Pirin-Like Protein from Pseudomonas stutzeri Zobell:Gene Cloning, Heterologous Expression, and Its Quercetinase Activity

Abstract of Dissertation

Studies on the Pirin-Like Protein from *Pseudomonas stutzeri* Zobell: Gene Cloning, Heterologous Expression, and Its Quercetinase Activity

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Date of Submission: July 3rd, 2015

Research Motivation

Cupin superfamily has a structure of single or repeated domain (cupin) of two β-stranded motifs which have consensus sequences containing His and Glu ligand site residues and an intervening loop [1,2]. The ligand coordinated metal ions give an enzymatic activation which is mostly on deoxygenation [3]. A kind of dioxygenase cupin, which is quercetinase (EC 1.13.11.24) catalyzes the oxygen incorporation into flavonols, especially quercetin and convert them into 2-protocatechuoylphloroglucinol (depside) and often along with CO released [4]. The enzyme is exuded by microbes as their defense against plants flavonols which are harmful for microbes because of antioxidant potency, which affect gyrase and prevent negative DNA supercoiling, so the DNA will be not replicated [5-7].

Quercetinases are possessed by fungi and bacteria. The fungal quercetinase are broadly studied, especially in products detection, environment dependency and enzyme kinetics by varieties of flavonols as part of catalytic activity; and structure profile according to crystallography or spectroscopy data or sequence homology [4-11]. In the contrary, bacterial quercetinase is rarely explored. Only kinetic in quercetin cleavage and structure examination are currently testified [12-15].

Additionally, other cupins of *E. coli* and human, which are pirin-like proteins, were reported to hold quercetinase competence. The study analyzed the competency by product and structure identification with a confirmation about the ligand site and innate metal ion cofactor [16]. However, the essential intention in ions dependency by a variety of bimetal compound supplementations and catalytic activity to other flavonols were uncertain and demanding an advance analysis.

These evaluated enzymes provided mechanism of flavonoids catabolism by terrestrial microorganism, but no study has been done among aquatic one. A pirin-like protein of a marine most respected denitrifier bacteria, *Pseudomonas stutzeri* Zobell (CCUG 16156), with no inquiry of the function has built the awareness for analyzing [17]. The marine surface with many plant detritus is an ideal condition for denitrification, but also alarming for the denitifier itself because of the detritus' flavonols antibacterial effect [18]. Thus, there is a chance that the pirin-like protein contribute in flavonols degradation in such mechanism as the bacteria defense.

The current report intended to examine this *Pseudomonas stutzeri* Zobell pirin-like protein qualification in quercetinase by managing the gene cloning, protein expression,

enzyme assay and mutagenesis. In order to get an accurate and reliable enzyme, firstly, the precise pirin gene sequence as a confirmation to the registered draft genome should be achieved, secondly this gene should be cloned into the finest vector for enzyme expression, and thirdly the expressed protein should be extracted from the cells, purified and verified. This accomplished enzyme was further analyzed to examine the structure, metal ion dependency as the cofactor, the catalytic activity in quercetin deoxygenation, the reaction product, the specific activity regarding a variety of flavonols along with particular kinetic values and the environmental reliance. Next, the low specific activity of the pirin-like protein in quercetinase which was assumable because of its insignificant active cavity has triggered a site-directed mutagenesis experiment for enlargement. The target of the mutation was Phe56 and the alternate amino acid was Ala56.

Result:

