การแสดงออกของรีคอมบิแนนท์เพปไทด์ที่มีฤทธิ์ต้านออกซิเดชันจากกากเมล็ดแมงลัก *Ocimum basilicum* L. ใน *Escherichia coli*

นางสาวอาภาพรรณ คงเจริญ

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EXPRESSION OF RECOMBINANT ANTIOXIDATIVE PEPTIDES FROM HAIRY BASIL *Ocimum basilicum* L. SEED WASTE IN *Escherichia coli*

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พืชเป็นสิ่งมีชีวิตที่มีการศึกษาคุณสมบัติการต้านอนุมูลอิสระอย่างแพร่หลาย เนื่องจากความสามารถในการยับยั้งอนุมูลอิสระและป้องกันการเกิดโรคต่างๆ งานวิจัยที่ผ่านมาได้แสดงให้เห็นถึงฤทธิ์การต้านออกซิเดชันของเพปไทด์จากกากเมล็ดแมงลัก เพปไทด์ที่มีลำดับกรดอะมิโน QTFQYSRGWTN ถูกนำมาสังเคราะห์ด้วยวิธีทางเคมี และใช้เทคนิคดีเอ็นเอสายผสมเพื่อสร้าง *Escherichia coli* สายพันธุ์ที่มีการแสดงออกของรีคอมบิแนนท์เพปไทด์ดังกล่าวจำนวน 7 ชุด บนเอ็กเพรสชันเวคเตอร์ pQE-30 Xa เรียกว่าสายพันธุ์ OSW ผลการทดลองพบว่ารีคอมบิแนนท์ *E. coli* สายพันธุ์ OSW มีการแสดงออกของเพปไทด์ที่ต้องการ โดยอยู่ในรูปของโปรตีนที่ละลายอยู่ในเซลล์ รีคอมบิแนนท์เพปไทด์ถูกทำให้บริสุทธิ์ด้วย Ni^{2+} คอลัมน์ซึ่งยังพบการปนเปื้อนของโปรตีนอื่นๆ จึงใช้การสกัดเพปไทด์ที่ต้องการจาก SDS เจลที่ขนาด 15 กิโลดาลตัน เพื่อให้ได้เพปไทด์ที่บริสุทธิ์ ซึ่งเกิดการสูญเสียเพปไทด์ที่ต้องการบางส่วนไประหว่างกระบวนการนี้ รีคอมบิแนนท์เพปไทด์นี้ถูกนำไปตรวจสอบด้วยด้วยวิธีการวิเคราะห์แบบ dot blot นอกจากนี้ยังมีการตรวจสอบรีคอมบิแนนท์เพปไทด์ด้วยวิธี qRT-PCR อีกด้วย แต่อย่างไรก็ตาม OSW เพปไทด์ที่ได้จากการทำให้บริสุทธิ์จากคอลัมน์ ได้ถูกนำไปทดสอบฤทธิ์การต้านอนุมูลอิสระต่อ DPPH และ ABTS รวมไปถึงการทดสอบการออกฤทธิ์ป้องกันการถูกทำลายของดีเอ็นเอ ผลการทดสอบแสดงให้เห็นว่า รีคอมบิแนนท์เพปไทด์ OSW ที่ได้จากการทำให้บริสุทธิ์จากคอลัมน์ มีฤทธิ์การต้านอนุมูลอิสระ ABTS ดีกว่าเพปไทด์ที่ได้จากการสังเคราะห์ทางเคมีอย่างมีนัยสำคัญทางสถิติที่ความเชื่อมั่น 95% และสามารถออกฤทธิ์ป้องกันการถูกทำลายของดีเอ็นเอได้ดีกว่าเช่นกัน ดังนั้นรีคอมบิแนนท์ *E. coli* สายพันธุ์ OSW ดังกล่าวนี้จึงสามารถนำไปศึกษาต่อในการหาปัจจัยที่เหมาะสมของแสดงออกและการทำให้บริสุทธิ์ เพื่อที่จะเป็นทางเลือกในการใช้ผลิตรีคอมบิแนนท์เพปไทด์ที่มีประสิทธิภาพมากยิ่งขึ้นต่อไป

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APAPUN KONGCHAROEN: EXPRESSION OF RECOMBINANT ANTIOXIDATIVE PEPTIDES FROM HAIRY BASIL *Ocimum basilicum* L. SEED WASTE IN *Escherichia coli* . ADVISOR: RUETHAIRAT BOONSOMBAT, Ph.D., pp.

Antioxidant activities in plants were widely investigated due to reduction of free radicals which are risks of some diseases. Previously report revealed a potential antioxidative peptide from seed waste of hairy basil. The peptide with amino acid sequence as QTFQYSRGWTN was selected for synthesizing recombinant antioxidant peptide and expressed in *Escherichia coli* MG1655. This recombinant *E. coli* named as OSW strain containing the DNA fragment encoding seven copies of the target peptide on pQE-30 Xa expression vector. The OSW strain could express the recombinant OSW peptide in the soluble fraction. The recombinant OSW peptide elution from Ni^{2+} affinity column was contaminated with other proteins. The expected 15 kDa OSW peptide band was further extracted from SDS gel, resulted in the loss of more target peptide, was verified by modified dot blot analysis with antiHis-HPR antibody. Moreover, the recombinant OSW peptide expression was verified by qRT-PCR. The elution of recombinant OSW peptide was examined for the DPPH and ABTS radical scavenging activities, and *in vitro* protective effect on DNA damage induced by hydroxyl radical. The recombinant OSW elution revealed significantly higher antioxidant activities that those of the chemical synthesized one, particularly the DNA damage prevention. However, for further study, this strain should be subjected for optimizing factors involved in recombinant peptide production and additional purification.

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CONTENTS

Page
THAI ABSTRACT iv
ENGLISH ABSTRACT v
ACKNOWLEDGEMENTS vi
CONTENTS vii
LIST OF FIGURE 1
LIST OF TABLE 3
LIST OF ABBREVIATIONS 5
CHAPTER I INTRODUCTION 6
CHAPTER II LITERATURE REVIEWS 10
2.1 Free radicals and Antioxidants 10
2.2 Oxidative stress 14
2.3 External antioxidants 15
2.3.1 Vitamin C 17
2.3.2 Vitamin E 18
2.3.3 Flavonoids 19
2.4 Hairy basil 22
2.5 Recombinant DNA technology 27
2.6 Expression and purification of recombinant proteins in *E. coli* 31
2.7 Measurement of antioxidant activities 34
2.7.1 DPPH radical scavenging activity 34
2.7.2 ABTS radical scavenging activity 35
2.7.3 Protective effects against hydroxyl radical inducing DNA damage 36
CHAPTER III METHODOLOGY 37
3.1 Materials 37
3.1.1 Chemical and Reagents 37
3.1.2 Equipments and supplies 39
3.1.3 Kits..... 42
3.1.4 Enzymes 42

Page

3.1.5 Antibiotics 43
3.1.7 Plasmids....43
3.2 Methods 44
3.2.1 Construction of recombinant *E. coli* for production recombinant OSW peptide……. 44
3.2.1.1 Maintenance and amplification of pIDTBlue-OSW plasmid....44
3.2.1.2 Construction of plasmid for expressing recombinant OSW peptide 45
3.2.1.3 Preparation of E. coli MG1655 competent cells 47
3.2.2 Recombinant peptide expression and purification 48
3.2.2.1 Recombinant peptide expression....48
3.2.2.2 Protein purification....50
3.2.3 Protein analysis....51
3.2.3.1 Determination of Protein concentration 51
3.2.3.2 Protein analysis by SDS-PAGE 51
3.2.3.3 Detection of recombinant peptide expression at transcription level by quantitative reverse transcription PCR (qRT-PCR)....52
3.2.3.4 Analysis of recombinant protein by modified dot blot analysis...53
3.2.4 Characterization of antioxidant activity 54
3.2.4.1 DPPH radical scavenging activity....54
3.2.4.2 ABTS scavenging activity....54
3.2.4.5 In vitro protective effect of the purified recombinant peptide against hydroxyl radical inducing DNA damage 55
CHAPTER IV RESULT AND DISSCUSION....56
4.1 Construction of recombinant *E. coli* strain for production of recombinant OSW peptide 56
4.2 Recombinant OSW peptide expression and purification....64
4.3 Protein analysis....67
4.3.1 Determination of Protein concentration 67
4.3.2 Protein analysis by SDS-PAGE 69

Page

4.4 Characterization of antioxidant activities 76
CHAPTER V CONCLUSION 83
REFERENCES 87
APPENDIX 92
APPENDIX A MEDIA AND SOLUTIONS 93
APPENDIX B DETERMINATION OF PROTEIN CONCENTRATION 99
APPENDIX C DETERMINATION OF ANTIOXIDANT ACTIVITIES 103
VITA 106

LIST OF FIGURE

Figure 2.1 Chain reaction of free radical set off. 11

Figure 2.2 Oxidative stress-induced diseases in humans. 15

Figure 2.3 Enzymatic antioxidant activity of SOD. 17

Figure 2.4 Chemical structure of vitamin C. 18

Figure 2.5 The α-tocopherol form of vitamin E. 18

Figure 2.6 Chemical structure of flavonoids 19

Figure 2.7 Chemical structure of corazonin. 25

Figure 2.8 Steps in making a recombinant DNA plasmid. 28

Figure 2.9 The extraordinary contribution to molecular genetics of bacterial plasmid 29

Figure 2.10 The antioxidant activity of DPPH free radical scavenging 35

Figure 2.11 The antioxidant activity of ABTS free radical scavenging 36

Figure 2.12 The generation of $OH^{\bullet}$ radical by Fenton reaction. 36

Figure 3.1 Expression of OSW peptide in *E. coli* MG1655 49

Figure 4.1 DNA sequence of synthesized DNA fragment for expressing recombinant OSW peptide 57

Figure 4.2 The plasmid pIDTBlue-OSW encoding recombinant OSW peptide with 7 repetitive sequences copies and ampicillin resistance gene. 58

Figure 4.3 The introduction of *Bam*HI and *Hin*dIII restriction sites into the DNA fragment through PCR. 58

Figure 4.4 1% agarose gel electrophoresis analysis of PCR products. 59

Figure 4.5 Map of plasmid pQE-30 Xa consisting of target *Bam*HI and *Hin*dIII restriction sites 60

Figure 4.6 Agarose gel electrophoresis analysis of expression vector and gene fragment after being digested by *Bam*HI and *Hin*dIII. 61

Figure 4.7 Agarose gel electrophoresis analysis of recombinant plasmids from the transformed clones after being digested by *Bam*HI and *Hin*dIII restriction enzyme ..62

Figure 4.8 Agarose gel electrophoresis analysis of recombinant plasmids from the transformed clones after being digested by *Bam*HI and *Hin*dIII restriction enzymes.....63

Figure 4.9 DNA sequence of target antioxidative OSW peptide. 64

Figure 4.10 The pH values, the absorbance at 600 nm at different time points (H) of OSW and CONT strains. 65

Figure 4.11 Total protein amount of each fraction from the OSW and the CONT strains (mg) at different time points after induction (H). 68

Figure 4.12 The SDS-PAGE analysis of the total insoluble protein fraction after IPTG induction from the OSW (A) and CONT (B) strains. 71

Figure 4.13 The SDS-PAGE analysis of the total soluble protein from the OSW and CONT strains. 72

LIST OF TABLE

Table 2.1 IC_{50} of antioxidant activity of hairy basil waste after oil extraction. 25

Table 2.2 Amino acid sequences of protein hydrolysates from hairy basil after permeated with 5,000 Dalton 26

Table 2.3 Comparison of general expression systems. 32

Table 4.1 Total protein amount of each fraction from 50 ml culture of the OSW strain.....68

Table 4.2 Crossing point value from qRT-PCR of the mRNA for the recombinant OSW peptide expression. 74

Table 4.3 The DPPH scavenging activity of the tested peptides at concentration of 1 mg/ml. 77

Table 4.4 The ABTS scavenging activity of the tested peptides. 78

Table B1 The insoluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 1st expression.....99

Table B2 The insoluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 2nd expression. 100

Table B3 The insoluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 3rd expression. 100

Table B4 The soluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 1st expression. 101

Table B5 The soluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 2nd expression. 101

Table B6 The total protein amount of recombinant OSW elution from 50 ml culture of the OSW strain cultured at 37 °C in LB broth medium after added IPTG for 4 hours. 102

Table B7 The total protein amount of each fraction from 50 ml culture of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours. 102

Table B8 The soluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 3rd expression. 102

Table C1 Comparison of the different of % inhibition DPPH between chemical synthesized OSW peptide and recombinant OSW elution…….104

Table C2 The % DPPH radical inhibition of chemical synthesized OSW peptide, recombinant OSW elution and ascorbic acid at concentration of 1 mg/ml. 104

Table C3 The IC50 of Antioxidant activity with ABTS of recombinant OSW elution and ascorbic acid.....105

LIST OF ABBREVIATIONS

bp	base pair
°C	degree Celsius
dNTP	deoxynucleoside triphosphates
DNA	deoxyribonucleic acid
g	gram
l	liter
LB	Luria-Bertani
µg	microgram
µl	microliter
mg	milligram
ml	milliliter
mM	millimolar
OD	optical density
rpm	revolutions per minutes
RNA	ribonucleic acid

CHAPTER I
INTRODUCTION

Antioxidant is a compound, substance or enzyme with an ability to protect oxidative damage from free radicals by pairing up with unpaired electron of free radical. Then, free radicals are eliminated, resulting in protection of biomolecules, including protein and DNA, from damage potentially being caused of pathos-physiological consequences. Normally, the human body produces a balance of antioxidants and free radicals. However, aging and pollutants increase risk of an oxidative stress state in the body. Therefore, additional antioxidants can significantly balance with accumulating free radicals. Previous reports revealed that uptaking of external antioxidants could provide additional protection for some diseases) Bagchi *et al.* 2000(. Currently, antioxidants can be both synthetic and natural, but the synthetic one is a kind of phenolic compound with higher stability and remains in the body longer than the natural one (Jenjira and Prasong 2011). From these reasons, the synthetic antioxidant has been more concerned about health. The natural antioxidants can be found in many organisms including plants, which are regarded as the major sources of antioxidants, fungi and algae. Thereby,

antioxidants from natural sources have become interested and widely studied.

Plant is an important source of natural antioxidants. Due to their applications in traditional treatments of many diseases, antioxidant compounds and antioxidant activities have been widely discovered in many kinds of Thai edible plants. Hairy basil (*Ocimum baisilicum* Linn.) is one of edible plants which is commonly used its seeds for food because of its concentrated nutrition that supports the digestion system. Moreover, extracted oil from hairy basil seeds is also used for aromatherapy purpose. It was revealed that *O. basilicum* contained higher phenolic contents than *A. graveolens* and *L. sativum* seeds. Moreover, the methanol extract of *O. basilicum* L. displayed significantly higher DPPH and H_2O_2 radical scavenging activities than the others (Aydemir and Becerik 2011). It was reported that small seed waste particle (<90 mm) after oil extraction found protein as 57% of total component (Sarintip *et al.* 2009). Protein hydrolysate from hairy basil seed waste after being permeated with 5 kDa found the potential antioxidative peptides with its sequence of QTFQYSRGWTN (Semanit 2013). However, natural product extraction poses several problems including requirement of a great number of raw materials, difficult quality control, high cost and time consuming.

Recombinant DNA technology is one of the alternatives to solve these problems. This technique is usually come with higher yield, easy manipulation, lower cost, and less time consuming (Keasling and Bang 1998). Many recombinant peptides have been successfully expressed through *E. coli* system such as a recombinant Tat-HA-NR2B9c peptide with ability to decrease in the infarction area and improve functions of neurological (Zhou *et al.* 2012). Previous study reported that Porcine beta defensin1 and a cationic antimicrobial peptide, was successfully expressed in *E. coli* after being induced by IPTG and recovered with 90% purity by Histidine-tagged affinity column (Li *et al.* 2013). This study also suggested the better production yield from prokaryotic system, especially in *E. coli* system.

In this research, recombinant *E. coli* strain for expressing the potential antioxidative peptide, originally found in hairy basil seed waste, was constructed. Recombinant peptide is expected to be either similar or better to the native one. In addition, this strain should be more useful, due to an easier genetic handling, for potential improvement of production scale in the future.

Objectives of this research:

To express a recombinant antioxidative peptide, originally found in hairy basil seed waste, in *E. coli*. Construction of this recombinant strain should be an alternative method for further improving recombinant peptide production.

CHAPTER II
LITERATURE REVIEWS

2.1 Free radicals and Antioxidants

Free radicals, reactive oxygen species (ROS), or reactive nitrogen species (RNS) are also called reactive species (Rss). They can be generated from aerobic metabolisms, endogenous systems as a byproduct in auto-oxidative, enzymatic and autoimmune, or transition of metal reaction. These mechanisms include reactions from respiratory system, phagocytosis, and prostaglandin synthesis. At low concentration, beneficial effects from free radical generation involve cellular responses, cellular signaling system, immune function to destroy invading pathogenic organisms and also mitogenic response induction (Valko *et al.* 2007) (Pham-Huy *et al.* 2008). Due to a permanent electron leak from oxygen, the reactive species mostly generates in mitochondria during energy transduction (Valko *et al.* 2007), particularly with unusual conditions such as inflection, radiation, air pollutant, and chemical contamination. Free radical is a molecule with one or more unpaired electron, highly unstable molecules than usual radicals, so it has high ability to react with electrons of normal neighbor atoms,

resulting in becoming a free radical continuously by oxidation chain reaction (Fig. 2.1) (Pham-Huy *et al.* 2008).

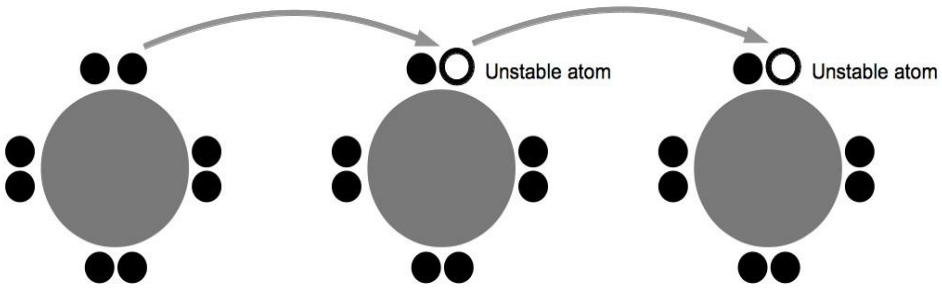

Figure 2.1 Chain reaction of free radical set off.

