Characterization of two thermophilic cellulases from Talaromyces leycettanus JCM12802 and their synergistic action on cellulose hydrolysis

PLOS One

Nov 16, 2019

Yuan Gu[1], Fei Zheng[1,2], Yuan Wang[1], Xiaoyun Su[1], Yingguo Bai[1], Bin Yao[1], Huoqing Huang[1], Huiying Luo[1]

[1]Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, People's Republic of China, [2]College of Biological Sciences and Biotechnology, Beijing Forestry University, Beijing 100083, People's Republic of China

1 Works for me dx.doi.org/10.17504/protocols.io.8g4htyw

Yuan Gu

ABSTRACT

The genes of two Thermophilic cellulases (*Ti*Cel5A and *Ti*Cel6A) belonging to GH5 and GH6 respectively were obtained from *Talaromyces leycettanus* JCM12802 and proteins of them were expressed in *Pichia pastoris*. The properties of the cellulases were investigated by determining their optimal temperature, optimal pH, temperature stability, pH stability, and kinetic parameters. Through the hydrolysis of oligosaccharides analysis, the effect of two cellulases (*Ti*Cel5A and *Ti*Cel6A) on oligosaccharides can be known. Then, under the optimal conditions, the synergistic effect between two cellulases (*Ti*Cel5A and *Ti*Cel6A) mixed in different ratios was determined.

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0224803

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

References 1. Larkin MA, Blackshields G, Brown NP, Chenna R, Mcgettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007; 23(21): 2947-2948. 2. Robert X, Gouet P. Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Research. 2014; 42: W320-W324. 3. Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Tobias S, et al. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Research. 2014; 42: W252-W258. 4. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis. 1997; 18(15): 2714-2723. 5. Miller G. Use of dinitrosalicyclic acid reagent for determination of reducing sugars. 1969; 31(3): 426-428.

Protocol.docx

MATERIALS TEXT

T. leycettanus JCM12802 from the Japan Collection of Microorganisms (JCM, Japan), *P. pastoris* GS115 (Invitrogen, Carlsbad, CA), the vectors pGEM-T Easy (Promega, Madison, WI) and pPIC9 (Invitrogen)

The restriction endonucleases, T4 DNA ligase and endo-β-*N*-acetylglucosaminidase H (Endo H) were purchased from New England Biolabs (Ipswich, MA).The *Taq* DNA polymerase was from TaKaRa (Dalian, China). The DNA isolation and purification kits were purchased from Tiangen (Beijing, China) and Omega (Cowpens, SC), respectively. RNeasy plant mini kit and ReverTra-α-™ kit were supplied by Qiagen (QIAGEN, Germany) and TOYOBO (Osaka, Japan), respectively. The substrates lichenan, barley β-glucan, laminarin, Avicel, carboxymethyl cellulose sodium (CMC-Na), birchwood xylan, xyloglucan, konjac glucomannan, locus bean gum, 4-nitrophenyl b-d-cellobioside (*p*NPC), *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlu), and 4-nitrophenyl-α-D-galactopyranoside (*p*NPGal) were purchased from Sigma-Aldrich (St. Louis, MO). PASC treated by 85% of phosphoric acid and SECS were supplied by the Institute of Process, Chinese Academy of Sciences. And all other chemicals were of analytical grade and commercially available.

1 Cloning of the cellulase-encoding genes

1.1 The full length cellulase-encoding genes, *Tlcel5A* and *Tlcel6A*, were identified in the genome sequence of *T. leycettanus* JCM12802, and obtained by PCR amplification with specific primers GH5F/GH5R and GH6F/GH6R (Table 1).

1.2 The PCR conditions were as follows: 95°C for 3min, followed by 30 cycles of 95°C for 30 s, 60°C for 60 s, and 72 ◦C for 1min.

1.3 cDNAs were then synthesized *in vitro* using the ReverTra Ace-a-™ kit. The cDNAs coding for the mature *Tl*Cel5A and *Tl*Cel6A were amplified with primers GH5F1/GH5R1 and GH6F1/GH6R1 harboring restriction sites, respectively. (Table 1).

1.4 The PCR products were inserted into plasmid pGEM-T Easy and then transformed into *E. coli* Trans1-T1.

2 Sequence and structure analysis

2.1 The nucleotide and amino acid sequences were used for BLAST analysis at NCBI (http:// www.ncbi.nlm.nih.gov/BLAST/).

2.2 Simplified sequence alignment and prediction of the isoelectric point and molecular weight were conducted by using the Vector NTI Suite 10.0 software.

