Enzymatic Characteristics of a Highly Thermostable β-(1-4)-Glucanase from *Fervidobacterium islandicum* AW-1 (KCTC 4680)

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Introduction

Extremophiles are defined as microorganisms that are tolerant of and thrive under severe environmental conditions such as extremes of pH, temperature, pressure, and salinity [1]. These microorganisms produce a variety of fascinating enzymes that exhibit their functional activity under extreme conditions [2, 3]. Microbial enzymes that function under extreme conditions have attracted attention as biocatalysts, since they are tremendously active and stable under conditions generally considered unsuitable for biological materials. There are many possible applications for these biocatalysts in food and biochemical industries [4].

Cellulose and hemicellulose are the basic structural components of plant cell walls and the most abundant organic compounds on Earth [4, 5]. Recent research efforts have focused on the transformation of these renewable bioresources into biofuels and other biologically valuable materials [6, 7]. Cellulose is a linear polysaccharide consisting of several hundreds to thousands of β-1,4-linked glucose molecules, whereas hemicellulose is a diverse heteropolymer containing many different sugar monomers such as xylose, mannose, galactose, rhamnose, and arabinose. Hydrolysis of cellulose requires three different enzymes, including endoglucanase, exoglucanase, and β-glucosidase [6]. Whereas endoglucanase cleaves randomly at internal amorphous sites in cellulose, exoglucanase liberates two or four glucose units from the ends of cellulose polysaccharide chains produced by endoglucanase in a processive manner. β-Glucosidase (E.C. 3.2.1.21) catalyzes the glucose hydrolysis reaction by acting on the soluble cellobextrin and cellobiose. Unlike cellulase, glucanases are not easily biochemically characterized owing to the complexity of their substrates. However, it is evident that glucanases break down diverse polysaccharides linked by various glycosidic bonds as in β-D-glucan and curdlan as well as cellulose. These

A highly thermostable β-(1,4)-glucanase (NA23_08975) gene (fig) from *Fervidobacterium islandicum* AW-1, a native-feather degrading thermophilic eubacterium, was cloned and expressed in *Escherichia coli*. The recombinant FiG (rFiG) protein showed strong activity toward β-D-glucan from barley (367.0 IU/mg), galactomannan (174.0 IU/mg), and 4-nitrophenyl-cellobioside (66.1 IU/mg), but relatively weak activity was observed with hydroxyethyl cellulose (5.3 IU/mg), carboxymethyl cellulose (2.4 IU/mg), and xylan from oat spelt (1.4 IU/mg). rFiG exhibited optimal activity at 90°C and pH 5.0. In addition, this enzyme was extremely thermostable, showing a half-life of 113 h at 85°C. These results indicate that rFiG could be used for hydrolysis of cellulosic and hemicellulosic biomass substrates for biofuel production.

Keywords: Cellulase, extremozyme, *Fervidobacterium islandicum*, β-glucan, β-(1-4)-glucanase, thermophilic enzyme

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heterogeneous enzymes have many applications in food industries as well as in biotechnology [8, 9].

In this study, we report the expression and characterization of an extremely thermostable β-glucanase that has strong glucanase activity towards β-D-glucan and galactomannan as well as comparatively low cellulase activity. The detailed studies on the enzymatic characteristics of this enzyme elucidate catalytic advantages and the possibility of potential applications in the hemicellulose hydrolysis process.