Free radicals and molecules that easily become free radical reaction, such as l, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) $ABTS^{\bullet+}$, superoxide radical, hydroxyl radical, lipid radical, nitric oxide radical, peroxynitrite radical, and metal, have potential to damage biomolecules including DNA, proteins, lipids and small cellular molecules associated with pathological processes (Valko *et al.* 2007, Sanjib *et al.* 2011). Free radicals are commonly grouped by their transferring mechanisms as either a hydrogen atom or an electron to convert the radical to a stable species. For the hydrogen atom transfer (HAT), one or more electrons transferred to reduce

target compounds resulting from possible secondary quenching by radical recombination and single electron transfer (SET) (Schaich *et al.* 2015). These free radicals can be aggregated in any parts of cells including nucleus, cytoplasm and membrane. Therefore, when they attack to important macromolecules, this can lead to chronic diseases. Many researches point out that this is an important cause of promoting several diseases. In 1956, Denham Harman proposed the concept of free radical's role in the aging process (Valko *et al.* 2007). It is also elucidated that ROS acts as a signaling mediator in normal cell including vascular smooth muscle cell (VSMC) contraction, relaxation and growth. Therefore, it plays an important role in several vascular diseases (Sanjib *et al.* 2011). Moreover, ROS also plays a key role in other newborns pathologies, classified as free radical-related diseases (FRD) (Perrone *et al.* 2010). Free radicals are also known as an important cause of oxidative DNA damage and secondary messengers in intracellular signaling cascades, which induce and maintain the oncogenic phenotype of cancer cells. Moreover, they can also induce cellular deterioration and apoptosis as an antitumourigenic function as well (Valko *et al.* 2006).

Substances for defensing against free radicals are called antioxidants including enzymatic such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and non-enzymatic system such as ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and others (Valko *et al.* 2007). Antioxidants can remove free radicals by various mechanisms, for example; radical scavenging, singlet oxygen quenching, metal chelation, chain breaking, antioxidant synergism, and enzyme inhibition (Jenjira and Prasong 2011). Most of antioxidants are phenolic compounds, which can pair up with unpaired electrons of free radicals, and then the radicals become oxidized resulting in termination of oxidation chain reaction. The process can divided into 2 types, one is chain breaking by stealing an electron and further forming a new radical until free radical being stabilized or decomposing into unobjectionable substances. Theo other is prevention process which an antioxidant reduce the rate of initiation by scavenging initial free radicals or stabilizing transition metal radicals (Pham-Huy *et al.* 2008).

2.2 Oxidative stress

Even the body has antioxidation system to eliminate these harmful radicals, but the production of antioxidant in the body may not enough since more accumulation of free radical by aging, pollution, and current lifestyle. An unbalance condition between free radical and antioxidants in the body is called oxidative stress. Oxidative stress is related to pathogenic causes of many diseases in several organisms such as cancer, diabetes, Alzheimer's disease, Parkinson's disease, age-related degeneration symptoms (Pham-Huy *et al.* 2008) (Valko *et al.* 2007), and others as in Fig. 2.2 (Pham-Huy *et al.* 2008). Therefore, up-taking external antioxidants, especially antioxidants from certain natural sources, will provide additional protection to free radical invaders.

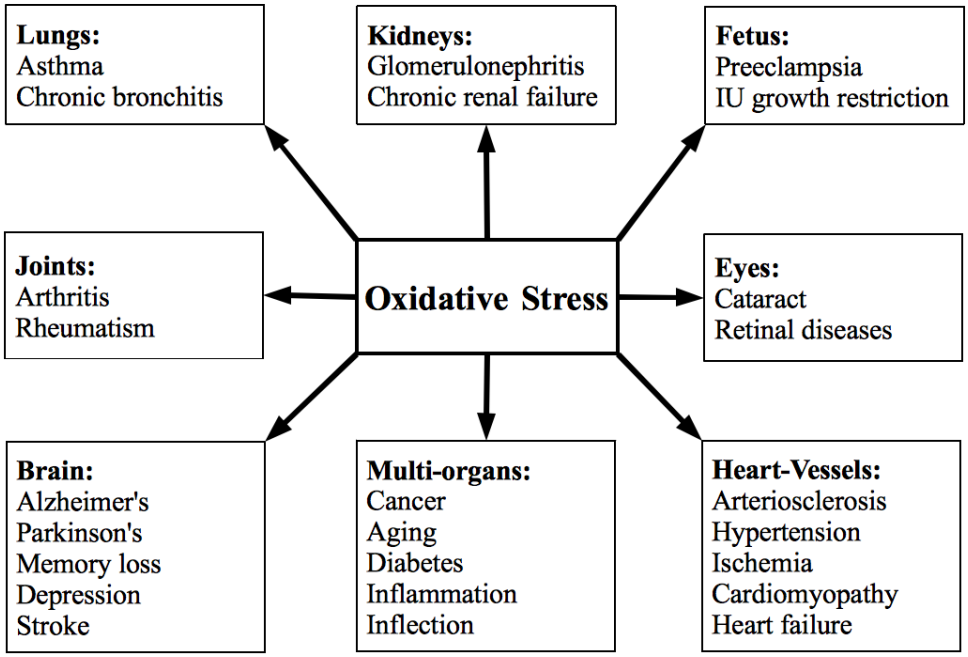

Figure 2.2 Oxidative stress-induced diseases in humans.

2.3 External antioxidants

Nowadays, external antioxidants become widely interested and various applied. They can be both chemical synthetized and natural substances. Many synthetic ones have better antioxidant activity than natural ones and was used in wide variety of product, especially supplements and food to protect the lipid oxidation, such as tertiary-butylhydroquinone (TBHQ), butylated hydroxylanisole (BHA), butylated hydroxyltoluene (BHT), propyl gallate (PG), octyl gallate (OG), and dodecyl gallate (DG) (Makahleh A *et al.* 2015).

Although the synthetic antioxidants are widely industrial applications, they are still controversial due to their potentially harmful long-term health effects. Moreover, chemical antioxidants are concerned to be injurious to the body system because they can be more durable which possibly remain in the body longer than the natural ones. Their artificial analogs might not be recycled and reused in human body leading to some possible adverse effects from harmful byproducts (Bagchi *et al.* 2000) (G.M. Williams *et al.* 1999). There are evidence supporting the carcinogenic effects, including liver and DNA damage, resulting from butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) in rats at high concentration, and also other synthetic food with phenolic antioxidants such as TBHQ and 2-tert-butyl-4-methylphenol (TBMP) (Embuscado 2015) (Fereidoon and Priyatharini 2015).

From all reasons mentioned previously, synthetic phenolic antioxidants are strictly used in various countries due to their considered toxicity. Therefore, antioxidants from natural products are currently more interested. The natural ones can be found in many organisms, including plants, fungi, and algae, and become potentially replaced the synthetic ones in food industry to reduce health risk.

The importance chemical structure of antioxidant peptide which involved with biological oxidation is phenolic compound consists of aromatic hydroxyl. This functional group can be reacted with free radical to protect oxidation reaction by hydrogen transferring [Jirum 2011 and Farhoosh 2016]. For instance, SOD detoxified hydrogen peroxide, an enzymatic antioxidant which catalase the dismutation of $O_2^{\bullet-}$ to O_2 by successive oxidation and reduction of the transition metal ion and to the less-reactive species H_2O_2 (Fig. 2.3) [Flora 2009]

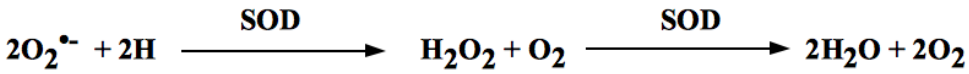

Figure 2.3 Enzymatic antioxidant activity of SOD.

Examples of antioxidants are listed as the following.

2.3.1 Vitamin C

Vitamin C or ascorbic acid (Fig. 2.4) is a water soluble compound. It is considered as antioxidant, antiaterogenic, anticarcinogenic and immunomodulator. Moreover, it plays an important role in many systems; for example, blood vessel development, collagen synthesis, and immune system (Dorota *et al.* 2015). Natural sources of Vitamin C are acidic fruits, green vegetables, tomatoes, and lemon.

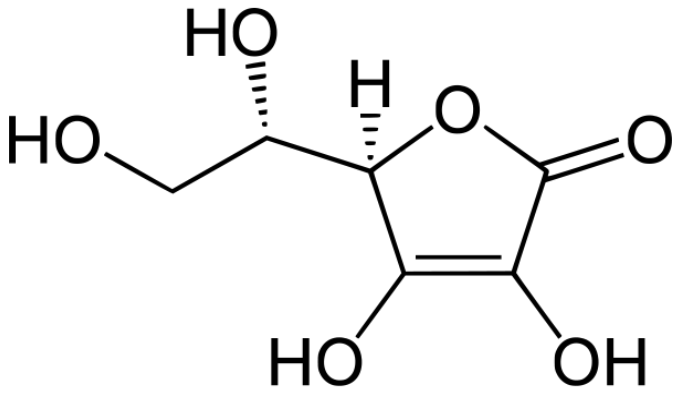

Figure 2.4 Chemical structure of vitamin C.

2.3.2 Vitamin E

Vitamin E is a fat soluble compound with strong antioxidant activities. Its chemical structure is similar to tocopherol derivative (Fig. 2.5). It is purposed to protect lipid peroxidation. Vitamin E can be found in vegetable oils, fruits, eggs, nut, cereal and others (Pham-Huy *et al.* 2008).

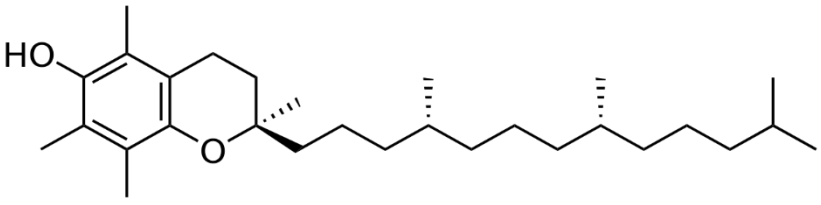

Figure 2.5 The α-tocopherol form of vitamin E.

2.3.3 Flavonoids

Flavonoids are phenolic compounds major found in plant. There are more than 6,000 kinds which its metabolites depend on its chemical structure. It has been suggested that this kind of phenolic compounds can prevent cancer, cardiovascular diseases, arthritis, aging, cataract, memory loss, stroke, Alzheimer's disease, inflammation, and infection (Pham-Huy *et al.* 2008). Flavonoids can be found in fruit skins, citrus, orange, lemon, broccoli, onion, buckwheat, soy bean, tea and others of which classes are varied (Fereidoon and Priyatharini 2015).

Plant is a major source of natural antioxidants, which may

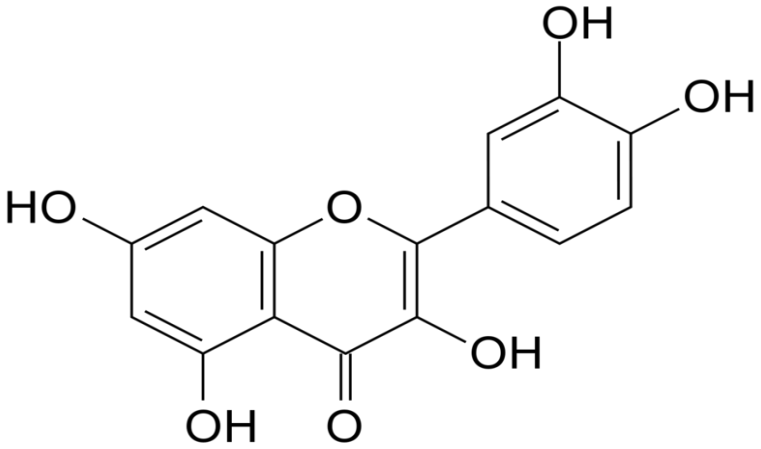

Figure 2.6 Chemical structure of flavonoids consists of 3 benzene rings with hydroxyl group.

found in any parts. Many scientific reports and studies reported many useful chemical substances in plants, so called phytonutrients, that comprise of antioxidant activities. They have over 7,000 edible plants which nutrients are varied. Many researchers have continuously discovered new sources of antioxidants from natural sources. For example, a wide range of antioxidant activities are found from the screening of antioxidant capacities and total phenolic contents of infusion from 223 medicinal plants. Ten plants; *S. miltiorrhiza* Bge., *P. multiflorum* Thunb. (Stem), *R. sacra* Fu, *S. cuneata* Rehd. et Wils., *F. rhynchophylla* Hance, *P. persica* Linn., Batsch., *C. foetida* L., *P. lactiflora* Pall., *T. farfara* L., and *S. officinalis* L. showed the highest antioxidant capacities and total phenolic contents with low toxicities with a tendency to be an interesting source of antioxidants (Li *et al.* 2013). Edible wild flowers in China also evaluated using ferric-reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC) assays and they point out that the flowers of *R. hybrida*, *L. sinuatum*, *P. hortorum*, *J. integerrima* and *O. fragrans* also could be potential rich sources of natural antioxidant (Li *et al.* 2014). When phenolic compounds and antioxidant activities a investigated in Thai edible flowers

which had long been consumed as Puangchompoo (*Antigonon leptopus*), Fueangfa (*Bougainvillea hybrida*), Kheelek (*Cassia siamea*), Aunchan (*Clitorea ternatea*), Dawkajay (*Cosmos sulphureus*), Chaba (*Malvaviscus arboreus)*, Kem *(Ixora chinensis*), Katin (*Leucaena leucocephala*), Bua Luang (*Nelumbo nucifera*), Leelawadee (*Plumeria obtusa* L.), Daao rueang (*Tagetes erecta*) and Kajorn (*Telosma minor*), Keelek revealed the highest of total phenolic content and DPPH inhibition and it also provided some useful information to utilize edible flowers as a potential sources of phytonutrients (Kaisoon *et al*. 2011). A great number of edible plants in Thailand used for food and traditional medical treatment have been studied for their antioxidant activities. From the screening for anti-oxidative activity of 83 species of Thai indigenous vegetables, 55.9% of them have high potential more than 100 mg of BHA in 100 g of fresh vegetable (Trakoonvitakorn G and J. 2000). This can be elucidated that there are abundant of medicinal plant containing useful and valuable compounds which could be treasure sources of natural antioxidants. An interesting group is one that is rich of phytochemicals and powerful antioxidant compounds; particularly flavonoids, containing in any parts of plants. They have been used as whole, ground, extraction,

encapsulation or as emulsion (Fereidoon and Priyatharini 2015). Furthermore, these plants are commonly used as food ingredient because of their ability to reduce oxidative rancidity of lipid. Several studies have demonstrated that spices such as rosemary, sage, and oregano have high antioxidant activities (Zheng *et al.* 2016).

2.4 Hairy basil

Hairy basil (*Ocimum basilicum* Linn.), a popular Lamiaceous plant known as sweet basil, is used as a culinary herb, ingredient for cooking and as an decoration plant in house gardens. It is probably Asian or African native plant which has been used traditionally for the treatment of anxiety, diabetes, cardiovascular diseases, headaches, nerve pain, digestive tonic, carminative, expectorant (Chanwitheesuk *et al.* 2005), headache relief and a variety of neurodegenerative disorders. It could prevent and treat cerebral damage by decreasing size of cerebral infract in mice brain (Bora *et al.* 2011). There are evidences indicating that active compositions of *O. basilicum* extract can be used as a therapeutic tool in hyperlipidaemic subjects and may lead to further study in the field of hyperlipidaemia and related diseases (Mahajan *et al.* 2013).

Hairy basil seeds are commonly used for many purposes. They have concentrated nutrition that supporting the digestion system which can be used for digestive disorders treatment by its carminative effects. They also have antispasmodic effects providing a relief from cold and whooping cough. Moreover, extracted oil from hairy basil seeds and seed consumption are concerned for stress reliever. Oil in basil seeds can be used for infection treatment due to its antimicrobial characteristic. Study of phenolic content and antioxidant activities of *O. basilicum*, *Apium graveolens* and *Lepidium sativum* seeds revealed that in all methanol, ethanol and water extracts, *O. basilicum* contained higher phenolic contents than *A. graveolens* and *L. sativum* seeds. Moreover, the methanol extract of *O. basilicum* L. had significantly higher DPPH and H_2O_2 scavenging effect than the others (Aydemir and Becerik 2011). This study also suggested the possible application of these seeds for medicinal uses and food preservation, especially *O. basilicum* seeds.

It was reported that small seed waste particle (<90 mm) after oil extraction found high protein component up to 57% which is higher than the others waste particle sizes (Sarintip *et al.* 2009). Therefore, in this part of waste was expected to contain potential

antioxidant peptides. The study of antioxidant activities in hairy basil seed waste by extraction after permeated with 5,000 Da and 10,000 Da found that the extraction solution size less than 5 kDa had the highest antioxidant capacities, especially with DPPH and ABTS radical scavenging activity. The IC_{50} of antioxidant activity is represented in Table 2.1. The highest antioxidant activity is DPPH radical scavenging activity of which IC_{50} value is 25.23 μg/ml, greater than a positive control, ascorbic acid, (10.95 μg/ml) (Semanit 2013). One of the peptide found in the extraction solution size less than 5 kDa expected to be a potential antioxidative peptides revealed the sequence of QTFQYSRGWTN (Table 2.2). It was conserved sequence of corazonin (Fig 2.7), a highly conserved neuropeptide found in many insects, in particular locusts and cockroaches. Corazonin plays role in extension the lifespan of neurons in *D. Melanogaster*, exposed to starvation, osmotic, and oxidative stress and also increases locomotion and dopamine levels in male flies (Žitňan and Daubnerová 2016).

