ฤทธิ์ต้านอนุมูลอิสระและยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งของเปปไทด์จากสาหร่ายผักกาดทะเล *Ulva rigida*

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Antioxidant and antiproliferative activities of peptide from SEA LETTUCE *Ulva rigida*

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สิริลักษณ์ แก้วมณี : ฤทธิ์ต้านอนุมูลอิสระและยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งของเปปไทด์จากสาหร่ายผักกาดทะเล*Ulva rigida* (Antioxidant and antiproliferative activities of peptide from SEA LETTUCE*Ulva rigida*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.พลกฤษณ์ แสงวณิช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.อภิชาติ กาญจนทัต, 68 หน้า.

ในการศึกษาครั้งนี้ได้ทำการเตรียมโปรตีนจากสาหร่ายผักกาดทะเลโดยการย่อยสลายด้วยโปรติเอส จากจุลินทรีย์ 3 ชนิด คือ แอลคาเลส นิวเทรส และฟลาโวไซม์ และตรวจสอบกิจกรรมยับยั้งอนุมูลอิสระด้วยสาร DPPH ABTS และ NO ผลที่ได้คือโปรตีนที่ย่อยด้วยฟลาโวไซม์ 5 เปอร์เซนต์ มีฤทธิ์ยับยั้งอนุมูลอิสระได้ดีกว่า จากนั้นนำเปปไทด์ผสมที่ได้มาทำบริสุทธิ์ โดยแยกตามขนาดด้วยเทคนิคอัลตราฟิลเตรชัน และเทคนิคโครมาโตกราฟีแบบเจลฟิลเตรชัน พบว่าเปปไทด์ผสม F5 มีฤทธิ์ยับยั้งอนุมูลอิสระดีที่สุด และได้นำเปปไทด์ผสมมาตรวจสอบกิจกรรมยับยั้งอนุมูลอิสระด้วยเอนไซม์ SOD Catalase และ GPx ด้วย ในการศึกษาผลของเปปไทด์ผสม F5 ต่อการยับยั้งการเจริญเซลล์มะเร็ง 5 ชนิดด้วยวิธี MTT พบว่ามีฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งกระเพาะอาหาร (KATO-3) และเซลล์มะเร็งลำไส้ (SW 620) และสามารถเหนี่ยวนำให้เซลล์เกิดการตายแบบอะพอพโทซิส โดยความเข้มข้นของเปปไทด์ที่สามารถยับยั้งการเพิ่มจำนวนของเซลล์ได้ครึ่งหนึ่ง คือ 1.23 และ 1.00 ไมโครกรัมต่อมิลลิลิตรตามลำดับ พิสูจน์เอกลักษณ์ของเปปไทด์ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงและเทคนิคแมสสเปกโตรเมตรี พบสายของเปปไทด์ 4 สาย ซึ่งทุกสายจะประกอบด้วยกรดอะมิโนชนิดที่ไม่ชอบน้ำ (Pro Leu Ala Trp และ Phe) และในบางสายประกอบด้วยกรดอะมิโนชนิด อะโรมาติค (Phe Trp และ Tyr) ซึ่งมีฤทธิ์ในการยับยั้งอนุมูลอิสระ ดังนั้นสาหร่ายผักกาดทะเลจึงถือว่ามี เปปไทด์ที่เป็นสารต้านอนุมูลอิสระจากธรรมชาติที่ดีแหล่งหนึ่ง และอาจนำไปสู่การใช้ประโยชน์ในอนาคตได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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5672215423 : MAJOR BIOTECHNOLOGY
KEYWORDS: ANTIOXIDANT / ANTIPROLIFERATION / PROTEIN HYDROLYSATES / SEA LETTUCE

SIRILUK KAEWMANEE: Antioxidant and antiproliferative activities of peptide from SEA LETTUCE*Ulva rigida*. ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., CO-ADVISOR: ASST. PROF. APHICHART KARNCHANATAT, Ph.D., 68 pp.

The purpose of this study was to preparation protein hydrolysates from sea lettuce by enzymatic hydrolysis of three microbial proteases (alcalase, flavourzyme, and neutrase) and investigates antioxidant activity by determination of the DPPH, ABTS, and NO scavenging activity. The result showed protein hydrolysate by five percent flavourzyme exhibited higher antioxidant activities than those other enzymes. These peptides were fractionated by ultrafiltration and gel filtration chromatography, low molecular weight (F5) of peptide was the highest antioxidant activity. Peptide fractions were observed for their antioxidant activities including enzymatic SOD, Catalase and GPx scavenging activity. Additionally, using cytotoxicity activity was determined by MTT assay in five cell lines. The results revealed that F5 had more inhibition against the proliferation of KATO-III cell lines and SW620 cell lines. The F5 could induce apoptosis cells in KATO-III cell lines of IC_{50}=1.23 µg/ml and SW620 cell lines of IC_{50}=1.00 µg/ml. These peptides were identified by HPLC technique and mass spectrometry as 4 peptide chains. All peptide chains contain hydrophobic amino acids (Pro, Leu, Ala, Trp and Phe) and some peptide chain had aromatic amino acids (Phe, Trp and Tyr) to possess high antioxidant activity. However, sea lettuce stills a good source of natural antioxidant and might be used in future.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

Abs	Absorbance
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BSA	Bovine serum albumin
CO_2	Carbon dioxide
DMSO	Dimethylsulfoxide
DPPH•	2,2-diphenyl-1-picrylhydrazyl radical
et al.	and others
FCS	Fetal calf serum
GPX	Glutathione peroxidase
GSH	Glutathione
h	Hour
H	hydrogen
H_2O	water
H_2O_2	Hydrogen peroxide
IC_{50}	Median inhibitory concentration, 50% maximum inhibition
kDa	Kilodalton
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NaCl	Sodium chloride

NED	N-(1-naphthyl) ethylene diamine
nm	Nanometer
NO•	Nitric oxide
O_2	oxygen
O_2•-	Superoxide anion
PBS	Phosphate buffered saline
Rpm	Round per minute
UV	Ultraviolet
α	Alpha
β	Beta
℃	Degree Celsius
γ	Gamma
g	Microgram
µl	Microliter
µM	Micromolar
/	Per
%	Percentage
:	Ratio

CHAPTER I

INTRODUCTION

Free radicals are molecules that have unpaired electrons and sensitive reaction with other stable molecule. The electron of this molecule is lost and free radical is created and a chain reaction is begun. Free radicals derived from normal aerobic metabolic process in the form of reactive oxygen species (ROS) such as hydroxyl radicals (HO·), superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2). Moreover, free radical can generate from external source such as UV rays, pollution, smoking, stress, herbicides, pesticides and food additive. Free radicals attacks biological molecules such as DNA, protein and lipid, and is associated with many diseases including aging, coronary heart disease, Alzheimer's disease, arthritis, allergies, diabetes and cancer (Ames, Shigenaga et al. 1993). Oxidative stress occurs as a result of an imbalance between the productions of ROS and availability of antioxidant compounds.

Antioxidants are molecules that inhibit the oxidation of other molecules from free radicals. Normally, human body generate enzymatic antioxidant : SOD, catalase, glutathione peroxidase. Nowadays, there is an increasing interest in natural antioxidants because of its safety and non-toxic as compared to synthetic antioxidants such as butylated hydroxylanisol (BHA) and butylated hydroxytoluene (BHT). Natural antioxidants can be derived from many foods, including fruits, vegetables and peptides from protein sources. Recently, bioactive peptides from enzymatic hydrolysis of various food proteins such as soy proteins, whey proteins, fish, shell and algae have been shown to possess antioxidant activity. Hydrolysate of proteins rich in peptides containing aromatic amino acids such as Phe, Trp and Tyr are believed to possess high antioxidant activity, followed by Cys and His (imidazole group) (Dávalos, Miguel et al. 2004). Moreover, hydrolysates rich in peptides containing hydrophobic amino acids, such as Pro, Leu, Ala, Trp and Phe, also show the highest antioxidant activity (Mendis, Rajapakse et al. 2005). Therefore, amino acid compositions of peptides in protein hydrolysates are critical factors in controlling the

antioxidant activity of protein hydrolysates dependent on the protein substrates and proteases used. Furthermore, some of these peptides may exhibit multifunctional bioactivities (Qian, Jung et al. 2008).

Antiproliferative activity is the ability of a compound to stop the growth of cells. Cancer cell growth is different from normal cell growth. Cancer cell grow out of control and invading other tissues are makes a normal cell to a cancer cell. Resulting in uncontrolled cell proliferation. Cancer is one of the most abundant diseases worldwide. The most common sites of cancer among men are lung, prostate, colon, rectum, stomach and liver. The most common sites of cancer among women are breast, colon, rectum, lung, cervix and stomach (WHO). Cancer treatment depends on the type of cancer of the following categories: surgery, radiation, chemotherapy, immunotherapy, hormone therapy, or gene therapy. Moreover, Natural compounds or bioactive peptide used in cancer therapeutics (Nobili, Lippi et al. 2009). As cancer cells have multiple mechanisms to resist the induction of programmed cell death (apoptosis), the apoptosis signaling pathways by natural compounds have been a key event in these antiproliferative activities. Many bioactive peptides with antiproliferative have been extracted from various marine source like sponges, tunicates, soft corals, nudibranchs, sea hares, bryozoans, sea slugs algae and other marine organisms (Newman and Cragg 2004).

There are wide sources of marine algae, which are rich in vitamins A, B, C, and E, minerals, dietary fiber, proteins, and polysaccharides (MacArtain, Gill et al. 2007). Marine algae produce diverse compounds that function as chemical defense systems to facilitate their survival in extremely competitive environments, many of which have a rich source of antioxidation. Antioxidant activity has been reported in numerous genera of marine algae, including *Enteromorpha prolifera, Grateloupia filicina*, *Porphyra tenera, Costaria costata* and *Ulva rigida* (Lee, You et al. 2005). Recently, active antioxidant compounds were identified as phlorotannin, ascorbic acid, carotenoids, bromophenols, catechins (Matsukawa, Dubinsky et al. 1997). Morever, *Ulva rigida*, *Enteromorpha clathrata, Jania adherens* and *Corallina elongate* exhibited antiproliferative effect on *Ehrlich ascites* carcinoma (EAC cell line) (Salem and Ibrahim 2011).

This research has been interested in sea lettuce *U. rigida* that can be found in Thailand. *U. rigida* is macroalgae in Chlorophyceae which contains 10-20 % protein (Fleurence, Le Coeur et al. 1995), and peptides containing amino acid exhibiting antioxidant activity are 47.83% of Phe and 37.83% of Try. There is little or no information on bioactive peptides with antioxidant in *U. rigida.* The objective is to determine the optimal conditions for the preparation of peptide from *U. rigida* with antioxidant and antiproliferative activities, purification, characterization and identification of the peptides.

CHAPTER II

LITERATURE REVIEW

2.1 Free radical

Free radicals are molecules that have unpaired electrons and are sensitive reaction with other stable molecule. The electron of this molecule is lost and free radical is created a chain reaction. Free radicals chain reactions is chemical reaction involving free radical can be divided into three processes consisting of initiation, propagation, and termination (shown in Figure 1) (Dunn 1997).

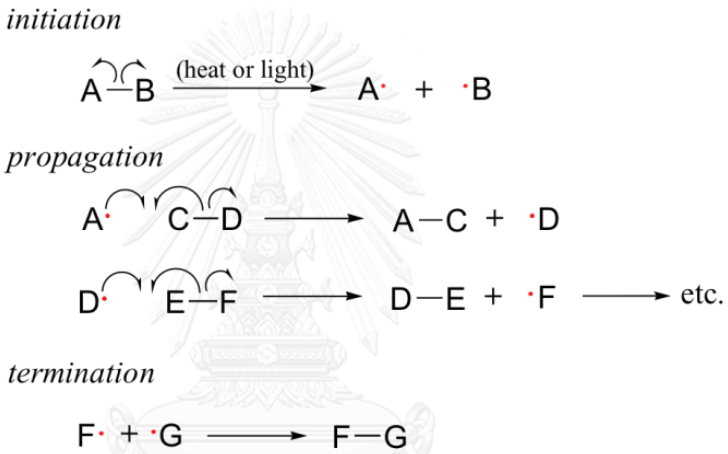


Figure 1 Free radicals chain reactions

- The **initiation phase** describes the step that initially creates a radical species. In most cases, this is a homolytic cleavage.
- The **propagation phase** describes the chain reactions. Once a reactive free radical is generated, it can react with stable molecules to form new free radicals.
- **The chain termination** occurs when two free radical species react with each other to form a stable, non-radical adduct

Free radicals in living systems derived from normal aerobic metabolic process in the form of reactive oxygen species (ROS) such as hydroxyl radicals (HO·), superoxide anion radicals (O_{2-}), hydrogen peroxide (H_2O_2) and Peroxyl radical ($ROO^{\bullet}$). ROS are summarized in Table 1 (Birben, Sahiner et al. 2012).

Table 1 Reactive oxygen species (ROS) (Ames, Shigenaga et al. 1993)

ROS	Formula	Reaction Equation
Superoxide anion radicals	$O_2^{-\cdot}$	$NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^{-\cdot} + H^+$
Hydrogen peroxide	H_2O_2	$Hypoxanthine + H_2O + O_2 \leftrightarrow xanthine + H_2O_2$
Hydroxyl radicals	HO•	$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO•$
Peroxyl radical	ROO•	$R• + O_2 \longrightarrow ROO•$

Moreover free radical can generate from external source such as Ionizing radiations (from industry, UV-ray, x-ray), Heavy metals (mercury, cadmium and lead), pollutants (cigarette smoke, air pollution), stress, herbicides, pesticides and food additive. The body human have over free radicals can occur oxidation stress. Oxidative stress can damage biological molecules to three major structures DNA, protein and lipid. The damage to DNA strands can occur directly by free radical. Alteration in DNA is a major factor in the development of cancer (Siti, Kamisah et al. 2015). Free radical could attack fatty acid side chains of intracellular membranes and lipoproteins. A chain reaction known as lipid peroxidation ensues. Lipid peroxidation is implicated in the development of arteriosclerosis. The last structures damaged by oxidative stress are cellular proteins. This plays a role in the causation of cataracts. Free radicals can interfere with protein function leading to irregular, abnormal metabolism and rapid aging (Elochukwu 2015). Oxidative stress is associated with many diseases including aging, coronary heart disease, Alzheimer's disease, arthritis, allergies, diabetes Hypertension and cancer (Ames, Shigenaga et al. 1993).