2.3 Signal peptide prediction was carried out with SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/).

2.4 Multiple alignment of protein sequences was performed with the ClustalX 2.1 (http://www.clustal.org, [1], and the results were demonstrated by ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi, [2].

2.5 The NetNglyc server was used to predict the putative N-glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.6 And the modeled structures were predicted and visualized by using the SWISS-MODEL [3] and Pymol [4].

3 Heterogeneous expression in *P. pastoris*

3.1 The cDNA fragments coding for the mature proteins of *Tl*Cel5A and *Tl*Cel6A were digested with restriction enzymes *Sna*bI or *Eco*RI and *Not*I and subcloned into plasmid pPIC9 to yield expression vectors pPIC9-*Tlcel5A* and pPIC9-*Tlcel6A*, respectively.

3.2 The recombinant plasmids were then linearized with *Bgl*II and individually transformed into *P. pastoris* GS115 competent cells by electroporation.

3.3 MD plates can be used to select the transformants and pick some positive colonies into 3 mL BMGY with 10 mL tubes for two days and change 1 mL BMMY for another two days at 30 °C and 220 rpm.

3.4 The culture supernatants were collected by centrifugation at 5,000 rpm for 10 min, followed by cellulase activity assay as described below.

3.5 The most active transformants of *Tl*Cel5A and *Tl*Cel6A were grown in 1 L shake-flasks containing 400 mL BMGY at 220 rpm and 30 °C for 2 days, respectively.

3.6 The cultures were pelleted by centrifugation and resuspended in 200 mL of BMMY for 3-day-growth at 30 °C and 200 rpm. Methanol was then added at the final concentration of 0.5 % every 24 h for continuous induction of 3 days.

3.7 The culture supernatants were collected by centrifugation at 4500× g for further analysis.

4 Purification of recombinant *Tl*Cel5A and *Tl*Cel6A

4.1 The viva flow 200 ultrafiltration membrane system (Sartorius, Germany) with 5 kDa cut-off was used for the concentration and buffer exchange (to 0.1 M Tris-HCl, pH 8.0) of the crude enzymes.

4.2 The HiTrapTM Desalting column was used to desalt and the HiTrap Q Sepharose XL FPLC column (GE Healthcare) pre-equilibrated with 0.1 M Tris-HCl (pH 8.0) was used to purify for the recombinant proteins.

4.3 The gradient NaCl of 0–0.7 M at the flow rate of 3 mL/min was used to elute the proteins.

4.4 Fractions showing cellulase activities were pooled and further desalted with a 5 kDa molecular cut-off concentration tube (Millipore) using 0.1 M McIlvaine buffer (pH 3.5 or 4.5).

4.5 The purified proteins were separated on 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for purity and molecular mass analysis.

4.6 Protein concentration was determined by using the Bradford method.

5 Deglycosylation of the purified recombinant *Tl*Cel5A and *Tl*Cel6A

To remove N-glycosylation, the purified recombinant *Tl*Cel5A and *Tl*Cel6A were treated with Endo H for 2 h at 37 °C following the manufacturer's instructions (New England Biolabs), and checked by SDS-PAGE.

6 Enzyme activity measurements

The cellulase activities were determined using 1 % (w/v) CMC-Na, barley β-glucan or lichenan as the substrate.

6.1 The reaction mixtures containing 100 µL of properly diluted enzyme solution and 900 µL substrate solution in 0.1 M McIlvaine buffer (pH 3.5 and 4.5) were incubated at 75 °C or 80 °C (optimum temperature) for 10 min. (When using 5 mg/mL Avicel as the substrate, the reaction period was lengthened to 60 min)

6.2 The amounts of reducing sugars were determined via the 3,5-dinitrosalicylic acid (DNS) method. [5] One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 µmol of reducing sugar per minute under the assay conditions.

7 Biochemical characterization of *Ti*Cel5A and *Ti*Cel6A

CMC-Na, barley-glucan and PASC were used as the substrates for enzyme characterization.

7.1 The optimal pH was determined at 75 °C for *Ti*Cel5A and 80 °C for *Ti*Cel6A, respectively, in 0.1 M McIlvaine buffer with pH ranging from 2.0 to 8.0.

7.2 To test the pH stability, the purified enzymes were pre-incubated at 37 °C for 60 min in buffers with pH ranging from 1.0 to 12.0 (0.1 M KCl-HCl for pH 1.0–2.0, 0.1 M McIlvaine buffer for 2.0 to 8.0, and 0.1 M glycine-NaOH for pH 9.0–12.0).

7.3 The residual enzyme activities were determined at 75 °C and pH 3.5 for *Ti*Cel5A and 80 °C (barley β-glucan) or 70 °C (PASC) and pH 4.5 for *Ti*Cel6A, respectively.

