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Cloning, expression and characterization of a thermotolerant endoglucanase from *Syncephalastrum racemosum* (BCC18080) in *Pichia pastoris*

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Abstract

Endoglucanase is a major cellulolytic enzyme produced by *Syncephalastrum racemosum* (BCC18080). Preliminary results showed that this endoglucanase is thermotolerant as it retained more than 50% of its activity after incubation at 80 °C for an hour. As this property may be of industrial use, we have cloned the full-length BCC18080 endoglucanase gene of 1020 nucleotides. Sequence analysis suggested that it belonged to the glycosyl hydrolase family 45. N-terminal sequencing and analysis by SignalP program suggested that the first 32 amino acid residues encoded the signal peptide. Expression of the recombinant clones with and without its own signal peptide in *Pichia pastoris* demonstrated that *P. pastoris* produced active 55 and 30 kDa secreted proteins. N-terminal sequencing suggested that the 55 kDa band was the mature protein while the 30 kDa band was the truncated protein. Glycoprotein analysis showed that the 55 kDa protein was glycosylated; while the smaller protein was not. All recombinant endoglucanases showed optimal temperature of 70 °C and optimal pH of 5–6. They retained more than 50% activity for 4 h at 70 °C. In addition, high k_{cat} and low apparent K_m of these recombinant proteins indicated good properties of this enzyme against carboxylmethylcellulose.

Keywords: *Syncephalastrum racemosum*; Endoglucanase; Thermotolerant; *Pichia pastoris* expression

Cellulose, one of the most important industrial cellulosic materials, can be degraded by cellulolytic enzymes. This group of enzymes are classified based on their amino acid sequence and substrate specificity [1,2] and can be broadly divided into three groups: (i) endoglucanases or endo-1, 4-β-D-glucanases (EC 3.2.1.4) (ii) exoglucanases or cellobiohydrolases (EC 3.2.1.91) and (iii) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21).

Since fungi produce large amount of extracellular cellulases, they have been widely studied and utilized for industrial applications in food, animal feed, paper and pulp, textile and chemical industries [3,4]. In industrial applications, themostable enzymes are ideal because they can withstand high temperature; thus, thermostable cellulases have been widely studied with the aim to improve their properties. For example, expression of thermostable cellulases has been reported in both bacteria and yeast [5–7]. Also, to improve the cellulase activity, hybrid protein was made by fusing the thermostable chitin-binding domain of *P. furiosus* to the endoglucanase of *P. horikoshii* which lacks the cellulose-binding domain [8].

Since some studies suggested that glycosylation effect thermostability of enzyme, for instance, deglycosylation in *Humicula insolens* cellulases lower their stability [9]. Therefore, most thermostable cellulases are expressed in yeast because besides providing high expression level, yeast has post-translational modification such as glycosylation.

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Our preliminary studies on more than two hundreds thermotolerant fungi collected throughout Thailand from the BIOTEC Culture Collection demonstrated that a fungus belonging to the *Zygomycotina* subdivision, *Syncephalastrum racemosum* (BCC18080) produced the highest level of thermotolerant cellulases, i.e. greater than 50% of its cellulase activity remained after incubation at 80 °C for an hour. To begin to understand the thermotolerant property of *S. racemosum* cellulases, the gene encoding one of the cellulolytic enzymes, endoglucanase, which is one of the major cellulolytic enzymes produced by this fungus was cloned, expressed in *Pichia pastoris* and biochemically characterized. In addition, the use of heterologous secretion signal of *S. racemosum* endoglucanase in *P. pastoris* was also investigated.

Materials and methods

Strains, plasmids, culturing conditions, and primers

Syncephalastrum racemosum (BCC18080), a filamentous fungus, was obtained from the BIOTEC Culture Collection (BCC).[1] It was grown on 5% wheat bran agar plate at 30 °C for 3 days, and then inoculated into 100 ml of 5% wheat bran broth in a 500 ml Erlenmeyer flask. The cell was grown for 5 days at 30 °C with shaking at 250 rpm. pGEM-T easy vector was purchased from Promega, USA. pPICZαA and pPICZB vectors were from Invitrogen, USA. All nucleotide primers were either synthesized by Bioservice Unit (BSU), BIOTEC, Thailand or Sigma-Proligo, Singapore. Cellulase from *Trichoderma reesei* used as the reference enzyme was purchased from Boehringer Mannheim, Germany. AZCL-HE cellulose was purchase from Megazyme, Ireland. Zeocin was purchased from Invitrogen, USA. Other chemicals and solvents (analytical grade) were purchased from Gibco BRL,USA; Fluka, Switzerland; Sigma, USA or Merck, Germany. Restriction enzymes and other modification enzymes were purchased from New England Biolabs, USA; Boehringer Mannheim, Germany; Promega, USA; Gibco BRL, USA; Stratagene, USA or Sigma, USA.

