A novel pH-stable, endoglucanase (JqCel5A) isolated from a salt-lake microorganism, *Jonesia quinghaiensis*

Ling Lin\(^a,\ast\), Xiaozhou Liu\(^a\), Yating Zhou\(^a\), Linyan Guan\(^a\), Jiajia He\(^a\), Weiqian Huang\(^b\)

\(^a\) Provincial Key Laboratory of the Conservation and Exploitation Research of Biological Resources, College of Life Sciences, Anhui Normal University, Wuhu, Anhui, People's Republic of China

\(^b\) Guangdong Rongda Bioengineering Company Limited, Qingyuan, Guangdong, People's Republic of China

**Abstract**

**Background:** Endoglucanase, one of three type cellulases, can randomly cleave internal β-1,4-linkages in cellulose polymers. Thus, it could be applied in agricultural and industrial processes. **Results:** A novel endoglucanase gene (*JqCel5A*) was cloned from *Jonesia quinghaiensis* and functionally expressed in *Escherichia coli* Rosetta (DE3). It contained 1722 bp and encoded a 573-residue polypeptide consisting of a catalytic domain of glycoside hydrolase family 5 (GH5) and a type 2 carbohydrate-binding module (CBM2), together with a predicted molecular mass of 61.79 kD. The purified JqCel5A displayed maximum activity at 55°C and pH 7.0, with 21.7 U/mg, 26.19 U/mg and 4.81 U/mg towards the substrate carboxymethyl cellulose, barley glucan and filter paper, respectively. Interestingly, JqCel5A exhibited high pH stability over a broad pH range of pH (3–11), and had good tolerance to a wide variety of deleterious chemicals including heavy metals and detergent. The catalytic mechanism of JqCel5A was also investigated by site mutagenesis and homology-modeling in this study. **Conclusions:** It was believed that these properties might make JqCel5A to be potentially used in the suitable industrial catalytic condition, which has a broad pH fluctuation and/or chemical disturbance.

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1. Introduction

Cellulases, including three types of cellulases, endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21), can completely hydrolyze cellulose to glucose by synergistic action. Thus, they could be applied in agricultural and waste treatment processes, and be employed as an environmentally bioenergy to replace depleting fossil fuels [1].

Currently, as a large volume industrial enzyme worldwide, endoglucanase possesses a great potential application in the textile industry, paper recycling, detergent industry, juice extraction, animal feed additives and renewable energy. However, the stability for specific processes and hydrolysis efficiency of endoglucanase seemed to be usually insufficient and also an important and difficult challenge in industrial process [2,3]. Besides, organic agents and divalent cations, which were used to solubilize the hydrophobic substrates and eliminate microbial contamination, led to a remarkable decrease in enzyme catalysis activity [4]. Therefore, high pH stability and resistance to metal ions and chemical reagents would make it a strong candidate for commercialization of endoglucanase in biofuel and detergent industry [5].

*Jonesia quinghaiensis* is a Gram-positive, non-acid-fast coryneform bacteria, which was isolated from mud of a soda lake in Qinghai, western province of China and grows optimally at pH 7–9 and salt concentration of 2.0–7.5% NaCl [6]. It is known that hypersaline habitat conditions might offer hyperproducers desirable properties meeting industrial demands. We have cloned and sequenced a novel endoglucanase gene (designated as *JqCel5A*) from *J. quinghaiensis*. The gene was heterogeneously expressed in *Escherichia coli* Rosetta (DE3), subsequently the recombinant enzyme was purified and characterized. The properties of organic solvent-tolerance and pH-resistance would make this enzyme’s potentials in industrial applications. Moreover, computer modeling of JqCel5A’s structure and the site-directed mutagenesis were constructed to demonstrate the importance of catalytic residues, Glu193 and Glu334, as providing evidence for double displacement mechanism originally suggested by Koshland [7].

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\(\ast\) Corresponding author.

E-mail address: linling8@mail.ahnu.edu.cn (L. Lin).

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2. Materials and method

2.1. Bacterial strains and plasmids

*J. quinghaiensis* conserved by our laboratory was routinely cultured in Luria-Bertani (LB) broth at 30°C. The genomic DNA was prepared using TIANamp Bacterial DNA Kit (TIANGEN BIOTECH). *E. coli* DH5α and *E. coli* Rosetta (DE3) were used as hosts for cloning and gene expression with pET28a vector (Novagen).