Materials and Methods

Bacterial Strains and Reagents

Ferredoxibacterium islandicum AW-1 (Korean Collection for Type Cultures (KCTC) 4680) was from the laboratory collection of Dr. Dong-Woo Lee (School of Applied Biosciences, Kyungpook National University, Daegu, Korea). Descriptions of the strain and growth conditions have been published previously [10]. Escherichia coli DH10B (F’ rpsL deoR Φ80lacZΔM15 endA1 nupG recA1 mcrA Δ(mrr hsdRMS mcrBC)) and E. coli BL21 (DE3) (F−, ompT, hsdSB(g−, m−), dcm, gal, k. (DE3)) were employed as hosts for gene manipulation and expression, respectively. The Escherichia coli strains were grown in Luria-Bertani (LB) medium (BD, USA) supplemented with ampicillin (100 μg/ml). The pGEM T-easy (Promega, USA), and pET-21a (Novagen, Germany) vectors were used for PCR cloning and expression of the glucanase enzyme, respectively. The various cellulolytic and hemicellulolytic substrates, including cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose, laminarin, mannan from Ceratonia siliqua seeds), laminarin, mannan from Saccharomyces cerevisiae, and 4-nitrophenyl β-D-cellobioside (pNPC). The reaction mixture contained 1% (w/v) substrate in 50 mM sodium acetate buffer (pH 5.0) with appropriate amounts of enzyme, which was then incubated at 80°C for 30 min. The reducing sugars released from the substrates were determined using 3,5-dinitrosalicylic acid reagent [13]. One unit (IU) of enzyme activity was defined as the amount of enzyme that released 1 pmol reducing sugars per minute. For pNPC, the reaction was performed at 80°C for 5 min and the resulting reaction products (pNP) were measured at 410 nm [14]. One unit (IU) of pNPC hydrolyzing activity was defined as the amount of enzyme that produced 1 pmol pNP per minute.

Thin-Layer Chromatography (TLC) Analysis

Reaction mixtures consisting of 0.5% (w/v) of each substrate (cellobiose, cellulbiose, cellobiose, cellopentaose, cellohexaose, and CMC) and 1.4 IU/mg of rFiG in 50 mM sodium acetate buffer (pH 5.0) were incubated at 80°C for 1 h. TLC with a precoated silica gel 60 F254 glass plate (Merck Millipore, Germany) was used to identify the reaction products. After applying 4 μl of reaction samples, the TLC plate was developed with a solvent system of 1-butanol: acetic acid: water = 2:1:1 (v/v) and reaction products were detected by spraying the plate with 0.5% (w/v) N-(1-
The NA23_08975 gene of *F. islandicum* AW-1 B1 (Reference Sequence Accession: CP 014334.1; region 1900845 to 1901909; called as FiG, hereafter) was annotated as an endoglucanase [11] that encodes 355 amino acid residues and showed amino acid identity to bacterial endoglucanases from *F. pennivorans* (79%), *Thermosipho africanus* (70%), and *Dictyoglomus thermophilum* (55%). Although the gene was predicted to encode a glycoside hydrolase from family 5 (GH5), which comprises putative cellulases, the sequence revealed that FiG does not harbor a carbohydrate-binding module (CBM). Lack of the CBM region could explain the lower specific activity observed for Avicel (see the data below) since this domain is considered responsible for the targeting and hydrolyzing of crystal cellulose [15]. The FiG gene product was predicted (SignalP 4.1; http://www.cbs.dtu.dk/services/SignalP) to function as an extracellular enzyme owing to a putative signal peptide sequence of 24 amino acids at the N-terminal end of the protein. The absence or presence of this signal peptide sequence did not affect expression of the recombinant gene in this experiment (data not shown). *Escherichia coli* BL21 CodonPlus-RP cells were used as the host for expression to eliminate possible codon usage problems. The recombinant gene product was well expressed in *E. coli* cells and purified by Ni-NTA column chromatography. SDS-PAGE analysis showed that a thick protein band appeared near 37 kDa (Fig. 1). The theoretical molecular mass of the rFiG protein is 41.4 kDa; however, discrepancy between the observed molecular mass in SDS-PAGE analysis and the theoretical molecular mass is not unusual [14].

