Isolation of Chitinase B [ChiB] gene from Serratia plymutica strain UBCF_13

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Abstract. The utilization of synthetic fungicides harms the environment. Several studies have been carried out to control disease in plants using biocontrol agents and bio fungicides that are more environmentally friendly. Laboratory tests showed that bacterial isolates UBCF_13 can act as biocontrol of pathogenic fungi that have been identified as Serratia plymutica bacteria. This bacteria is capable of producing chitinase enzymes that can degrade pathogenic fungal cell walls. The study was aimed to isolate the chitinase B gene from Serratia plymutica strain UBCF_13. Cloning of the chitinase B [ChiB] gene from the bacterial genome UBCF_13 was carried out using a PCR-based cloning strategy using specific primers. The UBCF_13 ChiB gene was successfully isolated and identified with a sequence of 1583 bp covering ORF of 1500 bp which encodes 499 amino acids. Domain analysis exhibited 2 functional domains, namely catalytic and the chitin-binding domain. The 3D analysis of the ChiB protein structure indicates a similar function with the c1ur88 template which is identified as ChiB. Further research is necessary to determine the optimal method to express and determine its detailed function.

Keywords: ChiB, PCR-based cloning, Serratia plymutica, bio fungicide

1. Introduction

In an effort to replace the role of synthetic pesticides, several studies have been carried out to control disease in plants due to fungal pathogens using biocontrol agents and bio fungicides. Bio fungicides are known to be more environmentally friendly compared to synthetic fungicides so that it can provide a safer impact on health and the environment [1]. Bio fungicides can be developed from the class of enzymes that can degrade pathogenic fungal cell walls. These enzymes are known as hydrolase enzymes, including protease, glucanase, cellulase, and chitinase. Of the four types of enzyme hydrolases, chitinase is known to be able to degrade chitin which plays a very important role in maintaining the strength of fungal cell walls. Utilization and development of chitinase as a bio fungicide should be an appropriate and effective choice for overcoming fungal pathogens in plants [2].

Chitinase is divided into two groups of enzymes based on the chitin degradation system, namely, exochitinase [ChiA and ChiB], and endochitinase [ChiC]. Exochitinase works or cuts the N-terminal ends on chitin polymer chain molecules. The ChiA enzyme will cut off at the reduced end, while ChiB will cut off the chitin from the non-reducing end, whereas endochitinase [ChiC] works randomly to cut chitin polymer chains [3].

Chitinase B has a similarity of products produced with chitinase A [GlcNAc] but ChiB has less attachment substrate compared to ChiA, therefore ChiB can be optimized for relatively short
chitooligosaccharides cuts. ChiB has a lower specific activity for chitin compared to ChiA and has a higher affinity for [GlcNAc] 3 compared to ChiA[4]. ChiB substrate has more residual domains than ChiA and ChiC substrates where this domain functions to attach substrate and efficiency of substrate hydrolysis. After β-chitin degradation by all three chitinases, the final dimer/trimer ratio of each enzyme was 7.3 for ChiA, 12.6 for ChiB and 4.1 for ChiC, where the ChiB enzyme had a greater final dimer/trimer ratio than ChiA and ChiC. From this ratio, it can be seen that the ChiB enzyme shows a more progressive work compared to ChiA and ChiC [3]. Therefore ChiB was chosen for further study as a candidate for alternative biocontrol agents or bio fungicides.

Isolation of the ChiB gene is expected to be the first step in the strategy of developing alternative bio fungicide compounds. ChiB gene isolation was performed using a PCR-based cloning strategy. The gene is isolated from Serratia plymutica, an antagonistic bacteria against pathogenic fungi that are known to be able to produce chitinase. So far, only ChiA gene sequences have been reported from Serratia plymutica [2]. Previous studies have succeeded in isolating some chitinase genes from Serratia plymutica UBCR_12 including ChiA [5], ChiC [6], ChiB gene and putative chitinase [7] characterized by 1781 bp, 1564 bp, 1681 bp, and 1299 bp in size respectively. The ChiB gene sequence information from Serratia plymutica strain UBCF_13 in this study will be the first report.

