

Cloning and Characterization of a Bifunctional Cellulase-Chitosanase Gene from *Bacillus licheniformis* NBL420

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Abstract A 1.3 kb cellulase gene encoding novel bifunctional cellulase-chitosanase activity was cloned from biopolymer-producing alkali-tolerant *B. licheniformis* NBL420 in *E. coli*. A recombinant cellulase-chitosanase, named CelA, was expressed and purified to homogeneity. The activity staining and the enzymatic characterization of the purified CelA revealed bifunctional activities on carboxymethyl cellulose (CMC) and glycol-chitosan. The similar characteristics of the enzymatic activities at the optimum pH, optimum temperature, and thermostability indicated that CelA used a common catalytic domain with relaxed substrate specificity. A comparison of the deduced amino acids in the N-terminal region revealed that the mature CelA had a high homology with the previously identified bifunctional cellulase-chitosanase of *Myxobacter* sp. AL-1.

Key words: *Bacillus licheniformis*, bifunctional enzyme, cellulase, chitosanase, gene cloning

Cellulose and chitosan are known to have a similar structure due to the β -1,4 glycosidic bond in their linkages (in chitosan, the C-2 hydroxyl groups are replaced by amino groups). These substrates are mainly degraded through the action of cellulase or chitosanase, respectively. Recently, microbial bifunctional cellulases, which exhibit dual activities in the utilization of both cellulose and chitosan as a substrate, have been purified and characterized in *Myxobacter* sp. AL-1, *Bacillus* species, and other bacteria [6, 10, 14, 16, 23]. These bifunctional cellulase-chitosanases are known to have relaxed or broadened substrate specificities, and often show biased hydrolyzing activities toward their preferred substrate, namely, one is a major while another is a minor between two enzymatic characteristics. Except for the case of *Myxobacter* sp., most known bifunctional enzymes

preferentially degrade chitosan as the main substrate and slowly degrade cellulose [6, 23].

Cellulases are modular enzymes composed of independently folding, structurally and functionally discrete units or domains [2]. Most reported bacterial cellulases assigned to class family 5 consist of a catalytic domain and one substrate binding domain with a typical alpha/barrel structure [21]. The major function of the cellulose binding domain is to deliver its resident catalytic domain to the cellulose substrate. The folding characteristics of the cellulose binding domain eventually determine whether the substrate is microcrystalline or an amorphous soluble substrate [21]. Many genes encoding cellulolytic enzymes have already been cloned from different organisms over the past decade [11, 16, 18, 20]. A single substrate binding domain is responsible for the specific interaction with a substrate. However, the mechanisms related to how a single substrate binding domain exhibits dual activities on different substrates are still unknown. Therefore, it is interesting to study the relationship between the structure and the function of the cellulose binding domain in a bifunctional enzyme, because such data could provide useful information in designing substrate alteration in hydrolases [10, 12].

Previously, a biopolymer-producing alkali-tolerant *B. licheniformis* NBL420 was isolated from soil and characterized [7]. *B. licheniformis* NBL420, which belongs to *Bacillus licheniformis*, secretes numerous extracellular hydrolases, including cellulase, xylanase, amylase, and chitosanase [7]. Genetic analysis of this *Bacillus* strain has resulted in the cloning of various hydrolase genes. Accordingly, the current paper reports on the cloning and characterization of a novel cellulase gene (*CelA*). It was also found that this cellulase gene contained another chitosanase activity due to its novel cellulose binding domain. The similarity in the N-terminal amino acid sequences with the bifunctional enzyme of *Myxobacter* sp. of AL-1 [16] further supports the finding of a bifunctional characteristic behavior in CelA.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

B. licheniformis NBL420 was grown aerobically in a medium at 30°C, as previously described by Horikoshi and Akiba [5]. The culture medium was comprised of 1.0% (w/v) glucose, 0.5% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.1% (w/v) KH₂PO₄, 0.02% (w/v) MgSO₄·7H₂O, and 1% (w/v) Na₂CO₃ buffered basal medium (pH 10.0). *E. coli* DH5α was used as the host strain for the plasmid maintenance and gene cloning. Ampicillin was added to the media at a final concentration of 50 µg/ml. Plasmid pBluescript II KS(-) (Stratagene) and pUC18 (Gibco BRL) were used for the cloning and subcloning.

Isolation of Genomic DNA

The *Bacillus* genomic DNA was isolated using the method of Miller *et al.* [9]. The *B. licheniformis* NBL420 was cultured in 400 ml of a suitable medium at 30°C with shaking at 100 rpm [5]. The cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C. The genomic DNA was purified by CsCl/ethidium bromide equilibrium density gradient centrifugation. The plasmid DNA was isolated by the alkaline lysis procedure of Sambrook *et al.* [19].