**Enzymatic Properties and Substrate Specificity of rFiG**

Since FiG showed a significant level of amino acid homology (55–79%) with various endoglucanases, many cellulosic and hemicellulosic substrates, including CMC, HEC, Avicel, xylan, β-D-glucan, soluble starch, galactomannan, laminarin, pNPC, and mannan, were tested as rFiG substrates. In addition, pNPC hydrolyzing activity was determined (Table 1). rFiG showed the highest activity on β-D-glucan from barley (367.0 IU/mg) followed by galactomannan (174.0 IU/mg) and pNPC (66.1 IU/mg). In addition, relatively weak activity was observed with HEC (5.3 IU/mg), CMC (2.4 IU/mg), and xylan from oat spelt (1.4 IU/mg). Even though Avicel did not dissolve well in aqueous solution, very low activity was detected with rFiG, and no activity was found with soluble starch, laminarin, and mannan. The only common structure among rFiG substrates was that they had a backbone connected by β-(1-
4)-linkages with no consistency in the length of side-chain molecules. These results indicate that rFiG is a glucanase with broad substrate specificity and does not function as an endoglucanase. Wang et al. [14] expressed and studied a thermostable cellulase from F. nodosum Rt17-B1, which has the most similar amino acid sequence homology to rFiG. The results showed that this enzyme has strong activity towards CMC (440 IU/mg), but very low activity with pNPC (0.15 IU/mg), which is quite different from our results. It will be interesting to study the specific catalytic residue(s) involved in determining the substrate specificity of these two cellulosic or hemicellulosic enzymes. The gene encoding FiG and related genes are typically annotated as endoglucanases [11]; however, the results of this research revealed that rFiG is an exo-type enzyme and has a broad substrate specificity toward various β-D-glucans and related carbohydrates. Therefore, it is evident that FiG is a β-(1-4)-glucanase.

Enzyme activity was determined with pNPC although rFiG showed its strongest activity towards β-D-glucan owing to its simple detection method. The optimal condition for pNPC was 90°C at pH 5.0. To examine the thermal stability, enzyme solutions were incubated at 70°C, 75°C, 80°C, and 85°C for various lengths of time and measured for residual

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**Table 1.** F. islandicum β-(1-4)-glucanase activity on various substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (IU/mg of protein)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC</td>
<td>2.4</td>
<td>100</td>
</tr>
<tr>
<td>β-D-Glucan</td>
<td>367.0</td>
<td>15,525</td>
</tr>
<tr>
<td>HEC</td>
<td>5.3</td>
<td>220</td>
</tr>
<tr>
<td>Galactomannan</td>
<td>174.1</td>
<td>7,363</td>
</tr>
<tr>
<td>Avicel</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Mannan</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Xylan</td>
<td>1.4</td>
<td>57</td>
</tr>
<tr>
<td>Laminarin</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>pNPC</td>
<td>66.1</td>
<td>2,756</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

*ND, not detectable.

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**Table 2.** Effects of various cations, chelating agents, and surfactants on rFiG β-(1-4)-glucanase activity.

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Cations (5 mM)</td>
<td></td>
</tr>
<tr>
<td>MnCl₂</td>
<td>108.3 ± 3.5</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>105.7 ± 3.9</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>28.6 ± 0.8</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>69.1 ± 3.8</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>100.8 ± 1.6</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>34.7 ± 0.2</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>122.0 ± 6.3</td>
</tr>
<tr>
<td>NaCl</td>
<td>92.8 ± 1.7</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>90.0 ± 4.9</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>116.2 ± 0.6</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>65.1 ± 3.5</td>
</tr>
</tbody>
</table>

Chelating agent (0.5% (w/v))
- EDTA: 17.6 ± 1.0

Surfactants (0.5% (w/v))
- Triton X-100: 98.6 ± 2.2
- SDS: 45.7 ± 3.7

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**Fig. 2.** Effects of pH (A) and temperature (B) on β-(1-4)-glucanase activity from F. islandicum.