The research was aimed to isolate the chitinase B gene from the isolate UBCF_13. The data of the ChiB gene obtained from this study can be used as a source of information for further research in the exploitation of the ChiB gene which will then be used as a bio fungicide.

2. Materials and methods

2.1. PCR based cloning
Genomic DNA of Serratia plymutica strain UBCF_13 was isolated as described in a previous study [8]. Then, the genomic DNA was amplified using chitinase B [ChiB] specific primers, namely ChiB15-FR [7]. Forward primer [ChiB15-F] sequence: 5'-CATTTAATAGCGCGAGAGAR-3' and reverse primer [ChiB-R] sequence: 5'-GGGTTACCTTGGTGCAATA-3' have an estimated product about 1,681bp in length.

The PCR reaction mostly was carried out in a total volume of 25 µL consisting of 12.5 µL KAPA2G Robust HotStartReadyMix PCR Kit [KapaBiosystems-USA], 3 µL genomic DNA [5 ng/µL], 1.5 µL of each primer ChiB-F [5 pmol/µL] and ChiB-R [5 pmol/µL] and 6.5 µL ddH2O. The condition of PCR amplification was performed as follows: pre-denaturation at 95°C for 3 min, followed denaturation at 95°C for 15seconds. The 35 cycles used 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 1 min. The final extension was run at 72°C for 1 min. The PCR product was separated using 1% agarose gel on 100 volts for 30 min and then was visualized with UV-transilluminator and gel documentation system [Biometra-Germany]. The amplicon was purified using GeneJET Gel Extraction Kit [ThermoFisher Scientific-USA] before being sent to 1st BASE-Singapore for sequencing with specific primers.

2.2. Bioinformatics analysis
The sequencing was edited manually and followed by comparing their homology using the BLASTn tool available at the NCBI website [http://blast.ncbi.nlm.nih.gov]. Then, the target sequence was analyzed in Mega6 software to build a phylogenetic tree. Furthermore, the target sequence was also analyzed using the ORF finder program [https://www.ncbi.nlm.nih.gov/orffinder/] and the Conserved Domain Database [https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi]. Finally, 3D-modeling of protein structure analysis was performed using Phyre2 [http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index].
3. Results and discussion

3.1. Amplification and purification of ChiB genes from UBCF_13

In this study, the ChiB gene sequence of Serratia plymuthica strain UBCF_13 was isolated using ChiB15-FR specific primers. The PCR produced two fragments that have a length of about 1681bp and 1400 bp respectively [Figure 1.A]. The concentration of the main fragment is estimated at 100 ng/µL. The occurrence of a second product probably was caused by the non-specific binding site of the primers. Another possibility could be from not optimal annealing temperature [9]. For that reason, gel purification was performed to isolate the target single fragment. Purification was carried using the GeneJET Gel Extraction Kit provided by Thermo Scientific. Electrophoresis analysis of purified fragment is shown in Figure 1B.

![Figure 1](image1.png)

**Figure 1.** Visualization of the ChiB gene from Serratia plymuthica strain UBCF_13. [A] The unpurified PCR product [B.] Purified PCR product.

3.2. Homology and cluster analysis

![Figure 2](image2.png)

**Figure 2.** Electrophoregram of the ChiB gene from Serratia plymuthica strain UBCF_13.

The electropherogram of sequencing data using a specific primer was shown in figure 2. The ChiB gene sequences obtained were sized 1,583 bp after trimming and editing. The forward and reverse primer sequences cannot be found since the sequencing results are derived from the PCR product without cloning. The 1,583 bp sequences were analyzed using BLASTn to identified the similarities level compare to other sequences in the data bank. From the BLAST results, the ChiB gene from UBCF_13 isolate has the highest identity with the ChiB gene sequence from the Serratia plymuthica strain UBCR_12 [KX863872.1] by 99% identity and 99% query coverage.

