Cloning and Overexpression of Pyrococcus Furiosus Endoglucanase A Gene (eglA) in Escherichia Coli

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Abstract

This study describes the cloning and high-level expression of an endoglucanase A gene (eglA) from a hyperthermophilic archeon Pyrococcus furiosus. An expression plasmid pET-EglA was constructed for the production of recombinant EglA in E. coli BL21 (DE3) under the control of T7lac promoter. Following induction, ~35kDa protein expressed at levels greater than 20% of the total E. coli cellular proteins. The expressed protein, however, was in the form of inclusion bodies with little enzymatic activity, which was solubilized using higher concentration of denaturing agent (8M urea) followed by its refolding to an active state. A 7-8 fold increase in enzyme activity corresponding to 285U/mg specific activity could be achieved after refolding. The refolded EglA, partially purified by heat treatment upto ~92%, is being investigated for applications like hydrolysis of cellulose, a major component of plant biomass. Local, upscale and cheap production of these cellulolytic enzymes can help in reducing the costs of many processes in various industries like poultry and textile.

Key words: Endoglucanase A, Pyrococcus furiosus, overexpression, inclusion bodies, refolding

Introduction

Pyrococcus furiosus is an obligately anaerobic, heterotropic hyperthermophilic archeon that grows optimally at 98-100°C and employs a fermentative type of metabolism utilizing a range of sugars such as cellobiose, laminarin, chitin, maltose, barley glucan and starch as primary carbon and energy sources [1-5]. Various enzymes like *Pfu* DNA polymerases, amylases, laminarases, α-, β-glucosidases, etc. have been isolated from this marine archaebacteria, till to date. These enzymes have attracted considerable research and commercial interest because of their evolutionary significance and potential applications in the biotechnology industry [6,7].

Cellulose, a major component of plant biomass and the most abundant polysaccharide on biosphere [8], is composed of D-glucose units that are linked together via β-1,4-glucosidic linkages to form linear chains. Earlier it was documented that *P. furiosus* is capable of degrading α-linked glucose polymers, such as starch and glycogen, only by the concerted action of alpha glucosidase, α-amylase and pullulanase [9]. But later it was found that *P. furiosus* is also able to grow on β-linked glucose polymer and has ability to hydrolyze laminarin to glucose by a combined action of extracellular endo-β-1,3-glucanase (LamA) and β-glucosidase (CelB). Some other researchers have identified a novel endoglucanase (EglA), capable of degrading β-1, 4 linkages of both cellulose and mixed-linkage β-glucans, in *P. furiosus* [10].

The two endoglucanases, LamA and EglA, have distinctly different amino acid sequences and substrate specificities and therefore, belong to two different enzyme families, i.e., family 16 and 12, respectively [11]. The nucleotide sequence of the EglA encodes a 319 amino acid long protein with a calculated molecular mass of 35.9 kDa and an N-terminal signal peptide of 19 amino acids. Cellulose binding domain, however, is absent in this enzyme.

In the present study, the gene encoding EglA from *P. furiosus* (ATCC 43587) was cloned and expressed in *E. coli* BL-21 (DE3) under the regulation of T7*lac* promoter. Refolding and partial purification of recombinant enzyme has also been described.

Materials and methods

Bacterial strains, DNA, enzymes and kits

E. coli strains DH5α and BL21 (DE3) were used as cloning and expression hosts, respectively. The genomic DNA of *P. furiosus* was obtained from the American Type Culture Collection (ATCC 43587). *Taq* DNA polymerase, T4 DNA ligase and restriction endonucleases were purchased from MBI Fermentas (MD, USA). QIAquick gel extraction and QIAprep miniprep kits, used for DNA extraction and plasmid miniprerparation, respectively were acquired from QIAGEN Inc. (CA, USA). All other chemicals used were of the analytical grade commercially available.