Genomic Library Construction and Screening

The genomic DNA of *B. licheniformis* NBL420 was partially digested with *Sau*3AI. Fragments ranging between 2 and 10 kb in length were purified using a QIAquick™ gel elution kit (QIAGEN, Germany) and ligated to *Bam*HI cut, CIP treated pBluescriptII KS(-) DNA. *E. coli* DH5α was transformed with the ligated DNA using the CaCl₂ method. About 5,000 transformants were tested on an LB ampicillin agar plate containing 0.1% (w/v) carboxymethyl cellulose (CMC). After incubation at 37°C for 12 h, positive CMC case clones were identified by the Congo-red dye staining method [22].

Nucleotide Sequence Analysis

The nucleotide sequence analysis was conducted at the Macrogen Co. DNA sequencing facility (Seoul, Korea) using the double-stranded dideoxy sequencing method on an ABI 377 DNA sequencer.

Southern Hybridization

Total genomic DNA of *Bacillus licheniformis* NBL420 was digested with *Hind*III-*Eco*RI, separated by 0.8% agarose gel electrophoresis, baked at 80°C for 2 h, transferred to nylon membrane by Turboblottter System (Schleicher & Schuell, NH, U.S.A.), and hybridized to random primed DNA labeled with digoxigenin-dUTP labeled DNA probes, which were prepared with the DIG DNA labeling and detection kit (Roche, Mannheim, Germany). *CelA* gene was used as probes. In order to prepare *CelA*, forward primer, 5'-CGG GAT CCG ATG TCA TAC ATG AAA-3',

and a reverse primer, 5'-CCG CTC GAG ATT GGA CGA ATT TGT-3', were synthesized and used for PCR using total genomic DNA as a template. PCR reaction was done by 28 cycle of PCR under the following reaction conditions: reaction volume, 25 µl; reaction composition, 1 µl of template, 50 pmol primer, and 0.125 µl of Ex-Taq™ DNA polymerase (Takara, Japan); cycle profile, 15 sec at 94°C, 30 sec at 56°C, 2 min at 72°C. Prehybridization and hybridization were done at 60°C for 4 h and 20 h, respectively, in the following buffer; 5× SSC, 1% blocking reagent, 0.1% N-lauroylsarcosine, and 0.02% SDS. The most stringent wash was done by 5× SSC and 0.1% SDS at room temperature for 25 min, and 0.1× SSC and 0.1% SDS at 60°C for 2×15 min. Detection was performed by the manufacturer's method (Roche, Mannheim, Germany).

Gel Electrophoresis and Activity Staining

The SDS-PAGE was performed in a 12% SDS polyacrylamide gel containing 0.5% CMC or 0.1% glycol-chitosan [6]. The samples were heat-treated for 5 min at 80°C before being loaded and run at 40 mA for 40 min using a Bio-Rad Mini-Protein III Gel Kit. After the electrophoresis, the gels were washed for 30 min at 4°C with shaking in a 10 mM sodium phosphate buffer (pH 7.0) and further incubated for 90 min at 40°C. The protein gel was stained with Coomassie brilliant blue G, while the activity gels were stained with 0.1% Congo red solution for 10 min and destained with 1 M NaCl solution.

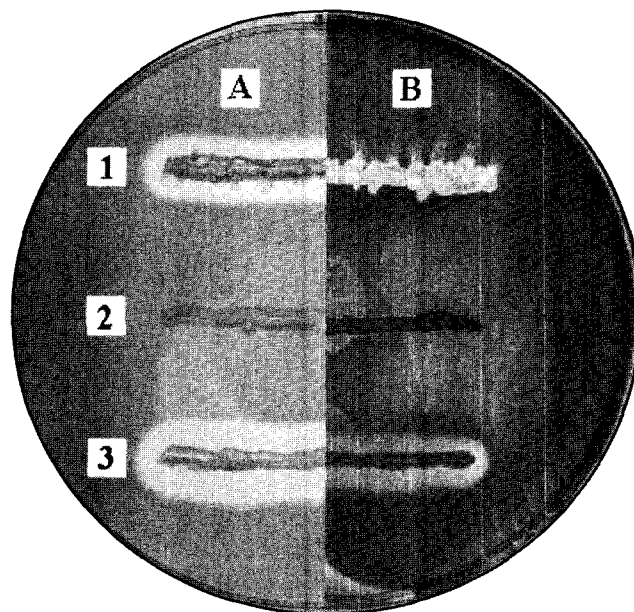


Fig. 1. Detection of bifunctional activities of NBL420 and recombinant *E. coli* clones by Congo-red staining of LB plates containing CMC (A) or glycol-chitosan (B).