1. DNA library of the whole pirin gene sequence.

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atggcccaacgggaaattctatccatcacgaccggccggcctacatccgatggcgctggc
 M  A  Q  R  E  I  L  S  I  T  T  G  R  P  T  S  D  G  A  G
gtcagtctgacacgtgttttcggcggcgtagcaccggagcgtttcgacccttttctgatg
 V  S  L  T  R  V  F  G  G  V  A  P  E  R  F  D  P  F  L  M
ctcgacgagttcggctcgaacgatccggaagagtacatcgccggcttcccgccgcacccg
 L  D  E  F  G  S  N  D  P  E  E  Y  I  A  G  F  P  P  H  P
catagaggtttcgaaaccatcacctatatgctcgaagggcggatgcgccacgaggatcac
 H  R  G  F  E  T  I  T  Y  M  L  E  G  R  M  R  H  E  D  H
atgggcaacgtcgggcggctggaaagcggcggtgtgcagtggatgaccgcagcacgcggt
 M  G  N  V  G  R  L  E  S  G  G  V  Q  W  M  T  A  A  R  G
gtgatccacagcgaaatgccggagcaggaggaaggcctcatgcgcggcttccagctgtgg
 V  I  H  S  E  M  P  E  Q  E  E  G  L  M  R  G  F  Q  L  W
ctgaacctgcccgcccacgccaagctcggagagccgggttaccgcgacttcgcacctgca
 L  N  L  P  A  H  A  K  L  G  E  P  G  Y  R  D  F  A  P  A
gagattccccaggtgcgcctcgaaaacggcgtacgggccaaggtcatcgccggaacattg
 E  I  P  Q  V  R  L  E  N  G  V  R  A  K  V  I  A  G  T  L
aaggcggaaggcatcgagcaccaaggcgtcgtgcagcggcccgataccgagccccagcta
 K  A  E  G  I  E  H  Q  G  V  V  Q  R  P  D  T  E  P  Q  L
ttcgatctgcacttgcccgccggtagcacgttctcgccgcagatcccggacggccacctg
 F  D  L  H  L  P  A  G  S  T  F  S  P  Q  I  P  D  G  H  L
ttgttgctctatgtatacgagggcgcgctgcaggtcggcgatcagccggtcggcaagggc
 L  L  L  Y  V  Y  E  G  A  L  Q  V  G  D  Q  P  V  G  K  G
cagctggtgcgcctgtccgaacagggtgagctgcaattacacagcgagaccggcgcacga
 Q  L  V  R  L  S  E  Q  G  E  L  Q  L  H  S  E  T  G  A  R
ctgatgctgctcgccggccgaccgctgagagaacccatcgtgcagtacggtccgtttgtg
 L  M  L  L  A  G  R  P  L  R  E  P  I  V  Q  Y  G  P  F  V
atgaatagccgcgaggaggtcgagcaagcgctgcgggattttcgcgatgggacactggcc
 M  N  S  R  E  E  V  E  Q  A  L  R  D  F  R  D  G  T  L  A
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Fig. 1. The nucleotides sequences of the pirin gene (written in lowercase) and the translated amino acid sequences of the pirin protein (written in uppercase).

Genome walking method by DNA Walking SpeedUp Premix Kit II has able to deliver a complete sequence of the pirin gene as detailed in Fig. 1. which is equivalent to the reported draft genome [17]. This method of gene amplification is recommended for

determination of nucleotide sequences adjacent to a known region because of its nucleotide highly recognition by some designed primers and its timesaving strategy [19]. This gene was then cloned into pGEM-T Easy Vector as a genome library for further experiment.

2. Expression vector.

The pUC18 vector was assigned as the finest vector because it preserved a suitable nucleotides sequence which deliver an appropriate pirin protein. This pUC has a *lac* promoter. This promoter might produce a low protein expression, but it can be implemented by many bacteria, including the ones without lactose transporter [20].

3. Extraction, purification and validation of the pirin-like ptotein.

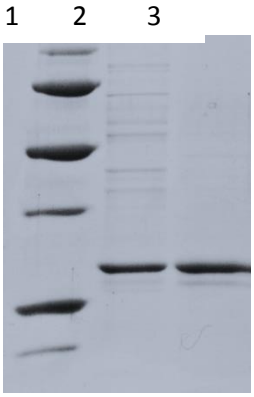

Fig. 2. The SDS-PAGE to check the purity of the pirin protein with 1 was the Precision Plus Protein Prestained Standard of Bio-Rad, 2 is the Ni-affinity chromatography protein purified, and 3 is the gel filtration protein purified.

The expressed pirin-like protein of the recombinant pUC 18 within the BL21 (DE3) cells was extracted by sonication. Two phases of purification, the Ni-NTA and the gel filtration column chromatography were able to provide the most reliable protein refinement as Fig. 2. This protein was authorized to be the pirin protein based on its N-terminus protein sequence.

4. The pirin-like protein structure of the pirin.

The pirin-like protein sequences of *Pseudomonas stutzeri* Zobell was aligned to the other enzyme sequences and gave result of identical relation of 25%, 29%, and 37% to *A. japonicus* quercetinase, *B. subtillis* quercetinase, and *E. coli* pirin respectively. Thus, the enzyme structure was built as a homology modelling toward *E. coli*, which indicate a bicupin appearance with N-terminal domain has the residues for metal ion ligand. The residues are His59, His, 61, His103 and Glu105.