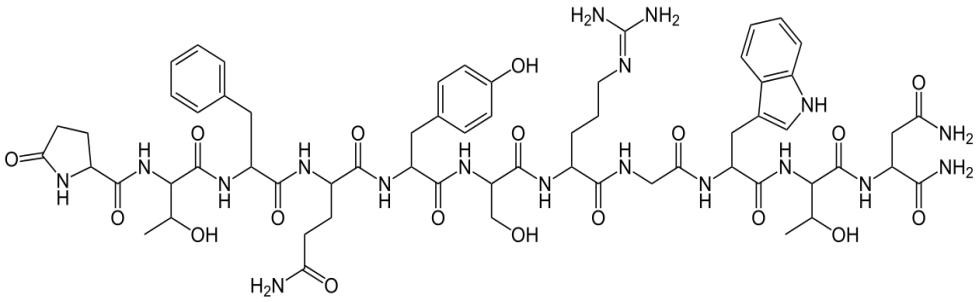

Figure 2.7 Chemical structure of corazonin.

Table 2.1 IC_{50} of antioxidant activity of hairy basil waste after oil extraction. The antioxidant activity was measured DPPH, ABTS, NO, H_2O_2 assays after permeated with 5,000 and 10,000 Da (Semanit 2013).

IC_{50} of Antioxidant activity (µg/ml)	Molecular weight (KDalton)				Positive Control µg/ml
	X<5	5<X<10	X>10	All size	
DPPH	25.23 ±0.1437	37.00 ±0.1316	41.17 ±0.1389	14.65 ±0.1347	14.28 ±0.0021
ABTS	44.50 ±0.0026	67.09 ±0.0021	161.80 ±0.0029	76.11 ±0.0033	15.49 ±0.0021
H_2O_2	798.30 ±0.0030	1411.00 ±0.0023	1177.00 ±0.0042	1048.00 ±0.0029	15.53 ±0.0091
NO	212.10 ±0.0070	220.80 ±0.0058	4301.00 ±0.0049	3262.00 ±0.0057	54.0 ±0.0049

Table 2.2 Amino acid sequences of protein hydrolysates from hairy basil after permeated with 5,000 Dalton (Semanit 2013).

Fraction	sequence	Protein name	Accession number	organism	score
1	SKVIETKQQVVTEIADKLRA	50S ribosomal protein L10	P83063	*Bacillus cereus*	21
2	MYEVPIVVVYAAQI	Unknown protein CP 4 from 2D-PAGE	P81349	*Clostridium pasteurianum*	41
3	QTFQYSRGWTN	Corazonin	B3A0J2	*Tyrannophasma gladiator (Gladiator) (Heel-walker)*	22
4	MYEVVIVVVYAAQI	Unknown protein CP 4 from 2D-PAGE	P81349	*Clostridium pasteurianum*	20
5	QPDIMIFTIGPA	Unknown protein CP 10 from 2D-PAGE	P81345	*Clostridium pasteurianum*	17
6	ASSAAAAFAPSTPLA	ATP synthase subunit beta, mitochondrial	P80083	Spinacia oleracea (Spinach)	Not sig
7	IALTV	N/A	N/A	N/A	
8	SESYTPISGPNGYEVDVK	Lectin	P33888	Euphorbia characias (Albanian spurge)	Not sig

However, commercial basil extraction method is steamed distillation which has some drawbacks such as thermal decomposition of labile components, high energy consumption, and being time consuming. Even new techniques, such as supercritical or pressurized fluid extraction and microwave or ultrasound assisted techniques are alternatives (Li and Chang 2016), utilization problems of natural extraction such as intensive labor, large

amount of material requirement, difficulty in controlling raw material quality, are still major problems.

2.5 Recombinant DNA technology

Recombinant DNA technology is one of the alternatives to solve problems of natural product extraction. Higher yield, easy manipulation, lower cost and less time consuming are expected from recombinant peptide production technique (Keasling and Bang 1998). This technique is involved of manipulating an expected DNA fragment in a host organism. The DNA fragment is *in vitro* cut and joined with the selected DNA vector, creating the recombinant DNA molecule, copying in host or vector system (Fig. 2.8).

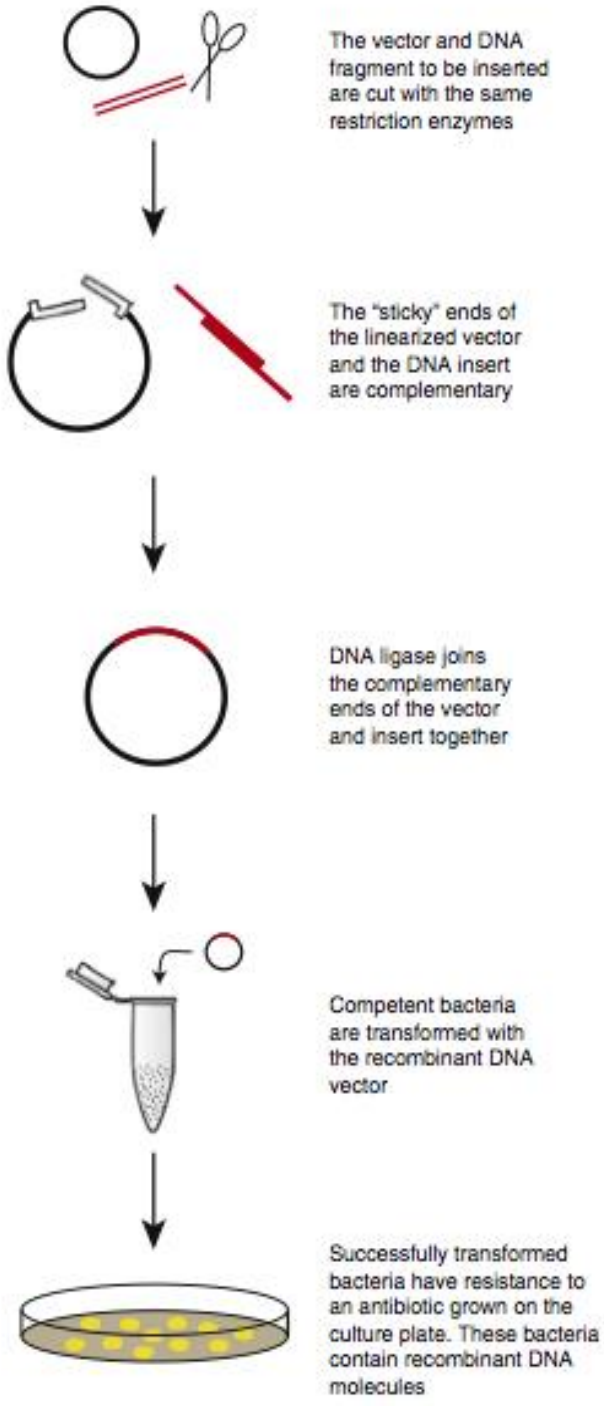

Figure 2.8 Steps in making a recombinant DNA plasmid (Carter and Shieh 2015).

A DNA vector, such as plasmid, phage and cosmid, is a nucleic acid storage system that can hold an isolated DNA sequence of interest. The essential features of a vector are ability to replicate autonomously in a host species (usually bacteria), and combine with other pieces of DNA. The inserted DNA fragment usually comes from different organisms from DNA vectors of which fusion is called recombinant DNA constructs (Carter and Shieh 2015). Generally, plasmid is used as a vector for several purposes as shown in Figure 2.9.

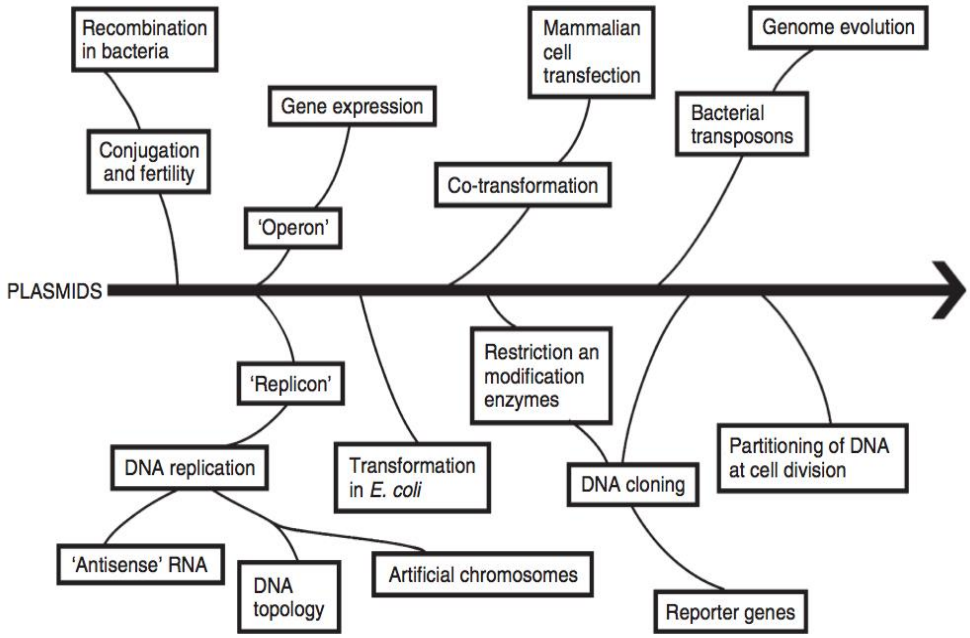

Figure 2.9 The extraordinary contribution to molecular genetics of bacterial plasmid (cohen 1993).

To reduce development cost and more effectively improve in pharmaceuticals, the recombinant DNA technique has been used for a long time. This method is also applies to identify human drug metabolism identification, and to characterize the role of an enzyme in drug metabolism and chemical carcinogenesis by introducing and expressing its genetic information in a cell, so called heterologous expression (Friedberg and Wolf 1996). Dengue vaccines for medical treatment of dengue disease (DEN), which is dramatically increasing and lead to infection and death up to 25,000 a year in South-East Asia and South America nations, has been developed by using recombinant DNA technology because the virus genetics and replication that can be specifically altered and rationally investigated. Recombinant DNA technology provides an alternative method to generate these candidate vaccines by switching/replacing the genes among each individual serotype with inexpensive price (Lee *et al.* 2012). The other example is Human serum albumin (HSA), a protein in human plasma which is widely used for treat hypoproteinaemia, foetal erythroblastosis, fluid loss due to burn injuries or haemorrhagic shock, and ascites caused by cirrhosis of the liver. Conventionally, it is produced by donation of human plasma which is limited and potentially risked on spreading

blood-derived pathogens. Therefore, the genetic engineering has been currently applied as an alternative method for large scale production of pathogen-free HSA (Chen *et al.* 2013).

2.6 Expression and purification of recombinant proteins in *E. coli*

Bacterial expression system such as *E. coli* expression system is widely used to start-up experiment in numerous laboratories due to its cost, simplicity, and easy genetic manipulation. Moreover, according to the structures of bacterial cell wall and membrane along with the lack of nucleus, it is easy to isolate DNA from cells. The bacterial heterologous expression has been frequently used for producing recombinant enzymes, and protein including peptides, as well. Although *E. coli* system has some disadvantages such as the lack of a post-translational modification (PTM) process and the presence of codon usage bias that some rare codon translation may be lost (Wu *et al.* 2004), the expression system is depended on several factors including protein, purity and quantity requirement, glycosylation and other PTM process requirement, functional activity, cost and other specifications. Most recombinant peptides do not require glycosylation or the PTM procedure, so this limitation is not a

major problem for *E. coli* system. Therefore, host system selection has to be considered with several factors such as target protein and research objectives. A comparison of general features and advantages for each expression are listed in Table 2.3 (www.genwaybio.com)

Table 2.3 Comparison of general expression systems.

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 - High
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
Yield (mg/l)	50-500	10-200	10-200	0.1-100
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 PTM	Longer time, less PTM	Longer time, higher cost	Highest cost, lower yield

Currently, the *E. coli* expression system is usually selected as the first choice for initial investigation and development in functional study. The recombinant protein technology have brought abundant therapeutic proteins into clinical applications over the past decades and still going on with a hundred and thousands of

recently investigated proteins (Chen *et al.* 2013). Many recombinant peptides have been successfully obtained through *E. coli* expression system such as a recombinant Tat-HA-NR2B9c peptide for clinical stroke therapy with ability to decrease the infarction area and improve neurological functions. Because this recombinant peptide is accumulated in inclusion bodies of *E. coli* cells at high level of expression, it is useful for further large scale production. Moreover, with high neuroprotective effects in rats, it is promising to enable use for stroke therapy in animals or humans in the future (Zhou *et al.* 2012).

A previous study reported that Porcine beta defensin1, a cationic antimicrobial peptide for prevent pathogens in pig diseases, was successfully expressed in *E. coli* after being induced by IPTG and recovery with 90% purity by Histidine-tagged affinity column. As expected, the recombinant peptide revealed antimicrobial properties against both Gram-positive *S. aureus* and Gram-negative *E. coli* with minimum inhibitory concentrations (MICs) of 80 μg/ml and 100 μg/ml, respectively (Li *et al.* 2013). This study also suggested the commonly higher yield from prokaryotic *E. coli* system. Furthermore, another peptide such as Lethal factor (LF), a virulence factor of *Bacillus anthracis*

infection, was also reported for successful expression from *E. coli* with full biological activity. The yield of 5 mg and over 95% purity was obtained from one liter of *E. coli* culture (Liu *et al.* 2013). These previously mentioned reports support the effective expression and purification of various recombinant peptides in *E. coli* system with high activities and purities.

2.7 Measurement of antioxidant activities

The Antioxidant activity normally represents the concentration of antioxidant required to provide a specified rate or extent of reaction. The activity generally corresponds with total phenolic content, as called antioxidant capacity, in fruits and vegetables (Schaich *et al.* 2015). Normally, 2,2′-diphenylpicryl hydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical scavenging activities are usually used to characterize antioxidative peptide.

2.7.1 DPPH radical scavenging activity

DPPH radical scavenging activity is one of the most extensive assays to determine antioxidative activity because of its simplicity and sensitiveness. This method is based on an ability of an antioxidant to reduce DPPH radical. The DPPH radical absorbs UV spectrum at 515-520 nm and reacts with hydrogen or electron

donor compound. In the reaction (Fig. 2.10), the color of the solution is depended on the number of electron paired. The purple chromogen radical is reduced by antioxidant reducing compounds (hydrogen-donating antioxidants), and then becomes pale yellow hydrazine (Musa *et al.* 2016). The IC_{50} value, the concentration of antioxidant that decreases 50% of DPPH absorbance, can be calculated (Bagchi *et al.* 2000).

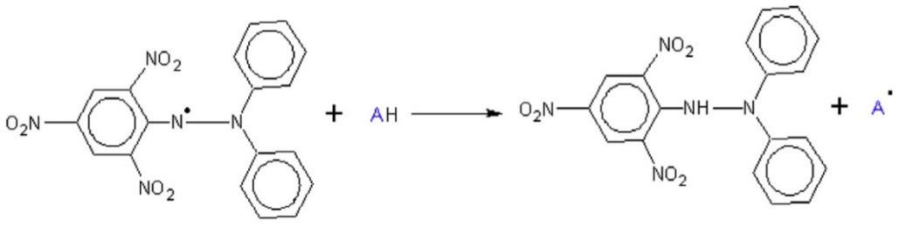

Figure 2.10 The antioxidant activity of DPPH free radical scavenging where AH is donor molecule and $A^{\bullet}$ is free radical produced (Musa *et al.* 2016).

2.7.2 ABTS radical scavenging activity

For the $ABTS^{\bullet+}$ scavenging assay, a blue/green $ABTS^{\bullet+}$ is generated by oxidation of ABTS with potassium persulfate. In the presence of antioxidants, the reduction of this free radical can be determined by the extent of decolorization at a fixed time point (Fig. 2.11) (Zheng *et al.* 2016). The $ABTS^{\bullet+}$ assay can be used to evaluate whether antioxidants are HAT or SET dominant in their reactions, and it can be used to compare changes in the same

antioxidant during processing or storage.

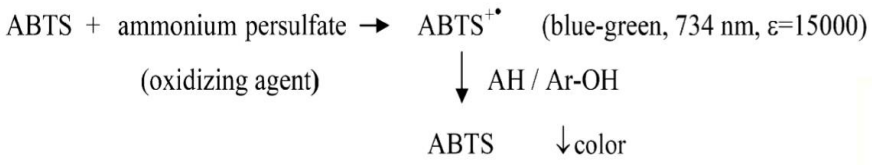

Figure 2.11 The antioxidant activity of ABTS free radical scavenging where AH/Ar-OH is donor molecule (Schaich *et al.* 2015).

2.7.3 Protective effects against hydroxyl radical inducing DNA damage

As mentioned above, DNA damage can lead to several diseases, so the protective effect against DNA damage should be an initial property of antioxidant peptide. Generally, in the assay, *in vitro* DNA damage is induced by hydroxyl radicals ($OH^{\bullet}$) which generated by Fenton reaction (Fig. 2.12). This reaction mimics the *in vivo* generation of free radicals by chemicals such H_2O_2 and transition metal (Chen *et al.* 2013).

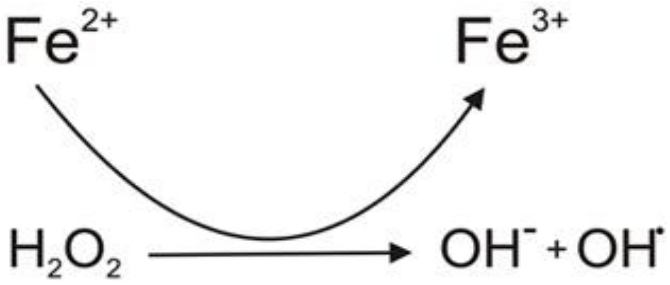

Figure 2.12 The generation of $OH^{\bullet}$ radical by Fenton reaction.