(1) *B. licheniformis* NBL420, (2) *E. coli* DH5α containing pUC18, (3) *E. coli* DH5α containing pBUC1.

Enzyme Assay of Cellulase and Chitosanase

The CMC hydrolyzing activity of the enzyme was determined by measuring the amount of reducing sugar ends in the

reaction mixture. The reaction mixture consisted of 1.0% CMC in 100 µl of a 20 mM sodium phosphate buffer (pH 7.0) and 10 µl of the enzyme solution. The reaction was

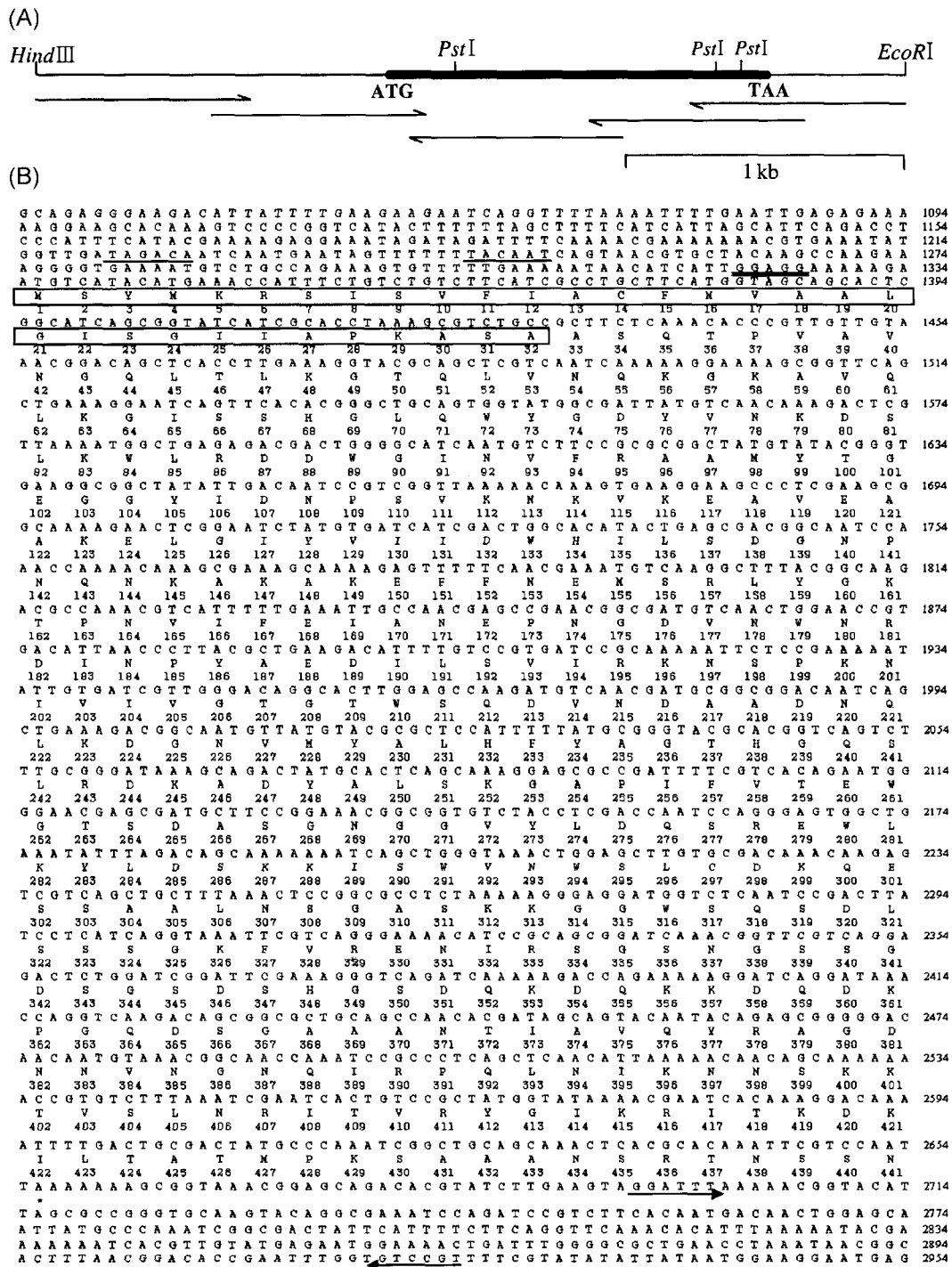


Fig. 2. DNA sequencing strategy and deduced amino acid sequences of *Cela*. (A) Physical map of entire 3.2 kb of *Cela*. The closed thick box indicates the coding DNA region of *Cela*. The relative DNA sequencing directions are indicated by arrows. (B) Partial nucleotide sequence of *B. licheniformis* NBL420 cellulase (*Cela*) around open reading frame. The full 3.2 kb DNA sequence was assigned as GenBank accession number AY039744. The putative -35, -10 regions and ribosome binding site (RBS) are marked by single and double underlines, while the palindromic transcriptional termination sequences are indicated by inverted arrows. The putative signal sequence is represented as an open box.

