

CLONING, SEQUENCING, AND IDENTIFICATION OF *rhd* α -SUBUNIT GENE OF HALOAROMATIC DEHALOGENASE TERMINAL OXYGENASE FROM *Pseudomonas aeruginosa* LOCAL STRAIN

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ABSTRACT

Haloaromatic is one of organohalogen pollutants found in the environment. These compounds are toxic, persistent, carcinogenic, and mutagenic. Accumulations of these compounds in the environment may cause serious diseases in organisms, including humans. Previous research results showed that breaking the bond of halogen atom from the aromatic ring could eliminate the toxicity of these compounds. Haloaromatic dehalogenase is an enzyme that possesses an ability to cut the halogen atom from the aromatic ring. In this research, a *rhd* α -subunit gene of haloaromatic dehalogenase terminal oxygenase has been isolated and sequenced. The gene was isolated by PCR approach from *Pseudomonas aeruginosa* local strain, which is a Gram-negative bacterium found in soil as a pioneer growth bacterium. The sequence obtained indicated that *rhd* α -subunit gene is 1290 bp in size. The RHD α -subunit deduced amino acid has Cys98-X1-His100-X17-Cys118-X2-His121 and His217-X4-His222-X149-Asp372 motifs, two important motifs in aromatic ring hydroxylating dioxygenase family, which is a large family of multi subunit and multifunctional proteins. One of its functions is in the breaking of halogen element from the aromatic carbon ring. Omori classification showed that RHD α -subunit is classified as Group II of aromatic ring hydroxylating dioxygenase family, which includes haloaromatic dehalogenases. The result of 3D structure prediction also indicated that this protein belongs to this large family with 54 percentile Mol Probit score.

Key words: *Pseudomonas aeruginosa*, haloaromatic dehalogenases, *rhd* α -subunit gene

INTRODUCTION

Haloaromatic is one group of many organohalogen pollutants found in the environment. These are also dominant pollutants ever found (Chaudry & Chapalamadugu, 1991). Haloaromatics are salubrious compounds because of its toxic, persistent, carcinogenic, mutagenic, and accumulated behavior. There are many environmental pollutant haloaromatics, such as chlorotoluene (Pierce *et al.*, 1983; Pierce *et al.*, 1984), chlorobenzene (DeBont *et al.*, 1986; Haigler *et al.*, 1988), and chlorophenol (Apajalahti *et al.*, 1986; Bruhn, 1988).

Dehalogenase is an enzyme that is able to release halogen from the organohalogen compounds. Some of the research results showed that the breaking of the halogen from the compounds could eliminate the toxicity of the organohalogen.

Dehalogenase that is able to break the bond of the halogen atom from the aromatic carbon ring is called haloaromatic dehalogenase (Pankaj *et al.*, 2014; Zhishu *et al.*, 2017). Most haloaromatic dehalogenase work by dihydroxylation reaction, that is the addition of two OH groups to aromatic ring using the energy of a redox reaction. Most of the haloaromatic dehalogenases are included in a huge family of enzyme called aromatic ring hydroxylating dioxygenase.

Enzymes catalyzing the dihydroxylation reaction typically consist of two or three subunits with its specific function. Three components system usually consist of reductase, ferredoxin, and terminal oxygenase (Singleton, 2012). Terminal oxygenase consists of α - and β -subunits, where the α -subunit is the most important part, as it not only has the catalytic site but also has a specific sequence that is used as a recognition area in enzyme classification.

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MATERIALS AND METHODS

Bacterial strains and plasmid

The bacterium used as a gene source was *Pseudomonas aeruginosa* local strain obtained from Microbiology Laboratory, SITH, Institut Teknologi Bandung. The *E. coli* TOP10 was used as the host cell in the cloning process, utilizing pGEM-T Easy as the cloning vector (Promega).