2.2. Gene cloning and site-directed mutagenesis construction

The predicted endoglucanase gene designated as *JqCel5A* was amplified from the genomic DNA of *J. quinghaiensis* DSM 15701 strain by PCR with PrimeSTAR DNA Polymerase (TaKaRa, Japan) and the primers (JQ-F GGAATTC CATATGTGGGGCATCGTGCTCG; JQ-R: CCGCTCG AGTCCGTGTGAGCCTAGTGTCG; The NdeI and XhoI sites are underlined), which designed using nucleotide sequence (GenBank accession no. NZ_AUHN01000006). PCR program was preformed as follow: 5 min 94°C followed by 30 cycles of 30 s 94°C, 30 s 56°C, 110 s 72°C, and finally 8 min 72°C. The obtained PCR products were purified with the AxyPrep DNA purification kit (Axygen) and cloned into the expression vector, pET-28a, which was further transformed into the competent cells of *E. coli* DH5α and submitted to Genscript (Nanjing, China) for sequencing. Subsequently, the recombinant plasmid was transformed into *E. coli* Rosetta (DE3) host strain for gene expression.

Point-mutant genes were prepared by site-directed mutagenesis using the overlap extension PCR method with some modification [8]. Initial PCRs, performed with mutagenic primers (E193A-F CCGTACCT AAAAAGTGGCAGCCAGTTCG; E193A-R GCCATGGGGCTGAGCA AATTATTAGCTACCG; E334A-F CCCCCCTCATTGGCCAGTTGGCAGTT CCTCG; E334A-R CGAGGACCCCGAGGAGTTGACAGAGGGG; the mutated sites are underlined), generated overlapping gene segments that were then used as template DNA for a subsequent PCR to create a full-length product. All mutant genes were inserted into the expression vector pET28a (Novagen). The constructed plasmids were introduced into *E. coli* Rosetta (DE3) for recombinant protein expression.

2.3. Protein expression and purification

Expression and purification of *JqCel5A* and its variants E193A and E334A were carried out following a method described previously by the protocol of WorkBeads™ 40Ni (TIANGEN) with slight modification. Respectively, *E. coli* Rosetta (DE3) cells harboring wild and mutant genes were grown at 20°C to an OD_{600} of 2.0 in LB medium containing 20 μg/mL kanamycin. Subsequently, IPTG was added to a final concentration of 0.1 mM. After 10 h induction at 20°C, cells were harvested and resuspended in lysis buffer (20 mM NaH₂PO₄, 0.5 M NaCl, 5 mM MgCl₂, 50 mM imidazole) and sonicated. The samples were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was loaded onto a Ni²⁺-NTA column. The column was washed with wash buffer (20 mM NaH₂PO₄, 500 mM NaCl, 0.5 M imidazole), and the recombinant protein was eluted using elution buffer (20 mM NaH₂PO₄, 500 mM NaCl, 10 mM imidazole). Fractions containing target proteins were identified by SDS-PAGE and subsequently dialyzed against lysis buffer before loading to WorkBeads™ 40Ni columns. The samples were eluted with elution buffer (20 mM NaH₂PO₄, 500 mM NaCl, 100 mM imidazole) and dialyzed against lysis buffer before storage at -20°C.

Fig. 1. Multiple sequence alignment between glycoside hydrolase domain of *JqCel5A* and similar family 5 endoglucanases. The multiple sequence alignment was performed using ClustalX program and ESPript 3.0. Framed areas indicate high amino acid similarity, in which the catalytic residues, Glu193 and Glu334, are marked with asterisks. The multiple endoglucanase sequences, Endocellulase (gi 37441445), Endoglucanase V (gi 74627299), Cellulase 3 (gi 13959390), Endoglucanase (gi 74627298) and Cellulase K (gi 15825907) belonging to GH5 were from *P. horikoshii*, *A. aculeatus*, *A. niger*, *M. phaseolina*, *H. insolens*, *R. solanacearum*, Robillarda sp. V-20, *M. phaeospora* and *Bacillus* sp. KSM-635, respectively.
2.4. Characterization of enzyme activity

The standard enzyme activity of JqCel5A, E193A and E334A was determined using 3, 5-Dinitrosalicylic acid (DNS) method. Fifty microliters of diluted enzymes was applied into 100 μl reaction mixture containing 0.5% CMC and 0.2 M Na2HPO4-NaH2PO4 buffer (pH 7.0). After incubation at 55°C for 30 min, 100 μl. DNS reagent was added, and the reaction mixture was further heated in a boiling water bath for 5 min. Later, 800 μl H2O was added into the mixture and the absorbance of the mixture was measured at 540 nm. The activity of the enzyme was further determined by using the glucose as the standard. One activity unit was defined as the amount of enzyme capable of releasing 1 μmol of reducing sugars per minute at 55°C [10].