stopped after 30 min of incubation at 55°C by boiling for 5 min, then 1 ml of DNS reagent (0.8 M NaOH, 4 mM dinitrosalicylic acid, and 1.5 M potassium sodium tartrate) was added. After boiling for 10 min, the absorbance was measured at 570 nm using a Shimadzu UV mini-1240 spectrophotometer. The enzyme activity was obtained from a calibration curve prepared following the same procedure with D-glucose as the standard. One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1.0 μ mol of reducing sugar as glucose per minute under the reaction conditions. For the chitosanase activity, glycol chitosan was used as the substrate with D-glucosamine as the standard. The reaction mixture consisted of 1.0% glycol chitosan in 300 μ l of a 20 mM glycine-NaOH buffer (pH 8.0) and 300 μ l of the enzyme solution. The reaction was stopped after 30 min of incubation at 50°C by boiling for 5 min. The protein concentration was determined using a Bio-Rad (Richmond, U.S.A.) protein assay kit with bovine serum albumin as the standard.

Purification of Recombinant CelA Protein

All purification steps were performed at 4°C. *E. coli* DH5 α harboring pBC1 was grown in 200 ml of a LB ampicillin medium at 37°C for 12 h, and the culture was centrifuged at 10,000 rpm for 20 min. The cells were washed twice in a 0.2 M sodium phosphate buffer and then resuspended in 5 ml of the same buffer. The cell suspension was placed on ice and subjected to sonication using an Artec Sys. Co-150 ultrasonicator. The sonicated cells were then centrifuged at 15,000 rpm for 10 min, and the supernatant was used to purify the protein. A KONTES FLEX-COLUMN™ (1.5 \times 5 cm) was used for this step. A pre-swollen DEAE-cellulose (DE52, Whatman) was equilibrated with 20 mM sodium phosphate buffer (pH 7.0) and packed in the column. The sample was then applied to the column. The column was washed several times with 20 ml of the same buffer. The protein was eluted with a gradient of NaCl (3–0 M) in the same buffer. The fractions exhibiting cellulase activity were analyzed on an SDS-PAGE gel and used for the enzymatic characterizations.

Effect of pH on the Enzyme Activity and Stability

The activity was measured by incubating the reaction mixture at different pHs for 30 min, as described in the enzyme assay. To measure the pH stability, the enzyme was incubated at room temperature for 30 min in different buffers. The residual activity was then measured, as described in the enzyme assay. The buffer solutions used were 20 mM citric acid-Na₂HPO₄ buffer (pH 5–7), 20 mM Na₂HPO₄-NaH₂PO₄ buffer (pH 7–8), and 20 mM glycine-NaOH buffer (pH 8–10).

Effect of Temperature on Enzyme Activity and Stability

The effect of temperature was measured by incubating the reaction mixture at different temperatures for 30 min, as

described for the enzyme assay. For the thermostability measurement, the enzyme was incubated at different temperatures for 30 min in 20 mM sodium phosphate buffer (pH 7.0) and the residual activity measured, as described above.

Phylogenic Analyses of *Bacillus* sp. NBL420

Bacillus sp. NBL420 was further analyzed using an API Bacterial Identification Kit and 16s DNA analysis [3], and identified as *Bacillus licheniformis* according to computer analyses of the API Kit (BioMerieux) results (99.9%) and the NCBI Blast search result of a partial 1.4 kb of the 16s rDNA sequence data (98%) (data not shown).

Computer Model Generation

The Swiss-Model, a knowledge-based protein modeling tool [17], was used to predict the tertiary structure of CelA from the known X-ray structure of *Bacillus* cellulase (Protein Data Bank entry 8A3H from the Brookhaven Protein Databank).

DNA Sequence Accession Number

The DNA sequence reported here was deposited in the GenBank under accession No. AY039744 (*CelA*).

