone consisted of a 5,293-bp DNA sequence and three ORFs. To determine which ORFs had the ability to degrade cellulosic compounds, the three ORFs were expressed heterologously in *E. coli* BL21. The expressed protein was then purified to homogeneity from the cellular extracts using Ni-NTA agarose slurry. A congo red overlay assay identified the presence of cellulase activity only in the recombinant protein (CelAM11) coded by ORF3 (Fig. 1C).

Characterization of CelAM11

The purified polypeptide product of CelAM11 enzyme was

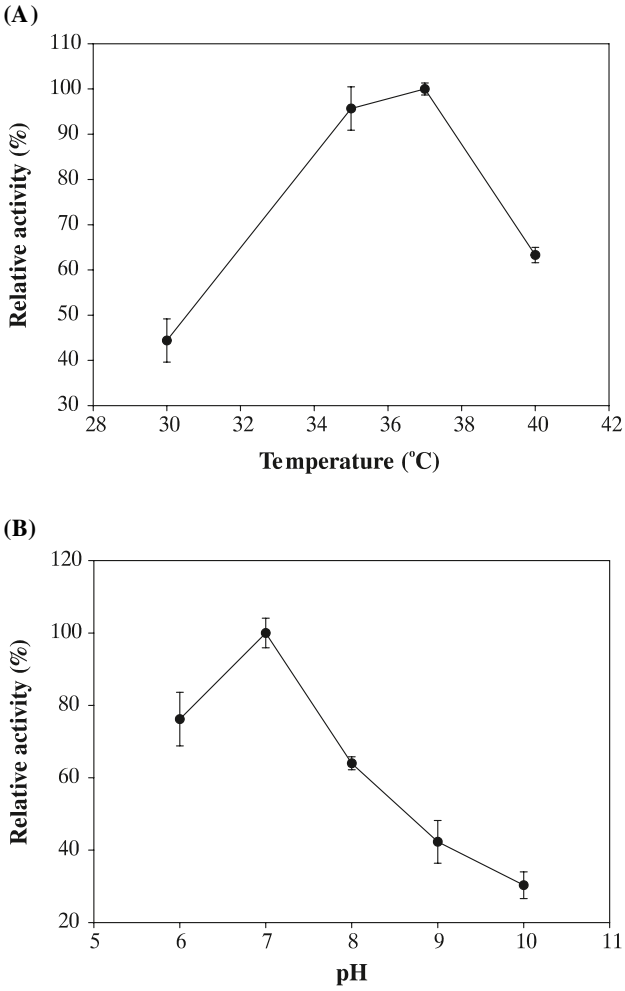

Fig. 3. The effect of temperature (A) and pH (B) of purified recombinant protein (CelAM11) on the cellulase activity in a reducing sugar assay.

37 kDa, which is in accordance with the theoretical molecular mass of 37 kDa (Fig. 2A). After SDS-PAGE, the proteins in the gel were analyzed by SDS-PAGE under renaturation conditions and then subjected to activity staining. The cellulase activity of CelAM11 was detected in a clear band with a molecular weight of approximately 37 kDa (Fig. 2B). The endoglucanase activity of the enzyme was determined by a reducing sugar assay in which a greater number of reducing sugars leads to increased color intensity in response to enzymatic hydrolysis of the glycosidic bonds of carbohydrates (Leemhuis *et al*., 2003). The reducing sugar assay revealed that the cellulolytic activity was higher at 30-37°C. However, the activity decreased rapidly at temperatures greater than 40°C. The absolute enzyme activity was 0.15 μmoles/min/mg protein and the maximum cellulolytic activity was obtained at pH 7 (Fig. 3). The effects of cofactors such as metal ions and NAD^+ on the activity of cellulase (CelAM11) are summarized in Table 2. The results revealed that the divalent cations Ca^{2+}, Co^{2+}, Fe^{2+}, and Mn^{2+} had at least a 3-fold higher stimulatory effects on the cellulase activity, while Cu^{2+} and Mg^{2+} had

Table 2. Effect of metal cations and NAD^+ on cellulase activity

Chemicals	Fold activity[a]		Fractional fold activity (FFA) index[b]
	Single	Combination (Cation + NAD^+)	
None	1.0±0.1	-	-
NAD^+ (0.1mM)	1.4±0.0	-	-
EDTA (1 mM)	0.8±0.1	-	-
$CaCl_2$ (1 mM)	3.0±0.6	3.3±0.4	0.9
$CoCl_2$ (1 mM)	3.1±0.6	3.6±0.1	0.9
$CuCl_2$ (1 mM)	1.6±0.4	2.8±0.1	1.0
$FeSO_4$ (1 mM)	3.1±0.1	4.3±0.2	1.1
KCl (1 mM)	1.8±0.2	2.6±0.1	0.8
$MgCl_2$ (1 mM)	2.0±0.2	5.0±0.0	1.5
$MnCl_2$ (1 mM)	3.4±0.5	3.4±0.2	0.9

[a] Fold activity was measured relative to the control reading as 1. These data are representative of the Mean±SD of three experiments.
[b] The fractional fold activity (FFA) index is defined as an interaction coefficient and the index is calculated by the formula: FFA=(A+B)/4, where A=(fold activity of combination X+Y) / (fold activity of chemical X alone), B=(fold activity of combination X+Y) / (fold activity of chemical Y alone). The combination of chemicals is synergistic when a FFA index of two chemical components is more than 1, additive: FFA=1, antagonistic: FFA<1.

