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Article

Molecular Characterization of a β-1,4-Endoglucanase Gene from *Bacillus subtilis* H12

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A β-1,4-endoglucanase gene from *Bacillus subtilis* H12 was cloned into *Escherichia coli* JM109 (pBC8) and sequenced. The endoglucanase gene with an insert DNA of 2.5 kb possessed an open reading frame of 1,500 bp encoding a mature protein of 499 amino acids with a calculated molecular mass of 55 kDa. The deduced amino acid sequence showed similarity to those of the known neutral cellulase genes of *B. subtilis* PAP115 (99.2%) and BSE616 (97.8%), as well as the alkaline gene of *Bacillus* sp. N4 (55.1%). The endoglucanase activity expressed by *E. coli* (pBC8) was localized in the periplasmic fraction (80%) and the cytoplasmic fraction (20%). An endoglucanase was purified from the periplasmic fraction by performing gel filtration and anion exchange chromatography. The molecular weight of the purified enzyme was estimated to be 31 kDa by SDS-PAGE, and the maximum activity occurred at pH 7 and 40°C. The enzyme easily hydrolyzed soluble substrates such as carboxymethyl cellulose and barely β-glucan, whereas the sigmacell and xylan, the known insoluble substrates, were not entirely hydrolyzed.

Key words: Bacillus subtilis *H12, cloning, β-1,4-endoglucanase, purification*

Cellulose, which consists of long chains of β-1,4-linked glucose units, is one of the most abundant renewable biomass on the earth. Thus, numerous studies have been performed on cellulases during the last few decades, because this carbohydrate can be converted by enzymatic degradation into useful products such as soluble sugars, bio-ethanol, and industrial chemicals [Lynd *et al*., 2002]. The cellulose is hydrolyzed into glucose by the synergistic action of endo-β-1,4-glucanase (EC 3.2.1.4), exo-β-1,4-glucanase (EC 3.2.1.91), and β-glucosidase (EC3.2.1.21) [Woodward, 1991], among which, the action mechanism of endoglucanase, as well as the substrate-binding site and the hydrolysis site, has been extensively studied [Murasima *et al*., 2002; Yoon *et al*., 2007]. A number of microbial endoglucanase genes have been cloned and sequenced from most bacteria and fungi [Ozaki *et al*., 1991; Wymelenberg *et al*., 2002]. The encoded enzymes were found to differ markedly in their substrate specificities. Furthermore, the sequence analysis revealed important information relating to the evolutionary relationship of the genes as well as the primary structure and the enzymatic function [Yoda *et al*., 2005].

The genus *Bacillus* is one of the representative cellulolytic bacteria. To date, cloning and characterization of several endo- or exo-glucanase genes from the genus *Bacillus* have been extensively reported from *B. subtilis* [MaCkay *et al*., 1986], *B. latus* [Hansen *et al*., 1992], and *B. polymyxa* [Baird *et al*., 1990]. Recently, several cellulase genes of the *Bacillus* strain have been cloned into *E. coli* to investigate the catalytic domain and the cellulose-binding domain of the genes [Lima *et al*., 2005; Bischoff *et al*., 2007].

The present study was undertaken to determine the neucleotide sequence of the gene coding the endoglucanase gene of *B. subtilis* H12 isolated from soil. In addition, the properties of the endoglucanase expressed in *E. coli* were investigated.

Materials and Methods

Bacterial strains, plasmids, and culture condition. *B. subtilis* H12 isolated from a green house soil was used as

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Abbreviations: CMC, carboxymethyl cellulose; LB, Luria-Bertani; ORF, open reading frame

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a source of DNA for the endoglucanase gene. *B. subtilis* H12 was grown at 30°C in Dubos' broth (0.1% $NaNO_3$, 0.05% $MgSO_4$, 0.05% NaCl, 0.001% $FeSO_4$, pH 7) containing 1% bacto soyton, 1% CMC, and 0.05% yeast extract. *E. coli* JM109, used as the host organism for transformations, was routinely cultured in LB medium at 37°C. The pBluescript II SK (+) and pGEM3Zf (-) were used as vectors for cloning.