CHAPTER III
METHODOLOGY

3.1 Materials

3.1.1 Chemical and Reagents

Chemical and Reagents	Company and country
100bp DNA Marker	BioExcellence, India
Acrylamide, C_3H_5NO	Invitrogen, USA
Agarose, low EEO	Research Organics, USA
Amersham Low-Range Rainbow Molecular Weight Markers	GE Healthcare, UK
Ammonium persulfate $(NH_4)2S_2O_8$	Bio Basic, USA
Bacto-agar	Himedia, India
Bis-Acrylamide $C_7H_{10}O_2N_2$	Bio Basic, USA
Bovine serum albumin	Sigma Aldrich, USA
Calcium Chloride	Bio Basic, Canada
Coomassie brilliant blue G-250	Merck, USA
Ethidium bromide	BioExcellence, India
Ethyl alcohol, C_2H_5OH	Merck, USA

Chemical and Reagents	Company and country
GangNum STAIN™	iNtRON Biotechnology
Gel Loading dye Purple (6X)	BioLabs, UK
GeneRuler™ 1kb DNA ladder	Fermentas, Canada
Glycerol	Sigma Aldrich, USA
Glycine NH_2CH_2COOH	Ajax, Australia
Hydrochloric acid (HCl)	Merck, USA
Imidazole $C_3H_4N_2$	Bio Basic, USA
Isopropanyl alcohol, C_3H_7OH	BDH, UK
Isopropyl-β-D-thiogalactoside (IPTG), $C_9H_{18}O_5S$	Bio Basic, USA
Manganese II Chloride	Sigma Aldrich, USA
Methanol, CH_3OH	Tedia, USA
3-[N-Morpholino] propanesulfonic acid (MOPS)	Sigma Aldrich, USA
N, N, N´, N´-tetramethylethylenediamine (TEMED)	BDH, UK
Peptone	Bio Basic, USA

Chemical and Reagents	Company and country
Rubidium chloride RbCl	Bio Basic, USA
Sodium chloride, NaCl	Merck, USA
Sodium dodecyl sulfate, SDS $C_{12}H_{25}O_4SNa$	Bio Basic, USA
Sodium phosphate, NaH_2PO_4	Bio Basic, USA
Tris $C_4H_{11}NO_3$	Bio Basic, USA
Urea NH_2CONH_2	Fluka, USA
Yeast extract	Bio Basic, USA

3.1.2 Equipments and supplies

Equipments and supplies	Company and country
- 4 °C Freezer	Sanyo, Japan
-20°C Freezer	Sanyo, Japan
-70°C Freezer	Thermo Fisher Scientific, USA
0.22 µm and 0.45 µm Acrodisc® Syringe Filter	PALL, USA

Equipments and supplies	Company and country
Aquatherm® water bath shaker	New Brunswick Scientific, USA
Autoclave HICLAVE™ HV-50	Hiyamaya, Japan
Automatic micropipette P20, P200, and P1000	PZ HTL S.A., Poland
Biological safety Cabinel	Heal force, China
C-MAG HS7	IKA®, Malaysia
Centrivap Concentrator	LABCONCO, USA
CentriVap Cold Trap	LABCONCO, USA
High Speed Refrigerate centrifuge 6500	KUBOTA, Japan
Hight Speed Micro Refigurated centrifuge MTX-150	TOMY, Japan
Incubator 37°C	Sanyo electric, Japan
Innova 4080 incubator shaker	New Brunswick Scientific, USA
Microcentrifuge Gyrospin	Gyrozen, Korea

Equipments and supplies	Company and country
Microcentrifuge TOMOS	Pacific science, Thailand
Microcentrifuge tube 1.5 ml	LABCON, USA
Mupid-exU submarine electrophoresis	ADVANCE, Canada
Orbital shaker OS-10	Biosan, Latvia
pH-meter	Mettler Toledo, Switzerland
Pipette tips 10, 100 and 1000 µl	LABCON, USA
Power supply, Power PAC Basic	BIORAD, USA
Sonicator	TOMY, Japan
T100^{TM} Thermocycler	BIORAD, USA
Water bath SB-9	EYELA, Japan
Votex geniez	Scientific industries, USA
UV transilluminator	Uvltec, UK
UV plattinum gel documentation	Uvltec, UK

3.1.3 Kits

Kits	Company and country
KOD-Plus-Neo	TOYOBO, Japan
MasterPure™ RNA Purification kit	Epicenter, USA
SpinClean™ Gel Extraction kit	Mbiotech, Korea
SpinClean™ plasmid DNA miniprep	Mbiotech, Korea
SYBR Lo-ROX One-step kit	Mbiotech, Korea
Protino Ni-IDA Packed column	MACHEREY-NAGEL, Germany

3.1.4 Enzymes

Enzymes	Company and country
*Bam*HI	BioLabs, UK
*Hin*dIII	BioLabs, UK
Lysozyme	Bio Basic, USA
T4 ligase	BioLabs, UK

3.1.5 Antibiotics

Antibiotic	Company and country
Ampicillin	TP DRUG Laboratories

3.1.6 Bacterial strains

Strain	plasmids	Reference
E. coli HIT-DH5α	-	RBC Bioscience, Taiwan
E. coli MG1655	-	RBC Bioscience, Taiwan
OSW-S	pIDTBlue-OSW	PIDTBlue-OSW/DH5α
CONT	pQE-30 Xa	pQE-30 Xa/MG1655
a9	pQE -OSW	pQE -OSW/DH5α
OSW	pQE -OSW	pQE -OSW/MG1655

3.1.7 Plasmids

Plasmid	Reference
pQE-30 Xa	Invitrogen, USA
pIDTBlue-OSW	IDT, USA
pQE-OSW	pQE-30 Xa containing DNA fragment encoding OSW peptide

3.1.8 Primers

Primer	Sequence
Ocimum1	5’-GGATCCAAAAGAGACCAAACTTTCC-3'
Ocimum2	5'-AAGCTTCGTACGATCGTTGGTCCAACC-3'

3.2 Methods

3.2.1 Construction of recombinant *E. coli* for production recombinant OSW peptide

3.2.1.1 Maintenance and amplification of pIDTBlue-OSW plasmid

The QTFQYSRGWTN peptide, originally found in hairy basil seed waste hydrolysate (Semanit 2013), was named as OSW. To construct DNA fragment for encoding OSW peptide, DNA fragment containing 7 copies and linked by aspartic acid residues was designed for easier genetic investigation. This fragment was synthesized and inserted in the plasmid pIDTBlue, named as pIDTBlue-OSW (Intergrated DNA Technologies, USA). The plasmid was chemically transformed into *E. coli* HIT-DH5α for maintaining. Chemical transformation was carried out by gently mixing 10 µl of plasmids with 90 µl of *E. coli* competent cells, and then stored on ice for 30 minutes. The tube was immediately heat

in water bath at 42 ^{o}C for 45 seconds without disturbance. Afterwards, the tube was back on ice for 3 minutes without shaking. The 900 µl of LB broth was added into the tube and incubated at 37 ^{o}C for 2 hours in incubation shaker. The cells culture was spread on LB agar medium with 100 µg/ml Ampicillin and then, incubated at 37 ^{o}C overnight.

3.2.1.2 Construction of plasmid for expressing recombinant OSW peptide

The plasmid pIDTBlue-OSW was extracted from the strain OSW-S by using SpinClean^{TM} plasmid DNA miniprep kit (Mbiotech, Korea). The purified plasmids was stored at -20 ^{o}C until use. The 279 base pair synthesized DNA fragment encoding OSW peptide was then purified by Polymerase Chain Reaction (PCR) by using plasmid pIDTBlue-OSW as template with primers named Ocimum1 and Ocimum2. The 50 µl of reaction composed of 5 µl of plasmid, 29 µl of ddIH_2O, 5 µl of 10X PCR Buffer for KOD-Plus-Neo, 3 µl of 25 mM $MgSO_4$, 5 µl of 2 mM dNTPs, 1 µl of forward primer, 1 µl of reverse primer, and 1 µl of KOD-Plus-Neo (*Taq* plomerase). The PCR reaction was carried out as the following: 95 ^{o}C for 5 minutes (pre-denaturation), 95 ^{o}C for 30 seconds (denaturation), 63 ^{o}C for 30 seconds (annealing), 72 ^{o}C for

1 minutes (extension), and 72 ^{o}C for 7 minutes (elongation). PCR product was analyzed by agarose gel electrophoresis. Agarose gel was prepared by dissolving the agarose gel powder in 1X TAE buffer pH 7.6 The DNA was separated by electrophoresis at 100 volts for 30 min in 1X TAE running buffer. The gel was stained with ethidium bromide solution for 15 minutes and de-stained in the water for 15 minutes and the DNA bands were visualized under the UV transilluminator. The expected DNA band 279 bp was extracted follow by the SpinClean^{TM} Gel extraction kit manual (Mbiotech, Korea), and followed by *Bam*HI and *Hin*dIII restriction enzyme digestion. The digested DNA fragment was ligated with pQE-30 Xa, previously digested with the same enzymes, by using T4 ligase enzyme at 16 ^{o}C overnight. The ligation reaction was chemically transformed into competent cells *E. coli* HIT-DH5α as described previously. The ampicillin resistant transformants were selected to culture, and then plasmids were extracted by SpinClean^{TM} plasmid DNA miniprep kit (Mbiotech, Korea), The purified plasmids was verified by *Bam*HI and *Hin*dIII restriction enzymes analysis and DNA sequencing (Macrogen, Korea). The verified plasmid was named as pQE-OSW and *E. coli* HIT-DH5α harboring this plasmid was named as a9 strain.

The purified plasmid pQE-OSW from a9 strain was transformed into *E. coli* MG1655 competent cells as mentioned above. The Ampicillin resistant colonies were also selected for plasmid extraction. The plasmids were verified by *Bam*HI and *Hin*dIII restriction enzyme digestion and DNA sequencing of the target OSW fragment (Macrogen, Korea). The verified recombinant *E. coli* expression strain harboring pQE-OSW plasmid was named as OSW strain.

3.2.1.3 Preparation of *E. coli* MG1655 competent cells

To prepare the competent cells, *E. coli* MG1655 from stock cells was cultured on LB agar medium at 37 ^{o}C overnight. After that, a single colony was selected and inoculated into 2 ml LB broth medium at 37 ^{o}C overnight. Then, 50 µl of the cells were inoculated into 5 ml LB broth medium until early-log phase (about 3 hours, OD_{600} = 0.3). The cell culture was incubated on ice for 20 minutes and centrifuged at 4,000 rpm, 4 ^{o}C for 10 minutes. The supernatant was then discarded. Afterwards, cell pellet was resuspended in 3.3 ml TB I buffer, gently swirled on ice water, and incubated for 2 hours on ice. Next, it was centrifuged at 4,000 rpm, 4 ^{o}C for 10 minutes. Finally, cell pellet was harvested and

resuspended in 1.1 ml TB II buffer. The 90 µl of competent cells was aliquoted into each tube, and stored at -70^{o}C until use.

3.2.2 Recombinant peptide expression and purification

3.2.2.1 Recombinant peptide expression

To prepare the inoculum, a single colony of the OSW and the CONT strains were inoculated into 2 ml LB broth medium with 100 µg/ml of ampicillin at 37 ^{o}C overnight. The 2 µl of inoculum was transferred into 50 ml of LB medium with 100 mg/ml ampicillin, and incubate at 37 ^{o}C until early-log phase, OD_{600} = 0.6-0.8 (about 2 hours). Then, IPTG was added to induce the expression (1mM final concentration). Cells were harvested after incubation for 0, 2, 4, and 6 hours of post induction by centrifugation at 8000xg, 4 ^{o}C for 30 minutes. The cell pellet was resuspended with 5 ml of lysis equilibrium wash (LEW) buffer, pH 8.0 with gently shaking for 5-10 minutes to ensure the completion of cell lysis. Cell lysis was frozen-thawed for 3 times before adding 1 mg/ml final concentration of Lysozyme. After being stirred on ice for 30 minutes, cell was further lysed by sonication (10 times of 15 second bursts at high intensity with a 15 second cooling period between each burst). The inclusion bodies and soluble fraction was separated by centrifugation at 10,000xg, 4 ^{o}C for 30 minutes. The

cell debris was kept for weighing, while supernatant, kept as soluble fraction, was transferred to a new tube. The inclusion bodies were resuspended in 5 ml of LEW buffer for washing and harvested by centrifugation at 10,000xg, 4 ^{o}C for 30 minutes. Then, pellet was resuspended in 3 ml of denaturation solubilization (DS) buffer, pH 8.0 and proteins in this insoluble fraction was then separated by centrifugation at 10,000xg, 4 ^{o}C for 30 minutes, the supernatant was kept as insoluble fraction (Fig. 3.1). All samples were stored at 4 ^{o}C until use.

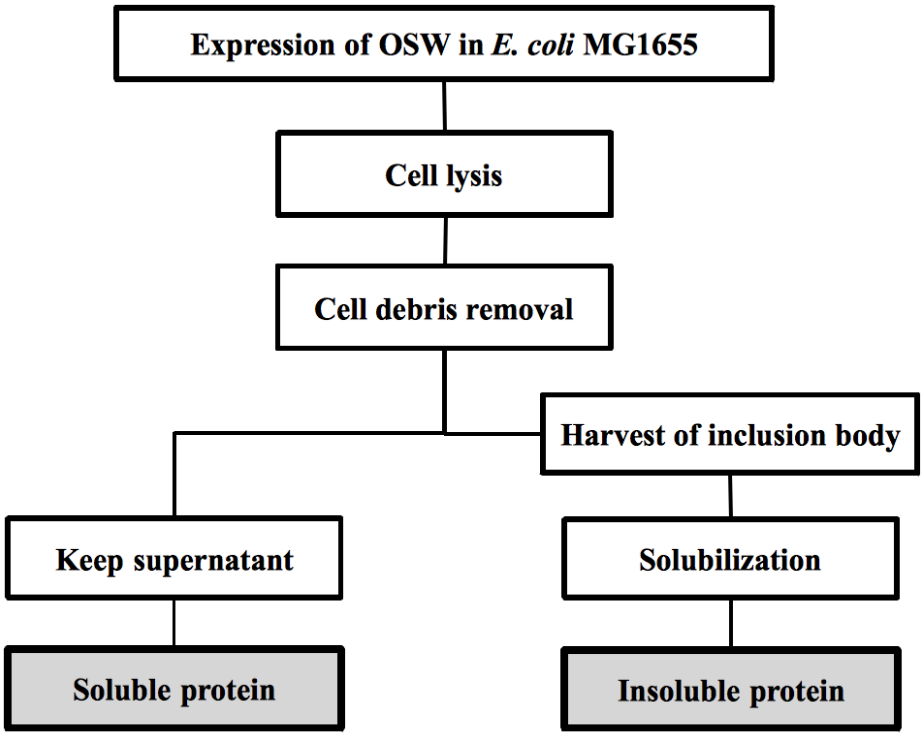

Figure 3.1 Expression of OSW peptide in *E. coli* MG1655

3.2.2.2 Protein purification

Due to the expression of 6xHistidine-tagged fusion from pQE-30 Xa vector, Protino Ni-IDA Packed column (MACHEREY-NAGEL, Germany) was used to purify proteins. The column was equilibrated by washing with 10 column volumes of distill water and 10 column volumes of LEW buffer. The cell lysate was added in to equilibrated column and incubated at 4 ^{o}C overnight. After that, sample was allowed to drain by gravity and kept as Flow through. Then, the column was washed with 10 column volumes of LEW buffer and harvested the fraction as Wash. Finally, polyhistidine-tagged protein was eluted to a new collecting tube for 6 factions with 3 ml elution buffer with 100 mM imidazole, pH 8.0 and collected as Elute. The purification profile of each collected fraction was analyzed by separating proteins on 18% SDS-PAGE.

The target peptide was eluted from SDS gel as the second step of purification to improved purity of eluted protein. The target band 15 kDa, approximately was cut into pieces and was soaked in elution buffer at 4 ^{o}C overnight with gently shaking. After that, eluted protein was analyzed by SDS-PAGE, immunoblotting, and also was characterized antioxidant activities.

3.2.3 Protein analysis

3.2.3.1 Determination of Protein concentration

The protein concentration was determined by UV absorption at 280 nm and 260 nm by Multiskan™ Go spectrophotometer (Thermo Sciencetific, USA) with 15X dilution for proteins from 0 hour culture after induction and 20X dilution for the others.

3.2.3.2 Protein analysis by SDS-PAGE

The gel solutions for 18% separating gel and 5% stacking gel were prepared. First, the separation gel solution was poured into the glass plates with 1 mm spacer and covered on top with distilled water, then gel was placed at room temperature until completely polymerized (about 1 hour). After distilled water was discarded, the stacking gel solution was poured on top of the separating gel and immediately put the comb in the glass plates. After the stacking gel was polymerized, the comb was removed and excess un-polymerized acrylamide was removed by rinsing with distilled water. The samples were mixed with Next Gel® Sample Loading buffer, 4X (AMRESCO, USA) and then, was boiled for 10 minutes before loading. Electrophoresis was run in 1X SDS running buffer with a constant current of 25 mA per gel until the dye front reaching the end of the gel. Afterwards, the gel

was stained with the Coomassie brilliant blue R250 staining solution at room temperature with gently shaking for an hour. Finally, the gel was destained by the destaining solution and shaken at room temperature.

3.2.3.3 Detection of recombinant peptide expression at transcription level by quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extract according to the MasterPure^{TM} RNA Purification Kit manual (Epicenter, USA). The purified total mRNA was used as the template for qRT-PCR (LightCycler 480 Real-Time PCR with SYBR Lo-ROX one-step kit) to detect 279 base pair DNA fragment encoding OSW peptide by using forward primer Ocimum1 and reverse primer Ocimum2. The total 20 µl of each qRT-PCR reaction was composed of 1 µg of purified total mRNA, 0.5 µM of primer Ocimum1 and Ocimum2, 10 µl of 2X Mbiotech SYBR Lo-ROX one-step mix, 0.2 µl of reverse transcriptase, 0.4 µl of RNase inhibitor, and DEPC-H_2O. The temperature of PCR reaction was carried out as the following: 45 ^{o}C for 10 minutes (reverse transcription) and 95 ^{o}C for 2 minutes (pre-denaturation and polymerase activation). Then, the amplification was followed by 40 cycles of 95 ^{o}C for 15 seconds

(denaturation), 60 ^{o}C for 15 seconds (annealing), and 72 ^{o}C for 20 minutes (extension). The fluorescent absorbance was detected in each cycle. The CP (crossing point) value of each sample was calculated.

3.2.3.4 Analysis of recombinant protein by modified dot blot analysis

The modified dot blot analysis analysis was used for identifying the target Histidine-tagged peptide adapted from G-Biosciences guidebook. The nitrocellulose membrane was striped and labeled. The 1 µl of 0.3 mg/ml of eluted target peptide, Histidine-tagged protein, bovine serum albumin (BSA), and ddIH_2O were spotted onto the center of each strips. After being absorbed (approximately 2 hours), 5% skim milk solution was used as blocking buffer and incubated at room temperature for an hour with gently shaking. The spotted membrane was washed by PBST buffer for 5 minutes (5 times) and antiHis-HRP was applied. Then, the system was incubated at room temperature for 2 hours. The membrane was washed again by PBST buffer for 5 minutes (5 times). Then, the color was developed by gently shaking with incubation of DAB solution for 5 minutes. Finally, the substrate was discarded and ddIH_2O was added to stop the color reaction.