1.6 and 2.0-fold higher stimulatory effects than the control, respectively. It has been reported that the glycoside hydrolase family, GH4, requires both NAD^+ and metal ions for hydrolysis of α-1,4 or β-1,4-glucosidic linkages (Varrot *et al*., 2005). NAD^+ caused a slight increase in the cellulase activity, but EDTA inhibited cellulase activity by about 20%. This might have been due to the chelation of required cations present in trace amounts that are necessary for enzyme activity (Kansoh and Nagieb, 2004). The combination of metal ions (Fe^{2+} or Mg^{2+}) and NAD^+ produced FFA indices of 1.1 and 1.5, respectively, indicating that the combination of these metals had a synergistic effect on the cellulase (CelAM11) activity, while the combination of other metal ions and NAD^+ produced an FFA index value of <1.0 (Table 2). The end products of the hydrolysis of cellulooligosaccharides (cellobiose, cellotriose, cellotetraose, and cellopentaose) by CelAM11 were determined by thin-layer chromatography (TLC) after 30 min (Fig. 4). Different product profiles for the cellulase (CelAM11) were obtained when cellotetraose and cellopentaose were used as the substrates. CelAM11 hydrolyzed G4 to G2, whereas the enzyme digested G5 to G2 and G3. However, CelAM11 was not able to cleave G2 and G3.

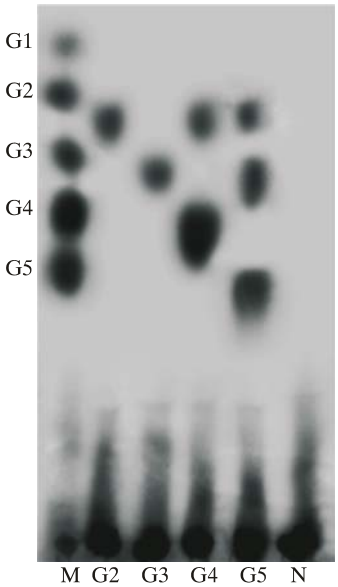

Fig. 4. Thin layer chromatography analysis of degradation products released by CelAM11. Hydrolysis of cellooligosaccharides (G2 to G5) after 30 min of incubation at 37°C. M is the standard mixture: G1 (glucose), G2 (cellobiose), G3 (cellotriose), G4 (cellotetraose), and G5 (cellopentaose). N is the negative control.

Amino acid sequencing analysis

pAM11L9 contains three ORFs and the deduced amino acid sequence for ORF1 (466 amino acids), ORF2 (374 amino acids) and ORF3 (323 amino acids) showed a 97%, 98%, and 100% similarity with pyridine nucleotide-disulphide oxidoreductase from *Vibrio alginolyticus* 12G01, carboxypeptidase from *Vibrio* sp. Ex25 and outer membrane protein A from *Vibrio alginolyticus* 12G01, respectively. Multiple amino acid sequence alignments of the ORFs showed no significant sequence homology to known cellulases. Many studies have identified outer membrane protein A (OmpA) as a multifaceted molecule with potential roles in initial cell attachment to cellulose and in cellulose digestion, as well as in clinical and biotechnological applications such as antimicrobial resistance, bacterial infection and bioemulsification (Lin *et al*., 2002; Abdel-El-Haleem 2003; Choi *et al*., 2005; Jun *et al*., 2007). Unlike the well studied mechanisms (free cellulase mechanism and cellulosomal mechanism) of cellulases by which they degrade the β-1,4-glucosidic linkages in cellulose, outer membrane protein complex of *Cytophaga hutchinsonii* and *Fibrobacter succinogenes* plays an important role in degradation of cellulose fibers. The genomic sequence of *C. hutchinsonii* identified one gene that was involved in cellulose utilization showed homology to a *Bacteroides thetaiotaomicron* outer membrane protein that may be involved in starch degradation (Xie *et al*., 2007). Additionally, *F. succinogenes* has different outer membrane proteins that are required for binding, removing and transporting cellulose molecules (Jun *et al*., 2007). The results showing complete amino acid sequence homology of CelAM11 with outer membrane protein A of *V. alginolyticus* suggest that further research would be necessary to investigate a novel mechanism of multifaceted cellulase ac-

tivity using outer membrane complex of different *Vibrio* species.

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