การแสดงออกของรีคอมบิแนนต์เพปไทด์ที่มีฤทธิ์ต้านออกซิเดชันจากสาหร่าย
ใน *Pichia pastoris* GS115 และ *Escherichia coli* MG1655

นางสาววรรณพรรณ ภู่เล็ก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EXPRESSION OF RECOMBINANT ANTIOXIDATIVE PEPTIDES FROM ALGA E IN *Pichia pastoris* GS115 AND *Escherichia coli* MG1655

Miss Wannapan Poolex

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	EXPRESSION OF RECOMBINANT ANTIOXIDATIVE PEPTIDES FROM ALGAE IN *Pichia pastoris* GS115 AND *Escherichia coli* MG1655
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Field of Study	Biotechnology
Thesis Advisor	Ruethairat Boonsombat, Ph.D.

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วรรณพรรณ ภู่เล็ก : การแสดงออกของรีคอมบิแนนต์เพปไทด์ที่มีฤทธิ์ต้านออกซิเดชันจากสาหร่ายใน *Pichia pastoris* GS115 และ *Escherichia coli* MG1655 (EXPRESSION OF RECOMBINANT ANTIOXIDATIVE PEPTIDES FROM ALGAE IN *Pichia pastoris* GS115 AND *Escherichia coli* MG1655) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ฤทัยรัตน์ บุญสมบัติ, หน้า.

งานวิจัยที่ผ่านมาได้มีการค้นพบเพปไทด์ที่มีคุณสมบัติเป็นสารต้านออกซิเดชันจากขบวนการย่อยน้ำเสียจากการผลิตสารสกัดจากสาหร่าย เพปไทด์ดังกล่าวมีกรดอะมิโนเป็นองค์ประกอบอยู่ 11 โมเลกุล ดังนี้ VECYGPNRPQF งานวิจัยนี้นำเพปไทด์นี้มาทำการศึกษาและเรียกเพปไทด์ชนิดนี้ว่า เพปไทด์ AW โดยมุ่งเน้นการผลิตเพปไทด์ AW ที่มีฤทธิ์ต้านออกซิเดชันในเชื้อจุลินทรีย์ 2 สายพันธุ์ ได้แก่ *P. pastoris* GS115 และ *Escherichia coli* MG1655 ซึ่งเชื้อจุลินทรีย์ทั้งสองสายพันธุ์ถูกสร้างเพื่อให้สามารถผลิตเพปไทด์ AW ที่มีฤทธิ์ต้านออกซิเดชัน ได้ในปริมาณมาก โดยใช้เทคนิคการสร้างดีเอ็นเอสายผสม ชิ้นดีเอ็นเอถูกออกแบบให้มีชุดข้อมูลของเพปไทด์ AW เรียงต่อกัน 6 ชุด เชื่อมต่อด้วยกรดอะมิโนไลซีน ในกรณีของ *P. pastoris* GS115 นั้น ชิ้นดีเอ็นเอที่สนใจจะถูกแทรกอยู่ในโครโมโซมของเซลล์ แล้วจึงชักนำให้มีการผลิตเพปไทด์ AW ออกนอกเซลล์ แต่อย่างไรก็ตามพบว่ารีคอมบิแนนท์เพปไทด์ AW ไม่มีการผลิตออกนอกเซลล์ อีกทั้งการตรวจสอบรีคอมบิแนนท์เพปไทด์ดังกล่าวจาก *P. pastoris* GS115 นั้นทำได้ยุ่งยากและซับซ้อนเนื่องจากไม่มีตัวติดตามโปรตีน ในส่วนของการผลิตรีคอมบิแนนท์เพปไทด์ AW ใน *E. coli* MG1655 ที่มีชื่อสายพันธุ์ว่า AW จากการตัดต่อชิ้นดีเอ็นเอที่สนใจเข้ากับเอ็กเพรสชันเวคเตอร์ pQE-30 Xa แล้วทรานสฟอร์มเข้าสู่เซลล์ พบว่าสามารถตรวจพบการผลิตรีคอมบิแนนท์เพปไทด์ AW ด้วยวิธี qRT-PCR และพบว่าเชื้อจุลินทรีย์สายพันธุ์ AW มีการผลิตรีคอมบิแนนท์เพปไทด์ได้ในปริมาณน้อย นอกจากนี้ยังพบว่ารีคอมบิแนนท์เพปไทด์อยู่ในส่วนของโปรตีนที่ละลายอยู่ในเซลล์ โดยรีคอมบิแนนท์เพปไทด์ AW สามารถวิเคราะห์ได้ด้วยการประยุกต์ใช้วิธี immunoblotting โดยใช้แอนติบอดี antiHis-HRP ในการจับจำเพาะ และรีคอมบิแนนท์เพปไทด์ AW สามารถทำให้บริสุทธิ์ได้โดยใช้ Ni^{2+} affinity column แต่อย่างไรก็ตามการทำให้เพปไทด์บริสุทธิ์นั้นยังพบการปนเปื้อนของโปรตีนขนาดใหญ่ในส่วนอีลูชัน สำหรับการตรวจสอบคุณสมบัติการต้านออกซิเดชันของรีคอมบิแนนท์เพปไทด์ AW พบว่า อีลูชันรีคอมบิแนนท์เพปไทด์ AW สามารถขจัดอนุมูลอิสระ ABTS ได้ดีกว่าการอนุมูลอิสระ DPPH นอกจากนี้ยังพบว่าอีลูชันรีคอมบิแนนท์เพปไทด์สามารถป้องกันดีเอ็นเอถูกทำลายเนื่องจากอนุมูลอิสระไฮดรอกซิลได้ แต่อย่างไรก็ตามการออกฤทธิ์ต้านอนุมูลอิสระของอีลูชันรีคอมบิแนนท์เพปไทด์ AW ต่ำกว่าเพปไทด์ AW ที่ได้จากการสังเคราะห์ทางเคมี ดังนั้นเพื่อพัฒนาการผลิตรีคอมบิแนนท์เพปไทด์ AW ใน *E. coli* สายพันธุ์ AW ควรมีการศึกษาสภาวะที่เหมาะสมต่อการผลิตเพปไทด์ การทำรีคอมบิแนนท์เพปไทด์ให้บริสุทธิ์ และการทดสอบการออกฤทธิ์ต้านออกซิเดชันอื่น ๆ ต่อไป

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5572098223 : MAJOR BIOTECHNOLOGY
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WANNAPAN POOLEX: EXPRESSION OF RECOMBINANT ANTIOXIDATIVE PEPTIDES FROM ALGAE IN *Pichia pastoris* GS115 AND *Escherichia coli* MG1655. ADVISOR: RUETHAIRAT BOONSOMBAT, Ph.D., pp.

The peptide extracted from algae protein waste hydrolysate with the 11 amino acid sequence VECYGPNRPQF, named as AW peptide in this research, was found to be a potential antioxidant. In this study, recombinant *Pichia pastoris* GS115 and *Escherichia coli* MG1655 were constructed to provide a high-level expression of this antioxidative peptide. For *P. pastoris*, the 234 base pair DNA fragment containing six copies of the target peptide linked by the codons of lysine was integrated to the chromosome. However, the recombinant peptide could not be obtained. It might be due to the intracellularly accumulation of target peptide which could not be extracted due to the lack of a tagged marker. The construction of recombinant *E. coli* for expressing the target peptide was done by cloning the same DNA fragment into pQE-30Xa expression vector before being transformed into *E. coli* MG1655. The verified strain, named as AW strain, showed the expression at transcriptional level detected by RT-PCR. Moreover, the target peptide was in the soluble protein fraction but the protein yield was still low. The target protein band was verified by immunoblotting with the detection of antiHis-HRP antibody. The crude fraction containing recombinant AW peptide elution from the Ni^{2+} affinity column. The DPPH and ABTS scavenging activities of the recombinant AW peptide elution were lower while the in vitro protective effect on DNA damage induced hydroxyl radicals was better than those of chemical synthesized one. To improve the recombinant AW peptide production, the optimization and purification, along with other antioxidant properties, should be further investigated.

Field of Study: Biotechnology Student's Signature .....

Academic Year: 2015 Advisor's Signature .....

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°C	degree Celsius
dNTP	deoxynucleoside triphosphates
g	gram
l	liter
LB	Luria-Bertani
µg	microgram
µl	microliter
mg	milligram
ml	milliliter
mmole	milimole
mM	millimolar
OD	optical density
rpm	revolutions per minutes

CHAPTER I
INTRODUCTION

Rationale

Free radicals can damage biological molecules such as DNA, protein and lipid. This potentially leads to many diseases such as atherosclerosis, cancer and Alziemer's. In normal cell, free radical can be eliminated by many mechanisms, for example, superoxide dismutase, peroxidase, and glutathione peroxidase. However, due to the nature of free radicals that are very reactive and the accumulation of free radical caused by pollution and life style, the mechanisms to scavenge them in the body are not enough. One of the alternatives to get rid of free radical in the body is obtaining antioxidants from external sources such as food. Moreover, antioxidants extracted from natural sources, which are considered being safe for consumers, are found in many kinds of medicines, food supplements and cosmetics.

However, protein extraction from natural sources usually encounters some problems such as requirement of large amount of sample and sample degradation. Furthermore, sample storage is a key process for extracting target protein with good quality and quantity. To improve the production of the target antioxidative peptide, recombinant DNA technology was applied in this project. The methylotrophic yeast *Pichia pastoris* GS115 and *Escherichia coli* MG1655 were selected as a host organism for producing the interested antioxidative peptides.

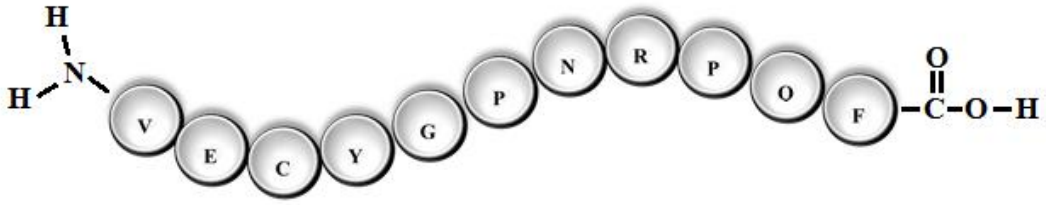


Figure 1.1 The amino acid sequence of antioxidative peptide found in algae protein waste hydrolysate.

In this research, an antioxidative peptide with amino acid sequence of VECYGPNRPQF (Figure 1.1), originally found in algae protein waste hydrolysate, was selected. Due to a small peptide, a DNA fragment encoding 6 copies of this antioxidative peptide which Lysine residue is chosen as a linker, was synthesized, and then express in *P. pastoris* and *E. coli*. Furthermore, the antioxidative characterization of this recombinant peptide was investigated.

Objective:

The aim of this study is focus on expression of a recombinant antioxidative peptide, originally found in algae waste hydrolysate, in *P. pastoris* GS115 and *E. coli* MG1655. Furthermore, antioxidant activities of the recombinant peptide, such as the protection effect of the purified peptide on oxidation-induced DNA damage, and ABTS, DPPH scavenging ability assay, are also investigated.

CHAPTER II
LITERATURE REVIEWS

2.1 Free radicals

Free radicals or reactive oxygen species (ROS) are unstable and independently existent atoms which are composed of unpaired electrons in the outermost shell configuration. Free radicals can be generated by an exposure to a variety of chemical and physical agents such as pollution, smoke, and radiations. Furthermore, ROS is produced by endogenous mechanisms as pathological conditions such as inflammation and production of toxic by-products during aerobic metabolism under non-completed process, defined as "oxidative stress" in normal cell. The effect of oxidative stress is involved in either viability of cell or induction of cellular responsibility through generation of secondary reactive species. It can lead to death cell by necrosis or apoptosis (Prior 2015)

2.1.1 Reactive oxygen species reactions

The most important reaction is oxidation occurring mostly within mitochondrial via electron transport chain mechanism (ETC).

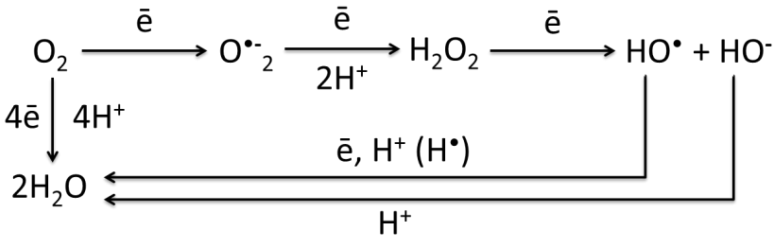


Figure 2.1 The schematic of reduction of oxygen through four and one-electron (Lushchak 2014).

Under an aerobic condition, more than 90% of oxygen molecule in living organisms is directly reduced to water by cytochrome oxidase through four-electron mechanisms without releasing of ROS (Ott *et al.* 2007) On the other hand, much less than 10% of oxygen, one electron is reduced resulting in conversion of oxygen to superoxide anion (O_2^{-}). This reaction is followed by the reduction of one electron and receiving two protons (H^+) concurring, and then a molecule of hydrogen peroxide (H_2O_2) is yielded. When H_2O_2 obtained one electron, the molecule splits up into hydroxyl radical ($OH^{\bullet}$) and hydroxyl anion (OH^-). Then, in the final stage, water molecule is formed by the interaction of hydroxyl radical with one electron and one proton (Figure 2.1). Probably, ROS are generated mainly in mitochondrial ETC by electron escaping from coenzyme Q to oxygen.

Normally, molecule is stabilized by sharing electrons to maintain equal pairs. However, when molecule splits out and then causes unpaired electron, it can attack other molecules; for example, DNA, lipids, and proteins, from living cell leading to a deterioration of biological molecules. This damage is potentially causative in many diseases such as aging, arthritis, atherosclerosis, cancer and Alziemer's. To stop the damage, antioxidants are required to neutralize these free radicals in the body (Figure 2.2).

In normal cell, free radicals can be eliminated by many kinds of mechanisms. However, due to the nature of free radicals that are very reactive and the accumulation of free radical caused by pollution and life style, the mechanisms to scavenge them in the human body are not enough. One of the alternatives to get rid of free radicals is obtaining antioxidants from external sources such as food.

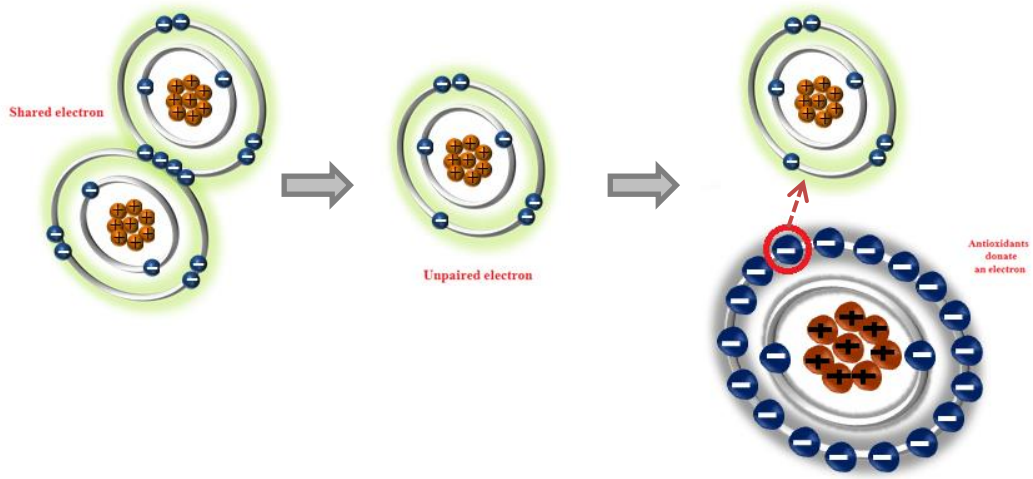


Figure 2.2 The neutralization of a free radical by an antioxidant molecule.

2.1.2 The kinds of reactive oxygen species

Examples of reactive oxygen species forms are listed as the following.

2.1.2.1 Superoxide anion (O_2^-)

Superoxide anion (O_2^-), a common form of oxygen free radicals, is generated by the reduction of one oxygen molecule to water. It can directly initiate lipid peroxidation. Although superoxide anion is quite toxic, it is also essential in the phagocyte of the immune system for killing bacteria cells. However, it still has some problems because simultaneous oxidation and reduction process released hydrogen peroxide (H_2O_2). Besides, superoxide anion dis-mutation and the coactive reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) generate more harmful reactive species afterward such as singlet oxygen and hydroxyl radical.

2.1.2.2 Hydrogen peroxide (H_2O_2)

Although H_2O_2 is collectively called reactive oxygen species, it is not a free radical (Lushchak 2014). It can be generated by the simultaneous oxidation and reduction process of superoxide anion. In plants, the hydrogen peroxide formation was found in photosystem under light stress in chloroplasts, mitochondria, and peroxisomes(Goiris *et al.* 2015)

2.1.2.3 Hydroxyl radical ($OH^{\bullet}$)

Hydroxyl radical ($OH^{\bullet}$) is classified as a type of ROS. It is occurred by cleaving oxygen-oxygen bond with UV radiation. This molecule may either destroy cell membrane by splitting covalent bonds out into proteins and carbohydrates, or may react with other biological molecules including sugars, amino acids, fatty acid, phospholipids and organic acids.

2.1.2.4 Peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals

In addition, peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals, oxidizing agent, are also a kind of reactive oxygen species by metal transition in biological systems of lipid peroxidation. The protonated form of superoxide anion is HO_2•, which usually termed as either hydroperoxyl radical or perhydroxyl radical. HO_2• can be regarded as the simplest form of the peroxyl radical (Prior 2015)

2.1.2.5 Singlet oxygen ($^1O_2$)

Although oxygen is important in biological systems, it can be a radical after exposure to irradiation, various chemicals, and biological processes, resulted in singlet oxygen ($^1O_2$).The excessive singlet oxygen can interact with DNA, lipids, and proteins in human body causing disease (Prior 2015) Furthermore, it also is highly generated from the photosystem II reaction center in the chloroplasts of photoautotrophic organisms under light stress (Goiris *et al.* 2015)

2.2 Antioxidants

Antioxidants are substances that defend biological molecules against deteriorating effects from free radicals, or inhibit the oxidation reaction. In human body, there are endogenous antioxidants which are non-enzymatic forms and low molecular mass chemical compounds such as uric acid, glutathione, albumin, vitamins and phenols, and other nutritional factors. Moreover, antioxidants which are high molecular mass enzymatic forms are usually found. For example, superoxide dismutase (SOD) transforms superoxide anion to hydrogen peroxide, and then catalase and glutathione peroxidase, revolutionizing enzymes, further react with hydrogen peroxide (Lushchak 2014).

Antioxidants play a pivotal in protection of healthy animals by preserving the balance of ROS production that strongly influences on the function of signal transduction of gene expression in immune cell, membrane lipids, cellular proteins, and nucleic acids. Moreover, to neutralize those free radicals, antioxidants binds with them to share electron instead of biological molecules in body.

Many kinds of antioxidants can be found in foods such as vitamin C (ascorbic acid), vitamin E (tocopherol), carotenoid, and polyphenols. Along with superoxide dismutase, catalase, and glutathione peroxidase, each nutritional antioxidant affects free radicals through different mechanisms including maintaining the roles of immune cells, directly neutralizing free radicals, and sustaining as a cofactor for stimulation of ROS-eliminating enzyme. In addition, they adjust NF-KB pathway which affects innate immunity and inflammation pathway.

2.2.1 Determination of antioxidant activities

Several *in vitro* methods for the evaluating of antioxidant activity are summarized in this research. The currently preferable methods, which are different according to the different chemicals, can be biological such as hydroxyl radical and hydrogen peroxide scavenging, or non-biological such as 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH• assay), 2,2-azinobis-(3-ethylbenzothaizoline-6-sulphonate) radical scavenging (ABTS assay) (Boligon 2014). Generally, a screening method of antioxidant activity is presented as a decolorization assay that absorbance at a certain wavelength can be measured by spectrophotometer. Moreover, there are some researches demonstrated the ability of antioxidant on oxidation-induced DNA damage protection. An antioxidant determination is applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, and plasma antioxidant. Each method concerns about the generation of different radicals acting through a variety of mechanisms. Two procedures of approaches have been observed. One is the inhibition assay related to the extent of the scavenging by hydrogen- or electron-donation of a pre-

formed free radical. The other is involved in the appearance of antioxidant system during the generation of the radical (Re *et al.* 1999).

2.2.1.1 ABTS scavenging ability assay

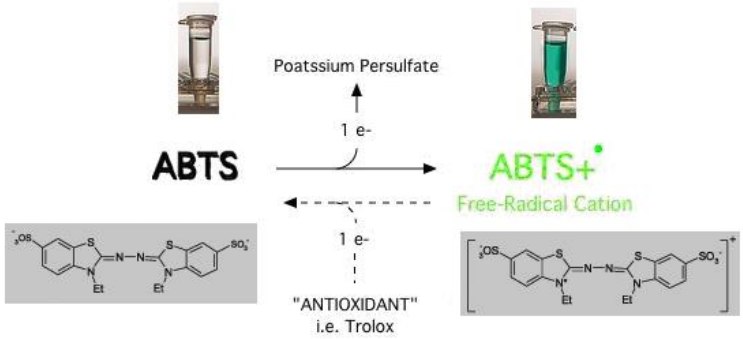


Figure 2.3 The ABTS scavenging reaction (Boligon 2014).

This method represents the relative ability of an antioxidant, including pure substance and aqueous mixture, to scavenge the $ABTS^{\bullet+}$ radical cations. This assay is useful for discriminating between additive and synergistic effect. ABTS assay is based on the interaction between a putative antioxidant and ABTS radical cation ($ABTS^{\bullet+}$), which is generated through reaction of a strong oxidizing agent as potassium permanganate or potassium persulfate with ABTS salt. The formation of ABTS radical cation presents the characteristic color that is the basis of spectrophotometer measurement. The developed technique for $ABTS^{\bullet+}$ generation is described as the reaction between ABTS and potassium persulfate which directly produces blue/green $ABTS^{\bullet+}$ chromophore. This chemical structure can be measured by the absorption maxima at 645 nm, 734 nm, and 815 nm. To investigate in an antioxidant activity, the reaction can be observed by the conversion of blue $ABTS^{\bullet+}$ radical cation

to colorless neutral form by hydrogen-donating antioxidant as summarized in figure 2.3 (Re *et al.* 1999, Boligon 2014).

The result of antioxidant activity is reported as percentage of the $ABTS^{\bullet+}$ radical cation inhibition associated with the relative concentration of antioxidant. The extent of decolorization can be monitored by spectrophotometer. The percentage of inhibition can be calculated by the following equation, of which Trolox or ascorbic acid can be used as a standard.

$$\% \text{ Inhibition} = \frac{(OD_{control} - OD_{blank}) - (OD_{sample} - OD_{blank}) \times 100}{(OD_{control} - OD_{blank})}$$

2.2.1.2 DPPH scavenging ability assay

DPPH assay is one of the simplest procedures for evaluating antioxidant activity because DPPH• radical is stable, commercially available and rapid. This method can be used for analyzing pure compounds as well as complex samples. Therefore, this is the most widely used among the methods. DPPH• radical is the purple chromogen. However, when it is reduced by a hydrogen-donating antioxidant or a reducing compound, the purple DPPH• radical is faded to pale yellow hydrazine (figure 2.4). The development of this reaction can be monitored by spectrophotometer at the wavelength 518 nm. The inhibition concentration (IC_{50}) is calculated to demonstrate the amount of antioxidant necessary to decrease 50% of the initial DPPH• concentration (Boligon 2014).

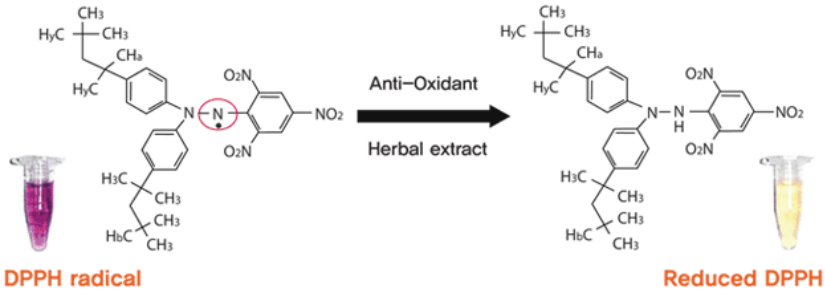


Figure 2.4 The reaction of antioxidant activity by DPPH radical scavenging.
(Reference:http://www.damocos.co.kr/damo/language/english/lab_paper3.php)

2.2.1.3 Hydroxyl radical scavenging assay

The hydroxyl radical (HO•) is generated by Fenton reaction, the combination of Fe (II) and hydrogen peroxide. Because most of substances in biological systems can be considered as HO• scavengers, this method is not performed by any specific molecules and enzymes. HO• scavenging ability assay requires very high concentration of an antioxidant to compete with adjacent molecules for HO• generated. However, this assay is useful to quantify the ability of an antioxidant to scavenge or block the precursors related to HO• creation (Boligon 2014). Furthermore, the functional objective of this reaction emphasizes on the protective property of antioxidant. Sheih and coworkers investigated the protective effect of antioxidant on DNA damage induced by hydroxyl radicals which are based on the Fenton reaction. They found that the addition of potential antioxidative peptide with amino acid sequence of VECYGPNRPQF at the concentrations of 84.9 µM, 42.4 µM, and 10.6 µM had significant *in vitro* protective effect on DNA plasmid damage (Sheih *et al.* 2009). Furthermore, Zhong-Ji Qian and collaborators

illustrated the protective effect of antioxidative peptide purified from gastrointestitinal digests of *Crassostrea gigas* on DNA damage free radicals derived from the Fenton reaction. The result indicated that treated DNA with dose dependent purified peptide could protect hydroxyl radical inducing DNA damage (Qian *et al.* 2008). In 2010, it was suggested that the lunasin purified from *Solanum nigrum* L. could prevent DNA damage block from Fenton reaction by chelating Fe^{2+} (Jeong *et al.* 2010).

