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Cloning and Characterization of Cellulase Gene (*cel*5C) from Cow Rumen Metagenomic Library

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A metagenomic library of cow rumen in the pCC1FOS phage vector was screened in *E. coli* EPI300 for cellulase activity on carboxymethyl cellulose agar plates. One clone was partially digested with *Sau*3AI, ligated into the *Bam*HI site of the pBluescript II SK+ vector, and transformed into *E. coli* DH5α. We obtained a 1.5 kb insert DNA, designated *cel*5C, which hydrolyzes carboxymethyl cellulose. The *cel*5C gene has an open reading frame (ORF) of 1,125 bp encoding 374 amino acids. It belongs to the glycosyl hydrolase family 5 with the conserved domain LIMEGFNEIN. The molecular mass of the Cel5C protein induced from *E. coli* DH5α, as analyzed by CMC SDS-PAGE, appeared to be approximately 42 kDa. The enzyme showed optimum cellulase activity at pH 4.0, and 50℃. We examined whether the *cel*5C gene comes from the 49 identified cow rumen bacteria using PCR. No PCR bands were identified, suggesting that the *cel*5C gene came from the unidentified cow rumen bacteria.

Key words : Rumen metagenome, cellulase, *cel*5C, unculturable bacterium, CMC-SDS-PAGE

Introduction

The anaerobic bacteria in the rumen have been a subject of intensive studies over the past 40 years, beginning with the recognition of the fact that large numbers of bacteria are present in the rumen [12,28,34]. Recently molecular diversity of the bacterial part of the system, which is mainly responsible for the plant fiber breakdown process, has been intensively studied during several years. The extreme bacterial molecular diversity uncovered in these investigations reflects the complex metabolic network in which the rumen bacteria are involved [8,11,13,15,23]. Traditional methods for culturing microorganisms limit analysis to those that grow under laboratory conditions. More than 99% microbes present in various environments cannot be cultured [6,21,29]. For overcoming this difficulty, metagenome technology was developed. There has been an increase in the number of studies using a metagenomic approach to investigate the catalytic potential of non cultured microorganisms [30].

Cellulose is not only the most abundant biopolymer on earth, primarily as a structural component of the cell wall of plants and marine algae, but can also be produced by other organisms such as bacteria [18]. The plant cell wall is composed primarily of fibril of cellulose, a hydrogen-bonded β(1-4)-linked D-glucan, which accounts for 20-30% of the dry weight of most plant primary cell walls [9,22]. The degree of crystallinity of the cell wall is highly variable. It may be as low as 20% in primary cell walls or as high as 70% in secondary cell walls [17]. Although terrestrial herbivores might be expected to have a set of enzymes that can digest the cellulose and other polysaccharide structures of plants, terrestrial animals have elaborated a different evolutive solution: symbiotic relationships with bacteria, protists, and fungi which carry out these activities in their own interests [8,11]. The bacterial population of the rumen is a complex ecosystem composed of many different species of bacteria, but it has about 15 to 20 predominant species. Ruminant animals depend on microorganisms to digest cellulose, but only a few species of rumen bacteria can degrade cellulose [12,31].

Biodegradation of cellulose is primarily performed by microbial action. Cellulose is digested by cellulose which was firstly classified by its mode of catalytic action and more recently by structural properties [22]. Three major types of enzymatic activities are found: (i) endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also

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known as cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and (iii) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21). Endoglucanases cut randomly at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends [19]. Exoglucanases act processively on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolases) as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure. β-glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose. Therefore, all three functional cellulases perform effective degradation of cellulose via consequently processing of individual cellulose [2,3].

In this study, the cellulase of a new type was isolated and characterized from bovine rumen metagenome by using cosmid library and subcloning by using shotgun method.

Materials and Methods

Bacterial strains and growth conditions

Escherichia coli DH5α was used as the host for transformation of plasmid DNA, and *E. coli* EPI300 was used for infection with λ phages. These strains were grown in Luria-Bertani (LB) liquid medium or on LB agar plates at 37°C. The media were supplemented with 50 μg of ampicillin per ml or 12.5 μg of chloramphenicol per ml for selection of plasmids. The vector pBluscript II SK+ was used for cloning and DNA sequencing, the vector pCC1FOS with arabinose-inducible promoter was used for expression of *cel* gene in *E. coli* EPI300.

