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Full Length Article

A Novel Neutral Endoglucanase from *Streptomyces* sp. H31 with Wide-pH-range Stability

Wei-Zhao Chen[1,2], Xiao-Yang Ou[2], Sen-Lin Liu[2*] and Miao Xing[1*]
[1]*College of Life Science, South China Agricultural University, Guangzhou 510642, P.R. China*
[2]*Shenzhen Key Laboratory for Microbial Gene Engineering, College of Life Sciences and Oceanography, Shenzhen University, Shenzhen 518060, P.R. China*
[*]For correspondence: xingmiao@scau.edu.cn

Abstract

An endoglucanase-producing strain *Streptomyces* sp. H31 was isolated from mangrove soil in Shenzhen, P.R.China, and the endoglucanase gene egH31 was cloned. The open reading frame (ORF) of this gene is 762bp and it encodes 253 amino acid residues, which showed the highest similarity (84%) with the endoglucanase from *Streptomyces davawensis*. The endoglucanase gene egH31 was successfully expressed in *E. coli*. We purified the recombinant EgH31 to homogeneity by affinity chromatography. Molecular mass of the recombinant EgH31 is about 31 kDa. The maximum activity of EG-H31 was determined at pH 7.0 and 45°C and shown high stability within a wide pH range (3.0–11.0). EgH31 had good resistibility to SDS, EDTA, EGTA and most of metal ions examined. Most interestingly, EgH31 was stable in commercial laundry detergents, indicating that this enzyme is useful in textile and detergent industry. © 2017 Friends Science Publishers

Keywords: Neutral endoglucanase; *Streptomyces* sp. H31; Wide-pH-range; Resistibility

Introduction

Cellulase-producing microorganisms have a wide distribution in the natural environment, and include fungi, bacteria and actinomycetes (Horikoshi *et al*., 1984; Ma *et al.*, 2015). During this past period, people pay close attention to cellulases produced by fungi, for their potential application in biofuel production (Gong *et al*., 1999). By comparison, the study of cellulases from bacterial and actinomyces was developed slowly (Sumitomo *et al*., 1992) because bacteria and actinomycetes mainly produce alkaline or neutral endoglucanases (Hakamada *et al*., 2002).

Horikoshi *et al*. (1984) discovered the alkaline cellulase (endoglucanase) for the first time in 1984 (Ito, 1997). During the last decade, the alkaline endoglucanase has been extensively researched for the revolutionary application prospect in detergent industry. The industrial detergent base on alkaline cellulases are now purchasable in Japan (Horikoshi, 1999).

In the most recent period, neutral endoglucanases, with optimum pH values is between 6.0–8.0, were found to be suitable for biology stonewashing and biopolishing during the wet processing stages of textile industry. (Godfrey, 1996; Galante *et al*., 1998; Bhat, 2000; Pazarlioglu *et al*., 2005). Neutral endoglucanases were also beneficial for enzymatic deinking of various pulps wastes (Gübitz *et al*., 1998).

The biological deinking treatment can be improved with the properties of recycling fibers from waste paper, such as fiber brightness, strength properties, higher pulp cleanliness, *et al.* (Gübitz *et al*., 1998; Gaur and Tiwari, 2015).

Because of many advantages, the neutral endoglucanases hold great commercial value in the increasingly fierce market competition. The demand of neutral endoglucanases growth rapidly in the past few years (Ding *et al*., 2002; Bansal *et al*., 2014), and yet more neutral endoglucanases studied would be crucial.

Recently, we cloned a neutral endoglucanase gene from *Streptomyces* sp. H31, which was isolated from mangrove soil in Shenzhen, P. R. China. Unlike many fungal endoglucanases with only acidic pH stability (Dong *et al*., 2010), this enzyme is unusual in that this neutral enzyme had a wide-pH-range stability under both acidic and alkaline conditions, contributing to its potential value in many industrial processes (Horikoshi, 1996; Oksanen *et al*., 2000). So far, neutral endoglucanase with high stability under both extreme alkaline and acidic conditions has never been reported. In this paper, we give a description of the cloning, expression and characterization of this novel neutral endoglucanase with high stability under both extreme acidic and alkaline conditions.