2.2.2 Natural sources of antioxidants

Antioxidants extracted from natural sources such as vegetables, fruits, berries, seeds and algae, are considered to be safe for applications in medicines, food supplements and cosmetics.

Currently, antioxidants extracted from natural sources have been widely interested. Many researchers reported the isolation of several natural antioxidants from different kinds of plant materials such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs. For many kinds of fruit, exceptionally natural edible vitamins that are benefit for health have been discovered. The antioxidants activities from all of fruit materials including pericarp, peel, pulp, and seed, are exhibited. Many reports suggest relatively high antioxidant capacity in fruit seeds. In 2003, Changjiang and collaborators investigated in the antioxidant activities of peel, pulp, and seed fractions of 28 fruits commonly consumed in china including hawthorn, date, guava, kiwifruit, purple mulberry, strawberry, white pomegranate, “Lukan” tangerine, “Honey” tangerine, orange, lemon, cherry, longan, “Banana flavored” apple, pineapple, banana, plum, lychee, kumquat, “Red rose” grape, pomelo,

mango, “Jiubao” peach, apricot, “Hami” melon, “Duck” pear, “Jingxin #1” watermelon, and persimmon by Ferric reducing antioxidants power assay (FRAP assay). They found that the most fruit seed fractions revealed the higher FRAP values than that of fruit pulps. The highest FRAP value among all fruit seeds in this research was the grape seeds. In addition, the longan and lychee seed also showed relatively high antioxidant activity respectively. Therefore, they might potentially be rich of natural antioxidant (Changjiang Guo and Jiang 2003) (Figure 2.5).

FRAP values of peel, pulp and seed fractions of 28 fruits (mmol/100g wet weight)

Fruits	Pulp	Peel	Seed	Total
Hawthorn	13.42 ± 0.74	29.25 ± 1.59	0.43 ± 0.03	43.10
Date	6.98 ± 0.29	16.69 ± 0.55	1.77 ± 0.13	25.44
Guava	6.07 ± 0.69	10.24 ± 0.24	4.71 ± 0.24	21.02
Kiwifruit	4.38 ± 0.20	11.13 ± 0.23	–	15.51
Purple mulberry	4.11 ± 0.25	–	–	4.11
Strawberry	3.29 ± 0.30	–	–	3.29
White pomegranate	3.10 ± 0.12	82.11 ± 4.01	0.72 ± 0.05	85.93
Lukan tangerine	2.29 ± 0.13	6.94 ± 0.40	1.15 ± 0.02	10.38
Honey tangerine	2.19 ± 0.08	5.44 ± 0.07	–	7.63
Orange	1.89 ± 0.19	5.69 ± 0.26	–	7.58
Lemon	1.43 ± 0.07	2.30 ± 0.12	0.91 ± 0.07	4.64
Cherry	0.99 ± 0.21	2.82 ± 0.29	0.77 ± 0.12	4.58
Longan	0.94 ± 0.05	3.98 ± 0.30	24.26 ± 2.79	29.18
Banana flavored apple	0.80 ± 0.05	3.24 ± 0.39	0.84 ± 0.09	4.88
Pineapple	0.80 ± 0.08	2.01 ± 0.03	–	2.81
Banana	0.73 ± 0.11	3.16 ± 0.16	–	3.89
Plum	0.71 ± 0.01	8.09 ± 0.55	0.65 ± 0.06	9.45
Lychee	0.59 ± 0.11	2.86 ± 0.12	22.36 ± 0.97	25.81
Kumquat	0.50 ± 0.05	0.25 ± 0.05	0.66 ± 0.03	1.41
Red rose grape	0.49 ± 0.04	11.02 ± 1.83	55.54 ± 1.62	67.05
Pomelo	0.39 ± 0.03	1.84 ± 0.04	–	2.23
Mango	0.38 ± 0.08	10.13 ± 0.37	14.59 ± 0.55	25.10
Jiubao peach	0.38 ± 0.03	0.95 ± 0.08	1.17 ± 0.03	2.50
Apricot	0.34 ± 0.07	0.79 ± 0.07	0.72 ± 0.09	1.85
Hami melon	0.24 ± 0.06	0.52 ± 0.07	0.31 ± 0.13	1.07
Duck pear	0.22 ± 0.03	0.89 ± 0.08	2.06 ± 0.09	3.17
Jingxin #1 watermelon	0.16 ± 0.01	0.42 ± 0.11	1.01 ± 0.07	1.59
Persimmon	0.14 ± 0.03	0.62 ± 0.03	1.48 ± 0.08	2.24
Total	52.75	213.14	131.40	397.29

Data are expressed as mean ± SD. Each fruit was analyzed five times.

Figure 2.5 The FARP values of peel, pulp, and seed fractions of selected 28 fruits (mmol/100g wet weight). (Changjiang Guo and Jiang 2003)

The other valuable natural sources of antioxidants are algae. Nowadays, natural products from marine organism are widely investigated for bioactive compounds to develop a new product certainly involving in antioxidants. For marine algae, they play a key role to produce secondary metabolites in the ocean by being served as primary producers. Furthermore, marine algae can be an important energy source for others organism in the ocean (Samarakoon and Jeon 2012). Because hydrogen peroxide and singlet oxygen are generated under light stress in both macro and microalgae, there are several ROS detoxification in these organisms including enzymatic and non-enzymatic forms (Goiris *et al.* 2015)

In 2009, Paul J. Janknegt and coworkers reported the important enzymes associated with ROS-disposal. By measurement of the quantity of SOD activity by the RF/NBT enzyme assay, *superoxide dismutase* (EC 1.15.1.1) presented as both Fe-SOD and Mn-SOD isoforms in Antarctic microalgae, temperate flagellates, and temperate diatoms including 15 low-light-acclimated species. In addition, *peroxidase* is an important one to catalyze the reduction of hydrogen peroxide into water. According to the different forms other catalases for their electron donor requirement, it is a diverse array; for example, glutathione peroxidase (EC 1.11.1.9) required glutathione for scavenging hydroxyl peroxidase (Janknegt *et al.* 2009). As a result of Régine D. *et al*. (2008) several isomers of glutathione peroxidase detected in *Chlamydomonas reinhardtii* showed some similarities to ones in human cells and other phytosynthetic organisms. In addition, two glutathione peroxidase homologous genes were identified in *Chlorella* sp (Dayer *et al.* 2008). The purified recombinant enzyme, named as NJ-18GPXs, revealed activities to protect

cell from oxidative damage by hydrogen peroxide and hydroperoxide reduction (Wang and Xu 2012).

Nonenzymatic antioxidants such as vitamin C have also been found in in cytosaol and chloroplast of alga cell. The extracellular production of L-ascorbic acid was detected in *Chlorella protothecoides* when various carbon and nitrogen sources of fermentation were supplied (Running *et al.* 2002). Moreover, α-tocopherol, quenching both of singlet oxygens and peroxides, is an active antioxidant form only found in chloroplasts of photosynthetic organisms (Lesser 2006, Goiris *et al.* 2015)

The high production of α-tocopherol was observed in green algae including *Dunaliella tertiolecta* and *Tetraselmis suecica*, when nitrogen-controlled concentration in batch cultivation was applied. The result indicated that a decrease in nitrogen concentration was resulted in an increase in α-tocopherol production versus growth cells (Carballo-Cardenas *et al.* 2003).

Carotenoids are pigmented antioxidative compounds synthesized by plants, algae, fungi and microorganisms, excepting for animals. Carotenoids have strong ability to scavenge singlet oxygen and catching up with free radicals. The major ingredients of carotenoids such as Fucoxanthin and astaxanthin, are potentially antioxidants especially in brown seaweed including *Undaria pinnatifida* and *Laminaria japonica* (Yasuhiro Nishida 2007, Ngo *et al.* 2011). According to the study from Sachindra and collaborators in 2007, marine carotenoids fucoxanthin, fucoxanthinol, and halocynthiaxanthin exhibited several biological effects measured by DPPH radical scavenging, singlet oxygen quenching (with EC 50 as 164.60, 153.78, and 826.39 μM, respectively.), ABTS radical scavenging (with EC 50 of fucoxanthin and fucoxanthinol as 8.94 and

2.49 microM, respectively.), and Hydroxyl radical scavenging activity by the chemiluminescence technique was higher than α-tocopherol. The higher radical scavenging activity of fucoxanthin and fucoxanthinol compared with halocynthiaxanthin is assumed from the presence of the allenic bond (Sachindra *et al.* 2007).

Another important nonenzymatic antioxidant is carotenoids which can be found in microalgae as primary and secondary carotenoids. One such carotenoid, "Lutein", was discovered in *Chlorella protothecoides*. The heterotrophic fermentation of this strain can produce lutein responding to singlet oxygen (Wei *et al.* 2008).

Many alga extracts are useful. For example, the extracted antioxidants from brown seaweeds were reported a distinct effect on hydrogen peroxide scavenging activity. In addition, the extracts from seven species of brown seaweeds have an effect on different reactive oxygen species (ROS) scavenging such as DPPH (1,1-diphenyl-2-pricrylhydrazyl) free radical, hydroxyl radical and superoxide anion. The extracts also have a protective DNA damage property (Ahn *et al.* 2004, Heo *et al.* 2005). The sulfated polysaccharides (SPs) extract from edible brown marine algae are by-product which are also important and non-toxic antioxidants to prevent oxidative damage which are causative carsinogenesis, and can be applied as a promising natural antioxidant in the food industry (Dai-Hung, N., 2011). Moreover, SPs exhibited the excellent antioxidant activities were extracted from five species of algae including one brown algae (*Laminaria japonica*), one red algae (*Porphyra haitanensis*), and three green algae (*Ulva pertusa*, *Enteromorpha linza*, *Bryopsis plumose*) (Zhang *et al.* 2010).

Furthermore, proteins derived from macro and microalgae with specific biological properties such as antioxidative, antihypertensive, anticoagulative, antitumor and immune-stimulative properties were also reported (Samarakoon and Jeon 2012).

The algae have been widely interested due to composition of essentially nutritional amino acids. In 2001, Wong and coworkers investigated the nutritional values of seaweed protein concentration (PCs). In two red seaweed species (*Hypnea charoides, Hypnea japonica*) and one green seaweed species (*Ulva lactuca*), high total amount of essential amino acids (36.2-40.2%) which were rich in leucine, valine, and threonine but lack in cysteine was report (Wong, H.K., *et al*.,2001). Due to these reasons, algae have been interested for food industry. Furthermore, highly valuable molecules, such as polyunsaturated fatty acid oil, supplements and pigments, could be found in some microalgae resulting in potential application of various industries (Spolaore *et al.* 2006, Sheih *et al.* 2009).

As information already provided, marine algae are suggested as a key rich source of secondary metabolites. For beneficial human, many scientists have attempted to find out bio-functionalities of protein. Therefore, algae have become as experimentally attractive to produce crude extract for natural food additives. Due to the chemical compositions, many species of microalgae are not only used for nutrient enrichment in food and animal feed, but also supplement in drug and many cosmetic products.

Interestingly, in 2009, Sheih and cowokers investigated algae waste hydrolysate from algae essence production. The hydrolysate was prepared by cleaving *Chlorella vulgaris* waste at the target hydrophobic

aromatic, amino acid residues, phenylalanine, leucine and tyrosine by pepsin, and then some special antioxidative properties were revealed. It was suggested that the algae waste hydrolysate peptide could successfully quench a variety of free radicals, involving hydroxyl radical, superoxide radical, peroxyl radical, DPPH radical and ABTS radicals with more efficiency than BHT, Trolox and peptides from marine protein sources in most cases. The amino acid the sequence containing 11 amino acids as VECYGPNRPQF was detected. Moreover, the purified peptide has significant protective effects on DNA and prevents cellular damage caused by hydroxyl radicals. The peptide also has gastrointestinal enzyme-resistance and no cytotoxicity observed in human lung fibroblasts cell lines (WI-38) in vitro. These results demonstrated that low-cost algae protein waste could be a new alternative antioxidative peptide. This information is useful for the further study.

However, extract of native seaweed protein might be destroyed because of phenolic compounds. Moreover, large amount of polyanionic cell wall mucilages the complicated procedures of extraction and purification are required. Nevertheless, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), were possibly hazardous to human health (Sheih *et al.* 2009). It has been suggested that synthetic medicines used to treat several disorders can possibly produce free radical, leading to oxidative stress and further tissue damage (Saikat Sen 2010). Furthermore, protein extraction from natural sources usually encounters some problems such as large amount of sample requirement and sample degradation. The sample storage is also a key process for extracting target protein with

good quality and quantity. To improve the production of antioxidative peptides, recombinant DNA technology is applied.

2.3 Recombinant DNA technology

The recombinant DNA technology was first proposed by Peter Lobban, a graduate student of Prof. Dale Kaiser in Biochemistry Department at Stanford University Medical School. This molecular biological method involves in construction of genetically modified organisms by bringing genetic materials from multiple sources for creating a new DNA sequences that cannot be found in nature. The, the target product can be generated through the expression system of the recombinant organisms. Organisms created by this technique are called GMOs. The recombinant DNA technique relates to the theory of George Beadle and Edward Tatum, whose explanation of gene direct protein synthesis as “There is a specific connection between gene and enzyme, the one gene-one enzyme”. By this mean, DNA and enzyme are coherent via RNA. The DNA of a gene is transcribed into a complementary RNA molecule. Then, The RNA molecule is translated to the corresponding amino acid sequence for protein formation. Therefore, to understanding in structure and function of protein, nucleic acid sequence is also required to reveal the information of its encoded protein by way of gene regulation (Principles of Biochemistry, Voet, *et al*.).

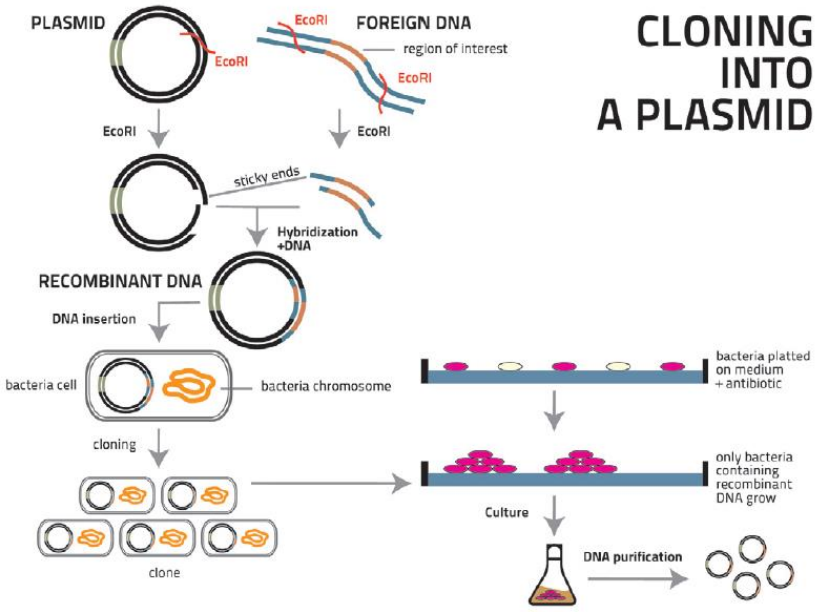


Figure 2.6 The recombinant DNA technique *(Stryjewska et al. 2013)*.

Normally, recombinant DNA technique is efficiently manipulated target gene including cloning, modifying and expressing gene in microorganisms such as bacteria and yeast. The procedure generally is composed of digesting plasmid vector and foreign DNA fragment with particular restriction enzymes. Then, digested plasmid and DNA fragment are ligated by DNA ligase enzyme, creating recombinant DNA. Subsequently, the recombinant plasmid is transformed into the other microorganisms (Figure 2.6). To determine the growth of transformant colonies, suitable selection; for instance, appropriate antibiotic, is necessary. In case of yeast cell, to verify integrated DNA onto chromosome, the colony PCR can be applied in experiment with the affinity primer. Moreover, the accuracy of DNA sequence can be confirmed by DNA sequencing method. Finally, the verified organism can be used for the next step such as larger scale cultivation.

2.3.1 Applications of recombinant DNA

Applications of recombinant DNA have been founded in various industries such as food production, human and veterinary medicine, agriculture, and bioengineering.

For a relatively long time, there are many experiments and productions carried out by applying recombinant DNA technique for various recombinant protein productions. The expansion of this field will probably not be terminated at any time soon. Pharmaceutical products and industrial enzymes were the first commercial bio-products made by the means of recombinant DNA applications. The production of human insulin was the first commercial innovation in recombinant protein production from microbial hosts. Examples of current recombinant protein products from the recombinant DNA technology are represented in Table 2.1. Admirable development in the recombinant DNA technology over the past decades has given hundreds of therapeutic proteins for clinical applications (Chen 2012).

Table 2.1 Examples of available recombinant proteins (Principles of Biochemistry, Voet, et al.).

Protein	Use
Human insulin	Treatment of diabetes
Human growth hormone	Treatment of some endocrine disorders
Erythropoietin	Stimulation of red blood cell production
Colony-stimulating factors	Production and activation of white blood cells
Coagulation factor IX and X	Treatment of blood clotting disorders (hemophilia)
Tissue-type plasminogen activator	Lysis of blood clots after heart attack and stroke
Bovine growth hormone	Production of milk in cows
Hepatitis B surface antigen	Vaccination against hepatitis B

The early success of recombinant DNA technology on biological possesses in microbial systems. Nowadays, yeasts and bacteria have been interested hosts for heterologous protein productions. Some proteins require the post-translational modification and more factors while some do not. Therefore, to use recombinant DNA technological, one of the priorities is determination of host for expression system (Stryjewska *et al.* 2013).

2.3.2 Host microorganisms for recombinant DNA technology

The hosts for recombinant DNA technology can be both prokaryotic and eukaryotic depending on the purpose of work. Expression of a cloned gene not only concerned about gene function, but also a considerable amount of protein and non-protein production. To express

valued proteins, the strategy to determine the most suitable vector with host cell depends on a diverse range of factors related to the properties of the target gene and its product. In addition, purification procedure of downstream product is another important factor. Furthermore, the compatibility of the appropriate host cell and vector is necessarily considered to ensure the translation of RNA transcription, the proper folding of the recombinant protein, and maintenance of the functional protein product.

2.3.2.1 *Escherichia coli*

Bacteria species, such as *Escherichia coli*, has been a preferable host for recombinant DNA technology because of several advantages. There is a great number of available information and easy manipulation. For *E. coli*, it can grow in cheap media with high cell density. Importantly, *E. coli* has high productivity (Table 2.2). Consequently, it has been the first dominating choice for bacterial expression system and continuously remaining to be the preferred system for an experiment design in laboratory scale which proceeds to a commercial activity or becomes to useful benchmark for comparison among various expression platforms. *E. coli* is an important host for protein engineering and high-throughput structural analysis.

Table 2.2 Comparison of expression systems (Reference:http://www.genwaybio.com/technologies/protein-expression)

Characteristics	*E. coli*	Yeast	Insect cells	Mammalian cells
Cell Growth	Rapid (30 Min)	Rapid (90 Min)	Slow (18-24 H)	Slow (24 H)
Complexity of Growth Medium	Minimum	Minimum	Complex	Complex
Cost of Growth Medium	Low	Low	High	High
Expression Level	High	Low - High	Low - High	Low - Moderate
Extracellular Expression	Secretion to Periplasm	Secretion to Medium	Secretion to Medium	Secretion to Medium
Protein Folding	Refolding Usually Required	Refolding May Be Required	Proper Folding	Proper Folding
N-linked Glycosylation	None	High Mannose	Simple, No Sialic Acid	Complex
O-linked Glycosylation	No	Yes	Yes	Yes
Phosphorylation	No	Yes	Yes	Yes
Acetylation	No	Yes	Yes	Yes
Acylation	No	Yes	Yes	Yes
gamma-Carboxylation	No	No	No	Yes
Yield (mg per liter culture)	50-500	10-200	10-200	0.1-100
Success Rate (%) (soluble or functional)	40-60	50-70	50-70	80-95
Project Cost	Low	Low	Middle	High
Recommended Use	Antigen protein, Protein standards, Functional proteins	Proteins with glycosylation, Vaccine, Secreted form, Alternative to insect cell system	Proteins with glycosylation, Assay standards, Secreted form, Alternative to yeast system	Functional study, PTM study, Assay standards, Characterization
Advantage	Simple, robust, lowest cost, highest yield	Simple, low cost, good for certain proteins	Relatively higher yield, better PTM	Natural protein configuration, best PTM
Disadvantage	Least PTMa	Longer time, less PTM	Longer time, higher cost	Highest cost, lower yield

PTM = Post-Translational Modification such as glycosylation.

From a previous research, the chimeric peptide Tat-HA-NR2B9c was investigated. This peptide is composed of a fragment of the cell membrane transduction domain of the human immune deficiency virus type1 (HIV-1) Tat, an influenza virus hemagglutinin (HA) epitope-tag, and 9 amino acids of NR2B (NR2B9c) at C-terminal end. The peptide and gene were designed and constructed by using polymerase chain reaction and ligated into a novel expression vector in *E. coli*. The researcher found that the recombinant fusion protein was expressed in inclusion bodies of *E. coli* under the induction of IPTG. Moreover, the recombinant peptide has ability to reduce infarct size and improve neurological functions (Zhou *et al.* 2012).

In 2013, under IPTG induction, the expression of recombinant porcine β defensin 1 ($pBD1_{48}$) in *E. coli* was studied. pBD1 is a cationic antimicrobial peptide with three pairs of disulfide bonds. The $pBD1_{42}$ peptide was originally found in pigs. The $pBD1_{48}$ gene was obtained by RT-PCR with the tongue total RNA as template, cloned into pET30a expression vector, and transformed into *E.coli* BL21 (DE3) plysS. After, they purified this peptide by His tag affinity column with 90% purity recovery. The recombinant $pBD1_{48}$ exhibited antimicrobial activity against both Gram-positive *Staphylococcus aureus* and Gram-negative *E.coli* comprising the multi-resistant *E. coli* strain (Li *et al.* 2013).

In 2014, the expression of recombinant Galectin-1 (Gal-1) was observed in *E. coli* through the use of recombinant DNA technique. Gal-1, a member of beta-galactoside-binding protein family, is implicated in modulating cell-cell and cell-matrix interactions and performs an autocrine negative growth factor that regulates cell proliferation. The monomer and concatemer Gal-1($Gal\text{-}1_2$) genes with two repeats was

amplified by PCR and cloned into pET-22b(+) vector, then transformed into *E. coli* DH5α strain. They found that both soluble forms of Gal-1 and Gal-1_2 were expressed by *E. coli* with 1 mM IPTG induction. The recovery was over 95% purity after purifying with ion-exchange chromatography (Shu *et al.* 2014).

In 2015, after induction of IPTG in *E. coli*, the successful high-level expression of recombinant interleukin (IL)-37, a novel member of the IL-1 cytokine family, with a C-hexahistidine tag, designated as IL-37b, was reported. The recombinant IL-37b was obtained in the soluble fraction with the yield of 90 mg from 1 L of bacterial culture (Gu *et al.* 2015). *E. coli* was also used to express soluble HECT domain truncation (WHP2) of WW domain-containing protein 2 (WWP2). With the difficulty of expression, protocols have not yet been developed for WWP2 preparation in large scale. However, approximately 60 mg/L of the soluble WHP2 was obtained from this study (Jiang *et al.* 2015). Furthermore, it was indicated that a sufficient amount of recombinant protein production with antigenic epitopes may be possible obtained by recombinant DNA techniques using *E. coli* as the expression host (Hoang *et al.* 2015).

However, Due to the nature of prokaryotes, bacteria including *E. coli* are not equipped with the full enzymatic machinery to accomplish post-translational modification or protein folding. Hence, multi-domains of eukaryotic proteins expressed in bacteria are often non-functional. Many recombinant proteins expressed in this kind of host become insoluble as inclusion bodies that are very arduous to recover without harsh denaturants. These chemicals can obstruct protein-refolding procedures. From these reasons, *E. coli* is not suitable for producing

recombinant proteins requiring post-translational modification, disulfide bond, disulfide isomerization, lipidation, glycosylation, protein cis/trans, phosphorylation, and sulfation (Lueking *et al.* 2000).

2.3.2.2 *Pichia pastoris*

Yeasts are the first eukaryotic organisms that could be served as models for systematic analysis of life, and also suitable references for human to inspect metabolic pathways by the combination of biochemical and genetic approaches at the same time. The other advantage of yeast is tractability to classical genetic techniques and simplified genetic mapping. Especially, the original isolated *Saccharomyces cerevisiae* has been widely employed in recombinant protein expression by means of laboratory scale, drug screening and analysis of drug action mechanism, Beside, it has been regarded as safe to industrial scale for brewing, wine making, baking, and a nutritional or food additive. Moreover, yeast is a unicellular organism which can be grown on determined media which can be controlled over culture condition. The highest glycosylation capacity among other yeast species has been discovered. However, it has some limitations. For example, *S. cerevisiae* secrets a low protein yield in medium rather than in periplasmic space, leading to a decrease in product yield via purification (Buckholz and Gleeson 1991). Also, N-linked glycosylation terminated with α-1,3-linked mannose residue proteins in yeast can cause allergic which consequently limits commercial product. Therefore, many research attempts to develop yeast strain for enhancing the recombinant proteins expression. For four decade years, Kochi Ogata has discovered new methylotrophic yeasts with a lot of advantages for

protein expression. The comparison of common expression systems was represented in Table 2.3.