Chromosome isolation and amplification of *rhd* α -subunit

The chromosome of *Pseudomonas aeruginosa* local strain was isolated according to Klijn method. The three solutions used in this method were solution I (5 mg lysozym in 500 μ L of 0.1 M Tris-Cl buffer at pH 8), solution II (40 μ L of 10% (w/v) SDS, 80 μ L of 0.5 M EDTA pH 8, 8 μ L of 20 mg/mL proteinase K, and 72 μ L ddH₂O), solution III (30 mL of 5 M CH₃COOK, 5,75 mL of glacial acetic acid, and 14,25 mL ddH₂O). The isolated chromosome was used as a template in PCR for the amplification of a *rhd* α -subunit gene, using ATGGACGTCACCTCCACCCTGAGT and TCAGCTGCCGCCACCTT as the forward and reverse primers. The experiment was performed in 20 μ L PCR cocktail that composed of 7.6 μ L ddH₂O, 0.8 μ L template, 0.8 μ L forward primer, 0.8 μ L reverse primer, and 10 μ L ready mix PCR (KAPA *Taq Extra Hot Start Ready Mix*, KAPA Biosystem). The amplification was carried out for 30 cycles, which consists of 5 minutes denaturation at 95°C, 30 seconds of annealing at 55°C, and 1 minute of elongation at 72°C.

Cloning of the *rhd* α -subunit

The obtained amplicon was cloned into the pGEM-T Easy vector according to Promega standard protocol. The recombinant plasmid carrying the *rhd* α -subunit, called pGEM-*rhd*, was used to transform the *E. coli* TOP10 by heat shock method, and the positive transformants were selected by blue white screening α -complementation (Sambrook, 2001). The transformants were cultured at 37°C on Lauria Bertani agar medium containing 100 μ g/mL ampicillin, 100 μ M IPTG, and 50 μ g/mL X-gal. The *p-rhd* recombinant plasmid was isolated from the white colonies by alkaline lysis according to Sambrook protocol (Sambrook, 2001), which was then sequenced and analyzed.

Characterization and 3D structure prediction of RHD α -subunit

The RHD α -subunit protein was deduced from the *rhd* α -subunit cloned gene sequence. Characterization of the RHD α -subunit was aimed to confirm that the *rhd* α -subunit was part of the terminal oxygenase of a haloaromatic dehalogenase.

The first approach was searching for Cys-X1-His-X15-17-Cys-X2-His and His-X3-6-His-X148-149-Asp motifs (Dong, 2005) to confirm that the RHD α -subunit was an enzyme that belongs to aromatic ring hydroxylating dioxygenase family. The second approach was applying Omori classification system (Nam *et al.*, 2001), where haloaromatic dehalogenase is put in group II based on amino acid sequence homology of the α -subunit terminal oxygenase of aromatic ring hydroxylating dioxygenase family. Two softwares used in this characterization were BioEdit and Mega 6. The 3D structure of RHD α -subunit was established using I-Tasser online software (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>) and some of the suggested structures were validated using Mol Probioty online software (<http://rutilio.biochem.duke.edu/>).

RESULTS AND DISCUSSION

Amplification, cloning, and sequencing of the *rhd* α -subunit gene

The *rhd* α -subunit amplified from the *Pseudomonas aeruginosa* local strain chromosome appeared to be about 1300 bp in size as shown by the agarose gel electrophoregram visualized under the UV light. The pGEM-*rfd* recombinant clone isolated from *Escherichia coli* TOP10 transformants was confirmed by re-PCR, which then sequenced using T7 and SP6 universal primers carried by the vector. The obtained sequence of the cloned *rhd* α -subunit is shown in Figure 1, which revealed to be 1290 bp in size.

