Molecular cloning, purification and characterization of thermostable β-1,3-1,4 glucanase from *Bacillus subtilis* A8-8

Youn-Ju Jung1, Yong-Seok Lee1, In-Hye Park1, M Subhosh Chandra1, Keun-Ki Kim2 and Yong-Lark Choi1*

1Dept. of Biotechnology, College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Republic of Korea
2Division of Applied Life Science, College of Natural Resources and Life Science, Pusan National University, Miryang 627-914, Republic of Korea

Received 02 February 2010; revised 16 July 2010

A gene encoding a β-1,3-1,4-glucanase (*CelA*) belonging to family 5 of glycoside hydrolases was cloned and sequenced from the *Bacillus subtilis* A8-8. The open-reading-frame of *celA* comprised 1499 base pairs and the enzyme was composed of 500 amino acids with a molecular mass of 55 kDa. The recombinant β-1,3-1,4 glucanase was purified by GST-fusion purification system. The pH and temperature optima of the enzyme were 8.0 and 60°C, respectively. The enzyme was stable within pH 6.0-9.0. It was stable up to 60°C and retained 30% of its original activity at 70°C for 60 min. It hydrolyzed lichenan, CMC, xylan, laminarin, avicel and pNPC, but was inactive towards cellobiose. The enzyme activity was markedly activated by Co²⁺ and Mn²⁺, but was strongly inactivated by Fe³⁺. The truncated gene, devoid of cellulose-binding domain (CBD) showed 60% of activity and bound to avicel.

**Keywords:** *Bacillus subtilis*, Glucanase, Cellulose-binding domain, Catalytic domain, Purification

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most abundant organic compounds in the biosphere with its estimated synthesis rate of 10¹⁰ tonnes per year. Effective utilization of cellulosic material through bioprocesses is important to overcome the shortage of foods, feed and fuels, because of the explosive increase in human population. Enzymatic hydrolysis has several advantages over acid hydrolysis, because enzymatic hydrolysis of cellulose results in release of soluble sugars from insoluble substrate, due to cleavage of the glycosidic bonds.

Cellulases or cellulolytic enzymes are responsible for hydrolytic cleavage of β-glycosidic bonds in cellulose. They can be broadly classified into three types based on their mode of action: i) endoglucanases or carboxymethyl cellulases (CMCases) (endo-1,4-glucanase, EC 3.2.1.4) bring out hydrolysis of internal glycosidic bonds in cellulose chains, creating more terminal ends in the fragments, ii) exoglucanases or cellobiohydrolylases (exo-1,4-glucanase, EC 3.2.1.91) liberate glucose or cellobiose from ends of cellulose chains/fragments due to hydrolysis of terminal glycosidic bonds, and iii) β-1,4-glucosidases (β-D-glucohydrases, EC 3.2.1.21) further hydrolyze cellobiose formed as a result of synergistic action of the above two enzymes.

Although cellulases produced by fungi have gained much attention over the past years due to their potential application in bioconversion of renewable lignocellulosic material to ethanol and other useful products, the studies on bacterial cellulases are lacking. *Bacillus* spp. such as *B. cereus*, *B. licheniformis*, *B. subtilis*, and *B. polymyxa* have been shown to produce cellulases. Among bacteria, *Bacillus* spp. produces a number of extracellular polysaccharide hydrolyzing enzymes. Bacilli have been commonly believed to lack the complete cellulase system, and the main activity is β-glucanase (endo-1,4-β-glcanase, EC 3.2.1.4) which only randomly hydrolyzes internal 1,4-β-bonds in cellulose and does not hydrolyze crystalline cellulose.

The cellulase is organized into two functional domains which contain information for binding to crystalline cellulose and for catalytic activity. Cellulose-binding domains (CBDs) potentially have wide range of applications in protein purification and enzyme immobilization. The CBD of the endoglucanase of *Cellulomonas fimi* is at the NH₂-terminus, while the cellulose-binding structure predictions suggest that the CBDs are linked to the catalytic domain via a short polypeptide of amino acids rich in proline and hydroxyl amino acids.
A thermostable β-1,3-1,4 glucanase with broad pH stability should be useful for several industrial applications, such as agricultural, food and biofuel, as these processes are performed in extreme environment. In the present study, we report the cloning and sequencing of the gene encoding β-1,3-1,4 glucanase, as well as the enzymatic properties.

Materials and Methods

Microorganisms and culture conditions

*Bacillus subtilis* A8-8 was isolated as described previously. *E. coli* JM109 and BL21 were used as a transformation and expression host. The plasmid pGEM-T easy (Promega) was used as a cloning vector. Luria Broth (LB) medium contained 50 µg/pGEM-T easy (Promega) was used as a cloning vector. The plasmid cloning and sequencing of the gene encoding the enzyme was performed in extreme conditions. In the present study, we report the cloning and sequencing of the gene encoding β-1,3-1,4 glucanase, as well as the enzymatic properties.