Table 2.3 Comparison of the features of commonly used expression systems

Feature	Expression system			
	E. coli	*S. cerevisiae*	Mammalian cells	Methylotrophic yeasts
Information available	++	++	+	++
Growth on cheap media	+	+	-	++
Protein secretion	+ -	+	++	++
Authentic glycosylation	-	+ -	++	+
Growth high cell density	+	+	-	++
Stringent control of expression by medium components	+ -	+ -	+ -	+
Productivity	++	+	+ -	++
Full biological activity	+ -	+	++	+

Table 2.3 summarizes superior properties of the methylotrophic yeasts. The doubling times of *E. coli* and yeast are approximately 30 minutes and 1-2 hours, respectively. However, considered with the bigger size, it can be implied that yeast can grow better. The methylotrophic yeasts can grow as 100 g dry weight per liter while *S. cerevisiae* as 30g dry weight per liter. According to higher growth rate of methylotrophic yeasts, it can be concluded that they have higher productivity than *S. cerevisiae*.

Methylotrophic yeasts can use methanol, which is a low cost material for carbon and energy sources. Methanol metabolism is initiated by alcohol oxidase (AOX) enzyme. AOX first catalyzes methanol oxidation to formaldehyde and hydrogen peroxide. Along with catalase, AOX is secluded within the peroxisome. In this organelle, catalase cleaves hydrogen peroxide molecule to oxygen and water. The formaldehyde molecule which generated by first AOX enzyme is transferred to cytosol and further oxidized to formate and carbon dioxide by the function of two cytoplasmic dehydrogenases. The residual formaldehyde is absorbed to form cellular components by a cyclic pathway. The dihydroxyacetone synthase (DHAS), a peroxisomal enzyme, initiates compression of formaldehyde with xylulose 5-monophosphate associated with catalyzed reaction. The produced glyceraldehyde 3-phosphate and dihydroxyacetone are transferred to cytoplasmic pathway for regeneration of xylulose 5-monophosphate molecule, and then one net glyceraldehyde 3-phosphate for every three cycles (Figure 2.7). The function of AOX and DHAS enzyme regulates yeast for properly selecting carbon source, in this case by directing to methanol rather than glucose, glycerol, or ethanol. They have been attracted as potential sources of single-cell protein (SCP) for commercial high-protein animal feed. Importantly, it can express high-level heterologous proteins (Cereghino and Cregg 2000).

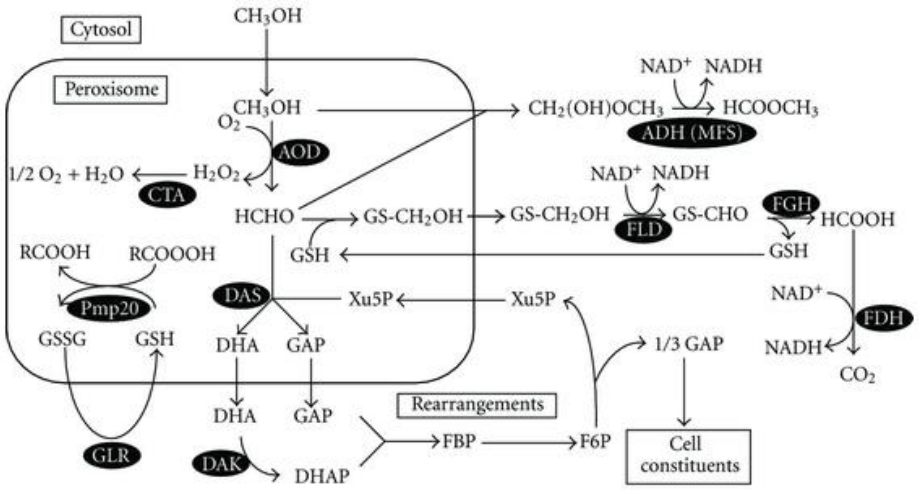


Figure 2.7 Schema of methanol metabolism in methylotrophic yeasts. Enzymes: AOD: alcohol oxidase; CTA: catalase; DAS: dihydroxyacetone synthase; Pmp20: peroxisome membrane protein which has glutathione peroxidase activity; GLR: glutathione reductase; DAK: dihydroxyacetone kinase; ADH (MFS): alcohol dehydrogenase (methylformate-synthesizing enzyme); FLD: formaldehyde dehydrogenase; FGH: S-formylglutathione hydrolase; FDH: formate dehydrogenase.

Abbreviations: DHA: dihydroxyacetone; DHAP: dihydroxyacetone phosphate; F6P: fructose 6-phosphate; FBP: fructose 1,6-bisphosphate; GAP: glyceraldehyde 3-phosphate; GS-CH_2OH: S-hydroxymethyl glutathione; GS-CHO: S-formylglutathione; GSH: reduced form of glutathione; GSSG: oxidized form of glutathione; RCOOOH: alkylhydroperoxide;Xu5P:xylulose5-phosphate

(Reference:http://www.hindawi.com/journals/ijmicro/2011/101298/fig1/)

Methylotrophic yeasts are classified into four genera *Hansenula*, *Candida*, *Torulopsis* and *Pichia*. For *Pichia pastoris*, it was first developed for experiment on methanol culture to obtain high cell density for an animal feed additive by Phillips Petroleum Company during the 1970s. Unfortunately, at that time, the cost of methanol was increased due to petroleum market. As a result, soybean became the alternative animal feed source, and commercial SCP from methanol was uninteresting. In the subsequent decade, Phillips Petroleum Company and the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA) cooperated to develop *P. pastoris* strain for heterologous protein expression by isolating available genes and alcohol oxidase promoter. After that, vectors, strains and corresponding protocol for the *P.pastoris* genetic manipulation were created. Because of the strong promoter of alcohol oxidase, high level of foreign protein expression can controlled. Phillips Petroleum Company licensed these products to Invitrogen Corporation (Carlsbad, CA) under Research Corporation Technologies. Therefore, since 1984, *P. pastoris* has been utilized for producing over 300 foreign intracellular and extracellular proteins of human, animal, and plant in large scale production (Cereghino and Cregg 1999). In the past few years, the expression system of *P. pastoris* has achieved much awareness as commercial kit by Invitrogen (Romanos 1995).

P. pastoris has many benefits such as efficiency of performing numerous eukaryotic post-translational modifications (Romanos 1995, Cereghino and Cregg 2000). In addition, the yeast has available genetic information. Due to the extracellular secretion of expressed proteins at high level thus, the procedure to harvest target protein is uncomplicated. The extracellular protein can save costs on breaking cells and can avoid

the loss of target protein. *P. pastoris* is easy to be experimentally manipulated because of the ability to be as stable single or multicopy integration of expression plasmids at specific sites onto *P. pastoris* genome (Cereghino and Cregg 1999). Importantly, it can grow with a simple nutrition in high cell density. Moreover, *P. pastoris* has a strong inducible promoter, named as *AOX1* promoter, originally regulates the transcription of alcohol oxidase (AOX) enzyme.

Normally, Alcohol oxidases in *P. pastoris* are encoded by two genes, *AOX1* and *AOX2*, but the majority is *AOX1* (Cregg *et al.* 1989). Successful protein expression of *AOX1* gene (5-30%) is tightly regulated and induced by methanol with much higher level than those for *AOX2* promoter regulation. The *AOX1* induction of wild-type *P. pastoris* has advised that maximal mRNA level would be produced by one copy of *AOX1* expression vector (Romanos 1995).

In 1987, the β-galactosidase expression of *lacZ* gene in repressing or inducing carbon sources controlling by the regulation of the *AOX* and *DAS* promoters in *P. pastoris* was investigated (Tschopp *et al.* 1987). This expression system contained the AOX-lacZ fusion and HIS4 gene, selectable marker. For glucose repression, the alcohol oxidase activity was measured by the levels of β-galactosidase. On the other hand, *P. pastoris* increasingly expressed both β-galactosidase (from 0 to 120 U/mg) and alcohol oxidase (from 0 to 17 U/mg) under no detectable glucose after 25 hours of growth. In addition, the yeast was able to grow in methanol with distinctly high level of expression within 20 hours (maximum 5 to $13x10^3$ U/mg of protein of β-galactosidase and $5x10^2$ U/mg of protein of alcohol oxidase activity). Moreover, there are some studies reviewed that the high-level expression of recombinant protein

was repressed when glucose or glycerol was in culture in high density. According to described reasons, *P. pastoris* is the advantageous for preventing selection of non-expressing mutant cells. Afterward, methanol medium could be used to shift the culture to induce rapid high-level expression.

GAP promoter is an alternative promoter of *P. pastoris* to solve the potential hazard of methanol to induce protein expression in food industry. GAP promoter provides constitutively high-level expression on glucose, glycerol, and methanol. However, it has not been widely utilized because of cytotoxic effects of constitutive GAP promoter during production of protein in *P. pastoris* (Macauley-Patrick *et al.* 2005)

P. pastoris also has another promoter for regulating metabolic pathway of methanol. *FLD1* promoter is a key one for regulating formaldehyde dehydrogenase (FLD) enzyme which protects cell from the toxic formaldehyde generated from methylamine metabolism. The *FLD1* gene can be induced by methanol and methylamine. The nitrogen sources is a limitation for FLD1 operation, however, several available carbon sources for inducing foreign protein expression such as glucose and glycerol are advantageous of using this promoter (Macauley-Patrick *et al.* 2005).

DAS1 and DAS2 are dihydroxyacetone synthase, regulating methanol metabolic pathway. The equally high induction of transcription of the two genes is found upon methanol production (Macauley-Patrick *et al.* 2005).

Because the requirement for optimizing protein production, selectable markers have been used to screen and select. Choosing the appropriate marker and promoter by considering phenotype and strain

(Table 2.4 and 2.5) is essential for recombinant protein production from *P. pastoris*.

Table 2.4 Phenotypes of *P. pastoris*

Phenotype	Characteristics
Mut^+	- Wild-type (*AOX1*,*AOX2* regulation) - Require high feeding rates of methanol
Mut^s	- Have a disruption in the AOX1 gene (*aox1*,*AOX2*) - Slow growth phenotype on methanol medium
Mut^-	- Have both AOX gene deleted (aox1, aox2) - Unable growth on methanol

Table 2.5 *P. pastoris* strains

Strain	Genotype	Application
GS115	*his4*	Selection of expression vectors containing *HIS4*
X-33	Wild type	Selection of Zeocin™-resistant expression vectors
KM71	*his4, aox1::ARG4, arg4*	Selection of expression vectors containing *HIS4* to generate strains with Mut^S phenotype
KM71H	*aox1::ARG4, arg4*	Selection of Zeocin™-resistant expression vectors to generate strains with Mut^S phenotype
SMD1168	*his4, pep4*	Selection of expression vectors containing *HIS4* to generate strains without protease A activity
SMD1168H	*pep4*	Selection of Zeocin™-resistant expression vectors to generate strains without protease A activity

However, others parameters such as signal sequence, processing, proteolysis, and glycosylation are also important for yield and quality of recombinant protein production from *P. pastoris*.

There are many researches that investigated in recombinant protein expression from *P. pastoris*; for example, the expression of the recombinant insulin-like growth factors (IGF) by Chen and collaborators in 2007. The IGFBP-6 was modulated by a family of six high-affinity IGF-binding protein (IGFBPs). In *P. pastoris* GS115, because 5' end of human IGFBP-6 encoding sequence was fused with α-factor, this recombinant peptide could enter into secretory pathway. Under methanol induction, the approximately 30 kDa of recombinant human IGFBP-6 from purified supernatant by ion-exchange chromatography and hydrophobic-interaction chromatography was recovered. In addition, rhIGFBP-6 could inhibit IGF-II-stimulated cell proliferation. Importantly, human IGFBP-6 is an *O*-glycosylated protein with eight disulfide bonds within the conserved amino- and carboxyl-terminal domains formed by pairing of adjacent cysteines. The formation of disulfide bonds in proteins is a result from post-translational modification gives a proper structure of protein for their biological activity. The PAS staining and SDS-PAGE results of this study confirmed that the secreted rhIGFBP-6 in *P. pastoris* was glycosylated and folded with correct disulfide bridges. This study also demonstrated that, for further investigation, the functional rhIGFBP-6 can be produced in sufficient quantities by using *P. pastoris* as the expression system (Chen *et al.* 2007).

In 2008, Guo and Ma revealed that the alkaline protease gene from *Aspergillus oryzae* was cloned into pPIC9K and successfully expressed in *P. pastoris* GS115 with native signal peptide, or α-factor secretion signal

peptide. They obtained the maximal yield of 513 mg/L. The 34 kDa of purified recombinant peptide was appeared on SDS-PAGE. Moreover, the activities of the recombinant alkaline protease were identical to native one. The recombinant alkaline protease displayed predominant enzyme properties and was essentially identical to the native enzyme. Therefore, these enzyme properties were suggested that the heterologously expressed enzyme could be utilized in commercial application (Guo and Ma 2008).

In 2010, the successful improvement of recombinant small cysteine-rich protein (Sm1) production in *P. pastoris* GS115was reported. Generally, Sm1, a member of the cerato-platanin family that was secreted from *Trichoderma virens*, induces defense responses in dicot and monocot plants. The *sm1* gene was cloned in the pPIC9K vector under the AOX1 promoter. The transformants highly expressed rSm1 protein approximately 55 mg/L after 4 day culture containing 1% final concentration of methanol. Although, the secreted rSm1 in *P. pastoris* was revealed higher molecular mass than the native peptide on the MALDI/TOF/MS because of six amino acids extension at N-terminal, the similar activities for the treatment of specialty crops to promote disease resistance of rSm1, compared with native Sm1 were still, was revealed (Buensanteai *et al.* 2010).

In 2013, Production of a fusion protein comprising of melittin genetically linked to a mutant human interleukin 2(^{88}Arg, ^{125}Ala), or M-IL-2(^{88}Arg, ^{125}Ala), was observed in *P. pastoris*. The gene encoding M-IL-2(^{88}Arg, ^{125}Ala) was cloned into pPICZαA vector. *P. pastoris* GS115 was used as the expression host with 1% v/v methanol induction. The secreted recombinant protein yield reached up to 814.5 mg/L, higher than the system in *E. coli*. In addition, According to *in vitro* bioassay result,

the fusion protein M-IL-2(^{88}Arg, ^{125}Ala), it was indicated the dramatic inhibition of the growth of human ovarian cancer SKOV3 cell and Hela cells. Interrestingly, the capability of inhibition effects was better than that of melittin. Therefore, the researchers claimed that, by using *P. pastoris* as the host, it was an alternative strategy for large-scale production of bioactive M-IL-2(^{88}Arg, ^{125}Ala) and a preparation for further clinical practice (Li *et al.* 2014).

The other example involved in the utilization of *P. pastoris* for recombinant laccase expression. The thermo-alkali-stable laccase gene from *Bacillus licheniformis* was cloned into pPICZαA vector and transformed in *P. pastoris*. The recombinant laccase was secreted in the culture medium after being induced by methanol. The recombinant laccase showed maximum activity of 227.9 U/L. Moreover, this study also revealed the estimated molecular 65 kDa peptide on SDS-PAGE after deglycosylation. For glycosylation, it is a common post-translational modification for recombinant protein secreted from *P. pastoris*. Some fungal laccases expressed in *P. pastoris* have been reported to be glycosylated. Hence, according to the lower molecular weight appearance of *B. licheniformis* LS04 laccase than the native (71 kDa by gel filtration) after treatment with PNGase F, it was concluded that the recombinant laccase was a monomeric glycoprotein. However, the lower molecular weight of recombinant laccase still displayed high tolerance to NaCl and organic solvents, similar to the native spore laccase. In addition, the recombinant laccase exhibited high efficiency in dry decolorization under alkaline conditions that was more difficult than fungal laccase (Lu *et al.* 2013).

CHAPTER III
METHODOLOGY

3.1 Materials

3.1.1 Strains and plasmids

The interested DNA fragment was synthesized by Integrated DNA Technologies (IDT), USA. The plasmid pCR®2.1-TOPO® (Invitrogen, USA) was used to maintain the synthesized DNA fragment. All plasmid derivatives were transformed into competent HIT-DH5α *E. coli* (RBC Bioscience, Taiwan) for amplification and maintenance.

P. pastoris GS115, cultured on YPD agar medium, was obtained from Dr. Sarintip Sooksai, The Institute of Biotechnology and Genetic Engineering (IBGE), Chulalongkorn University, Bangkok, Thailand. The expression vector for *P. pastoris* GS115, pPICZαA plasmid (Invitrogen, USA) was used.

Escherichia coli strains were cultured in LB broth media or on LB agar plate while strains harboring plasmids in this research were cultured with ampicillin supplemented. The recombinant *E. coli* for antioxidative peptide expression were *E. coli* MG1655 derivatives. The pQE-30Xa (Invitrogen, USA) was selected to be the expression vector in for target peptide production in *E. coli*. All strains and plasmids used in this research were listed as the following

Table 3.1 Strain list

Strain	Organism	Plasmid	Reference
HIT-DH5α	*E. coli*	-	RBC Bioscience, Taiwan
EWP2304	*E. coli*	pWP2304	pWP2304→ DH5α
EWPαO	*E. coli*	pWPTAαO	pWPTAαO → DH5α
MG1655	*E. coli*	-	Laboratory stock
MG1655/pQE-30 Xa	*E. coli*	pQE-30 Xa	pQE-30 Xa→ MG1655
DHAW	*E. coli*	pQE-AW	pQE-AW → DH5α
AW	*E. coli*	pQE-AW	pQE-AW → MG1655
GS115	*P. pastoris*	-	Dr. Sarintip Sooksai, IBGE
WPTA1	*P. pastoris*	-	This research (Integrated DNA fragment encoding AW peptide onto the chromosome)
αA	*P. pastoris*	-	This research (Integrated empty pPICZαA onto the chromosome)

Table 3.2 Plasmid list

Plasmid	Reference
pCR®2.1-TOPO®	Invitrogen, USA
pWP2304	This research (pCR®2.1-TOPO® containing DNA fragment encoding AW peptide)
pPicZαA	Invitrogen, USA
pWPTAαO	This research (pPICZαA containing DNA fragment encoding AW peptide)
pQE-30 Xa	Invitrogen, USA
pQE-AW	This research (pQE-30 Xa containing DNA fragment encoding AW peptide)

3.1.2 Chemical and reagents

Table 3.3 Chemical

Chemical and reagents	Company
2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)	Sigma-Aldrich Co, U.S.A.
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich Co, U.S.A.
2-hydroxylmethyl-2-methyl-1,3-propanediol (Tris)	Bio Basic, Inc., Canada
Acetic acid (glacial) 100% anhydrous	Merck, Germany
Acrylamide	Invitrogen, U.K.
Agar (Microbiology grade)	Merck, Germany
Agarose	Research Organic Inc.,

	U.S.A.
Ammonium persulfate	Bio basic, Inc., Canada
Ampicillin	TP DRUG Laboratory
Ascorbic acid	Sigma-Aldrich Co, U.S.A.
Biotin	Fluka, Germany
Bis-acrylamide (N,N'-methylenebisacrylamide)	Bio basic, Inc., Canada
Boric acid	Sigma Aldrich, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich Co, U.S.A.
Bromophenol blue	Sigma-Aldrich Co, U.S.A.
Calcium chloride dihydrate	Merck, U.S.A.
Cobalt chloride	Sigma Aldrich, Germany
Coomassie Blue R-20	Merck, Germany
Cupric sulfate pentahydrate	Carlo Erba Reagenti, Italy
Dextrose	Bio basic, Inc., Canada
Ethanol	Merck, U.S.A.
Ethylenediaminetetreacetic acid (EDTA)	Bio basic, Inc., Canada
Ferrous sulfate heptahydrate	Carlo Erba Reagenti, Italy
Glycerol	Sigma Aldrich, Germany
Glycine	Ajax Finechem Pty Ltd., Australia
Histidine	Fluka, Germany
Hydrochloric acid (HCl)	Merck, Germany
Isopropylβ-D-1-thiogalactopyranoside (IPTG)	Bio basic, Inc., Canada

Maganese sulfate monohydrate	Merck, Germany
Magnesium sulfate heptahydrate	Bio basic, Inc., Canada
Methanol	Merck, U.S.A.
Peptone	Bio basic, Inc., Canada
Phosphoric acid	Merck, U.S.A.
Potassium di-hydrogen phosphate	Merck, Germany
Potassium persulfate	Merck, Germany
Skim milk	Fonterra, New Zealand
Sodium chlorine	Ajax chemicals, Australia
Sodium dodecyl sulfate (SDS)	Bio basic, Inc., Canada
Sodium iodine	Merck, U.S.A.
Sodium molybdate dehydrate	Merck, U.S.A.
Sulfuric acid	Merck, Germany
TAE buffer premix powder	Bio basic, Inc., Canada
TEMED (N,N,N',N'-tetramethylene-ethylenediamine)	Bio basic, Inc., Canada
Tryptone powder	Bio basic, Inc., Canada
Tween-20	Sigma Aldrich, Germany
Yeast extract powder	Bio Springer, U.S.A.
Zeocin	Invitrogen, U.S.A.
Zinc chloride	Sigma Aldrich, Germany
MasterPure™ RNA Purification Kit	Epicentre®, U.S.A
Pichia EasyComp™ Transformation Kit	Invitrogen, U.S.A.
ReverTra Ace® qPCR RT Master Mix Kit	Toyobo, Japan

SpinClean™ Gel Extraction Kit	Mbiotech, Inc, Korea
SpinClean™ Plasmid miniprep Kit	Mbiotech, Inc, Korea
THUNDERBIRD® SYBR qPCR Mix Kit	Toyobo, Japan
TOPO® TA Cloning® Kit	Invitrogen, U.S.A.

3.2 Methods

3.2.1 Preparation of DNA fragment for expressing target antioxidative peptide

The interested antioxidative peptide from algae waste hyhrolysate, named as AW, contains only 11 amino acids: VECYGPNRPQF (Sheih et al., 2009), however, it is difficult for genetic handling. Therefore, DNA fragment for encoding this AW peptide, 6 copies of this antioxidative peptide separated by Lysine residues was designed. The more copied was expected to be easier genetic manipulation, and recombinant peptide should provide more antioxidant activity. These Lysine residues can be further digested by trypsin to obtain the small peptides with the same size as the original ones. The 234 base pair DNA fragment which flanked by *Xho*I restriction sites was synthesized as double-standed DNA oligonucleotides: gBlocks® Gene Fragments by Intergrated DNA technology (Figure 3.1).

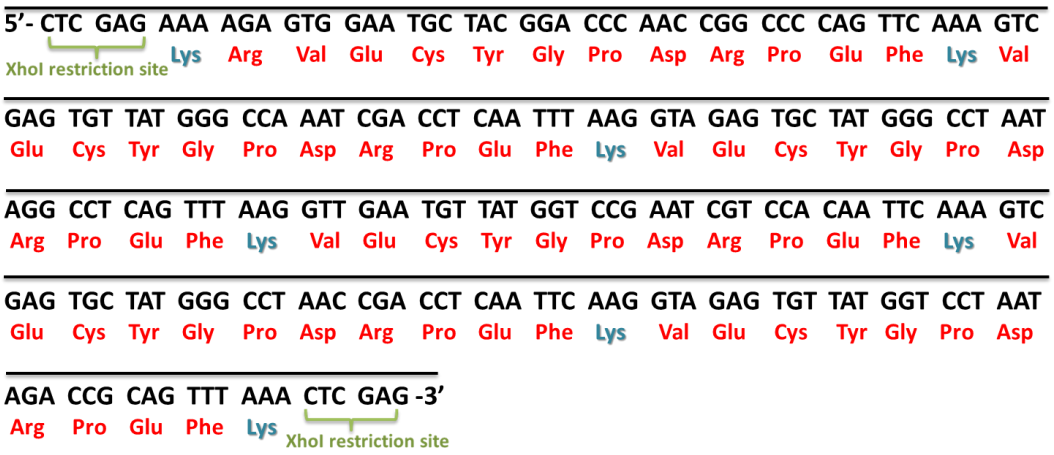


Figure 3. 1 The sequence of designed DNA fragment encoding 6 copies of algae waste hydrolysate (AW) peptide which linked by Lysine residue and flanked by *Xho*I restriction sites.

To amplify and maintain the synthesized DNA fragment, white powder of gBlocks Gene Fragment was resuspended in 20 µl TE buffer and then performed 15 µl reaction by adding 5 µl of diluted gBlocks Gene Fragments, 1 µl of *Taq* polymerase, 1.5 µl of 0.05 mM dNTP, 1.5 µl of 1X *Taq* polymerase Buffer with 1.5 mM MgCl at 70ºC for 15 to 30 minutes. The products were cloned into pCR®2.1-TOPO® to generate the plasmid named as pWP2304, and then transformed into HIT-DH5α *E. coli* according to TOPO® TA Cloning® Kit manual. To confirm transformed *E. coli* with pWP2304, the white colony was selected according to Blue/White screening by spread on LB agar with 40 µg/µl of X-gal and 100 µg/ml of ampicillin at 37˚C overnight. The plasmids were extracted from white colony by using SpinClean^{TM} Plasmid miniprep Kit and verified by *Xho*I digestion, resulting in expected 234 base pair of DNA fragment on 1% agarose electrophoresis. The verified strain was named as EWP2304

3.2.2 Construction of *P. pastoris* strain for target antioxidative peptide expression

The plasmids pWP2304 and pPICZαA were cut by *Xho*I at 37ºC overnight to obtain the complementary ends of specific DNA fragment and linear pPICZαA. After the analysis by 1% agarose gel electrophoresis, the correct sizes of 234 base pair DNA band from pWP2304 and 3500 base pair DNA band from pPICZαA were extracted by SpinClean™ Gel Extraction Kit, and then ligated by T4 DNA ligase enzyme. After being incubated at 16˚C overnight, the ligation reaction was transformed into *E. coli* DH5α competent cells and selected on low salt LB ager with 25 μg/ml zeocin at 37˚C for 16-18 hours. The recombinant expression vector was verified by *Xho*I digestion and DNA Sequencing (Macrogen, Korea). The verified plasmid was named as pWPTAαO. Then, pWPTAαO was cut at the 5' *AOX1* region by *Sac*I to linearize it. Linear DNA was transformed into *P. pastoris* GS115 competent cells by using *Pichia* EasyComp™ Transformation Kit and selected on YPD plate with 100 μg/ml zeocin at 30˚C for 3-5 days. The integration of target DNA from pWPTAαO onto *P. pastoris* chromosome was screened by colony PCR method with AOX1 forward and AOX1 reverse primers. The accuracy of PCR products were then verified by DNA Sequencing. The recombinant yeast strain for target AW peptide expression was named WPTA1.