Materials and Methods

Materials

A Ni-Sepharose 6 FF column was purchased from GE

Healthcare(General Electric Co., USA).We used vector pET-28a (+) and *E. coli* BL21 (DE3) for the expression of endoglucanase. We used vector pMD19-T and *E. coli* Top10F´for plasmid construction and propagation. Sodium salt of Caboxy Methyl Cellulose (CMCNa) and other reagents were of analytical grade.

Isolation of *Streptomyces* Strain

Soil samples were gathered from mangrove soil in Shenzhen, P. R. China. The Method and assay of enzyme activity was used as described previously (Liu *et al*., 2008). *Streptomyces* sp. H31 was conserved in China Center for Type Culture Collection (CCTCC No. M2015003) as a parent strain.

Cloning and Plasmid Construction

Extraction protocols of Genomic DNA and plasmid was used as described (Sambrook *et al*., 1989). The Primers used in this research were outlined in Table 1. The primes 27F and 1492R were used for the amplification of 16S rDNA. The conserved sequence of the endoglucanase gene egh31 was amplified by using primers E-H31-C-F and E-H31-C-R, which were based on the most highly conserved amino acid residues WDVVNE and QAGFEPW, through comparison of the amino acid sequence of family 10 endoglucanases from *Streptomyces* in the DNAssist 2.0 software. The upstream and downstream sequences were amplified by TAIL-PCR as described (Liu and Whittier, 1995) using the primers in Table 1. The coding sequence of egH31 was ligated into the vector pET-28a and then transformed into *E. coli* BL21 (DE3). The positive recombinants were picked by Congo red method as described (Liu *et al*., 2011).

Expression of the Recombinant EgH31

The selected recombinant was induced by IPTG and the cultures were harvested by centrifugation. The bacteria deposit was suspended in 20 mL McIlvaine buffer (20 mM, pH 7.9) and sonicated for 20 min with an ultrasonicator (Model SCIENTZ-IId, Ningbo Scientz Biotechnology Co., LTD). The cell-free extract was used for further purification. The recombinant enzyme (EgH31) was tested for hydrolysis of 1% CMC-Na (McIlvaine buffer, 20 mM, pH 7.0) at 40°C for 30 min.

Purification of the Recombinant Enzyme

The cell-free extract as prepared above was dialyzed and then was loaded onto a Ni-Sepharose 6 FF column pre-equilibrated with 20 mM McIlvaine buffer (pH 8.0). A linear gradient (i.e., 0.05, 0.1, 0.3 and 0.5 M) of imidazole containing 0.3 M NaCl (flow rate: 2.5mL/min) was used for the elution of adsorbed proteins. Fraction with endoglucanase activity were collected and dialyzed. The Recombinant EgH31 was subsequently examined by SDS-PAGE (Sambrook *et al*., 1989).

Biochemical Characterization of EgH31

Carboxymethylcellulose (CMC) was used as the substrate for the assay of the endoglucanase activity in different pH values and temperatures. One unit of the enzyme activity was defined as the amount of enzyme that produced 1mg product per hour. The Method was used as described previously (Liu *et al*., 2008).

Results

Isolation of the Endoglucanase-producing Strain

The alkaliphilic strain H31 was isolated from mangrove soil. The sequence of the 16S rDNA (1490 bp) showed the highest homology (99%) with that of *Streptomyces halotolerans* strain YIM 90017 (GenBank accession no. AY376166). This isolated strain H31, which was Gram-positive with branching and filamentous forms, was identified as *Streptomyces* and designated as *Streptomyces* sp. H31.