Construction of recombinant pET-EglA plasmid

T7*lac* promoter based pET-22b(+) vector was used for the construction of pET-EglA expression plasmid. A pair of forward (NSSZ-F: 5´GCAG*CATATG*ATATATTTTGTAGAAAAGTATCAT ACCTC-3´) and reverse (NSSZ-R: 5´-GA*GGATCC*TTGATAATTCCCAGGTTGCCGA-3´) primers was designed to amplify the *egl*A gene (GenBank Accession No. AF181032) from the genomic DNA of *P. furiosus*. *Nde*I and *Bam*HI sites were incorporated at 5´-

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termini of the designed primers (shown in italics) to facilitate the directional cloning. The conditions used for PCR were: 95°C for 4 minutes, 30 cycles of [94°C 45 sec., 60°C 45 sec., and 72°C 1 min.], and a final extension of 72°C for 20 min. The amplicon thus obtained was cloned in pTZ57R/T vector by employing dA.dT tailing technique to generate pTZ-EglA, followed by its transformation into *E. coli* DH5α competent cells according to the standard molecular biology protocols [12].

For the construction of pET-EglA, the pTZ-EglA plasmid was digested with *Nde*I and *Bam*HI restriction enzymes to release the *egl*A, which was then inserted into a similarly digested pET-22b(+) vector by ligase-mediated cloning. The resulting recombinant plasmid (pET-EglA) was used to transform *E. coli* BL21 (DE3) expression host and the transformants were selected on LB-agar plates containing 100µg/ml ampicillin. Presence of insert in positive transformants was confirmed either by colony PCR, restriction digestion or nucleotide sequencing using Beckman CEQ 8000 Genetic Analyzer.

Analysis of EglA expression

E. coli BL21 (DE3) harboring the pET-EglA were grown in 10ml LB-ampicillin medium overnight at 37°C in an orbital incubator shaker (150 rpm). The following day, 3ml of this overnight culture was used to inoculate 100ml LB-ampicillin broth, grown at 37°C until the OD_{600} reached 0.5-0.8. At this stage, the cells were either induced with 5-10mM lactose or 0.5mM IPTG and the fermentation was continued with constant shaking at 150rpm till the maximum cell density was achieved. The cells were thereafter harvested by centrifugation (6,500 rpm, 4°C, and 15 minutes), resuspended in 50mM Tris-Cl (pH 7.0) and subjected to sonication (15x30s bursts with 1 minute interval between the two successive pulses). The soluble and insoluble fractions were separated at this point and analyzed by 15% SDS-PAGE [13]. The inclusion bodies containing the recombinant EglA were washed twice with Triton X-100 and then solubilized using higher concentration of urea (8M).

Refolding and partial purification of EglA

The solubilized EglA was refolded in a renaturation buffer containing 50mM Tris-Cl (pH 9.0), 2mM EDTA, 5mM cysteine, 0.5mM cystine and 1mM PMSF. Solubilized protein solution was added to the renaturation buffer in pulses (1ml/hr, 4°C) with constant stirring on a magnetic stir plate. Following refolding, the sample was dialyzed against Tris-Cl (pH 7.0), concentrated in a freeze dryer and then subjected to heat treatment at 70°C for different time intervals ranging between 10-60 min., for partial purification of recombinant enzyme. Protein estimation and enzyme activity assessment was performed at each step by Bradford [14] and DNS methods [15], respectively.

Zymogram analysis

For zymogram analysis, 15% SDS-gel, after electrophoresis, was sequentially transferred to solution A [50mM Tris-Cl (pH 7.0) containing 20% isopropanol] and solution B [50mM Tris-Cl (pH 7.0)] for one hour each, at 4°C in a petridish. The SDS-gel was then placed on an agar-CMC gel containing 1.7% agar and 0.5% carboxy methyl cellulose (CMC) and incubated at 60°C for 1 hour. Following incubation, the SDS-gel was removed and the agar-CMC gel was stained with Congo red solution for 10 minutes, and destained with 1M NaCl.

Results and discussion

PCR amplification and cloning of eglA gene

The gene encoding full-length EglA protein (without signal peptide) was amplified from *Pyrococcus furiosus* genomic DNA using a pair of gene specific primers. PCR amplification yielded a single product of around 0.90 kb (Figure. 1). To obtain the maximum amplification of *egl*A gene, the PCR reaction conditions were optimized with respect to annealing temperatures and $MgCl_2$ concentrations and the best amplification could be achieved at an annealing temperature of 60°C using 2.5 mM $MgCl_2$.

The amplicon was gel purified, cloned in pTZ57R/T vector and then subcloned between *Nde*I and *Bam*HI sites of pET-22b(+) vector to generate pET-EglA expression plasmid (Figure 2). The recombinant plasmid was first maintained in *E. coli* DH5α for vector propagation and then transformed into BL21 (DE3) for expression studies.