3.2.3 Study of recombinant antioxidative peptide expression in *P.pastoris* GS115

The strain WPTA1 was grown in 25 ml YPG broth at 30˚C in shaking incubator (250-300 rpm) until the OD_{600} being 2-6 (approximately 16-18 hours). Cells were harvested by centrifugation at 1,500-3,000 x g for 5 minutes at room temperature and the pellet was resuspended in Basal medium pH 5.0 supplemented with 0.004% w/v Histidine and 0.435% v/v PTM1 to obtain an OD_{600} of 0.1. To induce the expression, 100% methanol was added to final concentration of 0.5% v/v every 24 hours for maintaining the expression for 48 hours. Sample was taken during process every 24 hour for monitoring the growth by measuring the absorbance at 600 nm, and pH value. The *P. pastoris* GS115 and αA (integration of empty pPICZαA onto *P. pastoris* GS115 chromosome) strains were served as negative controls in the same conditions. To observe the recombinant AW peptide, the supernatant without cell pellet was analyzed by 18% acrylamide gel on Tricine-SDS-PAGE and stained with Coomassie Blue R-20. The protein concentrations were determined by Bradford method (ref.). Furthermore, the expected protein band on SDS-PAGE method was selected to verified amino acid sequence by MS/MS.

3.2.3.1 Reverse Phase-HPLC analysis

The supernatant obtained from the WPTA1 strain production by using Basal medium and MMH medium was filtered through 0.45 µm filters (Whatman, GE, Buckinghamshire, UK) and separated using HPLC (Spectra System, Thermo Scientific) with reversed phase C_{18} column (250 × 4.6 mm, Luna 5U, Phenomenex, Torrance, CA, USA). Peptide

fractions were eluted by a rational gradient condition of mobile phase A (0.1% (v/v) trifluoric acetic acid) and mobile phase B (70% (v/v) acetonitrile in 0.05% (v/v) trifluoric acetic acid) at a flow rate of 0.7 ml/min. Ultraviolet absorbance was detected at 280 nm. Chromatographic analyses were completed with ChromQuest Software (Thermo Scientific).

3.2.3.2 Cell lysis

The whole wet cells from the expressed culture was initially disrupted by centrifugation at 3,000 x g for 5 minutes, and then the pellet was resuspended with 5 µl of LEW buffer After that, the suspension was frozen at -20 ºC. The frozen cell pellet in LEW buffer was then thawed. This freeze-and-thaw step was repeated for 3 times. Then, the 100 µg/ml of lysozyme was added to the suspension. After being kept on ice for 30 minutes, the suspension was sonicated on ice according to the instruction by manufacture using 15 x 15 s bursts with a 15 s cooling period between each burst. The crude lysate was centrifuged at 10,000 x g, 4 ºC for 30 minutes, then supernatant was kept as a total protein lysate. The total proteins lysate was operated on18% acrylamide gel on Glycine-SDS-PAGE and stained with Coomassie Blue R-20.

3.2.4 Construction of *E. coli* strain for target antioxidative peptide expression

The pWP2304 plasmid was amplified by PCR technique using AG1W-F and AG1W-R primers to gain the DNA fragment encoding 6 copies of AW peptide. Then, the 234 base pair of PCR product and the 3,509 base pair pQE-30 Xa expression vector were cut by *Bam*HI and *Hin*dIII restriction enzymes. The two digested DNA were ligated by T4

DNA ligase enzyme at 16ºC overnight. The reaction was transformed into DH5α *E. coli* to primary amplify and check the recombinant expression for AW peptide. The transformants were growth on LB agar added 100 µg/ml of ampicillin at 37ºC. After, the colony on the select medium was extracted plasmid to verify the ligation by *Bam*HI and *Hin*dIII. The digested reaction was operated on 1% agarose gel electrophoresis. The verified plasmid was transformed to *E. coli* MG1655. The plasmid was then extracted and verified by double digestion of *Bam*HI and *Hin*dIII and DNA sequencing. The verified recombinant expression vector was named as pQE-AW, and *E.coli* MG1655 derivative harboring this plasmid was named as AW strain.

3.2.5 Study of recombinant antioxidative peptide expression in *E. coli*

The single colony of AW strain was grown in 2 ml LB broth with 100 µg/ml ampicillin, and then incubated at 37˚C, 200 rpm in shaking incubator for approximately 16-18 hours. After that, the culture was transferred into 50 ml of new sterilized LB broth with 100 µg/ml ampicillin in 250 ml flask and incubated at 37ºC, 200 rpm until the OD_{600} being 0.6-0.8 (approximately 2 hours). Then, the expression was induced by 1 mM IPTG addition. The experiment was operated for 6 hours which samples were harvested every 2 hours. The cell growth of each sample, measured by absorbance at 600 nm, and pH value was monitored. The expression of recombinant antioxidative AW peptide was studied by using *E. coli* MG1655/pQE-30 Xa strain as the control.

3.2.5.1 Cell lysis

For *E. coli*, recombinant protein was expressed in cells. The cell lysis was required as the first step to obtain intracellular proteins. The whole wet cells from the expressed culture was initially disrupted by centrifugation at 8,000 x g for 30 minutes, and then the pellet was resuspended with 5 µl of LEW buffer After that, the suspension was frozen at -20 ºC. The frozen cell pellet in LEW buffer was then thawed. This freeze-and-thaw step was repeated for 3 times. Then, the 100 µg/ml of lysozyme was added to the suspension. After being kept on ice for 30 minutes, the suspension was sonicated on ice according to the instruction by manufacture using 15 x 15 s bursts with a 15 s cooling period between each burst. The crude lysate was centrifuged at 10,000 x g, 4 ºC for 30 minutes, then supernatant was kept as the soluble proteins. However, the pellet was also harvest and kept on ice for extracting inclusion bodies. The remaining pellet was resuspended in 5 ml of LEW buffer, and centrifuging at 10,000 x g, 4 C for 30 minutes. The pellet was further used to harvest the insoluble proteins by resuspending in 3 ml of DS buffer, stirring on ice for 60 minutes, and then centrifuging at 10,000 x g, 4 ºC for 30 minutes. At this time, the supernatant was kept as solution C (insoluble proteins). The solution A and C was analyzed on 18% acrylamide gel Glycine-SDS-PAGE stained with Coomassie Blue R-20 for investigating protein expression. The protein concentrations were determined by Bradford method (Bollag, D.M., Protein Methods).

3.2.5.2 Determination of the recombinant AW peptide expression in *E. coli.*

3.2.5.2.1 Real-Time PCR analysis

To measure the expression of recombinant AW peptide from the *E. coli* strain at the transcriptional level, Real-Time PCR method was applied with the specific primers, AG1W-F and AG1W-R. By using MasterPure™ RNA Purification Kit, the total RNA was extracted from the pellet cells harvested from the highest protein expression period. Then, the 50 ng of RNA solution was used as the template in 20 µl total volume reaction of reverse-transcription according to ReverTra Ace® qPCR RT Master Mix Kit to obtain cDNA. The reaction was conditions was performed as : incubating at 37ºC for 60 min, 50ºC for 5 min, heating to 98ºC for 5 min, and storing at 4ºC. After that, the cDNA solution was used as the template in the further reaction according to THUNDERBIRD® SYBR qPCR Mix Kit through 40 cycles of RT-PCR as: pre-denaturation at 95ºC for 10 min, denaturation at 95ºC for 15 sec, annealing 60ºC for 15 sec, and extension at 72ºC for 20 sec. The RT-PCR product could be verified on 1% agarose gel electrophoresis and DNA sequencing. The expression was compared with the control strains (MG1655 and MG1655/pQE-30 Xa).

3.2.5.2.2 Immunoblot analysis

From the 18% acrylamide gel Glycine-SDS-PAGE, the only expected protein band was cut from the gel and extracted in 500 µl of 1X Elution buffer by incubating the solution on stirrer at 4 ºC overnight. After that, the 3 µl eluted protein solution was transferred onto nitrocellulose membrane and incubated at room temperature for 2 hours.

The membrane was then blocked with 5% skim milk on rocking platform for 1 hour. After discarding the buffer, the membrane was rinsed with PBS buffer containing 20% tween for 4-5 times. To detect the recombinant peptide, due to the fusion of 6X Histidine tag at the N-terminal of recombinant peptide, the HRP conjugated anti-His tag was applied to the blotted membrane for 2 hours at room temperature. Then, the membrane was washed again in PBS buffer containing 20% tween for 4-5 times. Finally, colorimetric determination was provided by DAB solution.

3.2.6 Antioxidant characterization of recombinant antioxidative peptide

3.2.6.1 DPPH radicals scavenging activity assay

This method is based on DPPH radicals scavenging activity assay. In this research, ascorbic acid was used as the positive control. The 40 µl of each serial dilution of recombinant peptide was mixed with 160 µl of 1 mM DPPH radical dissolved in ethanol. The mixture was incubated in dark condition for 40 minutes. Then, the absorbance at 517 nm was monitored. The %inhibition and IC_{50} values (concentration of peptide scavenging 50% of DPPH radicals). In addition, the chemical synthesized peptide was also tested with the same protocol.

3.2.6.2 ABTS radical scavenging assay

The recombinant peptide was also tested for ABTS radical scavenging activity. The ABTS radical was generated by preparing 7 mM of solvable ABTS in aqueous solution of 2.45 mM $K_2S_2O_8$ at the ratio 1:1. The mixture was incubated for 12-16 hours in a dark condition.

Then, this solution was diluted with deionized water until absorbance at 734 nm reached to 0.7±0.2. The reaction was performed by adding 10 µl of sample into 300 µl of ABTS radical solution. The reaction was incubated at room temperature for 10 minute. Then, the absorbance at 734 nm was measured. The ascorbic acid was used as positive controls. The chemical synthesized peptide was also tested with the same protocol. The %inhibition and IC_{50} values (concentration of peptide scavenging 50% of ABTS radicals).

3.2.6.3 The *in vitro* protective effect of the recombinant peptide on oxidation-induced DNA damage

For this method, pBR322 was served as the DNA sample. The DNA damage was induced by hydroxyl radicals based on The Fenton reaction. The reactions were incubated with various concentrations of the recombinant peptide. Each reaction was performed in total volume 20 µl by mixing 3 µl of 50 ng pBR322 plasmid DNA, 3 µl of 50 mM phosphate buffer (pH7.4) and 4 µl of different recombinant peptide concentrations at room temperature. After, the reaction was mixed 3 µl of 2 mM $FeSO_4$, and 3 µl of 30% H_2O_2. The mixture was incubated at 37˚C for 30 minutes. The result was determined by 1% agarose gel electrophoresis to observe an appearance of DNA band the represented several form of plasmid DNA. The chemical synthesized peptide was also tested with the same protocol.

CHAPTER IV
RESULT AND DISCUSSION

The previous research of Sheih was revealed antioxidant properties of a new antioxidative peptide from the algae protein waste, which is a by-product during *Chlorella vulgaris* algae essence production hydrolyzed by pepsin. The peptide, consisting of 11 amino acid sequences as VECYGPNRPQF, could efficiently quench a various free radicals including ABTS radical measured by TEAC assay, according to the method of Arts in 2004. They found that the IC_{50} value of this purified peptide was 12.83±0.5 µg/ml. For the DPPH scavenging ability assay, the purified peptide, measured according to the method of Huang and Mau (2006), showed a concentration-dependent increase of antioxidative activity for concentrations up to 78.54 µg/ml. Its activity against DPPH radicals was higher than synthetic antioxidant BHT, but lower activity than trolox. Furthermore, this antioxidative peptide illustrated the positive role on protecting DNA damage from hydroxyl radicals based on the Fenton reaction (Qian *et al.* 2008). This protective ability was concentration-dependent, ranging from 13.88 to 111.13 µg/ml. Interestingly, Sheih et al. was also described that the peptide played a pivotal role in antioxidant more efficiently than peptide from other marine protein sources in most case. Moreover, it was suggested that this antioxidative peptide was also *in vitro* gastrointestinal enzyme-resistant without cytotoxicity in human lung fibroblasts cell lines (Wl-38).

Due to all interesting properties mentioned above, the peptide of VECYGPNRPQF, named as AW peptide, was selected to be studied in this research. The chemical synthesized AW peptide was tested for

antioxidant activities, including DPPH and ABTS radical scavenging ability, and *in vitro* protective effect on DNA damage induced by hydroxyl radicals. When each serial dilution of the synthesized AW peptide (1.95 µM up to 1000 µg/ml) was tested for DPPH radical scavenging ability, the IC_{50} could not be calculated. However, the 1 mg/ml of this synthesized peptide revealed 26.32 ± 0.92% inhibition against DPPH radical (Table 4.1). For the ABTS scavenging activity, the protocol in this research was applied from the method of Art et al. (2004) (Arts *et al.* 2004). The concentrations from 0.002 µg/ml up to 1000 µg/ml of this 1.309 kDa synthesized AW peptide were assayed. The IC_{50} value of the synthesized antioxidative peptide was 76.99 ± 1.62 µg/ml (Table 4.1).

Table 4.1 The IC_{50} values of DPPH and ABTS radical scavenging assay

Sample	**IC_{50} (µg/ml)**	
	DPPH scavenging assay	**ABTS scavenging assay**
Ascorbic acid	15.37 ± 2.4	43.44 ± 2.58
Previous study*	ND	12.83 ± 0.5
Chemical synthesized AW peptide	ND	76.99 ± 1.62

* From the study of Sheih et al, 2009.

ND Not determined as IC_{50}

The synthesized antioxidative AW peptide was also investigated for the *in vitro* protective effect on oxidation-induced DNA damage. This property is interested due to the *in vivo* generation of hydroxyl radicals. For this assay, the hydroxyl radicals were created by Fenton reaction (0.2 M $FeSO_4$ mixing with 30% H_2O_2). The plasmid DNA, served as DNA template) is normally in supercoil DNA form which mobile in agarose gel electrophoresis faster than other forms. The plasmid can change to open circular and linear DNA forms when DNA is attacked. These forms slower migrate through the agarose gel electrophoresis that the form of supercoil (Qian *et al.* 2008, Sheih *et al.* 2009). The result of this assay illustrated that the pBR322 plasmid DNA could be protected by the synthesized peptide from generated hydroxyl radicals (Figure 4.1). On the other hand, without the antioxidative peptide, pBR322 plasmid was damaged by hydroxyl radicals.

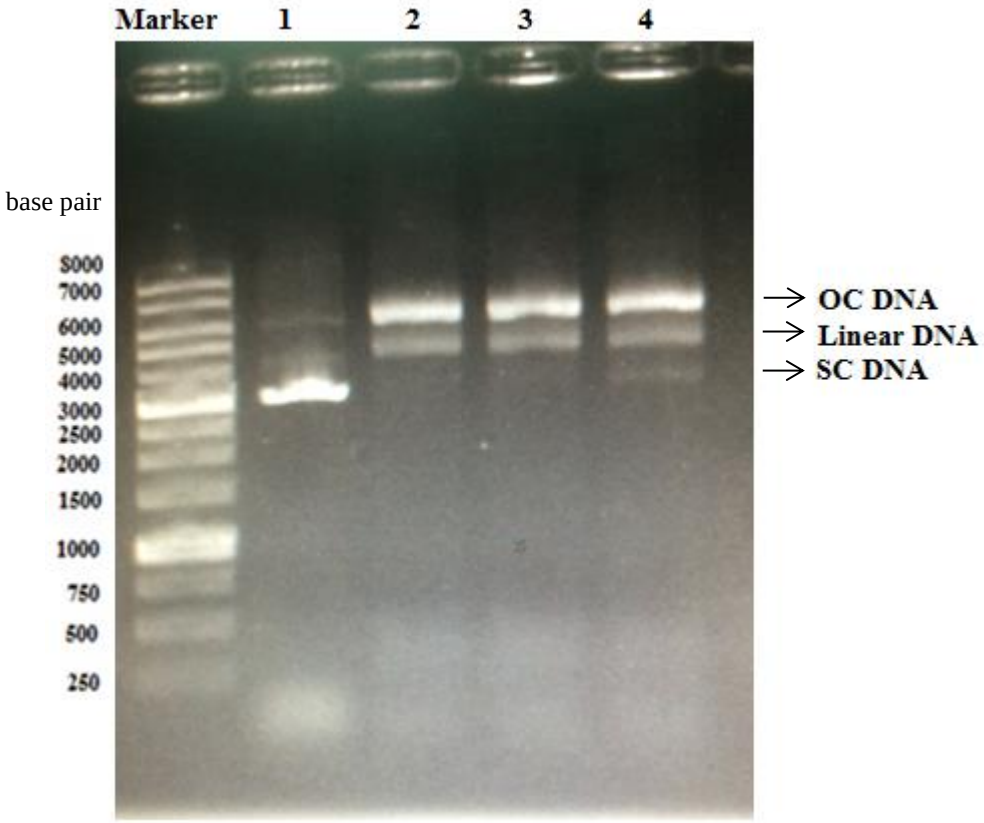


Figure 4.1 1% agarose gel electrophoresis analysis of the *in vitro* protective effect of the chemical synthesized AW peptide on oxidation-induced DNA damage. Lane 1, pBR322 plasmid DNA; Lane 2, pBR322 plasmid DNA treated with Fenton reaction, Lane 3-4, pBR322 plasmid DNA mixed with the chemical synthesized AW peptide at the concentrations of 0.005 µg/ml, 0.01 µg/ml, respectively, then treated with Fenton reaction (OC= open circular, SC= supercoil).

From the previously described results, the synthesized AW peptide revealed the satisfactory evidences of the antioxidant capacity. The different activity between this research and the previous research from Sheih et al, 2009 may resulted from the different assay protocols. To overcome some obstacles from natural product extraction, such as large

amount sample requirement, sample storage and relatively complicated procedure, recombinant DNA technology was applied to be served as an alternative to produce the target antioxidative peptide. The interested AW peptide consists of only amino 11 acids of VECYGPNRPQF which is difficult for genetic manipulation. Along with the purpose to increase effectiveness of antioxidative peptide, the DNA fragment for encoding this recombinant AW peptide was designed to contain 6 copies of this antioxidative peptide and each copy was separated by Lysine residues. The DNA sequence of this fragment was represented in Figure 4.2. The 234 base pair DNA fragment was flanked by *Xho*I restriction sites. With longer sequence, the easier genetic handling and also higher antioxidant activity than the single copy of the chemical synthesized one were expected. The expression systems for this research were *Pichia pastoris* and *Escherichia coli*.

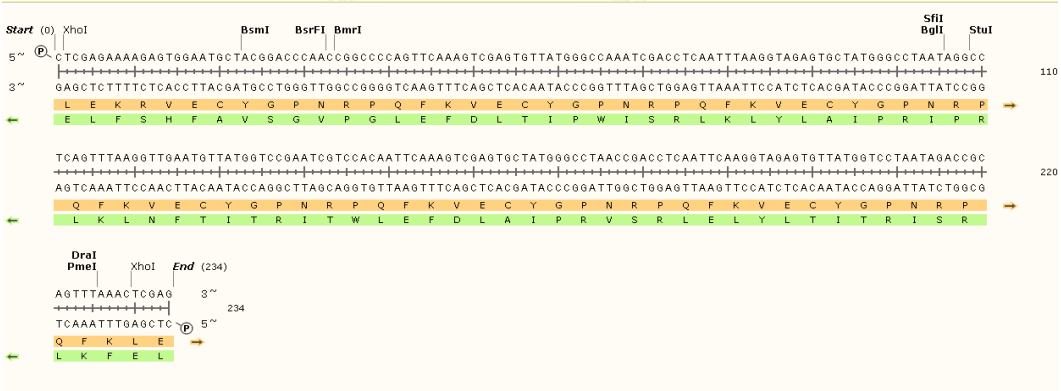


Figure 4.2 The sequence of DNA fragment encoding for 6 copies of the antioxidative AW peptide (VECYGPNRPQF). Each copy is linked by lysine codon. The fragment contains *Xho*I restriction site at 5' and 3' terminals for further molecular cloning.

4.1 Preparation of DNA fragment for expressing target antioxidative peptide

The synthesized DNA fragment (Figure 4.2) was obtained in the form of gBlocks® Gene Fragments (Integrated DNA Technologies, IDT, USA). After being amplified, the 234 base pair DNA fragment was cloned into pCR®2.1-TOPO® to generate plasmid named as pWP2304, then the plasmid was transformed into *E. coli* DH5α strain. The white ampicillin resistant transformants on LB added X-gal were selected. The inserted DNA fragment in pCR®2.1-TOPO was verified by *Xho*I digestion. The approximately 230 base pair of target and 3,900 base pair of TOPO plasmid were visualized on agarose gel electrophoresis (Figure 4.3). This DNA fragment was further extracted from the gel to clone into pPICZαA expression vector at *Xho*I restriction site, then plasmid named as pWPTAαO was generated.

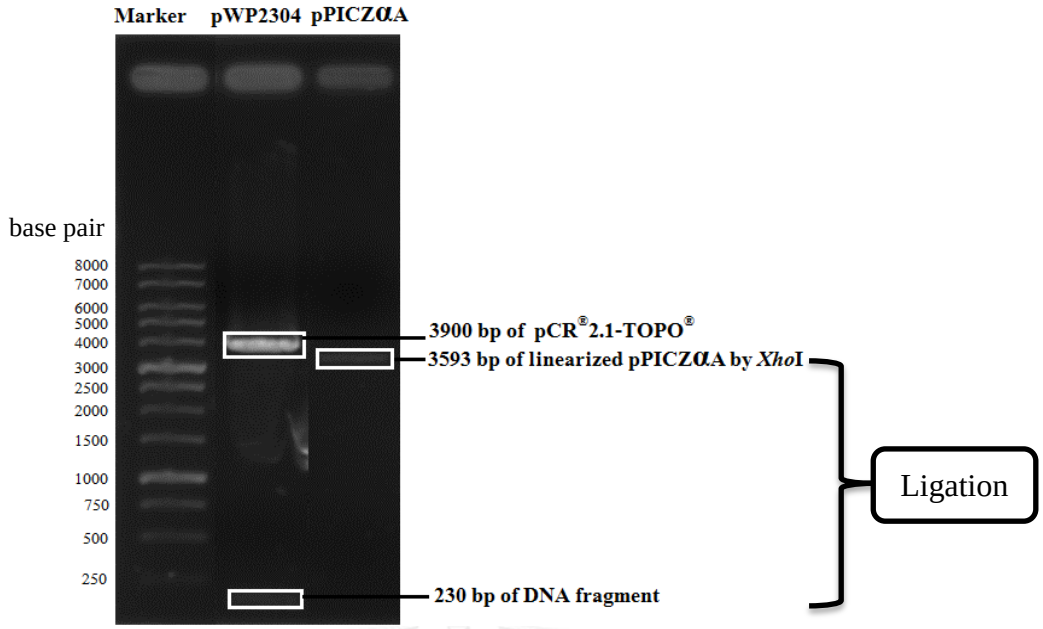


Figure 4.3 The *Xho*I digestion of pWP2304 and pPICZαA plasmids analyzed by 1% agarose gel electrophoresis analysis. The approximately 230 base pair fragment from pWP2304 and 3593 base pair fragment from pPICZαA were extracted from the gel for further ligation to generate the plasmid named pWPTAαO.

The pWPTAαO plasmid was first kept into *E.coli* DH5α on low salt LB agar with zeocin. The plasmid was primary verified by *Xho*I digestion (Figure 4.4). Then, the pWPTAαO was checked for the DNA sequence accuracy by DNA sequencing (Macrogen, Korea). The *E.coli* DH5α harboring pWPTAαO was named as EPWαO strain.

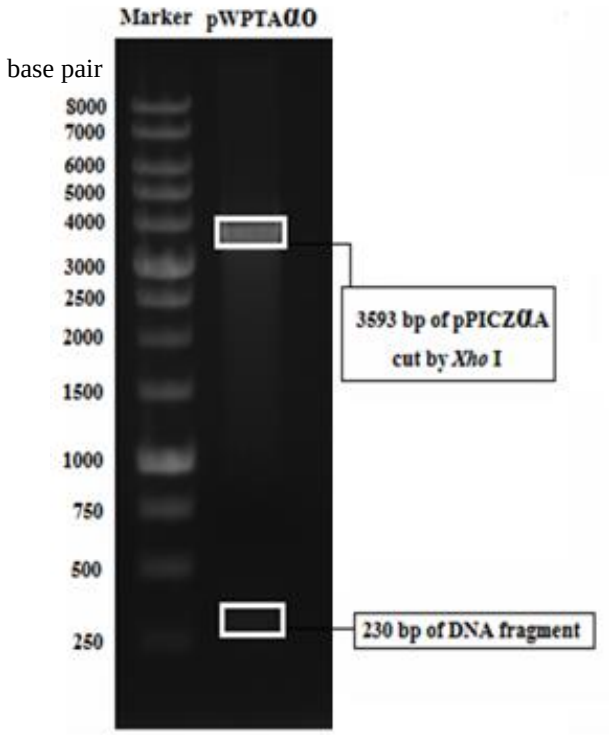


Figure 4.4 Verification of pWPTAαO by *Xho*I digestion. The 1% agarose gel electrophoresis analysis revealed expected 230 base pair and 3,600 DNA bands.

4.2 Construction of *P. pastoris* strain for target antioxidative peptide expression

The *P. pastoris* was the first selected expression system due to many advantages including availably accessed information, extracellular secretion of recombinant protein at high level, easy manipulation and simple nutrition requirement, post-translation modification and strong inducible *AOX1* promoter.