The optimal working pH of JqCel5A was determined by evaluating their maximal activities in the buffer conditions ranging from pH 3.0–11.0 (0.2 M Na2HPO4-citric acid (pH 3.0–8.0) and 0.05 M Glycine-NaOH (pH 9.0–11.0)). The optimal working temperature was evaluated at the temperatures ranging from 10°C to 90°C. Thermal stability of JqCel5A was determined by assessing residual activities after incubation at the temperature from 20°C to 90°C for 1 h. For pH stability, relative activity was determined after the enzyme had been incubated with different pH buffers (pH 3–11) at 4°C for 1 h.

For the determination of the \( K_m \) and \( V_{\text{max}} \) values, the enzymatic activities towards various concentrations (ranging from 0.25–10 mg/mL) of CMC were measured. The data was fitted into the Michaelis–Menten equation using GraphPad Prism software (v. 5.1, GraphPad Software, Inc.) and the \( K_m \) and \( V_{\text{max}} \) values were calculated.

To investigate the substrate specificity of the enzymes, the activities were determined under the optimal conditions by replacing CMC with following substrates: 0.5% barley glucan (Sigma), 1% avicel (Sigma), 1% filter paper (Whatman) and 1% laminarin (Sigma) [11].

2.5. Molecular modeling of protein

Structural modeling of JqCel5A was performed by “Swiss-model Workspace” [12,13] using the reported crystal structure of EGPh from Pyrococcus horikoshii (Protein Data Bank code 3QHO) and type 2 carbohydrate-binding module from Cellulomonas fimii (Protein Data Bank code 1EXG) as the templates [14,15]. The hypothetical cartoon diagrams were predicted and illustrated by PyMol v 1.2.1.

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**Table 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JqCel5A</strong></td>
<td><strong>E193A</strong></td>
</tr>
<tr>
<td>CMC</td>
<td>21.72 ± 1.32</td>
</tr>
<tr>
<td>Barley glucan</td>
<td>26.19 ± 1.36</td>
</tr>
<tr>
<td>Filter paper</td>
<td>4.81 ± 0.21</td>
</tr>
<tr>
<td>Avicel</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Laminarin</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Assay was performed at the optimum condition.
* Standard deviations were shown behind the specific activities.
Fig. 4. The effects of pH and temperature on the activity and stability of JqCel5A. (a) Effect of temperature on enzyme activity. The reaction was carried out in 0.2 M Na₂HPO₄-NaH₂PO₄ buffer (pH 7.0) containing 0.5% CMC at indicated temperatures. The maximum activity observed was taken as 100%. (b) Effect of temperature on enzyme stability. Enzymes were incubated for 1 h at indicated temperatures. Then samples were measured under the standard conditions. The activity without treatment was taken as 100%. (c) Effect of pH on enzyme activity. Enzyme activity was measured at 55°C in the different buffers with pH ranging from 3 to 11. The maximum activity observed was taken as 100%. (d) Effect of pH on enzyme stability. Enzymes were incubated at 4°C for 1 h at indicated pHs in various buffers. All the reactions were measured under the same condition of CMC activity assay. The activity without treatment was taken as 100%. Error bars represent the SD of the mean calculated for three replicates.

Fig. 5. Effects of metal ions and reagents on the activity of JqCel5A. Error bars represent the SD of the mean calculated for three replicates.
Table 1, among the different polysaccharide substrates, the JqCel5A measured using the DNS method as described above. As shown in SDS-PAGE (Fig. 3), which showed the same target protein bands of and molecular weight of the proteins was analyzed and observed with 6His-tag and puri
coding proteins were produced in E. coli.

