Improving Endoglucanase Activity by Adding the Carbohydrate-Binding Module from Corticium rolfsii

Zizhong Tang, Hui Chen, Lijiao Chen, San Liu, Xueyi Han, and Qi Wu

College of Life and Physical Sciences, Sichuan Agricultural University, Yaan 625014, China

Introduction

With the inevitable depletion of the world's energy supply, there is increasing global interest in alternative forms of energy. The application and transformation of cellulosic biomass can solve the issues of agriculture, renewable energy, and environmental pollution. Because cellulase is responsible for the conversion of renewable cellulosic biomass to simple sugars for fermentation to ethanol, H₂, and microbial oils, this enzyme has been the subject of much research [25]. Cellulase is the main component of the plant cell wall and also the most abundant renewable carbon resource in the biosphere [11]. It is the main product of photosynthesis, making up half of the plant's mass from this process. Cellulose is a linear homopolymer of D-glucose units linked by β-(1,4)-glycosidic bonds. Therefore, the most important step for cellulose application is the cleavage of the β-1,4 linkages by cellulase [8]. Cellulases comprise a group of enzymes that can convert cellulose to glucose, mainly endo-1,4-β-glucanase (E.C. 3.2.1.4), 1,4-β-D-cellobiohydrolase (E.C. 3.2.1.9) and 1,4-β-glucosidase (E.C. 3.2.1.21). These three enzymes cooperate for the total conversion of cellulose to glucose.

Endoglucanases (EGs), especially from fungi, have garnered the most attention in studies on cellulose [19]. Recently, bacterial cellulases have shown satisfactory application performance and economic value in the energy industry, due to their relative stability under neutral and basic conditions [3]. However, the large-scale industrial application of these enzymes is restricted by their low activity and high cost. Improving their activity through genetic engineering and protein engineering is considered an efficient approach to cutting down on these costs.

Most cellulases contain three domains: the cellulase domain, a carbohydrate-binding module (CBM), and a linker peptide, which are usually structurally and functionally independent [9]. More than 20 CBM families have been defined by comparing their primary structures, and some three-dimensional structures have also been elucidated [13]. CBMs help cellulase bind to the enzyme, thereby promoting enzymatic activity without having any catalytic activity themselves. Reconstruction of structural domains is an effective approach to studying these modules' roles. It has been reported that CBMs can change the characteristics of the cellulase, allowing for improvement in the degradation of cellulose.

The carbohydrate-binding module (CBM) is an important domain of most cellulases that plays a key role in the hydrolysis of cellulose. The neutral endoglucanase (EG1) gene was reconstructed. A redesigned endoglucanase, named EG2, was constructed with a CBM containing a linker from Corticium rolfsii (GenBank Accession No. D49448). The redesigned EG genes were expressed in Escherichia coli, and their characteristics are discussed. Results showed that the degradation of cellulose by EG2 was about double that by EG1. The specific activities of EG1 and EG2 were tested under optimal conditions, and EG2 had higher activity (169.1 ± 2.74 U/mg) toward CMC-Na than did EG1 (84.0 ± 1.98) in the process of cellulose degradation. The optimal pH and temperature, pH stability, and heat stability of EG1 and EG2 were similar. Results indicated that the CBM plays an essential role in the hydrolysis of cellulose. We can improve EG's catalytic power by adding the CBM from Corticium rolfsii.

Keywords: Endoglucanase, carbohydrate-binding module (CBM), gene fusion, enzyme property, Corticium rolfsii
or improve the enzymatic activity of endoglucanases [2, 4]. Bae et al. [2] fused EG IV (Cel5) and CBM6 to obtain the hybrid protein Cel5–CBM6, which showed higher activity with insoluble substrates. Sunna et al. [20] and Meissner et al. [16] showed that CBMs in some enzymes can thermostabilize their attached catalytic domain.

In a previous study, we isolated a strain of EG-producing Bacillus subtilis and cloned the gene encoding the EG (GenBank Accession No. DQ782954) [21, 23]. Another EG-encoding gene from the fungus Corticium rolfsii (GenBank Accession No. D49448) was also analyzed [24]. Like most cellulases, these EG-encoding genes were composed of a cellulose domain, a CBM, and a linker peptide. Therefore, this study aimed at fusing the fungal CBM (FCBM) to the mature EG to improve its catalytic activity. The signal peptide was removed from the EG, producing EG1, and the reconstructed EG with enhanced FCBM was named EG2. These EGs were reconstructed and expressed in Escherichia coli and the characteristics of these two enzymes were analyzed.