2.2 Antioxidation

Antioxidants are molecules that inhibit the oxidation of other molecules from free radicals**.** These have several mechanisms: (1) scavenging species that initiate peroxidation, (2) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, (3) quenching O2 – preventing formation of

peroxides, (4) breaking the autoxidative chain reaction, and (5) reducing localized O_2 concentrations (Brewer 2011). Antioxidants can be divided into 2 categories such as endogenous antioxidants and exogenous antioxidants(Noori 2012).

2.2.1 Endogenous antioxidants

Endogenous antioxidants can be categorized into enzymatic and non-enzymatic antioxidant (Bouayed and Bohn 2010).

2.2.1.1 Enzymatic antioxidant

Enzymatic system directly/indirectly contributes to defense against the ROS such as superoxide dismutase (EC 1.15.1.11) that catalyze the dismutation of the superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2), Catalase (EC 1.11.1.6) is the ferroprotoporphyrin (heme) containing enzyme which converts hydrogen peroxide (H_2O_2) to water and oxygen molecule and glutathione peroxidase (EC 1.11.1.9) to reduce H_2O_2 and lipid peroxides (Birben, Sahiner et al. 2012). Enzymatic antioxidant shown in Table 2.

Table 2 Enzymatic antioxidant

Enzymatic Antioxidant	Acronym	Catalyzed Reaction
Superoxide dismutase	SOD	$M^{(n+1)+}\text{-SOD} + O_2^- \longrightarrow Mn^+\text{-SOD} + O_2$
		$M^{n+}\text{-SOD} + O_2^- + 2H^+ \longrightarrow M^{(n+1)+}\text{-SOD} + H_2O_2$
Catalase	CAT	$2H_2O_2 \longrightarrow O_2 + 2H_2O$
		$H_2O_2 + \text{Fe(III)-E} \longrightarrow H_2O + \text{O = Fe(IV)-E(.1)}$
		$H_2O_2 + \text{O = Fe(IV)-E(.1)} \longrightarrow H_2O + \text{Fe(III)-E} + O2$
Glutathione peroxidase	GPx	$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$
		$2GSH + ROOH \longrightarrow GSSG + ROH + H_2O$

2.2.1.2 Non enzymatic Antioxidant

Non-enzymatic antioxidants (principal intracellular reducing agents) Glutathione (GSH), lipoic acid, NADPH, coenzyme Q, albumin, bilirubin

2.2.2 Exogenous antioxidants

The most effective antioxidants are those that interrupt the chain reaction. Usually containing aromatic or phenolic rings, these antioxidants donate H to the free radicals formed during oxidation becoming a radical themselves (Noori 2012).

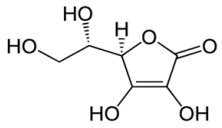
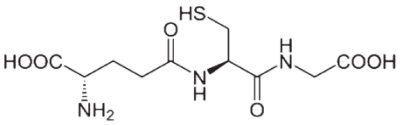
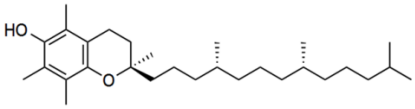
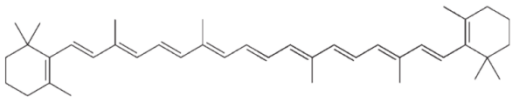
2.2.2.1 Dietary antioxidants

Antioxidants are broadly divided into two group; depends on its solubility.

1) Hydrophilic antioxidants**:** Antioxidants react with oxidants in the cell cytoplasm and the blood plasma. For example: Ascorbic acid, Glutathione and Uric acid.

2) Hydrophobic antioxidants**:** Protect cell membranes from lipid peroxidation. For example: Carotenes, **α**-tocopherol and Ubiquinol. These compounds may be synthesized in the body or obtained from the diet (Halliwell 2000). Non enzymatic antioxidant shown in Table 3.

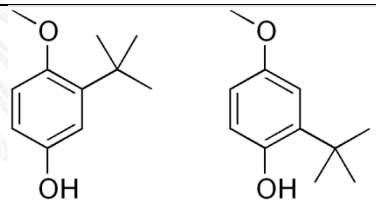
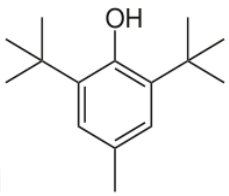
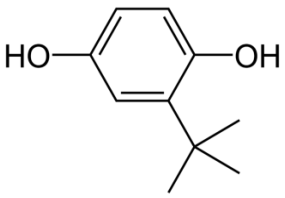
Table 3 Dietary antioxidants

Antioxidant	structure
Ascorbic acid (vitamin C)	
Glutathione	
α-tocopherol(vitamin E)	
b-Carotene	

2.2.2.2 Synthetic antioxidants

A range of synthetic antioxidants are available to restore or even improve the oils natural protection against oxidative degradation and thus increasing their shelf life considerably. Other use of antioxidants are found in the rendering of animal fats, the meat industry, cosmetics, in baked goods and practically all foods with a high oil content such as mayonnaise and margarine. Synthetic antioxidants include Butylated Hydroxyanisole, Butylated Hydroxytoluene, Propyl Gallate, and Tertiary Butyl Hydroquinone (Bouayed and Bohn 2010). Synthetic antioxidants shown in Table 4.

Table 4 Synthetic antioxidant

Synthetic antioxidants	Structure
Butylated Hydroxyanisole (BHA)	
Butylated Hydroxytoluene(BHT)	
Tertiary Butyl Hydroquinone(TBHQ)	

2.2.2.3 Antioxidative protein hydrolysate

Natural antioxidants can be derived from many foods, including fruits, vegetables and peptides from protein sources. Recently, bioactive peptides from enzymatic hydrolysis of various food proteins such as soy proteins, whey proteins, fish, shell and algae have been shown to possess antioxidant activity. Protein

hydrolysate is obtained through hydrolysis of protein, and is a mixture of peptones, peptides, and amino acids. Some protein hydrolysates have been found to have specific functional activities(Cempel, Piot et al. 1995). Antioxidant activity of protein hydrolysates mainly relies on peptides in the hydrolysate. Hydrolysate of proteins rich in peptides containing aromatic amino acids such as Phe, Trp and Tyr are believed to possess high antioxidant activity, followed by Cys and His (imidazole group) (Dávalos, Miguel et al. 2004). Aromatic amino acids convert radicals to stable molecules by donating electron, improving the radical-scavenging properties of the amino acids residues (Sarmadi and Ismail 2010). Hydrolysates rich in peptides containing hydrophobic amino acids, such as Pro, Leu, Ala, Trp and Phe, also show the highest antioxidant activity (Mendis, Rajapakse et al. 2005). Enhancing the solubility of peptide in lipid which facilitates accessibility to hydrophobic radical species and to hydrophobic PUFAs. In addition, acidic (Asp and Glu) and basic amino acids(Lys) play an important role in the chelation of metal ions by carboxyland amino groups in their side chains (Mendis, Rajapakse et al. 2005) Therefore, amino acid composition of peptides in protein hydrolysates is a critical factor in controlling the antioxidant activity of protein hydrolysates. Follow by Table 5. The molecular weight of a peptide has been demonstrated to be one of the most important factors in producing protein hydrolysates with antioxidant properties. The higher antioxidant potential of low-molecular-weight peptides has more effectively than large (Ranathunga, Rajapakse et al. 2006).

Table 5 Antioxidant activity of protein hydrolysate

Source of protein	Enzyme	Protein sequence	Reference
Jumbo squid (*Dosidicus gigas*)	Trypsin	Phe–Asp–Ser–Gly–Pro–Ala–Gly–Val–Leu	Mendis, Rajapakse et al. 2005
Chlorella vulgaris	Pepsin	Val-Glu-Cys-Iyr-Gly-Pro-Asn-Arg-Pro-Glu-Ph	Sheih, Fang et al. 2009
Sea cucumber	Trypsin	Gly-Pro-Glu-Pro-Thr-Gly-	Zhou, Wang et al.

(*Stichopus japonicas*)		Pro-Thr-Gly-Ala-Pro-Gln-Trp-Leu-Arg	2012
Conger eel *(Conger myriaster*)	Trypsin	Leu-Gly-Leu-Asn-Gly-Asp-Asp-Val-Asn	Ranathunga, Rajapakse et al. 2006
Prawn *(Penaeus japonicas*)	Pepsin	Ile-Lys-Lys, Phe-Lys-Lys, and Phe-Ile-Lys-Lys	Suetsuna, 2000
Alaska pollock (*Theragra chalcogramma*)	Alcalase	G-P-Hyp-G-P-Hyp-G-P-Hyp-G-P-Hyp-G	Kim, Kim et al. 2001

2.3 Protein hydrolysates

Bioactive peptides usually contain 3–20 amino acid residues, and their antioxidant activities are based on amino acid composition of sequence and molecular weight of a peptide (Ranathunga, Rajapakse et al. 2006). Bioactive peptides can be produced by three methods such as solvent extraction, enzymatic hydrolysis, and microbial fermentation. However, especially in food and pharmaceutical industries the enzymatic hydrolysis method is preferred because enzymatic hydrolysis is develop under mind condition of pH (6-8) and temperature (40-60 c) , non-toxic chemicals in the products, minimizing side reactions and specific in residue peptide to breakdown it (Kim, Je et al. 1999).

Proteolytic enzymes are classified by their hydrolyzing mechanism: endopeptidase and exoproteases. Endopeptidase hydrolyse peptide bonds with in protein molecules at random to produce relatively large peptide. Exoproteases remove amino acids from either N terminus or C terminus by hydrolyzing the terminal peptide bonds (Clemente 2000). The enzymatic hydrolysis is performed at controlled pH and temperature follow by Table 6 shown Proteolytic enzymes, characteristics and optimum conditions (Samarakoon and Jeon 2012)

Table 6 Proteolytic enzymes, characteristics and optimum conditions

Enzyme	Type of protease	Source of origin	Conditions	
			pH	Temp (°C)
Pepsin	Digestive, acid protease and endo-peptidase	Porcine gastric mucosa	2	37
Trypsin	Digestive, Serine protease and endo-peptidas	Bovine, porcine or human pancreas	5	37
Alcalase	Serin endo-protease	*Bacillus licheniformis*	7	50
Papain	Cysteine protease, endopeptidase	*Carica papaya* (papaya latex)	6	37
Flavourzyme	Endo-exo-peptidase	*Bacillus stearothermophilus*	7	50
Neutrase	Bacterial protease, metallo-endo-protinase (Zn)	*Bacillus amyloliquefaciens*	8	50

2.4 DNA damage

ROS damage to three major structures: DNA, Lipids and Proteins. The damage to DNA strands can occur directly by free radical in close proximity to the DNA or indirectly, by impairing production of protein needed to repair DNA. Alteration in DNA is a major factor in the development of cancer (Ames, Shigenaga et al. 1993).The oxidative damage to DNA overtime could cause changes to both the structure and functions of chromosomes, which can lead to cancer and chronic diseases. The peptide hydrolysate had the protective capacity to protect hydroxyl radical induced DNA damage. Follow by (Sheih, Wu et al. 2009antioxidant) : they study on oxidation-induced DNA damage was analyzed to elucidate the positive role of antioxidative peptide from *Chlorella vulgaris*. In this study, hydroxyl radicals generating system

was based on the Fenton reaction that make the super coil (SC) form in DNA converted to the open circular (OC) form due to the hydroxyl radical damage. But DNA treat with peptide shown DNA was not damage. So, the purified peptide had the protective capacity in oxidation-induced DNA damage.

2.5 Antiproliferative avtivity

Cancer is one of the most abundant diseases worldwide. In 2008, there were 12.6 million new cases of cancer, approximately 28.8 million people living with cancer and 7.6 million deaths from cancer worldwide (IARC, 2010). Antiproliferative activity is the ability of a compound to stop the growth of cells. As cancer cells have multiple mechanisms to resist the induction of programmed cell death (apoptosis), the apoptosis signaling pathways by natural compounds have been a key event in these antiproliferative activity. According to recent studies, the antiproliferative avtivity of hydrolysate peptides has been evidenced by induction of apoptosis and inhibition of cell proliferation *in vitro* and *in vivo*. These peptides have been obtained from sea hare (Madden, Tran et al. 2000), sea slug (Wesson and Hamann 1996), squid (Alemán, Pérez-Santín et al. 2011), tuna dark muscle(Hsu, Li-Chan et al. 2011), and shrimp shell (Kannan, Hettiarachchy et al. 2011).

2.6 Apoptosis

Apoptosis has been recognized and accepted as a distinctive and important mode of "programmed" cell death, which involves the genetically determined elimination of cells. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson 2001). Irradiation or drugs used for cancer chemotherapy results in DNA damage in some cells, which can lead to apoptotic death through a *p53*-dependent pathway. Some hormones, such as corticosteroids, may lead to apoptotic death in some cells (e.g., thymocytes) although other cells are unaffected or even stimulated.

Apoptosis is characterized by cell membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Most importantly, the cell are subsequently phagocytosed by macrophages. (Su, Yang et al. 2015) Figure 2 shown Apoptosis pathway

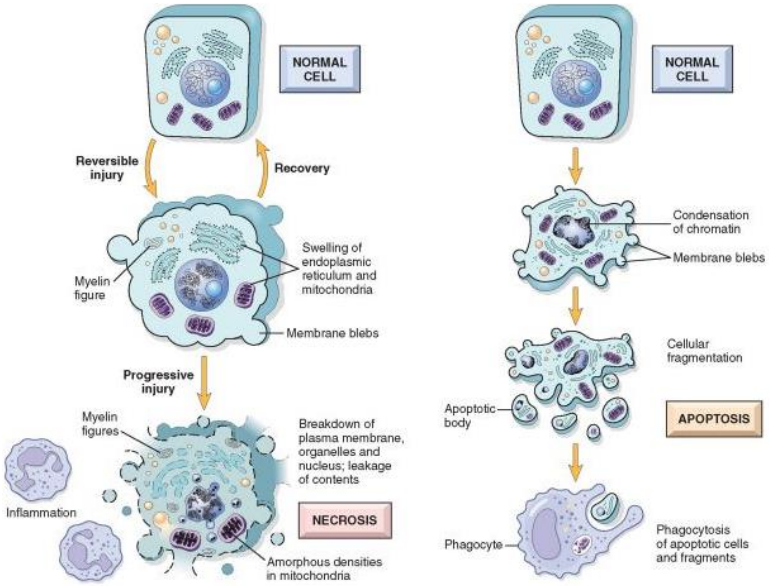


Figure 2 Apoptosis pathway

2.6.1 The mechanisms of apoptosis

The mechanisms of apoptosis are highly complex, involving an energy dependent cascade of molecular events. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway(Igney and Krammer 2002).