7.4 To determine the optimal temperature, the cellulase activities were determined at temperatures ranging from 30 °C to 90 °C and pH 3.5 for *Ti*Cel5A or pH 4.5 for *Ti*Cel6A for 10 min, respectively.

7.5 For thermal stability assays, *Ti*Cel5A and *Ti*Cel6A were incubated at temperatures of 70 °C, 75 °C or 80 °C and pH 3.5 or 4.5 for different periods of time.

7.6 For half-life determination, 0.05 mg of *Ti*Cel5A or 0.2 mg of *Ti*Cel6A was incubated at 60 °C, 65 °C or 70 °C for 0.5–24 h

7.7 Residual activities were determined as described above.

8 Substrate specificity and kinetic parameters

Substrate specificity of *Ti*Cel5A and *Ti*Cel6A were determined by using 1 % (w/v) CMC-Na, barley β-glucan, Avicel, lichenan, laminarin, xyloglucan, birchwood xylan, konjac flour, locus bean gum, glucomannan, or 0.2 mM of *p*NPGlu, *p*NPGal, *p*NPC as the substrate.

8.1 The kinetic parameters (Km, Vmax and kcat/Km) of *Ti*Cel5A were determined by incubating the enzyme with 0.25 to 10 mg/mL CMC-Na at pH 3.5 and 75 °C for 5 min.

8.2 For *Ti*Cel6A, the kinetic parameters were determined at pH 4.5 and 80 °C for 5 min with 0.25–10.0 mg/mL barley β-glucan as the substrate.

8.3 The enzyme activities were determined by using the DNS method. The kinetic constants were calculated using the Lineweaver-Burk plots by GraphPad Prism 6.0 (http://www.graphpad.com/scientific-software/prism/).

9 Analysis of the cellooligosaccharides hydrolysis products

The hydrolysis products of cellooligosaccharides by *Ti*Cel5A and *Ti*Cel6A were detected by high-performance anion exchange chromatography (HPAEC, model 2500, Dionex, Sunnyvale, CA).

9.1 Purified *Ti*Cel5A (0.05 U) or *Ti*Cel6A (0.02 U) was added into 1 mL of cellooligosaccharide solution containing 200 μg of cellotetraose, cellopentaose, or cellohexaose and incubated at 60 °C for 0 min, 1 min, 5 min, 30 min, 1 h, 4 h or 5 h.

9.2 After enzyme inactivation by boiling water bath, the hydrolysates were diluted 100 times with ddH_2O, and 100 μL of each sample was injected into the column of HPAEC.

9.3 The oligosaccharides were eluted by 100 mM NaOH. And the standards of oligosaccharides consisted of glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose.

9.4 The amount of each hydrolysate was calculated based on the peak area.

One enzyme unit (U) was defined as the amount of enzyme to hydrolyze 1 μmol of substrate per minute under the assay conditions. The catalytic efficiency values (kcat/Km) of *Ti*Cel6A against cellooligosaccharides were calculated according to Xia et al. (2016).

10 Synergistic of *Ti*Cel5A and *Ti*Cel6A on cellulose hydrolysis

10.1 When using Avicel as the substrate, *Ti*Cel5A and *Ti*Cel6A were combined at the ratios of 0.4:3.6, 0.8:3.2, 1.2:2.8, 1.6:2.4, 2.0:2.0, 2.4:1.6, 2.8:1.2, 3.2:0.8, 3.6:0.4 in the total amount of 4.0 mM and incubated with 5 mg/mL of Avicel in 50 mM McIlvaine buffer, pH 4.0 at 60 °C for 24 h. The amounts of reducing sugars released were determined using the DNS method.

10.2 When using SECS or Avicel as the substrate, 8 μM of *Ti*Cel5A or *Ti*Cel6A alone, or 4 μM *Ti*Cel5A and 4 μM *Ti*Cel6A was incubated with 5 mg/mL substrate at pH 4.0 and 60 °C for 12 or 18 h.

10.3 With 2.5 mg/mL PASC as the substrate, 2 μM of *Ti*Cel5A or *Ti*Cel6A alone or 1 μM *Ti*Cel5A and 1 μM *Ti*Cel6A was added.

10.4 The amounts of reducing sugars released were determined by using the DNS method.

10.5 The amounts of glucose released were analyzed by a one-factor ANOVA of SPSS19.0 to assess the synergistic effects of *Ti*Cel5A and *Ti*Cel6A. Statistical differences were considered to be significant at $P < 0.05$.