RESULTS AND DISCUSSION

Cloning and Nucleotide Sequencing of *CelA*

One positive cellulase clone harboring 4.2 kb genomic DNA was obtained after Congo-red staining of about 5,000 *E. coli*

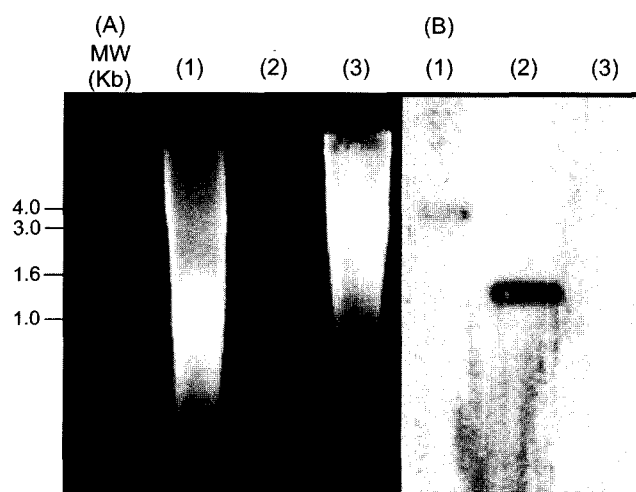


Fig. 3. Southern hybridization of *Bacillus licheniformis* NBL420 chromosomal DNA.

The DNA digested with *Hind*III-*Eco*RI was electrophoretically separated and transferred onto nylon membrane. After hybridization with DIG-labeled *CelA* probe, the membrane was treated as described in Materials and Methods. (A): Agarose gel pattern with ethidium bromide, (1) *Bacillus licheniformis* NBL420 chromosomal DNA; (2) PCR product of *CelA* as a positive control; (3) *E. coli* DH5 α chromosomal DNA as a negative control. (B): DNA pattern of Southern blot.