Sampling of rumen metagenome

Cow's rumen contents were obtained from a closed herd at the Gyeongnam National University of Science and Technology (Jinju, Korea). The animals were rumen-fistulated Korean cows (HANWOO) with the body weight 400±10 kg, and fed twice by a mixed ration (rice hull and concentrate in a 4:1 ratio) for a day. The concentrate feed was purchased from Daehan Food (Ulsan, Korea). Representative samples of total rumen contents were collected from the animal via the ruminal fistula before the morning feeding. The samples were immediately transferred into an anaerobic box and were used for construction of the metagenomic library [4,14].

Recombinant DNA techniques

Standard procedures for agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, restriction endonuclease digestion, and other cloning-related techniques were used as described by Sambrook *et al.* [27].

Construction of cosmid library from cow rumen metagenome

The cosmid library of rumen metagenomic DNA was constructed in pCC1FOS [32]. The rumen metagenomic DNA was shorn by syringe. The sheared DNA (100 μg) was layered directly onto a 12 ml of 12.5 to 45% sucrose gradient in 20 mM Tris-HCl, pH 8.0/10 mM EDTA/50 mM NaCl, centrifuged for 22 hr at 22°C in 80,000× *g* using SWi-41 rotor (Beckman), and fractionated according to sizes. The fractionated DNA was recovered twice by ethanol precipitation and the sizes of DNA fragments in each fraction were estimated on a 0.7% agarose gel. Among these fractions, fractions of DNA molecules around the sizes 40 kb were used for construction of a cosmid library. The vector pCC1FOS was ligated with size-fractionated DNA fragments. After ligation reaction, this sample was *in vitro* packaged by lambda phage, and packaged sample was transformed into EPI300 by infection. White colonies were picked onto plates gridded to be compatible with 384-well microtiter plates. The library was replicated into duplicate sets of 384-well microtiter plates with freezing medium and stored at -80°C [5,14].

Subcloning using shotgun method

Metagenomic DNA of cow rumen was partially digested into 2 to 5 kb fragments with *Sau*3AI. The plasmid SK+ DNA was digested with *Bam*HI and dephosphorylated by CIP treatment. The SK+/*Bam*HI/CIP DNA (10 μg) was ligated with 2 to 5 kb DNA fragments and transformed into *E. coli* DH5α. The active clones were selected from above constructed subclones.

Assay of extracellular cellulase

In order to detect cellulase activity in *E. coli* harboring cloned *cel* gene, bacterial colonies were grown on a cellulase activity indicator medium [LB agar plates containing appropriate antibiotics and 15% (v/v) Cellomix]. After growth at 37°C for 24 hr, positive clones for extracellular cellulase activity were surrounded by a clear zone against a blue

background. The optimal pH and temperature for the cellulase activity were determined in citric/sodium phosphate buffer containing 1% (w/v) carboxymethyl cellulose. The cellulase activity in the recombinant DNA clones was measured using the dinitrosalicylic acid method. Cellulase activity was measured at temperature 10-80°C and at pH 3-11 over 30 min in absorbance at 510 nm.

DNA sequencing of the *cel5C* gene

Nucleotide sequencing was done by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer, USA). The samples were analyzed with an automated DNA sequencer (Applied Biosystems, USA). Assembly of the nucleotide sequences and the amino acid sequence analysis were performed with the DNAMAN analysis system (Lynnon Bisoft, Canada). The BLAST program was used to find the protein coding regions.

Primers and PCR condition

Specific PCR primers used to examine whether the *cel5C* gene comes from the unidentified cow rumen bacteria. An internal primer set from the ORF region of *cel5C*, 5′-TCAAGGACTGCGAGGACTGG-3′ (forward/#1378F) and 5′-ACGGGAGTCGCTCTTGTGA-3′ (reverse/#1379R) were used to amplify the corresponding region from the genomic DNA of 49 identified cow rumen bacteria (Table 1). PCR conditions were as follows: 30 sec at 94°C for denaturation, 30 sec at 55°C for annealing and 30 sec at 72°C for extension (35 cycles), except for 5 min denaturation in the first cycle and 10 min extension in the last cycle.

