Sequence Variation of Metalloprotease Genes from Three *Serratia plymuthica* Isolates Collected from Rhizosphere and Pylloplant for Sustainable Agricultural Practices

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Abstract: The effectivity of proteolitic enzyme for biopesticide is determined mainly by the nature of biological sources from which they have been isolated. Three isolates identified as *Serratia plymuthica* were isolated from rhizsosphere of onion and phylosphere of cabbage. In order to maximize their capability as biopesticide we isolated and characterized their metalloprotease encoding gene and looked deeper for genetic manipulation possibility. All the three strains have similar length of 1059 bp which is shorter than that previously expected 1107 bp. Seven point mutations along the gene sequences were observed. However, only 780 bp could be predicted as ORF and gave 259 amino acids sequences for each strains. Domain analysis predicted that the gene contained M48C_loiP-like motif started from 115-762 base. The domain also contains Zn binding motif (HEXXH) indicating that their activity might be influenced by the presence of Zn ion. The core ORF covering 250 amino acids contains three mutations events, causing amino acid changing on Ser-35-Phe, Ser-52-Pro and Ile-208-Asn. However, three dimentional structure and ligand binding analysis did not show any significant variation among them. The data found here, indicated that the two strains *S. plymuthica* share similar proteolitic capability eventhough they isolated from two different habitats.

1 INTRODUCTION

Protease is considered to be one of the most effective proteolytic enzymes as biofungicides (Dunne *et al.*, 1997). For this reason, the exploration of proteolytic bacteria has been widely reported by several researchers. Some species such as *Bacillus subtilis*, *Bacillus amyloliquefaciiens*, and *Bacillus vallismortis* are known to produce proteolytic enzymes capable of inhibiting the growth of pathogenic fungi *Colletotrichum gloeosporioides*, *Colletotrichum capsici*, *Fusarium solani*, and *Septobasidium* spp. (Ann, 2012). Palaniyandi *et al.*, (2013) isolated *Streptomyces phaeopurepureus* ExPro138 which is antagonistic to the fungus *C. coccodes*, the cause of anthracnose disease in tomato plants, while *Paenibacillus polymyxa* APEC128 is known to suppress anthracnose disease in apples caused by *C. gloeosporioides* and *C. acutatum* (Kim *et al.*, 2016).

Syafriani *et al.* (2016) identified one strain of rhizobacteria which is capable of suppressing the development of the phytopathogenic fungus *C. gloeosporioides*, while Aisyah et al (2016) successfully isolated two phylobacteria showing similar actrivity. All the three strains are coded as UBCR_12, UBCF_01 and UBCF_13. Using the 16S rRNA gene sequence, the three species were identified as *Serratia plymuthica* and their nucleotide sequences have been deposited in NCBI sequence databases with access codes KU299959.1, KX394778.1 and KX394779.1 respectively. Moreover, isolation and characterization at the molecular level, such as isolation of genes associated with chitinase activity have also been performed (Syafriani, 2017) as well as their pathogenic potency. Therefore, in the effort to

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exploit these three isolates making as one of the component in the sustainable agricultural practices ie: as biopesticide or plant growth promoting agent, further characterization of their genetic potential becomes a high urgency.

In this article, we report the characteristics and comparison of metalloprotease gene sequences isolated from 3 isolate *Serratia plymuthica* species collected from the rhizosphere and pyloplan regions.

2 MATERIALS AND METHODS

2.1 Bacterial Isolate Identity

One rhizsopheric and two phyllopant bacterial isolates designated as UBCR_12; UBCF_01 and UBCF_13 were used in this study. Collection procedures and their morphological identity have been previously described by (Aisyah *et al.*, 2016; Syafriani, 2017). Molecular identity based on their 16S rRNA gene from the isolates was also reported by both above mentioned authors and was deposited in the NCBI nucleotide database designated with the accession number KU299959.1, KX394778.1 and KX394779.1 respectively.