3.2.4 Characterization of antioxidant activity

3.2.4.1 DPPH radical scavenging activity

The DPPH radical scavenging activity was applied from Mohsen (Mohsen and Ammar 2009), the recombinant peptide at 10 different concentrations (1-0.0020 mg/ml) were mixed with 0.1 M DPPH in ethanol for 30 minutes in the dark. The absorbance at 517 nm was measured by using Multiskan^{TM} Go spectrophotometer (Thermo Sciencetific, USA). The IC_{50} value was determined from linear regression of the DPPH inhibition against the concentration of proteins. Water was used as the negative control and ascorbic acid was used as a standard.

3.2.4.2 ABTS scavenging activity

The ABTS scavenging activity, the method was adapted from Cai (Cai *et al.* 2004). 6 mM of sulphonic acid-6-ethylbenzthiazoline-3(bis-azino-2' ,2) (ABTS) and 0.245 mM potassium persulfate was mixed and incubated in the dark for 16 hours. The solution was diluted with 80% ethanol or pure water until the absorbance at 734 nm was exactly 0.7 ± 0.02. Then, 990 µl of ABTS working solution was mixed with 10 µl each sample (10 dilutions of each sample was tested) and placed at room temperature for 10 minutes. Ascorbic acid was used as a positive

control as the same condition. The absorbance at 734 nm was measured by Multiskan™ Go spectrophotometer (Thermo Sciencetific, USA).

3.2.4.5 *In vitro* protective effect of the purified recombinant peptide against hydroxyl radical inducing DNA damage

The *In vitro* protective effect of target peptide on DNA damage from hydroxyl radical induction was modified from Qian's method (Qian *et al.* 2008). The pBR 322 plasmid DNA was used as DNA template. The reaction to induce DNA damage by hydroxyl radical was based on Fenton reaction. The mixture of 3 µl of plasmid DNA (50 -100 ng/ml), 3 µl of 50 mM phosphate buffer (pH 7.4), and 4 µl of purified peptide at 4 different concentrations were mixed thoroughly and incubated for 20 minutes at room temperature. Next, the 3 µl of 2 mM $FeSO_4$, and 30% H_2O_2 were added and incubated at 37 ^oC for 30 minutes. The DNA forms were analyzed by 1% agarose gel electrophoresis.

CHAPTER IV
RESULT AND DISSCUSION

4.1 Construction of recombinant *E. coli* strain for production of recombinant OSW peptide

Due to the small size of OSW peptide (11 amino acids as QTFQYSRGWTN, it is difficult for recombinant DNA technology to be applied. Therefore, the DNA fragment for expressing recombinant OSW peptide was designed to contain nucleotide sequence encoding 7 copies of OSW peptide and each copy being linked by aspartic acid codons (Fig. 4.1). With this strategy, DNA fragment was long enough for the technique of recombinant DNA technology. Moreover, recombinant peptide consisting of the 7 copies was expected to provide higher antioxidant activity than the single one copy of the native one. This DNA fragment was synthesized and inserted in the plasmid (Integrated DNA technologies, IDT, USA), named as pIDTBlue-OSW (Fig.4.2) with ampicillin resistance gene served as a selective marker. Codon optimization was also analyzed to avoid codon usage problem that could lead to fault expression of heterologous protein, particularly, the rare arginine (AGG, AGA, CGG, and CGA), isoleucine (AUA), leucine (CUA), and proline codons (CCC) (Wu *et al.* 2004) (Seo *et*

al. 2012). Thus, the sequence for translated protein was designed to contain 2.5% of rare codons in *E. coli*. The plasmid with synthesized DNA fragment was initially maintained in *E. coli* strain called OSW-S by transformation procedure. After that, the DNA fragment for expressing recombinant OSW peptide was amplified by PCR with use of the pIDTBlue-OSW plasmid as the template (Fig. 4.3). The terminal sites of the PCR products could be modified by the specific primers during PCR process (Li *et al.* 2013). In this case, the terminal ends was change from *Xho*I restriction sites to *Bam*HI and *Hin*dIII restriction sites at 5' terminal and 3' terminal, respectively. Thus, after ligation step, this DNA fragment was expected to translate with correct open reading frame.

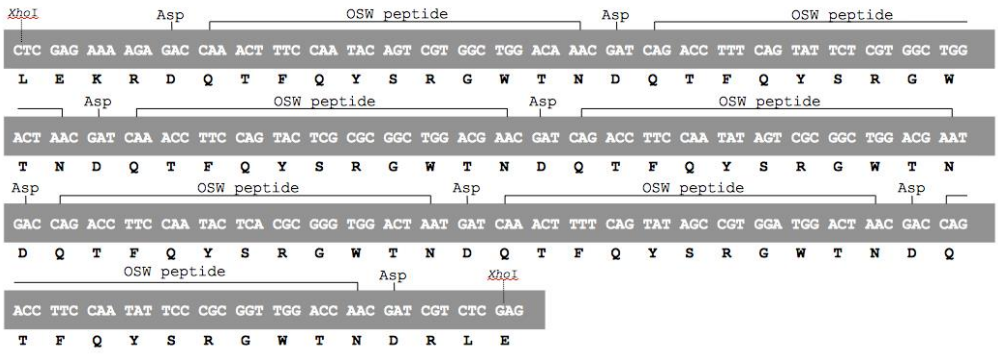

Figure 4.1 DNA sequence of synthesized DNA fragment for expressing recombinant OSW peptide.

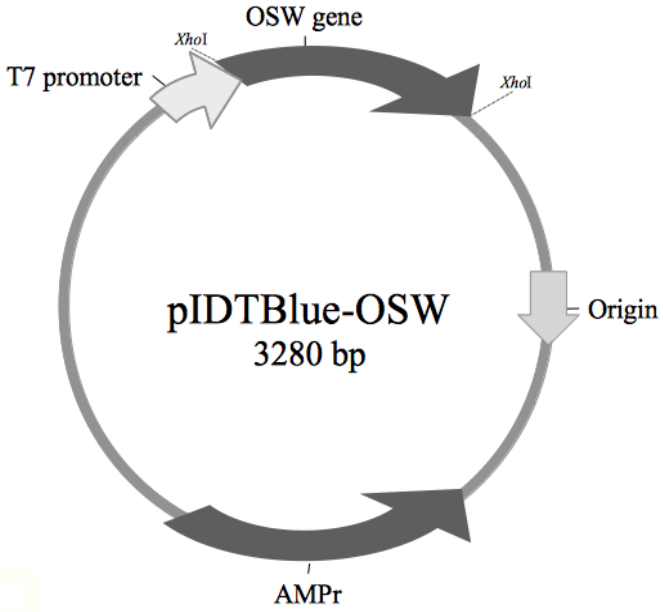

Figure 4.2 The plasmid pIDTBlue-OSW encoding recombinant OSW peptide with 7 repetitive sequences copies and ampicillin resistance gene.

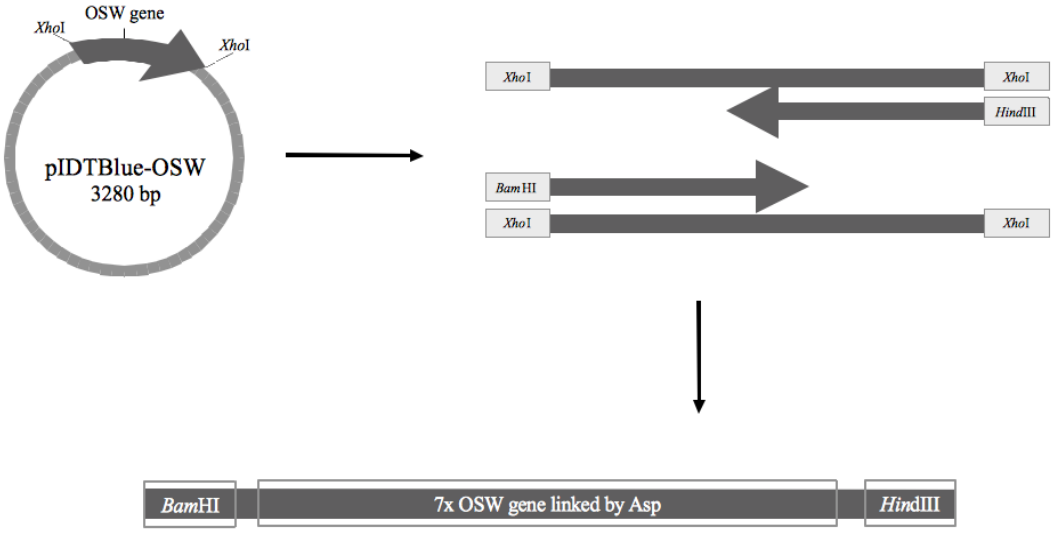

Figure 4.3 The introduction of *Bam*HI and *Hin*dIII restriction sites into the DNA fragment through PCR.

The PCR product was analyzed by 1% agarose gel electrophoresis with the expected size of 279 bp (Fig. 4.4).

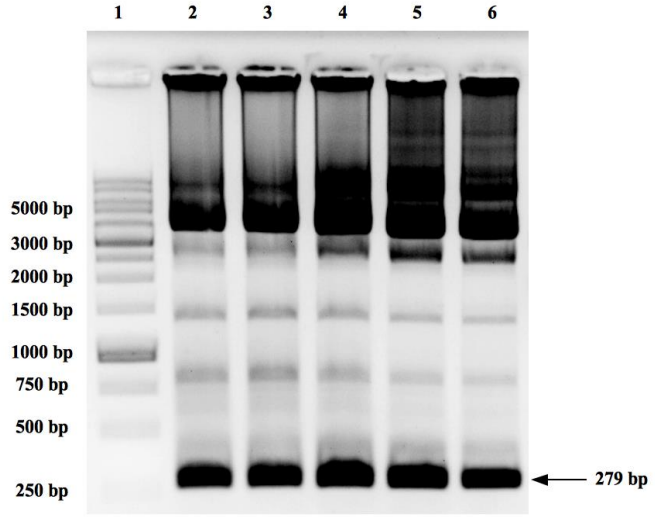

Figure 4.4 1% agarose gel electrophoresis analysis of PCR products. Lane 1: 1 kb DNA ladder. Lane 2-6: PCR products with expected 279 bp DNA band of the DNA fragment for expressing recombinant OSW peptide.

The expected DNA band was then extracted from agarose gel. The DNA fragment and expression vector, pQE-30 Xa (Fig. 4.5), were further digested with *Bam*HI and *Hin*dIII restriction enzymes. The expected 273 bp of OSW fragment and 3479 bp of pQE-30 Xa vector were revealed on 1.5% agarose gel electrophoresis, as shown in Figure 4.6. Then, these two bands were eluted from the gel and applied for ligation reaction.

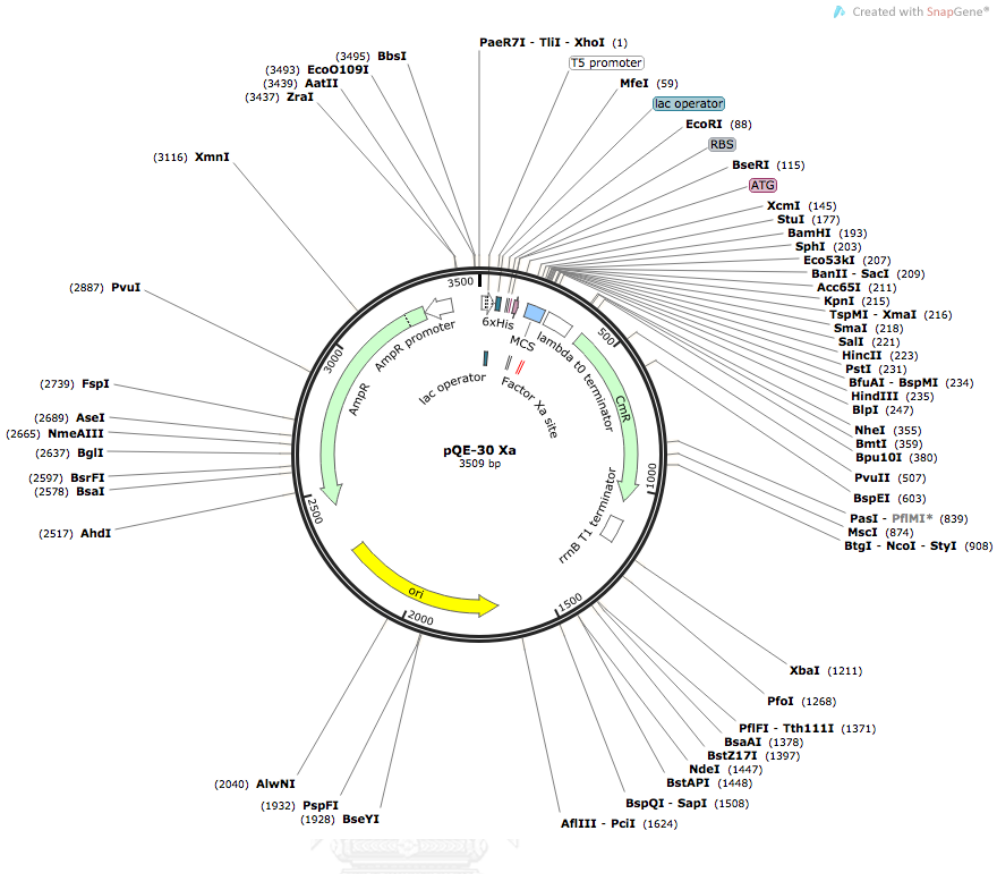

Figure 4.5 Map of plasmid pQE-30 Xa consisting of target BamHI and HindIII restriction sites [http://www.snapgene.com].

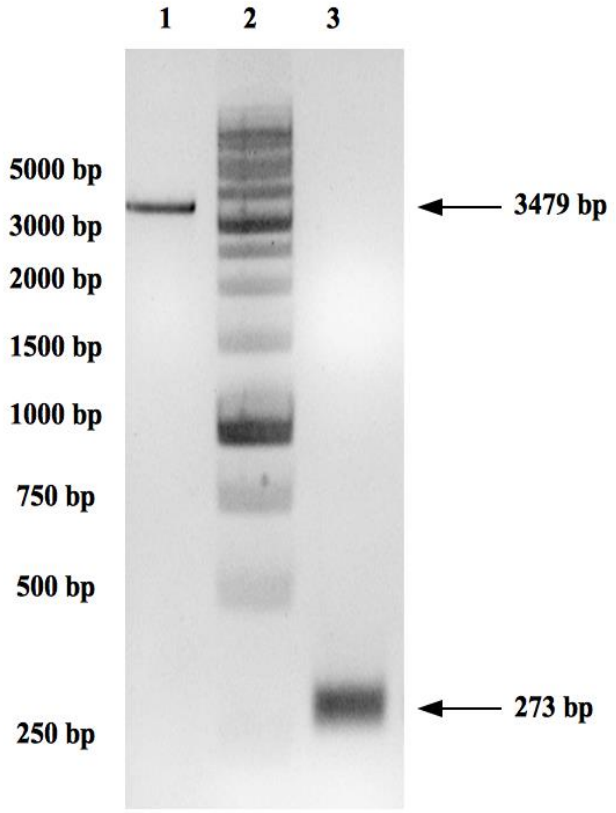

Figure 4.6 Agarose gel electrophoresis analysis of expression vector and gene fragment after being digested by *Bam*HI and *Hin*dIII. Lane 1: the expected 3479 bp of pQE-30 Xa, Lane 2: 1Kb DNA ladder, Lane 3: the expected 273 bp of target DNA fragment for expressing recombinant OSW peptide.

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After transforming ligation reaction to the *E. coli* HIT-DH5α, the ampicillin resistant clones were picked up, and extracted plasmids were checked by *Bam*HI and *Hin*dIII double digestion (Fig. 4.7). It was found that clone a9 contained the possible target DNA fragment. The recombinant plasmid was named pQE-OSW.

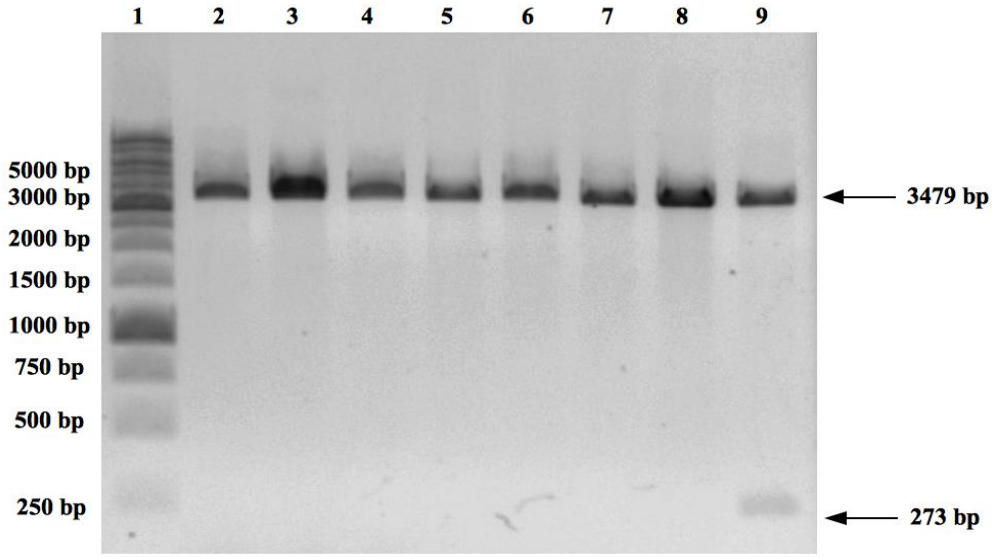

Figure 4.7 Agarose gel electrophoresis analysis of recombinant plasmids from the transformed clones after being digested by *Bam*HI and *Hin*dIII restriction enzymes. Lane 1: 1 kb DNA ladder, and Lane 2-9: selected clones a2-a9, orderly.