2.6.1.1 The extrinsic or death receptor pathway

The extrinsic pathway can be induced the activation of death receptors by members of the TNF family such as TNFR1 and Fas. As ligands bind to these receptors, the death inducing signaling complex (DISC) is formed leading to initiation of the caspase cascade through caspase 8 via the adaptor protein FADD. Activated caspase-8 stimulates apoptosis (Elmore 2007).

2.6.1.2 The intrinsic or mitochondrial pathway

The intrinsic apoptotic pathway is activated by various stimuli, including DNA damage and oxidative stress to induced release of cytochrome c from mitochondria. It relies on the levels of pore-forming pro-apoptotic Bcl-2 family proteins such as Bax, Bak, Bcl-2, and Bcl-xL. In the cytosol, cytochrome c binds and activates Apaf-1, allowing it to bind and activate pro-caspase-9. Active caspase-9 (intrinsic) and

caspase-8 (extrinsic) have been shown to directly cleave and activate the effector protease, caspase-3 (Reed 2000). (Figure 3)

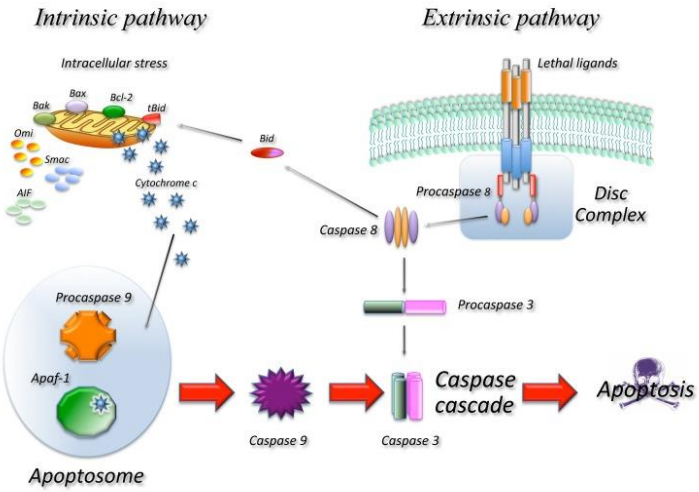


Figure 3 The mechanisms of apoptosis (Favaloro, Allocati et al. 2012)

2.6.2 The caspase activity

The progress of apoptosis is regulated in an orderly way by a series of signal cascades under certain circumstances. The caspase-cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals. A group of intracellular proteases called caspases are responsible for the deliberate disassembly of the cell into apoptotic bodies during apoptosis. Caspases are present as inactive pro-enzymes that are activated by proteolytic cleavage. Caspases 8, 9 and 3 are situated at pivotal junctions in apoptosis pathways. Caspase 8 initiates disassembly in response to extracellular apoptosis-inducing ligands and is activated in a complex associated with the cytoplasmic death domain of many cell surface receptors for the ligands. Caspase 9 activates disassembly in response to agents or insults that trigger the release of cytochrome *c* from mitochondria and is activated when complexed with apoptotic protease activating factor 1 (APAF-1) and extra-mitochondrial cytochrome *c*. Caspase 3 appears to amplify caspase 8 and caspase 9 initiation signals into full-fledged commitment to disassembly. Caspase 8 and caspase 9 activate caspase 3 by proteolytic cleavage and caspase 3 then cleaves vital cellular proteins or other caspase (Fan et al 2005).

2.7 Marine macroalgae

Marine macroalgae or seaweeds as they are commonly known, are a diverse group of marine organisms that have developed complex metabolic pathways to survive in highly competitive marine environments such as high radiation, desiccation, freezing, low temperature, heavy metals and variability in salinity, promote the production of excess ROS within macroalgae cells (Cornish and Garbary 2010). Marine macroalgae can be classified into three group base on their pigmentation such as red algae (Rhodophyceae), green algae (Chlorophyceae) and brown algae (Pheaophyceae).

There are wide sources of marine algae, which are rich in vitamins A, B, C, and E, minerals, dietary fiber, proteins, and polysaccharides (MacArtain, Gill et al. 2007). In fact, marine algae have protective enzymes (superoxide dismutase, glutathione reductase, catalase) and antioxidative molecules such as phlorotannins, ascorbic acid, tocopherols, carotenoids, phospholipids, chlorophyll related compounds, bromophenols, catechins, mycosporine-like amino acids, polysaccharides (Rupérez, Ahrazem et al. 2002). So, marine algae produce diverse compounds that function as chemical defense systems. Morever, components of proteins in marine algae contain sequences of bioactive peptides that can exert a physiological effect in the body such as antihypertensive activity, ACE-I inhibititory, anticoagulant,antibecterial antityrosinase and antiproliferative activity. Furthermore, researchers of bioactive peptides from marine algae as shown in Table 7.The hydrolysate of *Undaria pinnatifida* investigated by 1,1- diphenyl-2-picrylhydrazyl (DPPH), hydroxyl and superoxide radicals. Hydrolysate exhibited strong radical scavenging activity on DPPH and hydroxyl radical. *U pinnatifida* was rich in Glu, Asp, Ala, and His also show the highest antioxidant activity (Je, Park et al. 2009). Heo, Park *et al* (2005) identify enzymatic hydrolysate from seven brown seaweeds, the hydrolysate exhibited more effects in hydrogen peroxide scavenging activity compared to the other scavenging activities. Athukorala, Kim et al (2006) isolate AMG hydrolysate of *Ecklonia cava, which* showed a potential antiproliferative activity. The hydrolysate of *Porphyra yezoensis* contain peptides that shown ACE-I inhibititory. The peptides have aromatic (Phe, Trp, Tyr) also show the highest activity (Suetsuna 1998).

Table 7 Bioactivity of Marine algae

Marine algae	Bioactivity	Protein sequence	References
Porphyra yezoensis	ACE-I inhibititory	Ile-Tyr Met-Lys-Tyr Ala-Lys-Tyr-Ser-Tyr Leu-Arg-Tyr	Suetsuna, 1998
Undaria pinnatifida	Antihypertensive	Ala-Ile-Tyr-Lys Tyr-Lys-Tyr-Tyr Lys-Phe-Tyr-Gly Tyr-Asn-Lys-Leu	Suetsuna and Nakano 2000
Palmaria palmata.	Antihypertensive	Ile-Arg-Leu-Ile-Ile-Val-Leu-Met-Pro-Ile-Leu-Met-Ala	Fitzgerald, Mora-Soler et al. 2012
Undaria pinnatifida (Wakame)	Antihypertensive	AIYK, YKYY, KFYG,YNKL	Suetsuna et al. 2000
Chlorella vulgaris	Anticancer	Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe	Sheih, Fang et al. 2010
Codium fragile	Anticoagulant	-	Athukorala, Lee et al. 2007
Saccharina longicruris	Antibacterial	Thr-Ile-Thr-Leu-Asp-Val-Glu-Pro-Ser-Asp-Thr-Ile-Asp-Gly-Val-Lys	Beaulieu, Bondu et al. 2015
Ecklonia cava	ACE-inhibitory	Ala-Ile-Tyr-Lys, Tyr-Lys-Tyr-Tyr, Lys-Phe-Tyr-Gly and Tyr-Asn-Lys-Leu,	Suetsuna & Nakano, 2000
Hizikia fusiformis	ACE-inhibitory	Ile-Tyr, Met-Lys-Tyr, Ala-	Suetsuna

		Lys-Tyr-Ser-Tyr and Leu-Arg-Tyr	1998
Laminaria japonica	Antioxidant	-	Park, Kim et al. 2009
Sargassum horneri	Anticoagulant	-	Athukorala, Lee et al. 2007
Polysiphonia urceolata	ACE-inhibitory	-	He, Chen et al. 2007
Grateloupia filicina	Antityrosinase	-	Lee, You et al. 2005

2.7.1 Ulva rigida

Taxonomy of Ulva rigida

Division	Chlorophyta
Subdivision	Chlorophytina
Class	Ulvophyceae
Order	Ulvales
Family	Ulvaceae
Genus	Ulva Linnaeus, 1753
Species	*Ulva rigida* C. Agardh

Key of *Ulva rigida* (Dhargalkar and Kavlekar 2004)

Division Chlorophyta

class Ulvophyceae

Uninucleate cells with a parietal chloroplast; thallus with a hollow cylinder or asheet,

one or two cells thick..... .....order Ulvales

Tubular or blade, 1 – 2 celled thick.....family Ulvaceae

Thallus membranous, 2 celled thick.....*Ulva*

Plants not reticulate, clearly bladelike, mostly attached.....

Blades without holes.....

Blades orbicular, margins with teeth or spines.....*Ulva rigida*

Figure 4 *Ulva rigida*

Sea lettuce, *U. rigida* that can be found in Thailand. *U. rigida* is macroalgae in Chlorophyceae which contains 10-20 % protein (Fleurence, Le Coeur et al. 1995) which are rich in vitamins, minerals, Moisture, dietary fiber, and polysaccharides and peptides containing amino acid exhibiting antioxidant activity as shown in Table 8. *Moreover, U. rigida* was reported Anticancer activity. The report shown the cytotoxic activity of different exaction of algae on Ehrlich ascites carcinoma (EAC) cell line. The methanol extract of *U. rigida* exhibited the most potent antiproliferative effect on EAC cell line (Salem and Ibrahim 2011).

Table 8 Nutritional value of U.rigida (วรสิงห์, ศรีวีระชัย et al. 2552)

Nutrition	Nutritional value (%)
Protien	23
Carbohydrate	25.35
Fat	2.76
Fiber	9.79
Moisture	39.1

There is little or no information on bioactive peptides with antioxidant in *U. rigida.* The objective is to determine the optimal conditions for the preparation of peptide from *U. rigida* with antioxidant and antiproliferative activities, purification, characterization and identification of the peptides.

CHAPTER III
MATERIALS AND METHODS

3.1 Preparation of protein hydrolysate

The green alga (*U. rigida*) was collected from Aquatic Resources Research Institute, Chulalongkorn University, Koh Srichang, Chonburi province, Thailand. Algae (1.5 kg) were cleaned, pared, and blended in 5 l of phosphate buffered saline (PBS; 20 mM phosphate buffer with 0.15 M NaCl pH 7.2) using a mixer and subsequently stirred overnight at 37 $^{\circ}$C. The samples were digested with alcalase, flavourzyme and nuetrase at an enzyme to substrate ratio of 1%, 2.5% and 5%, respectively at temperature of 50ºC for 4 h. After digestion, the enzyme was inactivated by boiling for 10 min and the undigested proteins were precipitated by centrifugation at 10000 xg for 15 min. Collected supernatant, in supernatant containing target peptides.

3.2 Determined protein contents

The protein contents were determined using the Bradford method (Bradford 1976). This method is used to measure the concentration of total protein in a sample. The principle is the binding of protein molecules (basic amino acid residues, arginine, lysine and histidine) to formation of the protein-dye complex results in a color change from brown to blue. Bradford dye is easy to use, is fast and sensitive, but several compounds can interfere with the assay.

Bradford working buffer; 95% ethanol, 88% phosphoric acid, Bradford stock solution with bovine serum albumin as a standard protein in range of sensitive: 0-200 µg/ml protein solution. Mix 20 µl protein hydrolysate with 200 µl Bradford working buffer incubates 2 min. The absorbance was measured at 595 nm using a microplate reader spectrophotometer. Plot graph concentrations of standard BSA on X axis and absorbance 595 nm in Y axis. The protein hydrolysates were determine compare with standard curve.

3.3 Determined amino acid contents

Amino acid contents of algae were determine by acid hydrolysis method. Algae were pestle with liquid nitrogen. Add 5 ml of 6 N HCl and placed in a heating block at 110 ℃ for 24 h to calibrate the individual amino acids. Then, added the

internal standard (10 ml of 2.5 mM L-α-amino-n-butyric acid in 0.1 M HCl) diluted with deionized water to 250 ml and placed in a heating block at 55 ºC for 10 min. Amino acids were separated by reversed-phase high performance liquid chromatography (RP-HPLC) analysis on a Hypersil GOLD C18 column with sodium acetate buffer pH 4.90 and 60% acetonitrile, at a flow rate of 0.3 ml/min.

3.4 Isolation and purification

Ultrafiltration is a separation process using membranes with pore size and pressure lead to separation trough. The protein hydrolysate was passed through ultrafiltration membranes with molecular weight cut-off (MWCO) of 10 kDa.

The protein hydrolysate < 10 kDa were separated on a column (0.8×60 cm) of Sephacryl S-200 gel filtration chromatography using an automatic liquid chromatography system (AKTA prime Amersham Pharmacia Biotech,Sweden). The column was operated in downward flow at room temperature. Phosphate buffer was used to equilibrate the column and to elute the proteins at a flow rate of 5 ml/min. The sample (5 ml) was applied to the column, and 5-ml fractions were collected. The absorbance of the effluent was measured at 280 nm.

3.5 Antioxidant activity assay

3.5.1 DPPH scavenging activity assay

DPPH (2,2-diphenyl-1-picrylhydrasyl) radical scavenging activity was measured using the method described by Zhang (Zhang, Duan et al. 2007). Briefly, 40 µl of each protein hydrolysate at various dilutions were mixed with 160 µl of a 0.1 M DPPH. The mixture was mixed and allowed to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a microplate reader spectrophotometer. Calculate percentage inhibition and IC_{50}. Ascorbic acid was used as a positive control.