Construction of endoglucanase gene library. The gene encoding endoglucanase from *B. subtilis* H12 was isolated from a shotgun genomic library prepared in the pBluscript vector. Chromosomal DNA from *B. subtilis* H12 was prepared by the method of Saito and Miura [1963], digested with *Pst*I, and electrophoresed on 0.8% agarose gel. The fragments of 2-5 kb were ligated with pBluscript II SK(+), previously digested with *Pst*I, and dephosphorylated with the calf alkaline phosphatase. The ligated DNA was used to transform the $CaCl_2$-treated competent *E. coli* JM109 [Cohen *et al*., 1973]. *E. coli* transformants were spread on the LB agar containing ampicillin (50 μg/mL), X-gal, and IPTG, and the selection of the positive clones expressing endoglucanase activity was performed by using the Congo red test [Teather and Wood, 1982]. The recombinant clones were tooth-picked onto the LB agar containing ampicillin and incubated at 37°C overnight. The colonies were overlaid with 0.6% top agar supplemented with 0.5% CMC in 0.1 M sodium acetate buffer (pH 6.4), 0.0002% Congo red, and 0.02% triton X-100, and incubated at 45°C for up to 12 h. The positive clone developing a clear zone on LB agar was selected into the transformant with endoglucanase gene.

Nucleotide sequence analysis. The insert DNA fragment of 2.5 kb (pBC8), which was a real gene expressing endoglucanase from the selected recombinant plasmid (pBC5) with a 5.4-kb insert gene, was deleted in both orientation by using exonuclease III and SI nuclease (Promega, Madison, WI) to sequence the middle region of the insert DNA. The ends of the fragments were subcloned into the pGEM3Zf(-) vector after blunting with the DNA polymerase (Klenow fragment). The nucleotide sequence of the pBC8 clone was determined by sequencing the entire 2.5-kb insert of the recombinant plasmid using a Big Dye Terminator cycle sequencing kit (Perkin Elmer Applied-Biosystems, Forster city, CA) and an automated DNA sequencer (Model 377, Perkin Elmer Applied-Biosystems).

Preparation of cell extract enzymes. The extraction of periplasmic and cytoplasmic enzymes was performed by using the cold osmotic shock procedure of Nossal *et al*. [1966] to examine the cellular localization of endoglucanase in *E. coli* carrying pBC8. The *E. coli* transformant cells were suspended in 30 mM Tris-HCl buffer (pH 8) containing 20% sucrose and 1 mM EDTA and centrifuged at 4°C for 10 min at 12,000×g. The cell pellet was suspended in ice-cold water and slowly shaken for 10 min for osmotic shock. After centrifugation, the supernatant was used as the periplasmic enzyme. To prepare the cytoplasmic fraction, the osmotically shocked cells were sonicated on ice for 2 min, followed by centrifugation at 4°C for 10 min at 12,000×g. The supernatant was used as the cytoplasmic enzyme.

Enzyme purification. The periplasmic enzyme (30 mL) was concentrated by ultrafiltration using a PM10 membrane (Millipore, Beverly, MA) and a 2-mL aliquot of the concentrated solution was separated on a Superdex R-200 column (1.6×100 cm; bed volume, 115 mL; Amersham Bioscience, Uppsala, Sweden) equilibrated with 20 mM phosphate buffer, pH 7.0. The active fractions containing endoglucanases were pooled and concentrated by ultrafiltration using a PM10 membrane (Millipore). The enzymatically active filtrate was applied onto a DEAE-Sephadex A50 column (2.3×30 cm; bed volume, 130 mL) equilibrated with 50 mM Tris buffer, pH 7.5, and the elution was performed at a linear gradient of NaCl from 0 to 0.5 M in the same buffer. One active fraction was separated, and the purified enzyme was identified as homogeneous by SDS-PAGE.

Enzyme assay. β-1,4-Endoglucanase activity was determined by measuring the amount of reducing sugars released from CMC as described previously [Yoon *et al*., 2007] using the method of Nelson and Somogyi [1956]. A unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of glucose per min under the assay condition.

Results and Discussion

Isolation of *B. subtilis* H12. A strain of genus *Bacillus*, which produces high level of endoglucanase and amylase, was isolated from green house soil with a high salt content. The isolate was classified as a novel species belonging to *B. subtilis* based on chemotaxanomic and phylogenetic analyses (data not shown).