To construct *P. pastoris* strain for expressing the recombinant AW peptide, pWPTAαO was extracted from the EWPαO strain. Then, the recombinant expression vector was digested with *Sac*I restriction enzyme

to. The 3,823 base DNA band was eluted 1% agarose electrophoresis gel (Figure 4.5) and transformed into *P. pastoris* according to the *Pichia* EasyComp™ Transformation Kit manual (Invitrogen, USA).

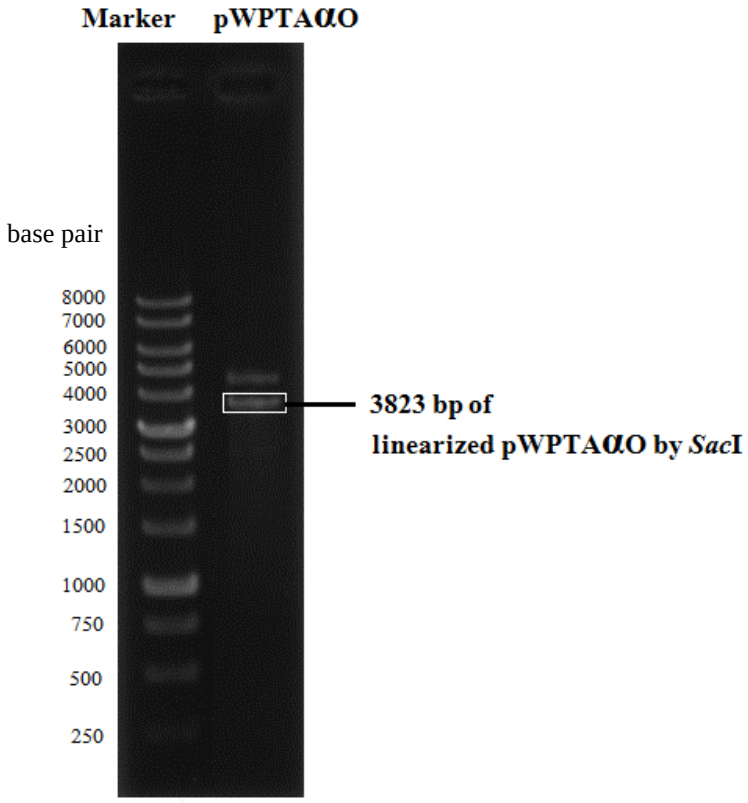


Figure 4.5 Linearization of pWPTAαO by *Sac*I. The 3,823 base pair fragment from pWPTAαO was further used for integrating the target DNA onto *P. pastoris* chromosome.

The zeocin resistant transformants on YPD plates with 100 µg/ml were selected. The colony PCR with AOX1 forward and AOX1 reverse primers was performed to screen for the integration of target DNA from pWPTAαO onto *P. pastoris* chromosome. The approximately 730 base pair PCR product indicated the integration of target DNA fragment,

compared with the approximately 500 base pair PCR product of empty pPICZαA served as a control (Figure 4.6). The selected clones were subjected for DNA sequencing (Marogen, Korea). The correct integration resulted in the recombinant *P. pastoris* named as WPTA1.

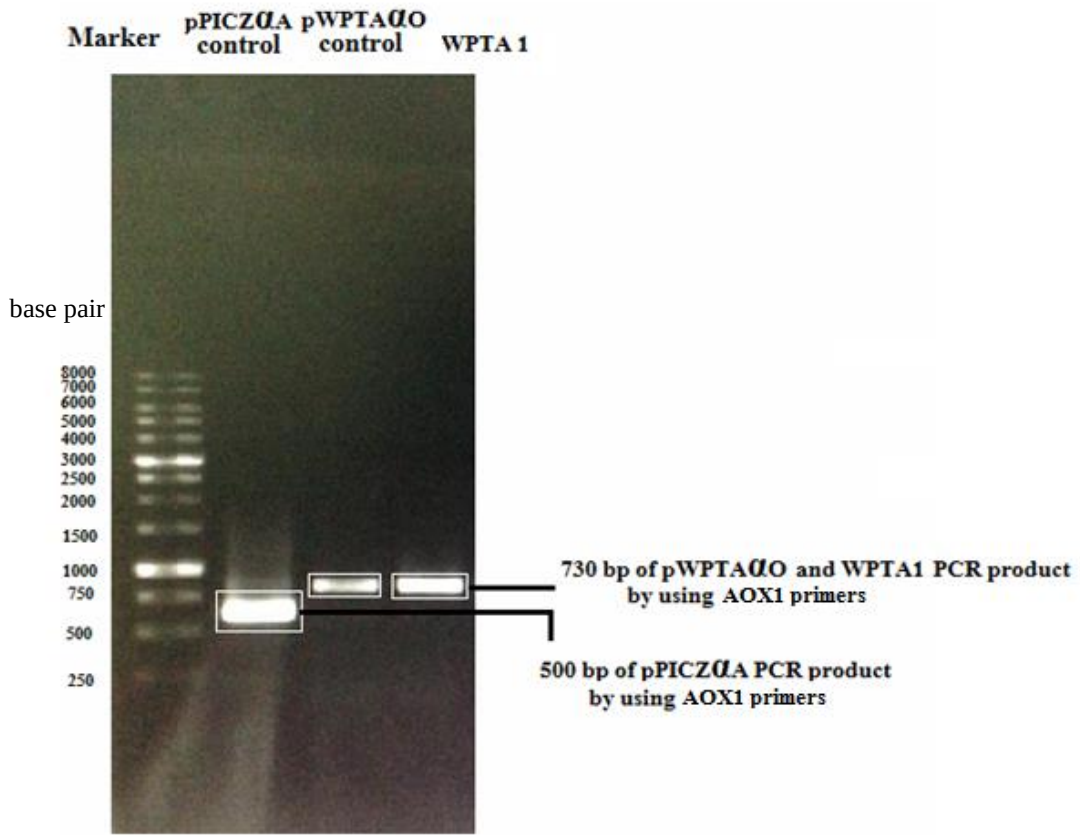


Figure 4.6 The colony PCR products from pPICZαA, pWPTAαO and WPTA strain using AOX1 forward and AOX1 reverse primers analyzed by on 1% agarose gel. The presence of the target DNA fragment added approximately 230 base pair to the 500 base pair of DNA region amplified by these AOX1 primers.

4.3 Study of recombinant antioxidative peptide expression in *P.pastoris* GS115

To express the recombinant AW from *P. pastoris* system, the WPTA1 strain was cultured in YPG medium at 30 ºC, 250 rpm for 16 hours. After that, the pre-culture pellet was transferred to 50 ml Basal medium with 0.004% w/v histidine and 0.435% v/v PTM1. Then, to induce the expression, 0.5% final concentration of methanol was added to the culture every 24 hours.

The growth profile of the AW strain was represented in Figure 4.7. The pH value was changed from 7 to 6.49 (Figure 4.7). Moreover, the cell-free culture was investigated for the highest total protein concentration, and 13.53 mg/ml of protein was obtained at 24 hour after being cultured in Basal medium. The result corresponded to the previous study of the expressed insulin from the strain NGEN2 which the highest recombinant protein was obtained at 24 hour in Basal medium induced by methanol (Ngenprasertsiri, S., 2012(.

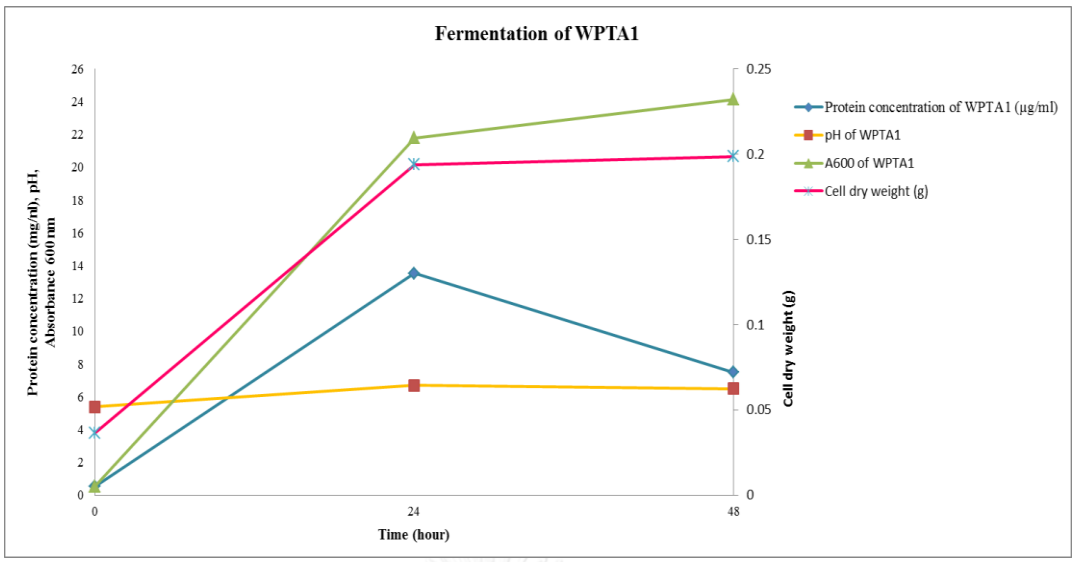


Figure 4.7 The growth profile of WPTA1, the recombinant *P. pastoris* strain for expressing the recombinant AW peptide. The strain was grown in Basal medium with methanol inducer at 30 C, 250 rpm for 48 hours.

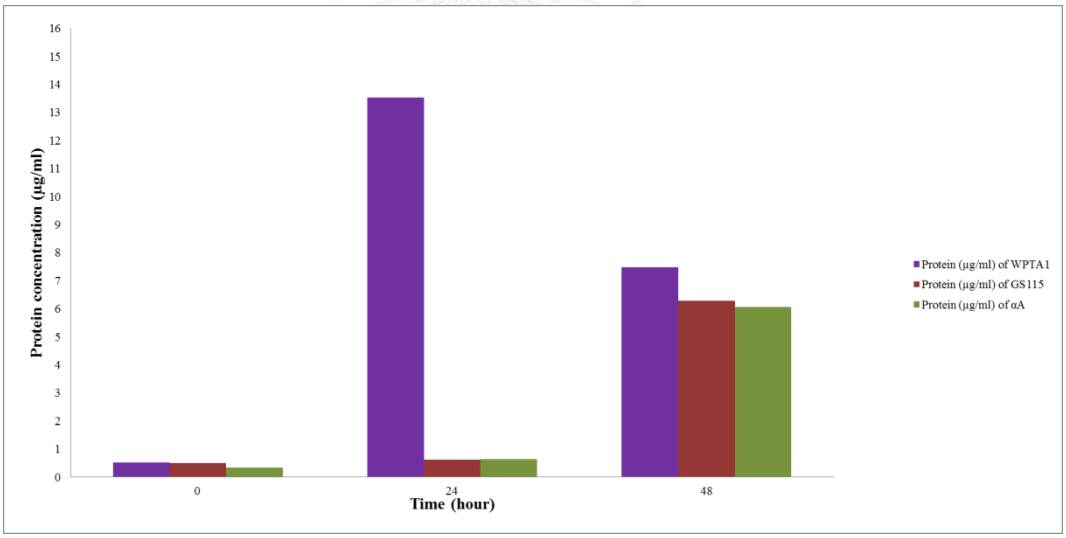


Figure 4.8 The protein concentration of WPTA1, GS115, and αA strains. The strains were grown in Basal medium with methanol inducer at 30 C, 250 rpm for 48 hours.

The recombinant AW peptide expressed from *P. pastoris* was expected to be extracellularly secreted due to the fusion of α-mating factor. However, the total protein concentration from WPTA1strain, measured by Bradford method, might not directly represented the yield of the target recombinant AW peptide due to the interference of others proteins. Then, the SDS-PAGE was applied to investigate the recombinant AW expression. Initially, the culture supernatant was run on 18% acrylamide gel of Glycine-SDS-PAGE system. The expected size of the recombinant antioxidative peptide with α-factor signal sequences was approximately 18.52 kDa. The target amino acid sequences of the recombinant AW peptide expressed from *P. pastoris* WPTA1 strain was represented in Figure 4.9.

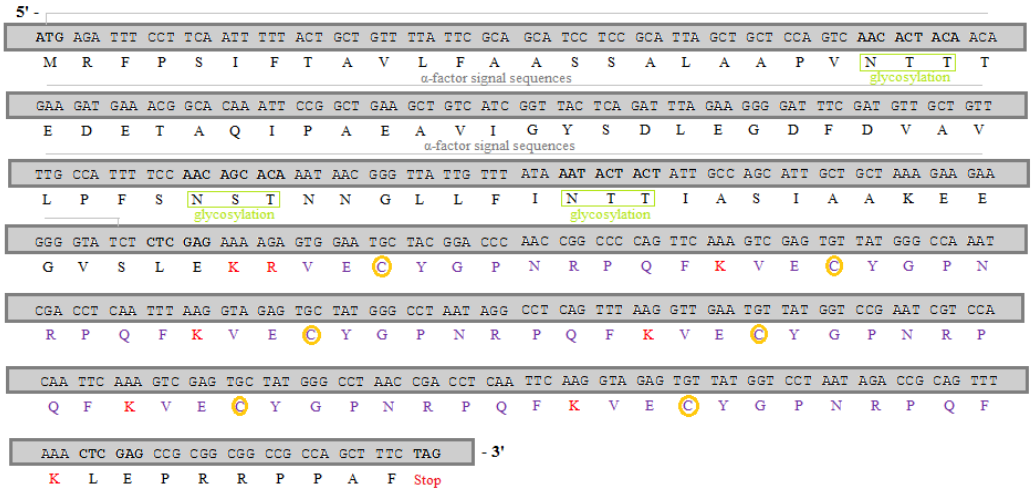
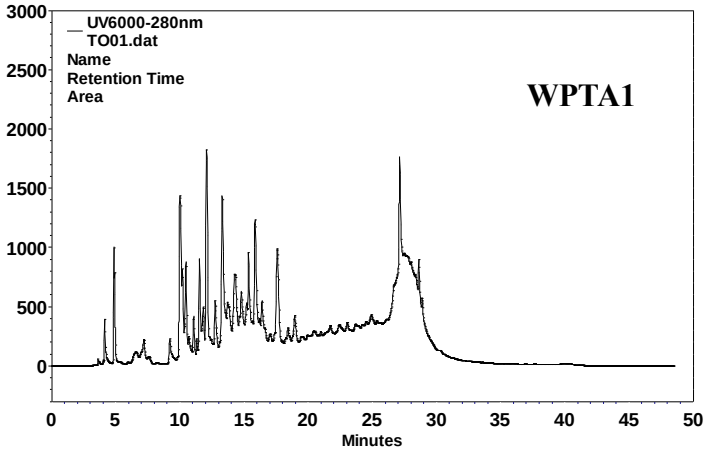
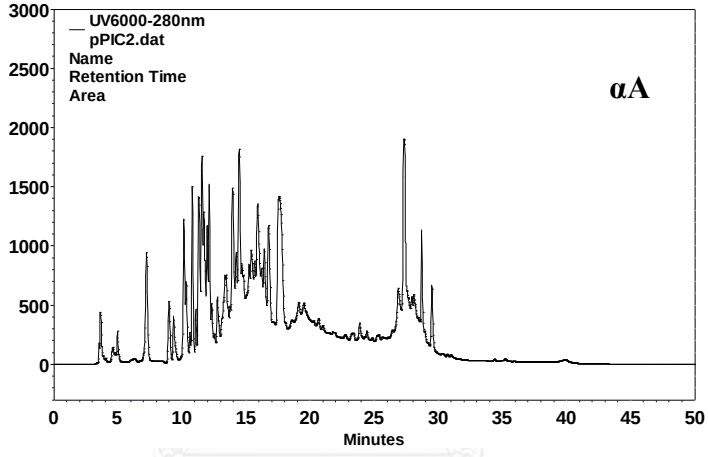


Figure 4.9 The expected DNA and amino acid sequences of the recombinant AW peptide expressed in *P. pastoris* WPTA1 strain.

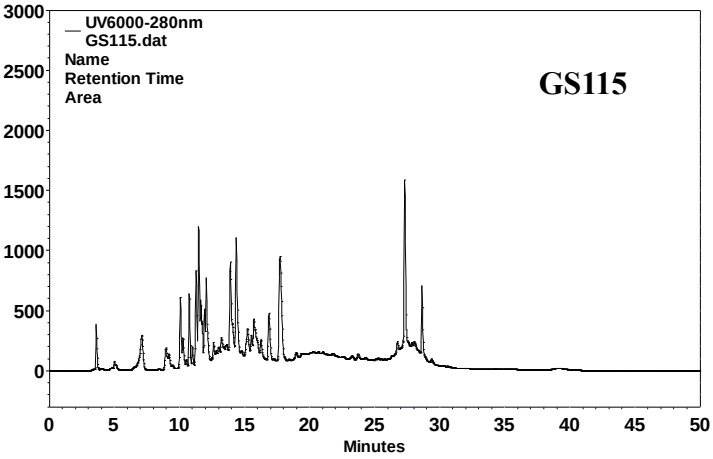
After staining gel by Coomessie Blue, the very light bands proteins were displayed. It might be resulted from the low-level protein expression. To increase in protein concentration, the samples were subjected to the speed vacuum at 25ºC. The additionally concentrated samples were again run on 18% acrylamide gel of Glycine-SDS-PAGE. However, the absence of target 18.52 kDa band was still revealed (Figure 4.13B). It was thought that this SDS-PAGE may not suitable to monitor low concentration of proteins. Therefore, the samples were further analyzed by the more sensitive method of HPLC for searching the interesting peak of target protein, compared with the production of GS115 and αA strains. The HPLC result was demonstrated in figure 4.10. However, it was complicated to further analyze due to the interference of other molecules.



A



B



C

Figure 4.10 The result of HPLC analysis of (A) the supernatant from WPTA1, (B) the supernatant from αA, (C) the supernatant from GS115. All strains were cultured in Basal medium with methanol inducer for 24 hours.

Due to low concentration of expressed proteins from WPTA1 strain in Basal medium, the optimization was performed. According to previous many researches on the protein production by using *P. pastoris* as the host cell, there were several important factors needed to be concerned. When a new recombinant *P. pastoris* strain was constructed and prepared for protein expression, it was suggested that process control and optimization should be investigated. According to the review of Minjie and Zhongping in 2013, they reported that optimization of foreign protein expression in *P. pastoris* had several crucial steps including genetic modification and expression cassette, fermentative process control for high level of recombinant product, and downstream purification. They stated that the optimization of process was in the middle stage of the crucial step, thus the bioprocess control directly influenced to the efficiency of recombinant strain and cost of downstream process for successful recombinant proteins production Moreover, they described that the key and necessary techniques of protein production in *P. pastoris* should focus on fermentation media, a condition of operation, temperature, optimal glycerol concentration for gaining a high cell density, an effective protein induction by methanol, etc (Gao and Shi 2013). The research of Çalık P. and coworker was demonstrated that the recombinant human growth, rhGH, was achieved the highest productivity by *P. pastoris* when maintain the pH value to 5 since the growth step until induction (Çalık P. et al., 2010). Furthermore, the review from Sue Macaulay-Patrick provided an interested example in optimization (Sue Macaulay-Patrick, et al., 2005). In 2001, Li and collaborators described that the yield of herring antifreeze protein was high-expressed in *P. pastoris* by decreasing the temperature of process from 30ºC into 23ºC

(Li *et al.* 2001). In addition, Hong and coworkers reported , for the production of laccase in *P. pastoris*, the higher activity of laccase was obtained by decreasing the temperature from 30ºC to 20ºC and reducing methanol concentration from 1% to 0.5% methanol in production (Hong *et al.* 2002). In 1999, Chauhan *et al*. studied the production of hepatitis B virus surface antigen (HBsAg) by recombinant *P. pastoris*and found the effects of semi-synthesized growth/induction media on recombinant substance production. The two-fold increase in HBsAg expression was found when, along with the addition of limited amounts of casamino acids in methanol-based induction medium, basal salts in glycerol-based growth medium was used (A.K. Chauhan 1999). The concentration of methanol is also an important factor for a recombinant peptide expression in *P. pastoris*. The optimization of methanol concentration can be varied in a range of 0.1%-3.0% v/v. The research from Damasceno and coworker reported that the highest concentration (4.3 g/l) of humanized single-chain variable domain fragment antibody (A33scFv) in *P. pastoris* was achieved by using 0.5% of methanol concentration after 72 h induction (Damasceno *et al.* 2004).

Due to the undesirable results of the recombinant AW expression by using Basal medium, the medium was first optimized. It was thought that the Basal medium of which nitrogen source was ammonium sulfate may not suitable for the recombinant AW expression. Therefore, the WPTA1 was subjected to be cultured in MMH medium, under the same conditions as Basal medium. However, the protein production of WPTA1 strain was very low proteins when analyzed by SDS-PAGE (Figure 4.11).

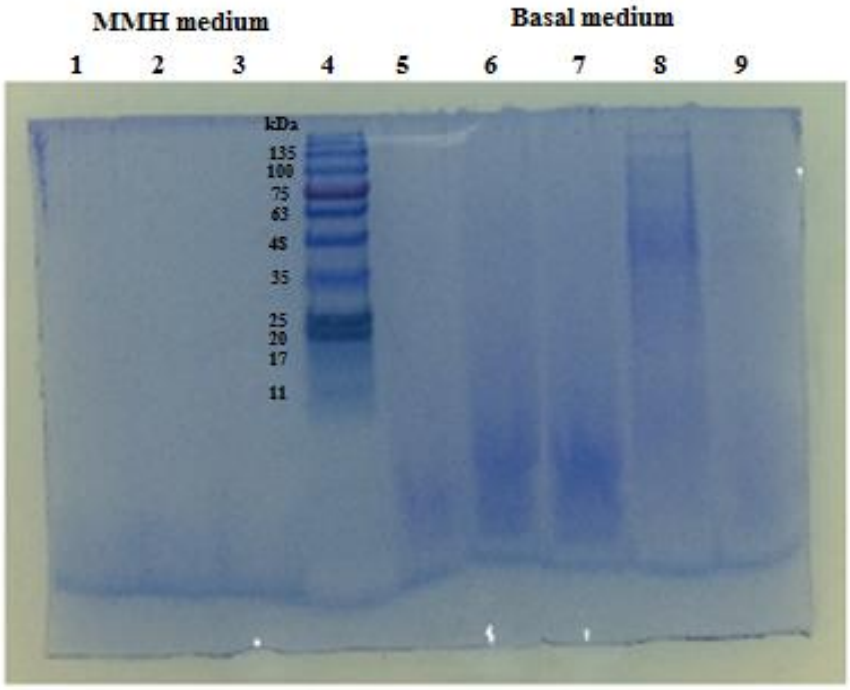


Figure 4.11 Protein expression from the supernatant of WPTA1 strain in MMH and Basal media on 18% acrylamide gel of Glycine-SDS-PAGE. Lane 1-3, protein expression from WPTA1 strain in MMH medium at 0, 24, and 48 hours, respectively; Lane 4, pre-strain protein marker; Lane 5, protein expression from GS115 strain in Basal medium at 48 hours; Lane 6 protein expression from αA strain in Basal medium at 48 hours; Lane 7-9, protein expression from WPTA1 strain in Basal medium at 0, 24, and 48 hours.

The supernatant of the WPTA1 expression in MMH medium was also analyzed by HPLC. As in figure 4.12, the interesting peak at 30 minute of analysis was collected. This fraction was further verified by MS/MS to confirm the amino acid sequences. However, the MS/MS result did not harmonized with the recombinant AW peptide sequences.

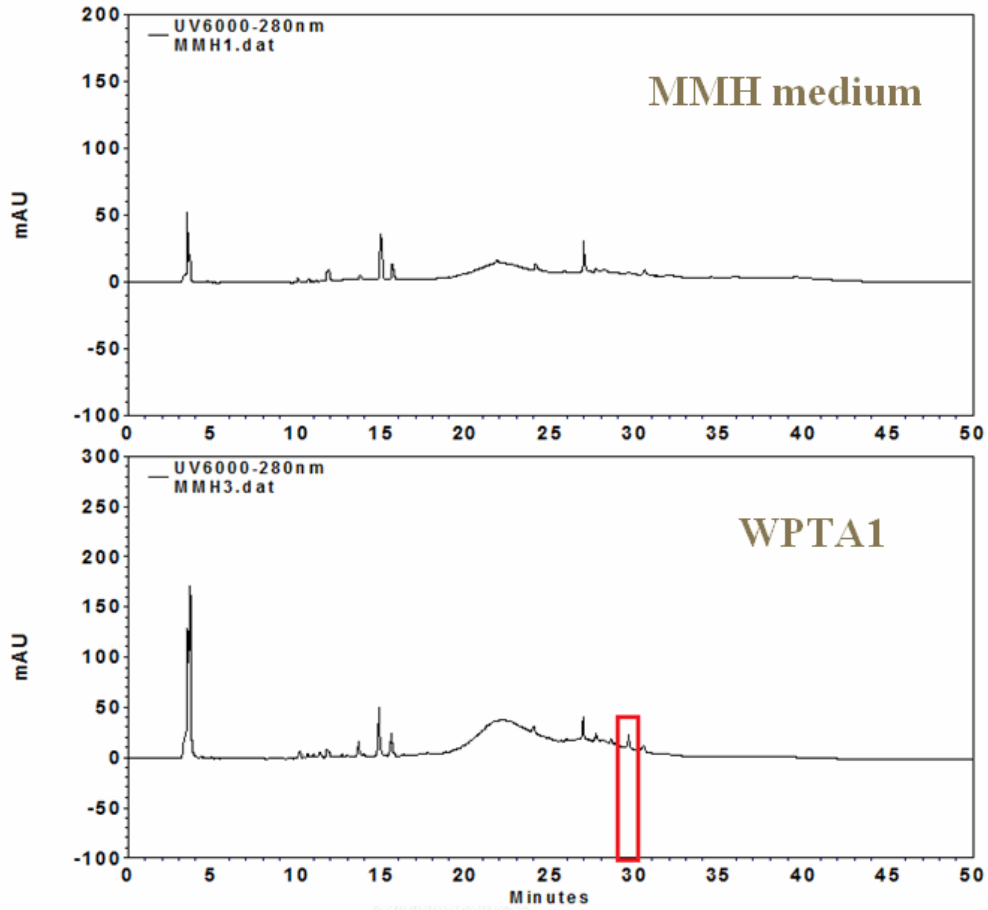


Figure 4.12 The result of HPLC analysis of the supernatant from WPTA1 strain in MMH medium at 24 hours.

