In-vivo expression of chitinase-A from *Serratia plymuthica* UBCR_12

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**Abstract.** The chitinase-A [ChiA] encoding gene isolated from *Serratia plymuthica* UBCR_12 was cloned into *E. coli* DH5α using pGEM-T Easy vector and expressed in *E. coli* BL21 using pET-28a (+) vector. The length of the open reading frame [ORF] is 1692 bp composed of 563 amino acid residues precursor with a molecular weight of 61 kDa. The protein structure composed of three domains: signal peptide, FnIII-like, and catalytic. The signal peptide domain was cleavages during transport through the periplasmic membrane, therefore the molecular weight of secreted ChiA is about 58 kDa. The recombinant ChiA *Serratia plymuthica* UBCR_12 that expressed in *E. coli* BL21 could hydrolyze colloidal chitin.

1. **Introduction**

Chitin is a major structural component of the fungal cell wall [1]. Based on this, many chitinolytic enzymes are considered to have their role in degrading fungal cell walls, including some important phytopathogenic fungal such as *Colletotrichum gloeosporioides*, anthracnose causing agent in chili pepper [*Capsicum annuum*] cultivation. The pathogen can decrease yield up to 50% [2], [3], [4], [5].

Syafriani *et al* [6] previously have successfully isolated a rhizobacterium. It can suppress *C. gloeosporioides* growth *in vitro*. The isolate is characterized as *Serratia plymuthica* UBCR_12. This species also exhibited suppression activity against *Fusarium oxysporum* and *Sclerotium rolfsii* [7]. Further analysis showed that this bacteria can produce a chitinolytic enzyme. The crude extract of extracellular chitinase inhibited about 31.33 ± 6.12% of *C. gloeosporioides* growth [8].

Most bacterial chitinases belong to glycoside hydrolases [GH]-18 family based on their amino acid similarities [9]. *Serratia marcescens* have chitinolytic enzyme subfamily: Chitinase-A, B, and C. Those genes have been cloned and expressed in *E. coli* BL21 [10], [11]. It showed that the three kinds of enzymes had a synergistic mechanism to degrade chitin.

In order to further detail the characterization of its capability and its potential downstream engineering, the chitinase-A [*chiA*] gene from *S. plymuthica* UBCR_12, was cloned and expressed in *E. coli*. Here we report the characterization of the cloned and expressed of the *chiA* gene and its detailed characteristic *in silico*. 
2. Materials and methods

2.1. Culturing and DNA genomic isolation
The *S. plymuthica* UBCR_12 was recultured from the collection of Biotechnology Laboratory, Agriculture Faculty, Andalas University. It was grown on Nutrient Agar, at room temperature [about 27°C] overnight and the single colony was recultured in Luria Broth for 16 hours under continuous shaking at 160 rpm. The genomic DNA was isolated from *S. plymuthica* UBCR_12 by using Wizard® Genomic DNA Purification Kit [Promega-USA].

2.2. Cloning chiA gene and homology modeling
The gene fragment encoding chiA was amplified by specific primers chiA-F and chiA-R. The primers were designed according to the initial and terminal conserved regions of several sequences available in the NCBI database. PCR was performed to amplify the whole open reading frame [ORF] sequence of the chiA gene in a total volume of 25 µL. It was containing 50 ng genomic DNA of *S. plymuthica* as a template, 10 pmol each primer, 12.5 µL of 2×KAPA 2G Robust Hot Start®, and filled until 25 µL with PCR-grade H2O.

The PCR condition was set as follows: initial denaturation at 95°C for 3 minutes; followed by 35 cycles that composed of denaturation at 95°C for 15 seconds, annealing at 54.7°C for 15 seconds, and extension at 72°C for 90 seconds. The final extension at 72°C was set for 3 minutes. The PCR product was examined by electrophoresis using a 1% agarose in 0.5xTE buffer.

The amplified PCR product was ligated into the pGem-T Easy cloning vector [Promega-USA] and further transformed into *Escherichia coli* DH5α. The recombinant clones were selected based on blue-white assay and ampicillin. Further verification of recombinant clones was performed by the PCR colony using T7/SP6 primers combination. The verified recombinant DNA vectors containing the chiA gene were purified using Wizard Plus SV Minipreps DNA Purification System [Promega-USA] and sent for sequencing service provided by 1st Base, in Singapore. The sequencing process was done from both termini using T7 and SP6 combinations.