2.2 DNA Preparation and in-Vitro Amplification

Genomic DNA of the three isolates was prepared as described previously by Syafriani, *et al.*, (2016). Integrity of the genomic DNA was controlled with standard electrophoresis method. Isolation of metalloprotease gene sequences was performed by PCR technique applying primer combination Mtlpro-F (ATGCCAAATGAGAGCGAGTT) and Mtlpro-R (TGGCGGAAGCGATTAACTAT). Amplification was conducted in 25 µL of total volume, containing 3 µL (5 ng/µL) of genomic DNA, 15 pmol of each primer, 12, 5 µL DNA Polymerase (KAPA2G-Japan) and 6,5 µL of ddH_2O. Amplification was performed on T-Personal Thermocylcer (Biometra-Germany) using PCR condition set on 30 cycles amplification containing: 95 ºC-15 seconds of denaturation; 55 ºC- 30 seconds for annealing and 72 ºC - 60 second. Initial denaturation was set on 95 ºC for 3 minutes and final extension was performed on 72 ºC for 60 seconds.

2.3 Cloning and Sequencing

PCR product showing a single unique band of 1107 bp in size was isolated from the gel and subjected for sequencing step. Prior sequencing step, cloning of PCR product was performed into pGem-T Easy Vector (Promega-USA) using *E. coli* strain DH5α as host by means of the heatshock technique. Positive recombinant clones were verified directly using T7/SP6. The positive recombinants clones from each transformation were subjected for plasmide DNA isolation. Sequencing was undertaken at the sequencing service company (1st-BASE-Singapore) from both termini using T7 and SP6 primers.

2.4 Sequence Analysis and Bioinformatics

Nucleotide sequences data generated from the sequencing step was verified using Bioedit software (Hall, 1999) in order to fix their status. The editing steps was also aimed to fix the length of complete sequence of metalloprotease gene started from start codon to terminator codon which was verified by ORF finder tool available at NCBI website. The final edited sequence data was then subjected to the homology search by means of BLASTn (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990) provided at NCBI database in order to verify their sequence homology status with other metaloproteinase gene sequences available worldwide. Conserved Domain analysis (Marchler-Bauer *et al.*, 2015) was also run to map the position of every domain which is possibly harboring along the sequences. Three dimentional structure was also constructed in order to predict their tertiary structure based on their deduced amino acid sequences. This analysis was performed by using Pyre2 software (Kelley *et al.*, 2015). Finally the sequences was compared among the three isolated sequences and also with some metaloprotease sequence in both nucleotide level as well as in amino acid level using Mega6 software (Tamura *et al.*, 2013).

3 RESULTS AND DISCUSSION

3.1 Gene Amplification and Cloning

In-vitro amplification of Metalloprotease gene sequence using primer combination Mtlpro-F and Mtlpro-R from the three strain UBCR_12, UBCF_01 and UBCF_13 successfully produced

fragment of 1.107 bp in size. However, the fragments are not specific. Some additional faint fragments of 700-800 bp in size are still visible (Figure.1a). For that reason, cloning of PCR product into pGem-T Easy Vector was undertaken.

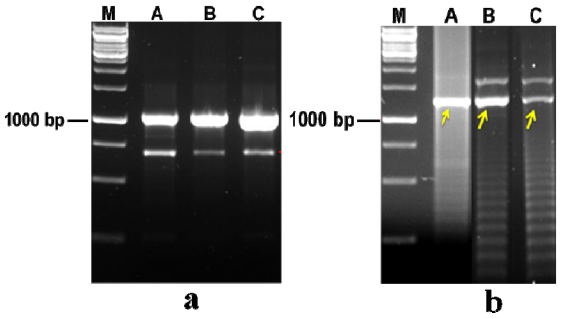

Figure. 1: PCR product of metalloprotease gene generated with Mtlpro-F/R primer combination obviously produced major fragment of about 1107 bp in size (a), while amplification of putative recombinant clones with T7/SP6 primer combination exhibited of about 1248 bp fragment in size (b). A=UBCR-12, B=UBCF_01 and C=UBCF_13. M = 1 kb ladder.

