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Characterization of the recombinant cellulase A from *Thermotoga maritima*

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Abstract A gene encoding thermostable cellulase A (TmCelA) was isolated from *Thermotoga maritima*. The open reading frame of TmCelA gene was 774 bp long which predicted to encode 257 amino acid residues with a molecular weight of 29,732 Da. To examine the biochemical properties, the TmCelA was overexpressed in *E. coli* BL21, and expressed protein was purified. The optimum temperature of recombinant TmCelA was 90-95 °C, and the optimum pH of recombinant TmCelA was approximately pH 5.0. Recombinant TmCelA was stable at temperature below 90 °C.

Keywords Cellulase · Cellulose · Thermostable · *Thermotoga maritima*

Introduction

Cellulose is one of the most affluent polysaccharides on Earth and is an important alternative energy source. It consists of D-glucose residues linked together to make linear polysaccharide chains via β-1,4-glycosidic linkages [1]. Enzymes that hydrolyze biopolymers have been studied in many species of fungi [2], as well as in mesophilic and hyperthermophilic bacteria [3,4], mainly because of their substantial economic potential for the conversion of biopolymers into chemicals and biofuel, and their application in the food and detergent industries [5]. *Thermotoga maritima* is anaerobic and hyperthermophilic bacteria, and grows at temperature up to 90 °C (optimal growth at 80 °C). *T. maritima* metabolizes various biopolymers, such as pectin, cellulose, and xylan [6,7], and hyperthermophilic enzymes isolated from *T. maritima* provide very useful catalysts for industrial applications.

The genome of *T. maritima* was completely sequenced [8], disclosing several genes encoding hyperthermostable enzymes for biopolymer utilization including a candidate gene for a cellulase A (TmCelA). In this study, the cellulase A gene of *T. maritima* was cloned into the *E. coli* expression vector, pRSET, overexpressed in *E. coli*, and purified using His-tag affinity column. The biochemical properties of the expressed recombinant TmCelA were examined.

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Materials and Methods

T. maritima genomic DNA and Bacterial strains

T. maritima genomic DNA was obtained from ATCC (ATCC 43589D, Manassas, VA, USA). For plasmid propagation and transformation, *E. coli* TOP10 was used. *E. coli* BL21 was used for the overexpression of recombinant TmCelA gene.

Enzymes and reagents

DNA-modifying enzymes and restriction enzymes were obtained from Promega (Madison, WI, USA). Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) agarose resin was obtained from Qiagen Inc. (Germantown, MD, USA). Carboxymethylcellulose and other reagents and materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Construction of pBRTmCelA expression vector

The open reading frame (ORF) of the TmCelA gene was cloned into the *E. coli* expression vector, pRSET-B, through polymerase chain reaction (PCR) amplification and expressed in *E. coli* BL21. The PCR primers for amplification of TmCelA gene were 5'-AAATTTGAATTCGTGGTACTGATGACAAAA-3' and 5'-AAATTTGAATTCTCATTCTCTCACCTCCAG-3' containing translation initiation and termination codons (underlined), respectively. After digested with *EcoR*I, the PCR product was introduced into the *E. coli* expression vector, pRSET-B, to produce pRBTmCelA.

Thereafter PCR was carried out on a total volume of 50 μL containing template DNA (100 ng), dNTPs (0.2 mM), each primer (100 pmol), $MgCl_2$ (1.5 mM), Tris-HCl (10 mM, pH 8.3), and Taq DNA polymerase (2.5 U). PCR was carried out as follows: 3 min at 94 °C: followed by 30 cycles of 1 min at 94 °C, 1 min at 52 °C, 2 min at 72 °C, and extension at 72 °C for 5 min.

Overexpression of recombinant TmCelA

E. coli BL21 was transformed with pRBTmCelA and induced by adding IPTG (0.7 mM) at 37 °C for 4 h. After centrifugation, the *E. coli* BL21 cell was resuspended in 4 mL of lysis buffer (10 mM imidazole, 50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl) and the sonication was carried out for 4 min on ice. The total crude extract including the recombinant TmCelA was purified by Ni^{2+}-NTA-agarose column chromatography and eluted with a 10-250 mM imidazole gradient. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to analyze protein samples [9]. Bradford method were used to determine the purified recombinant TmCelA protein concentration using BSA as a standard [10].

Enzyme assay of recombinant TmCelA

Cellulose-degrading activity of recombinant TmCelA was assayed in a final reaction volume of 300 μL containing 1.0% carboxy-methylcellulose, 2 μL of purified recombinant TmCelA, and 50 mM McIlvaine buffer (pH 5.0). After incubation at 90 °C for 30 min, DNS method was used to analyze the reducing sugars released from carboxymethylcellulose by recombinant TmCelA.

Determination of biochemical properties of recombinant TmCelA

To determine the optimum temperature of recombinant TmCelA for cellulose-degrading activity, the reaction mixtures were incubated in a series of temperatures ranging from 80 to 100 °C.