Cloning and Sequence Analysis of Gene egH31

The partial egH31 gene (569 bp) was generated by a touch-down PCR, using primer F and R (Table 1). DNA sequence assays showed that this fragment has 89% similarity with the endo-1, 4-beta-glucanase gene *celA1* from *Streptomyces davawensis* JCM 4913 (Sequence ID: HE971709.1), indicating that it belongs to a partial endo-1, 4-beta-glucanase gene. Based on the partial identified sequence, the rest of the 5′ and 3′ regions were obtained via TAIL-PCR using the specific primers E-H31-Up-sp 1, 2, 3 and E-H31-Down-sp 1, 2, 3, as well as random primers. Two fragments of the rest 5′ and 3′regions were isolated and sequenced which were 425 and 280 bp, respectively.

The complete egH31 gene (762bp) has an open reading frame (ORF) was successfully cloned by PCR from the total DNA extracted from *Streptomyces* sp. H31, which encoded 253 amino acid residues, including a putative 11-amino-acid signal peptide composed of many hydrophobic amino acids.

Expression and Purification of Recombinant Endoglucanases EgH31

The vector pET-28a-egh31 was introduced into *E. coli* BL21 (DE3) and expressed. Under optimum conditions, the endoglucanase activity of EgH31 was 4.7 U/mL. The purification of recombinant EgH31 was based on affinity chromatography using a Ni-Sepharose column (General Electric Co., USA). SDS-PAGE indicated that the enzyme was purified to electrophoretic homogeneity as a single

band (including extra 36 amino acids of plasmid expression) (Fig. 1). The recombinant endoglucanase in apparent molecular mass of 31 kDa was identical with theoretical value.

Characteristic of the Recombinant EgH31

Effect of pH on the Activity of Endoglucanase EgH31: The optimal pH of EgH31 was determined at a broad pH values ranging from 2.0 to 12.0. Fig. 2a illustrates that the optimal CMCase activity of EgH31 was approximately pH 7.0, and remains more than 70% of peak activity between pH 6.0 and 9.0.

Effect of pH on the stability of Endoglucanase EgH31: The purified EgH31 was incubated in various pH values at 37°C for 60 min. As demonstrated in Fig. 2b, the recombinant EgH31was stable over a wide pH range from 3.0 to 11.0, indicating good stability under both extreme alkaline and acidic conditions.

Effect of temperature on the activity of Endoglucanase EgH31: The optimal temperature on enzyme activity of the purified EgH31 was measured from 30 to 80°C. As demonstrated in Fig. 3a, the endoglucanase EgH31 displayed maximum activity at around 60°C.

Effect of temperature on the stability of Endoglucanase EgH31: The thermal stability of the purified EgH31 was determined by incubating for 20 min at 30–80°C. As demonstrated in Fig. 3b, The EgH31 retained more than 98% of its initial activity at 50°C, indicating the endoglucanase EgH31 was stable up to 50°C.

The effects of different metal ions and chemical reagents: The purified EgH31 was determined at 10 mM different metal ions (e.g., Ni^{2+}, Ca^{2+} and Zn^{2+}, Fe^{3+}) at 30°C for 20 min in McIlvaine buffer (pH 7.0). As shown in Table 2, Ni^{2+}, Ca^{2+} and Zn^{2+} were the three most effective activators and significantly enhanced EgH31 activity by about 47.1%, 33.9% and 32.5%, respectively. Fe^{3+} strongly inhibited the enzyme activity of EgH31 by about 76.9%. All other metal ions tested in this study had little effect on the enzyme activity of EgH31.

To examine the effect of reagents on the activity of EgH31, 0.01% SDS, 0.05% SDS, 0.2% EDTA, 0.5% EDTA and 0.2% (w/v) commercial laundry detergents were used. As Table 2, EgH31 had good resistibility to SDS, EDTA, EGTA and commercial laundry detergents. Generally, EgH31 showed outstanding stability in wide pH ranging from 3.0 to 11.0 and meanwhile had excellent thermo-stability and resistibility, thus showing market potential in textile and detergent industry.

