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Identification and characterization of a new acid-stable endoglucanase from a metagenomic library

La Xiang [a], Aiying Li [c], Chaoguang Tian [b], Yuling Zhou [a], Guimin Zhang [a,*], Yanhe Ma [b]

[a] The Collaborative Innovation Center of Bio-resource Green Transformation, The College of Life Sciences, Hubei University, Wuhan 430062, China
[b] Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China
[c] The College of Life Sciences, Central China Normal University, Wuhan 430079, China

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ABSTRACT

A new endoglucanase gene *cel124* was cloned from a metagenomic library and expressed in *Escherichia coli*. Catalytic triad analysis showed that the catalytic triad sites were different from the known endoglucanases. Cel124, a 34 kDa protein, exhibited a specific activity (29.08 U mg^{-1}) toward 1% of sodium carboxymethyl cellulose and was stable at 50 °C for 30 min. The optimal temperature and pH for its catalytic activity were 50 °C and pH 5.5 respectively. Cel124 could hydrolyze soluble cellulose, but not insoluble cellulose or other polysaccharides. The kinetic parameters (5.63 mg ml^{-1} for K_m and 0.0397 mmol min^{-1} mg^{-1} for V_{max}) were measured. 3 M NaCl in the system could increase its activity by 2 fold. Site-directed mutation and circular dichroism spectra test suggested that the residue (Glu41) was essential for its activity, might be a potential active site. Based on our data, we proposed that Cel124 might represent a new type of endoglucanase.

Introduction

Cellulose is one of the most abundant natural renewable polymers, which can be hydrolyzed by cellulase. Based on their different catalytic mechanisms, cellulases can be divided into endoglucanase (E.C.3.2.1.4), exoglucanase (E.C.3.2.1.91) and β-glucosidase (E.C.3.2.1.21) [1].

Endoglucanase possesses a great potential use in food processing, textile industry, paper industry, animal feed preparation, renewable energy and so on [2]. For example, addition of endoglucanase was found to improve significantly the quality of animal feeding and benefit beer brewing, for that random hydrolyzation of glycosidic bonds in β-glucan by endoglucanase could decrease the viscosity of the β-glucan solution [3]. So it is important to discover various endoglucanases with novel enzymatic properties.

Though endoglucanases are widely distributed in nature and produced by bacteria, fungi and animals, endoglucanases that are applied widely in industry are obtained from fungi, mostly from the genera of *Trichoderma*, *Aspergillus* and *Penicillium* [4]. It is worth noticing that endoglucanases are mostly intracellular enzymes when heterologous expressed in *Escherichia coli* and cannot be secreted into media, which largely restrict their applications in industry [5], and most endoglucanases used in industry today could not meet the requirements of different applications. Thus, screening of new endoglucanases from other sources other than fungi is still attracting researchers' interests.

Since metagenomic approaches are quite promising to search for new endoglucanase genes from uncultured microorganisms, in present study, a new endoglucanase gene *cel124* was cloned from the metagenomic library by plate screening. The highest amino acid sequence identity of Cel124 shared with all previously-characterized cellulases is 47%. Here, the gene *cel124* was heterologously expressed in *E. coli* and the enzymatic properties of recombinant enzyme Cel124 were investigated. Finally, given the sequence analysis, computer modeling of its structure and site-directed mutagenesis data, we proposed Cel124 to represent a new type endoglucanase and may have a different catalytic triad from other known endoglucanases.

Materials and methods

Plasmids, strains, chemicals and media

E. coli XL10-Gold and BL21 (DE3) were obtained from Stratagene as the cloning and expression hosts respectively. The plasmid pET28a from Stratagene was used for construction of the

* Corresponding author. Address: The College of Life Sciences, Hubei University, Wuhan 430062, China. Tel.: +86 27 88661746; fax: +86 27 88663882.
E-mail addresses: zhangguimin@hubu.edu.cn, zhangguimin6@hotmail.com (G. Zhang).

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expression plasmid. The plasmids and strains were preserved on LB plates containing ampicillin (100 μg ml^{-1}) or kanamycin (50 μg ml^{-1}).

Synthesis of PCR primers and DNA sequencing were performed by GenScript Co. Ltd. Restriction enzymes, ExTaq DNA polymerase and T_4 DNA ligase were purchased from TakaRa. All chemicals were of analytical grade and obtained from commercial suppliers.

Cloning of endoglucanase gene cel124 from a metagenomic library

A metagenomic library was constructed with the plasmid pHBM803 by digestion with *Ear*I in our previous job [6]. In this study, 24,000 transformants in this library were transferred to the LB plates supplemented with ampicillin (100 μg ml^{-1}), 1% CMC (sodium carboxymethyl cellulose) and 0.025% trypan blue (Sigma). Among these transformants, only one colony showed haloes indicating the activity of endoglucanase. The plasmid isolated from this colony was named pHBM121. Plasmid pHBM124, a subclone derived from pHBM121, bore a 1.59 kb inserted fragment, was further sequenced.

Bioinformatics analysis of DNA and protein sequences

The sequence of the inserted fragment in the plasmid pHBM124 was released in the GenBank (accession number: AY859541.1). Sequence analysis was performed by BLAST programme. The signal peptide was identified with the SignalP 4.1 sever (http://www.cbs.dtu.dk/services/SignalP). The ExPASy-PROSITE was performed in http://prosite.expasy.org/. The secondary structure of the Cel124 protein was predicted with PredictProtein (https://www.predictprotein.org/).