To determine the optimum pH of recombinant TmCelA for cellulose-degrading activity, the reaction was carried out in a series of 50 mM McIlvaine and sodium phosphate buffers with pH ranging between 2.5-6.0, and 6.0-8.0, respectively.

For determination of the temperature effect on the cellulose-degrading activity of recombinant TmCelA, recombinant TmCelA was incubated at 80, 85, 90, 95, and 100 °C. The recombinant TmCelA was taken every 30 min over a 3 h period, and the cellulose-degrading activity was determined.

Results and Discussion

Construction and overexpression of pBRTmCelA

To isolate the thermostable cellulose A (TmCelA) gene from *T. maritima*, PCR was performed with TmCelA-specific primers designed in accordance with the genome analysis of *T. maritima*.

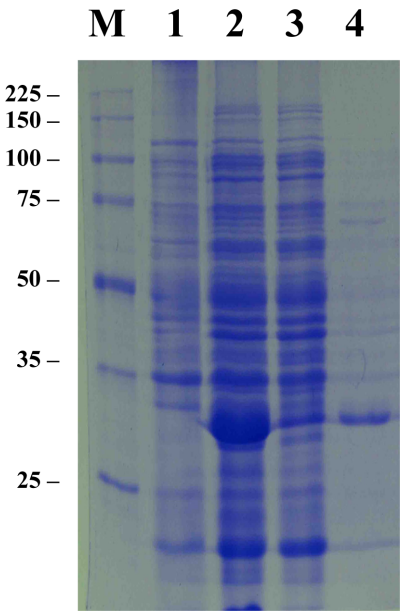

Fig. 1 Expression of recombinant TmCelA in *E. coli.* The open reading frame of the TmCelA gene was introduced into the pRSET expression vector and was expressed in *E. coli.* Protein extracts prepared from induced *E. coli* were analyzed by 10% SDS-PAGE and stained with Coomassie Blue. Lane M: Molecular weight marker. Lane 1: total extract of *E. coli.* harboring pRSET plasmid only. Lane 2: total extract of *E. coli* harboring pRBTmCelA. Lane 3: soluble fraction of *E. coli* extract harboring pRBTmCelA. Lane 4: purified recombinant TmCelA.

A candidate gene for cellulose hydrolysis has previously been studied in the *T. maritima* genome [8]. The ORF of TmCelA gene was 774 bp long and encoded 257 amino acid residues (molecular weight 29,732 Da). The PCR was carried out to amplify TmCelA gene and the amplified product of TmCelA gene was treated with *EcoR*I and inserted into the *E. coli* expression vector, pRSET-B, to make pRBTmCelA.

To characterize the biochemical properties and enzymatic activity of recombinant TmCelA, pRBTmCelA was expressed in *E. coli* BL21, and the overexpressed TmCelA was analyzed by discontinuous SDS-PAGE. Recombinant TmCelA was expressed in *E. coli* BL21, and the overexpressed TmCelA was purified by Ni^{2+}-NTA-agarose column chromatography (Fig. 1). The molecular weight of recombinant TmCelA including the 6-His domain derived from the pRSET-B plasmid was approximately 31,000 Da, which is in accordance with the molecular weight value calculated from the amino acid sequence.

The cellulase activity of recombinant TmCelA was determined in a reaction mixture including 2 μL of purified recombinant TmCelA, 1.0% carboxymethylcellulose, and 50 mM McIlvaine buffer (pH 5.0). The reducing sugars released from carboxy-methylcellulose by recombinant TmCelA were detected by DNS method, which revealed that the recombinant TmCelA had cellulose degradation activity.