3.5.2 ABTS radical scavenging activity assay

ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) radical scavenging activity was measured using the method described by Re (Re, Pellegrini et al. 1999). ABTS solution was generated by mixing 7 mM ABTS with 2.45 mM potassium persulfate stand at room temperature in the dark for 12 h. The ABTS solution obtained an absorbance of 0.7± 0.02 units at 734 nm. 10 µl of each protein

hydrolysate at various dilutions were mixed with 300 µl of ABTS solution and the mixture incubated at room temperature for 10 min. The absorbance was measured at 734 nm using a microplate reader spectrophotometer. Calculate percentage inhibition and IC_{50}. Ascorbic acid was used as a positive control.

3.5.3 Nitric oxide radical scavenging assay

Nitric oxide radical scavenging activity was measured using the method described by Alam (Alam, Bristi et al. 2013). Nitric oxide radical was generated by sodium nitroprusside solution to produce nitrite ions which were measured by the Griess reaction. 25 µl of each protein hydrolysate at various dilutions were mixed with 10 mM Sodium nitroprusside in phosphate buffer (pH 7.2). The mixture incubated at room temperature for 150 min. Then, add 100 µl of 0.33% sulphanilamide in 20% acetic acid stand for 5 min for completing diazotization. 0.1% naphthylethylenediamine dichloride was added, incubated at room temperature for 30 min. The absorbance was measured at 546 nm. Calculate percentage inhibition and IC_{50}. Curcumin was used as a positive control.

The percentage inhibition

The percentage inhibition of antioxidant activity was calculated as follows:

$$\left[\frac{(\text{Abs}\,control - \text{Abs}\,blank) - (\text{Abs}\,\text{sample} - \text{Abs}\,background)}{(\text{Abs}\,control - \text{Abs}\,blank)}\right] x\,100$$

where *Abs* control is the absorbance of the control (no sample), *Abs* sample is the absorbance of the sample, *Abs* background is the color absorbance of the sample, and *Abs* blank is the absorbance of deionized water. The IC_{50} was calculated using GraphPad Prism version 6. All tests were performed in triplicate. The values are expressed as mean ± standard deviation of the triplicate data.

3.5.4 Superoxide radical scavenging activity assay

Superoxide radical scavenging activity was measured using 19160 SOD determination kit by sigma-aldrich. SOD catalyzes the dismutation of the superoxide anion (O_2.-) into hydrogen peroxide and molecular oxygen. SOD Assay Kit-WST allows very convenient SOD assaying by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2Htetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with

a superoxide anion. Briefly , 20 µl of each protein hydrolysate were mixed with 200 µl WST working solution. Add 20 µl Enzyme working solution and mix thoroughly. The mixture incubated at 37°C for 20 min. The absorbance was measured at 450 nm

3.5.5 Catalase activity assay

Catalase activity was measured using Catalase assay kit by sigma-aldrich. Catalase is an antioxidant enzyme that is present in all living organisms. It functions to catalyze the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. The assay is based on the measurement of the hydrogen peroxide substrate remaining after the action of catalase. The assay involves spectrophotometrically following the decrease in absorbance of hydrogen peroxide at 240 nm with a kinetic program. This kit may also be used to perform a quick and direct UV assay. Briefly, add 20 µl of protein hydrolysate to quartz plate mixing with 80 µl of 1xAssay Buffer. Start the reaction by adding of 100 ml of UV Assay Substrate Solution (20 mM H_2O_2). Follow the decrease of A_{240} for 30 sec with the kinetic program, the following parameters:

Initial delay = 3 seconds

interval = 5 seconds

readings = 7

The activity was calculated as follow:

$$\text{Unites/ml} = \frac{[\Delta A/\min(\text{Blank}) - \Delta A/\min(\text{Sample})] \times d \times 1}{V \times 0.0436}$$

d = dilution of original sample, V= sample volume (x µl = 0.00x ml), 0.0436 = ε^{mM} for hydrogen peroxide , 1= reaction volume in ml

3.5.6 Glutathione peroxidase activity assay

Glutathione peroxidase activity was measured using Glutathione peroxidase cellular activity assay kit sigma-aldrich. The assay kit uses an indirect determination method base on the oxidation of glutathione(GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH (b-Nicotinamide Adenine Dinucleotide Phosphate, Reduced). Pipette 180 µl Glutathione Peroxidase Assay Buffer into quartz plate. Add 10 µl of NADPH Assay Reagent and 8 µl of protein hydrolysate and mix by

inversion. Star the reaction by addition of 2 µl of 30 mM *tert*-Butyl Hydroperoxide Solution. And mix by inversion. Follow the decrease in absorbance at 340 nm with the kinetic program, the following parameters:

Initial delay = 15 seconds

interval = 10 seconds

readings = 6

The activity was calculated as follow:

$$\text{Unites/ml} = \frac{[\Delta A/\min(\text{Blank}) - \Delta A/\min(\text{Sample})] \times DF}{V \times 6.22}$$

DF = dilution factor of original sample, V= sample volume in ml, 6.22 = ε^{mM} for NADPH

3.6 Protection effect of the purified peptide on oxidation-induced DNA damage

3.6.1 Preparation plasmid from *E.coli*

Streaking plate *E.coli* containing plasmid puc19 and pks in LB agar (2% agar : peptone, yeast, NaCl and agar powder) and antibiotic (ampicillin) is needs in agar to maintain plasmid, growth over night at 37°C. Picking of a single colony from streaked plate of *E.coli* containing plasmid puc19 and pks. Inoculate with the picked colony to 5 ml of LB broth containing ampicillin, incubate for 12-16 h at 30°C while shaking at 200 rpm. Harvest the *E.coli* culture by centrifugation at 13000 rpm in a microcentrifuge for 2 min at room temperature. Decant the supernatant.

3.6.2 Plasmid DNA purification

Plasmid DNA purification using Spinclean plasmid miniprep kit

1. Resuspend the pelleted cells in 250 µL of the Resuspension Solution(Sol I) by pipetting up and down until no cell clumps remain.

   Add 250 µL of the Lysis Solution (Sol II) and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear.

   Add 350 µL of the Neutralization Solution (Sol III) and mix immediately and thoroughly by inverting the tube 4-6 times.

2. Centrifuge at 13000 rpm for 10 min to pellet cell debris and chromosomal DNA.
3. Transfer the supernatant to the spin column tube by decanting.
4. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
5. Wash the spin column by adding 400 µL of Wash Solution I and centrifuge for 2 min. Discard the flow-through.
6. Add 750 µL of Wash Solution to spin column. Centrifuge for 2 min and discard the flow-through. Place the column into 1.5 mL microcentrifuge tube.
7. Add 50 µL of the Elution Buffer to the center of spin column membrane to elute the plasmid DNA. Incubate for 2 min at room temperature and centrifuge for 2 min.

3.6.3 Determined DNA concentration

Running plasmid DNA on agarose gel electrophoresis by mix 5 µl of DNA with 2µl loading dye. Load sample into a well in 1%w/v agarose gel. Run the gel in 1xTAE buffer at 100 V for 30 min. Stain the gel in ethidium bromide for 10 min. Wash the gel with water and check DNA band by UV light. And measure DNA concentration by 260 nm. Dilute DNA solution 1:10 (DNA 10µl: Elution buffer 90 µl). The absorbance was measured at 260 nm using a microplate reader spectrophotometer.

DNA concentration can be determined by the equation: 1 OD_{260} unit = 50 µg/ml

Concentration (µg/ml) = (A_{260} DNA –A_{260} Blank) x dilution factor x 50 µg/ml

3.6.4 Protection effect of the purified peptide on DNA damage

DNA damage was induced by hydroxyl radicals base on the Fenton reaction using the method described by Sheih (Sheih, Wu et al. 2009). Briefly, mixing 8 µl of DNA (puc19 2686 bp. and pks- 2958 bp in concentration of 16-20 ng/ml) with 4µl of various concentrations of protein hydrolysate (12.5, 6.25, 3.13, 1.56 and 0.78 µg/ml) incubated for 20 min.Then, mixed 3 µl of 2 mM $FeSO_4$, and 4 µl of 0.06 mM H_2O_2.

The mixture incubated at 37 ^{o}C for 30 min. After that, check DNA bands by 1% agarose gel electrophoresis. Plasmids band will appear 2000-3000 bp.

3.7 Antiproliferative activity assay

3.7.1 Cancel cell culture

Human cell line including BT474 (human breast ductal carcinoma ATCC no.HTB 20), HEP-G2 (human liver hepatoblastoma ATCC no.HB8065), CHAGO (human undifferentiated lung carcinoma), SW620 (human colon adenocarcinoma ATCC no.CCL227) and KATO-III (human gastric carcinoma ATCC no.HTB 103) were used in an antiproliferative assay. The cacinoma cell lines were cultured in 25 cm^{2} culture flask using RPMI 1640 containing 10% fetal calf serum. Cultured cells were incubated at 37°C in the presence of 5% CO_2.

3.7.2 Cytotoxicity assay

The antiproliferative assay (cytotoxicity) of protein hydrolysate on (BT474) (breast), HEP-G2 (hepatoma), CHAGO (lung), SW620 (colon) and KATO-III (gastric) was evaluated by the MTT assay (Mosmann 1983) MTT assay is based on the ability of active mitochondrial of living cells to cleave the tetrazolium rings of yellow MTT and form dark blue insoluble formazan crystals, resulting in its accumulation within healthy cells.

Trypsinization cells by 0.05% Trypsin-EDTA. The cells were plated at 2.5 x 10^{4} cells per well (200µl/well) in 96-well plates. After 24 h, the plates were incubated with protein hydrolysate (6.0, 2.4, 1.2, 0.6, 0.3, 0.15, 0.01 µg/ml) for 72 h at 37°C 5% CO_2. Then, 10 µl of MTT stock solution (5 mg/ml) was added to wells and incubated at 37°C for 4 h. Remove solution each well. The insoluble purple formazan crystal was dissolved in 150 µl of DMSO and 10 µl of glycine buffer pH 10.4. The absorbance was determined at 540 nm using a microplate reader spectrophotometer. The percentage of cell viability was calculated as follow:

$$\% \text{ Cell Survival} = \frac{Abs\ sample}{Abs\ control} \times 100$$

The concentration of the hydrolysate causing 50% inhibition of cancer cell growth was considered as IC_{50}.

3.7.3 Apoptosis

Apoptosis was determinate by Membrane Alterations (Elmore 2007). Externalization of phosphatidylserine residues on the outer plasma membrane of apoptotic cells allows detection via FITC- Annexin V in tissues.

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer of the plasma membrane. Annexin V binds to exposed apoptotic cell surface PS (early apoptosis). In late apoptotic and necrotic cells, the integrity of the plasma and nuclear membranes decreases allowing PI to pass through the membranes, intercalate into nucleic acids.They can be measure fluorescence with flow cytometer.

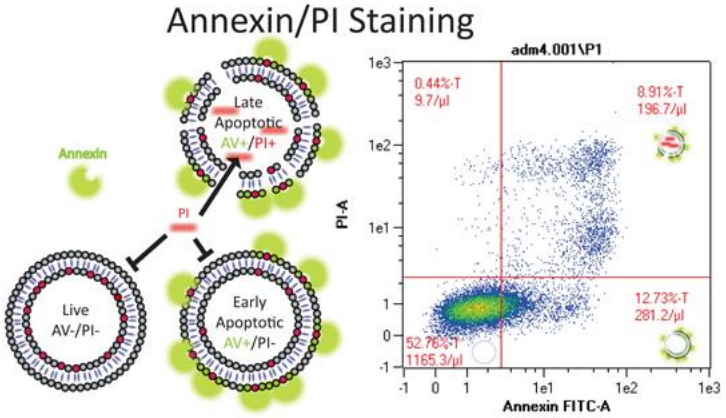


Figure 5 Annexin V and PI

SW620 and KATO-III were used in this assay. Cell lines were cultured in 25 cm^2 culture flask at a density of 1×10^6 cells / flask / 5ml with RPMI 1640 containing 10% fetal calf serum. Cultured cells were incubated at 37°C in 5% CO_2. After 24 h, the flasks were incubated with protein hydrolysate in concentration of IC_{50} value for 2, 4, 8, 12, 24, 48 h at 37°C 5% CO_2. Positive control is the cells incubate with Doxorubicin. Negative control is the cells incubate with RPMI 1640 containing 10% fetal calf serum. Then, the cells were harvested by trypsinization and washed with 20 mM cold PBS containing 1% fetal calf serum. The cells were determine with FITC Annexin V Apoptosis Detection Kit with PI. Suspended cell in Annexin V binding buffer

100 µl and transfer cell suspension in 1.5 ml micro centrifuge tubes. Then, add 2.5 µl of FITC Annexin V and 5 µl of PI solution to each tube. Incubated for 15 min at room temperature in the dark. After that, 200 µl of Annexin V binding buffer was added to each tube and incubated 20 min on ice, in the dark. Analyzed by flow cytometry with cytomics EC 500 MPL, analyzed data by Flowjo software.

3.7.4 Caspase 3 and Caspase 8 activity assay

3.7.4.1 Preparation of cell lysates from apoptosis cells

Induce apoptosis in SW620 and KATO-III cell in a density of $5x10^6$ cells by addition of protein hydrolysate at a concentration of IC_{50} value. Incubate at 37 °C in a 5% CO_2 for 4 and 24 h. The induced cells and the control cells were harvested by trypsinization and washed with 20 mM cold PBS. Centrifugation at 1000 x g for 5 min and remove the supernatant. Suspend the cell pellets in 1X lysis buffer 100 µl. Incubate the cells on ice for 15-20 minutes. Centrifuge the lysed cells at 13,000 for 15 min at 4 °C. Transfer the supernatants to new tubes.