Assay for the binding of cellulose to microcrystalline cellulose (Avicel)

One ml of avicel solution (30 mg/ml) was mixed with 0.5 ml of intracellular fraction of *E. coli* JM109 carrying recombinant plasmids and gently shaken for 30 min at room temperature. The avicel powder was then pelleted by centrifugation at 15,000 × g for 10 min. After removal of the supernatant, the pellet was first re-suspended in the same volume of 1 M NaCl and left for 15 min at room temperature. The mixture was again centrifuged at 15,000 × g for 5 min and the supernatant was analyzed by SDS-PAGE. The pellet was suspended in the same 1 M NaCl and centrifuged at 15,000 × g for 5 min. Finally, the proteins were released from the avicel powder in the presence of 8 M urea. At each step, released enzyme activity from the avicel powder was also assayed.

Characterization of β-1,3-1,4 glucanase

Enzyme assay

The enzyme activity was measured by incubating 0.1 ml of the enzyme sample with 0.9 ml of 1% CMC in 0.1 M Tris-HCl buffer (pH 7.0) at 50°C for 40 min. The amount of reducing sugar released was measured by 3,5-dinitrosalicylic acid method. One unit of β-1,3-1,4 glucanase activity was defined as the amount of enzyme releasing 1 µl of glucose equivalents per min.

Assay of *p*-nitrophenyl β-D-celllobioside (pNPC) degrading activity

The reaction mixture containing 400 µl of 1% *p*-NPC in 50 mM sodium acetate buffer (pH 5.8) and 100 µl of diluted enzyme was incubated at 45°C for 20 min. The reaction was stopped by adding 500 µl of 2% sodium carbonate and the yellow coloured *p*-nitrophenol liberated was determined at 420 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µl of *p*-nitrophenol per min.

Assay of β-1,3-1,4 glucanase

The standard assay for β-1,3-1,4 glucanase activity was carried out at 80°C for 10 min, using 0.5% (w/v) laminarin (Sigma) as a substrate in 0.1 M phosphate (Na2HPO4, K2HPO4) buffer (pH 6.5). The reducing sugar released was determined by 3,5-dinitrosalicylic acid method with glucose as a standard. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmoll of reducing sugar per min. The concentration of protein was determined according to the method of Bradford using bovine serum albumin (BSA) as the standard.

Determination of CBD

In order to determine the function of CBD of *celA*, deletion mutants were made from pTA150 by constructing a series of *celA* using exonuclease-III and mung bean nuclease.

Purification of β-1,3-1,4 glucanase

*E. coli* BL21 (DE3) clone harboring pTA150 gene was induced for overexpression with 0.1 mM
isopropyl-ß-D-thiogalacto-pyranoside (IPTG) at the mid-exponential growth phase and incubated for 3 h at 37°C. Cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C, washed with 1× phosphate buffered-saline (PBS) (diluted 10× PBS, 1.4 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄ and 18 mM KH₂PO₄ at pH 7.3) and then re-suspended in 1×PBS buffer. The cells were disrupted by sonication and the supernatant was obtained by centrifugation at 13000 rpm for 30 min at 4°C. The supernatant solution was loaded on to a GSTrap FF column (Amersham Pharmacia Biotech), equilibrated with 1× PBS and eluted with 10 mM reduced glutathione in the 50 mM Tris-HCl (pH 7.5) at a flow rate of 1 ml/min.

The eluted fractions were dialyzed overnight against PreScission cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA and 1 mM dichlorodiphenyltrichloroethane at pH 7.0) and concentrated by using Amicon Ultra-4 (Millipore, Bedford, MA, USA). The purified fusion protein was digested with PreScission protease for 12 h at 5°C to remove the GST region and then digested fusion protein was loaded on to a GSTrap FF column chromatography as described above. The unbound fraction was collected and used as a purified enzyme.

**Effect of temperature and pH on enzyme activity and stability**

The optimal temperature of β-1,3-1,4-glucanase was determined by performing the standard assay within the temperature range of 30-90°C. The thermal stability of purified enzyme was examined by measuring the residual activity after incubating the enzyme mixture at each desired temperature for 30-120 min. The optimal pH was determined by performing the standard assay in the appropriate buffers: 100 mM acetate buffer (pH 2.0-6.0), phosphate buffer (pH 6.0-8.0), glycine-NaOH buffer (pH 8.0-11.0) and phosphate buffer (pH 11.0-13.0) at 50°C. The pH stability of purified enzyme was determined by measuring the residual activity after incubating the enzyme mixture at each desired pH for 30 min.