Active staining on CMC-SDS-PAGE

Carboxymethyl cellulose-SDS-PAGE was carried out as described by Park *et al.* [20,25]. The *E. coli DH5α* cells harboring *cel5C* gene were cultured at 37°C for 12 hr in LB medium supplemented with 0.1% (w/v) CMC and ampicillin (50 μg/ml). Whole cell extracts from these cultures were prepared by sonication (three times for 30 sec each at 4°C). The cell extracts and supernatants were mixed at 1:1 (v/v) with sample buffer [62 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 0.025% (w/v) bromophenol Blue, 5% (v/v) β-mercaptoethanol, and 2% (w/v) SDS], and the mixtures were heated at 95°C for 5 min. The protein samples (35 μg) were electrophoresed in Carboxymethyl cellulose-SDS-Polyacrylamide gel. Subsequently the protein was renatured by incubation in three changes of 250 ml of 10 mM Tris/HCl buffer (pH 7.5), 1% (v/v) Triton X-100 with shaking for 10 hr. Finally the gel was incubated in 10 mM Tris/HCl buffer (pH 7.5) at 70°C for 8 hr. The gel was immersed in 0.5% (w/v) Congo Red for 15 min and destained with 1 M NaCl for 15 min. Active band appeared as yellow halos on a red background. A final treatment with 0.1 M HCl turned the background dark blue, which facilitated photographic documentation.

Results and Discussion

Screening for cellulase activity

A shotgun method was used to clone the cellulase gene. The pooled DNA of library clones were selected the active clones on a cellulase activity indicator medium for cellulase activity. One positive colony showing the clear zone indicated CMCase activity was isolated. By subsequent subcloning from this 1.5 kb fragment, designated *cel5C*, which had the activity of hydrolyzation of carboxymethyl cellulose. A 1.5 kb fragment (pRCS300) of cow rumen metagenomic DNA was found to contain a *cel* gene with CMCase activity. The inserted DNA, pRCS300, contains the restriction sites for *Sma*I, *Eco*RV, *Apa*I, *Eco*RI, *Pst*I, and *Nco*I (Fig. 1). This fragment was sufficient for cellulase activity as determined on CMC indicator medium (Fig. 2). Also it is reported that a metagenome expression library of bulk DNA extracted from the rumen content of a dairy cow was established in a phage lambda vector and activity based screening employed to explore the functional diversity of the microbial flora. Nine endo-β-1,4-glucanase clones from twenty-two clones specifying distinct hydrolytic activities were identified in the library and characterized. Ferrer M. *et al* [7], Wang F. *et al* [33] studied that four different environmental DNA libraries were prepared from microbial consortium collected from forest soil, dung of elephant, cow rumen and rotted tree. Five endo-β-1,4-glucanases were isolated and identified.

Nucleotide sequence of the *cel5C* gene and PCR

The 1.5 kb inserted fragment was sequenced using the dideoxy chain-termination method. It contains one complete open reading frame. Fig. 3 depicts the *cel5C* structural gene with its flanking regions. The open reading frame contains 1,125 nucleotides and encodes a protein of 374 amino acid residues with a predicted molecular mass of about 42 kDa,

Table 1. List of culturable rumen bacteria and the results of confirmed PCR using internal primer of *ce*5C gene