3.7.4.2 Caspase 3 activity assay

Caspase 3 was determined by Caspase 3 assay kit, Colorimetric. The caspase 3 assay base on the hydrolysis of peptide substrate acetyl-Asp-Glu-Val-Asp p-Nitroaniline to p-nitroanililine (pNA) by caspase 3. p-nitroanililine has a high absorbance at 405 nm. Place 5 µl of cell lysate or Caspase 3 Positive Control in 96 wells. Add 1X Assay Buffer 85 µl. Add the Caspase 3 Inhibitor to the appropriate wells. Start the reaction by adding 10 µl of caspase 3 substrate to each well and mix gently by shaking. Incubate at 37 °C for 70 to 90 minutes. Read Absorbance at 405 nm. Calculate the results using a p-nitroaniline calibration curve. The caspase activity was calculated as follow:

$$\text{nmol pNA/min/ml} = \frac{\text{nmol pNA x d}}{\text{t x v}}$$

v = volume of sample in ml, d = dilution factor, t =reaction time in minutes

3.7.4.3 Caspase 8 activity assay

Caspase 8 was determined by Caspase 8 assay kit, Colorimetric. The assay based on the hydrolysis of the peptide substrate Acetyl-Ile-Glu-Thr-Asp p-nitroaniline (Ac-IETD-pNA) to a p-Nitroaniline (pNA) moiety by caspase 8. p-Nitroaniline has absorbance at 405 nm.

Place 10 µl of cell lysate or Caspase 8 Positive Control in 96 wells. Add the 1X Assay Buffer 80 µl. Add the Caspase 8 Inhibitor to the appropriate wells. incubated 5 min at room temperature. Start the reaction with the addition of 10 µl of Caspase 8 Colorimetric Substrate. Measure the initial absorbance at 405 nm with the kinetic program, the following parameters:

interval = 5 min

readings = 12

The activity was calculated as follow:

$$\text{nmol pNA/min/ml} = \frac{(A_t - A_0)\ \text{x d}}{(A_{1\ nmole})\ \text{x t x v}}$$

v = volume of sample in ml, d = dilution factor, t = reaction time in minutes, $A_{1\ \text{nmole}}$ = absorbance of 1 nmole of pNA in the well, A_t = absorbance at time t minutes, A_0 = absorbance at zero time

3.8 RP-HPLC

The purification of peptide from protein hydrolysate was described by High-performance liquid chromatography (HPLC). The protein hydrolysates from gel filtration chromatography were purified using reversed-phase high-performance liquid chromatography (RP-HPLC) on a Luna C18 (4.6 mm × 250 mm) column with a linear gradient of 88% in elute B; of 0.1% trifluoroacetic acid (TFA), and 12% in elute C; acetonitrile (ACN) containing 0.05% trifluoroacetic acid (TFA) at a flow rate of 0.7 ml/min. The protein peaks eluted were monitored at 280 nm. Bioactive peaks were pooled and the purity of peptide was assayed by mass spectrometer.

3.9 Mass spectrometry

Characterization of protein hydrolysate was performed by LC-MS/MS Q-TOF mass spectrometer (Bruker). The LC-MS/MS Q-TOF consisted of a liquid

chromatography and quadrupole time of flight mass spectrometer. Analysis peptide chain in mass range 25-20,000 m/Z. All collected LC-MS/MS Q-TOF data were analyze by de novo peptide sequencing. The principle of *de novo* sequencing is to use the mass difference between two fragment ions to calculate the mass of an amino acid residue on the peptide chain. The mass can usually determine the residue.
Definition for de novo peptide sequencing is peptide sequencing without data base of the amino acid sequence.

3.10 Statistics

Data were expressed as means $\pm$ standard errors of three replicate determinations. Statistical analyses by Duncan's new multiple range test were applied to the result at 0.05 level of significance ($P < 0.05$). GraphPad Prism (Version 6.00, GraphPad Software Inc, La Jolla, CA,USA) for windows was used to calculate the IC_{50} values

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Amino acid contents

Amino acid profile of *Ulva rigida* shown in table 9. Protein of *U. rigida* contain 18 essential amino acid was rich in Aspartic Acid, Glutamic Acid, Leucine, Alanine, Valine, Lysine, Glycine and Proline. *U. rigida* contain aromatic amino acids such as Phe, Trp and Tyr are 0.14%, 0.02%, 0.01%, respectively to possess high antioxidant activity (Dávalos, Miguel et al. 2004). And hydrophobic amino acids, 0.11%, 0.17%, 0.17%, 0.02% 0.14% of Pro, Leu, Ala, Trp and Phe are also show the highest antioxidant activity (Mendis, Rajapakse et al. 2005).

Table 9 Amino acid Profile of *Ulva rigida*

Amino acid	%
Alanine	0.17
Arginine	0.11
Glycine	0.13
Aspartic Acid	0.21
Valine	0.14
Cystine	0.03
Glutamic Acid	0.20
Leucine	0.17
Isoleucine	0.09
Histidine	0.04
Threonine	0.09
Proline	0.11
Lysine	0.13
Methionine	0.04
Hydroxyproline	Not Detected
Serine	0.10

Phenylalanine	0.14
Hydroxylysine	Not Detected
Tyrosine	0.10
Tryptophan	0.02

4.2 Antioxidant activity

4.2.1 DPPH radical scavenging activity

DPPH radical scavenging activity assay has been widely used to evaluate antioxidative properties of compounds as hydrogen donors. The violet compound of DPPH radical turns yellow when neutralized by antioxidant which could be determined by the decreasing in absorbance at 517 nm. The results were shown in Table 10. The IC_{50} value is a parameter used to measure the efficiency, and a low IC_{50} means high scavenging antioxidant activity. All the enzymatic protein hydrolysate showed the DPPH scavenging activity. Protein hydrolyte by 5% Neutrase (IC_{50} of 4.59 ± 0.65 µg/ml) showed the highest activity and higher activity than ascorbic acid (IC_{50} = 24.6±0.01µg/ml). And protein hydrolyte by 5% flavourzyme (IC_{50} of 5.19±0.23 µg/ml) was good activity too. The DPPH radical scavenging activity did not differ significantly between protein hydrolysis by 5%neutrase and protein hydrolysis by 5% flavourzyme.

4.2.2 ABTS radical scavenging activity

The assay of ABTS radical scavenging activity can be applied to both lipophilic and hydrophilic compounds. The green compound of ABTS radical turns colorless by antioxidant reduction of ABTS radical to ABTS which could be determined by monitoring the absorbance at 734 nm. The results were shown in Table 10. protein hydrolysate by 5%flavourzyme (IC_{50} of 2.32 ± 0.03 µg/ml) was shown to yield the highest antioxidant activity and this was higher activity than ascorbic acid (IC_{50} = 51.46±0.01 µg/ml).

4.2.3 Nitric oxide radical scavenging activity

The compound sodium nitroprusside converts nitrate to nitrite and which can be determined using Griess reagent to a deep purple azo compound, and can be monitored by measurement of the absorbance at 540 nm. Nitric oxide radical

scavenging activity of protein hydrolyte by 5% flavourzyme was 1.13 ± 0.26 µg/ml (IC_{50}) which is the highest antioxidant activity (Table 10) and this was higher activity than curcumin (IC_{50} = 37.26±0.04 µg/ml). Decreasing of the IC_{50} value indicates higher antioxidant activity.

The antioxidant activity results of protein hydrolysis by 5% flavourzyme showed the highest antioxidant activity on three assay compared to the other protease. The *U.rigida* hydrolysis by 5% flavourzyme can be used to evaluate in another bioactivity.

Table 10 The IC_{50} values DPPH, ABTS, nitric oxide activity of protein hydrolysate from *U. rigida*. Values are the mean ± standard deviation (SD; n = 3)

Protein	Hydrolyzed by protease (%concentration)		IC_{50}(µg/ml)		
			DPPH	ABTS	NO
Ulva rigida	Alcalase	1%	16.84±0.93^{c}	6.50±0.20^{cc}	2.98±0.30^{aaa}
		2.5%	24.32±1.70^{d}	4.75±0.55^{aacc}	2.82±0.74^{aaa}
		5%	18.35±0.83^{c}	4.09±0.14^{aabbcc}	6.09±0.93^{bbb}
	Flavourzyme	1%	14.80±1.10^{c}	10.67±2.61^{dd}	2.19±0.52^{aaa}
		2.5%	18.72±0.86^{c}	10.07±0.20^{dd}	2.64±0.69^{aaa}
		5%	5.19±0.23^{a}	2.32±0.03^{aa}	1.13±0.26^{aaa}
	Neutrase	1%	15.37±3.93^{c}	5.74±0.17^{cc}	1.21±0.08^{aaa}
		2.5%	9.98±0.35^{b}	5.31±0.16^{bbcc}	1.88±0.07^{aaa}
		5%	4.59±0.65^{a}	2.65±0.04^{aabb}	1.15±0.07^{aaa}
	Positive control		24.6±0.01	51.46±0.51	37.26±0.04

a, b, c, d, aa, bb, cc, aaa, bbb Statistic analysis by SPSS variance (ANOVA) with post hoc comparison (one-way) by using Duncan's multiple Range Test. The same of superscripts alphabet are not significantly different at ($p < 0.05$).

4.2.4 Superoxide dismutase radical scavenging activity

Superoxide radical is one of the important free radicals in vivo that could promote oxidative reactions due to its ability to reduce transition metals and react with the hydroxyl radical (Elias, Kellerby et al. 2008). Superoxide radical scavenging activity catalyzes the dismutation of the superoxide anion (O_2.-) into hydrogen peroxide and molecular oxygen. Protein hydrolyze by 5% neutrase gave 69.58±2.39 % inhibition, which is the highest Superoxide radical scavenging activity. The second is protein hydrolyze by 5% flavourzyme about 64.66±0.21% inhibition. Another protein hydrolysate showed the percent inhibition 30-60% (Table 11).

Table 11 The result of SOD activity of protein hydrolysate from *U. rigida*. Values are the mean ± standard deviation (SD; n = 3)

Protein	Hydrolyzed by protease	Enzyme concentration	SOD (% inhibition)
Ulva rigida	Alcalase	1%	34.49±1.46[e]
		2.5%	48.50±1.86[c]
		5%	50.85±3.44[c]
	Flavourzyme	1%	41.37±2.03[d]
		2.5%	56.24±2.16[b]
		5%	64.66±0.21[a]
	Neutrase	1%	36.64±1.13[e]
		2.5%	60.96±1.94[b]
		5%	69.58±2.39[a]

[a, b, c, d, e] Statistic analysis by SPSS variance (ANOVA) with post hoc comparison (one-way) by using Duncan's multiple Range Test. The same of superscripts alphabet are not significantly different at ($p < 0.05$).

4.2.5 Catalase activity

Catalase is an important antioxidant enzyme that defends against free radical. It protects the cell from toxic effects of hydrogen peroxide (H_2O_2) by catalyze its

decomposition into molecular oxygen and water. Catalase activity assay catalyze the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. The result shown protein hydrolyze by 5% flavourzyme gave the highest catalase activity (25.73±3.46 unit/ml).

Table 12 The result of catalase activity of protein hydrolysate from *U. rigida*. Values are the mean ± standard deviation (SD; n = 3)

Protein	Hydrolyzed by protease	Enzyme concentration	Catalase (unit/ml)
Ulva rigida	Alcalase	1%	3.53±0.27[e]
		2.5%	13.72±3.89[c]
		5%	19.21±1.39[b]
	Flavourzyme	1%	7.49±2.29[d]
		2.5%	18.00±0.37[b]
		5%	25.73±3.46[a]
	Neutrase	1%	7.07±1.94[d]
		2.5%	12.76±0.44[c]
		5%	20.22±1.91[b]

[a, b, c,] Statistic analysis by SPSS variance (ANOVA) with post hoc comparison (one-way) by using Duncan's multiple Range Test. The same of superscripts alphabet are not significantly different at ($p < 0.05$)

4.2.6 Glutathione peroxidase activity

Glutathione peroxidase (GPx) plays a critical role in the reduction of lipid and hydrogen peroxides. GPx catalyze the reduction of hydrogen peroxide (H2O2) and a wide variety of organic peroxides (R-OOH) to the stable alcohols (R-OH) and water (Espinoza, Guo et al. 2008). The protein hydrolyze by 5% flavourzyme showed the highest antioxidant activity of 1.91±0.15 unit/ml.

Table 13 The result of GPx activity of protein hydrolysate from *U. rigida*. Values are the mean ± standard deviation (SD; n = 3)

Protein	Hydrolyzed by protease	Enzyme concentration	GPx (unit/ml)
Ulva rigida	Alcalase	1%	0.29±0.03[f]
		2.5%	0.51±0.08[e]
		5%	0.74±0.04[cd]
	Flavourzyme	1%	0.66±0.01[d]
		2.5%	1.03±0.12[c]
		5%	1.91±0.15[a]
	Neutrase	1%	0.74±0.09[cd]
		2.5%	1.43±0.06[b]
		5%	1.84±0.11[a]

[a, b, c, d, e, f] Statistic analysis by SPSS variance (ANOVA) with post hoc comparison (one-way) by using Duncan's multiple Range Test. The same of superscripts alphabet are not significantly different at ($p < 0.05$)

Superoxide dismutase, catalase and glutathione peroxidase are antioxidant enzyme play an important role in scavenging free radicals. The assay to measure their enzyme activities by spectrophotometry showed protein hydrolyte by 5% flavourzyme was high in superoxide dismutase activity, catalase activity and glutathione peroxidase activity.

The antioxidant properties of these protein hydrolysate depend on enzyme specificity of enzymatic hydrolysis, degree of hydrolysis, nature of peptide from sample(Sarmadi and Ismail 2010, Heo 2003 and Kang 2011). The antioxidant activity results demonstrated that *U.rigida* protein hydrolyse by 5% flavourzyme showed the strongest antioxidant activity for six assay assessments compared to other protein hydrolysate from various protease. Therefore, mw < 3 kDa can be further improved and used in many applications such as functional food ingredients and cosmetic additives.