When expected amino acid sequence of the recombinant AW peptide was concerned Cysteine amino acid was composed in every copy of this peptide (Figure 4.8). According to the characteristic of *P. pastoris*, it can perform the post-translational modification protein. The existence of Cysteine is an important key to modify protein structure by creating disulfide-bond between Cysteine molecules. Hence, it is possible that the recombinant AW becomes folding, and then effects directly on protein secretion in *P. pastoris*.

From these reason, it was hypothesized that the target peptide was accumulated intracellularly. Consequently, after being centrifuged at 3,000 x g for 5 minutes, the cell pellets from the 24 hour methanol induced culture were lysed by 3 repeats of freeze-and-thaw step in LEW buffer. The 100 µg/ml of lysozyme was also added before cell breaking by sonicating. The lysate proteins were analyzed on 18% acrylamide gel Glycine-SDS-PAGE. The results displayed many protein bands including the approximately 18 kDa. It was possible that the target peptide was trapped in the *P. pastoris* cells (Figure 4.13C).

However, it was difficult to purify this target 18 kDa band for further application. Due to the initial purpose, it was thought that *c-myc* epitope sequence might affect antioxidant activity of the recombinant peptide. Moreover, if the recombinant peptide was further cut at the Lysine linker by trypsin to obtain the short peptide as the originally for further application such as encapsulation, the purification could be complicated due to the generation of other similar sized peptide. Along with the expected property of extracellular secretion of recombinant peptide, the polyhistidine tag on the pPicZαA plasmid was thought to be unnecessary. The inserted of designed DNA fragment therefore did not allow polyhistidine tag to frame at C-terminal of the recombinant AW peptide. The lack of this tag resulted in difficult purification and detection of target peptide among the various intracellular proteins. In addition, during plasmid cloning, the digestion of *Xho*I enzyme causes the absence of flushed Kex2 cleavage site. Therefore, the α-factor signal may not be cut by Kex2 enzyme, and then this region still remained on the secreted protein. Interestingly, there are possible 3 glycosylation sites at the α-factor signal sequence (Figure 4.14). The glycosylation may cause the

slower migration on SDS-PAGE gel than it should be. This may resulted in misleading to expected target protein bands.

Although *P. pastoris* has actually been used to high-level expression of an interesting protein, it has some limitations. It was suggested that not every recombinant protein could be produced intracellularly or secreted. Lower protein yield could be obtained frequently in case of complex proteins that were hetero-oligomers, membrane-attached or prone to proteolytic degradation (Ahmad *et al.* 2014). Moreover, even though the ability to extracellularly secret recombinant protein is one of the main reasons for selecting *P. pastoris* as an expression host, endoplasmic reticulum protein folding, correct glycosylation, vesicular transport to the plasma membrane, gene dosage, secretion signal sequences, and secretome studies have been suggested to be considered for improving recombinant protein production (Damasceno *et al.* 2012).

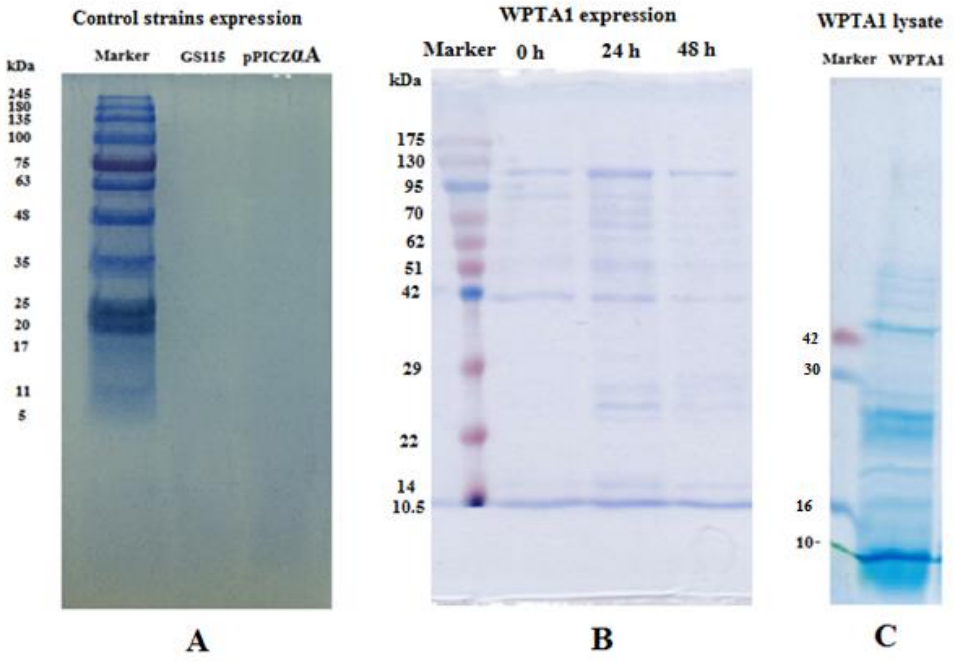


Figure 4.13 The protein expression from cultures in basal medium with methanol inducer analyzed on 18% acrylamide gel of Glycine-SDS-PAGE medium. (A) The supernatant cultures from 48 hours of GS115 and αA strains. (B) The supernatant from WPTA1 strain cultures at 0, 24, and 48 hours, respectively which concentrated by speed vacuum. (C) The cell lysate of WPTA1 strain.

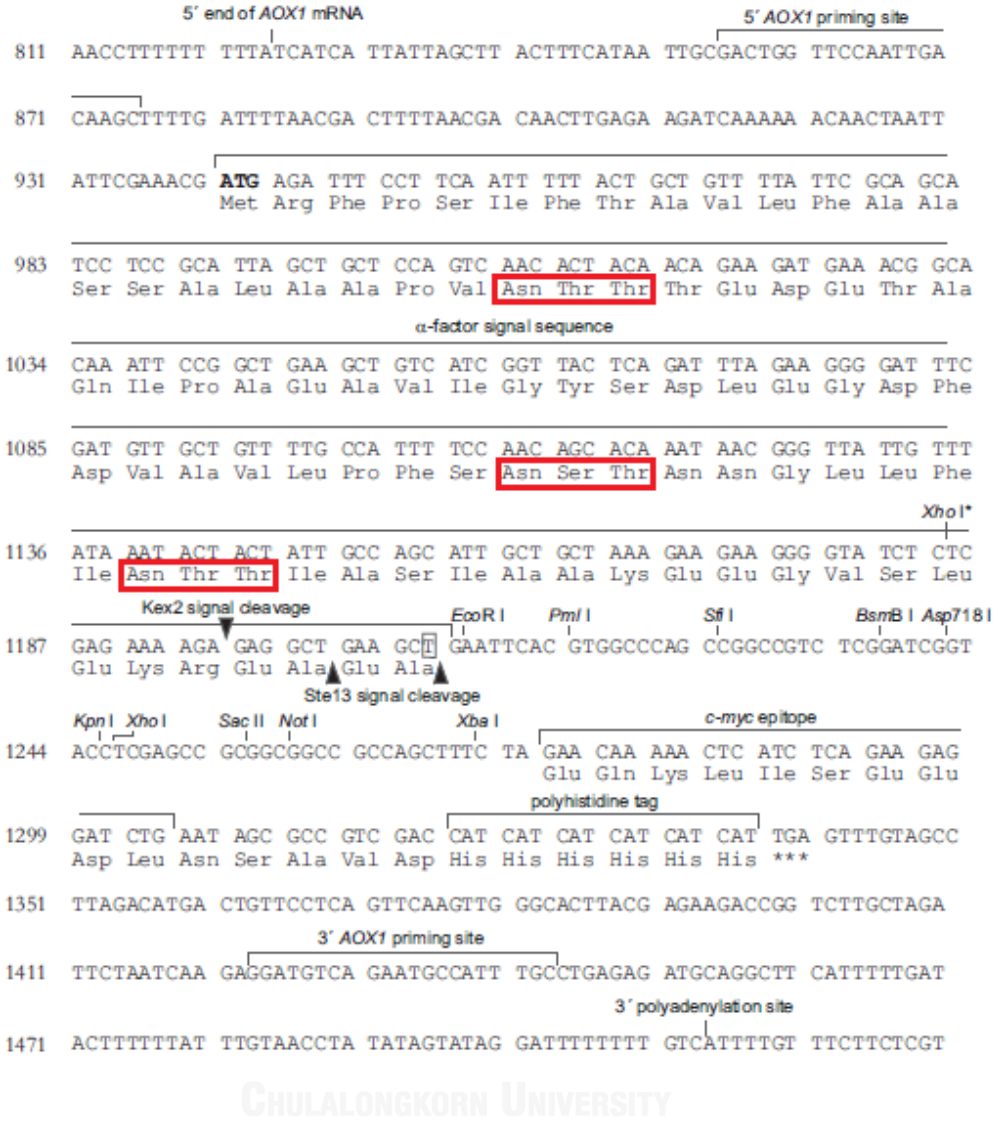


Figure 4.14 The expression vector, pPICZαA sequences. The squares represent possible glycosylation sites. (Reference: Invitrogen, Manual part no. 25-0148.)

For this recombinant AW peptide, *P. pastoris* seemed not to be effective host. To solve this problem, the other expression system, *E. coli* was selected because the recombinant peptide did not require post-translational modification. The disulfide-bond of Cysteine residues on the

peptide sequence could be avoided. Additionally, *E. coli* was suggested from many researches for high level expression of recombinant proteins.

4.4 Construction of *E. coli* strain for target antioxidative peptide expression

First, PCR technique was carried out by using pWP2304 as the template to amplify the DNA fragments encoding 6 copies of AW peptide, as previously described. However, in this experiment the AG1W-F and AG1W-R primers were used to change the *Xho*I to *Bam*HI and *Hin*dIII restriction sites, respectively (Figure 4.15). Then, the 234 base pair of PCR product and the 3509 base pair pQE-30Xa expression vector were cut by *Bam*HI and *Hin*dIII restriction enzymes (Figure 4.16 and 4.17). These two DNA bands were extracted from the agarose gel and consequently ligated together. This ligation reaction was transformed into *E. coli* DH5α strain. The ampicillin resistant colonies were selected to be preliminary verified by *Bam*HI and *Hin*dIII double digestion as in Figure 4.18 before being subjected for DNA sequencing (Macrogen, Korea). The verified recombinant expression plasmid was named as pQE-AW. The pQE-AW plasmid was then extracted and transformed into *E. coli* MG1655. The transformants on LB agar with ampicillin were selected. To verify the transformants, plasmids were extracted and consequently checked by the digestion of *Bam*HI and *Hin*dIII enzyme. The corrected transformant harboring pQE-AW plasmid, named as AW strain, was confirmed by the appearance of approximately 230 and 3500 of DNA fragments on 1% agarose gel electrophoresis (Figure 4.19).

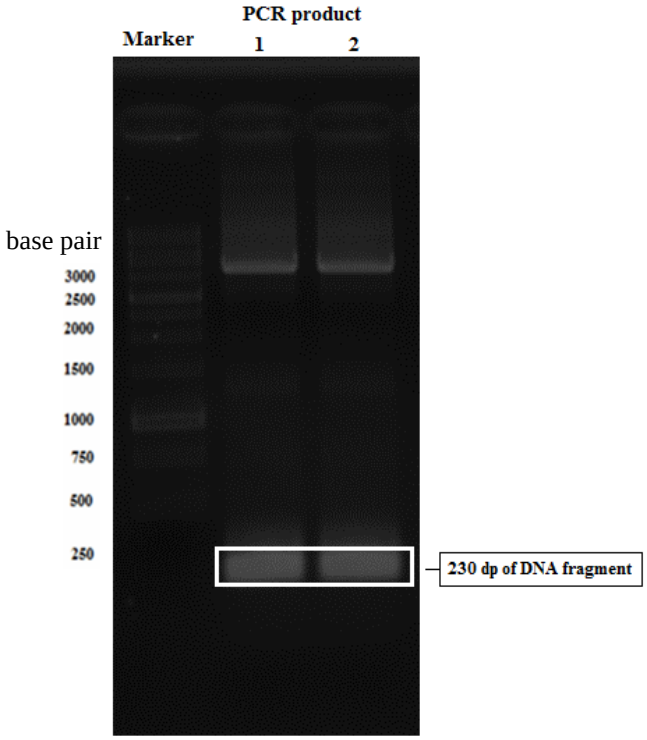


Figure 4.15 Amplification of DNA fragment encoding recombinant AW peptide for expressing in *E. coli* host. The approximately 230 base pair PCR product, when AG1W-F and AG1W-R primers were used, was appeared on 1% agarose gel electrophoresis.

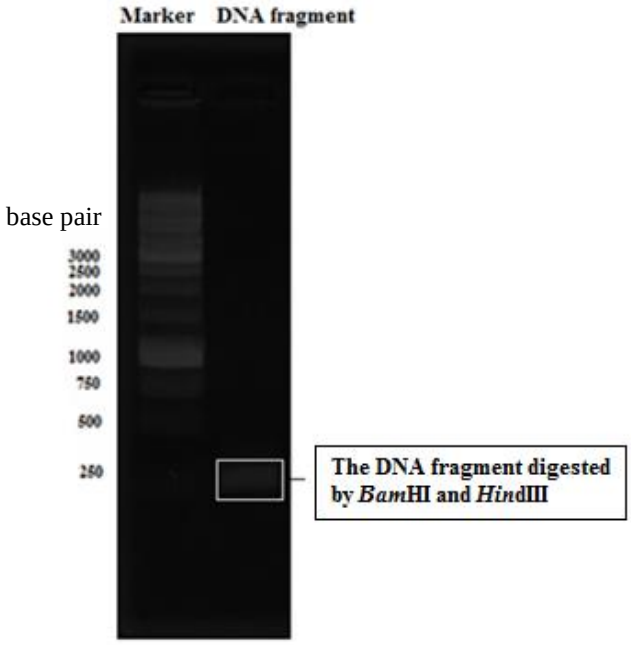


Figure 4.16 The 1% agarose gel electrophoresis analysis of the *Bam*HI and *Hin*dIII digestion of the PCR product (from Figure 4.13).

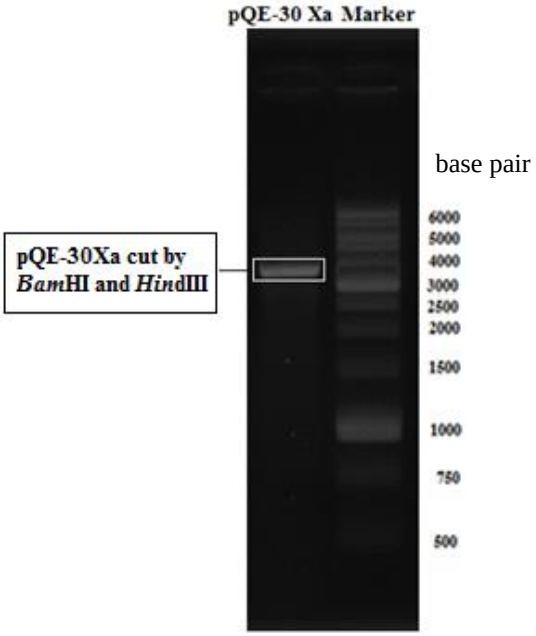


Figure 4.17 The 1% agarose gel electrophoresis analysis of the *Bam*HI and *Hin*dIII digestion of pQE-30 Xa expression vector.

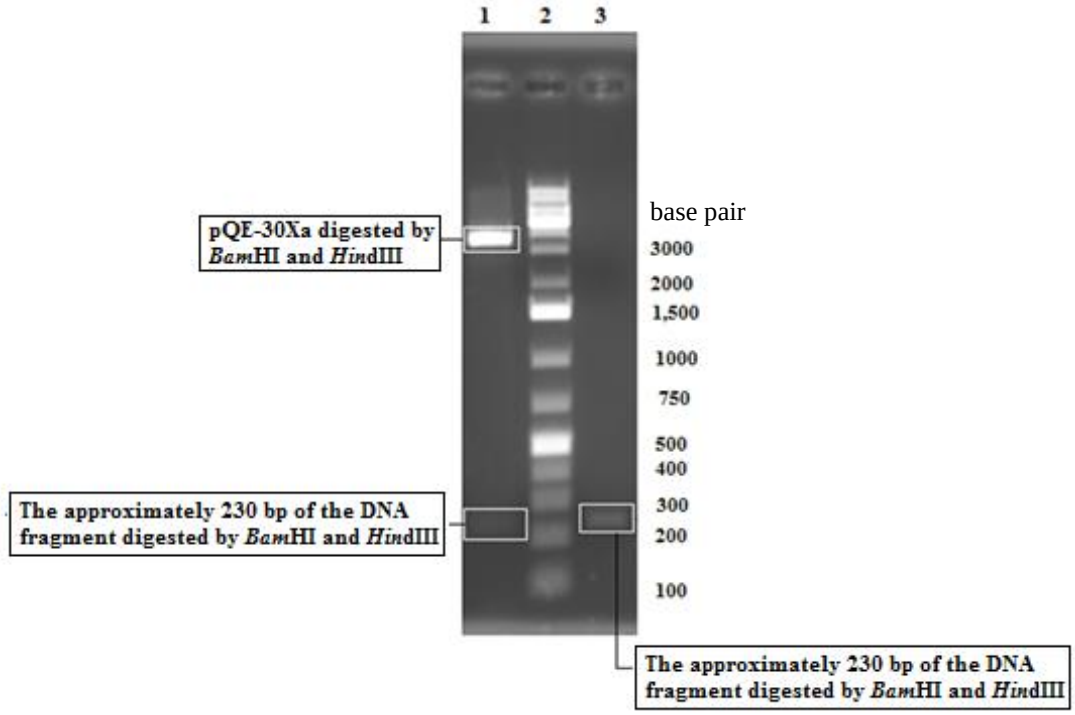


Figure 4.18 The *Bam*HI and *Hin*dIII digestion to verify the generation of pQE-AW plasmid from ligation reaction, visualized on 1% agarose gel electrophoresis. Lane 1: the approximately 230 and 3500 base paired was expected for the correct plasmid, Lane 2: The 1 Kb DNA ladder, Lane 3: the PCR product (from Figure 4.13) digested by *Bam*HI and *Hin*dIII served as a control.

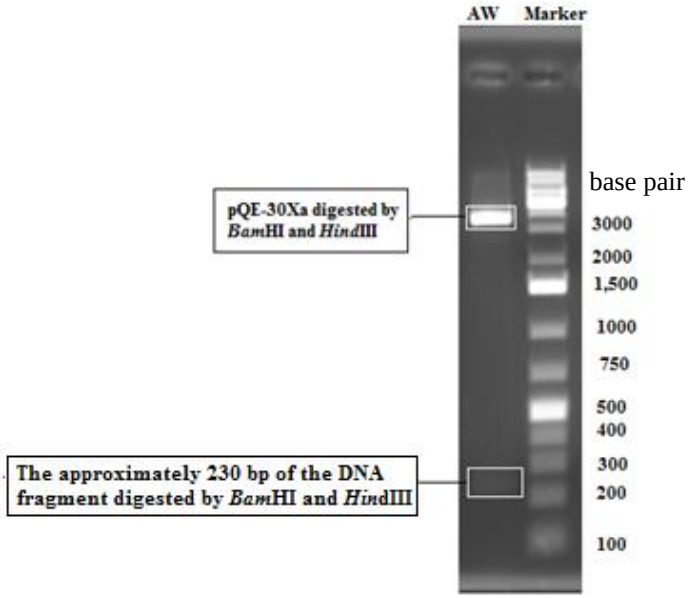


Figure 4.19 The digestion of pQE-AW extracted from *E. coli* MG1655 derivative by *Bam*HI and *Hin*dIII to verify the generation of AW strain. The 1% agarose gel electrophoresis was used to analyze the result.

4.5 Study of recombinant antioxidative peptide expression in *E. coli*

To express the recombinant AW peptide in *E.* coli, the inoculum was prepared by growing AW strain in 2 ml LB broth with 100 µg/ml of ampicillin, and incubated at 37˚C, 200 rpm in shaking incubator for approximately 16-18 hours. After that, the 2 ml of culture was transferred into 50 ml of new sterilized LB broth with 100 µg/ml of ampicillin in 250 ml flask, and incubated at 37ºC, 200 rpm until the OD_{600} being 0.6-0.8 (approximately 2 hours). Then, the expression was induced by adding 1 mM IPTG. The MG1655/pQE-30Xa strain was served as the control.

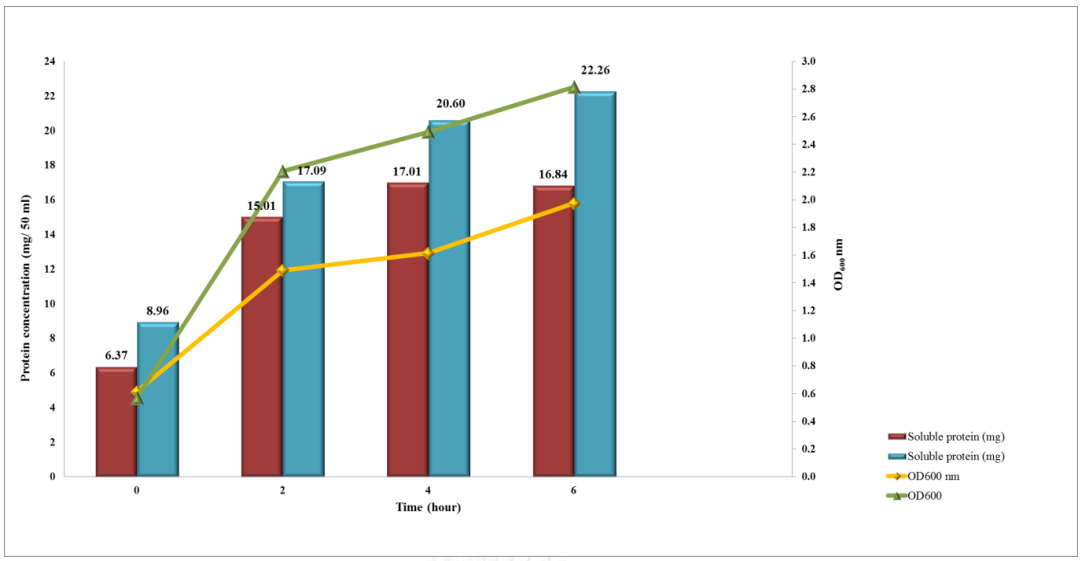


Figure 4.20 The total soluble protein production and cell growth of the AW strain for expressing the recombinant AW peptide after IPTG induction. The MG1655/pQE-30 Xa strain was served as the control.

The total protein production of AW strain was compared with the MG1655/pQE-30Xa production. The growth rate of AW strain distinctly reached up after 2 hour induction into the log phase. After that, the relatively slow growth rate was observed until approaching the stationary phase (at 4-6 hours). An increase in total protein concentration was investigated since the 2 hour of process, measured by UV absorbance at 260 and 280 nm. The concentration of soluble protein from 50 ml culture was raised from 17.09 mg at 2 hours to 22.26 mg at 6 hours after induction. The cell growth rate and the total protein concentration from the AW strain seemed to be higher than those from MG1655/pQE-30Xa (Figure 4.20).

According to metabolic strategy of growth of *E. coli* MG1655 on LB medium reported by Mark V. *et al*. (2006), the metabolism of acetate, produced in significant amount for assimilation of amino acid and

carbohydrate, and cellular stress responses, triggered by deterioration of nutrition in medium, are important factors. It was suggested that concentration of acetate was reached during first 3.5 hours which was the physiological state II of cell and consequently reassimilation in the next 1.5 hour. In Physiological state I, the abundant nutrients from the medium supported the unlimited grow. The maximum growth rate at the end of Physiological state I could be obtained. Corresponded to this research, the cell was supported by the culture at 0-2 hour of process which the steep growth rate was observed. For the Physiological state II, it was indicated that acetate was produced in medium broth and the content of medium was gradually diminished. Additionally, membrane protease was produced in this state. Then, in Physiological state III, 40% of cell growth was decreased, the cell started to reassimilate the previously produced acetate for biomass synthesis. Finally, Physiological IV was suggested that the most catabolic pathway was expressed (Baev *et al.* 2006). Therefore, in this experiment the culture after 4 hour induction was selected for recombinant target protein expression. The expected molecular weight of the recombinant AW in *E. coli* was approximately 12.28 kDa. To observe the expression of the recombinant AW peptide, the cell pellet was disrupted and then, the total soluble proteins and insoluble proteins was analyzed by 18% acrylamide gel Glycine-SDS-PAGE. After straining gel by Coomassie Blue, the expected recombinant AW protein was found in the total soluble protein fractions, (figure 4.21) which was higher than the MG1655/pQE-30 Xa strain (Figure 4.22). However, the low protein yield from the AW strain was observed. This may be caused by many conditions. Therefore, it is necessary to optimize several factors that can influent the recombinant protein production. It

was suggested that the ability of high heterologous protein expression in *E. coli* system could be limited, for example, codon usage, toxicity, mRNA stability, and protein stability (Kane 1995).