Alignment of *rhd* α -subunit and characterisation of RHD α -subunit

The cloned *rhd* α -subunit gene showed high homology to genes of *Pseudomonas aeruginosa* with 99% identity. The deduced RHD α -subunit also showed high homology to some *Pseudomonas aeruginosa* protein particularly to Gbc A protein, ring hydroxylating α -subunit, and Rieske (2Fe-2S) protein. According to the official homepage of Pseudomonas Genome Database, GbcA protein of *Pseudomonas aeruginosa* is a ring hydroxylating dioxygenase protein predicted to have the same function with anthranilate dioxygenase, a protein with haloaromatic dehalogenase activity (Tsoi *et al.*, 1999).

Further characterization of RHD α -subunit indicated that the RHD α -subunit could be classified into Group II based on Omori classification. The motif of Cys-X1-His-X15-17-Cys-X2-His appeared at Cys98-X1-His100-X17-Cys118-X2-His121 and the motif of His-X3-6-His-X148-149-Asp appeared at His217-X4-His222-X149-Asp372. The Xn indicated number of amino acids that separated the

> *rhd* α -subunit A
 TGGACGTCACCTCCACCTGAGTCTCGGCATCCCCTGGAACCGGCCCGCAAGGCCACCGCCGACATGCTG
 CGACCGCGACCACAGCTTCTCGTGCCGACGCCCTTCTACTGCGACCAGCGCTGTTTCGAGATCGACAT
 GCAGGAGATCTTCCACAAGGAATGGCTGATCGCCGGCATGACCTGCGAGATCCCGCCAAGGGCAATTC
 CTCACCCTGCAGATCGGCAAGAACCCTGCTGCTGATCCGTGGCGCCGAAAGGCCAGGTGCATGCCTTCC
 ACAATGTCTGCCGGCACCAGCGGCTCGCGCTGTGCGTGAGCGAGAAAGGCAAGGTGGCCAAGCTGGTCT
 GCCCCACCACAGTGGACCTACGAGCTGGATGGCCGCTGCTGTTTCGCCGGCACCAGATGGGCGCCG
 ACTTCGACATGAAGGAGTACGGCCTGAAGCCGATCCAGGTGAAGACCGCCGCGGGCTACATCTTCATCA
 GCCTGGCGGAGAACCCTGCCGCCATCGACGACTTCTGGCGACGCTCGAGCACTACATGGAGCCGTACG
 ACATGGAGAACGCCAAGGTGGCGGTGCGGACCACCATCCGCGAAGCCGCCAACTGGAAGCTGGTGATC
 GAGAACAACCGCGAGTGCTACCACTGCAACGGCTCCCACCCGGAAGTCTGAAGACCCTGCTGGAGTG
 GGACGACGTCACCGACCCGCGCCGACCCAGGCGCTCAAGGACCAGGTGGCGGCTGCACCAGCGCCT
 GGGACCGCGAGAAGATCCCGTACGCCATGCCAGTTCGGCCTGCGCAATCGCATCGTGCATCGCCGC
 TGCTGGACGGCAGGTGTCGATGACCATGGACGGCAAGCAGGGCAGCAAGAAGCTCATGGCCGCGATC
 AAGAATCCCGACCTCGGCTCGATGCGCATCCTGCACCTGCCGCACTCCTGGAACCACTGCATGGGCGACC
 ACCTGATCGTCTTACCCTGTGGCCGATCAGCGCCCAGGAAACCCTGGTACCACCAATGGCTGGTGCA
 CAAGGACGCGGTGGAAGGCGTGCAGTACGACGTGGCGCGCCTGCGCGAGGTATGGGACGCGACCAACG
 ACCAGGATCGTCGGCTGGCCGAGGAGAACCAGCGCGGGATCAACTCCGACGCCTACCAGCCTGGTCCAT
 ACTCGAAGACCTACGAGTTCGGTGTGATCAATTCCTCGACTGGTACAGCGAGCGCATGCTGAACAACCT
 CGGCGAGGAGTCCGCCACGTGCGCAAGGTGGCCGGCAGCTGA

Fig. 1. The nucleotide sequence of the *rhd* α -subunit.