Cloning of an endoglucanase gene from *B. subtilis* H12. DNA libraries of *B. subtilis* H12 were constructed in pBluescript SK(+), and their recombinant DNAs were transformed into *E. coli* JM109. The *E. coli* transformants were screened by Congo red test, in which the positive clones developed a clear zone on the LB agar plate containing CMC. Only one clone among 2,500 clones showed halo, indicating the activity of CMC hydrolysis, and the transformant was named as pBC5 (Fig. 1). Plasmid analysis revealed that the insert gene of pBC5 had restriction sites of *Eco*RI, *Hin*cII, *Ava*I, *Xba*I, and

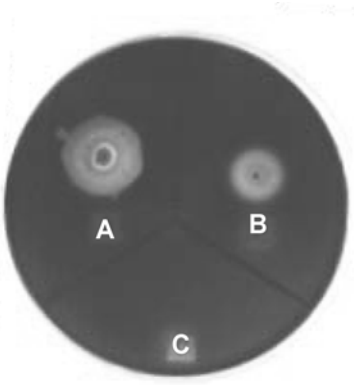

Fig. 1. Comparison of endoglucanase activity encoded by *E. coli* transformants harboring pBC5. The cells grown on LB agar were overlaid with 8 mL of top agar containing 0.5% CM-cellulose at 37°C for 12 h, followed by Congo red staining. A: *Bacillus* sp. H12; B: *E. coli* JM109/pBC5; C: *E. coli* JM109/pBluescript II SK(+).

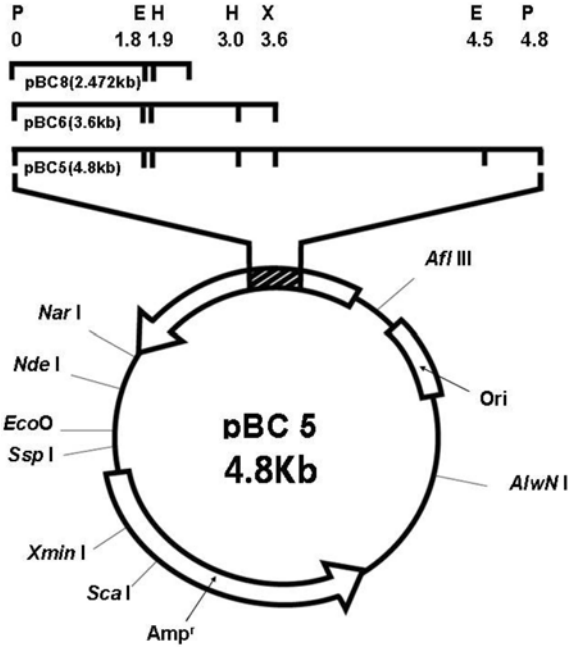

Fig. 2. Restriction map of the recombinant plasmid pBC5 and subclones. Restriction endonuclease: X, *Xba*I; E, *EcoR*I; H, *Hinc*II; P, *Pst*I.

*Sau*3AI within 4.8 kb of the *Pst*I DNA insert (Fig. 2).

Subcloning. The insert DNA of pBC5 was separately restricted with *EcoR*I and *Xba*I, and each fragment was subcloned into the pGEMZf vector to determine the location of the gene expressing endoglucanase at 4.8 kb. The subclones, pBC6 and pBC8, harboring the foreign DNAs of 3.6 and 2.47 kb, respectively, were finally obtained by estimating the endoglucanase activity (Fig. 2). The endoglucanase activities of both pBC6 and pBC8 appeared to be similar to that of pBC5, suggesting the