4.3 Sephacryl S-200 gel filtration chromatography

The most efficiency antioxidant activity of protein hydrolysate from *U. rigida* observed in 5% flavourzyme was passed through ultrafiltration membranes with molecular weight cut-off (MWCO) of 10 kDa, followed by separation on a column of Sephacryl S-200 gel filtration chromatography. The 5% flavourzyme hydrolysates of *U.rigida* showed a complex chromatogram containing 5 fractions; four high molecular weight (F1, F2, F3 and F4) and one low molecular weight fraction (F5) (Fig 5). The antioxidant activity of all the fractions was determined for DPPH, ABTS, and NO scavenging activity. The results were showed in Table 14. Fraction 5 gave higher antioxidant activity than the other fraction. For DPPH, the activity was >0.02 mg/ml. The IC_{50} value of ABTS was 0.01±0.001 mg/ml, and the IC_{50} value from nitric oxide assay was 0.05±0.02 mg/ml. Fraction 5 (low molecular weight and high peak) has highest antioxidant activity follow by reported of Sarmadi, Ismail 2010, the antioxidant properties depend on molecular weight and amino acid composition. Most of the reported peptides exhibiting antioxidative activity were those with low molecular weights (Rajapakse, Mendis 2005 and Ko 2012). And Wang et al. (2013) reports that high peak was provided the good effect because there are many amino acid.

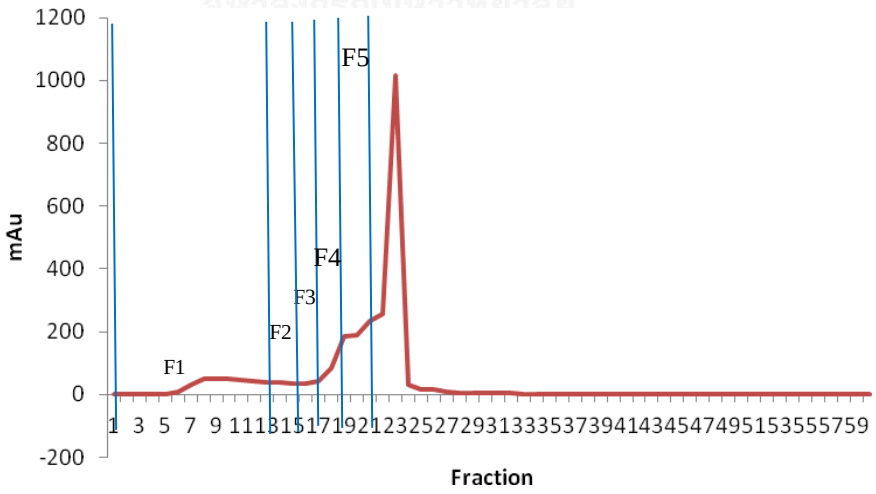


Figure 6 Chromatogram of protein hydrolysate by 5% fravourzyme on Sephacryl S-200 gel filtration column.

Table 14 The antioxidant activity of fractions (F1-F5) from a Sephacryl S-200 gel filtration column

Activity assay	Fraction (IC_{50} mg/ml)				
	F1	F2	F3	F4	F5
DPPH	>0.03	>0.21	>0.14	>0.08	>0.02
ABTS	>0.03	0.16±0.02	0.06±0.002	0.04±0.001	0.01±0.001
Nitric oxide	>0.03	>0.21	>0.14	>0.08	0.05±0.02

4.4 Protection effect of the purified peptide on oxidation-induced DNA damage

Protection effect of the purified peptide on DNA damage was induced by hydroxyl radicals base on the Fenton reaction and protect DNA by various concentrations(12.5, 6.25, 3.13, 1.56 and 0.78 µg/ml) of purified peptide hydrolysate from protein hydrolyze of *U.rigida* with 5% flavourzyme. The results showed that the super coil (SC) form in DNA was converted to the open circular (OC) form due to the hydroxyl radical damage based on the Fenton reaction (Fe^{2+} + H_2O_2). The purified peptide hydrolysate (F5) at 0.78-12.5 µg/ml (LAN 4-8) also protected supercoiled DNA strand in three plasmid DNA pKS^- and pUC19 induced by Fenton reaction. As shown in Figure 6 and 7. According to the research of Sheih et al (2009) and Qian et al (2008), the SC form in DNA was completely converted to the OC form due to hydroxyl radical generated from the Fenton reaction when DNA was treated with purified peptide. Since a DNA is another major sensitive biotarget of oxidative damage, these results clearly explain the protective effect of the purified peptide against oxidative damage.

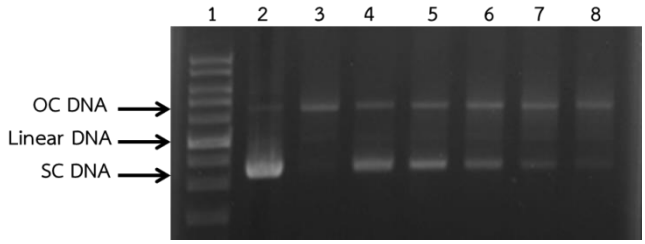


Figure 7 Protective effect of purified peptide on hydroxyl radicals-induced oxidation of pKS plasmid DNA.

Lane 1: marker 1 kbp, Lane 2: pKS^- plasmid DNA 2,958 bp (DNA=18.8 µg/ml), Lane 3: $FeSO_4$ and H_2O_2 treatment (as DNA damage control), Lane 4-8 : PKS^- plasmid DNA with $FeSO_4$ and H_2O_2 treatment in the presence of purified peptide at the concentrations of 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.56µg/ml and 0.78µg/ml respectively.

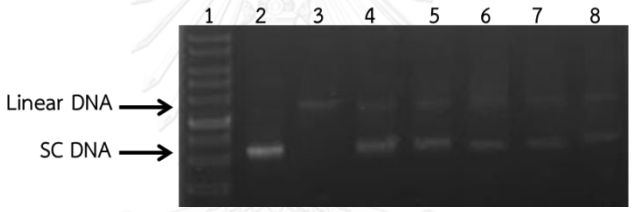


Figure 8 Protective effect of purified peptide on hydroxyl radicals-induced oxidation of pUC19 plasmid DNA.

Lane 1: marker 1 kbp, Lane 2: pUC19 plasmid DNA 2,686 bp (DNA=16.5 µg/ml), Lane 3: FeSO4 and H2O2 treatment (as DNA damage control), Lane 4-8 : PUC19 plasmid DNA with FeSO4 and H2O2 treatment in the presence of purified peptide at the concentrations of 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.56µg/ml and 0.78µg/ml respectively.

4.5 Cytotoxicity assay

MTT assay is based on the ability of an active mitochondrial enzyme of living cells to cleave the tetrazolium rings of yellow MTT and form dark blue insoluble formazan crystals which are largely impermeable through cell membrane, resulting in its accumulation within healthy cells (Hansen, Nielsen et al. 1989). The purified

peptide hydrolysate(F5) with the most effective antioxidant activity was used to test the cytotoxicity assay with five cancer cell lines (Figure 8). Treatment of human cell line including BT474 (breast), HEP-G2 (hepatoma), CHAGO (lung), SW620 (colon) and KATO (gastric) cell lines with purified protein hydrolysate from F5 produced inhibition of cell proliferation. Thus, F5 purified protein more inhibition against the proliferation of SW620 (colon) with IC_{50} of 1.00±0.08 µg/ml and KATO (gastric) with IC_{50} of 1.23±0.02 µg/ml (Figure 9). Also, SW620 (colon) with IC_{50} of 1.00±0.08µg/ml and KATO (gastric) with IC_{50} of 1.23±0.02 µg/ml were should to determined function about program cell death or apoptosis.

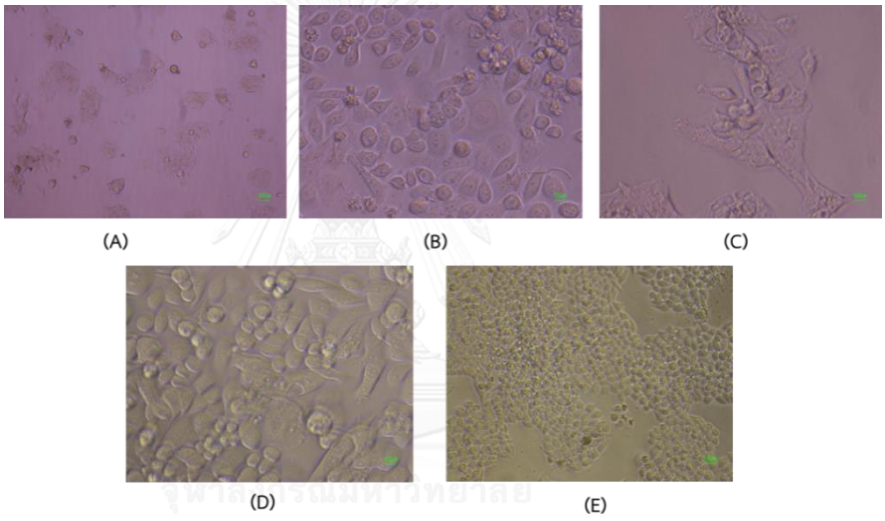


Figure 9 Image from microscope show morphology of cancer cell lines (A) BT474, (B) Chago, (C) HEP-G2, (D) KATO-III, (E) SW620

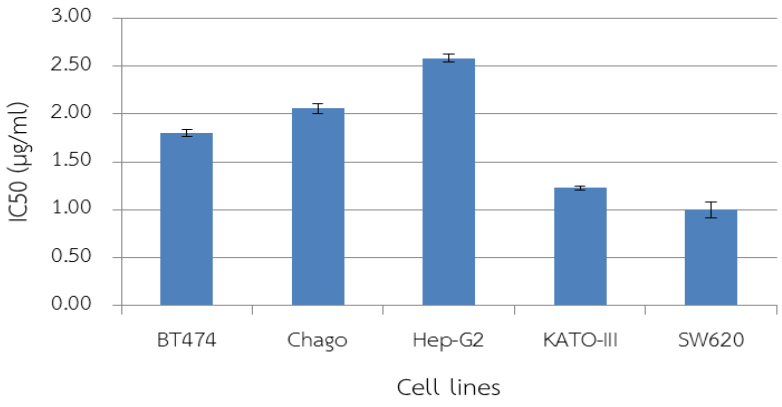


Figure 10 MTT assay of purified peptide hydrolysate(F5) on different cell lines.

Table 15 MTT assay of purified peptide hydrolysate(F5) on different cell lines.

Cell lines	BT474	Chago	Hep-G2	KATO-III	SW620
IC_{50} (µg/ml)	1.80±0.03	2.06±0.05	2.58±0.04	1.23±0.02	1.00±0.08

4.6 Apoptosis, caspase 3 and caspase 8

Apoptosis is the process of programmed cell death that occur in multicellular organisms when cells are damaged by disease or toxic agents . The assay to determine apoptotic cells allows detection via FITC- Annexin V in tissues and measured with flow cytometer. Caspase 3 and Caspase 8 are synthesized in the inactive cell or damage cell, which are usually detect in the process of apoptosis. Apoptosis cells stained with Annexin V-FITC and PI were classified as necrotic cells (the upper left quadrant; Annexin−/PI+), late apoptotic cells (the upper right quadrant; Annexin+/PI+), intact cells (the lower left quadrant; Annexin−/PI−) or early apoptotic cells (the lower right quadrant; Annexin+/PI−) (Yang, Zhao et al. 2013). As shown in Figure 10 and 11. Figure 10 (B), IC_{50} (1.23 µg/ml) of purified protein hydrolysate (F5) could induce apoptosis in KATO-III cells. Apoptosis ratios (the early and late apoptosis ratios) were obtained at 12-24 h with apoptosis ratios 10.37% and 10.38%, respectively, compared with the population of control and positive control (Figure 10 (A)). Caspase 3 and 8 activity results in KATO-III treat with 1.23 µg/ml of purified protein hydrolysate for 24 h shown 0.43 nmol pNA/min/ml and 1.03 nmol pNA/min/ml, respectively. Figure 11B shown apoptosis cell was induced by IC_{50} of purified protein hydrolysate (F5) (1.00 µg/ml). Apoptosis ratios (the early and late apoptosis ratios) were obtained at 2 h with 11.69%, compared with the population of control and positive control (Figure 11 (A)). Results of caspase 3 and 8 activity were 1.29 nmol pNA/min/ml and 0.45 nmol pNA/min/ml, respectively. Thus, the results show that the increased caspase activity by peptide to induce apoptosis can inhibit SW620 cell growth. This result same as Chen et al. (2014), study in Baicalin, a flavone derivative isolated and purified from the dry root of *Scutellaria*, was assessed for its antitumor effects in human SW620 CRC cells. Baicalin (200 µM) inhibited proliferation

of SW620 cells. Baicalin (200 μM) increased activities of caspase-3, -8, and -9 in SW620 cells.

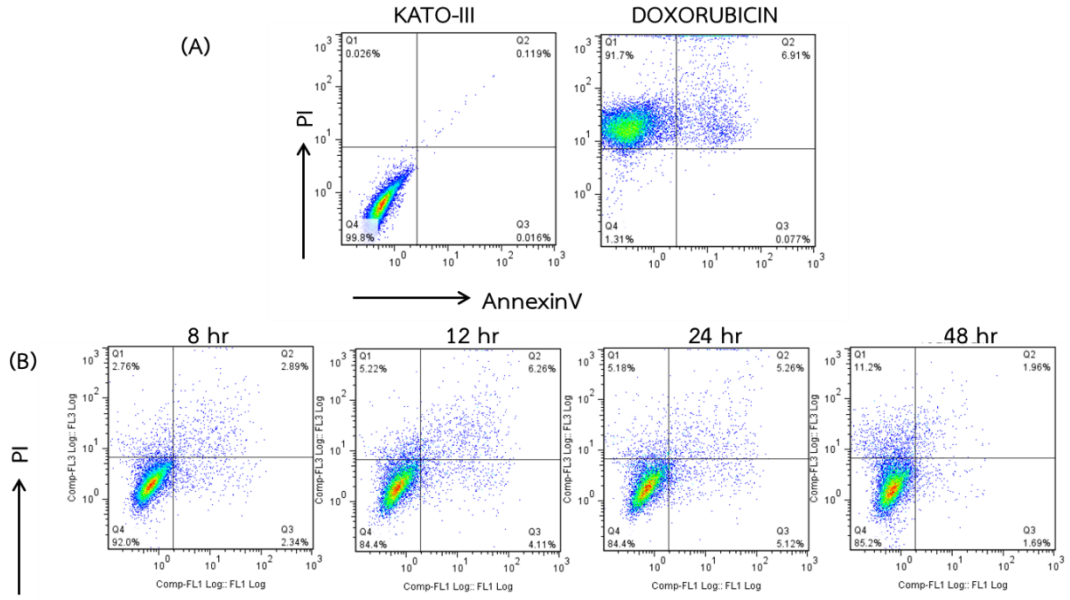


Figure 11 Apoptosis of Kato-III. (A) KATO-III cell lines and KATO-III treated with DOXORUBICIN (positive control). (B) KATO-III treated with purified protein hydrolysate in 8, 12, 24 and 48 hours by flow cytometry.