Characterization of enzyme structure was done by in silico analysis. The nucleotide sequence was translated to amino acids by Expasy Proteomics Server [http://www.expasy.ch/tools/dna.html]. The nucleotide sequence was translated to amino acids by Expasy Proteomics Server [http://www.expasy.ch/tools/dna.html]. Sequence alignments and the conserved domains were searched by using the online BLAST search engine at the NCBI [http://www.ncbi.nlm.nih.gov/BLAST/]. The 3D structure prediction was performed by Phyre2 [12] and active site prediction was performed by 3D Ligand Site [13].

2.3. Subcloning the *S. plymuthica* UBCR_12 chiA gene into the pET-28a[+] expression vector
The chiA gene previously ligated in pGEMT-Easy vectors was double digested by BamHI and SacI restriction enzymes. The fragment was further ligated with the pET-28a[+] vector [Merck-Germany]. The construct was then transformed into the *E. coli* DH5α competent cells to construct recombinant plasmids pET-ChiA. The clones containing recombinant molecule were selected based on kanamycin plate [30 µg/mL], and verified by colony PCR using T7-PF/T7-TR primers.

Recombinant DNA vectors pET-ChiA were purified by Wizard Plus SV Minipreps DNA Purification System [Promega-USA]. Recombinant pET-ChiA was transformed into *E. coli* BL21. They were spread onto LB agar plates containing kanamycin [30 µg/mL] and the plates were incubated at 37 °C for 16 h. The colony *E. coli* BL21 containing pET-ChiA was confirmed by PCR amplification by using T7-PF/T7-TR primers and inoculated in LB broth and LB agar containing kanamycin [30 µg/mL] followed by incubation at 37 °C in an incubator for 16 h. Sterile glycerol 50% was added to the culture of *E. coli* BL21 containing pET-ChiA in LB broth and stored in -80°C for further analysis and application. The sequences of PCR primers used in this study are listed in Table 1.
### Table 1. Primers used in this study

| Primer ID | Sequence [5’-3’] |
|-----------|------------------|
| ChiA-F    | GG G↓GACTC ATG CGC AAA TTT AAT AAA CCG |
| ChiA-R3   | CC GAGCT↓C TTG CGT GCC GGC GCT ATT GC |
| T7        | TAA TAC GAC TCA CTA TAG GGC GA |
| SP6       | ATT TAG GTG ACA CTA TAG AAT AC |
| T7-PF     | TAA TAC GAC TCA CTA TAG GG |
| T7-TR     | GCT AGT TAT TGC TCA GCG G |

Restriction sites were added to the primer to assist cloning to pET-28a[+]. The *Bam*HI restriction site was underlined while the *Sac*I restriction site was written in bold.

2.4. Expression of ChiA gene in E. coli BL21

The hydrolysis ability of The ChiA enzyme expressed using pET-28a[+] in *E. coli* BL21 was assayed by using 3% of colloidal chitin in LB agar that also containing 1 mM isopropyl β-D-1-thiogalactoside [IPTG]. *E. coli* BL21 was used as a negative control.

Harvesting enzyme produced in intracellular and secreted was performed by collecting ChiA enzyme from the cells and culture supernatant after induction expression for 15 h. Seed culturing was grown in 10 mL LB broth containing kanamycin [30 µg/mL] grown at 37°C for overnight. Further propagation step was grown seed culture in a volume of 100 mL LB broth containing kanamycin [30 µg/mL]. The expression activity of the ChiA gene was induced by 1 IPTG when the OD<sub>600</sub> reached 0.8. The culture was incubated for 15 h in 27°C. The culture supernatant and the cells were separated by centrifugation at 12,000 rpm for 10 minutes at 4°C. The cells containing expressed proteins with His-tag were purified by using QIAexpress Ni-NTA Fast Start [Qiagen-USA] according to the instructions in the manual. The culture supernatant was filtered [0.22 µm] and secreted ChiA was harvested by ammonium sulfate to final saturation 40%, 60%, and 80% respectively. The precipitated protein was obtained by incubation at 4°C overnight and then centrifugation at 12,000 rpm for 15 minutes at 4°C and dissolved in phosphate buffer pH 7.0. The purified proteins were separated by 15% sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis [PAGE].

3. Results and discussion

3.1. ChiA gene structure

An approximately 1.7 kb ChiA putative gene was cloned using the pGem-T Easy vector and transformed to *E. coli* DH5α [data is not shown]. The 1.7 kb fragment contained the recognition site of *Bam*HI at the 5’-end, and recognition site of *Sac*I at the 3’-end. Both of restriction enzyme sites were flanking ORF ChiA without stop codon. The aim of excluding the stop codon of the ChiA gene was to continue translation until polyhistidine at C-terminal. The expression of ChiA was performed in pET-28a[+].