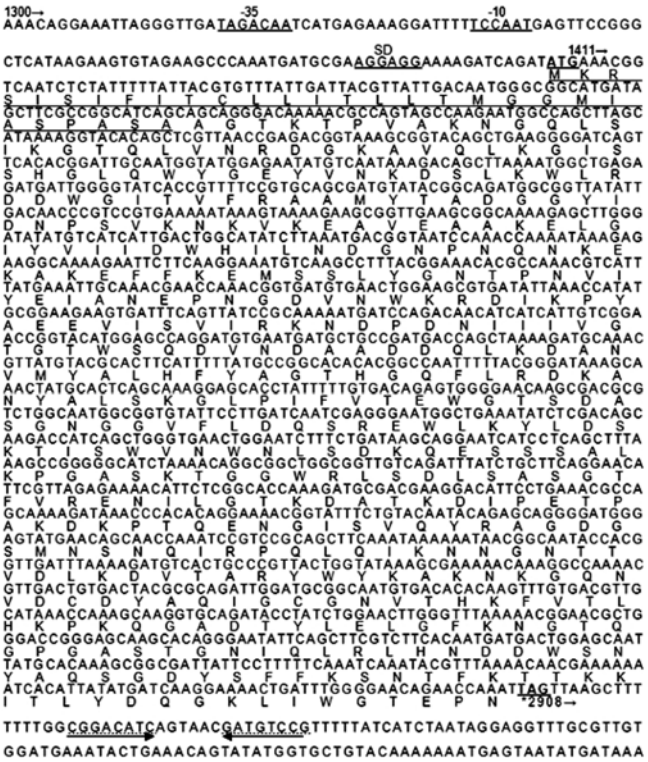

Fig. 3. Nucleotide sequence of the endo-β-1,4-glucanase gene and deduced amino acid sequence. The deduced amino acid sequence is shown below the nucleotide sequence. Possible -35 and -10 regions of the promoter and a possible Shine-Dalgarno sequence are indicated. A putative signal sequence of the enzyme is underlined. Long arrows indicate the inverted repeats with the potential to form a rho-independent transcription termination structure.

2.47 kb fragment of pBC8 is the gene encoding endoglucanase.

Nucleotide sequence. The insert DNA of the recombinant plasmid (pBC8) consisting of 2,472 bp was sequenced, and a single ORF of 1,500 bp encoding for a protein of 499 amino acid residues with a calculated molecular mass of 55 kDa was found in the inserted DNA. Shine-Dalgarno sequence, presumptive as a possible ribosome-binding site (5'-AGGAGG-3'), was found in 12 bases upstream of the putative ATG initiation codon. In addition, a putative promotor consensus was predicted using the Neural Network Promoter Prediction v. 2.2 software [Reese, 2001] with 5'-TAGACA-3' as the potential -35 region and 5'-TCCAAT-3' as the potential -10 region in the upstream region of ORF (Fig. 3). An inverted repeat sequence (5'-CGGACATC-3'), which could act as the transcriptional termination signal for the gene was followed by downstream of the stop codon (TAG). The 499-bp amino acid sequence of the ORF protein was compared with those of several bacterial endoglucanases by using the FASTDB alignment program. Comparison

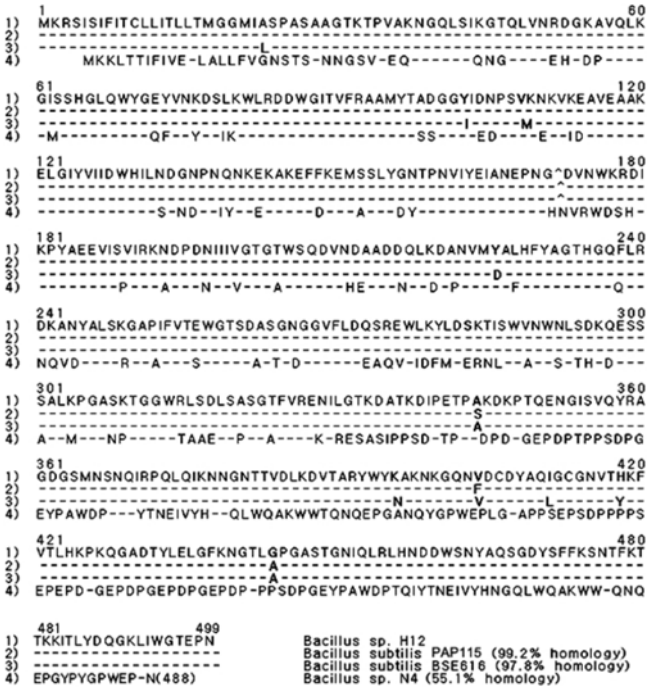

Fig. 4. Amino acid sequence homology between deduced sequences of the endo-β-1,4-glucanases from: 1) *Bacillus* sp. H12; 2) *B. subtilis* PAP115; 3) *B. subtilis BSE*616; 4) *Bacillus* sp. N4.