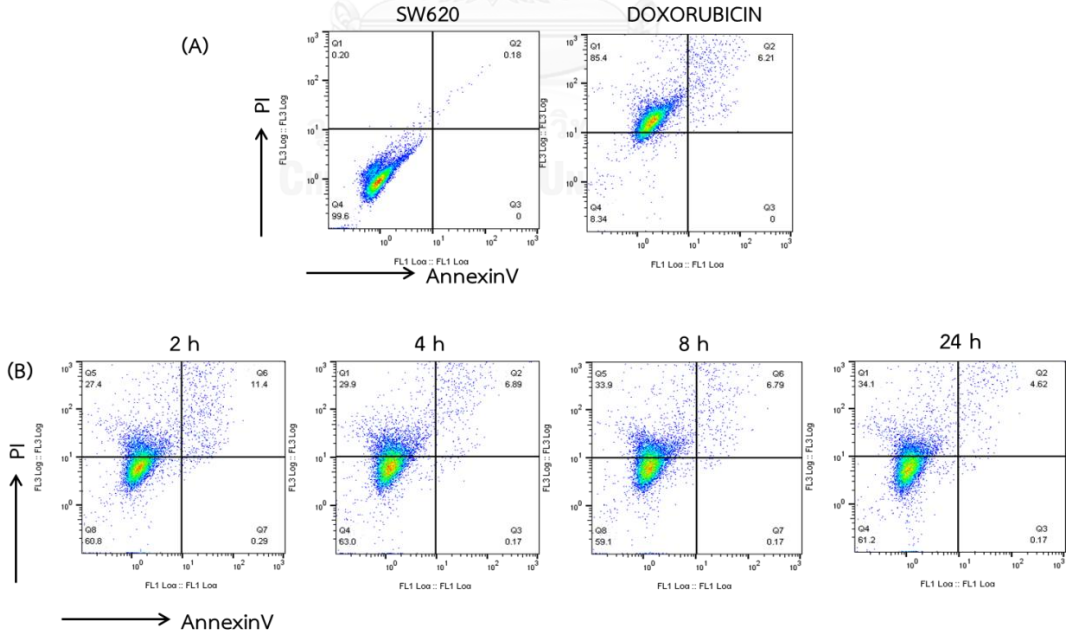


Figure 12 Apoptosis of SW620. (A) SW620 cell lines and SW620 treated with DOXORUBICIN (positive control). (B) SW620 treated with purified protein hydrolysate in 2, 4, 8 and 24 hours by flow cytometry.

Table 16 Caspase 3 activity and caspase 8 activity from apoptosis cells of KATO-III and SW620.

Enzyme activity	nmol pNA/min/ml					
	KATO-III			SW620		
	F5 peptide	positive	negative	F5 peptide	positive	negative
Caspase 3	1.03±0.05	12.6±0.2	0.4±0.1	1.29±0.06	13.7±0.12	0.5±0.08
Caspase 8	0.43±0.05	6.8±0.4	0.13±0.2	0.45±0.08	6.50±0.18	0.17±0.07

4.7 HPLC

Fraction 5 from protein hydrolysate was further purified using reversed-phase high-performance liquid chromatography (RP-HPLC) on a Luna C18 column with a linear gradient. The elution peaks were monitored at 280 nm. The result showed 2 bioactive peaks; F51 and F52 (Figure 12). These fractions were collected for characterization by mass spectrometry.

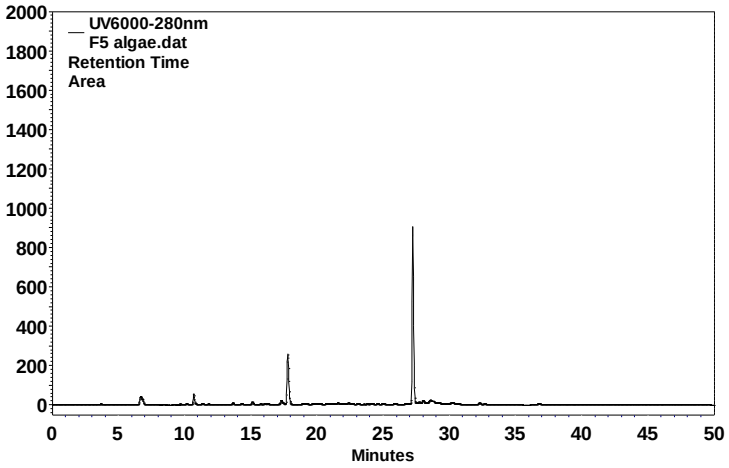


Figure 13 Reversed-phase high-performance liquid chromatography (RP-HPLC) of F5 from protein hydrolysate.

4.8 Mass spectrometry

The 2 fractions from RP-HPLC characterized by LC-MS/MS Q-TOF mass spectrometer. All data analyze by de novo peptide sequencing. Table 17 show

peptide sequence of protein hydrolysate. The fraction F51 wasn't analyzing protein sequence. The fraction F52 showed 4 peptide chains. In previous research Try, Typ, Phe, His and Cys showed the highest antioxidant activity (Zhang *et al.*,2014; Zhou *et al.*,2012). Aromatic amino acids in peptides consisting of Phe, Tyr, and Trp could make active oxygen stable through direct electron transfer and very good radical scavenging activity (Qian et al.2008; Kim et al.2001). Mendis et al. (2005) reported that hydrolysates rich in peptides containing hydrophobic amino acids, such as Pro, Leu, Ala, Trp and Phe, also show the highest antioxidant activity, and hydrophobic amino acids might increase the affinity and reactivity to the cell membrane in the living cells. Qian et al.(2008) reported that alanine, leucine, proline with non-polar aliphatic groups have high reactivity to hydrophobic radicals, and hydrogen donors such as aspartic and glutamic acid are able to quench unpaired electrons or radicals by supporting protons. And Park.(2001) reported peptides contained a leucine residue at their N terminal positions have been demonstrated to be antioxidative. The result showed all peptide chains contained a hydrophobic amino acids such as Pro, Leu, Ala, Trp and Phe, also show the highest antioxidant activity (Mendis, Rajapakse et al. 2005). And WLNGLFGLNPA sequence contains Phe and Trp, which are aromatic amino acids (Phe, Trp and Tyr) believed to possess high antioxidant activity.

Table 17 Peptide sequence of F5 identified by LC-MS/MS Q-TOF

Sequence	Organism	Mass	Query cover	Identity	Accession
1. PATPKTACPSP	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [*Ulva rigida*]	1069.61	54%	67%	AKA64646.1

	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [*Ulva rigida*]	1069.61	54%	67%	AAR32160.1
	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [*Ulva rigida*]	1069.61	54%	67%	ACD10900.1
	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [*Ulva rigida*]	1069.61	54%	67%	ACD10913.1
	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [*Ulva rigida*]	1069.61	54%	67%	CDU85284.1

2. GLLGPLGLTGGHA	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [*Ulva lactuca*]	1162.65	69%	64%	AAR32142.1
	chloroplast photosystem II subunit S [*Ulva linza*]	1162.65	38%	100%	AER58182.1
	chloroplast photosystem II subunit S [Ulva prolifera]	1162.65	38%	100%	AFW18037.1
	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [*Ulva intestinalis*]	1162.65	61%	62%	ABP82427.1
	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) [*Ulva* sp.	1162.65	61%	62%	AGS11259.1

	SD.10.06.04]				
3. WLNGLFGLNPA	Ycf1 (chloroplast) [Ulva sp. UNA00071828]	1201.65	45%	100%	AKC35200.1
	photosystem I P700 apoprotein A2, partial (chloroplast) [*Ulva arasakii*]	1201.65	54%	83%	BAJ21402.1
	P700 chlorophyll a-apoprotein A2, partial (plastid) [*Ulva lactuca*]	1201.65	54%	83%	ACB30051.1
	P700 chlorophyll a-apoprotein A2, partial (plastid) [*Ulva intestinalis*]	1201.65	54%	83%	ACB30050.1
	photosystem I P700 chlorophyll a apoprotein A2 (chloroplast) [*Ulva* sp. UNA00071828]	1201.65	72%	83%	AKC35156.1

4. LATLGPLGVGLLGP	photosystem II CP43 apoprotein [*Ulva arasakii*]	1277.78	85%	80%	BAJ21425.1
	photosystem II CP43 reaction center protein [*Ulva* sp. UNA00071828	1277.78	85%	80%	AKC35158.1
	chloroplast photosystem II subunit S [*Ulva linza*]	1277.78	78%	100%	AER58182.1
	chloroplast photosystem II subunit S [*Ulva prolifera*]	1277.78	78%	100%	AFW18037.1
	NADH-ubiquinone oxidoreductase chain 2 [*Ulva* sp. UNA00071828]	1277.78	64%	57%	AKF33554.1

CHAPTER V
CONCLUSION

The results of this study showed *Ulva rigida* protein that hydrolysis by 5% flavourzyme was high antioxidant activity than other protease. The purified protein from sephacryl S-200 gel filtration chromatography separated into 5 fraction. Also, the low molecular weight peptide of fraction 5 was the highest antioxidant activity. The purified peptide F5 also has significant protective effects on DNA damage caused by hydroxyl radicals. And this peptide was applied to antiproliferative activity against five cancer cell lines by MTT assay, the result showed the purified peptide (F5) had more inhibition against the proliferation of KATO-III cell lines and SW620 cell lines. Moreover, F5 could induce apoptosis in KATO-III cell lines and SW620 cell lines and that showed caspase 3 activity and caspase 8 activity in apoptosis cells. The best protein hydrolysate (F5) was purified by RP-HPLC, identified by LC-MS/MS Q-TOF mass spectrometer and analyzes peptide sequence. The protein showed 4 peptide chains, in all peptide chains contained the effective antioxidant amino acid include hydrophobic amino acids such as Pro, Leu, Ala, Trp and Phe and some peptide chain has aromatic amino acids (Phe, Trp and Tyr) believed to possess high antioxidant activity. At last, *U.rigida* is a potential natural antioxidants and ability to inhibition proliferative cancel cells. This might be used in future potentialities including pharmaceuticals, cosmeceuticals and nutraceutical.

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APPENDIX

APPENDIX A

Preparation of buffer and reagents

1. 20 mM Phosphate buffer pH 7.2

20mM KH_2PO_4	2.72 g
20mM K_2HPO_4	8.71 g
150mM NaCl	8.77 g
0.001% N_3Na	0.065 g
Deionized water	1000 ml

Protocal: Mix 1000 ml deionized water with all reagents. Adjust the pH to 7.2 with KOH

2. Bradford solution and protocal

2.1 Bradford stock solution

95% Ethanol	100 ml
88% Phosphoric acid	200 ml
SERVA Blue G	350 g

2.2 Bradford working buffer

Deionized water	425 ml
95% Ethanol	15 ml
88% Phosphoric acid	30 ml
Bradford stock solution	30 ml

Note: Before using, Bradford working buffer must be filtered through the Whatman No.1 paper. It is stored in a brown glass bottle at room temperature.

Bradford's protocal: 1. Pipet protein solution into 96 well plates 20 µl.

2. Add Bradford working buffer 200 µl and shake.

3. Read A_{595} after 2 minutes but before 1 hour.

3. DPPH solution and protocal

0.1 M DPPH 0.004 mg

Methanol 100 ml

DPPH protocal: 1. Pipet protein solution into 96 well plates 40 µl.

2. Add DPPH solution 160 µl and incubate 20 min in the dark room.

3. Shake and Read the absorbance at 517 nm.

4. ABTS solution and protocol

4.1 7 mM ABTS (solution A)

Dissolve 0.096 g ABTS in 25 ml deionized water.

4.2 2.45 mM potassium persulphate (solution B).

Dissolve 0.016 g potassium persulphate in 25 ml deionized water

4.3 ABTS solution

Mix solution A and solution B in the dark room for 12 – 16 hours before using.

Before use it, dilute ABTS solution with distilled water to obtain an absorbance value of 0.7 ± 0.02 at 734 nm

Protocal: 1. Pipet protein solution into 96 well plates 10 µl.

2. Add ABTS solution 300 µl and incubate 10 min.

3. Shake and Read the absorbance at 734 nm

5. Nitric oxide method

5.1 10 mM sodium nitroprusside (SND) in PBS pH 7.2

Dissolve 0.29 g Sodium nitroprusside in 100 ml Phosphate buffer pH 7.2

5.2 0.33% (w/v) sulfanilamide in 20% acetic acid

Start with dissolve 0.33 g sulfanilamide in 100 ml 20% acetic acid (20 ml acetic in 80 ml distilled water.

5.3 0.1% (w/v) N- (1-Napthyl) ethylenediamine dihydrochloride (NED)

Dissolve 0.1 g NED in 100ml deionized water.

Protocal : 1. Pipet protein solution into 96 well plates 25 µl.

2. Add SNP 25 µl and incubate 2.5 h

3. Add 0.33% (w/v) sulfanilamide 100 µl and incubate 5 min

4. Add 0.1% NED 100 µl and incubate 30 min

5. Shake and read the absorbance at 540 nm

6.SOD

WST Working Solution **-** Dilute 1 ml of WST Solution with 19 ml of Buffer Solution.

Enzyme Working Solution **-** Centrifuge the Enzyme Solution tube for 5 sec and dilute 15 µl of Enzyme Solution with 2.5 ml of Dilution Buffer

Protocal : 1. Pipet protein solution into 96 well plates 20 µl.