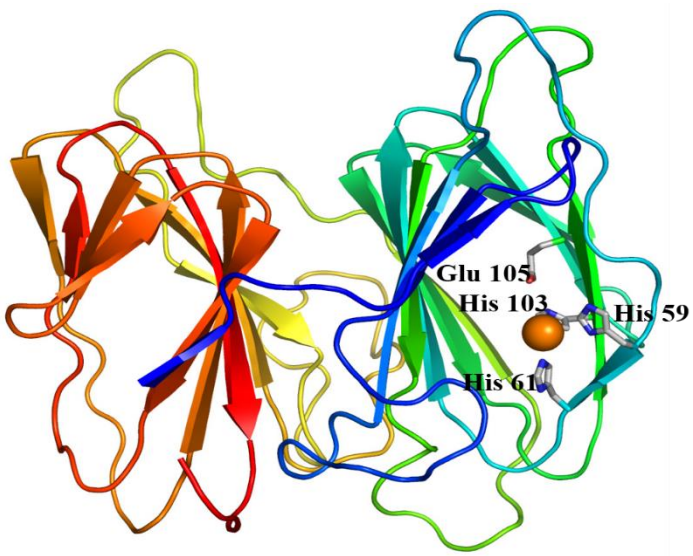

Fig. 3. Structural model of *Pseudomonas stutzeri* strain Zobell pirin-like protein which was constructed based on the *E. coli* pirin-like protein structure [PDB ID: 1TQ5] with red-yellow as the C-terminal domain and blue-green as the N-terminal domain. The labelled amino acid of the N-terminal domain are the ligand site residues.

5. Metal ion cofactor.

Divalent metal ions adjustment was combined to the apo pirin-like protein to induce such activity in flavonols degradation. Among the additional metal ions, copper has become the most reliable one. Additionally, the ratio of other metals of Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} in activity deduction to Cu^{2+} are 4.04-, 4.28-, 6.93-, 9.63-, and 11.46-fold of reduction, respectively. Thus, copper and iron were considerable proper as representative to explore the number of the coordinated metal ion in the ligand site which was done by titrations experiment. The result gave an agreement to the fact that only a single ligand site is present in the pirin-like protein.

The spectra analysis sustain the coordinated ions and ligand site details. The UV-VIS spectra as in Fig. 4 (A) with its 650 nm band was assigned to be the bound copper to the pirin-like protein. The intensity is in the range of the other Cu-proteins with type II Cu. This type II Cu was also confirmed by the g_{II} = 2.26, g_{I} = 2.064 A_{I} = 16.7 mT values of the gained EPR spectra as in Fig. 4 (B). The correlation of the A_{I} and g_{II} gave an indication of the bound three histidines and one glutamine residues to the copper ion.

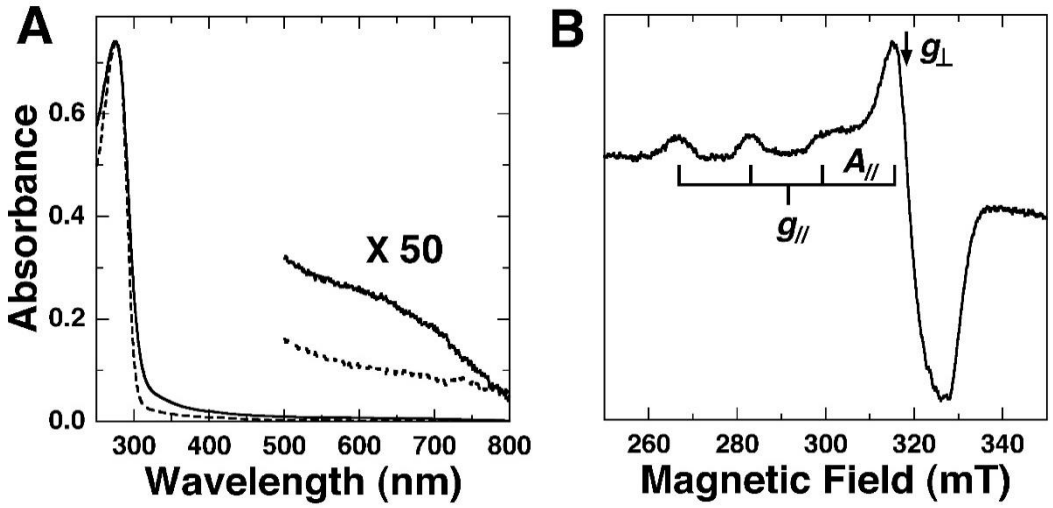

Fig. 4. The UV-visible (A) and X-band EPR at 77 K and 5 mW microwave power (B) of the pirin protein within 100 mM phosphate buffer pH 8.0. Note: the solid line is the Cu-pirin and the dotted line is the apo-pirin.