Species	Source & Strain	Confirmed PCR[1)]
RSC300 (Positive clone)	CRMGL[2)]	+
Acetitomaculum ruminis	ATCC43876	-
Actinobacillus succinogenes	ATCC55618	-
Bifidobacterium adolescentis	DSM20087	-
Bifidobacterium boum	ATCC27917	-
Bifidobacterium merycicicum	ATCC49391	-
Bifidobacterium pseudolongum subsp. *Globosum*	ATCC25864	-
Bifidobacterium ruminantium	ATCC49390	-
Bifidobacterium thermophilum	ATCC25866	-
Butyrivibro fibrisolvens	strainOB156C	-
Clostridium aminophilum	Unknown	-
Clostridium cellobioparum	DSM1351	-
Clostridium clostridioforme	strain tB316	-
Clostridium longisporum	ATCC49440	-
Clostridium proteoclasticum	ATCC29084	-
Corynebacterium vitaeruminis	ATCC10234	-
Eubacterium cellulosolvens	strain 2388	-
Eubacterium limosum	ATCC10825	-
Eubacterium oxidoreducens	DSM3217	-
Eubacterium ruminantium	strain GA195	-
Fibrobacter succinogenes	strain S85	-
Lachnobacterium bovis	strain YZ87	-
Lachnospira multipara	strain L14-8	-
Lactobacillus ruminus	ATCC27780	-
Lactobacillus vitulinus	ATCC27783	-
Megasphaera elsdenii	ATCC25940	-
Methanobrevibacter ruminantiu	DSM1093	-
Methanomicrobium mobile	DSM1539	-
Mitsuokella jalaludinii	ATCCBAA-307	-
Oxobacter pfennigii	DSM3222	-
Prauserella rugosa	ATCC43014	-
Prevotella brevis	ATCC19188	-
Prevotella ruminicola subsp. *Ruminicola*	ATCC19189	-
Pseudobutyrivibrio ruminis	strain C78	-
Rumincoccus albus	strain Sy3	-
Rumincoccus flavefaciens	strain 007	-
Rumincoccus hansenii	DSM 20285	-
Rumincoccus productus	ATCC27340	-
Schwartzia succinivorans	DSM10502	-
Selenomonas ruminantium subsp. *Lactilytica*	strain HD4	-
Selenomonas ruminantium subsp. *Ruminantium*	ATCC12561	-
Stenotrophomonas maltophilia	ATCC13637	-
Streptococcus bovis	ATCC33317	-
Succiniclasticum ruminis	DSM9236	-
Succinivibrio dextrinsolvens	strain 24	-
Succinomonas amylolytica	DSM2873	-
Syntrophococcus sucromutans	DSM3224	-
Treponema bryantii	strain $B_2$5	-
Treponema saccharophilum	DSM2985	-
Wolinella succinogenes	ATCC29543	-

[1)]0.46 kb PCR product with the internal primers, #1378F and #1379R, from *ce*5C (pRCS300).
[2)]CRMGL: Cow rumen meta-genomic library

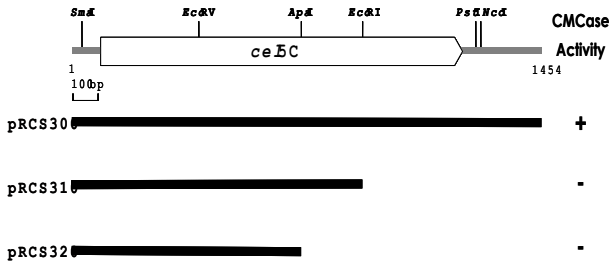

Fig. 1. Physical maps of the *cel*5C gene. The cleavage sites of restriction enzymes *Sma*I, *Eco*RV, *Apa*I, *Eco*RI, *Pst*I, and *Nco*I are shown. pRCS300 was constructed by cloning a 1.5 kb fragment of cow rumen metagenome into pBluescript II SK+ vector.

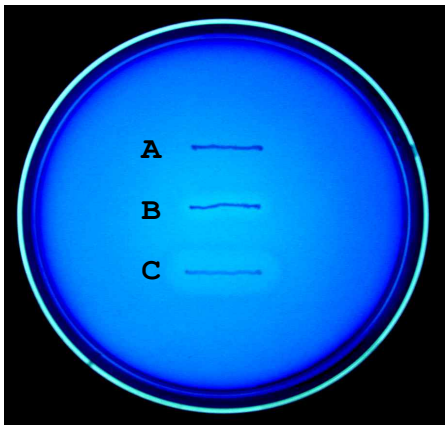

Fig. 2. Detection of enzyme CMCase activity by the agar diffusion method. Lane A, *E. coli* as a negative control; Lane B, *E. coli* harboring CelB of *Pectobacterium carotovorum* as a positive control; Lane C, *E. coli* harboring pRCS300.

and a calculated p*I* of 5.40. The ORF *cel*5C starts the ATG initiation codon and the reading frame ends with the opal stop codon TGA at position 1206. This sequence is likely to function in *E. coli* DH5α in the export of Cel5C to the extracellular environment. It was examined whether the *cel*5C gene comes from the metagenomic library of cow rumen by using PCR and DNA sequencing. The PCR product was electrophoresed on a agarose gel. A gel slice that