**Effect of metal ions and chemical reagents on enzyme activity**

To determine the effect of metal ions and chemical reagents on the enzyme activity, each reagent was added to the enzyme solution at a final concentration of 1, 5 and 10 mM, respectively. The mixture was then incubated at 50°C for 40 min and the residual β-1,3-1,4 glucanase activity was assayed using CMC as a substrate.²⁴,²⁵

**Results and Discussion**

**Cloning and sequencing of the celA gene from B. subtilis A8-8**

Analysis of 1.5 kb DNA sequence from B. subtilis A8-8 shows the presence of a 1499 bp open-reading-frame (ORF) which encodes for 500 amino acids along with a putative signal peptide of 29 residues. The first 29 amino acids of celA separated from the rest of the protein by a potential cleavage site in front of Ala29 have typical features of a prokaryotic signal peptide (Fig. 1). The molecular mass of the predicted mature protein is found to be 55 kDa. The deduced amino acid sequence of the protein has the conserved region VIYEIYNEPL, indicating that it belongs to the glycoside hydrolase family 5. The celA gene of B. subtilis A8-8 consists of a typical catalytic domain of 256 amino acids and CBD of 82 amino acids.

**Amino acid sequence similarities between celA and other cel proteins**

The comparison of predicted amino acid sequence of celA to sequences deposited in the GenBank and NCBI reveals that celA shares significant sequence similarity with Bacillus subtilis BSE616 (JNO111), Bacillus sp. IFO3034 (A27198), B. amyloliquefaciens (AF363635) and Pectobacterium carotovorum (AAC02964), showing 99, 93, 87 and 57%, respectively as shown in Fig. 1. The catalytic site of celA appears to involve Glu¹²⁹ and Glu¹³⁵ (Fig. 2). The C-terminal flanking sequence is found to be highly homologous to the non-catalytic domain of Clostridium stercorarium and Caldocellum saccharolyticum endoglucanase B²⁶.

**Determination of CBD and catalytic domain of celA**

In order to determine the function of CBD of celA, we made a series of celA deletion mutants from pTA150 and these mutants have been deleted at the C-terminal. A summary of adsorption assayed with a series of celA deletion mutants is shown in Fig. 3. Although 170 amino acids are removed from pTAC7 carboxyl-terminal, deletion mutants still able to bind CMC. When 171 amino acids are deleted from carboxyl terminal end of celA, the protein produced by this deletion mutant (pTAC11) loses its binding ability to CMC. These results show that CBD does not affect the ability to hydrolyze CMC. No significant difference are observed in optimum pH and temperature among these truncated CMC (pTAC11, pTA110 and pTA140) and native celA (pTA150) by preliminary analysis using the extracellular fraction (data not shown).
Enzymatic hydrolysis of cellulose consists of three steps: adsorption of CelA on to the surface of cellulose, biodegradation of cellulose to fermentable oligosaccharides, and desorption of CelA. CBDs are found in most of cellulose-degrading enzymes and are essential for the enzymes to bind to the solid cellulose substrate. Cel6A isolated from Trichoderma reesei and some bacterial CBDs have shown irreversible binding at 4°C\textsuperscript{27,29}. The irreversible adsorption of cellulase on to cellulose is partially attributed to the enzyme deactivation\textsuperscript{30}.

Avicel-binding ability of carboxyl-terminal truncated cellulose

The CBD of celA from B. subtilis A8-8 has been reported\textsuperscript{26}. All the plasmids pTA140, pTA120 and pTA110 still possess enzymatic activity to hydrolyze CMC as well as a native pTA150 enzyme. We have examined the ability of truncated celA bind to avicel using the extra fractions prepared from E. coli cells containing pTA140, pTA120 and pTA110. The celA and truncated protein produced by pTA140 have been found to bind the cellulose, whereas the proteins produced by pTA120 and pTA110 do not bind to the cellulose.
JUNG et al.: CLONING OF β-1,3-1,4 GLUCANASE GENE FROM BACILLUS SUBTILIS A8-8

207

... cellulose. Its crystalline cellulose-binding ability is independent of catalytic activity to hydrolyze CMC. These results suggest that pTA120 and pTA110 lack the CBD.