Materials and Methods

Experimental Materials

E. coli strain DH5α was used as a host for DNA manipulations and transformation. Strain DH5α containing the EG gene was constructed in our laboratory, using plasmid pET32a to construct the EG-expressing vector. E. coli strain BL21, stored in our laboratory, was used as a host for transformation and expression. PrimeSTAR HS DNA polymerase and the restriction enzymes were purchased from TaKaRa (Dalian, China). The primers and genes encoding the CBM of the EG from Corticium rolfsii (GenBank Accession No. D49448) were synthesized by Invitrogen (Shanghai, China).

Construction of Recombinant EGs

To improve enzymatic activity, two redesigned EGs were constructed in this study. The signal peptide was removed from the EG, giving EG1. EG1 was fused with the FCBM of C. rolfsii and was named EG2. The structures of the two EGs are shown in Fig. 1.

All fragments were amplified by PCR using PrimerSTAR HS DNA polymerase and several designed and synthesized oligonucleotide primers (Table 1). The PCR products were purified and digested with the corresponding restriction enzymes for the sites designed in the primers. Plasmid pET32a was digested with KpnI and XhoI, and the corresponding products were ligated to this vector fragment for 12 h at 16°C. E. coli strain BL21 (DE3) was transformed with these vectors and plated on Luria-Bertani (LB) medium containing ampicillin. The positive colonies were screened by colony PCR and digested with the corresponding restriction enzymes.

Hydrolytic Zone Analysis

Positive colonies were picked from the selective plate (with Amp, CMC-Na, and IPTG added) and incubated at 37°C for 3 days. To visualize the hydrolytic zone, the plates were flooded with an aqueous solution of 0.1% (w/v) Congo red for 30 min and washed with 1 M NaCl [1].

Expression of the Two Recombinant Enzymes in E. coli Strain BL21 (DE3) by the Shake-Flask Method

The positive colonies were picked and inoculated in 10 ml of LB medium with ampicillin (100 µg/ml), and cultivated overnight at 37°C with vigorously shaking at 190 rpm. The cell cultures were added into 100 ml of LB medium by 1:100 dilutions and grown to an OD600 of 0.6–0.8. IPTG was added to the medium at a final concentration of 0.1 mM and the culture was reincubated at 37°C for 3 h. The cells were harvested by centrifugation 5 h later. The pellets were resuspended in 10 ml of 1/15 M PBS buffer (pH 6.0) followed by ultrasound to release EG protein, and then analyzed by SDS-PAGE. The supernatants were used for determination of enzymatic activities and purification of the target proteins [17].

Table 1. Primers and corresponding restriction enzymes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Characteristic</th>
<th>Sequence</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Upstream primer to amplify mature EG</td>
<td>5’-cgcgGTACCCgcaagacaataaagccagct-3’</td>
<td>KpnI</td>
</tr>
<tr>
<td>P2</td>
<td>Downstream primer to amplify mature EG</td>
<td>5’-tggcGTCGACATTttggtctgctccccc-3’</td>
<td>SaI</td>
</tr>
<tr>
<td>P3</td>
<td>Downstream primer to amplify cellulase</td>
<td>5’-tggcGTCGACATTttggtctgctccccc-3’</td>
<td>XhoI</td>
</tr>
<tr>
<td>P4</td>
<td>Upstream primer to amplify carbohydrate-binding module</td>
<td>5’-gcgtGTCGACGgatakctactct-3’</td>
<td>SaI</td>
</tr>
<tr>
<td>P5</td>
<td>Downstream primer to amplify carbohydrate-binding module</td>
<td>5’-ctctCTCGAGttgctgctccccc-3’</td>
<td>XhoI</td>
</tr>
</tbody>
</table>

![Fig. 1. Structure of the reconstructed endoglucanase forms.](image)
EG Activity Assays

EG activity was determined by a revised classical method [15], using CMC-Na as the substrate. The amount of reducing sugars released from the assay was detected by the DNS method: 200 µl of supernatant was diluted to 1ml in 1/15 M PBS buffer (pH 6.0); 100 µl of diluted enzyme and 1 ml of 1% (w/v) CMC-Na were mixed in a 10 ml tube and incubated at 50°C for 30 min. The reaction was terminated with 2.5 ml of DNS, and then boiled for 5 min. Blanks were obtained by terminating the EG activity with DNS before adding CMC-Na. Glucose was used for the standard curve. One unit (U) of activity was defined as the amount of enzyme that released 1 µmol of glucose equivalents per minute under the assay conditions. The protein concentration was determined using a BCA protein assay kit (Pierce, USA) with BSA as the standard.