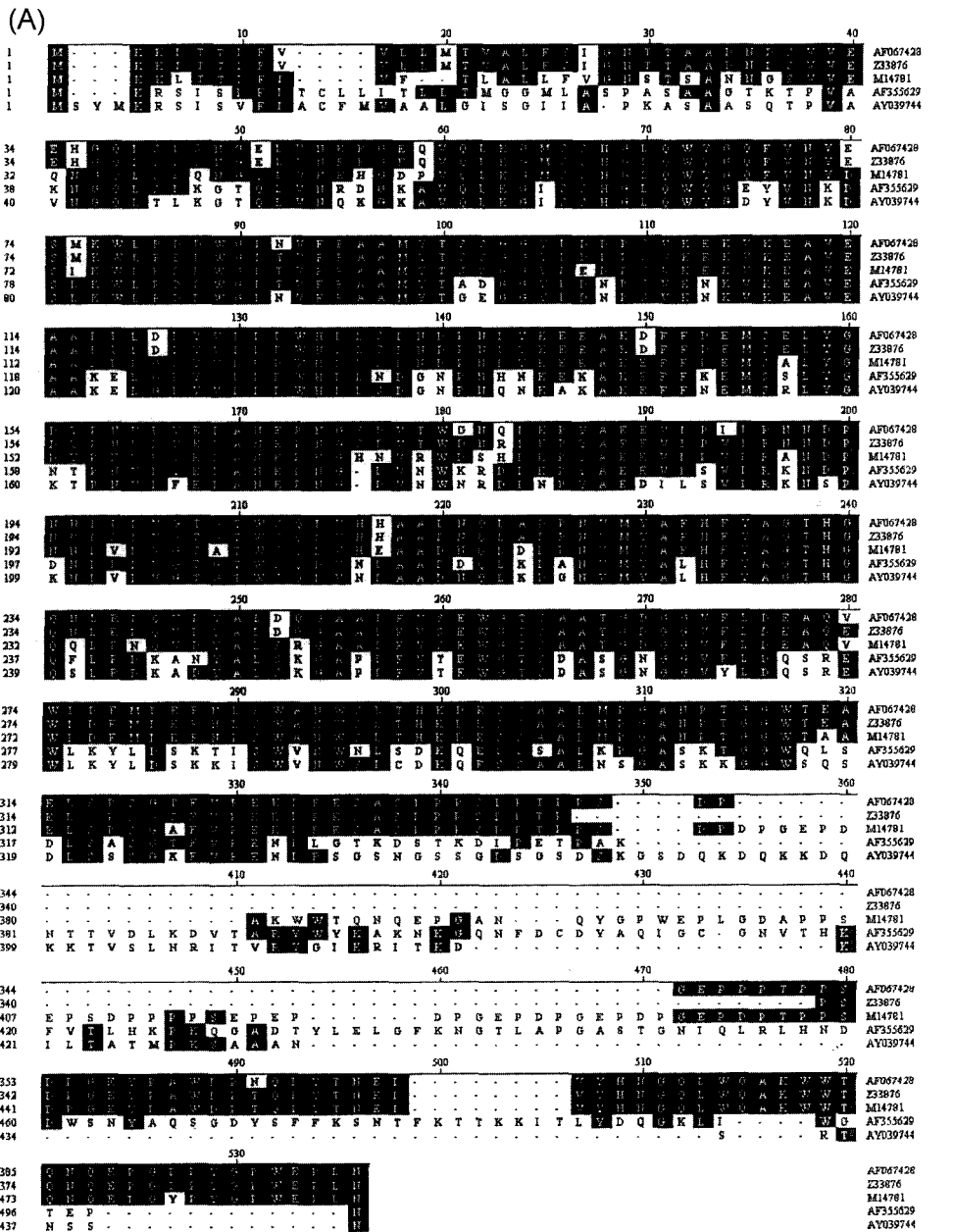


Fig. 4. (A) Multiple sequence alignment of deduced amino acid sequences for alkali-tolerant *Bacillus* cellulases. The sequences shown here are AF067428 from *Bacillus agaradhaerens*, Z33876 from *Bacillus* sp. N186-1, M14781 from *Bacillus* sp. N-4 (ATCC21833), AF355629 from *Bacillus subtilis* Y106, and AY039744 from *Bacillus licheniformis* NBL420 (current study). (B) Phylogenetic tree of CelA from alkaline-tolerant *Bacillus* species.



Fig. 5. Comparison of deduced amino acids sequences of the N-terminal region of mature cellulases from *Bacillus licheniformis* NBL420, *Myxobacter* sp. AL-1, and *Bacillus subtilis* 168.

transformants, and it was named as pBC1. The restriction map of pBC1 was obtained by a restriction enzyme analysis. The DNA fragment responsible for the cellulase activity was further reduced to a 3.2 kb *HindIII-EcoRI* fragment and subcloned into pUC18. The resulting plasmid, pBUC1, which harbored the 3.2 kb insert, was tested for cellulase activity (Fig. 1A) and subsequently used for dideoxy DNA sequencing analysis (Fig. 2A). The DNA sequencing results showed that it encoded a 1.3 kb open reading frame (ORF) representing 440 deduced amino acids of typical *Bacillus* endoglucanase (Fig. 2B). The computer graphic N-terminal analyses of CelA using a signal sequence prediction program (Swiss-prot, Geneva, Switzerland) revealed the existence of a putative signal sequence cleaved between Ala-32 and Ala-33. This putative signal peptide (32 amino acids) was similar to those of other *Bacillus* species. The estimated molecular mass for the mature CelA protein was about 44.8 kDa, which was identical to the actual band of *Bacillus licheniformis* NBL420 cellulase determined by SDS-PAGE and an activity gel (data not shown). The consensus *Bacillus* elements -35 (TAGACA) and -10 (TACAAT), plus the ribosome binding site (GGAGG), all appeared in the upstream region [8, 15]. An inverted repeated sequence (5'GGATTT-AAATCC3') was also shown after the termination codon.

Hybridization of the DIG-labeled *CelA* probe with the chromosomal DNA of *Bacillus licheniformis* NBL420 revealed an about 3.2-kb DNA band which corresponded to the same size as shown in the *HindIII-EcoRI* fragment of pBUC1 (Fig. 3B). This result demonstrated that the inserted fragment of pBUC1, representing cellulase activity, was originated from this chromosome.

Comparison of Deduced Amino Acids Sequences of CelA with Other *Bacillus* Cellulases

The deduced amino acid sequence of CelA was aligned with those of previously reported *Bacillus* cellulases (Fig. 4A). The multiple alignments showed that CelA had a significant homology with the cellulase of the recently cloned alkali-tolerant *Bacillus subtilis* Y106 (AF355629, unpublished result). The catalytic domain of CelA exhibited the typical alpha/barrel structure of class family 5 when its deduced amino acids were aligned with a previously known *Bacillus* cellulase crystal structure [4]. The Glu-259 and Glu-300 of CelA would appear to be possible active sites, because these two regions are also conserved among other *Bacillus* sp. cellulases [21]. The putative C-terminal substrate binding

domain of CelA appeared somewhat different from other cellulose binding domains, because that of CelA did not share with other cellulases (Fig. 4A). These facts suggest that the C-terminal region of CelA may be a novel substrate binding domain. A phylogenetic tree was constructed for CelA based on the Clustal method with a weighted residue weight table using Lasergene software (DNASTAR, U.S.A.). The numbers on the baseline refer to the divergence between the cellulase genes (Fig. 4B).