activity (Fig. 3). Hydrolyzing activity was not diminished up to 10 days at 70°C or 75°C. The half-lives of the rFiG were about 200 and 113 h at 80°C and 85°C, respectively; however, its activity was drastically reduced at 90°C. The effects of various metal ions at 5 mM concentration, chelating agent 0.5% (w/v), and surfactants 0.5% (w/v) on enzyme activity were examined at 80°C and pH 5.0 (Table 2). In fact, no significant effect was observed in the presence of various metal ions, including Ca²⁺, Mn²⁺, Co²⁺, Mg²⁺, and Na⁺, except for Cd²⁺, Fe²⁺, Zn²⁺, and Cu²⁺. EDTA noticeably inhibited enzyme activity, whereas the surfactant Triton X-100 did not eliminate catalytic activity. About half of the enzyme activity remained in 0.5% (w/v) SDS.

Cellooligosaccharide Hydrolysis Patterns
The best substrate for rFiG was β-D-glucan from barley; however, barley is not a homogeneous substrate, making it difficult to determine the enzyme action pattern. Therefore, the action pattern of rFiG was determined using various cellooligosaccharides (cellobiose–cellohexaose) and analyzed by TLC. Cellobiose and cellotriose were not good substrates for rFiG. Other cellooligosaccharides were efficiently degraded into cellobiose and cellotriose (Fig. 4A), and the reaction on CMC resulted predominantly in cellobiose and cellotriose. These results indicate that rFiG is an exo-type enzyme that liberates two or three glucan units from the substrate. When a time-course reaction was performed with cellohexaose as a substrate, cellobiose, cellotriose, and cellotetraose were initially generated (Fig. 4B), and as the reaction continued, the resulting cellotetraose was degraded into cellobiose whereas cellotriose was not changed. This action pattern is somewhat different from endoglucanases from various Clostridium strains, which release cellotetraose as the major product [16, 17]. However, the rFiG cleavage pattern on CMC was similar to those of GH5 endoglucanase from Volvariella volvacea [18] and the processive endoglucanase (Cel5H) from Saccharophagus degradans [19]. It has been

Fig. 3. Thermostability of the β-(1-4)-glucanase from F. islandicum.
The purified β-(1-4)-glucanase was incubated for various lengths of time at 70°C (●), 75°C (○), 80°C (▼), and 85°C (△). The half-life of purified rFiG was measured with pNPC at 80°C in 50 mM sodium acetate buffer (pH 5.0).

Fig. 4. TLC analysis for hydrolysis patterns of β-(1-4)-glucanase from F. islandicum.
(A) TLC of hydrolysis products from cellooligosaccharides hydrolyzed by rFiG. The substrates tested were as follows: cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose, and CMC (Lanes 1–6, respectively). The reaction was performed at 80°C for 1 h. Lane S, glucose to cellohexaose standards. (B) Time-course experiment of rFiG with cellohexaose as a substrate. The reaction was carried out with 2.5 mM cellohexaose at 80°C for 0, 15, 30, 60, and 120 min.
suggested that processive endoglucanases such as Cel5H are functionally equivalent to the endoglucanases and cellobiohydrolases that together comprise cellulolytic systems, thereby providing a cellobiohydrolase-independent cellulose-degrading mechanism [18]. In fact, F. islandicum AW-1 does not contain cellobiohydrolase or cellobiohydrolase-like gene products in its genome; therefore, there is a possibility that FiG may play a role in a cellobiohydrolase-independent cellulose-degrading system in F. islandicum.

In conclusion, β-(1-4)-glucanase cleaves β-(1-4) glycosidic linkages in cellulosic or hemicellulosic polymer chains. A wide range of β-(1-4)-glucanase applications are important in manufacturing fields such as biofuel, food, textile, and paper and pulp industries. In food industries, β-(1-4)-glucanases can be used not only in the extraction of beer, fruit juice, vegetable juice, and seed oil, but also in improving the nutritive quality of bakery products [20, 21]. These industrial applications require stable enzymes under harsh operational conditions [22, 23]. Therefore, rFiG is a good candidate for use in many industries since it has highly stable characteristics under extreme conditions.

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References