The pQE-OSW plasmid was then transformed into *E. coli* MG1655. The ampicillin resistant colony was also verified by *Bam*HI and *Hin*dIII restriction enzyme digestion. The 3479 bp and the 273 bp of DNA fragments were found as expected (Fig. 4.8). The verified recombinant plasmid pQE-OSW from *E. coli* MG1655 was also confirmed by DNA sequencing (Macrogen, Korea) (Fig 4.9). This MG1655 derivative strain was named as OSW strain. The gene expression is under the control of *lac* operator which is

induced by IPTG. Moreover, the 6xHis-tagged is fused to the target recombinant protein at its N-terminal which benefits for

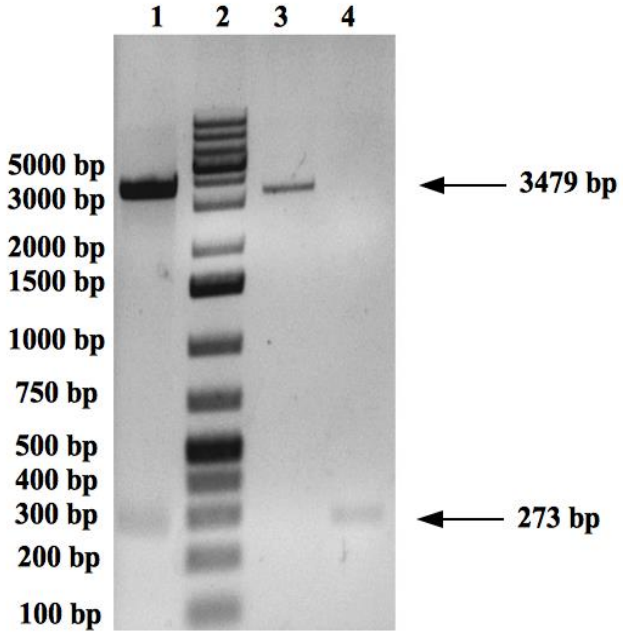

Figure 4.8 Agarose gel electrophoresis analysis of recombinant plasmids from the transformed clones after being digested by *Bam*HI and *Hin*dIII restriction enzymes. Lane 1: recombinant plasmid pQE-OSW extracted from recombinant OSW clone, Lane 2: 100 bp DNA ladder, Lane 3: the 3479 bp of pQE-30 Xa, and Lane 4: the 273 bp of DNA fragment for expressing recombinant OSW peptide.

purification purpose, as same as the successfully constructed recombinant defensin gene (Li *et al.* 2013).

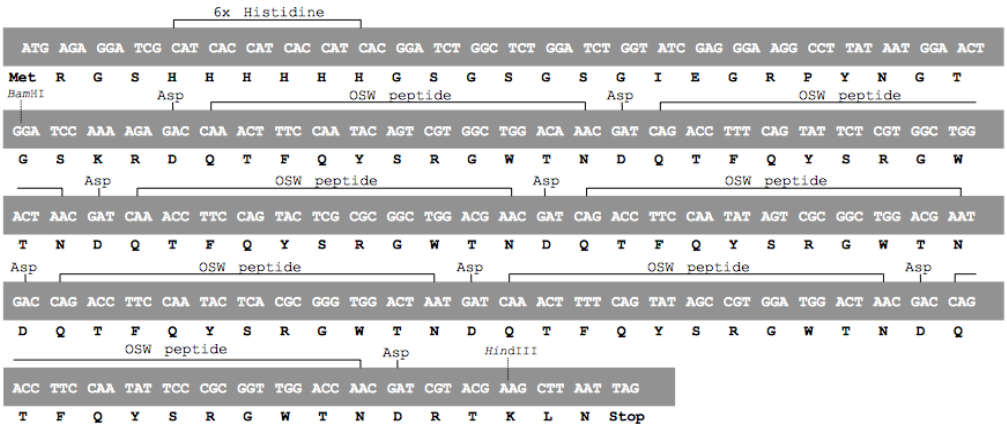

Figure 4.9 DNA sequence of target antioxidative OSW peptide.

4.2 Recombinant OSW peptide expression and purification

The cell culture of OSW strain was incubated at 37 ^{o}C, 200 rpm until the OD_{600} reaching 0.6-0.8 (approximately 2 hours). The cell culture was harvested every 2 hours for 8 hours after being induced by IPTG. Growth profile after induction, OD_{600} and pH, was recorded. The growth rate of recombinant strain rose dramatically until up to 2 hours of incubation, continuously increased and then dropped slowly after 6 hours. The trend of the CONT strain was similar to the OSW strain, but the rate was less than OSW strain as represented in Figure 4.10. While pH values of both strains were similar, being increased steeply at the first 2 hours, and then slightly increased until 8 hours of IPTG induction.

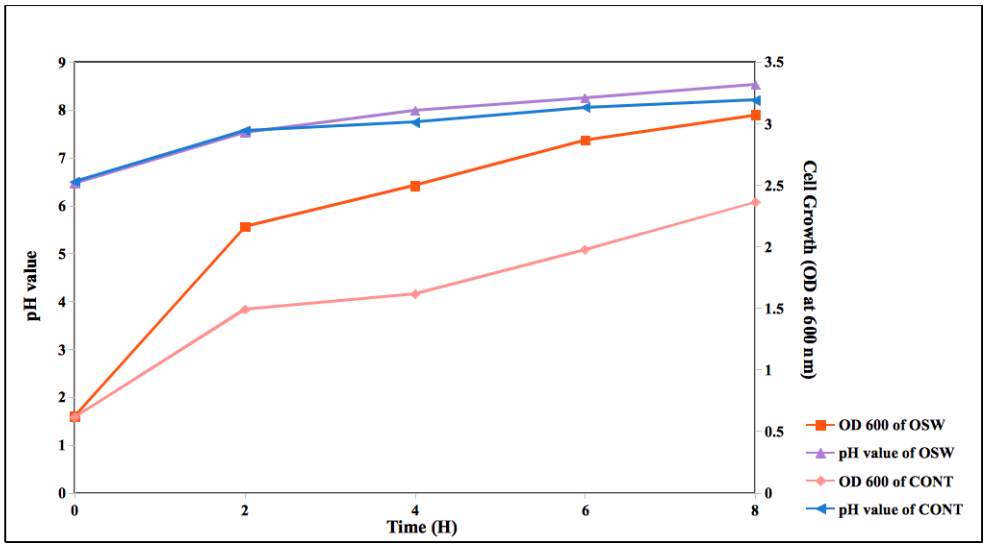

Figure 4.10 The pH values, the absorbance at 600 nm at different time points (H) of OSW and CONT strains.

These results were corresponded to the analysis of *E. coli* growth on LB medium (Baev *et al.* 2006). At the first state (0-2 hours), nutrients was available for all cells to grow. Then, the growth rate was increased rapidly in the second state (2-4 hours) until reaching the third state (4-5 hours) which nutrients became limited. The fourth state (5-7 hours) was important for the expression because expression level of catabolic pathways was mostly reached their highest level. The last state (7-8 hours) was so called stationary phase

Thus, the cell culture at 4 hours after IPTG induction (or total 6 hour culture) was selected for further harvesting the recombinant peptide which was suggested in the study of the time-

course in expression of recombinant human 6PGD in *E. coli* (Chan and Sukhatme 2013). The harvested sample was separated into 2 fractions as soluble protein and insoluble protein (Fig.3.1). The total protein amount from each fraction was represented in Figure 4.11. The expected molecular weight and pI value of the recombinant OSW peptide were 15.05 kDa and 9.39, respectively. The peptide should be expressed with 6xHis-tagged protein at N-terminal, so was subsequently purified by Protino Ni-IDA Packed column, a Ni^{2+} affinity column, using 100 mM imidazole in LEW buffer to elute 6xHis-tagged target proteins. The eluted protein was collected into 500 µl for 6 fractions. Most of eluted protein was found in the soluble protein fraction; however, the last 3 elution fractions seemed to contain no protein (Fig. 4.14). To gain the maximum binding capacity, purification was also further optimized by adjusting the amount of total protein loading as manual suggestion at 5 mg protein. It was found that 1.5 ml of total protein loading (~ 4.67 mg protein) yielded the best result to reduce another unspecific binding protein (Fig. 4.15). However, the elution still contained other protein contamination, especially at higher molecular weight. Therefore, more purification step was necessary. However, the limitation of adding more purification

steps was the loss of some protein, particularly protein expressed in low level. The direct extraction of target protein band and then, elution by diffusion was chosen to additionally purify target recombinant peptide for further work. However, low recovery was found. Moreover, this technique is suitable for purify protein for small scale requirement. The other purification procedures are required for the larger scale production.

4.3 Protein analysis

4.3.1 Determination of Protein concentration

The total protein concentration of each sample was determined by UV absorbance at 260 and 280 nm. The soluble and insoluble protein fractions from the OSW strain were increased gradually from 0 to 6 hours after induction (Figure 4.11) Moreover, the total protein concentrations from the OSW strain seemed to be higher than those of the CONT strain in most fractions. The increase in proteins concentration was corresponded to the cell growth which is increased continuously from 2 to 4 hours after induction. As previously mentioned, the expression after 4 hour induction was harvested for further analysis. Ater affinity column purification, 1.03 mg protein from 50 ml culture (approximately

0.2 g wet cell pellet), or 0.34 mg/ml was obtained (Table 4.1). This is accounted for 6.56% of the total soluble proteins.

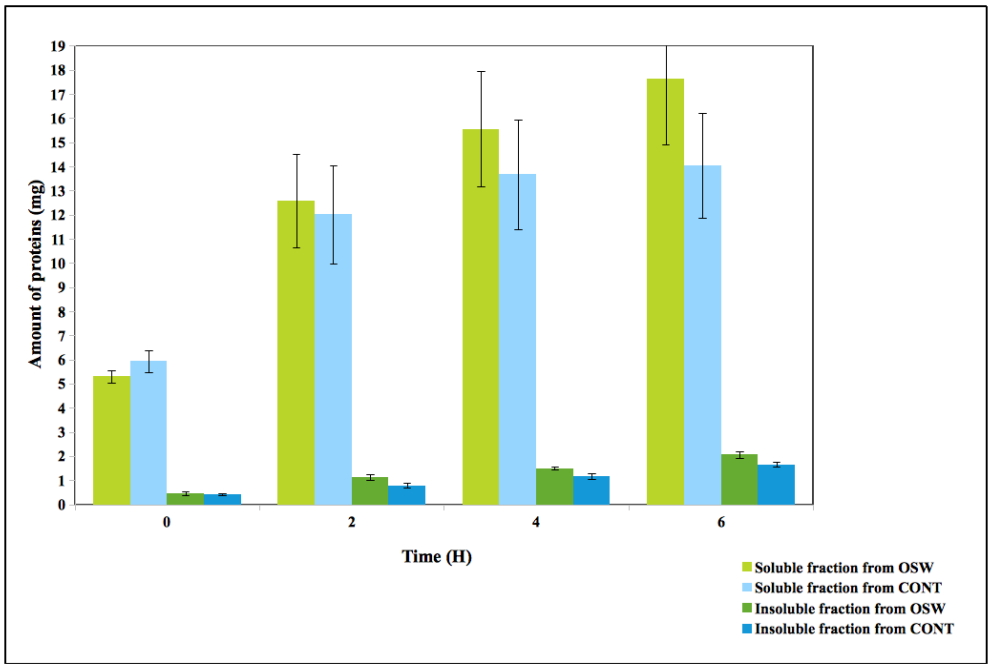

Figure 4.11 Total protein amount of each fraction from the OSW and the CONT strains (mg) at different time points after induction (H).

Table 4.1 Total protein amount of each fraction from 50 ml culture of the OSW strain.

Fraction	Proteins from OSW strain (mg)			
	0 hour	2 hours	4 hours	6 hours
Soluble protein	5.30 ± 0.26	12.57 ± 1.93	15.56 ± 2.39	17.63 ± 2.73
Insoluble protein	0.46 ± 0.08	1.13 ± 0.11	1.50 ± 0.07	2.06 ± 0.14
Purified protein	ND*	ND*	1.03 ± 0.08	ND*

* Not determined

However, it was suggested the low expression profile of the recombinant OSW peptide compared with other researches such as the 1.5 – 4 mg of recombinant human 6PGD expressed in soluble fraction in *E. coli* with the same culture volume (Chan and Sukhatme 2013). To gain more OSW recombinant peptide, there are many parameters needed to be optimized. It was reported that the optimization of the recombinant Tat-HA-NR2b9c peptide production could obtain 86 mg of the target peptide from with 6.5 g of wet cell pellet (Zhou *et al*. 2012). In addition, the expression could be improved by adding the sequence of peptide degradation inhibitor to prevent the low expressed products from the disruption of soluble protein during cell lysis (Wu *et al*. 2013).

4.3.2 Protein analysis by SDS-PAGE

SDS-PAGE analysis of total protein from the OSW strain was used to investigate the expected 15 kDa band. Insoluble protein fraction seemed not to contain the expected band (Fig.4.12A and B), while it was presented in the soluble protein fraction (Fig.4.13). Although it was suggested that the recombinant OSW peptide may in the soluble protein in cytoplasm, the expression level was not as high as expected. So, the target peptide was not found in the insoluble fraction. Ideally, recombinant

proteins expressed in *E. coli* can remain soluble in the cytoplasm and can be purified directly from supernatant of cell lysis; however, 70% of highly expressed recombinant proteins were accumulated in the inclusion bodies (Wu *et al.* 2013). *E. coli* was revealed the ability of high expression level, moreover, causes of heterologous protein expression trouble, such as codon usage, toxicity, mRNA stability, protein stability, was reported (Kane 1995).

However, after Ni-IDA column purification, the elution fraction still contained other higher molecular weight proteins (Fig 4.14 and Figure 4.15). Therefore, the additional purification was performed by extracting the expected 15 kDa bands from the SDS gel, then diffusing from the gel (Fig. 4.16).

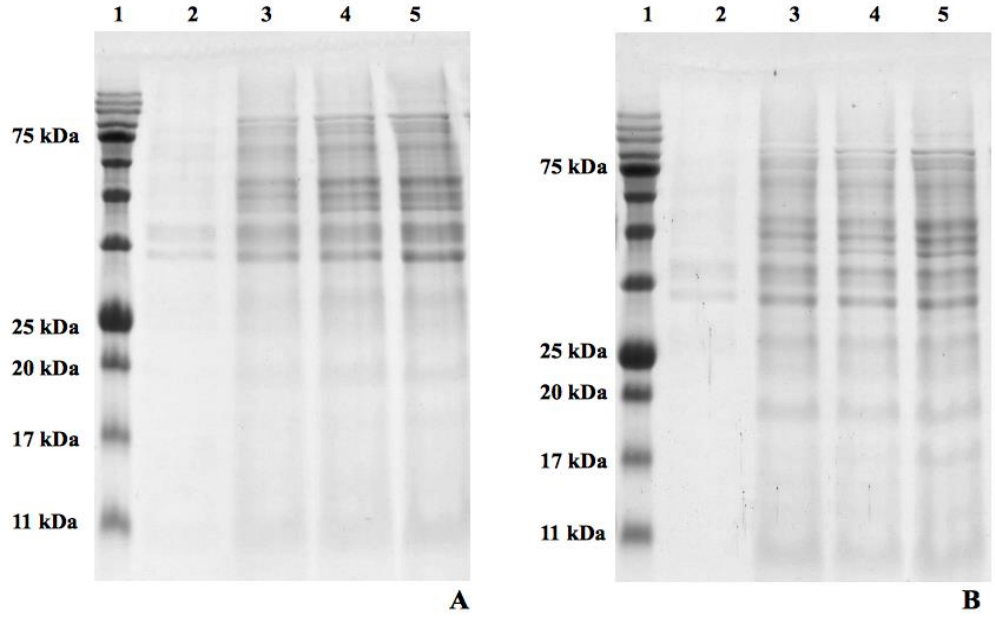

Figure 4.12 The SDS-PAGE analysis of the total insoluble protein fraction after IPTG induction from the OSW (A) and CONT (B) strains. The gel was stained by Coomassie blue after electrophoresis. (A) Lane 1: protein marker. Lane 2-5: the insoluble fractions from the OSW strain at 0, 2, 4, and 6 hours after IPTG induction, respectively. (B) Lane 1: protein marker. Lane 2-5: the insoluble proteins from the CONT strain at 0, 2, 4, and 6 hours after IPTG induction, respectively.

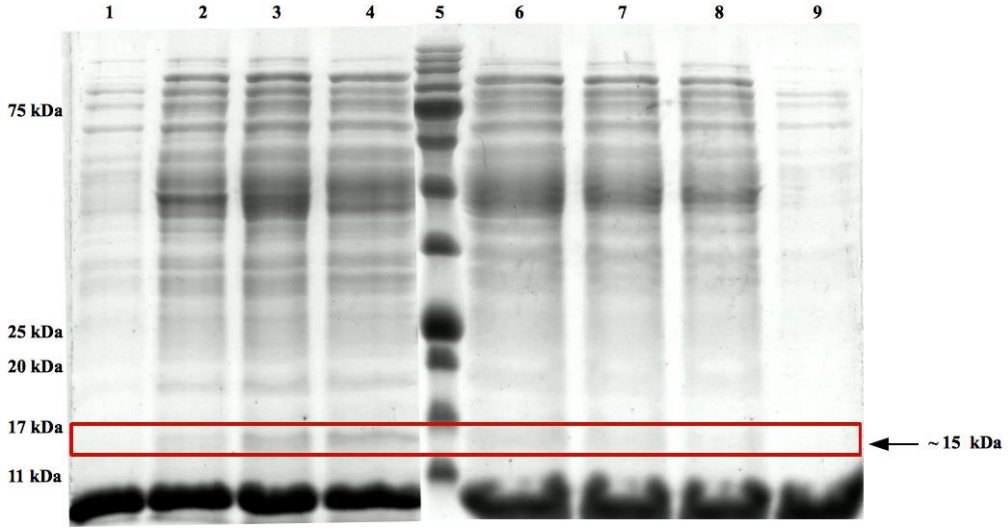

Figure 4.13 The SDS-PAGE analysis of the total soluble protein from the OSW and CONT strains. Lane 1-4: proteins from the OSW strain at 0, 2, 4, and 6 hours after IPTG induction, respectively. Lane 5: protein marker. Lane 6-9: proteins from the CONT strain at 6, 4, 2, and 0 hours after IPTG induction, respectively.