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1       TTACCCAGGCTGCGGCCGAAGAACCTAAGCCCGGGGAGGACACCAACTGGGAGTGGATCTAAGCTGGAATATGGGAAATCACTTCGACGCCTTCTACA
1                                                                                   M  G  N  H  F  D  A  F  Y  N  Y  G
121     AGAAGGATGGCTATCCCGACGAGACGGCCTGGCAGCCCAACAAAGCCACCAAGGGCAAAATGGAACTGGGCTTTTCCAGTGTCCGTATTCCCATCTCC
13      E  K  D  G  Y  P  D  E  T  A  W  Q  P  N  K  A  T  Q  A  T  F  D  G  L  K  E  L  G  F  S  S  V  R  I  P  I  S  W  L  K
241     TGATTGGCCCCGCCCCGGATTACAAGATTGACGAGACCTGGATGAACCGCGTGGTACTAGGCAATACGGCCGGTCTTAAGGTAATCATCAACACCCATC
53      M  I  G  P  A  P  D  Y  K  I  D  E  T  W  M  N  R  V  Y  E  V  V  G  F  A  H  T  A  G  L  K  V  I  I  N  T  H  H  D  E
361     ATCACGGTGTCAACAATGATTATCAGTGGCAGGATATCAAGAATGCAGCCAAAAAGGAGTGGCAACAAAGCCGAAATCAAAGCGGTCTGGACTCAGATTC
93      N  H  G  V  N  N  D  Y  Q  W  Q  D  I  K  N  A  A  N  N  S  A  K  N  E  A  I  K  A  E  I  K  A  V  W  T  Q  I  A  N  K
481     TCAAGGACTGCGAGGACTGGCTCATTATGGAGGGCTTCAATGAAATCAACGAGTGGAGGGGTAGGATTTCCGGGCCAATCCTGAGAAGCAGTGCAACATC
133     F  K  D  C  E  D  W  L  I  M  E  G  F  N  E  I  N  D  G  G  W  G  W  S  A  D  F  R  A  N  P  E  K  Q  C  N  I  L  N  E
601     GGAACCAGGTGTTCGTGAACGCCGTGCGTGCTACCGGTGGTAACAATGCCGACGTCGTCGACCTGATGCGGCCAATCCTACCTTTGAACAGTACTGGGCC
173     W  N  Q  V  F  V  N  A  V  R  A  T  G  G  N  N  A  T  R  W  L  G  C  P  T  Y  A  A  N  P  T  F  E  Q  Y  W  A  L  P  E
721     ACCCGGCCAAGAAGGTTATGATGGCCGTTCATTTCTACGATCCGTCCAGCAAGCACTCAGTGCGACTGGGGCCATACCGCTGCGGCCGACAAGAAAC
213     D  P  A  K  K  V  M  M  A  V  H  F  Y  D  P  S  S  Y  T  I  G  D  E  Q  Y  S  D  W  G  H  T  A  A  A  D  K  K  A  A  G
841     GTGACGAGCCTTATGTAAACGAAGTGTTCGGCAATCTGAACCTCAAGTATGTTCTCCACAGAAGGGAGAGTTTGGCTGCTCCATGCGTGACAAGAGCC
253     S  D  E  P  Y  V  N  E  V  F  G  N  L  N  L  K  Y  V  S  H  G  I  P  V  Y  L  G  E  F  G  C  S  M  R  D  K  S  D  T  R
961     CTTGGGCCTTTTACAAGTATTATCTGGAGTATATCGTGAAGGCGGCCAAAACTTGATGGCCTTGGGATAATGGTGCCACGGGCTCCGGAAAAGAGCAC
293     A  W  A  F  Y  K  Y  Y  L  E  Y  I  V  K  A  A  K  T  Y  G  L  P  C  Y  L  W  D  N  G  A  T  G  S  G  K  E  H  H  G  Y
1081    TAAACCACGCCACCGGAAAGCCTATGGGTAATAGTGCGGAAGTGCTCAAAGTCGTGGACCAATGACTCCGAAAGCTATACCCTTCAGTCTGTTTACAGCA
333     I  N  H  A  T  G  K  P  M  G  N  S  A  E  V  L  K  V  M  A  N  A  W  N  N  D  S  E  S  Y  T  L  Q  S  V  Y  S  K  A  P
1201    AGTTCTGATGAAAAGATTACTTATACTCGCTGCCCTGGCCCTCGCTGCCTGGAAGAACGGAAGGAAACGCCCAAAGACCAGCTGGTGCATACCCTGT
373     K  F  -
1321    GGAGAGCGGAAAGATTGCCTACGGGCATCAGGACGACCTCTTCTACGGCCAGAGGAGTGGAGGTAACGATGCCCTGGAACGTTCCGACGTCAAGGATG
1441    CTATCCCATGATCC
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Fig. 3. Nucleotide and deduced amino acid sequence of the *cel*5C gene. The stop codon is indicated by bar. The boxed region was indicated consensus region of cellulase family 5 and underlined was catalytic active site.