Due to the expression from the pQE-30 Xa vector, the N-terminal of the recombinant AW peptide was fused with 6x Histidine protein which can be purified by the affinity Protino Ni-IDA column using 1x LEW buffer with 100 mM imidazole. The eluted fraction was collected into 500 µl for 3 fractions. The elution was analyzed on 18% acrylamide gel Glycine-SDS-PAGE as showed in figure 4.23. The recombinant AW peptide elution from 50 ml culture was calculated to 0.95 mg. However, the expected protein band seemed to appear at higher size compared with the pre-stain protein marker. According to the manufacturing manual (Amersham Low-Rang Rainbow Molecular Weight Marker, GE healthcare companies), in some gel/buffer systems, the mobility of bands may differ from the original. It is possible that the different system and buffer from the company instruction may effect on the protein band mobility. However, although the higher than expected protein band should be the target protein because only this band was disappeared in the unbinding protein and wash samples collected during the affinity column purification.

However, the recombinant AW elution from this Ni^{2+} affinity column was contaminated with some proteins, especially those with higher molecular weight. Therefore, the additional purification methods are necessary for the recombinant AW peptide production. However, due to the low yield of the target peptide, the additional purification such as gel filtration, ion exchange column, can causes the loss of target protein. Therefore, as previously mentioned, it is necessary to optimize

recombinant protein production to obtain higher amount of the recombinant AW peptide, along with additionally suitable purification process.

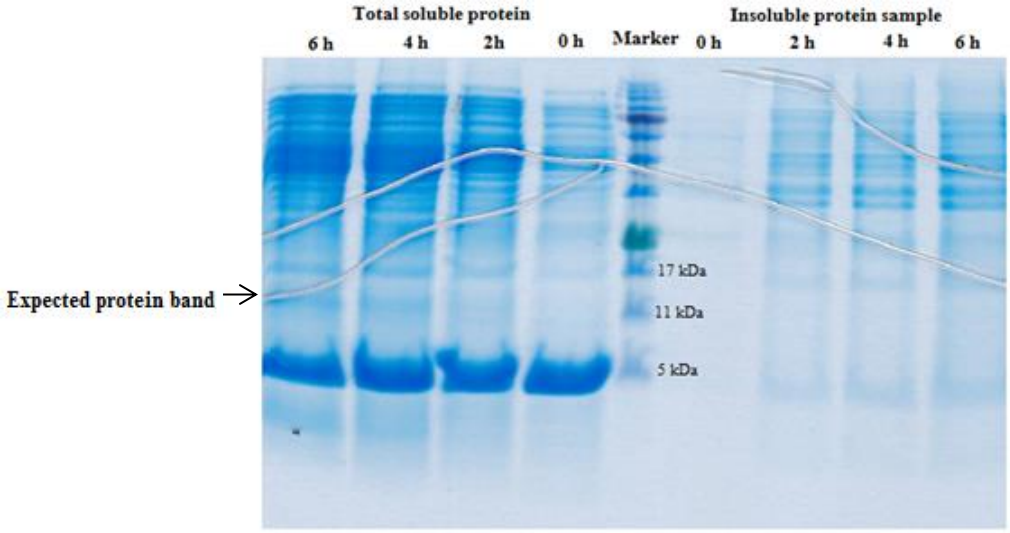


Figure 4. 21 The soluble proteins and insoluble proteins from the AW cell lysate analyzed on 18% acrylamide gel Glycine-SDS-PAGE. Lane 1-4, total soluble protein from the AW strain at 6, 4, 2, 0 hour, respectively; Lane 5, pre-strain Protein marker; Lane 6-9, total soluble protein from the AW strain at 0, 2 , 4 , 6 hour, respectively.

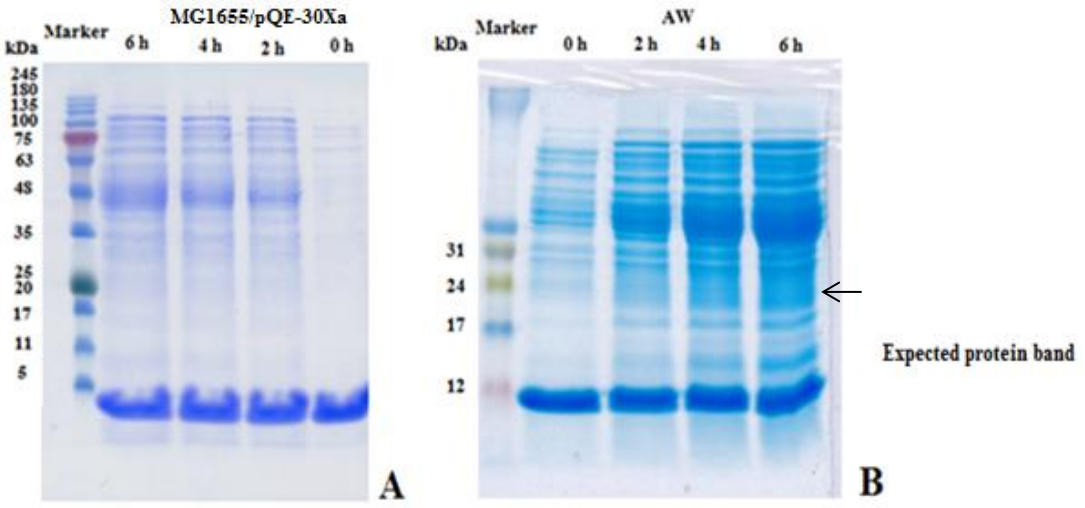


Figure 4.22 The total soluble protein from the AW and MG1655/pQE-30 Xa strains analyzed on 18% acrylamide gel Glycine-SDS-PAGE (A) Lane 1, protein marker; Lane 2-4, soluble proteins from the MG1655/pQE-30 Xa strain at 6, 4, 2, and 0 hours, respectively, after IPTG induction,. (B) Lane 1, protein marker; Lane 2-4: soluble proteins from the AW strain at 0, 2, 4, and 6 hours, respectively, after IPTG induction.

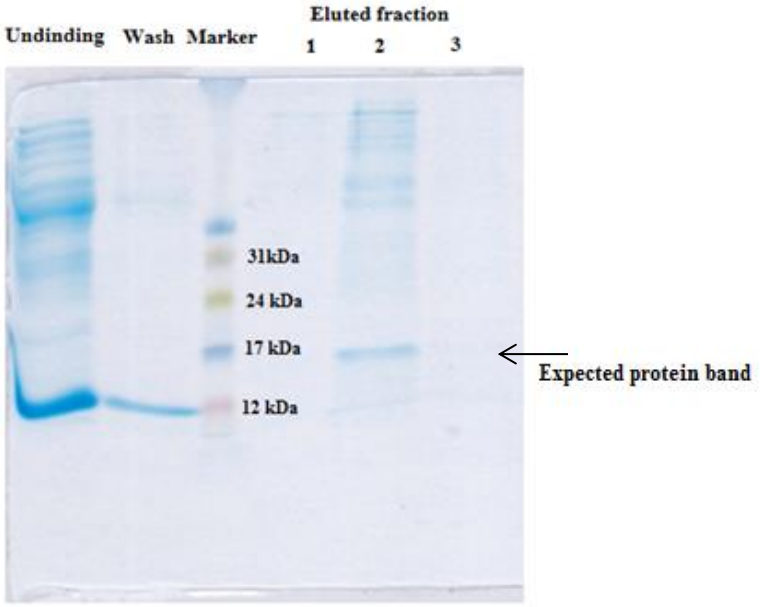


Figure 4.23 Purification of the recombinant AW peptide by Ni-IDA column, analyzed on 18% acrylamide gel Glycine-SDS-PAGE. Lane 1: Unbinding fraction, Lane 2, Wash fraction; Lane 3, Protein marker; Lane 4-6, Elute fraction.

4.5.1 Determination of the recombinant AW peptide expression in *E. coli*.

4.5.1.1 Real-Time PCR analysis

Due to the low yield of the expected band of the recombinant AW peptide was observed, the Real-Time PCR (LightCycler® 480 software) using SYBR® Green was applied to detect the expression at transcriptional level. This technique based on the reverse transcription, then the quantity and quality of mRNA transcription can be detected. The crossing point value (CP), the cycle number when the reaction crosses the threshold of light emission above background (Corthell 2014), was calculated. Moreover, Real-time PCR methods with SYBR® Green have

been suggested to be less cost than other methods (Carlos Sacristan, *et al*., 2015). The crossing point values of the AW strain was 15.1 while the MG1566/pQE-30 Xa strain with was 40.0, or could not be detected (Table 4.2). This result demonstrated the expression of the recombinant AW peptide at transcriptional level.

Table 4.2 The CP values of the AW strain and MG1566/pQE-30 Xa strain from the Real-time PCR analysis targeted the expression of recombinant AW peptide.

Strains	CP value
AW	15.1 ± 0.42
MG1655/pQE-30 Xa	ND

4.5.1.2 Immunoblot analysis

The eluted protein through Ni^{2+} affinity column still included of other large proteins. To verify the target peptide, due to the fusion of 6x-Histidine tag at N- terminal of the recombinant Aw peptide, the antiHis-HRP antibody was used. The Western-blot analysis was primary used, but there were several problems occurred during the procedure. The small peptide was not completely transferred onto the membrane with semi-dry rapid blotting system and heat of system possibly damage protein. Therefore, the modified immunoblotting analysis was applied to confirm the expected recombinant AW protein. The expected band of the recombinant AW protein on SDS-PAGE (Figure 4.23) was extracted and diffused from the gel, directly blotted on membrane, and consequently incubated with the antiHis-HRP. As the result in figure 4.24, the

recombinant His-tagged AW could be confirmed. However, the amino acid sequences of the recombinant AW peptide in AW strain will need to further verified by MS/MS analysis. However, the extraction and diffusion from the gel encountered the problem of low recovery and may not suitable for large scale production.

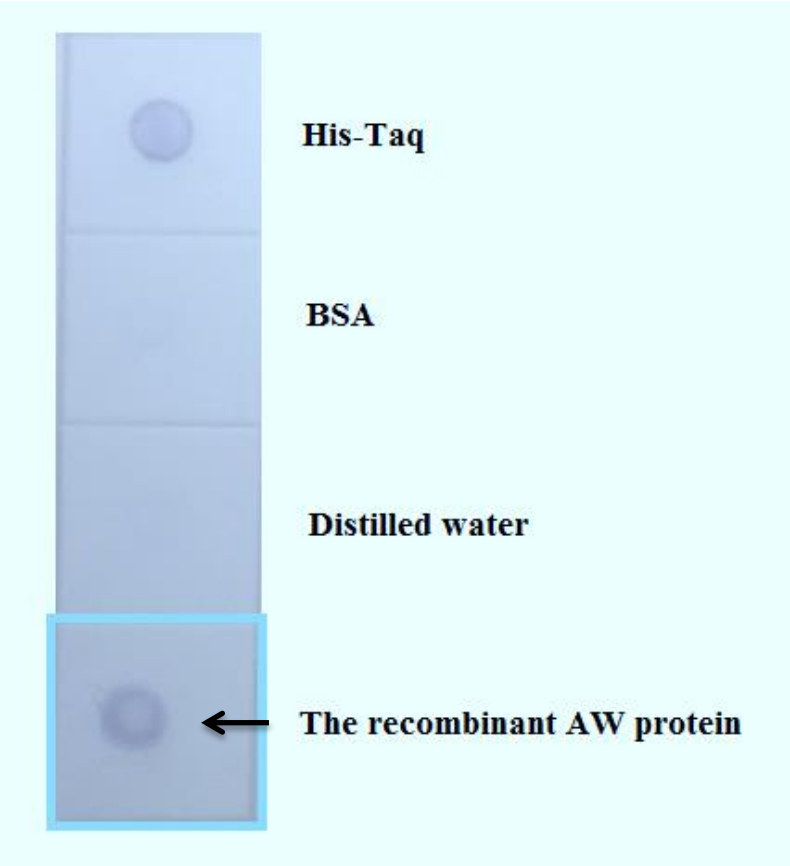


Figure 4.24 The verification of the recombinant AW peptide by employing the binding of polyhistidine tag on recombinant peptide and antiHis-HRP by modified immunoblotting method.

4.6 Antioxidant characterization of recombinant antioxidative peptide

4.6.1 DPPH radicals scavenging activity assay

DPPH radical was the one of stable radical sources. It was widely used to analyze the ability of antioxidant in various samples. In this study, DPPH radical was also utilized to test the capability of the recombinant AW peptide elution. However, the IC_{50} value could not be calculated. The 1 mg/ml of recombinant AW elution through NI^{2+} column presented 12.58 ± 0.7 % of inhibition. However, the recombinant AW peptide elution exhibited less DPPH radical than the chemical synthesized peptide (Table 4.3).

Table 4.3 The percentage of inhibition against DPPH radicals when using 1 mg/ml of samples.

Sample	% inhibition
Ascorbic acid	96.89 ± 0.4
Recombinant AW peptide elution	12.58^{a} ± 0.7
Chemical synthesized AW peptide	26.31^{a} ± 0.9

[a] Statistic analysis by SPSS independent-sample T-Test. The same subscripts are not significantly different from each other ($p < 0.05$)

4.6.2 ABTS radical scavenging assay

ABTS radical scavenging ability assay was widely used to test an antioxidant activity. In this study, the recombinant AW peptide elution in AW strain was determined by generated ABTS radical from the reaction of 7 mM of solvable ABTS in aqueous solution of 2.45 mM $K_2S_2O_8$ at the ratio 1:1. The recombinant AW peptide elution exhibited 50% of ABTS radicals, or IC_{50} value, at concentration of 1009.43 ± 9.1 ug/ml (Table 4.4). This result presented in the same trend as in case of the DPPH radical scavenging ability in which the recombinant AW peptide elution could lower against DPPH radical than the chemical synthesized peptide. The chemical synthesized peptide exhibited significantly more ABTS radical than that of the recombinant AW peptide elution (determined by t-test with $p < 0.5$)

Table 4.4 The IC_{50} values of ABTS radical scavenging assay

Sample	IC_{50} value (µg/ml)
Ascorbic acid	43.44 ± 1.5
Recombinant Aw peptide elution	1009.43^{a} ± 9.1
Chemical synthesized AW peptide	76.99^{b} ± 1.6

[a,b] Statistic analysis by SPSS independent-sample T-Test. The same subscripts are not significantly different from each other ($p < 0.05$)

By the result of ABTS and DPPH radicals scavenging assays, the antioxidant activity of the recombinant AW peptide elution might be interfered from the production process. The possible factors could be the

uncontrolled pH in shake flask level production and the disqualifiable elution buffer. The pH value of the purified recombinant AW elution was reached to 8. Moreover, the purified peptide was mixed with the denaturing elution buffer (Appendix A). On the other hand, the peptide in the research of Shieh et al (2009) and the chemical synthesized antioxidative peptide were dissolved by distilled water, of which the purity was up to 95.5%. The effects of pH and buffers on the stability of DPPH• and its reduced product (DPPHH) in the ethanol-buffer solution was investigated by Zeng L. *et al.* in 2015. It was suggested that the spectrophotometric measurement at 515-525 nm of the deprotonation of DPPHH could be interfered under basic condition. It was also suggested that the mixture of reaction should be maintained the final pH value at 5.0-6.5 in 1:1 ethanol-acetate/citrate buffer for appraising the activities of peptide. Additionally, the DPPH assay was sensitive for some amino acid including Cysteine. Conversely, the Try/Typ-containing presented high reactivity toward ABTS radicals but inert to the DPPH radicals (Zheng *et al.* 2015). This could be explained why the DPPH radical scavenging ability was lower activity than ABTS radical one in this study. Moreover, the effect of Arginine existence on DPPH radical scavenging activity was also observed. When Arginine was added in the reaction mixture, DPPH radical which presented absorption at 517 nm had red-shift to maximum at 525 nm. Therefore, the value was calculated to 94% slightly less than 100% owing to the limited absorption of DPPHH at 525 nm (Bertalanic *et al.* 2012). It is possible that the more Cysteine, Arginine, and tyrosine residue in the recombinant AW peptide than the native may interfered the DPPH and ABTS radical assays.

4.6.3 The *in vitro* protective effect of the recombinant peptide on oxidation-induced DNA damage

This research also investigated in the in vitro protective effect of the recombinant antioxidative peptide, expressed from *E. coli*, on DNA damage induced by hydroxyl radical with the same method described previously. The the recombinant peptide could also protect DNA damage. As the result in Figure 4.25, the efficiency of protective effect was not concentration dependent. The supercoil form of pBR322 was not completely changed into the open circular or linear, caused by the hydroxyl radical damage, when the recombinant AW peptide elution was added in the reaction. On the other hand, the reaction when chemical synthesized peptide was mixed into the reaction, the supercoil form of DNA templates were completely converted to linear DNA and open circular form, the damage form caused by the hydroxyl radical, even though using high protein concentration.

This assay illustrated that the recombinant peptide, six copies of VECYGPNRPQF amino acid sequences, could be protect the DNA with more efficiency than the only 1 copy of the chemical synthesized peptide.

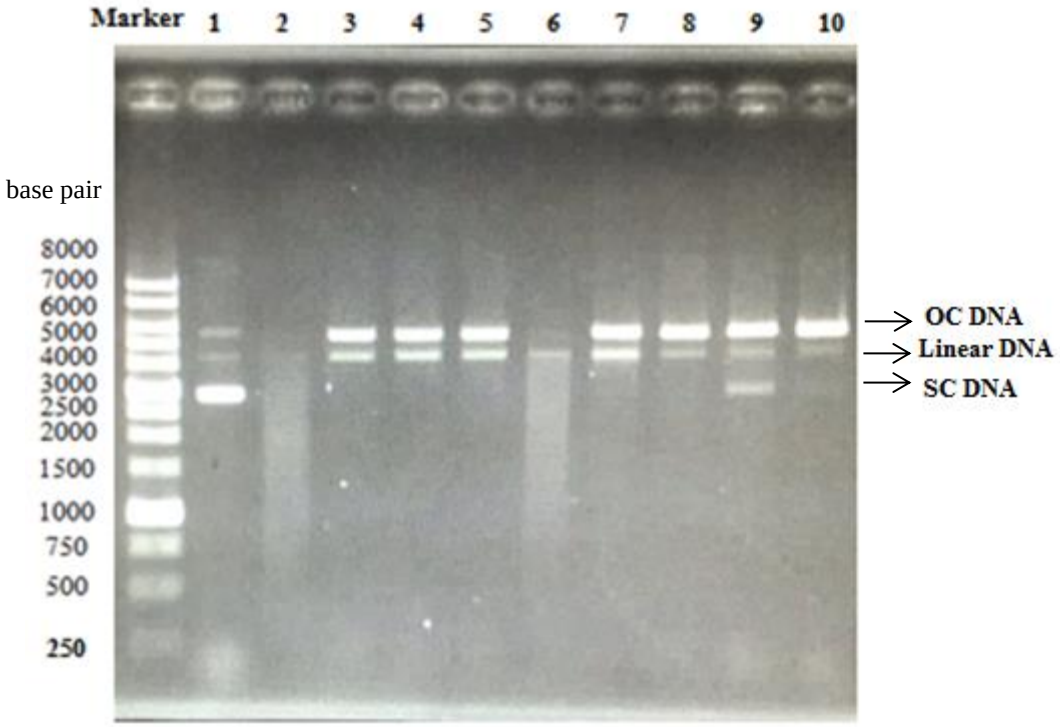


Figure 4.25 The in vitro protective effect of recombinant and chemical synthesized AW peptide on DNA damage induced by hydroxyl radicals. The 1% agarose gel electrophoresis was applied to analyze DNA forms. Lane 1, pBR322 plasmid; Lane 2 and 6, pBR322 plasmid treated with Fenton reaction; Lane 3-5, pBR322 plasmid mixed with chemical synthesized AW peptide at the concentrations of 1000 µg/ml, 500 µg/ml, 250 µg/ml, and 125 µg/ml, respectively before being treated with Fenton reaction; Lane 7-10, pBR322 plasmid mixed with recombinant AW peptide elution at the concentrations of 1000 µg/ml, 500 µg/ml, 250 µg/ml, and 125 µg/ml, respectively before being treated with Fenton reaction. (OC= open circular, SC= supercoil).

The result of this study corresponded to some of previous researches. For example, in 2008 Qian and coworkers investigated the protective effect of antioxidative peptide from gastrointestinal digests of oyster, *Crassostrea gigas* against free radical induced DNA damage. They found that the peptide including the amino acid sequence Leu-Lys-Gln-Glu-Leu-Glu-Asp-Leu-Leu-Glu-Lys-Gln-Glu (1.60 kDa) significant dose dependently protected on hydroxyl radical induced DNA damage. pBR322 plasmid DNA was treated with the reaction between 2 mM Fe^{2+} and 30% H_2O_2 and the purified peptide at concentrations ranging from 9.12 µg/ml to 72.96 µg/ml (Qian *et al.* 2008).

In this research, although the recombinant peptide did not display high DPPH and ABTS scavenging activities, the satisfactory result of protective effect on DNA damage induced by hydroxyl radicals was observed. It has been suggested that the antioxidant activity cannot be summarized by one single assay. This recombinant AW peptide possibly displays others properties determined by other methods, for example, Nitric oxide scavenging assay and Ferric reducing antioxidant power assay. Therefore, it is interesting to explore other antioxidant activity of the recombinant AW peptide.

CHAPTER V
CONCLUSION

Conclusion

Nowadays, antioxidants from natural sources become interested. One of a potential antioxidative peptide, in this research, named as AW peptide, with amino acid sequence is VECYGPNRPQF, is found in algae waste hydrolysate. However, natural production extraction has been encountered several problems including large amount of sample requirement, sample storage and relatively complicated procedure. To solve such problems, the recombinant DNA technique was used. In this study, the 234 base pair of DNA fragments encoding for 6 copies of AW peptide linked by Lysine residues was synthesized and successfully cloned into two microorganism systems including *P. pastoris* GS115 and *E. coli* 1655 strains. The strain of *P. pastoris* was named as WPTA1 while, the strain of *E. coli* was named AW.

However, the recombinant AW peptide could not be obtained from the *P. pastoris* host, WPTA1 strain. It was hypothesized that, due to the Cysteine residue on the recombinant peptide, disulfide bonds from post-translational modification could be occurred. This may be resulted in recombinant peptide misfolding and intracellular accumulation. In addition, the recombinant AW peptide was not designed to contain any tags, including 6xHisitide tag, so it is difficult to purify the target peptide.

Due to the recombinant AW peptide was not require modification for antioxidant activities, *E. coli* 1655 was selected to be the alternative expression host. The result illustrated that the recombinant AW protein was successfully expressed in the AW strain using 1 mM IPTG induction.

However, the protein yield was still low. Furthermore, the polyhistidine tagged recombinant AW elution from Ni^{2+} affinity column with protein was not pure enough. However, the target peptide band was verified by modified immunoblotting with AntiHis-HRP antibody detection.

The recombinant AW elution was displayed some antioxidant activities, especially, *in vitro* protective effect on oxidation-induced DNA damage by hydroxyl radicals. The lower DPPH and ABTS scavenging activities than those of the chemical synthesized and previous study may come from the other protein in the elution and test systems. Therefore, optimization and suitable purification technique are necessary for the further study. Moreover, other antioxidant capacity from the recombinant AW peptide is needed to be explored.

Suggestion

To improve the recombinant AW peptide in *P. pastoris* GS115, the 6 copies of AW peptide linked by Lysine residues should be modifying the sequences to be fused with the polyhistiding tag for the purpose of purification by Ni^{2+} column. In addition, the DNA fragment should be designed to contain the Kex2 signal cleavage site for cleaving α-mating factor before extracellular protein secretion. Moreover, for expressing the recombinant AW peptide from the engineered strain, several factors such as temperature, methanol concentration, and production media have to be optimized.

The optimization of the recombinant AW peptide production in the *E. coli* MG1655 derivative, AW strain, should be further investigated to increase protein yield. In addition, the purification by only Ni^{2+} affinity column is not enough. The additionally suitable purifying techniques,

such as gel filtration, are required. In this research, compare to the chemical synthesized AW peptide, the recombinant AW peptide elution displayed lower DPPH and ABTS scavenging activities, but higher DNA protection for hydroxyl radicals. Therefore, other antioxidant activities from the recombinant AW peptide, such as Nitric oxide scavenging assay and Ferric reducing antioxidant power assay, are worth to be characterized.

REFERENCES

A.K. Chauhan, D. A., N. Khanna (1999). "A novel feeding strategy for enhanced protein production by fed-batch fermentation in recombinant *Pichia pastoris*." Process Biochemistry **34**: 139-145.

Ahmad, M., M. Hirz, H. Pichler and H. Schwab (2014). "Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production." Appl Microbiol Biotechnol **98**(12): 5301-5317.

Ahn, C.-B., Y.-J. Jeon, D.-S. Kang, T.-S. Shin and B.-M. Jung (2004). "Free radical scavenging activity of enzymatic extracts from a brown seaweed *Scytosiphon lomentaria* by electron spin resonance spectrometry." Food Research International **37**(3): 253-258.

Arts, M. J. T. J., J. Sebastiaan Dallinga, H.-P. Voss, G. R. M. M. Haenen and A. Bast (2004). "A new approach to assess the total antioxidant capacity using the TEAC assay." Food Chemistry **88**(4): 567-570.

Baev, M. V., D. Baev, A. J. Radek and J. W. Campbell (2006). "Growth of *Escherichia coli* MG1655 on LB medium: determining metabolic strategy with transcriptional microarrays." Appl Microbiol Biotechnol **71**(3): 323-328.

Bertalanic, L., T. Kosmerl, N. Poklar Ulrih and B. Cigic (2012). "Influence of solvent composition on antioxidant potential of model polyphenols and red wines determined with 2,2-diphenyl-1-picrylhydrazyl." J Agric Food Chem **60**(50): 12282-12288.

Boligon, A. A. (2014). "Technical Evaluation of Antioxidant Activity." Medicinal Chemistry **4**(7).

Buckholz, R. G. and M. A. Gleeson (1991). "Yeast systems for the commercial production of heterologous proteins." Biotechnology (N Y) **9**(11): 1067-1072.