The transformants were selected on LB-ampicillin plates and the presence of *eglA* gene was confirmed by restriction digestion and/or colony PCR. Five different colonies were screened for the presence of insert by colony PCR and the results are shown as Figure 3. A prominent band of 0.90kb in all the screened colonies confirmed the successful transformation.

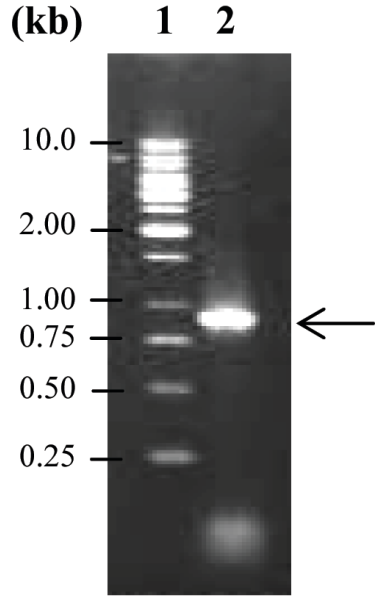

Fig. 1. PCR product of eglA gene analyzed on 1% agarose gel. Lane 1, 1kb DNA ladder; lane 2, ~0.90kb PCR amplified product. Arrow indicates the position of amplicon.

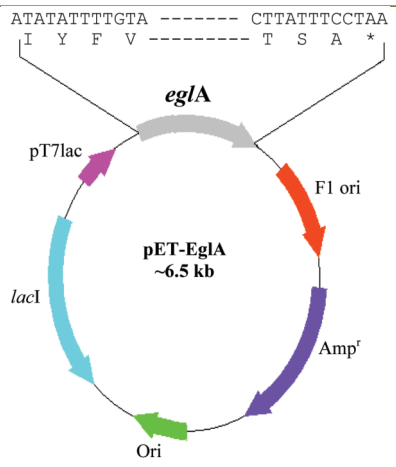

Fig. 2. Construction of pET-EglA recombinant plasmid. 0.90 kb amplicon was cloned in pET-22b(+) plasmid containing origin of replication (ori), T7-lac promoter (pT7lac), ampicillin resistance gene (Ampr) and gene for LacZ (lacI).

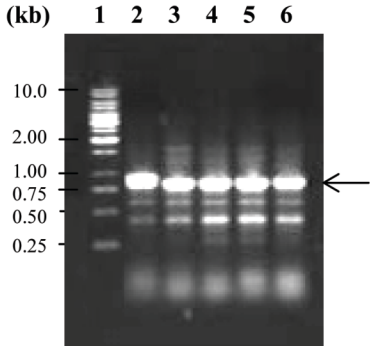

Fig. 3. Agarose gel showing colony PCR results of eglA gene after cloning in pTZ57R/T vector. Lane 1, 1kb DNA ladder; lanes 2-6, eglA gene from different colonies after transformation into E. coli competent cells. Arrow indicates the position of amplified PCR product.

Expression analysis

E. coli BL-21 (DE3) cells transformed with pET-EglA plasmid were grown in LB-ampicillin broth till an OD_{600} of 0.5-0.8 and induced with IPTG or lactose to analyze the expression of recombinant EglA in transformed *E. coli*. Sample fractions were collected at different time intervals during the fermentation and subjected to 15% SDS-PAGE.

Analysis of total cell protein revealed the presence of ~35 kDa protein (expected molecular mass of EglA) in both lactose- and IPTG-induced cells (Figure 4). A band of similar size, however, was absent in control and uninduced cells. It was further observed that the expression of recombinant EglA was better in lactose-induced cells where it accounted for ~25% of the total *E. coli* cellular proteins as compared to ~20%, when induced with IPTG (compare lane 1 of Figure 4 A and B). Owing to the expensiveness of IPTG as inducer and also to the effectiveness of lactose in achieving high level of expression, lactose was used in all subsequent expression studies.

SDS-PAGE analysis of soluble and insoluble fractions revealed that the recombinant EglA was expressed in the form of inclusion bodies (IBs), as a band of 35kDa corresponding to EglA protein was absent in the fraction obtained after centrifugation of the sonicated sample (Fig. 4B, lanes 2 and 3). This probably was the result of high level expression of recombinant EglA under the T7lac promoter providing not enough time for the folding of expressed protein in *E. coli*. Since expression of EglA was undetectable in soluble fraction, we proceeded with IBs for subsequent purification and refolding of expressed enzyme.