Fig. 4. Alignment of the predicted amino acid sequence of Cel5C with those of corresponding proteins in other organisms. Alignment was done by the DNAMAN program. Asterisks and dots indicate identical and similar amino acid in equivalent positions, respectively. The cellulase sequences compared to Cel5C are as follows: NP_347462, AAA26467, CAA73113, IEDG, and S22458.

contained a 1.5 kb fragment was cut and extracted with a gel extraction kit. The extracted DNA was analyzed with an automated DNA sequencer. It was conformed that the *cel*5C gene comes from the unidentified cow rumen bacteria by using PCR. An internal primer set from the ORF region of *cel*5C was used to amplify the corresponding region from the genomic DNA of identified cow rumen bacteria in Table 1 in a 50 µl reaction mixture with PCR. No PCR band was confirmed from culturable genomic DNAs. This fact suggests that *cel*5C come from the unculturable cow rumen bacteria (Table 1). For characterizing biomass-degrading genes and genomes, Hess, M. A. *et al.* [10] sequenced and analyzed 268 gigabases of metagenomic DNA from microbes adherent to plant fiber incubated in cow rumen and identified 27,755 putative carbohydrate-active genes and expressed 90 candidate proteins, of which 57% were enzymatically active against cellulosic substrates.

Amino acid sequence similarities between *cel*5C and other *cel* genes

A phylogenetic tree of the cellulase proteins by DNAMAN analysis system using amino acid sequences was constructed. The comparison of the predicted amino acid sequence of Cel5C to sequences deposited in the GenBank and SwissProt revealed that Cel5C shares significant sequence similarity with *Clostridium acetobutylicum* endoglucanase family 5 (NP_347462), *Ruminococcus albus* CelA (AAA26467) *Bacillus* sp. BP-23 cellulase (CAA73113), *Clostridium cellulolyticum* endoglucanase A (1EDG), and *Prevotella ruminicola* cellulase (S22458). The results of the alignment with the amino acid residues of Cel5C of cow rumen metagenome aligned with those of *Clostridium acetobutylicum* endoglucanase family 5, of *Ruminococcus albus* CelA, of *Bacillus* sp. BP-23 cellulase, of *Clostridium cellulolyticum* endoglucanase A, and of *Prevotella ruminicola* cellulase were shown 29.2%, 29.6%, 27.9%, 29.9%, and 40.4% identity, respectively (Fig. 4,5) [16,24,26]. The availability of sequencing data of several *cel* genes allowed us to identify family 5 of glucosidase from EC 3.2.1.4. with the conserved region, LIMEGFNEIN, localized close to N-terminal of Cel5A (Fig. 3). Ferrer M. *et al* [7] investigated that sequence analysis of the retrieved enzymes from the metagenome library of the dairy cow rumen

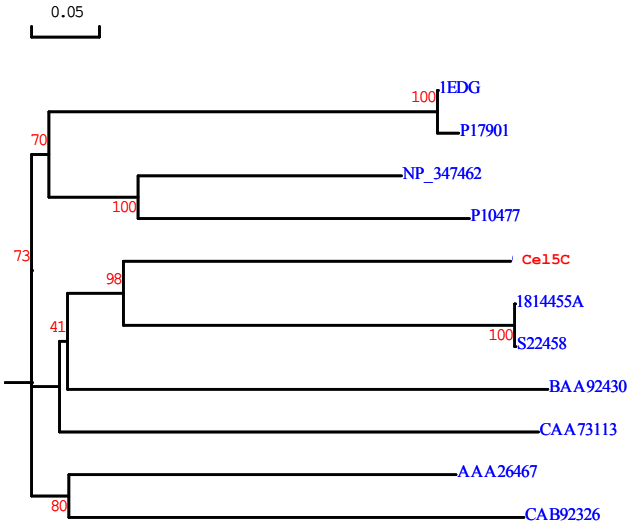

Fig. 5. Phylogenetic tree showed the evolutionary relatedness of the endoglucanse amino acid sequences. The estimated genetic distance between sequences is proportional to the length of the horizontal lines connecting one sequence to another. Accession numbers for these sequences shown in this tree obtained the GenBank and SwissProt.