Bifunctional Enzymatic Properties of CelA Protein

A comparison of the deduced amino acid sequences of CelA with previously known cellulases [16, 24] revealed that CelA was almost identical with the bifunctional cellulase-chitosanase of *Myxobacter* sp. AL-1 in the N-terminal region, except for one amino acid (Fig. 5). Since the bifunctional cellulase-chitosanase gene in *Myxobacter* sp. AL-1 has not yet been cloned, the whole amino acid sequence could not

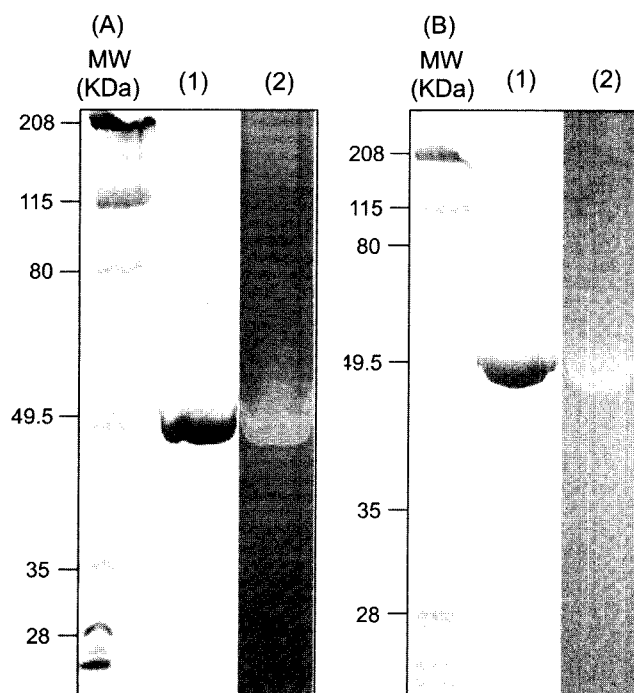


Fig. 6. Detection of cellulase-chitosanase activities of CelA on CMC/Chitosan SDS-PAGE.

(A) Activity staining of cellulase. MW: Protein size standards; (1): CelA protein band; (2): Activity staining band of CelA on CMC. (B) Activity staining of chitosanase. MW: Protein size standards; (1): CelA protein band; (2): Activity staining band of CelA on glycol-chitosan.