6. Pirin-like protein competence in quercetinase and product recognition.

There was a degradation of quercetin after the enzyme addition which was observed as a reduce spectra of the quercetin maximum absorbance. This degradation was measured as a specific activity of the Cu^{2+}-acted pirin-like protein against quercetin which was 1.2 U/mg. In order to confirm that this degradation was quercetinase activity, product recognition of depside (in this respect is the intermediate) and carbon monoxide has been made. This experiment has a fall of quercetin peak at 380 nm which was followed by an increase of peak at 330 nm after the Cu-pirin-like protein was combined into the quercetin reaction solution. 330 nm peak emergence was probably exposed by an intermediate in quercetin oxygenation [21]. This intermediate was most probably oxohydroperoxide or cyclic oxoperoxide which are the intermediates of quercetin base- or enzyme- catalyzed oxygenation [22]. Another product, which is carbon monoxide was detected by the formation

of black precipitate upon $PdCl_2$ soaked filter paper which was placed in the top of the enzyme reaction solution. The precipitate is presumed to be an elemental palladium as the outcome of Pd^{2+} reduction [23].

7. Pirin-like protein specific activity and particular kinetic values.

Among the 8 flavonols applied as the Cu- pirin-like protein substrates, only quercetin, myricetin and fisetin are able to be oxidized by the pirin- like protein. However, the rate upon fisetin is considerable low, so the K_m and V_{max} value are measured using quercetin and myricetin. The occupied kinetic parameters, the K_m and V_{max}, of both oxidation are 13 µM and 1.2 U/mg for quercetin substrate respectively, and 9.4 µM and 5.3 U/mg for myricetin substrate respectively. The *Km* values by Cu-pirin-like protein deoxygenation are comparable to the given value of other quercetinases while the V_{max} values are lower. This offers a sign that pirin homologue of the *P. stutzeri* has habitually affinity to quercetin compared to quercetinase but might be the activity is not intrinsically appeared in the current reported pirin.

8. Optimal temperature and pH for quercetinase activity of the pirin-like protein.

In order to define the appropriate environment condition for the pirin-like protein in quercetinase activity, several temperature adjustment and pH arrangement by Britton-Robinson buffer have been made. The result of this optimum environment was 40°C for temperature and 7.24 for pH as depicted on the following figure.

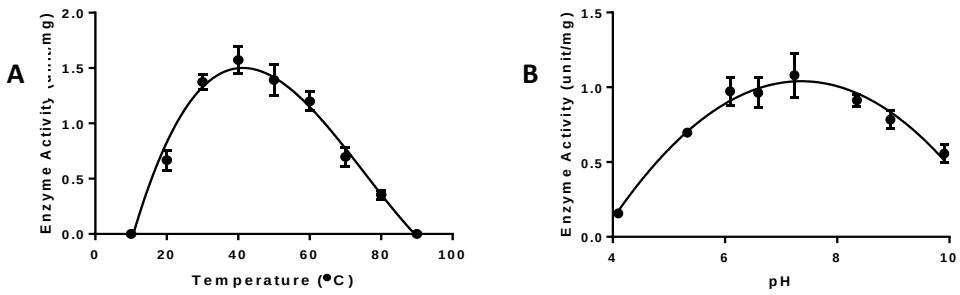

Fig. 5. Cu-pirin-like protein characterization of (A) optimum temperature in 50 mM Tris-HCl ph 7.5 and (B) optimum pH by using 50 mM Britton-Robinson buffer arrangement.

9. Site-directed mutagenesis.

The experiment was successfully managed a site-directed mutagenesis upon the pirin-like gene in which the expressed protein has an alternation of Phe56 to Ala56. However, the purpose of getting an active cavity enlargement was thought to be neglected because the mutant enzyme was unable to cleave the quercetin substrate.