The phylogenetic trees showing relatedness with other ChiB gene sequences isolated from Serratia plymuthica are shown in Figure 3. ChiB gene from UBCF_13 has 1,583 bp size in length, while other 9 chitinases gene sequence length ranges from 1,578 to 1,583 bp.
Figure 3. Phylogenetic tree of the ChiB gene from UBCF_13 with 9 other sequences.

The phylogenetic tree shows that the ChiB gene from the Serratia plymutica strain UBCF_13 has close relatedness with the ChiB gene from Serratia plymutica strain UBCR_12 using a 1000 bootstrap value on the neighbor-joining method.

3.3. Open reading frame and conserved domain database analysis

The ORF finder analysis was performed to reveal the number of ORF spanning along the 1,583bp ChiB gene sequence. This analysis was also purposed to predict the functional domain of the sequence. The ORF finder analysis shows that the UBCF_13 ChiB gene has 6 ORFs [Figure 4], whereas the ORF 1 [blue box] has the longest coverage of about 1,500 bp. The ORF of a gene normally starts with the start codon in the form of ATG, GTG and TTG, and the stop codon in the form of TAA, TAG or TGA [10], [11]. In our UBCF_13 ChiB gene, the start position of the ATG codon is in the base 19 and the TGA stop codon in the 1,518 bases. This ORF 1 has 1,500 bp in length encoding 499 amino acids [data not shown]. This is similar to a study provided by Ozgen et al.[12] that also found a 1,500 bp ChiB ORF from Serratia marcescens XdI.

Figure 4. ORF pattern of the ChiB gene from Serratia plymutica UBCF_13

The domain motif in the ChiB gene from UBCF_13 [shown in Figure 5] shows that there are two domains contained in the gene. These two domains are catalytic domain found in the N-terminal and the chitin-binding domain in the C-terminal [13]. The catalytic domain in the ChiB gene from UBCF_13 is classified into the Glyco_hydro_18 [Glycoside hydrolase 18] superfamily domain, based on Perrakis et al.[14] classification. They explained that chitinases in the Serratia plymutica bacteria belong to the Glycoside hydrolase family 18 groups.

The chitin-binding domain shown by our sequence is the ChtBD3 Super domain which will bind and hydrolyze the chitin substrate [Figure 5].
Figure 5. Two domains of the ChiB gene from Serratia plymutica plymutica strain UBCF_13.

3.4. 3D analysis
Analysis of the 3D structure of deduced ChiB-UBCF13 polypeptide sequence using Phyre2 exhibited a similar structure with template c1ur88 which is identified as ChiB with a 100% confidence level. Figure 5 shows the structure of the catalytic domain and chitin-binding. The active site from both two proteins seemed similar. The ChiB protein has an attachment gap in the substrate with an aromatic residue where the α + β domain is inserted in the formation of the substrate attaching gap wall. The ChiB enzyme has a linear pathway on the surface of amino acids starting from the chitin-binding domain and extending to catalytic domains [3]. Based on that structure, the ChiB -UBCF_13 has high potential as bio fungicides to hydrolysis chitin substrate in fungi and insect cells wall.

Figure 6. Structure of ChiB protein from UBCF_13 [A] and template c1ur88 ChiB protein [B].

4. Conclusion
The UBCF_13 ChiB gene was successfully isolated and identified with a sequence of 1,583 bp in size covered of ORF with 1,500 bp and encoding of 499 amino acids. The UBCF_13 ChiB gene composed of 2 domains, catalytic domain, and the chitin-binding domain. The 3D structure of ChiB protein analysis showed similarity with the c1ur88 template, which is also identified as ChiB. Further research is necessary to obtain an optimal method to express the ChiB gene and to determine its function in detail.

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