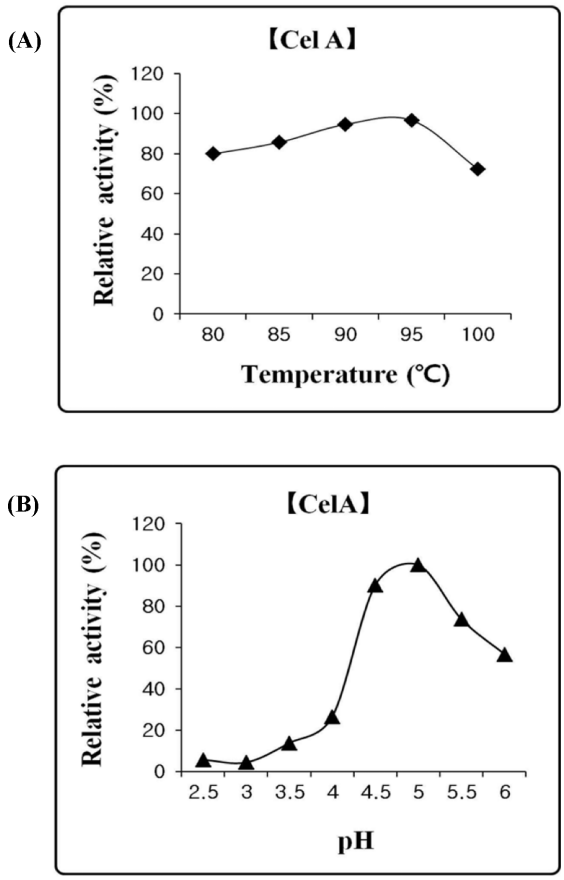

Fig. 2 Relative activities depending on temperature and pH of recombinant TmCelA. (A) Optimum temperature of recombinant TmCelA. A 1.0% of carboxymethylcellulose was incubated for 30 min with 2 μL of purified recombinant TmCelA at various temperatures in a final volume of 300 μL of a reaction mixture containing 50 mM McIlvaine buffer (pH 5.0). (B) Optimum pH of recombinant TmCelA. For the pH test, 50 mM McIlvaine buffer (pH 2.5-6.0) and sodium phosphate buffer (pH 6.0-8.0) were used. Carboxymethylcellulose (1.0%) was incubated at 90 °C for 30 min with 2 μL of purified recombinant TmCelA at various pHs in a final volume of 300 μL. The data represent the mean of three independent experiments

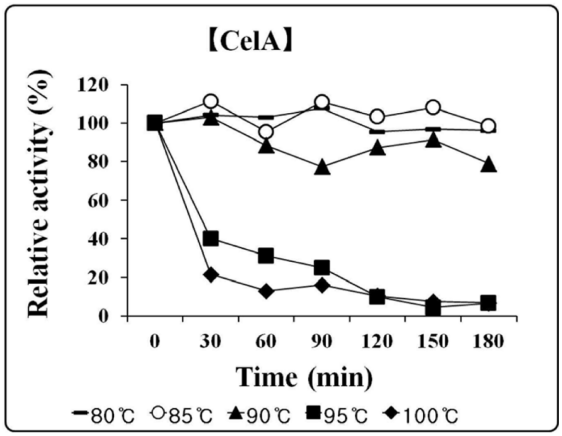

Fig. 3 Thermostability of recombinant TmCelA. Recombinant TmCelA was incubated at 80, 85, 90, 95, and 100 °C. The enzyme was extracted every 30 min over a 3 h period, and the residual activity was determined. The data represent the mean of three independent experiments

Biochemical properties of recombinant TmCelA

Figure 2 showed the optimum temperature of recombinant TmCelA (Fig. 2A) and optimum pH of recombinant TmCelA (Fig. 2B). To determine the optimum temperature of recombinant TmCelA for cellulose-degrading activity, the reaction mixtures were incubated in a series of water baths at temperatures ranging from 80 to 100 °C. The highest activity of the recombinant TmCelA was at around 90-95 °C, and recombinant TmCelA retained more than 65% of its activity up to 100 °C (Fig. 2A).

To determine the optimum pH of recombinant TmCelA for cellulose-degrading activity, the reaction was carried out in a series of 50 mM McIlvaine and sodium phosphate buffers with pH ranging between 2.5-6.0, and 6.0-8.0, respectively. The highest activity of the recombinant TmCelA was at approximately pH 5.0, and retained more than 55% of its activity up to pH 6.0 (Fig. 2B).

Thermostability of recombinant TmCelA

The thermostability of recombinant TmCelA is shown in Fig. 3. For determination of the effect of temperature on the cellulose-degrading activity of recombinant TmCelA, recombinant TmCelA was incubated at 80 to 100 °C. The enzyme was taken every 30 min over a 3 h period, and the residual cellulose-degrading activity was determined. The recombinant TmCelA was stable over 3 h at temperature up to 85 °C and had over 80% of cellulose-degrading activity after 3 h at 90 °C; however, the cellulose-degrading activity decreased after 30 min at 95 °C (Fig. 3).

The TmCelA gene from *T. maritima* was amplified by PCR, and expressed in *E. coli* through recombination into the *E. coli* expression vector, pRSET-B. There were extra amino acids at the N-terminal region of the recombinant TmCelA, including polylinker site , T7 gene 10 leader, the 6-His region derived from the pRSET plasmid. The extra amino acids might affect the biochemical properties of recombinant TmCelA. Recombinant TmCelA showed the highest activity at around 90-95 °C, and at approximately pH 5.0. These results were comparable to those of previous studies reporting that the optimum temperature was approximately 90 °C and the pH was between 4.5-6.5 [4]. The recombinant TmCelA retained over 80% of its activity after 3 h at 90 °C, but decreased after 30 min at 95 °C. These results are also comparable to those of previous studies [4].

Owing to its thermostability, recombinant TmCelA has very useful potential for industrial-scale applications of biomasss refining and degradation. Studies on industrial applications and other biochemical properties of recombinant TmCelA are underway.

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