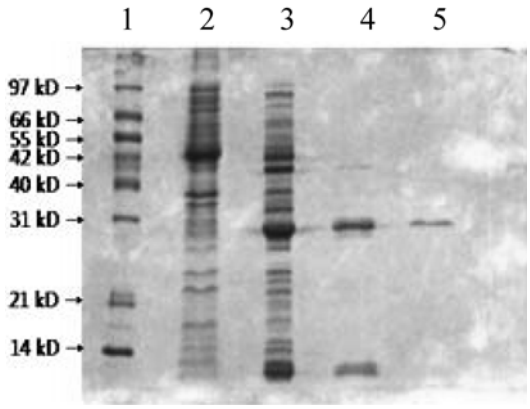

Fig. 5. SDS-PAGE of each protein samples during purification step of endoglucanase from *E. coli* (pBC8). Lane 1. Protein markers. Lane 2. Cytoplasmic fraction of *E. coli* (pBC8). Lane 3. Periplasmic fraction of *E. coli* (pBC8). Lane 4. Active fraction from gel filtration. Lane 5. Enzyme purified by anion chromatography.

of the deduced amino acid sequence showed significant similarities with the neutral cellulase genes of *B. subtilis* PAP115 (99.2%) [MacKay *et al*., 1986], *B. subtilis* BSE616 (97.8%) [Park *et al*., 1991], and *Bacillus* sp. N4 (55.1%) [Fukumori *et al*., 1986] known as the alkaline cellulase gene (Fig. 4).

Localization of endoglucanase activity expressed in *E. coli*. The cellular localization of endoglucanase in *E. coli* carrying pBC8 was examined in extracellular, periplasmic, and cytoplasmic fractions by measuring the endoglucanase activity. The endoglucanase enzymes produced by the *E. coli* transformant (pBC8) were localized in the periplasmic (80%) and the cytoplasmic (20%) fractions. In contrast, no enzyme activity was observed in the extracellular fraction. (Table 1). In general, extracellular enzymes are known to contain a probable leader signal peptide for membrane transport into the periplasmic space or the outer membrane [Wickner, 1979]. However, the leader signal sequence was not found in the N-terminal region of ORF, supporting the confinement of the endoglucanase activity of pBC8 in the cytoplasmic space without further excretion into the culture medium through the inner membrane.

Purification of endoglucanase. An endoglucanase from the periplasmic fraction of the *E. coli* transformant (pBC 8) was purified to homogeneity by means of gel filtration and anion exchange column chromatography. The molecular weight of the purified enzyme was estimated to be 31 kDa on SDS-PAGE (Fig. 5), and the optimal pH and temperature were 7.0 and 40°C, respectively (data not shown). The apparent molecular weight of 31 kDa was somewhat low compared with the putative enzyme of 55 kDa encoded from the ORF, presumably because the carboxyl-terminus of the soluble enzyme was hydrolyzed by the post-translocation modification or proteolysis of the mature protein. The proteolysis of the recombinant proteins expressed in *E. coli* is not unusual, and similar proteolytic events have been observed in other recombinant cellulases expressed

Table 1. Localization of endoglucanase in *E. coli* transformant (pBC8)

Strains	Endoglucanase activity (unit/min/mg of protein)			
	Total cell extracts	Extracellular fraction	Periplasmic fraction	Cytoplasmic fraction
E. coli (pBC8)	1.5	-	1.2(80)[a]	0.3(20)[a]
B. subtilis H12	1.06	1.06	N.D[b]	N.D[b]

[a]Numbers in parentheses indicate the percent distribution of activity between the cytoplasmic and the periplasmic compartments.
[b]N.D: not determined.

Table 2. Substrate specificities of endoglucanase purified from *E. coli* (pBC8)

Substrates	Enzyme activity (units min^{-1} mg^{-1})
CM-cellulose	4.50±0.6
Barley-β-Glucan	3.35±0.9
Sigmacell	Nd
Xylan	Nd
Chitosan	Nd
pNP-Cellobioside	2.34±0.3
pNP-Glucoside	Nd

Nd: not detected

in *E. coli* [Reverbel-Leroy *et al*., 1996].

Substrate specificities of endoglucanase. Substrate specificity of the enzyme purified from the *E. coli* transformants harboring pBC8 was investigated by assessing its ability to hydrolyze various cellulosic substrates (Table 2). Specific activity of the enzyme was very high toward CMC and barely-β-glucan, whereas the enzyme was completely inactive toward the microcrystalline celluloses such as xylan and sigmacell. These results suggest that endoglucanase encoded from pBC8 prefers the endo-type to the exo-type glycosides as the substrate, based on the report that CMC and barely-β-glucan are typical substrates for endoglucanase [Yoon *et al*., 2007].

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