Purification of EGs Expressed in E. coli Strain BL21 (DE3)

The recombinant protein was purified with Ni²⁺-nitrilotriacetate (NTA) resin (Qiagen, USA). The column was equilibrated with 50mM Tris-HCl (pH 8.0, buffer A). The crude supernatant was concentrated 30-fold by ultrafiltration through a 10kDa cut-off membrane (Millipore, USA). The crude extract–NTA mixture was loaded onto a chromatographic column and washed with 200 ml of buffer A. The recombinant protein was eluted with a linear imidazole gradient (100 ml of 0 to 500 mM in buffer A). The eluted fractions containing the target protein were pooled, concentrated, and assayed for EG activity. SDS-PAGE was then performed to estimate the molecular weight and concentration of the expressed products [5].

Substrate Specificity

The EGs’ hydrolytic activity against cotton, filter paper, carboxymethylcellulose (CMC), cellobiose, CMC-Na, p-nitrophenyl-β-D-glucopyranoside (pNPG), and Avicel was determined under optimal assay conditions to evaluate the substrate specificity of the purified cellulase, as described previously.

Enzyme Characterization Assays

The activities of the two reconstructed EGs at different temperatures and pH values were determined with the purified enzymes. Optimal temperature was determined in a range of 30°C to 90°C in PBS buffer (pH 6.0). To determine thermostability, the enzyme was preincubated at 30°C to 90°C in PBS buffer (pH 6.0) for 30 min, and then a standard activity assay was performed. The enzyme and CMC-Na were diluted in buffer with pH values ranging from 3.0 to 10.0 to estimate the optimal pH, and the enzyme was preincubated at 50°C in these same buffers to determine its pH stability. The standard DNS activity assay was then performed [6, 10].

Statistical Analysis

All of the data are reported as the mean ± SEM of three replicates. All data were subjected to the Student’s t-test at a significance level of p < 0.05 using the SPSS software package.

Results

Construction of Recombinant EGs

All PCR products were purified and inserted into the pET32a vector, and DNA sequence analysis of the recombinant plasmids confirmed cloning of the target segments. Recombinant plasmids EG1 and EG2 were confirmed by corresponding restriction enzyme analysis. As shown in Fig. 2, the anticipated bands were generated in the digested products. A 1,400 bp fragment was obtained after double digestion of EG1 with KpnI and XhoI. Fragments of 1,400 bp and 250 bp (CBM domain) were released after double digestion of EG2 with KpnI and SalI, and XhoI and SalI.

Hydrolytic Zone Analysis

pET32a-EG1, pET32a-EG2, and pET32a (control) were inoculated on a LB-ampicillin plate containing 1% CMC-Na and 0.1 mM IPTG. Three days later, the plate was flooded with 1% (w/v) Congo red solution for 30 min and then rinsed several times with 1 M NaCl. All of the strains showed a colorless hydrolytic ring except the control (Fig. 3). The hydrolytic zone of EG2 was larger than that of EG1.

Purification and Activity Analysis

A comparison of the expressed protein with the negative

![Fig. 2. Identification of EG1 and EG2 by enzyme digestion.](image-url)
control on an SDS-polyacrylamide gel (Fig. 4) showed that the molecular mass of EG2 was about 61 kDa, that of EG1 was about 53 kDa, and that of the negative control was only about 20 kDa. SDS-PAGE of the expressed products purified on a Ni²⁺-NTA agarose affinity chromatography column gave the same results (Fig. 5). We used the purified protein to analyze its specific activity. The concentration of the purified protein was determined by BCA protein assay kit, using BSA as a standard. All of the proteins were assayed for EG activity by the DNS method under optimal assay conditions. A brief summary of the purification results is presented in Table 2. EG2 had higher specific cellulose-degrading activity toward CMC-Na than did EG1 (Table 2).