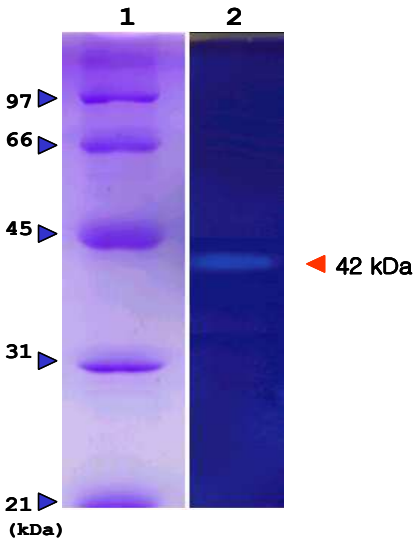

Fig. 6. Detection of enzyme activity bands by CMC-SDS-PAGE. Lane 1: contains molecular weight standards; phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Lane 2: Cel5C (pRCS300).

revealed that eight (36%) were entirely new and formed deep branched phylogenetic lineages with no close relatives among known glycosyl-hydrolases. Bioinformatic analyses of the hydrolase gene sequences, and the sequences and contexts of neighbouring genes, suggested tentative phylogenetic assignments of the rumen organisms producing the retrieved enzymes. The phylogenetic novelty of the hydrolases suggested that some of them may have potential for new applications in biocatalysis. Also Wang F. *et al* [33] reported that sequence analysis of the retrieved genes from four kinds of consortium metagenomic library revealed that the encoded products of these cellulase genes shared less than 50% identities and 70% similarities to cellulases in the databases. Domain analysis predicted that four endo-β-1,4-glucanases conform to glycolsyl hydrolase family 5 (GHF5) and one endo-β-1,4-glucanase to glycolsyl hydrolase family 9 (GHF 9), while both β-glucosidases belong to glycolsyl hydrolase family 3 (GHF3). Further sequence analysis indicated that although a solid affiliation could be made for the two endo-β-1,4-glucanases to the typical ruminal microbe *Prevotella ruminicola*, the rest formed deep-branched lineages with no close relatives.

Identification and characterization of the *ce*l5C gene product

An active protein band with the expected apparent molecular weight of about 42 kDa, as determined in comparison with the mobility of protein standards, produced a halo on a blue background (Fig. 6). Since the predicted *ce*l5C gene product consists of 374 amino acids with estimated molecular mass of 42.221 kDa and the size of the protein identified on the zymogram corresponds well with the predicted size.

The effect of pH on the activities of Cel5C against CMC was determined at 50°C in various buffers ranging from pH 3.0 to 11.0. The maximal activity was observed at pH 4.0 (Fig. 7A). The effect of temperature on Cel5C activity was also determined at pH 4.0 by measuring its activity at various temperatures. The maximal activity was observed at 50°C (Fig. 7B).

On the other hand, Cho K. M. et al [5] suggested that less than 30% of clones corresponded to previously identified bacteria, excluding duplicated and nonruminal clones, while more than 70% of clones corresponded to unidentified isolates. Based on these numbers, they estimated that there might be more than 300 different bacterial species in the rumen. How many different ruminal bacteria are there? It is now commonly accepted that the cultured species of bacteria represent only a minor fraction of the diversity existing. At present, more than 60 species of ruminal bacteria are stocked in the American Type Culture Collection (ATCC),