2. Add WST Working Solution 200 µl.

3. Add Enzyme Working Solution 20 µl and incubate at 37 °C for 20 min

4. Read the absorbance at 540 nm

7.Catalase

Color Reagent

UV Assay Substrate Solution (20 mM H_2O_2) **-** Dilute 200 ml of the 3% H_2O_2 (to 10 ml with 1X Assay Buffer

Protocal :1. Pipet protein solution into quartz 96 well plates 10 µl.

2. Add 240 ml of 1X Assay Buffer to the quartz 96 well plates and mix by inversion.

3. Start the reaction by adding of 500µl of UV Assay Substrate Solution and mix.

4. Follow the decrease of A_{240} for 30 seconds with the kinetic program

8. Glutathione Peroxidase

Glutathione Peroxidase Assay Buffer

NADPH Assay Reagent - Reconstitute 1 vial in 1.25 ml of water. Store the solution at 2–8 °C.

30 mM tert-Butyl Hydroperoxide Solution - Dilute 21.5 ml of Luperox TBH70X to a total volume of 5 ml with water.

Protocal:1. Pipette the volume of Glutathione Peroxidase Assay Buffer into quartz 96 well plates 180 µl.

2. Add 10 µl of the NADPH Assay Reagent and 8 µl of protein.

3. Start the reaction by addition of 2 µl of the 30 mM tert-Butyl Hydroperoxide Solution

4. Follow the decrease of A_{340} for 15 seconds with the kinetic program

9. LB Broth for *E.coli*

Peotone	1 g
Yeast extract	1 g
NaCl	2 g

Protocal: Mix all reagent with deionized water and sterile in 121°C.

10. DNA damage

10.1 2mM $FeSO_4$

Dissolve $FeSO_4$•$7H_2O$ 0.0278 g in 50 ml deionized water.

10.2 0.06 mM H_2O_2

Pipette 18% H_2O_2 0.57 µl mixing with 50mM Potassium phosphate buffer pH7.4

Protocal: 1. Pipet DNA plasmid into PCR tube 8 µl.

2. Add 8 µl of protein and incubate 30 min.
3. Add 3 µl of 2 mM $FeSO_4$.
4. Add 4 µl of 0.06 mM H_2O_2 and check DNA bands by 1% agarose gel electrophoresis.

11. MTT solution

5 mg/ml MTT solution

MTT	5 mg
Deionized water	1 ml

Protocal: dissolve 5 mg MTT with 1 ml deionized water.

12. Mobile phase in RP-HPLC analysis

12.1 Eluent A: 0.1% trifluoroacetic acid (TFA), 1000 ml

start with add 1 ml TFA into 999 ml double deionized water followed by filtration using a cellulose acetate membrane.

12.2 Eluent B: 70 % acetonitrile containing 0.05% TFA, 1000 ml

start with adding 300 ml 0.05% TFA in double deionized water into 700 ml 70% acetonitrile and mixing followed by filtration using PTFE membrane.

APPENDIX B

Amino acid abbreviations and structures

Amino acid	Three-letter	One-letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	v

APPENDIX C

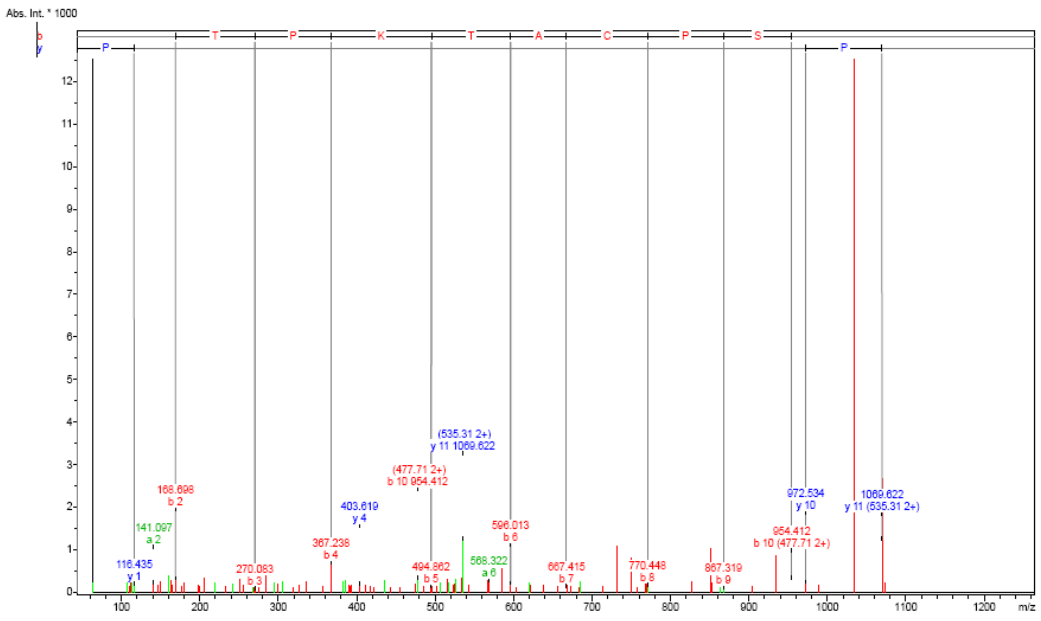
Sequence 1: PATPKTACPSP

Parentmass: 1069.614

Mass Error: 0.079 MH+ (mono): 1069.535 MH+ (avg): 1070.243

Threshold (a.i.): 0.000 Tolerance (Da): 0.500 Number of Peaks: 112

Above Threshold: 112 Assigned Peaks: 15 Not assigned Peaks: 97



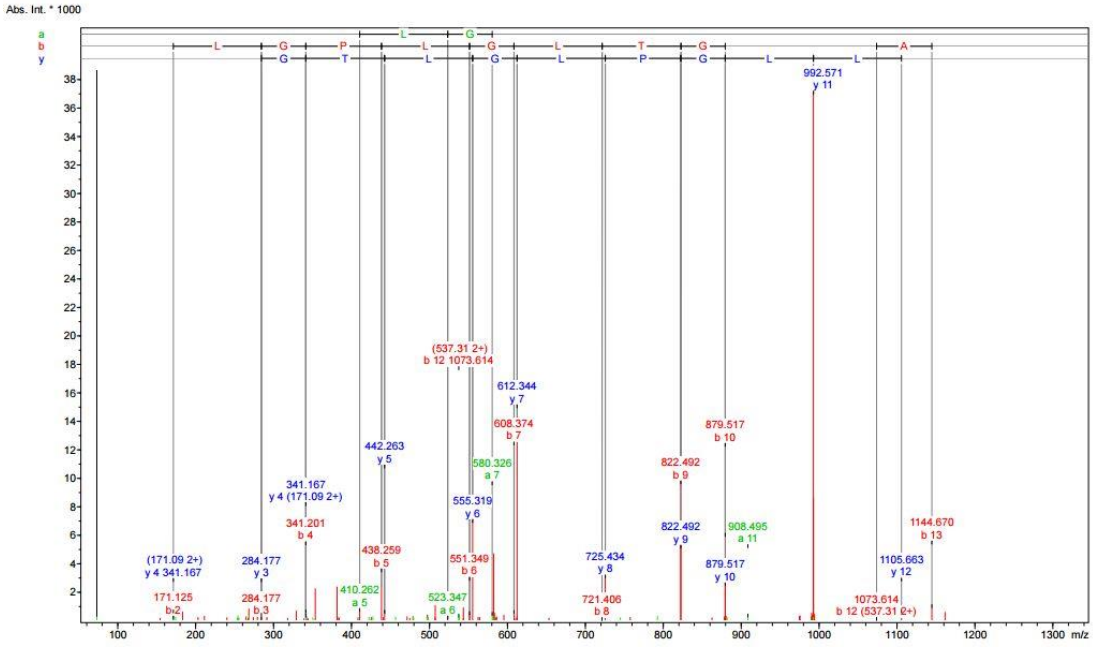
Sequence 2: GLLGPLGLTGGHA

Parentmass: 1162.687

Mass Error: 0.029 MH+ (mono): 1162.658 MH+ (avg): 1163.349

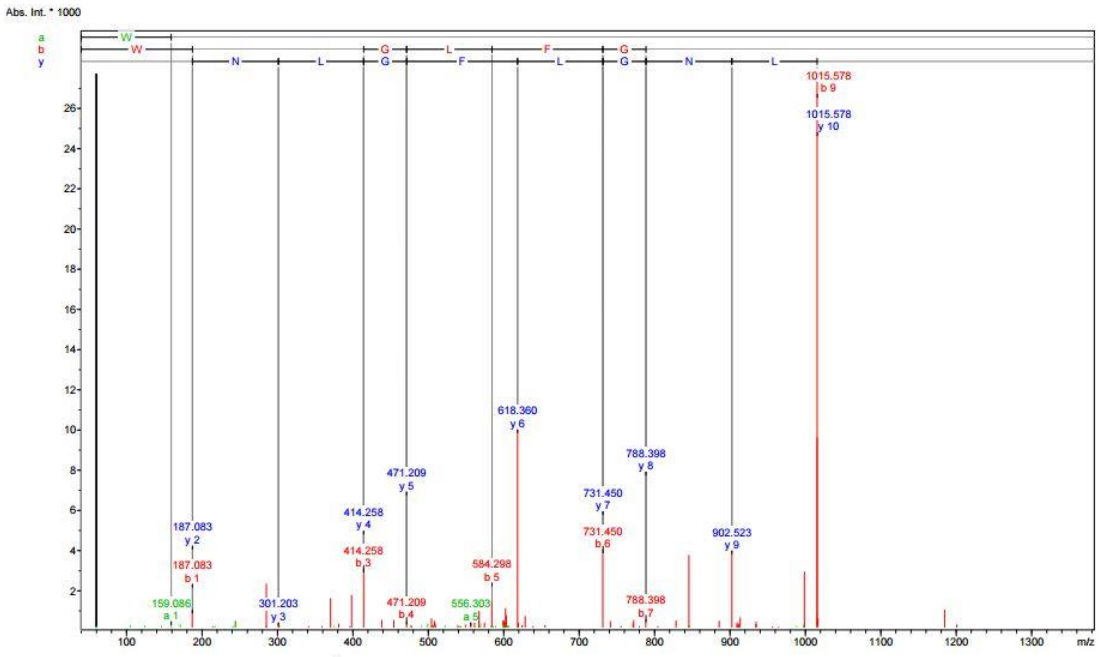
Threshold (a.i.): 0.000 Tolerance (Da): 0.500 Number of Peaks: 107

Above Threshold: 107 Assigned Peaks: 24 Not assigned Peaks: 83



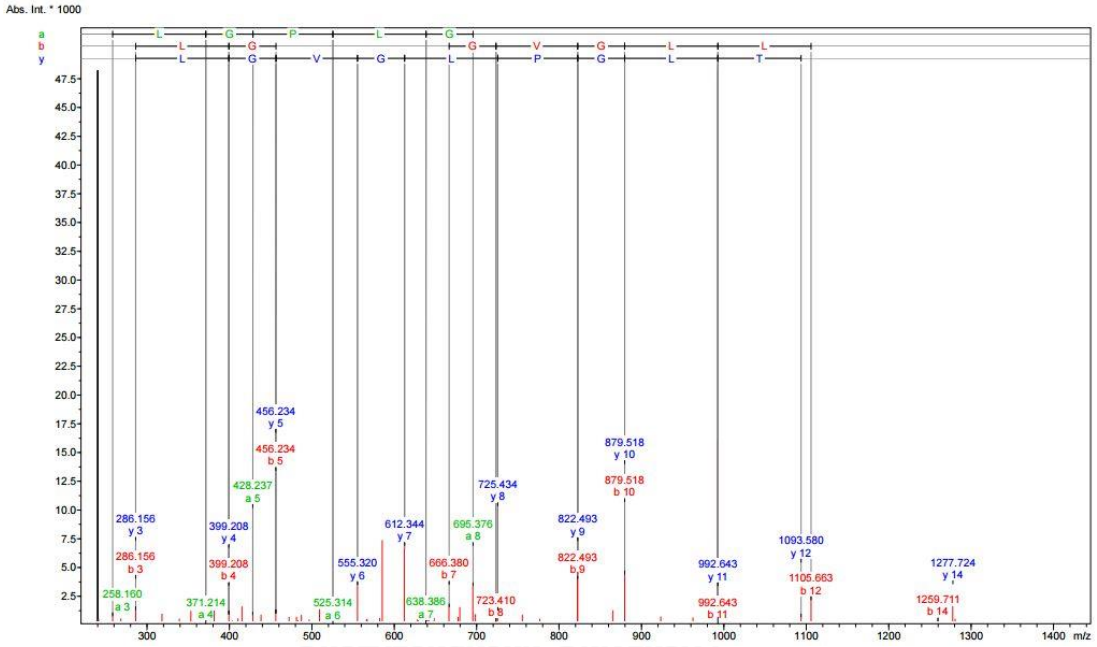
Sequence 3: WLNGLFGLNPA

				Parentmass:	1201.653
Mass Error:	0.016	MH+ (mono):	1201.637	MH+ (avg):	1202.384
Threshold (a.i.):	0.000	Tolerance (Da):	0.500	Number of Peaks:	177
Above Threshold:	177	Assigned Peaks:	12	Not assigned Peaks:	165



Sequence 4: LATLGPLGVGLLGP

				Parentmass:	1277.721
Mass Error:	-0.062	MH+ (mono):	1277.783	MH+ (avg):	1278.563
Threshold (a.i.):	0.000	Tolerance (Da):	0.500	Number of Peaks:	120
Above Threshold:	120	Assigned Peaks:	21	Not assigned Peaks:	99



VITA

Miss Siriluk Kaewmanee was born on September 21st, 1990 in Bangkok, Thailand. She graduated with Bachelor Degree of Science from Department of Marine Science, Faculty of Science, Chulalongkorn University in 2012. She has further studied to the Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University in 2013.

Academic presentations;

1. Kaewmanee, S., Sangvanich, P., and Karnchanatat, A. Antioxidation and antiproliferative activities of peptide from sea lettuce Ulva rigida. The 10th International Symposium of the Protein Society of Thailand, 15-17th July 2015, Convention center, Chulabhorn Research Institute Bangkok, Thailand.