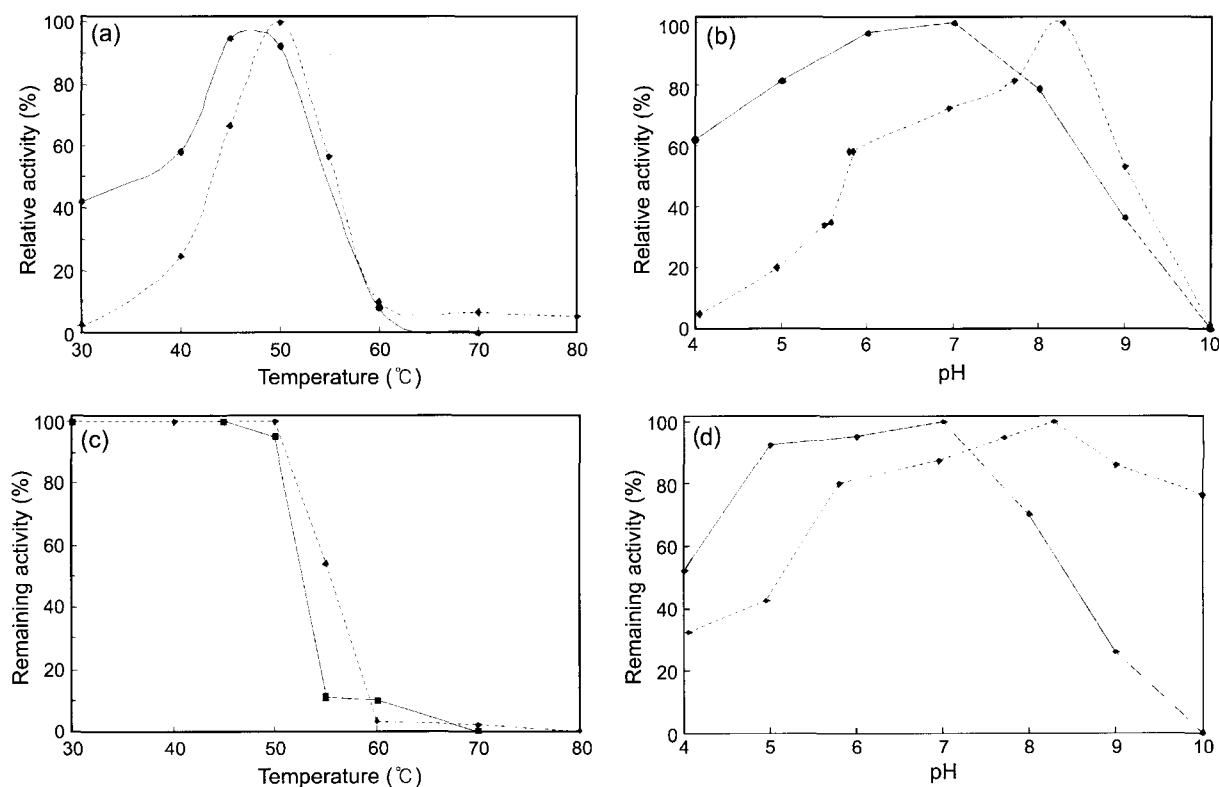


Fig. 7. Enzymatic properties of CelA protein. (a) Optimum temperature, (b) optimum pH, (c) thermostability, (d) pH stability. The solid lines indicate CMCase activity while the dotted lines indicate chitosanase activity.

be compared with the current data. In order to test a potential bifunctional activity of CelA, the recombinant *E. coli* clone was grown on LB ampicillin plates containing glycol-chitosan or soluble chitosan. The *E. coli* DH5 α clone harboring pBUC1 exhibited a clear halo on the chitosan plate as well as on the CMC plate (Fig. 1). Chitosanase activity staining of the purified CelA also revealed its bifunctional property (Fig. 6). To confirm the chitosanase activity of CelA, a DH5 α competent cell was retransformed with plasmid pBUC1. The transformed *E. coli* clone also exhibited both chitosanase and cellulase activities. The enzymatic characterization, including the optimum pH, optimum temperature, thermostability, and pH stability, further confirmed its bifunctional cellulase-chitosanase activity (Fig. 7). As reported for *Myxobacter* sp. AL-1 cellulase, CelA also preferentially degraded CMC and slowly degraded chitosan [16]. The optimum temperatures for both enzymatic characterizations were about 50°C (Fig. 7A). The optimum pH for cellulase activity was near neutral (pH 7), while that for chitosanase activity was slightly shifted towards an alkaline range (pH 8) (Fig. 7B). The thermostability of CelA was not high, because half of the total activity was lost at near 50°C (Fig. 7C). The study of pH stability revealed that the chitosanase activity was more stable than the cellulase activity (Fig. 7D). Consequently, the overall bifunctional enzymatic characteristics of CelA appeared to

be similar, except for a slight difference in the optimum pH. Accordingly, these findings suggest that a common catalytic domain with the relaxed substrate specificity was responsible for the dual activities in CelA. The substrate specificity of CelA was also tested with other substrates, such as avicel, alpha-cellulose, colloidal chitin, and oat spelt xylan. None of these were degraded, except for CMC, glycol-chitosan, and soluble chitosan (data not shown). Therefore, these facts suggest that CelA specifically degrades a soluble form of cellulose and chitosan. Currently, it has been reported that at least five different cellulases, including a bifunctional cellulase-chitosanase and other cellulases, exist in *Myxobacter* sp. AL-1. One of these five cellulases has already been cloned and characterized [1]. As such, the cloning and characterization of the remaining bifunctional cellulases in *Myxobacter* sp. AL-1 may provide more detailed information regarding the bifunctional cellulase-chitosanase activities in bacterial cellulases.

Computer Model Generation of CelA

In order to determine the possible three-dimensional structure of CelA, the Swiss-Model, a knowledge-based protein modeling tool [17], was used to predict the tertiary structure of CelA from the known X-ray structure of *Bacillus* cellulase (Protein Data Bank entry 8A3H from the Brookhaven Protein Databank). The resulting model (Fig.

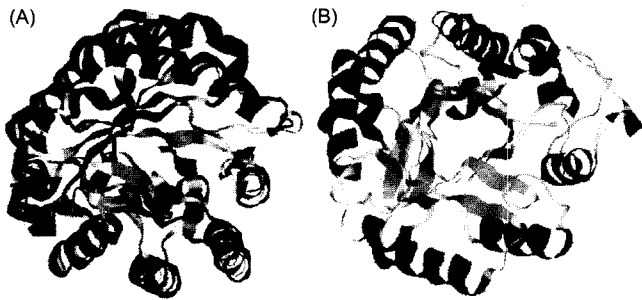


Fig. 8. 3-D Modeling of CelA. (A) *Bacillus licheniformis* NBL420 CelA, (B) *Bacillus agaradhaerens* Cel5A.

Homology modeling of CelA was produced using the Swiss-Model program (Glaxo, Geneva, Switzerland) based on the tertiary structure of previously identified *Bacillus agaradhaerens* Cel5A (PDB Number:8A3H). The homology of amino acid sequence between CelA and Cel5A was about 66 percent.

8A) also supported that CelA is a typical endoglucanase belonging to the class family 5 of bacterial cellulases [2].

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