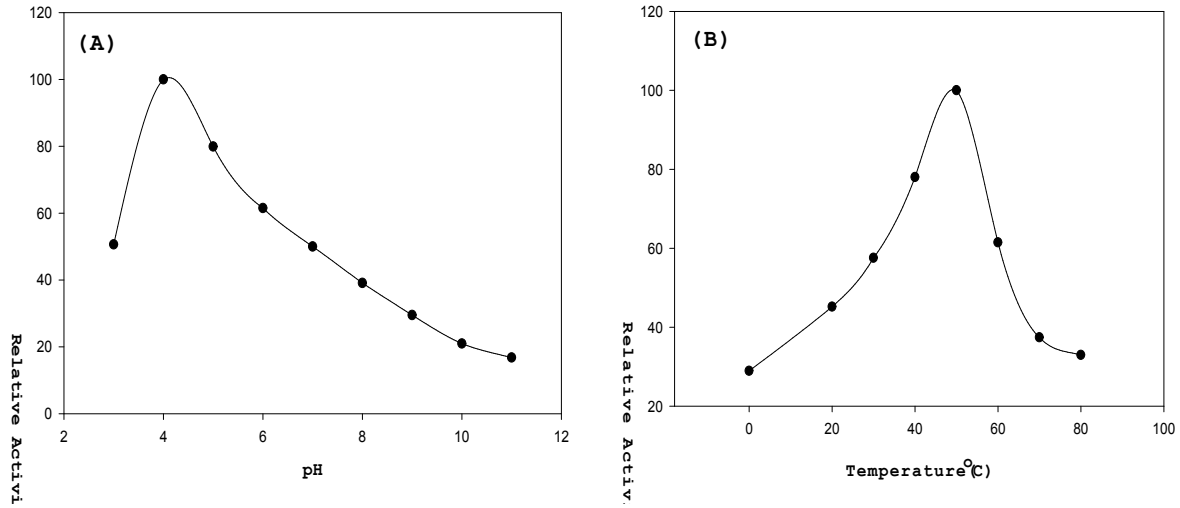

Fig. 7. Temperature and pH optimal activity of Cel5C. Charateristics of the activity of Cel5C enzyme expressed in *E. coli DH5a.* The Cel5C enzyme was assayed for its activity using CMC as a substrate at different pH values at 50°*C* for 30 min. The Cel5C enzyme was assayed for its activity using CMC as a substrate at different temperature values at pH 4.0 for 30 min.

the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and the Japan Collection of Microorganisms (JCM). Moreover, genomics technologies, including metagenomics, now provide rumen microbiologists with their best opportunity in both a functional and comparative studies. This is an exciting prospect. Clearly, genomics will greatly accelerate the rate of information acquisition, and both novel and conventional methodologies and techniques must flourish, when we fully realize the potential of genomics. Mining of ''metagenomes''for novel enzymatic activities has been successful in a number of reports, which univocally demonstrated the importance and necessity of searching different environments to dig out new activities potentially applicable for biotechnology.

Further study is anticipated in order to find the reason for multiple cell wall degrading enzymes. The cloning and characterization of more *cel* genes from cow rumen metagenome should bring us closer to an answer for this. The experimental results shown in this paper demonstrate that the possibility of the breeding of rumen bacteria and novel useful genes from rumen. The ability to the breeding of rumen bacteria and novel useful genes from rumen will expand the use of enzymes in industrial application.

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초록 : 소 반추위 메타게놈에서 새로운 섬유소분해효소 유전자(cel5C) 클로닝 및 유전산물의 특성

김민근[3] · 디렌 바르만[1] · 강태호[1] · 김정호[4] · 김훈[1] · 윤한대[1,2]*

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한우의 반추위에서 게놈 DNA를 분리하여 메타게놈 은행을 구축한 다음 섬유소분해효소를 암호화하는 유전자를 클로닝 및 유전자를 선별하였다. 선별된 유전자의 DNA 염기서열 및 아미노산 서열을 분석하고 유전산물의 생화학적인 특성을 조사하였다. *cel*5C 유전자는 1,125 bp로 374개의 아미노산 잔기를 가진 단백질을 암호화하였으며 이 단백질 분자량은 42 kDa이었다. 이 효소의 최적 pH는 4 근방이었으며 최적 온도는 50°C 부근이었다. *cel*5C 유전자의 internal primer를 사용하여 인공적으로 배양할 수 있는 49종의 반추세균에서 분리한 게놈 DNA을 주형으로 PCR 분석한 결과 해당하는 밴드를 확인할 수 없었다. Cel5C는 현재로서는 배양할 수 없는 반추 미생물로 추정된다.