**Purification of β-1,3-1,4 glucanase by the GST-fusion system**

The β-1,3-1,4 glucanase has been purified using glutathione affinity chromatography. The β-1,3-1,4 glucanase is expressed by using fusion vector pGEX-6P-1, which possesses the cytosol fraction of E. coli BL-21 (DE3) cells. Significant β-1,3-1,4 glucanase activity is detected from cells harboring the recombinant fusion plasmid pTA150 after induction by IPTG. The protein is purified by GSTrap FF column and PreScission protease after disrupting the

Fig. 2—Multiple alignment of the deduced amino acid sequence of β-1,3-1,4 glucanase with those of endoglucanase from other organisms Bacillus subtilis pTA150 (AY183475), B. subtilis BSE616 (JNO111), B. subtilis (AA22307), B. subtilis (CAA97610), B. amyloliquefaciens (AAL99668), and Pectobacterium carotovorum (AAC02964) [Numbering of the amino acid starts at the N termini of the proteins. Gaps are indicated by dashes. Amino acid identical of the sequences aligned are shown in black boxes]
cells by sonication. The purified enzyme appears to be homogenous as evident by the SDS-PAGE (Fig. 4) and has the molecular mass of approximately 55 kDa. The purified β-1,3-1,4 glucanase is used for further enzymatic characterization.

Effect of temperature and pH on enzyme activity and stability

The effect of temperature on the activity of purified β-1,3-1,4 glucanase is studied between 30-90°C. The optimal temperature purified enzyme of is 60°C, beyond which the enzyme activity is rapidly declined (Fig. 5A). Thermal stability is investigated by incubating the pure enzyme for up to 120 min at different temperatures (Fig. 6A). The enzyme retains 40% of its activity at 60°C for 120 min. At higher temperatures, thermostability decreases and the enzyme retains 30% of its original activity at 70°C for 60 min. The activity is completely lost after 70°C. The optimal pH for the purified enzyme is 8.0, but more than 80% of activity is observed between pH 2.0-8.0 (Fig. 5B). The enzyme demonstrated broad pH stability within a pH range of 6.0-9.0 (Fig. 6B).

The β-1,3-1,4 glucanase produced from B. subtilis A8-8 has shown relatively higher optimum temperature (60°C) when compared to endoglucanases from other Bacillus spp. A unique feature is its broad alkaline pH stability between pH 6.0-9.0; below pH 6.0, the low activity is observed. The high optimum temperature and high stability at alkaline pH indicate that the enzyme may find wide industrial applications. We have found no significant difference in optimum pH and temperature among the truncated genes CBD (pTAC7, pTAC9 and pTA1017) and native celA (pTA150), when enzyme activities are determined using the extracellular fraction (data not shown).

Substrate specificity of purified β-1,3-1,4 glucanase and celA

The activity of purified β-1,3-1,4 glucanase and celA is assayed with various cellulosic substrates (Table 1). The β-1,3-1,4 glucanase has been purified from Bacillus sp. A8-8 in the earlier study. Among the tested substrates, CMC and lichenan are efficiently hydrolyzed by the two enzymes. When the
endoglucanase of *Clostridium thermocellum*, *Cellulomonas fimi* and other *Bacillus* spp. hydrolyze CMC, swollen cellulose, cellotetraose and cello pentose, but not the exoglucanase substrate like pNPC\(^{38}\), while endoglucanase (Ba-EGA) from *Bacillus* sp.AC-1 only catalyze hydrolysis of CMC-Na\(^{38}\). The *celB* gene product of *Paenibacillus* sp. BP-23 synthesized in *E. coli* shows maximal activity on CMC and lichenan, whereas low activity is observed on avicel\(^{39}\). Unlike these endoglucanases, \(\beta\)-1,3-1,4 glucanase of *Bacillus* sp. A8-8 has specificity towards broad substrates.

**Effect of metal ions and chemical reagents on enzyme activity**

The effect of various metal ions and EDTA on the purified enzyme activity is shown in Table 2. The enzyme activity is significantly increased by Co\(^{2+}\) and Mn\(^{2+}\) and strongly inactivated by Fe\(^{3+}\). Other metal ions (Ni\(^{++}\), Ca\(^{2+}\), Mg\(^{2+}\)) and EDTA weakly affect the enzyme activity. A similar trend is observed from *Neisseria sicca* SB\(^{36}\) and the enzyme activity is not affected by Fe\(^{2+}\) and Cu\(^{2+}\)\(^{40}\).

In conclusion, the present study reveals that CBD is affected by Avicel binding ability and does not play an important role of binding ability towards the enzyme activity. Also, our results show that CBD does not affect activity of \(\beta\)-1,3-1,4 glucanase at optimum pH and temperature. The enzyme exhibits optimum activity at pH 8.0 and 60°C and shows broad temperature and pH stabilities, thus may find potential applications in the industry.

**Acknowledgment**

This paper was partially supported by the Dong-A University Research Fund.

**References**