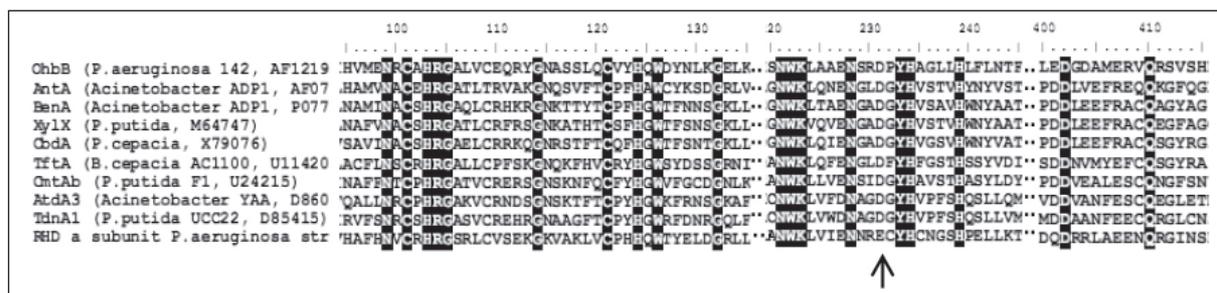


Fig. 2. The alignment of RHD α -subunit with some subunit α -subunit proteins of terminal oxygenase from Group II.

two residues in the motif. Cys98, His100, Cys118, and His121 residues were the binding sites of Rieske iron sulphur cluster (2Fe-2S) of RHD α -subunit of *Pseudomonas aeruginosa* local strain. On the other hand, His217, His222, and Asp372 residues were the binding sites of Fe atom, which is the redox centre of dihydroxylation reaction. The appearance of these two amino acid sequence motifs on the RHD α -subunit suggested that this protein was included into the aromatic ring hydroxylating dioxygenase family.

The result of protein alignment of RHD α -subunit with some α -subunit proteins of a terminal oxygenase component from Group II, showed the presence of conserved areas of proteins as depicted in Figure 2. Every single group of the Omori classification has different specific pattern and this pattern was the specific pattern of the Group II family.

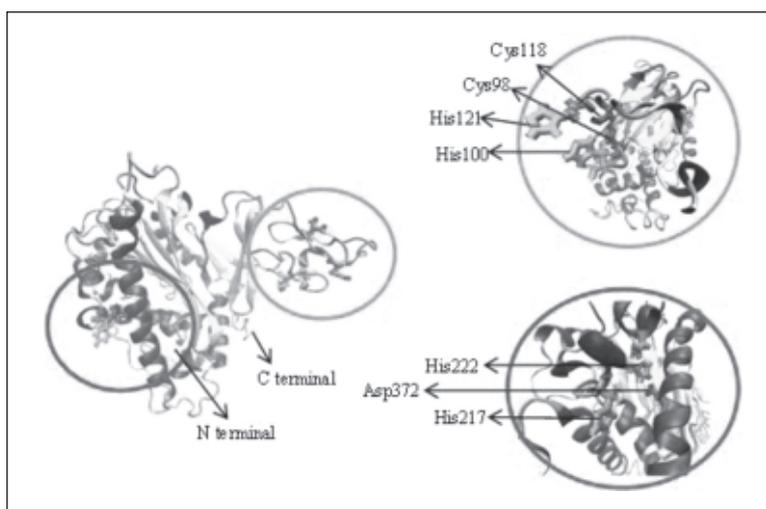
Previous research stated that there is a negatively charge amino acid residue in the region of about two amino acid before His217, which is the

Fe binding site (Kauppi *et al.*, 1998). In most bacteria, this negative residue is Asp. However, the amino acid number 214 in the deduced RHD α -subunit was Glu (mark with \uparrow in Figure 2), though the other residues that are conserved in aromatic ring hydroxylating dioxygenase namely Asn-Trp-Lys are still present. These residues were considered as the residues connecting α -subunit and β -subunit in RHD α -subunit. In Omori classification, the main characteristic is the existence of seven amino acids between Asn-Trp-Lys conserved residues and the first His of Fe binding site. As shown in Figure 5, these seven amino acids were present in the RHD α -subunit. Thus, it is evident that the RHD α -subunit is included in Group II, which also includes the haloaromatic dehalogenase.