Moreover, a study on a bacterial Ig-like protein mutations with Phe shift to the Ala as one of it, give a happening of a disruption toward the original enzyme folding. This mutation of Phe to Ala decrease the packing density and stability of the protein domain, which in turn reduce the size and the hydrophobicity, and finally the energy for enzyme β sheet folding within its sandwich structure was declined and the substrate cannot accurately come into the protein cavity [24].

Conclusion:

The study was able to provide a comparable pirin-like protein gene to the reported *Pseudomonas stutzeri* Zobell draft genome. The gene was achieved by genome walking and successfully cloned into pUC18 as the finest vector for the pirin-like protein expression. The expressed protein was extracted by sonication for breaking the host cells which is BL21 (DE3). Column chromatography procedure by Ni-NTA and gel filtration have delivered a considerable pure enzyme which has been N-terminal amino acid sequence confirmed to be the pirin-like protein. This enzyme was approved for having quercetinase competence as depside intermediate and carbon monoxide produced in the catalytic quercetin degradation. The enzyme quercetinase activity has an optimum temperature of 40°C and pH of 7.24.

Further analysis of this reliable enzyme has invented three contemporary results. The first was the pirin-like protein metal dependency which was taken by a separated addition of bimetal ion of Fe^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+} in which copper was settled to be the most suitable cofactor for the enzyme deoxygenation. Copper was feasibly bound to the ligand residues of His59, His61, His103 and Glu105 as elucidated by sequence alignment and homology modelling structure regarding to *E. coli* pirin-like protein. An adjusting type II Cu coordination and the proposed ligand residues were checked by UV-VIS spectrophotometer and EPR.

The second was the substrates specificity against myricetin, kaempferol, fisetin, galangin, taxifolin, morin, and luteolin, in which myricetin shows a higher specificity than quercetin. The last one was managing a site-directed mutagenesis over the pirin gene to

expand the expressed enzyme active site cavity but unfortunately could not achieve the intention which was to increase the rate of the pirin-like protein in quercetinase activity.

Finally, this study of pirin-like protein could not gain a clear quercetinase mechanism, but it stipulated a valuable marine bacteria representative of enzymatic degradation of flavonoid.

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平成２７年　８月　６日

学位論文審査報告書（甲）

１．学位論文題目（外国語の場合は和訳を付けること。）

Studies on the Pirin-Like Protein from *Pseudomonas stutzeri* Zobell: Gene Cloning, Heterologous Expression, and Its Quercetinase Activity.

Pseudomonas stutzeri Zobell 株由来のピリン様タンパク質に関する研究：遺伝子クローニング，異種発現，およびケルセチナーゼ活性

２．論文提出者　(1) 所　　属　物質科学　専攻

(2) 氏（ふり）　名（がな）　Talitha（たりさ） Widiatningrum（ういでぃあとにんぐらむ）

３．審査結果の要旨（600～650 字）

提出学位論文に関して各審査委員が個別に審査を行なうとともに，平成 27 年 7 月 14 日に，申請者の口頭説明に対する予備審査会を実施した。その後，平成 27 年 8 月 6 日に行なわれた公聴会の後に論文審査委員会を開催し，以下のように判定した。

植物が根圏に放出するケルセチン等のフラボノイドは抗菌活性を示す。このため微生物はフラボノイドを代謝分解するが，カビ以外の微生物の代謝については不明な点が多い。本論文は，海洋性脱窒菌 *Pseudomonas stutzeri* Zobell 株を材料に，フラボノイド酸化分解活性を示すピリン様タンパク質について次のことを明らかにしたものである。(1) *P. stutzeri* 染色体 DNA からピリン様タンパク質遺伝子をクローン化し，アミノ酸配列を決定した。(2) 大腸菌を宿主とするピリン様タンパク質の異種発現系を構築し，組換え型タンパク質を均一に精製した。(3) 活性中心に銅イオンを結合したピリン様タンパク質が，フラボノイド酸化活性（ケルセチナーゼ活性）持ち，同じタンパク質ファミリーに属する真菌ケルセチナーゼとは異なる特異性を示すことを初めて明らかにした。

これら本研究の成果は，ピリン様タンパク質のフラボノイド分解活性を初めて定量的に評価したものであり，細菌，特に海洋性細菌におけるフラボノイド代謝の解明に資するものである。従って，本博士論文は博士（学術）の学位に値するものと判断した。

４．審査結果　(1) 判　　定（いずれかに○印）　（合　格）・　不合格

(2) 授与学位　　博　士（　学術　）