ฤทธิ์ต้านออกซิเดชันและฤทธิ์ต้านมะเร็งของโปรตีนไฮโดรไลเสตจากเมล็ดแมงลัก *Ocimum basilicum* L.

นางสาวคริษฐา เสมานิตย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIOXIDANT AND ANTICANCER ACTIVITIES OF PROTEIN HYDROLYSATES FROM SEEDS OF HAIRY BASIL *Ocimum basilicum* L.

Miss Karittha Semanit

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	ANTIOXIDANT AND ANTICANCER ACTIVITIES OF PROTEIN HYDROLYSATES FROM SEEDS OF HAIRY BASIL *Ocimum basilicum* L.
By	Miss Karittha Semanit
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Jittra Piapukiew, Ph.D.

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คริษฐา เสมานิตย์ : ฤทธิ์ต้านออกซิเดชันและฤทธิ์ต้านมะเร็งของโปรตีนไฮโดรไลเสตจากเมล็ดแมงลัก *Ocimum basilicum* L.. (ANTIOXIDANT AND ANTICANCER ACTIVITIES OF PROTEIN HYDROLYSATES FROM SEEDS OF HAIRY BASIL *Ocimum basilicum* L.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. จิตรตรา เพียภูเขียว , 59 หน้า.

ในปัจจุบันประเทศไทยกำลังเข้าสู่สังคมสูงอายุ การบริโภคอาหารเพื่อสุขภาพเป็นที่น่าสนใจโดยกลุ่มผู้บริโภคซึ่งกำลังมีแนวโน้มเพิ่มมากขึ้น อนุมูลอิสระเป็นอะตอมหรือสารประกอบที่มีอิเล็กตรอนเดี่ยวอยู่ในออร์บิทอลวงนอกสุดซึ่งมีระดับพลังงานสูง จึงมีความว่องไวในการทำปฏิกิริยากับโมเลกุลต่างๆในร่างกาย ปกติแล้วเซลล์มีกระบวนการที่จะสามารถกำจัดอนุมูลอิสระออกไปได้ แต่จะมีเพียงส่วนน้อยเท่านั้นที่ไม่สามารถกำจัดได้ จึงทำให้เกิดการเสื่อมสภาพของเซลล์ นำไปสู่โรคต่างๆ เช่น โรคหลอดเลือด โรคมะเร็ง โรคอัลไซเมอร์ และโรคข้ออักเสบ เป็นต้น ดังนั้นงานวิจัยนี้ มีจุดประสงค์ในการหาสภาวะที่ดีที่สุดในการผลิตโปรตีนไฮโดรไลเสตที่มีสมบัติต้านอนุมูลอิสระจากเมล็ดแมงลัก ด้วยการย่อยโดยใช้เอนไซม์ 3 ชนิด คือ โปรติเอสจี 6, เปปซิน-เพนครีเอติน และปาเปน รวมถึงประเมินความสามารถในการยับยั้ง การเกิดอนุมูลอิสระของโปรตีนไฮโดรไลเสตที่ผ่านการย่อยจากเอนไซม์แต่ละชนิด หลังจากนำโปรตีนไฮโดรไลเสตที่ได้มาผ่านการกรองด้วยเทคนิคอัลตราฟิลเตรชันแล้ว นำมาทดสอบฤทธิ์ต้านออกซิเดชันด้วยเทคนิคที่ต่างกัน 4 เทคนิคคือ DPPH, ABTS, NO และ H_2O_2 ซึ่งจากผลการทดลองพบว่า เอนไซม์ที่ให้โปรตีนไฮโดรไลเสตที่มีสมบัติต้านออกซิเดชันที่ดีที่สุดคือ เอนไซม์โปรติเอสจี 6 ความเข้มข้น 580,000 Du ใช้ระยะเวลาในการย่อย 270 นาที เทคนิค DPPH และ ABTS แสดงให้เห็นว่า โปรตีนไฮโรไลเสตจากเมล็ดแมงลัก สามารถลดปริมาณอนุมูลอิสระโดยปฏิกิริยาการส่งผ่านอิเล็กตรอนและปฏิกิริยาลูกโซ่ได้ดี นอกจากนี้ เมื่อนำมาทดสอบความสามารถในการยับยั้งการเจริญเติบโตของเซลล์มะเร็ง พบว่าสามารถยับยั้ง เซลล์มะเร็งกระพาะอาหาร เซลล์มะเร็งตับ เซลล์มะเร็งปอด เซลล์มะเร็งลำไส้ และเซลล์มะเร็งเต้านมได้ดีกว่าด็อกโซรูบิซินซึ่งเป็นยาที่ใช้อยู่ปัจจุบัน

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS: PROTEIN HYDROLYSATE/ ANTIOXIDANT/ HAIRY BASIL

KARITTHA SEMANIT: ANTIOXIDANT AND ANTICANCER ACTIVITIES OF PROTEIN HYDROLYSATES FROM SEEDS OF HAIRY BASIL *Ocimum basilicum* L.. ADVISOR: ASST. PROF. JITTRA PIAPUKIEW, Ph.D., 59 pp.