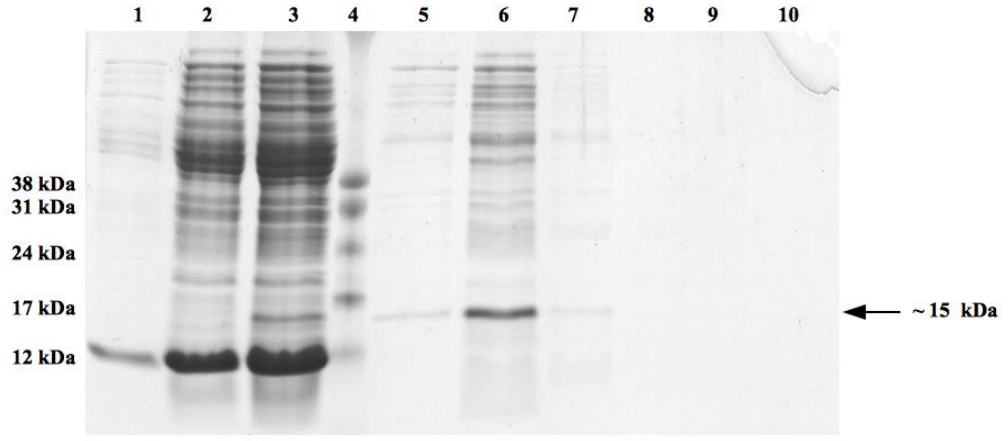

Figure 4.14 The SDS-PAGE analysis of each fraction from the OSW strain using Ni-IDA column purification. Lane 1: Wash fraction, Lane 2: Flow through fraction, Lane 3: Total protein loading, Lane 4: Protein marker, and Lane 5-10: Elute fraction.

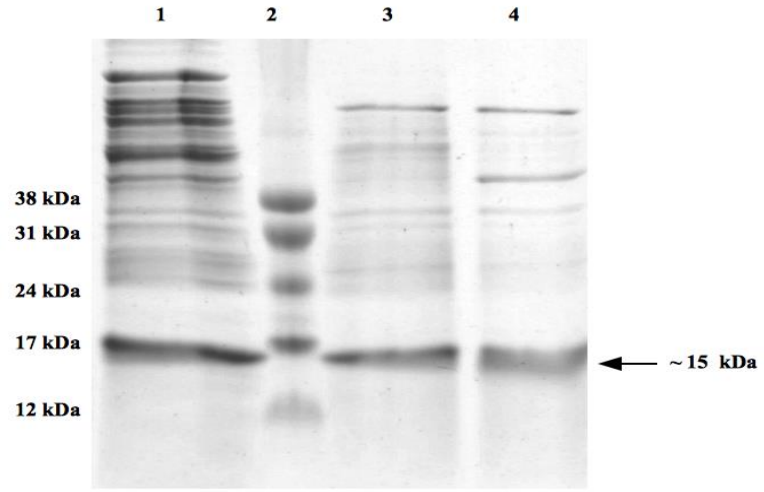

Figure 4.15 The SDS-PAGE analysis of eluted proteins with different protein loadings. Lane 1: Elute fraction with 3 ml of protein loading, Lane 2: Protein marker, Lane 3: Elute fraction with 2 ml of protein loading, and Lane 3: Elute fraction with 2 ml of protein loading.

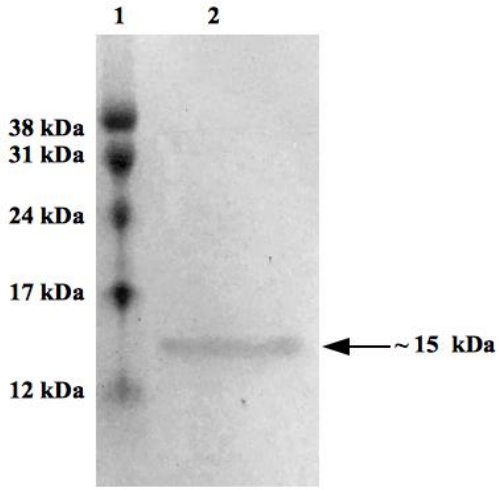

Figure 4.16 The recovery of the purified target 15 kDa from gel diffusion on 18% SDS-PAGE.

4.3.3 Detection of recombinant OSW peptide expression at transcription level by quantitative reverse transcription PCR (qRT-PCR)

Because the recombinant peptide expression level from the OSW strain was not as high as, qRT-PCR (LightCycler® 480 software) was used to detect the expression of target protein at the transcriptional level. The qRT-PCR can both quantify and qualify the mRNA in the sample. The Crossing point value, the cycle number when the reaction crosses the threshold of light emission above background, is the key number to determine the expression level (Corthell 2014). Table 4.2 shown crossing point value (CP), the OSW strain was 17.10 ± 0.22 while that of CONT strain could not be detected. This result indicated that mRNA for the target OSW peptide was transcribed. On the other hand, the negative control, the CONT strain, did not contain mRNA for the target OSW peptide expression.

Table 4.2 Crossing point value from qRT-PCR of the mRNA for the recombinant OSW peptide expression.

Sample	CP
OSW	17.10 ± 0.22
CONT	ND*

* Not determined

4.3.4 Detection of the recombinant OSW protein by modified dot blot analysis

As mentioned above, the elution from the Ni^{2+} affinity column still contained the others proteins. Previously, the Western blot analysis was applied to verify this recombinant peptide. However, there were several problems found during protein transfer. The small peptide was bound poorly to the transfer membrane with semi-dry rapid blotting system and it also was possibly damage from heat of system, therefore, the modified Dot blot analysis was used. First, the expected 15 kDa band was extracted from the SDS-PAGE gel, and then protein diffusion was carried out. The recovery of this peptide band was revealed, as in the Figure 4.16. The purified peptide was directly dotted onto the nitrocellulose membrane. To verify the recombinant OSW peptide, the antiHis-HRP was used by detecting the 6xHistidine-tagged fusion at the N-terminal of the peptide by immunoblotting. The result revealed that the recombinant His-tagged OSW peptide could be detected by this method (Fig. 4.17). A His-tagged protein was used as the positive control, ddIwater and BSA as the negative control.

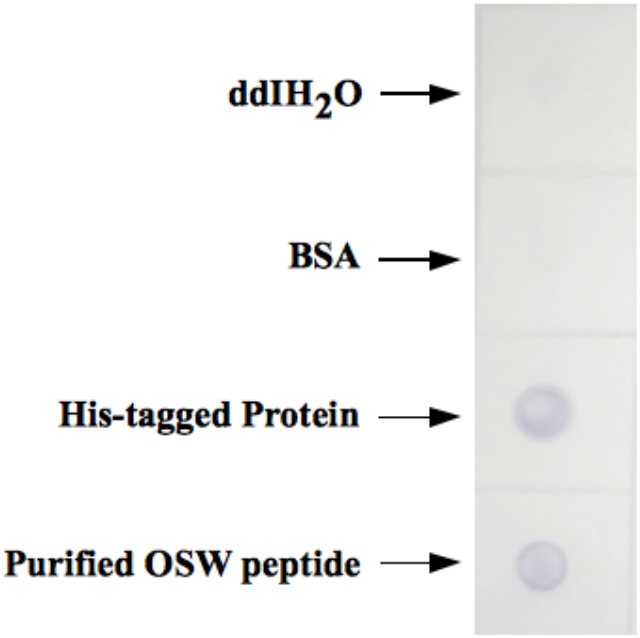

Figure 4.17 Detection of the recombinant OSW peptide by modified dot blot analysis with the antiHis-HRP.

4.4 Characterization of antioxidant activities

According to the previous research, antioxidant activities of protein hydrolysates from hairy basil seed waste with less than 5 kDa revealed satisfactory DPPH and ABTS radical scavenging activities, as shown in Table 2.1 (Semanit 2013). So, these 2 activities, along with the *in vitro* protective effect to against hydroxyl radical induced DNA damage, were selected to characterize the antioxidant activities in this research.

In the previous study of Semanit in 2013, there were several peptides found in the hydrolysate including the peptide with the

sequence of QTFQYSRGWTN (Table 2.2). However, the chemical synthesized OSW peptide and OSW peptide elution revealed the lower DPPH radical scavenging activity than the previous study (IC_{50} 25.23 ± 0.1437 μg/ml) Moreover, the 50% inhibition value (IC_{50}) from the chemical synthesized and the OSW peptide elution could not be calculated because the % inhibition was lower than 50%. The % DPPH radical inhibitions from the 1 mg/ml of chemical synthesized and OSW peptide elution were 4.70 ± 1.84 and 17.19 ± 1.88, respectively (Table 4.3). However, for the DPPH scavenging activity, the % inhibition of the OSW peptide elution was significant higher than that of the chemical synthesized one (p < 0.05) determined by SPSS independent-samples T Test.

Table 4.3 The DPPH scavenging activity of the tested peptides at concentration of 1 mg/ml.

Sample (concentration of 1 mg/ml)	**% inhibition of DPPH radicals**
Ascorbic acid	96.90 ± 0.24
Chemical synthesized OSW peptide	4.70^{a} ± 1.84
OSW peptide elution	17.19^{b} ± 1.88

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

The scavenging activity to ABTS radicals was also observed, the IC_{50} value of the chemical synthesized OSW peptide could not be calculated. However, at 1 mg/ml of chemical synthesized OSW peptide concentration, 28.59 ± 0.58 % inhibition was revealed. The IC_{50} value of the OSW peptide elution was 801.37 ± 53.24 µg/ml (Table 4.4), which suggested the more ability to scavenge ABTS radicals than that of the chemical synthesized peptide. However, the activity was still lower than of the hydrolysate from the previous study (Semanit 2013).

Table 4.4 The ABTS scavenging activity of the tested peptides.

Sample	IC_{50} of ABTS radicals (µg/ml)
Ascorbic acid	43.44 ± 1.49
Chemical synthesized OSW peptide	ND **
OSW peptide elution	801.37 ± 53.24
Hydrolysate *	44.50 ± 2.00

* The less than 5 kDa hydrolysate of hairy basil waste studied by Samanit, 2013.
** Not determined due to the lower than 50% inhibition at the 1 mg/ml of the peptide

Another antioxidative activity, determined by *in vitro* protective effect on DNA damage induced by hydroxyl radical, was tested. The damage was based on Fenton reaction with 0.2 M $FeSO_4$ and 30% H_2O_2. Most plasmid DNA is in form of supercoil DNA, which can migrated in the agarose gel electrophoresis faster than other forms. When it was damaged, plasmid could change to open circular and linear DNA forms, migrating slower that the supercoil form, and could be visualized by agarose electrophoresis gel (Qian *et al.* 2008) (Sheih *et al.* 2009). The study was indicated that, without antioxidative peptides, pBR 322 plasmid, as the DNA template, was damaged by hydroxyl radicals. Damage could also degrade plasmid DNA as seen in some experiments. Conversely, with chemical synthesized and OSW peptide elution, DNA was protected from the hydroxyl radical damage (Fig 4.18 and 4.19). The protective effect was found from the chemical synthesized OSW peptide at concentration of 5-10 mg/ml. Likewise, in this research, the OSW peptide elution exhibited the best suppression of DNA damage against hydroxyl radicals at the concentration of 42.5 – 85 μg/ml. It was demonstrated that the OSW peptide elution had ability to protect hydroxyl radical inducing DNA damage at the lower concentration, suggesting the more effectiveness, than the

synthesized peptide. DNA is a major sensitive biomolecule being a target of oxidative damage (Martinez 2003). This OSW peptide elution has a similar ability to protect DNA from oxidative damage to the peptide derived from oyster (Qian *et al.* 2008).

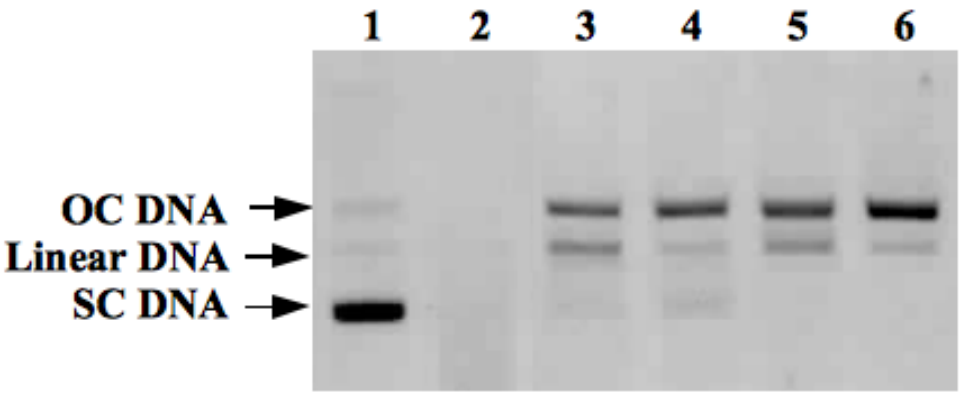

Figure 4.18 The *in vitro* protective effect of chemical synthesized OSW peptide on DNA damage induced by hydroxyl radicals. OC is open circular DNA form and SC is supercoil DNA form. Lane 1: pBR 322 plasmid DNA with hydroxyl radicals, served as a DNA damage control, Lane 2: empty pBR 322 plasmid DNA without DNA damage, Lanes 3–6: hydroxyl radicals inducing plasmid DNA damage treated with the chemical synthesized OSW peptide at the concentrations of 10, 5, 2.5, 1.25 mg/ml, respectively.

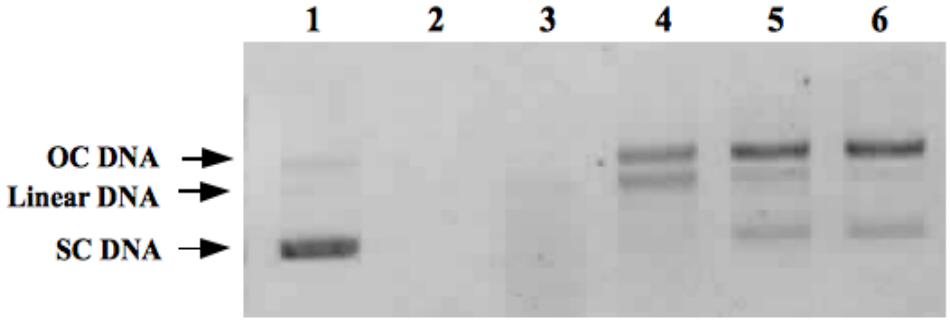

Figure 4.19 The *in vitro* protective effect of OSW peptide elution on DNA damage induced by hydroxyl radicals. OC is open circular, DNA form, and SC is supercoil DNA form. Lane 1: empty pBR 322 plasmid DNA without DNA damage, Lane 2: pBR 322 plasmid DNA with hydroxyl radicals, served as a DNA damage control, Lanes 3–6: hydroxyl radicals inducing plasmid DNA damage treated with the OSW peptide elution at the concentrations of 340, 170, 85, 42.5 μg/ml, consequently.

From measurement of the DPPH and ABTS scavenging activities, the recombinant OSW peptide elution showed lower these antioxidant activities than that of the hydrolysate reported by Semanit, in 2013. This may be the resulted from the other potential antioxdative peptides in the hydrolysate. However, the recombinant OSW peptide elution revealed the higher activities than that of the chemical synthesized one in all measurement. This may resulted from 7 copies of QTFQYSRGWTN peptide compared to the only one copy in the chemical synthesized one. This can be a benefit from using recombinant DNA technology for producing the target

antioxidative peptide. However, some problems, including low protein expression and protein purification, are required to be solved for the further application in the larger scale.

CHAPTER V
CONCLUSION

Conclusion

The previous study reported antioxidant activities of the small molecular weight hydrolysate from hairy basil seed waste after oil extraction. The activities included; DPPH and ABTS free radical scavenging. One of the interesting peptide found in such hydrolysate has its amino acid sequence as QTFQYSRGWTN, named as OSW peptide in this research. This peptide is a potential antioxidant due to its aromatic hydroxyl group and also polyphenol group. To overcome several problems of natural product extraction, recombinant DNA technology was applied to construct an *E. coli* strain for expressing OSW peptide. *E. coli* system has played a key role on recombinant protein production due to its benefit such as effective cost and easy genetic manipulation.

In this research, *E. coli* MG1655 derivative was successfully constructed for expressing recombinant OSW peptide. However, due to a short peptide, DNA fragment encoding 7 copies of QTFQYSRGWTN was designed, and a codon of aspartic acid was linked to each copy. With several copies, the longer DNA fragment was easier for genetic manipulation. Moreover, the recombinant peptide was expected to show

higher antioxidant activity, compared with the only single copy of the original one. The DNA fragment was inserted into the pQE-30 Xa plasmid which can be induced by IPTG. Furthermore, the 6xHis-tag fused at the N-terminal end of the recombinant protein was served for purification purpose by Ni^{2+} affinity column. Although, the r OSW strain could express the recombinant OSW peptide, verified by qRT-PCR, expected 15 kDa band on the SDS-PAGE gel, and immunoblotting with antiHis-HPR antibody, the yield of recombinant protein was low. Moreover, when the target peptide, expressed as soluble protein in cytoplasm, was purified by Ni^{2+} affinity column, there were higher molecular weight protein also eluted. Therefore, to purify this recombinant peptide, the expected peptide band was further extracted from SDS gel and then, performed gel diffusion. These additionally purifying steps resulted in the loss of more target peptide.

The eluted recombinant OSW was further examined for antioxidant activities including DPPH and ABTS radical scavenging activities, and *in vitro* protective effect on DNA damage induced by hydroxyl radical. Compared with the previous report, this recombinant peptide had lower DPPH and ABTS activities than those of the permeated 5 kDa hairy basil seed hydrolysate. It could be caused by other potential antioxidative peptides in the hairy basil seed hydrolysates. However, the

recombinant OSW peptide revealed higher antioxidant activities that those of the chemical synthesized one. This may resulted from the composition of 7 copies of QTFQYSRGWTN on one single recombinant peptide.

Due to the higher tested antioxidant activities of the recombinant OSW peptide than the chemical synthesized peptide, by using the recombinant *E. coli* strain, it is a potential candidate to improve antioxidative peptide production. Moreover, each copy of QTFQYSRGWTN is linked by the codon of aspartic acid that can be further cleaved by Endoprotinase AspN enzyme to obtain small peptide as the original which can be benefit in the case of some application such as encapsulation. However, there are some problems need to be overcome including optimization of production and protein purification.