The verification of pET-ChiA recombinant plasmid was performed by PCR and restriction [Figure 1]. The recombinant plasmid pET-AS12 is 7,066 bp in length. The open circular conformation caused it to migrated slower than linear DNA [14]. Restriction pET-ChiA by *Sac*I and *Bam*HI restriction enzyme resulted in fragment 1,705 bp and 5,361 bp. The result of PCR amplification by T7-PF/ T7-TR primer was 2,004 bp in length and by ChiA-F/ ChiA-R was the same with restriction fragment, 1,705 bp. Therefore, it verified the success of pET-ChiA construction in *E. coli*.
Figure 1. Verification of plasmid recombinant pET-AS12. T7: amplification with T7-PF/T7-TR primer, A: amplification with ChiA primer, R: restriction with BamHI/ SacI, and P: recombinant plasmid pET-AS12.

The length of ORF \textit{chiA} without stop codon is 1,689 bp [accession number KX826799]. It is expected to express 563 amino acid residues precursor excluded polyhistidine-tagging from the pET-28a[+] system. The gene encoding \textit{chiA} from \textit{Serratia marcescens} also expressed amino acid residues in 563 aa [11]. Multialignment this expressed gene with predicted structure from the \textit{chiA} enzyme from \textit{S. marcescens} BJL200 [15] indicated that the structure of the \textit{ChiA} enzyme consists of a signal peptide, fibronectin type III-like [FnIII-like] domain, and the catalytic domain [Figure 2]. The first N-terminal 23 amino acids of the gene product belong to signal peptide. Some chitinases have immunoglobulin-like fold domains, Fibronectin type 3 [FnIII] [9], [16], [17]. It contained exposed aromatic residues and contributed to substrate binding and the efficiency of substrate hydrolysis [18].

Figure 2. Modeling structure of ChiA enzyme \textit{Serratia plymuthica} UBCR_12 and domain characterization.

The 3D Ligand Site analysis showed that W167, I207, G274, W275, T276, E315, D391, R446, E473, and W539 were predicted as chitinase-A active sites. The amino acids of the \textit{ChiA} are 94% similar to \textit{ChiA} \textit{S. marcescens}. Therefore, It is possible that W245 and F232 were important residues in catalytic cleft [18], [19].

3.2. Expression of \textit{chiA} gene in \textit{E. coli} BL21
The colloidal chitin agar plate assay was performed to observe the enzymatic activity of \textit{chiA} that expressed using the pET-28a[+] system [Figure 3]. Clear zone did not appear around of colony \textit{E. coli} BL21, while \textit{E. coli} BL21 transformant pET-ChiA did. It indicated that originally \textit{E. coli} BL21 did not produce chitinolytic enzyme and the recombinant \textit{chiA} in \textit{E. coli} BL21 transformant was successfully expressed. The \textit{chiA} enzyme could hydrolyze the colloidal chitin around the colony \textit{E. coli} BL21 transformant.
The recombinant ChiA gene was controlled by the T7 promoter allowing induction of gene expression using 1 mM IPTG. The molecular weight of the ChiA precursor protein was about 61 kDa [Figure 4A]. But, the molecular weight of the secreted recombinant chiA enzyme was decreased to be 58 kDa [Figure 4B]. It indicated that the signal peptide domain was cleavage during transportation through the periplasmic membrane.

**Figure 3.** Chitinolytic activity test of ChiA-\textit{E. coli} BL21 transformant. A. \textit{E. coli} BL21 and B. \textit{E. coli} BL21 transformant pET-ChiA

**Figure 4.** Profiling of protein expression. A. Intracellular protein after induction of expression for 3 h, B. Purification ChiA recombinant after induction of expression for 15 h. M: Protein ladder SMBio 2700, C0: \textit{E. coli} BL21, Ci: \textit{E. coli} BL21 with IPTG, P0: \textit{E. coli} BL21 transformant ChiA, P1: \textit{E. coli} BL21 transformant ChiA with IPTG Induction, I: Pure ChiA recombinant from intracellular, and E40, 60, and 80: ammonium sulfate protein precipitation with saturation 40, 60, and 80%
4. Conclusion

The chitinase-A gene from S. plymuthica UBCR_12 was successfully cloned, expressed, and characterized in this study. The ORF of ChiA excluded stop codon is 1,689 bp. It expressed a 563 amino acid residue precursor with molecular weight 61 kDa. It consisted of three domains protein: signal peptide, FnIII- like, and catalytic. The signal peptide domain was cleavage during transport through the periplasmic membrane. The recombinant ChiA Serratia plymuthica UBCR_12 that expressed in E. coli BL21 could hydrolyze colloidal chitin.

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