Classification of RHD α -subunit into Group II could be seen further in the constructed phylogenetic tree based on the homology of α -subunit amino acid sequences of terminal oxygenase from aromatic ring hydroxylating family, as shown in Figure 3. This phylogenetic tree was established using three

Table 1. Validation of the predicted RHD α -subunit structure by MolProbity

All-Atom Contacts	Clashscore, all atoms:	2.52	98 th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.		
Protein Geometry	Poor rotamers	28	7.63% Goal: <1%
	Ramachandran outliers	21	4.92% Goal: <0.05%
	Ramachandran favored	360	84.31% Goal: >98%
	MolProbity score ^o	2.38	54 th percentile* (N=27675, 0Å - 99Å)
	C β deviations >0.25Å	18	4.49% Goal: 0
	Bad backbone bonds:	0 / 1715	0.00% Goal: 0%
Bad backbone angles:	4 / 2142	0.19% Goal: <0.1%	

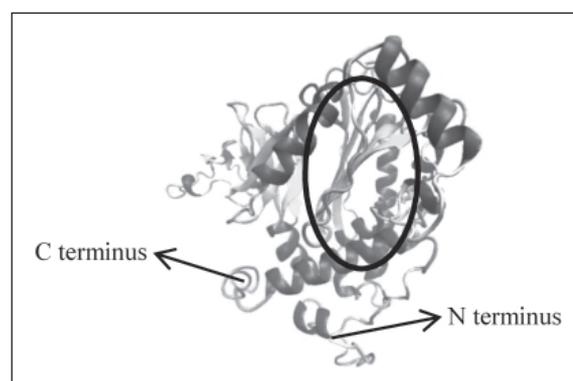
**Fig. 5.** The binding site of iron sulphur cluster [2Fe-2S] (blue circle) and the binding site of Fe atom (red circle).

score (Table 1). The Mol Probity score indicated the quality of the suggested structure, which was analyzed by comparing the determined structure with the published structures, where the highest score is 100%. Therefore, the predicted RHD α -subunit structure is a tolerable structure.

This structure has high homology with several proteins from aromatic ring hydroxylating dioxygenase family that has been published in Protein Data Bank. Some proteins that have high homology with the predicted RHD α -subunit structure are *ring hydroxylating dioxygenase* from *S. meliloti* 1021, *naphthalene 1,2-dioxygenase* from *Rhodococcus* sp., and *biphenyl dioxygenase* from *C. testosteroni*. Unfortunately, among those proteins, there is no haloaromatic dehalogenase, probably because there is no published 3D structure of haloaromatic dehalogenase.

The predicted RHD α -subunit structure has a binding site of Rieske iron sulphur cluster [2Fe-2S] which appeared as Cys98-X1-His100-X17-Cys118-X2-His121 on its primary structure. This binding site has a position on the head of RHD α -subunit. The predicted RHD α -subunit also has a binding site of Fe atom, which appeared as His217-X4-His222-X149-Asp372 on its primary structure (Figure 5).

This binding site has a position at the back of RHD α -subunit. Those parts appear on all proteins of aromatic ring hydroxylating dioxygenase family (Kauppi *et al.*, 1998; Ferraro *et al.*, 2005). At this predicted structure, there is a space under the binding site of Fe atom (Figure 6). This space is the catalytic pocket of the enzyme from aromatic ring hydroxylating dioxygenase family. Based on the results it can be concluded that the isolated *rhd* α -subunit cloned is encoding for the aromatic dehalogenase of *Pseudomonas aeruginosa*.

**Fig. 6.** Catalytic pocket of the RHD α -subunit.

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