Suggestion

In this research, low yield of target peptide was still revealed. It is important to improve recombinant peptide production by optimizing factors involved such as concentration of IPTG inducer, temperature, and cell culture media. Moreover, the upscale production is expected to obtain more yields due to the control of parameters such as the pH values, oxygen limitation and rpm in bioreactor. The purification is also necessary to be improved. It was indicated that purification by affinity

only column was not enough due to the remaining of unspecific protein in the elution. However, the additional purifying steps in this study, gel extraction and diffusion, can not only cause the loss of target protein, but also are not convenient especially, for the higher level of production. To solve this purification problem, gel filtration or preparative High Performance Liquid Chromatography (HPLC) with suitable loop injection are considered.

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APPENDIX

APPENDIX A
MEDIA AND SOLUTIONS

1. Ampicillin (100 µg/ml)

Dissolve 1000 mg of ampicillin in 10 ml dIH_2O. Prepare in 1 ml aliquots and store at -20 ^oC.

2. LB broth

Peptone	1.0	g
Yeast Extract	0.5	g
NaCl	1.0	g

Adjust final volume to 100 ml with dIH_2O and autoclave at 121 ^oC for 15 minutes.

3. LB agar

Peptone	1.0	g
Yeast Extract	0.5	g
NaCl	1.0	g
Bacto-agar	2.0	g

Adjust final volume to 100 ml with dIH_2O and autoclave at 121 ^oC for 15 minutes.

4. TB I (Transformation buffer I)

KOAc	1.47	g
$MnCl^2$	5.0	g
RbCl	5.0	g
$CaCl_2$	0.74	g
15% glycerol	75	ml

Adjust final volume to 500 ml with dIH_2O, sterilize by filtering thorough 0.22 µm and store at 4 ^oC.

5. TBII (Transformation buffer II)

MOPS	1.04	g
$CaCl_2$	5.5	g
RbCl	0.6	g
15% glycerol	75	ml

Adjust final volume to 500 ml with dIH_2O, sterilize by filtering thorough 0.22 µm and store at 4 ^oC.

6. Tris-Glycine buffer

Tris	3.0	g
Glycine	14.4	g
SDS	1.0	g

Adjust final volume to 1000 ml with dIH_2O and store at 4 ^oC.

7. 18% Separating gel

Acrylamide/bisacrylamide 30:0.8	6.0	ml
1.5 M Tris-HCl	2.5	ml
10%SDS	0.1	ml
H_2O	1.35	ml
TEMED	10	µl
10% Ammonium Persulphate (w/v)	50	µl

8. 5% Stacking gel

Acrylamide/bisacrylamide 30:0.8	0.67	ml
1.0 M Tris-HCl	0.5	ml
10%SDS	0.04	ml
H_2O	2.75	ml
TEMED	4.0	µl
10% Ammonium Persulphate (w/v)	40	µl

9. 50X TAE buffer pH 8.0

Trizma base	485	g
Disodium dihydrate EDTA	36	g
$NaOAc \cdot 7H_2O$	1.0	g

Dissolve in 1,000 ml with dIH_2O and bring pH to 8.0 using glacial acetic acid (approximately 180 ml). Adjust volume to 2,000 ml with with dIH_2O and store at room temperature.

10. LEW buffer pH 8.0

NaH_2PO_4	6.0 g
NaCl	17.5 g

Dissolve in 500 ml with dIH_2O and bring pH to 8.0 using NaOH. Adjust volume to 1,000 ml with with dIH_2O and store at room temperature.

11. DS buffer pH 8.0

NaH_2PO_4	6.0 g
NaCl	17.5 g
Urea	480.5 g

Dissolve in 500 ml with dIH_2O and bring pH to 8.0 using NaOH. Adjust volume to 1,000 ml with with dIH_2O and store at room temperature.

12. Elution buffer pH 8.0

NaH_2PO_4	6.0 g
NaCl	17.5 g
Imidazole	17.0 g

Dissolve in 500 ml with dIH_2O and bring pH to 8.0 using NaOH. Adjust volume to 1,000 ml with with dIH_2O and store at room temperature.

13. PBS buffer pH 7.4

NaCl	8.0	g
KCl	0.2	g
NaH_2PO_4	1.44	g
NaH_2PO_4	0.24	g

Dissolve in 800 ml with dIH_2O and bring pH to 7.4 using HCl. Adjust volume to 1,000 ml with with dIH_2O and store at room temperature.

14. PBST buffer

PBS buffer	1,000	ml
TWEEN 20	5.0	ml

15. DAB solution

PBS buffer	20	ml
DAB	6.0	ml
1% $CoCl_2$	50	µl
30% H_2O_2	20	µl

15. Ferrous sulphate solution

FeSO4	0.0278	ml

Adjust volume to 50 ml with with dIH_2O and store at room temperature.

16. 50 mM Potassium phosphate buffer pH 7.4

K_2HPO_4 17.42 g

Adjust volume to 10 ml with with dIH_2O as K_2HPO_4 solution.

KH_2PO_4 0.6804 g

Adjust volume to 5 ml with with dIH_2O as KH_2PO_4 solution.

Mix 8.02 ml of KH_2PO_4 solution and 1.98 ml of KH_2PO_4 solution. Adjust volume to 5o ml with with dIH_2O and store at room temperature.

APPENDIX B
DETERMINATION OF PROTEIN CONCENTRATION

The absorbance at 260 and 280 nm was used for calculated protein concentration (Table B1- B6) with possible nucleic acid contamination calculated by using the following formula;

$$\text{Concentration (mg/ml)} = [(1.55 \times A280) - 0.76 \times A260]$$

Table B1 The insoluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 1st expression.

Sample		Blank	Insoluble fraction 1st							
			OSW				CONT			
			0 hour	2 hours	4 hours	6 hours	0 hour	2 hours	4 hours	6 hours
A260	1.00	0.07	0.40	0.46	0.51	0.60	0.35	0.36	0.44	0.58
	2.00	0.08	0.38	0.46	0.50	0.60	0.35	0.37	0.44	0.58
	3.00	0.08	0.36	0.45	0.52	0.60	0.33	0.37	0.44	0.63
	Average	0.08	0.38	0.46	0.51	0.60	0.34	0.36	0.44	0.60
	A260 w/o Blank		0.31	1.91	2.16	2.63	0.27	1.44	1.82	2.60
A280	1.00	0.07	0.36	0.32	0.35	0.42	0.28	0.25	0.27	0.39
	2.00	0.07	0.34	0.31	0.36	0.44	0.29	0.26	0.33	0.40
	3.00	0.07	0.36	0.31	0.35	0.43	0.28	0.25	0.32	0.43
	Average	0.07	0.35	0.32	0.35	0.43	0.28	0.25	0.31	0.40
	A280 w/o Blank		0.28	1.23	1.40	1.78	0.21	0.92	1.18	1.67
	Concentration (mg/ml)		0.21	0.45	0.54	0.77	0.13	0.32	0.45	0.62
	Total protein (mg)		0.62	1.35	1.61	2.31	0.38	0.97	1.35	1.86

Table B2 The insoluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 2nd expression.

Sample		Blank	Insoluble fraction 2nd							
			OSW				CONT			
			0 hour	2 hours	4 hours	6 hours	0 hour	2 hours	4 hours	6 hours
A260	1.00	0.07	0.38	0.44	0.50	0.58	0.34	0.38	0.49	0.58
	2.00	0.08	0.36	0.45	0.50	0.60	0.33	0.37	0.50	0.52
	3.00	0.08	0.35	0.45	0.52	0.59	0.32	0.38	0.48	0.55
	Average	0.08	0.37	0.45	0.50	0.59	0.33	0.37	0.49	0.55
	A260 w/o Blank		0.29	1.87	2.14	2.57	0.25	1.49	2.06	2.37
A280	1.00	0.07	0.31	0.31	0.34	0.40	0.30	0.24	0.31	0.33
	2.00	0.07	0.31	0.29	0.35	0.40	0.30	0.25	0.34	0.39
	3.00	0.07	0.28	0.28	0.35	0.40	0.29	0.25	0.30	0.40
	Average	0.07	0.30	0.30	0.35	0.40	0.30	0.25	0.31	0.37
	A280 w/o Blank		0.23	1.13	1.38	1.65	0.23	0.89	1.22	1.51
	Concentration (mg/ml)		0.13	0.32	0.51	0.61	0.16	0.25	0.32	0.54
	Total protein (mg)		6.74	16.12	25.46	30.48	7.82	12.63	16.24	26.95

Table B3 The insoluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 3rd expression.

Sample		Blank	Insoluble fraction 3rd							
			OSW				CONT			
			0 hour	2 hours	4 hours	6 hours	0 hour	2 hours	4 hours	6 hours
A260	1.00	0.12	0.37	0.43	0.50	0.58	0.32	0.39	0.49	0.55
	2.00	0.13	0.36	0.44	0.48	0.58	0.32	0.38	0.48	0.52
	3.00	0.12	0.34	0.42	0.50	0.58	0.32	0.37	0.46	0.52
	Average	0.12	0.36	0.43	0.49	0.58	0.32	0.38	0.47	0.53
	A260 w/o Blank		0.24	1.55	1.85	2.29	0.20	1.28	1.76	2.04
A280	1.00	0.07	0.27	0.28	0.32	0.38	0.25	0.23	0.31	0.33
	2.00	0.08	0.28	0.27	0.31	0.39	0.24	0.22	0.28	0.33
	3.00	0.07	0.25	0.26	0.31	0.38	0.23	0.23	0.30	0.36
	Average	0.07	0.27	0.27	0.31	0.39	0.24	0.23	0.30	0.34
	A280 w/o Blank		0.20	0.99	1.20	1.56	0.17	0.76	1.11	1.32
	Concentration (mg/ml)		0.12	0.36	0.46	0.68	0.11	0.20	0.39	0.50
	Total protein (mg)		0.37	1.08	1.37	2.03	0.34	0.61	1.17	1.50

Table B4 The soluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 1st expression.

Sample		Blank	Soluble fraction 1st							
			OSW				CONT			
			0 hour	2 hours	4 hours	6 hours	0 hour	2 hours	4 hours	6 hours
A260	**1.00**	0.06	0.65	0.67	0.68	0.98	0.67	0.61	0.66	0.66
	2.00	0.07	0.65	0.68	0.70	0.99	0.68	0.70	0.66	0.67
	3.00	0.06	0.65	0.53	0.69	0.82	0.67	0.69	0.71	0.66
	Average	0.06	0.65	0.62	0.69	0.93	0.67	0.67	0.68	0.66
	A260 w/o Blank		8.78	11.26	12.53	17.33	9.16	12.07	12.27	12.03
A280	**1.00**	0.05	0.39	0.45	0.49	0.66	0.41	0.41	0.45	0.46
	2.00	0.06	0.39	0.46	0.50	0.66	0.41	0.49	0.47	0.47
	3.00	0.05	0.40	0.37	0.49	0.55	0.41	0.44	0.48	0.45
	Average	0.06	0.39	0.43	0.49	0.62	0.41	0.45	0.47	0.46
	A280 w/o Blank		5.00	7.42	8.68	11.35	5.31	7.86	8.21	8.07
	Concentration (mg/ml)		1.09	2.94	3.93	4.42	1.27	3.00	3.40	3.37
	Total protein (mg)		5.43	14.72	19.63	22.09	6.37	15.01	17.01	16.84

Table B5 The soluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 2nd expression.

Sample		Blank	Soluble fraction 2nd							
			OSW				CONT			
			0 hour	2 hours	4 hours	6 hours	0 hour	2 hours	4 hours	6 hours
A260	**1.00**	0.10	0.53	0.65	0.70	0.82	0.63	0.63	0.64	0.73
	2.00	0.07	0.55	0.65	0.69	0.81	0.65	0.66	0.67	0.73
	3.00	0.07	0.56	0.66	0.70	0.81	0.65	0.60	0.66	0.74
	Average	0.08	0.55	0.65	0.70	0.81	0.64	0.63	0.66	0.73
	A260 w/o Blank		6.97	11.45	12.35	14.59	8.42	10.94	11.58	13.03
A280	**1.00**	0.10	0.36	0.44	0.48	0.56	0.40	0.43	0.44	0.50
	2.00	0.07	0.35	0.45	0.48	0.55	0.41	0.45	0.46	0.49
	3.00	0.06	0.35	0.46	0.48	0.55	0.41	0.40	0.46	0.50
	Average	0.08	0.35	0.45	0.48	0.55	0.41	0.43	0.45	0.49
	A280 w/o Blank		4.15	7.46	8.08	9.49	4.95	7.03	7.57	8.39
	Concentration (mg/ml)		1.13	2.86	3.14	3.62	1.28	2.57	2.93	3.09
	Total protein (mg)		5.67	14.28	15.68	18.11	6.40	12.86	14.64	15.47

Table B8 The soluble protein concentration of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours from 3rd expression.

Sample		Blank	Soluble fraction 3rd							
			OSW				CONT			
			0 hour	2 hours	4 hours	6 hours	0 hour	2 hours	4 hours	6 hours
A260	1.00	0.05	0.55	0.60	0.64	0.84	0.54	0.54	0.53	0.57
	2.00	0.05	0.59	0.61	0.66	0.84	0.55	0.54	0.53	0.58
	3.00	0.05	0.58	0.62	0.67	0.85	0.55	0.55	0.53	0.57
	Average	0.05	0.57	0.61	0.66	0.84	0.55	0.54	0.53	0.57
	A260 w/o Blank		7.82	11.19	12.16	15.84	7.43	9.83	9.52	10.44
A280	1.00	0.05	0.33	0.37	0.41	0.52	0.33	0.34	0.35	0.37
	2.00	0.05	0.36	0.38	0.42	0.52	0.34	0.34	0.34	0.37
	3.00	0.05	0.35	0.39	0.43	0.52	0.34	0.34	0.34	0.36
	Average	0.05	0.35	0.38	0.42	0.52	0.33	0.34	0.34	0.37
	A280 w/o Blank		4.46	6.61	7.43	9.40	4.29	5.87	5.87	6.38
	Concentration (mg/ml)		0.96	1.74	2.27	2.54	1.01	1.63	1.87	1.95
	Total protein (mg)		4.80	8.71	11.36	12.68	5.03	8.16	9.36	9.75

Table B7 The total protein amount of each fraction from 50 ml culture of the OSW and CONT strains cultured at 37 °C in LB broth medium after added IPTG for 0, 2, 4, and 6 hours.

Sample	Value	Total protein from OSW strain (mg)				Total protein from CONT strain (mg)			
		0 hour	2 hours	4 hours	6 hours	0 hour	2 hours	4 hours	6 hours
Soluble fraction	1	5.43	14.72	19.63	22.09	6.37	15.01	17.01	16.84
	2	5.67	14.28	15.68	11.11	6.40	12.86	14.64	15.57
	3	4.8	8.71	11.36	12.68	5.03	8.16	9.36	9.75
	Average	5.30 ± 0.26	12.57 ± 1.93	15.56 ± 2.39	17.63 ± 2.73	5.93 ± 0.45	12.01 ± 2.02	13.67 ± 2.26	14.05 ± 2.18
Insoluble fraction	1	0.62	1.35	1.61	2.31	0.38	0.97	1.35	1.86
	2	0.40	0.97	1.53	1.83	0.47	0.76	0.97	1.62
	3	0.37	1.08	1.37	2.03	0.34	0.61	1.17	1.50
	Average	0.46 ± 0.08	1.13 ± 0.11	1.50 ± 0.07	2.06 ± 0.14	0.40 ± 0.04	0.78 ± 0.10	1.16 ± 0.11	1.66 ± 0.11

Table B6 The total protein amount of recombinant OSW elution from 50 ml culture of the OSW strain cultured at 37 °C in LB broth medium after added IPTG for 4 hours.

Sample	Total recombinant OSW elution (mg)
1st	1.19
2nd	0.91
3rd	1.00
Average	1.03 ± 0.08

APPENDIX C
DETERMINATION OF ANTIOXIDANT ACTIVITIES

The percentage of DPPH radical scavenging activity was calculated as the following.

$$\text{DPPH radical scavenging activity \%} = \frac{(AC-ACB) - (AS-ASB) \times 100}{(AC-ACB)}$$

Where AC was A_{517} of water mixed with DPPH in ethanol, ACB was A_{517} of water mixed with ethanol without DPPH (blank), AS was A_{517} of the sample mixed with DPPH in ethanol, and ABS was A_{517} of sample mixed with ethanol without DPPH. Dilution of samples was used for calibration curves and also for calculating IC_{50} values.

Table C2 The % DPPH radical inhibition of chemical synthesized OSW peptide, recombinant OSW elution and ascorbic acid at concentration of 1 mg/ml.

Sample		The % DPPH radical inhibitions
Chemical synthesized OSW peptide	1st	8.16
	2nd	4.05
	3rd	1.89
	Average	4.70 ± 1.84
Recombinant OSW elution	1st	20.86
	2nd	16.07
	3rd	14.64
	Average	17.19 ± 1.88
Ascorbic acid	1st	97.33
	2nd	96.84
	3rd	96.52
	Average	96.90 ± 0.24

Table C1 Comparison of the different of % inhibition DPPH between chemical synthesized OSW peptide and recombinant OSW elution by SPSS independent-samples T-Test (p = 0.05).

Sample	N	Mean	SD	t	Sig
Chemical synthesized OSW peptide	3	4.70	3.19	-4.748*	0.009
Recombinant OSW peptide	3	17.19	3.26		

* Significant at 95% confidence interval

The percentage of ABTS radical scavenging activity and calculated as the following formula:

$$\text{ABTS radical scavenging activity \%} = \frac{(AC-ACB) - (AS-ASB) \times 100}{(AC-ACB)}$$

Where AC was A_{734} of water mixed with ABTS working solution, ACB was A_{734} of water (blank), AS was A_{734} of the sample mixed with ABTS working solution, and ABS was A_{734} of sample mixed with water.

Table C3 The IC50 of Antioxidant activity with ABTS of recombinant OSW elution and ascorbic acid.

Sample		**The IC_{50} of Antioxidant activity with ABTS (µg/ml)**
Recombinant OSW elution	1st	702.60
	2nd	885.20
	3rd	816.37
	Average	801.37 ± 53.24
Ascorbic acid	1st	41.35
	2nd	42.64
	3rd	46.32
	Average	43.44 ± 1.49

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Academic Presentation;

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