Discussion

Endoglucanases attract a lot of attention and have been widely used in various industrial application such as textile and detergent industry (Gaur and Tiwari, 2015), which maily catalyzes the hydrolysis of the cellulose by breaking the internal bonds of glucose molecules. Recently, neutral

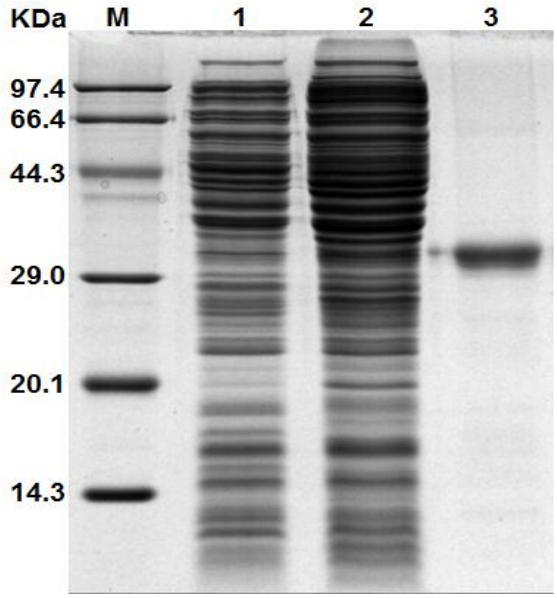

Fig. 1: SDS-PAGE analysis of recombinant EgH31: lane M: Premixed Marker (Low), lane 1: total crude protein from pET-28a-egH31 without IPTG induction, lane 2: total crude protein from pET-28a-egH31 with IPTG induction, lane 3: purified recombinase (EgH31) through Ni-Sepharose columns

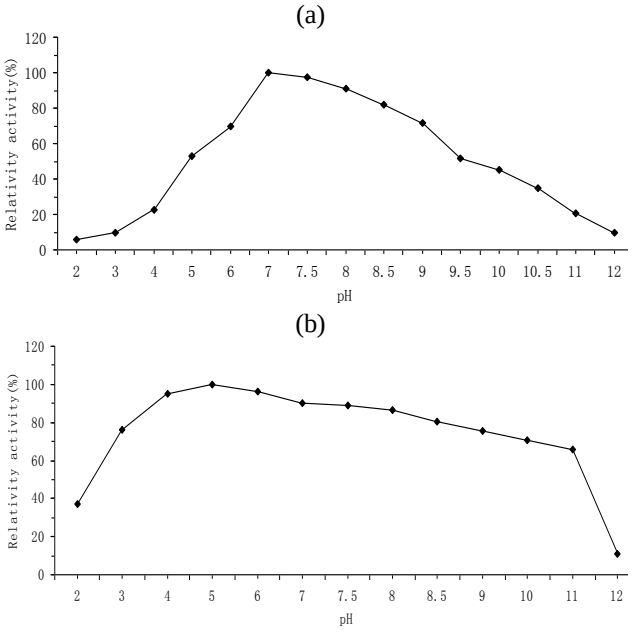

Fig. 2: Effects of pH on the recombinant endoglucanase EgH31

(a) Relative effect of varied pH values. The recombinant EgH31 was incubated at 55°C with pH ranging from 2.0 to 12.0 for 30 min. The maximum value was set as 100%. (b) Relative pH stability of recombinant EgH31. The recombinant EgH31 was incubated at 37°C for 60 min in pH 2.0 to 12.0, and then was assayed in 1% CMC-Na (pH 7.0, 55°C). The maximum value was set as 100%