Total RNA isolation, cDNA synthesis, and nested PCR

Total RNA was extracted and purified as described [10]. First-strand cDNA was synthesized using random hexamer and Superscript III reverse transcriptase (Invitrogen, USA) according to the supplier. The obtained first-strand cDNAs served as template for nested PCR. Four degenerate oligonucleotides (EndoF1, EndoF2, EndoR1, and EndoR2; Table 1) were designed based on the conserved regions of the endoglucanase genes of *Rhizopus oryzae*.

The first round PCR was performed in a total volume of 50 μl mixture containing 20 μM each of primers EndoF1 and EndoR2, 1× PCR buffer (Promega, USA), 2.5 mM each of dNTPs, 2 mM $MgCl_2$, 4 μl cDNA and 1 U of in-house *Taq* DNA polymerase. Amplification cycles were: one cycle of 5 min at 94 °C, 35 cycles of 94 °C for 30 s, 40 °C for 30 s and 72 °C for 45 s, then 10 min at 72 °C. The second round of PCR was as above, except that the template consisted of 4 μl of the first PCR products and primers, EndoF2 and EndoR1. The amplicon of the expected size was gel-purified [10] and cloned into pGEM-T Easy vector (Promega, USA). Sequencing was performed by Macrogen Inc. (Korea).

Table 1
Oligonucleotides used in the studies

Primer name	Sequence (5′→3′)
EndoF1	CARTGYGGNGGNAARAATTGG[a]
EndoF2	TGYAAYGAYAAYCARCCNTGG[a]
EndoR1	RTTRTCNGCRTTYTTRAACCA[a]
EndoR2	YTTNCGNGARCANCCNGTYTT[a]
PM1 oligo(dT)	CCGGAATTCAAGCTTCTAGAGGATCCTT TTTTTTTTTTTTT
PM2	CCGGAATTCAAGCTTCTAGAGGATCC
3′RaceEndo	GAGGGCAAGAAGATGGTCGT
5′RaceEndo1	ACGACCATCTTCTTGCCCTC
5′RaceEndo2Nested	CCGCTGATGCTTGCAGCAGC
expressEndoF1	GCGAATTCATGATCCTTCACCGCACA
expressEndoF2	GCCTCGAGAAAAGAGCCGATTGCAGC
expressEndoR	GCTCTAGATTAGGAGGTGCGTTCGCA

[a] For degenerate primer, the following abbreviations are used; N: A, T, C, G; H: A, T, G; Y: C, T; M: A, C; S: C, G; R: A, G.

Isolation of the full-length BCC18080 endoglucanase gene by 3′ and 5′RACE

For 3′ RACE, first-strand cDNAs were constructed as above from 1 μg of BCC18080 total RNA using an oligo-dT adaptor (PM1 primer). PCR was performed using 1 U of DyNazyme EXT DNA polymerase (Finnzyme, Finland), 20 μM of 3′RaceEndo, a gene-specific primer and PM2, an adaptor primer, in a volume of 50 μl containing 1.5 mM $MgCl_2$, 2.5 mM each of dNTPs, and 4 μl of cDNA. The PCR conditions were: one cycle at 94 °C, 3 min; 35 cycles of 30 s at 94 °C, 45 s at 52 °C, 45 s at 72 °C; and finally 10 min at 72 °C. For 5′RACE, first-strand cDNAs were generated using the partial heat denaturation reverse transcription method [11] using 10 μM of 5′RaceEndo1. Finally, nested PCR was performed as described [12] using primers, 5′RaceEndo1 and PM1, in the first round PCR at 47 °C annealing temperature; in the second round of PCR, the primers were 5′RaceEndo2 Nested and PM2, and the annealing temperature was 54 °C for 45 s. The obtained PCR products were purified, cloned and sequenced.

The obtained 3′ and 5′RACE DNA sequences were assembled *in silico* using Vector NTI. Next, gene-specific primers were designed to amplify the BCC18080 endoglucanase gene with or without its signal peptide (the signal peptide was predicted by SignalP 3.0 prediction program; http://www.cbs.dtu.dk/services/SignalP/). The primers were: expressEndoF1 and expressEndoR for amplifying

[1] *Abbreviations used:* BCC. BIOTEC Culture Collection; PVDF, polyvinylidene difluoride; DNS, dinitrosalicylic acid.