Purification and refolding

The IBs obtained from the lysate of cells grown in 2liter LB-ampicillin medium were washed twice with Triton X-100 to obtain relatively pure IBs. The purification achieved at this stage was around 80% (Figure 5). Washed IBs were thereafter solubilized in 8M urea and subjected to refolding in a renaturation buffer as detailed in materials and methods.

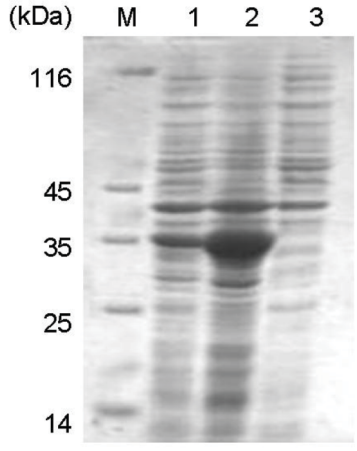

Fig. 4. 15% SDS-gel electrophoresis of total E. coli cellular proteins showing the expression of recombinant EglA. Lane M, molecular weight marker; lane 1, total cell protein; Lane 3, insoluble fraction; Lane 4, soluble fraction.

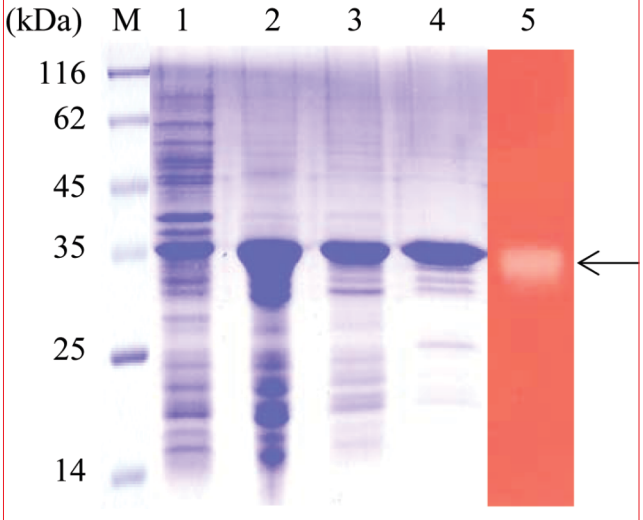

Fig. 5. SDS gel electrophoresis of recombinant EglA at different stages of processing. Lane M, protein size markers; lane 1, total E. coli cell proteins; lane 2: inclusion bodies (IBs); lane 3: IBs after washing with Triton X-100; lane 4: recombinant EglA after refolding; lane 5, zymogarm analysis of refolded protein.

Table 1. Comparison of specific activities of recombinant EglA of *Pyrococcus furiosus* before and after refolding.

Sample type	Volume	EglA (% of total protein)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)
Total cell protein	2 liter	17	-	-	-
Sonicated sample (insoluble fraction)	35 ml	65	1.66	0.045	
Insoluble fraction after washing with Triton X 100	35 ml	80	1.70	0.043	
Refolded sample (after dialysis)	75 ml	85	6.2	0.026	
Heat treated sample	75ml	~92	6.8	0.024	283.33

The addition of IBs in a pulsatile manner and the presence of cysteine and cystine in the renatuartion buffer helped in proper refolding of EglA while preventing the enzyme from precipitation/aggregation.

Zymogram analysis revealed that following refolding, the recombinant EglA was not only active but also sufficiently pure (Fig. 5, lanes 4 and 5). Heat treatment of refolded sample at 70°C for 15-60 min. further improved its purification without activity loss.

Activity assay

The specific activity of recombinant EglA was estimated by DNS method at each stage of purification and refolding. The results obtained are summarized in Table 1. The data reveals that the specific activity enhanced almost 8 fold after proper folding of the recombinant EglA.

Conclusion

Locally produced cellulase enzymes have the advantage of being cheap and readily available for use at various levels in industrial processes. The current study describes high level expression of a cellulase enzyme from a hyper thermophilic organism, which has the added benefit of use in industrial applications where heat generation has been reported to inactivate the enzymes being used.

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