Selection of recombinant using *Lac-Z* gene expression platform enriched with amphicillin (100 mg-/mL) succesfully differentiated non recombinant and recombinant plasmide containing inserted target sequence. Only one colony could be confirmed as recombinant from each UBCR_12 and UBCF_01, while 2 recombinant colonies were produced from UBCF_13. Verification of all recombinant colonies was performed using T7/SP6 primer combinations. Representative clone from each strain produced a major fragment of about 1248 bp in size (Figure 1b). The additional length of fragment (about 141 bp) generated from T7/SP6 amplification is caused by an additional segment flanked by T7 and SP6 primers located in the plasmid sequence. Furthermore, amplification with T7/SP7 of putative recombinant DNA plasmid also proved the correctness of the recombinant clone (data not shown).

3.2 Sequence Homology Analysis

Sequence data generated from sequencing procedure was edited and trimmed for their integrity and finally was built as a contig from each terminusi. Trimming the resulted contig produced a gene sequence of 1059 bp in size from each of the three strain. However, the gene length found in this study is shorter than what was expected of 1107 bp, a gene length which is commonly found in *S. plymuthica*. Assuming that, a number of deletion events has occured in our strain. In order to find out such hypothesis we performed homology search using BLAST tool provided at NCBI database. All the three sequences, showed significant homology ranging from 94,33 % to 96.00 % with metalloprotease gene sequence available in the NCBI database.

3.3 Distribution of Domains Along Metaloprotease Gene Sequence

Domain motif of all three metaloprotease gene was predicted using ORF finder software provided at: https://www.ncbi.nlm.nih.gov/orffinder/. The longest ORF motif from each strain showed similar pattern. All the three strain have a 780 bp ORF motif, encoding 259 amino acids started with ATG and ended with TAA. Conserved Domain analysis (CDD) (Marchler-Bauer *et al.*, 2015) indicated that the gene contained M48C_loiP-like motif started from 115-762 base (Figure 2).

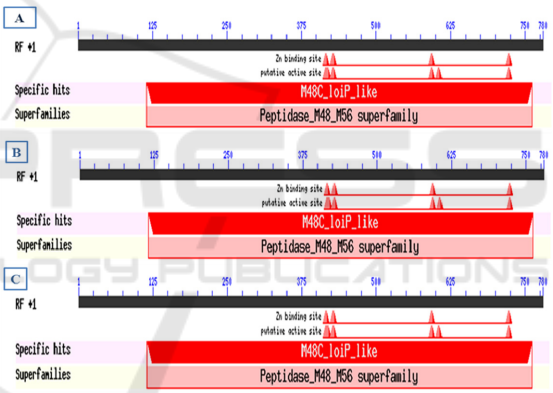

Figure 2: Conserved domain motif of metalloprotease gene from three *S. plymuthica* strain. A= UBCR_12, B = UBC-F_01, C = UBCF_13.

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Domain *M48C_loiP_like* belongs to the super-family of Peptidase M48C_M56 which has close relationship with the family *Ste24 endopeptidase*. Some others members belong to this family is *Ste24 protease* (*Peptidase M48A*), *protease htpX homolog*

(*peptidase M48B*) atau *CAAX prenly protease* 1, dan *mitochondrial metallopeptidase OMA1* (*peptidase M48C*). Most of them are proteins connected to the endoplasmic reticulum and golgi apparatus complex. The domain contains *Zn binding motif* (HEXXH) and COOH-terminal ER. The HEXXH motif plays a very important role in their catalytic and proteolytic activity (Fujimura-Kamada *et al.*, 1997). Mutation in that region will omit their catalytic activity especially for the family of protease Ste24p dan HtpX. The *peptidase M56* covers domain of *Zinc-metalloproteases* of some protein *MecRI* and *BlaRI*. That domain has significant similarity with the catalytic activity of *metallopeptidase membrane integral M48* and M56 and belongs to the new group of protein called minigluzincin.

3.4 Sequence Homology

In order to get the overview of homology structure among those three metalloprotease gene, multi-alignment analysis in nucleotide as well as in aminoacid level was run under the Clustal Omega (Sievers *et al.*, 2011) tool provided at https://www.ebi.ac.uk/Tools/msa/clustalo/. Six substitution events were detected in the nucleotide levels along the compared sequence of metaloproteases gene.