**Characterization of Recombinant EGs**

The effects of temperature and pH on the enzymatic activities of EG1 and EG2 were evaluated. The optimal temperature for both EG1 and EG2 activity was 60°C, and their activity dropped rapidly at temperatures above 65°C (Fig. 6). To determine thermostability, EG activity was

**Table 2.** Specific activities of EG1 and EG2.

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG1</td>
<td>127.0 ± 0.04</td>
<td>10,668 ± 1.52</td>
<td>84.0 ± 1.98</td>
</tr>
<tr>
<td>EG2</td>
<td>136.2 ± 0.01</td>
<td>23,031 ± 3.09</td>
<td>169.1 ± 2.74</td>
</tr>
</tbody>
</table>

The experiment was carried out in triplicate.
measured under optimal assay conditions after incubation at various temperatures for 30 min. As shown in Fig. 7, the relative activities of the two enzymes remained at over 85% after incubation at 30°C to 70°C for 30 min, but dropped to below 40% when the temperature was raised to 80°C. Activity levels of EG1 and EG2 were highest at pH 7.0 (83.0 ± 0.78 U/mg) and pH 6.5 (166.1 ± 1.83 U/mg), respectively, and dropped rapidly at pH less than 4 or higher than 8.5 (Fig. 8). The pH stabilities of EG1 and EG2 were similar. EG1 retained about 80% of its original activity in the pH range of 4.0–10.0, and EG2 retained over 80% of its original activity in the pH range of 4.5–10.0 (Fig. 9).

The purified recombinant EG enzymes were assayed for their activities toward different substrates (Table 3). The substrate specificities of EG1 and EG2 were quite similar, except that EG2 activity was higher than that of EG1 toward the different substrates under optimal conditions. EG1 and EG2 showed relatively high activity toward CMC-Na, intermediate activity with CMC, and lower activity on cotton and filter paper. Thus, both enzymes showed higher activity toward the soluble cellulose substrate (CMC-Na) than insoluble cellulose substrates. No activity was detected against pNPG, cellobiose, or Avicel, indicating that the enzymes lacked exoglucanase and β-glucosidase activities. Again, the specific activity of EG2 was always higher than that of EG1 in the process of soluble-cellulose degradation.

Discussion

As already mentioned, most cellulases contain a cellulase domain, CBM, and linker peptide, which are usually structurally and functionally independent [22]. The CBM
Table 3. Substrate specificities of the purified endoglucanases.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EG1</td>
</tr>
<tr>
<td>CMC-Na salt</td>
<td>84.0 ± 1.98</td>
</tr>
<tr>
<td>CMC</td>
<td>56.1 ± 2.17</td>
</tr>
<tr>
<td>Cotton</td>
<td>28.7 ± 1.48</td>
</tr>
<tr>
<td>Filter paper</td>
<td>23.83 ± 0.77</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0</td>
</tr>
<tr>
<td>Avicel</td>
<td>3.2 ± 0</td>
</tr>
<tr>
<td>pNPG</td>
<td>0</td>
</tr>
</tbody>
</table>

The experiment was carried out in triplicate.

and linker peptide play an important role in the process of cellulose degradation. Therefore, fusion of FCBM to EG might change the enzyme’s characteristics or improve its activity. Consequently, the effect of the CBM on cellulase activity has been studied with a number of EGs [12, 14, 21]. Some studies have suggested that the CBM enhances the enzymatic activity of cellulases by increasing the effective enzyme concentration at the substrate surface or by disrupting the crystalline cellulose structure, thereby improving substrate accessibility [18, 21]. A recent study on family 2 CBMs points to the role of surface aromatics in binding with the staircase-like cellulose surface [9]. Most of the work done to date on hybrid cellulases has been on EGs fused to CBM from the same organism. These hybrid proteins have shown enhanced activity on cellulose in some cases [11], whereas fusion of CBM to the catalytic center has had little or no effect in others [7]. CBM removal occurs naturally in some cellulases, accompanied by a reduction in their ability to degrade cellulose [2, 7, 17]. A recent study on family 6 CBMs found that multiple CBMs can bind cooperatively to enhance their binding ability by 20- to 40-fold relative to individual modules [7, 21]. For instance, Li and Shao [12] fused the CBM of the xylanase XynA from *Thermotoga maritima* and the EG Cel12B from the same species and obtained new crystalline cellulose-degrading activity. In our study, EG from the bacteria *B. subtilis* was fused with the CBM from the fungus *C. rolfsii* and expressed in *E. coli* strain BL21. The resultant cellulose-degrading ability of the fusion enzyme EG2 was double that of the control, EG1. The optimal temperature, pH stability, and heat stability of EG1 and EG2 were quite similar, but the optimal pH values of the two enzymes were 7.0 and 6.5, respectively. Substrate specificity and specific activity assays showed that EG2 had higher activity than EG1 in the process of soluble-cellulose degradation. Overall, we can conclude that fusion of a CBM to an EG serves to enhance the activity of the enzyme, as we had anticipated. This work shows the significance of protein engineering of EG to improve its enzymatic activity and the feasibility of designing a novel EG that is better suited to industrial applications.

Acknowledgments

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References

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