Table 1: Primers

Primer	sequence (5'----3')	Size (bp)
27F	AGAGTTTGATCCTGGCTCAG	20
1492R	TACGGCTACCTTGTTACGACTT	22
Degenerate primer F	TNCARAAYAAYMGNTGGGG	19
Degenerate primer R	CCANGGYTCRAANCCNGCYTG	21
E-H31-Up-sp1	AGCATCTCCTACGGCTACGTGC	22
E-H31-Up-sp2	ACGGGCAGCAACGGCAC	17
E-H31-Up-sp3	CCATCACGAGCTGGAGCTTCG	21
E-H31-Down-sp1	GCACGTAGCCGTAGGAGATGCT	22
E-H31-Down-sp2	GGCGAACAGTTGGTGTAGTGGC	22
E-H31-Down-sp3	GTGTAGTGGCAGCCGTTGAAGA	22
AD1	NTCGASTWTSGWGTT	15
AD2	NGTCGASWGANAWGAA	16
AD3	WGTGNAGWANCANAGA	16
AD4	TGWGNAGWANCASAGA	16
Endoglucanase F	CCG*gaattc*ATGCTCCTCGCCGCCGCCACTC (*EcoR* I)	31
Endoglucanase R	CCC*aagctt*CTACACCACCTGGCAGGCGGGCG (*Hind* III)	32

endoglucanases, with optimum pH values of 6–8, were found to be well applied in bio-stoning and bio-polishing of denim fabrics industry in a controlled and desired manner, and also successfully applied both in biological de-inking processes and for improving the properties of recycled pulp. Consequently, there has been a rapid growth in demand for neutral endoglucanases, especially those enzymes with particular biochemical properties. Unfortunately, the neutral endoglucanases have been less studied so far.

Neutral endoglucanase gene egH31 was cloned from *Streptomyces* sp. H31 (newly isolated microorganism).The recombinant enzyme EgH31 is a neutral endo-β-1, 4-endoglucanase possessing high activity toward CMC. The amino acid sequence of EgH31 through Blast, Sequence Analysis showed that this enzyme has the highest identity (84%) with the endoglucanase from *Streptomyces davawensis* (Sequence ID: WP_015656886.1), belonging to the Glycoside Hydrolase Family 12. EgH31 has the typical bacterial family-12 cellulose-binding domain (Gly^{26}–Asn^{232}) (pfam01670), with an E value of 2.2e-75. As shown in Fig. 4, the dimensional structure of EgH31 was constructed based on the crystal structure of the endoglucanase from *Streptomyces lividans* endoglucanase CelB2 (PDB: 1NLR (80.26% sequence identity) using SWISS-MODEL homology modeling, demonstrating that EgH31 only contains CD catalytic domain in N-terminal, yet does not contain CBM binding domains in the C-terminal region (Biasini *et al*., 2014).

The value of pH is critical for the stability and activity of endoglucanases, as it impacts the enzymatic active site and thus affects the binding of amino acids (Kupski *et al*., 2014). The pH value thus influences the use of various endoglucanases in industry process. However, most of the presently used endoglucanases, especially fungal endoglucanase, usually displayed their highest activity at acidic condition. Their activities are greatly reduced in case of the pH value above 7.0, limiting the application of those fungal enzymes under neutral and alkaline condition (Wang *et al*., 2014; Zeng *et al*., 2016; Ibrahim *et al*., 2017). Therefore, there has a large market and great demand for the endoglucanases which can be effective at broad pH with high activity. GH12 endoglucanases usually presented low resistance to high pH, for example, cel12B (GH12) from *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) showed a pH optimum of 6.0 (Ibrahim *et al*., 2017), AcCel12B (GH12) from *Acidothermus cellulolyticus 11B* remained less than 10% of max enzyme activity at pH 7.0 (Wang *et al*., 2015). Especially, the neutral endoglucanase EgH31 showed outstanding stability in wide pH ranging from 3.0 to 11.0, and remained more than 70% of peak activity between pH 6.0 and 9.0, while most endoglucanases reported did not functionally work under the wide range of pH (Galante *et al*., 1998; Bhat, 2000; Trivedi *et al*., 2011; Yang *et al*., 2016; Zeng *et al*., 2016).