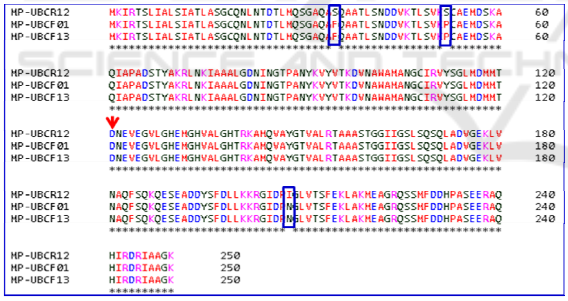

Figure 3: Multiple alignment of deduced amino acid sequences of metaloproteinase gene from three strain *S. plymuthica*. Position of amino acid changes were indicated by blue box, while red arrowhead shows position of possible ligand binding site as analyzed by 3DligandSite software.

The substitution events were detected at base 122, 148, 265, 315, 520, 784 and 952 (data not shown). However based on the analysis of ORF finder, only 780 bp is the most likely might be involved in the translational process. Therefore further analysis was focused on that segment. Interestingly, after translating the segment only 250 amino acids could be produced. Multiple alignment analysis among their amino acid sequence, exhibited three position showing amino acid change, ie: Ser-35-Phe, Ser-52-Pro and Ile-208-Asn.

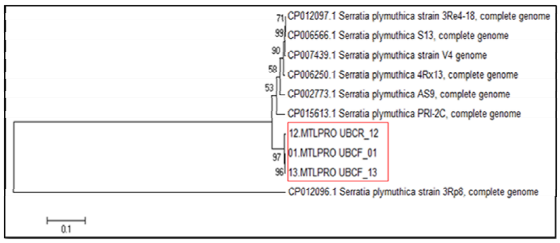

Figure. 4: Phylogenetic tree of three metaloprotease gene from three *S. plymuthica* strains among 7 other metalloproteases.

Multiple alignment of amino acid sequences obviously showed that UBCF_01 and UBCF_13 shared 100% homology, but they differentiated with UBCR_12 by three amino acid. Both UBCF_01 and UBCF_13 were collected from the phyloplant zone, while UBCR_12 was isolated from rhizosphere zone (Syafriani *et al.*, 2016; Aisyah *et al.*, 2016; Syafriani, 2017). This comparison is also in line with the result of cluster analysis shown in the phylogenetic tree (Figure 4) where all the tree sequences closely groupped in one cluster.

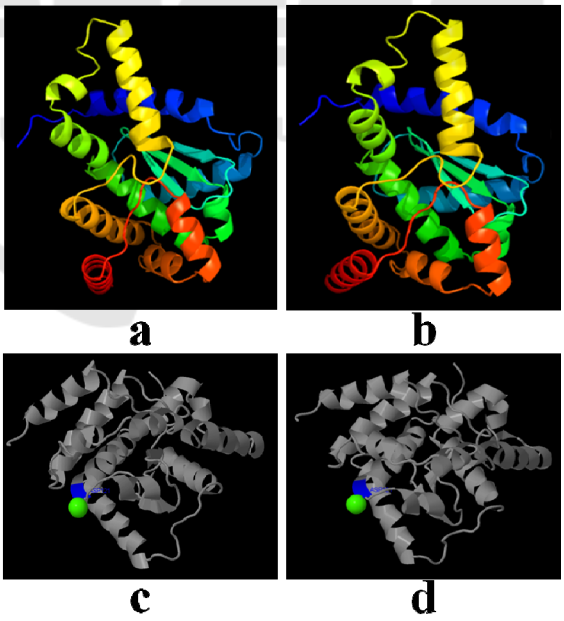

Figure 5. Three dimentional structure of possible metalo-protease enzyme from rhizosphere and phyloplan. Tertiary structure prediction of metalopreotease enzyme from UBCR_12 (a) and UBCF_01 (b) as predicted by Pyre2, while c-d showing possible ligand binding site as predicted by 3DligandSite software.

Three dimentional structure prediction by Phyre2 showed no significant differentiation between UBCR_12 and UBCF_01 (Figure 5 a-b.). Prediction of ligand binding site from both enzymes also indicate to aspartic acid (Asp) locating in amino acid number 121 (Figure 5c-d)

4 CONCLUSIONS

Data obtained form this research indicated that the two strains of *S. plymuthica* share similar proteolitic capability even though they isolated from two different habitats.

ACKNOWLEDGEMENTS

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