3.3. Expression and characterization of the enzymes

In order to characterize the JqCel5A and mutant enzymes, the gene coding proteins were produced in E. coli Rosetta with H-terminal 6His-tag and purified using an affinity chromatography. The purity and molecular weight of the proteins was analyzed and observed with SDS-PAGE (Fig. 3), which showed the same target protein bands of 62 kDa, quite close to the predicted molecular weight (61.79 kDa).

The activities of the purified JqCel5A, E193A and E334A were measured using the DNS method as described above. As shown in Table 1, among the different polysaccharide substrates, the JqCel5A exhibited the hydrolysis activity towards CMC, barley glucan and filter paper, and did not acquire activity against avicel and laminarin. In contrast to the purified wild type enzyme, the purified mutants E193A and E334A showed no detected activities towards all of the substrates. This observation was in agreement with the previous results as described in the study of halo-forming activities.

In order to calculate $K_m$ and $V_{max}$ of JqCel5A, the activities towards different concentrations of CMC substrates were measured and the data was plotted according to the Michaelis–Menten equations. The $K_m$ value of JqCel5A was 8.699 mg/mL and the calculated $V_{max}$ was 0.0642 mmol/min·mg. Comparison of CelB with the $K_m$ values of 6.6 mg/mL [18]; Cel124 with $K_m$ and $V_{max}$ values of 5.63 mg/mL and 0.0397 mmol/min·mg [19], it indicated that JqCel5A had medium affinity and catalytic efficiency for CMC substrate.

3.4. Effects of temperature and pH on the activity of JqCel5A

As shown in Fig. 4, the JqCel5A was optimally active at 55 to 65°C, and gained less than 60% relative activity at temperature below 40°C, rapidly decreased activity above 70°C, and completely lost activity at 80°C. These observations are compatible with the reports that endoglucanases possess optimum temperatures of 40–70°C such as from Chrysosporium lucknowense, A. niger and Bacillus subtilis [20,21,22].

Thermal stability assay shows that JqCel5A was very stable below 50°C, and retained nearly 80% of its initial activity after 1 h incubation at 60°C for 1 h. However, the activity decreased rapidly to less than 5% after incubation at 70°C. Similar to this, some other endoglucanases also showed stability under the same temperatures, examples include endocellulase from Chaetomium thermophilum, EG from Trichoderma reesel and cellulose from A. niger were thermal stable up to 60°C, but loses its activity after treatment at 70°C [23,24,25].

Maximal activity of the purified enzyme was observed at pH 7.0, showing more than 50% relative at pH range of 4–9, which satisfies the fundamental conditions of the application in food, paper and textile industries. Notably, it was very stable over a wide range of pH, maintaining over 95% activity after incubation in at the pH range of 3–11 for 1 h. These properties were similar to some previously reported endoglucanases, such as EG27 from Ampullaria crossean and Cel8H from Halomonas sp. both exhibited stability at acidic and alkaline condition, showing nearly full activity after incubation at pH range of 4–11 [5,26]. Moreover, this high pH stability in both acidic and alkaline region would make the neutral JqCel5A a strong candidate for production of bioethanol, detergents, fabrics and feed additives [27].
3.5. Effects of metal ions and reagents on enzyme activity

As shown in Fig. 5, the relative activity of the JqCel5A in the presence of different metal ions and chemical reagents was determined. Rb⁺, K⁺ and Co²⁺ at 1 mM concentration slightly stimulated enzyme activity, while Ca²⁺, Mn²⁺ and β-mercaptoethanol reduced the activity, and Cu²⁺ could bind the thiol groups and interact with imidazole or carboxyl groups of amino acids resulting in the dramatically decreased enzyme activity. It must be noted that the anionic surfactant SDS and the chelating agent EDTA showed nearly no effect on the activity of the enzyme. This resistance to the denaturant of protein suggests JqCel5A as a potential candidate for industrial detergents, in which divalent cations, detergents and chelating agent were commonly used [28].

3.6. Protein modeling

In order to more clearly understand the GH5 and CBM2 modules of JqCel5A at the tertiary levels, structural characteristics of the enzyme was predicted and determined by SWISS-MODEL workspace and illustrated using PyMOL v1.2.1, based on the reported crystal structure of EGPh from P. horikoshii [14]. As shown in Fig. 6, a typical clan GHA structure with a (β/α)₈ and a β-sandwich fashion are finally predicted by homology [15,29].