Buensanteai, N., P. K. Mukherjee, B. A. Horwitz, C. Cheng, L. J. Dangott and C. M. Kenerley (2010). "Expression and purification of

biologically active Trichoderma virens proteinaceous elicitor Sm1 in *Pichia pastoris*." Protein Expr Purif **72**(1): 131-138.

Carballo-Cardenas, E. C., P. M. Tuan, M. Janssen and R. H. Wijffels (2003). "Vitamin E (alpha-tocopherol) production by the marine microalgae *Dunaliella tertiolecta* and *Tetraselmis suecica* in batch cultivation." Biomol Eng **20**(4-6): 139-147.

Cereghino, G. P. and J. M. Cregg (1999). "Applications of yeast in biotechnology: protein production and genetic analysis." Curr Opin Biotechnol **10**(5): 422-427.

Cereghino, J. L. and J. M. Cregg (2000). "Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*." FEMS Microbiol Rev **24**(1): 45-66.

Changjiang Guo, J. Y., Jingyu Wei, Yunfeng Li, Jing Xu, and Y. Jiang (2003). "Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay." Nutrition Research **23**: 1719–1726.

Chen, R. (2012). "Bacterial expression systems for recombinant protein production: *E. coli* and beyond." Biotechnol Adv **30**(5): 1102-1107.

Chen, Z., H. Chen, X. Wang, X. Ma and B. Huang (2007). "Expression, purification, and characterization of secreted recombinant human insulin-like growth factor-binding protein-6 in methylotrophic yeast *Pichia pastoris*." Protein Expr Purif **52**(2): 239-248.

Corthell, J. T. (2014). "Immunoblotting (Weatern blot) basic molecular protocols in neuroscience:Tips, Tricks, and Pitfalls." 55-75.

Cregg, J. M., K. R. Madden, K. J. Barringer, G. P. Thill and C. A. Stillman (1989). "Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*." Mol Cell Biol **9**(3): 1316-1323.

Damasceno, L. M., C. J. Huang and C. A. Batt (2012). "Protein secretion in *Pichia pastoris* and advances in protein production." Appl Microbiol Biotechnol **93**(1): 31-39.

Damasceno, L. M., I. Pla, H. J. Chang, L. Cohen, G. Ritter, L. J. Old and C. A. Batt (2004). "An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*." Protein Expr Purif **37**(1): 18-26.

Dayer, R., B. B. Fischer, R. I. Eggen and S. D. Lemaire (2008). "The peroxiredoxin and glutathione peroxidase families in *Chlamydomonas reinhardtii*." Genetics **179**(1): 41-57.

Gao, M. and Z. Shi (2013). "Process Control and Optimization for Heterologous Protein Production by Methylotrophic *Pichia pastoris*." Chinese Journal of Chemical Engineering **21**(2): 216-226.

Goiris, K., K. Muylaert and L. De Cooman (2015). "Microalgae as a Novel Source of Antioxidants for Nutritional Applications." 269-280.

Gu, J., X. Gao, X. Pan, X. Peng, Y. Li and M. Li (2015). "High-level expression and one-step purification of a soluble recombinant human interleukin-37b in *Escherichia coli*." Protein Expr Purif **108**: 18-22.

Guo, J. P. and Y. Ma (2008). "High-level expression, purification and characterization of recombinant *Aspergillus oryzae* alkaline protease in *Pichia pastoris*." Protein Expr Purif **58**(2): 301-308.

Heo, S. J., E. J. Park, K. W. Lee and Y. J. Jeon (2005). "Antioxidant activities of enzymatic extracts from brown seaweeds." Bioresour Technol **96**(14): 1613-1623.

Hoang, V. V., Y. Zou, K. Kurata and K. Enomoto (2015). "Expression of recombinant T-cell epitopes of major Japanese cedar pollen allergens fused with cholera toxin B subunit in *Escherichia coli*." Protein Expr Purif **109**: 62-69.

Hong, F., N. Q. Meinander and L. J. Jonsson (2002). "Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*." Biotechnol Bioeng **79**(4): 438-449.

Janknegt, P. J., C. M. De Graaff, W. H. Van De Poll, R. J. W. Visser, J. W. Rijstenbil and A. G. J. Buma (2009). "Short-term antioxidative responses of 15 microalgae exposed to excessive irradiance including ultraviolet radiation." European Journal of Phycology **44**(4): 525-539.

Jeong, J. B., B. O. De Lumen and H. J. Jeong (2010). "Lunasin peptide purified from *Solanum nigrum* L. protects DNA from oxidative damage by suppressing the generation of hydroxyl radical via blocking fenton reaction." Cancer Lett **293**(1): 58-64.

Jiang, J., J. Zheng, Y. She and Z. Jia (2015). "Expression and purification of human WWP2 HECT domain in *Escherichia coli*." Protein Expr Purif **110**: 95-101.

Kane, J. F. (1995). "Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*." Curr Opin Biotechnol **6**(5): 494-500.

Lesser, M. P. (2006). "Oxidative stress in marine environments: biochemistry and physiological ecology." Annu Rev Physiol **68**: 253-278.

Li, C. L., T. T. Xu, R. B. Chen, X. X. Huang, Y. C. Zhao, Y. Y. Bao, W. D. Zhao and Z. Y. Zheng (2013). "Cloning, expression and characterization of antimicrobial porcine beta defensin 1 in *Escherichia coli*." Protein Expr Purif **88**(1): 47-53.

Li, L., D. Qian, G. Shao, Z. Yan, R. Li, X. Hua, X. Song and B. Wang (2014). "High-level expression, purification and study of bioactivity of fusion protein M-IL-2((88)Arg, (125)Ala) in *Pichia pastoris*." Protein Expr Purif **101**: 99-105.

Li, Z., F. Xiong, Q. Lin, M. d'Anjou, A. J. Daugulis, D. S. Yang and C. L. Hew (2001). "Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*." Protein Expr Purif **21**(3): 438-445.

Lu, L., T. N. Wang, T. F. Xu, J. Y. Wang, C. L. Wang and M. Zhao (2013). "Cloning and expression of thermo-alkali-stable laccase of *Bacillus licheniformis* in *Pichia pastoris* and its characterization." Bioresour Technol **134**: 81-86.

Lueking, A., C. Holz, C. Gotthold, H. Lehrach and D. Cahill (2000). "A system for dual protein expression in *Pichia pastoris* and *Escherichia coli*." Protein Expr Purif **20**(3): 372-378.

Lushchak, V. I. (2014). "Free radicals, reactive oxygen species, oxidative stress and its classification." Chem Biol Interact **224C**: 164-175.

Macauley-Patrick, S., M. L. Fazenda, B. McNeil and L. M. Harvey (2005). "Heterologous protein production using the *Pichia pastoris* expression system." Yeast **22**(4): 249-270.

Ngo, D.-H., I. Wijesekara, T.-S. Vo, Q. Van Ta and S.-K. Kim (2011). "Marine food-derived functional ingredients as potential antioxidants in the food industry: An overview." Food Research International **44**(2): 523-529.

Ott, M., V. Gogvadze, S. Orrenius and B. Zhivotovsky (2007). "Mitochondria, oxidative stress and cell death." Apoptosis **12**(5): 913-922.

Prior, R. L. (2015). "Oxygen radical absorbance capacity (ORAC): New horizons in relating dietary antioxidants/bioactives and health benefits." Journal of Functional Foods **18**: 797-810.

Qian, Z. J., W. K. Jung, H. G. Byun and S. K. Kim (2008). "Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, Crassostrea gigas against free radical induced DNA damage." Bioresour Technol **99**(9): 3365-3371.

Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans (1999). "Antioxidant activity applying an improved ABTS radical cation decolorization assay." Free Radic Biol Med **26**(9-10): 1231-1237.

Romanos, M. (1995). "Advances in the use of *Pichia pastoris* for high-level gene expression." Current Opinion in Biotechnology **6**: 527-533.

Running, J. A., D. K. Severson and K. J. Schneider (2002). "Extracellular production of L-ascorbic acid by *Chlorella protothecoides*, *Prototheca* species, and mutants of *P. moriformis* during aerobic culturing at low pH." J Ind Microbiol Biotechnol **29**(2): 93-98.

Sachindra, N. M., E. Sato, H. Maeda, M. Hosokawa, Y. Niwano, M. Kohno and K. Miyashita (2007). "Radical scavenging and singlet oxygen quenching activity of marine carotenoid fucoxanthin and its metabolites." J Agric Food Chem **55**(21): 8516-8522

Saikat Sen, R. C., C. Sridhar, Y. S. R. Reddy, Biplab De (2010). "FREE RADICALS, ANTIOXIDANTS, DISEASES AND PHYTOMEDICINES: CURRENT STATUS AND FUTURE PROSPECT." International Journal of Pharmaceutical Sciences Review and Research **3**(1).

Samarakoon, K. and Y.-J. Jeon (2012). "Bio-functionalities of proteins derived from marine algae — A review." Food Research International **48**(2): 948-960.

Sheih, I. C., T. K. Wu and T. J. Fang (2009). "Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems." Bioresour Technol **100**(13): 3419-3425.

Shu, Z., J. Li, N. Mu, Y. Gao, T. Huang, Y. Zhang, Z. Wang, M. Li, Q. Hao, W. Li, L. He, C. Zhang, W. Zhang, X. Xue and Y. Zhang (2014). "Expression, purification and characterization of galectin-1 in *Escherichia coli*." Protein Expr Purif **99**: 58-63.

Spolaore, P., C. Joannis-Cassan, E. Duran and A. Isambert (2006). "Commercial applications of microalgae." J Biosci Bioeng **101**(2): 87-96.

Stryjewska, A., K. Kiepura, T. Librowski and S. Lochynski (2013). "Biotechnology and genetic engineering in the new drug development. Part I. DNA technology and recombinant proteins." Pharmacol Rep **65**(5): 1075-1085.

Tschopp, J. F., P. F. Brust, J. M. Cregg, C. A. Stillman and T. R. Gingeras (1987). "Expression of the lacZ gene from two methanol-regulated promoters in *Pichia pastoris*." Nucleic Acids Res **15**(9): 3859-3876.

Wang, X. and X. Xu (2012). "Molecular cloning and functional analyses of glutathione peroxidase homologous genes from *Chlorella* sp. NJ-18." Gene **501**(1): 17-23.

Wei, D., F. Chen, G. Chen, X. Zhang, L. Liu and H. Zhang (2008). "Enhanced production of lutein in heterotrophic *Chlorella protothecoides* by oxidative stress." Sci China C Life Sci **51**(12): 1088-1093.

Yasuhiro Nishida, E. Y. a. W. M. (2007). "Quenching Activities of Common Hydrophilic and Lipophilic Antioxidants against Singlet Oxygen Using Chemiluminescence Detection System." Carotenoid Science **11**: 16-20.

Zhang, Z., F. Wang, X. Wang, X. Liu, Y. Hou and Q. Zhang (2010). "Extraction of the polysaccharides from five algae and their potential antioxidant activity *in vitro*." Carbohydrate Polymers **82**(1): 118-121.

Zheng, L., L. Lin, G. Su, Q. Zhao and M. Zhao (2015). "Pitfalls of using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay to assess the radical scavenging activity of peptides: Its susceptibility to interference and low reactivity towards peptides." Food Research International **76**: 359-365.

Zhou, H. H., A. X. Zhang, Y. Zhang and D. Y. Zhu (2012). "Cloning, expression, and purification of a recombinant Tat-HA-NR2B9c peptide." Protein Expr Purif **85**(2): 239-245.

APPENDIX

APPENDIX A
MEDIA AND SOLUTIONS

1. Luria-Bertani medium (LB)

Yeast extract	5	g
Trypsin	10	g
NaCl	10	g

Dissolve in 1000 ml of dH_2O and adjust pH to 7.5 then autoclave at 121 ºC for 15 minutes

2. Luria-Bertani ager (LB)

The ingredient is similarly to LB medium by adding 2% (w/v) ager and autoclave at 121 ºC for 15 minutes.

3. Low Salt LB medium

Yeast extract	5	g
Trypsin	10	g
NaCl	5	g

Dissolve in 1000 ml of dH_2O and adjust pH to7.5 then autoclave at 121 ºC for 15 minutes

4. Low Salt LB ager

Add 2% (w/v) ager in Low Salt LB medium and sterilize at 121 ºC for 15 minutes

5. Yeast Peptone Dextrose medium (YPD)

Yeast extract	10	g
Peptone	20	g
Dextrose	20	g

Dissolve in 1000 ml of dH_2O and adjust pH to 5.0 then autoclave at 121 ºC for 15 minutes

6. Yeast Peptone Dextrose ager (YPD)

Add 2% (w/v) ager in YPD medium and sterilize at 121 ºC for 15 minutes

7. Yeast Peptone Glyceral medium (YPD)

Yeast extract	10	g
Peptone	20	g
Glycerol	20	g

Dissolve in 1000 ml of dH_2O and adjust pH to 5.0 then autoclave at 121 ºC for 15 minutes

8. Basal medium

KH_2PO_4	10	g
$MgSO_4.7H_2O$	3.2	g
$CaCl_2.2H_2O$	0.35	g
Ammonium sulfate	20	g

Dissolve in 1000 ml of dH_2O and adjust pH to 5.0 then autoclave at 121 ºC for 15 minutes

9. PTM[1] Solution

Cupric sulfate.5H2O	6.0	g
Sodium iodine or potassium iodide	0.08	g
Manganese sulfate.H2O	3.0	g
Sodium molybdate.2H2O	0.2	g
Boric acid	0.02	g
Cobalt chloride	0.5	g
Zinc chloride	20.0	g
Ferrous sulfate.7H2O	65.0	g
Biotin	0.2	g
Sulfuric acid	5.0	ml

Adjust the volume to 1000 ml by water and filter through 0.45 µm nitrocellulose membrane.

10. MMH medium

10X YNB	100	ml
500X Biotin	2 ml	
100X Histidine	10	ml

Dissolve in 1000 ml of dH_2O autoclave at 121 ºC for 15 minutes

11. Lysis-Equilibration-Wash Buffer (1 x LEW Buffer)

50 mM NaH_2PO_4	7.8	g

300 mM NaCl	17.5	g

Dissolve in dH_2O and adjust pH to 8.0 suing NaOH, Take the solution to 1000 ml with dH_2O

12. Denaturing Solubilization Buffer (1x DS Buffer)

50 mM NaH_2PO_4	7.8	g
300 mM NaCl	17.5	g
8 M urea	480.5	g

Dissolve in dH_2O and adjust pH to 8.0 suing NaOH, Take the solution to 1000 ml with dH_2O

13. Denaturing Elution Buffer (1 x Elution Buffer)

50 mM NaH_2PO4	7.8	g
300 mM NaCl	17.5	g
8 M urea	480.5	g
250 mM imidazole	17.0	g

Dissolve in dH_2O and adjust pH to 8.0 using NaOH, Take the solution to 1000 ml with dH_2O

14. 50X TAE Buffer

Trizma Base	121.25	g
Disodium Dihydrate EDTA	9.0	g
$NaOAc.3H_2O$	4.452	g
Glacial acetic acid	45.0	ml

Dissolve in dH_2O by making to 1000 ml

15. Electrophoresis Buffer (Glycine buffer)

Tris	3.0	g
Glycine	14.4	g
SDS	1.0	g

Dissolve in dH_2O by making to 1000 ml, pH should be to 8.3

16. Coomassie Gel Stain

Coomassie Blue R-250	1.0	g
Methanol	450	ml
dH_2O	450	ml
Glacial acetic acid	100	ml

17. Coomassie Gel Destain

Methanol	100	ml
Glacial acetic acid	100	ml
dH_2O	800	ml

18. PBS solution

200 mM Phosphate buffer, pH 7.4	1.0	L
Sodium chloride	175.2	g
Double distilled water	18	L

<u>Stock solutions, 200 mM</u>

Stock A: $NaH_2PO_4.H_2O$	27.6	g

Stock B: $NaHPO_4.12H_2O$ 71.63 g

- Separately dissolve in dH2O and adjust the volume to 1 L
- Mix solution A (774 ml) with solution B (226)
- Adjust pH to 7.4 by titration with 5 M HCl

19. 50 mM Phosphate Buffer Potassium Phosphate Buffer, pH 7.4 for 50 ml

1 M Phosphate Buffer Potassium Phosphate Buffer 2.5 ml

Agjust by distillated water to 50 ml

Stock solution, 1M for 10 ml

Stock A: 1 M K_2HPO_4 8.02 ml

(K_2HPO_4 1.742 g dissolved in dH_2O to 10 ml)

Stock B: 1 M KH_2PO_4 1.98 ml

(KH_2PO_4 1.742 g dissolved in dH_2O to 10 ml)

20. DAB solution

DAB	6	mg
PBS solution	20	ml
30% H2O2	20	µl
1% $COCl_2$	50	µl

APPENDIX B
DETERMINATION OF PROTEIN CONCENTRATION BY BRADFORD METHOD

1. Standard curve of Bovine Serum Albumin concentration

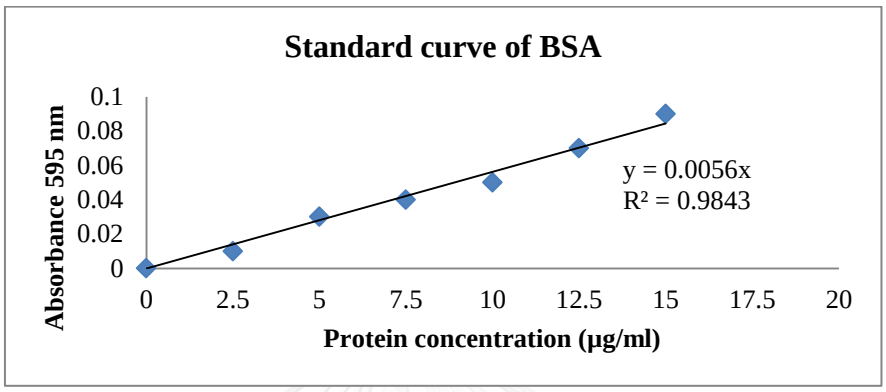


Figure B1 A standard Bovine Serum Albumin (BSA) curve from Bradford analysis for protein concentration determination.

The equation from the graph in figure B1 was used to calculate the protein concentration in each sample of WPTA1 strain fermentation and cell lysate sample of AW strain fermentation. From this graph, the protein concentrations were calculated as the following:

$$y = 0.0056x$$

Which x was the average OD_{575} value of each sample and y was the protein concentration in each sample. An example of protein concentration calculated from this equation is represented in Table B1 and B2. With this calculation, the average protein from WPTA1 and αA expression at 0, 24, and 48 hour including the average protein of the recombinant AW peptide elution from the 24th hour of the production (from 3 repeats of each sample) are represented in Table C1, C2, and C3, respectively.

Table B1 The total protein concentration from the production of WPTA1 strain

WPTA1 strain	OD_{575} nm			$OD_{average}$	$OD_{average}$ - Blank	Protein (µg/ml)
	1	2	3			
0	0.1833	0.1834	0.1864	0.1844	0.0029	0.52
24	0.2572	0.2500	0.2645	0.2572	0.0758	13.53
48	0.2235	0.2237	0.2229	0.2234	0.0419	7.48

When the OD_{575} value of blank was 0.1815

Table B2 The total protein concentration of the recombinant AW peptide elution through Ni^{2+} affinity column expressed by AW strain

the recombinant AW elution	OD_{575} nm	Blank	$OD_{average}$ - Blank	Protein (mg/ml)	Average
1	0.3485	0.221	0.1275	1.14	0.95 ± 0.17
2	0.3172	0.2279	0.0893	0.80	
3	0.3162	0.2143	0.1019	0.91	

APPENDIX C
GROWTH, pH AND PROTEIN CONCENTRATION

Table C1 The growth profile of WPTA1 stain for the recombinant AW peptide production induced by 0.5% methanol in 50 ml Basal medium culture at 30ºC, 250 rpm for 48 hours.

Time (hour)	WPTA1 strain			
	pH	OD_{600}	Cell dry weight (g)	Protein concentration (µg/ml)
0	5.38	0.52	0.0363	0.52
24	6.69	21.78	0.1938	13.53
48	6.49	24.13	0.1986	7.48

Table C2 The growth profile of αA stain at 30ºC, 250 rpm for 48 hours in Basal medium using 0.5% methanol.

Time (hour)	αA strain		
	pH	OD_{600}	Protein concentration (µg/ml)
0	5.32	0.63	0.34
24	5.37	0.81	0.64
48	6.60	22.10	6.06

Table C3 The cell growth rate, pH value, and the recombinant AW peptide expressed in *E. coli* AW strain by using 1 mM IPTG as inducer at 37ºC for 6 hours and the growth profile of MG1655/pQE-30Xa strain fermentation.

Time (hour)	MG1655/pQE-30Xa strain			AW stain		
	OD_{600} nm	pH	Soluble protein (mg)	OD_{600}	pH	Soluble protein (mg)
0	0.62	6.5	9.23	0.57	6.46	10.22
2	1.49	7.6	16.40	2.21	7.52	17.09
4	1.62	7.7	18.06	2.49	7.96	20.26
6	1.98	8.1	18.41	2.82	8.25	21.65

Table C4 The soluble protein concentration of AW and MG1655/pQE-30Xa strain at 0, 2, 4, and 6 hour after added IPTG.

Soluble Protein concentration										
Sample		Blank1	AW strain				MG1655/pQE-30Xa			
			0 hour	2 hour	4 hour	6 hour	0 hour	2 hour	4 hour	6 hour
A_{260}	1.00	0.06	0.6497	0.7860	0.8794	0.9662	0.6691	0.6065	0.6570	0.6621
	2.00	0.07	0.6198	0.7724	0.8777	0.8885	0.6799	0.6977	0.6626	0.6699
	3.00	0.06	0.6190	0.7786	0.8362	0.9531	0.6678	0.6916	0.7068	0.6571
	Mean	0.06	0.63	0.78	0.86	0.94	0.67	0.67	0.68	0.66
	A260 w/o Blank		8.52	14.34	16.05	17.48	9.16	12.07	12.27	12.03
A_{280}	1.00	0.05	0.4294	0.5240	0.5926	0.6498	0.4091	0.4149	0.4508	0.4569
	2.00	0.06	0.4050	0.5139	0.5911	0.5976	0.4141	0.4932	0.4718	0.4691
	3.00	0.05	0.4013	0.5173	0.5651	0.6388	0.4085	0.4396	0.4787	0.4537
	Mean	0.06	0.41	0.52	0.58	0.63	0.41	0.45	0.47	0.46
	A280 w/o Blank		5.33	9.24	10.53	11.45	5.31	7.86	8.21	8.07
Concentration (mg/ml)			1.79	3.42	4.12	4.45	1.27	3.00	3.40	3.37
Total protein (mg)			8.96	17.09	20.60	22.26	6.37	15.01	17.01	16.84

APPENDIX D
DETERMINATION OF ANTIOXIDANT ACTIVITIES

1. Calculation of the % inhibition of free radicals scavenging assay

The % inhibition value of each sample in DPPH and ABTS assay was calculated as the following:

$$\% \text{ Inhibition} = \frac{(OD_{control} - OD_{blank\ of\ control}) - (OD_{sample} - OD_{blank\ of\ sample}) \times 100}{(OD_{control} - OD_{blank\ of\ control})}$$

The equation was used to calculate the % inhibition in each sample of the recombinant AW peptide elution through the Ni2+ affinity column. An example of %inhibition of the ascorbic acid in DPPH and ABTS assay is represent in Table D1 and D2

With this calculation, the % inhibition value of ascorbic acid, the chemical synthesized peptide, and the recombinant AW peptide in DPPH and ABTS assay was calculated and determined into IC_{50} correlated to Table 4.3 and 4.4, in Chapter IV, respectively.

Table D1 The % inhibition value of ascorbic acid in DPPH assay.

Concentration (µg/ml)	OD_{517} of sample	OD_{517} of Background	%Inhibition
1000.00	0.0376	0.0274	97.33
500.00	0.0373	0.0286	97.73
250.00	0.0386	0.0279	97.20
125.00	0.0384	0.0277	97.20
62.50	0.0394	0.032	98.07
31.25	0.1123	0.0278	77.91
15.63	0.2575	0.0284	40.12
7.81	0.2877	0.0276	32.01
3.91	0.3226	0.0272	22.78
1.95	0.3362	0.0278	19.39
0.98	0.3535	0.0276	14.81
0.49	0.3602	0.0289	13.40
0.24	0.3629	0.0285	12.59
0.12	0.3767	0.0281	8.88
0.06	0.3656	0.0281	11.78
0.03	0.3721	0.0281	10.08

Table D2 The % inhibition value of ascorbic acid in ABTS assay.

Concentration. (µg/ml)	OD_{734} of sample	OD_{734} of Background	%Inhibition
1000.0	0.0359	0.0359	100.00
500.0	0.0362	0.0370	100.12
250.0	0.0369	0.0365	99.94
125.0	0.0922	0.0362	91.71
62.5	0.4286	0.0359	41.87
31.3	0.4863	0.0354	33.25
15.6	0.5666	0.0363	21.50
7.8	0.6020	0.0363	16.25
3.9	0.5942	0.0374	17.57
2.0	0.6292	0.0341	11.90
1.0	0.6408	0.0359	10.45
0.5	0.6419	0.0341	10.02
0.2	0.6404	0.0351	10.39
0.1	0.6440	0.0356	9.93
0.1	0.6477	0.0351	9.31
0.0	0.6476	0.0338	9.13

2. T-test of each sample in antioxidant activities

Table D3 Comparison of the different of %inhibition DPPH between the chemical synthesized peptide and the recombinant AW peptide elution.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Sum	Equal variances assumed	.136	.731	11.878	4	.000	13.73333
	Equal variances not assumed			11.878	3.721	.000	13.73333

Table D4 Comparison of the different of IC_{50} ABTS between the chemical synthesized peptide and the recombinant AW peptide.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Sum	Equal variances assumed	9.655	.036	-100.776	4	.000	-932.44333
	Equal variances not assumed			-100.776	2.128	.000	-932.44333

VITA

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