Construction of an expression plasmid

The resulting 993 bp open reading frame (ORF) region that might encode an endoglucanase on pHBM124 was named as *cel124*. The endoglucanase gene *cel124* without signal peptide encoding sequence was amplified by PCR with specific primers Cel124-FP (5′ CGCGGATCCGCAACTCCGGGCGATCCGGG 3′) and Cel124-RP (5′ CCCAAGCTTTCATAGGCAGCCCGGGCGGC 3′). The PCR product was digested with *Bam*HI and *Hin*dIII, and then cloned into the expression vector pET28a. The recombinant plasmid (named as pET28a-*cel124*) was confirmed by PCR and restriction enzymes digestion.

Expression and purification of recombinant endoglucanase Cel124

The recombinant plasmid pET28a-*cel124* was transformed into *E. coli* BL21 (DE3). Transformants were selected on LB-kanamycin plates containing 1% CMC, 0.025% trypan blue and IPTG to induce the expression of *cel124*. The transformant showing haloes was randomly selected for liquid cultivation in kanamycin-containing LB medium at 37 °C and 200 rpm till the OD_{600} reached 0.6, then added with 0.2 mM IPTG for another 16-h cultivation at 18 °C to induce the expression of *cel124*. Cells were collected by centrifugation, followed by ultrasonication in the 20 mM Tris–HCl buffer (pH 7.9) containing 0.5 M NaCl and 5 mM imidazole. Then, following the manufacturer's instructions, His-Bind® Kits (Novagen) was used to purify the His-tagged Cel124. All active fractions were collected and loaded onto GE HiTrap Q FF anion exchange resin equilibrated with 50 mM sodium phosphate buffer (pH 6.5) and eluted with a gradient concentration of NaCl solution ranging from 0 to 1.0 M. The molecular weight and homogeneity of the protein were evaluated by 12% SDS–PAGE. The concentration of protein was determined with BCA Protein Assay Kit.

Enzymatic assay of recombinant endoglucanase Cel124

The endoglucanase activity was tested by measuring the amount of reducing sugars produced from CMC using the 3, 5-dinitrosalicylic acid (DNS)[1] method and glucose as the standard [7]. The reaction mixture composed of 50 μl of the enzyme and 450 μl of reaction substrate (0.5% of CMC in different pH buffers) were incubated at 50 °C for 10 min. One unit of endoglucanase activity was defined as the amount of enzyme to form 1 μmol reducing groups per minute under the above conditions.

The optimal temperature for the activity of Cel124 was assessed at different temperatures ranging from 20 to 70 °C for 10 min in sodium phosphate buffer (25 mM, pH 7.4). The thermo-stability was determined by pre-incubating the enzyme for 30 min under different temperatures (30 °C, 40 °C, 50 °C, 60 °C), followed by residual activity determination at pH 5.5 and 50 °C.

The effects of pH on the enzyme activity were determined at different pH values by varying the buffer used to dissolve the substrate in the standard assay: 50 mM Na_2HPO_4-citrate (pH 3.0–8.0) and 50 mM Tris–HCl buffer (pH 8.0–9.0). The pH stability of endoglucanase was measured by incubating the Cel124 at different pH values ranging from 3.0 to 9.0 at 4 °C for 16 h. The residual activity was then measured at pH 5.5 and 50 °C.

To determine the kinetic parameters, all reactions were performed at 50 °C for 5 min using soluble CMC as substrate at different concentrations (4.0, 5.0, 6.0, 7.0 and 8.0 mg CMC ml^{-1}), in which the velocity of the enzyme reaction remained linear. The Eadie–Hofstee plots were used to calculate kinetic parameters K_m and V_{max} according to the enzyme reactions.

The effects of various metal ions and organic reagents on the activity of Cel124 were assayed under the optimal conditions (pH 5.5 and 50 °C) at the final concentrations of 5 mM, 0.25% or 0.5% (w/w). The activity of enzyme without any addition of metal ions or organic reagents was set as 100% as the control group. The degree of inhibition or activation of enzymatic activity was described as the percentage of enzyme activity in the control group.

The substrate specificity of the endoglucanase Cel124 was determined at 50 °C with CMC, konjac powder, chitosan, pectin, xylan, starch, dextran T500, methyl cellulose, cellulose powder and cellulose microcrystalline at the concentration of 0.5% (w/v) in 50 mM Na_2HPO_4-citrate (pH 5.5) buffer as substrates.

1% CMC dissolved in pure water was hydrolyzed at 50 °C for over 12 h. The hydrolyzates of CMC were qualitatively analyzed by liquid chromatography–mass spectrometry (LC/MS).

Site-directed mutagenesis

Inverse PCR amplification of the entire circular pET28a-*cel124* was performed with mutagenic primers Glu41AlaF (5′ GCACA GCGcGGGCCAGGGCT 3′) and Glu41AlaR (5′ TGGCCCgCGCTGTGCGA GATC 3′). The PCR product was purified with BioSpin Gel Extraction Kit, and then digested with *Dpn*I. The *Dpn*I-treated product was transformed into competent *E. coli* XL10-Gold. A mutated plasmid pET28a-*cel124E41A*, isolated from a randomly-picked-out transformant, was sequenced and further transformed into *E. coli* BL21 (DE3), followed by enzymatic assay as the procedures mentioned above.

Circular dichroism spectra

The mutant E41A and wild type Cel124 protein were purified according to the methods mentioned above and dissolved in the

[1] *Abbreviations used:* DNS, 5-dinitrosalicylic acid; CD, Circular dichroism.