Table 2: Effects of metal ions and chemical reagents

Metal ions or control	Concentration	Residual activity (%)
Control	-	100.0±0.3
Ni^{2+}	10 mM	147.1±2.1
Ca^{2+}	10 mM	133.9±3.0
Zn^{2+}	10 mM	132.5±5.3
Cu^{2+}	10 mM	113.0±2.2
Co^{2+}	10 mM	108.2±0.3
Li^{+}	10 mM	106.1±1.7
Ag^{+}	10 mM	105.4±2.5
Fe^{3+}	10 mM	23.1±0.3
Mg^{2+}	10 mM	81.0±0.9
Al^{3+}	10 mM	85.6±0.4
Mn^{2+}	10 mM	100±0.6
SDS	0.01%	99.4±0.9
SDS	0.05%	95.9±3.7
EDTA	0.2%	99.2±0.3
EDTA	0.5%	98.0±0.5

Data are expressed as mean ± SD (n = 3) relative to control samples

Neutral endoglucanase EgH31 also had good resistibility to SDS, EDTA, EGTA and commercial laundry detergents. These results indicated that there were significant similarities between this novel enzyme EgH31 and some peculiar endoglucanases that also have resistibility to various metal ions and reagents (Ding *et al.*, 2002). Endoglucanase must retain their activity in laundry detergents if enzymes are to be applied to commercial use in

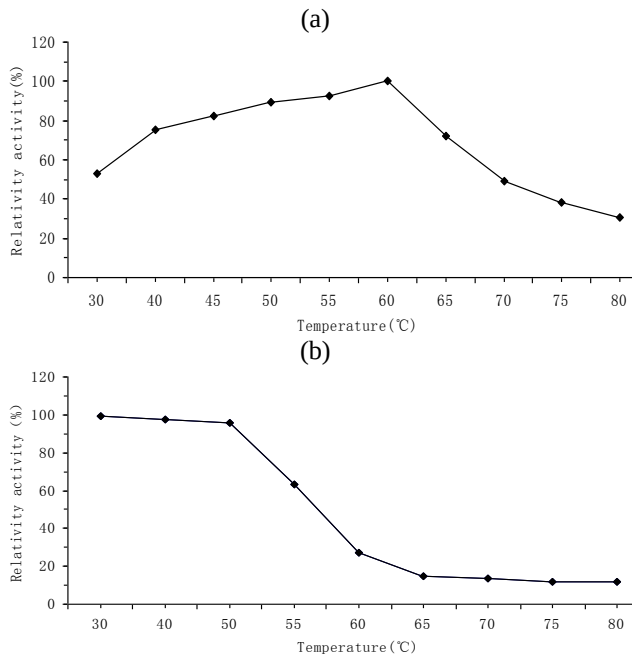

Fig. 3: Effects of temperature on recombinant EgH31
(a) Effect of temperature on recombinant EgH31 activity. The maximum value was set as 100%. (b) Effect of temperature on thermostability. The EgH31 was incubated in 20 mM buffers (pH 7.0) without substrate for 1 h at different temperature. Residual enzyme activities were measured at 55°C for 30 min. The 0 min value was set as 100%

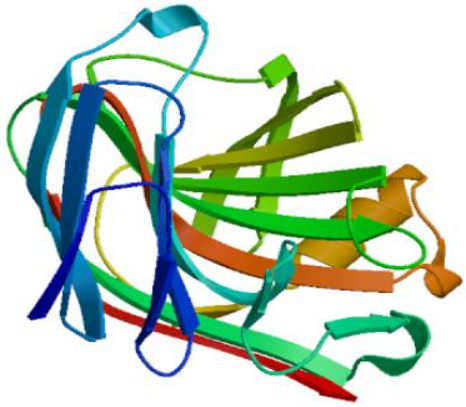

Fig. 4: Homology modeling of EgH31

detergent industry (Hakamada *et al*., 1997). In this study, commercial laundry detergent was added to evaluate the possibility of the application of EgH31, and no significant effect on activity was detected, indicating that this enzyme has potential commercial production prospects.

Conclusion

A novel neutral endoglucanase gene egH31 was cloned from *Streptomyces sp.* H31 and successfully expressed. This novel neutral enzyme EgH31 had a wide range pH stability under both acidic and alkaline conditions (pH 3.0–11.0). The EgH31 also has good resistibility to SDS, EDTA, EGTA and most of metal ions examined, which was stable in commercial detergents.

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