Nowadays, Thailand is going to be aging society. Consuming healthy food and dietary supplement are interesting and becoming increasing by consumer. Free radicals are atoms or compounds which have unpair electron at the high-energy outest ring of orbital. Therefore, they quickly react with other molecules within cells in human body. Normally, there are mechanisms to get rid of them out of human cells but there are also partial free radicals that cannot be eliminated. These uneliminated free radicals causes cell damages which lead to many diseases such as coronary thrombosis, cancer, Alzheimer's and arthritis. Thus this research aimed to find an optimized condition in production of antioxidant protein hydrolysates from hairy basil seeds by digestion of 3 enzymes, proteaseG6, pepsin-pancreatin, and papain, and evaluate ability of free radical scavenging of obtained proteins from each enzyme digestion. After ultrafiltration the protein hydrolysates were detained. The antioxidant activities were analyzed by 4 techniques, DPPH, ABTS, NO and H_2O_2. From results, digestion with 5.8 x 10^5 Du proteaseG6 for 270 min produced highest effective protein hydrolysates from hairy basil seeds. Besides, DPPH and ABTS analysis revealed that protein hydrolysates from hairy basil seeds could decrease free radical by electron transfer and chain breaking. In addition, The study in 5 cancer cell lines, human breast carcinoma cell line (BT4740), human liver carcinoma cell line (HEP-G2), human lung bronchus carcinoma cell line (CHAGO), human colon carcinoma cell line (SW620) and human stomach carcinoma cell line (KATO-III), showed that obtained hydrolysis proteins could inhibit cell proliferation better than doxorubicin which is commercial medicine used in the present.

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

Academic Year: 2013 Advisor's Signature .....

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LIST OF ABBREVIATION

Ab	absorbance
ml	millilitre
l	litre
µ	microlitre
g	gram
mg	milligram
mg/l	milligram per litre
M	molar
Mm	millimolar
mmol	millimole
min	min
hr	hour
IC_{50}	Median inhibitory concentration 50%
w/v	weight per volume
w/w	weight per weight
v/v	volumn per volumn
MW	molecular weight
rpm	revolution per min
NaCl	sodium chloride

BSA	bovine serum albumin
EDTA	ethylenediamine tetraacetic acid
NED	N-(1-naphthyl) ethylene diamine
S.E.	standard error
et al.	and others
%	percentage
SOD	Superoxide dismutase
SODs	Superoxide dismutase enzymes
O_2•–	Superoxide anion
H_2O_2	Hydrogen peroxide
GPX	Glutathione peroxidase
GSH	Glutathione
BHT	Butylated hydroxyl toluene
BHA	Butylated hydroxyl anisole
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
NO	Nitric oxide

CHAPTER I
INTRODUCTION

Protein hydrolysates are product of hydrolysis reaction from protein. Mixtures of free amino acid are prepared by splitting protein with chemicals or enzymes. Chemical protein degradation is the low cost precedure in protein hydrolysates process, but limitations in its use in food product or pharmaceutical, agitated to control product quality. (Lamsal 2006) The enzymatic hydrolysis by controlling condition such as hydrolysis time, temperature, pH, enzyme unit is method that is more effective to provide the maximum among of peptides. The protein hydrolysates from enzymatic reaction is a specific substrate, (Mahmaod 1994) which is a mild condition can control by choosing types of enzyme and optimized condition. The obtained protein hydrolysates have the required properties. The demand for the use of protein hydrolysates increase due to the low cost materials from agricultural products derived from plants or animals, safety and their inherent high nutritional value. (Franek 2000) Protein hydrolysates from plants are the products of hydrolyzed materials that are high in proteins as amino acids, peptides and other compounds with aromatic ring types, such as phenylalanine, tyrosine, tryptophan, histidine and cysteine. (Wang 2006) These amino acid group have demonstrated antioxidant activity because they can donate protons to the free radicals. Reported short polypeptides consist of histedine, tryptophan and tyrosine showed high antioxidant activity. (Xie 2008)

Oxidation reaction is one of the main causes for many diseases and pathogenesis in human such as free radical attack on proteins, lipids, nucleic acids, results in cell damage and apoptosis. Free radical plays important role in pathophysiological process such as cardiovascular diseases, Alzheimer's disease, inflammation and certain cancer (Berlett 1997). Reactive oxygen species (ROS) are chemically reactive species because of their unpaired electron sometimes referred to as "free radicals" other such as reactive nitrogen species (RNS), Hydrogenperoxide, singlet oxygen, hydroxyl radicals (OH), peroxyl radicals (OOR), superoxide anion (O_2) and peroxyl nitrile (ONOO-) are non-radical species but their allowing to oxidize biomolecules with some ROS and have an important physiological functions. (Halliwell 2007)

Antioxidant plays a vital role in human body, reducing oxidative processes. The human body has endogenous antioxidants including enzymes such as superoxide dismutase, catalase and glutathione peroxidase where the nonenzymatic compounds such as selenium, α-tocopherol and vitamin C may affect free radicals by an indirect way, for example by inhibiting the activity of prooxidation enzymes (Evgeny 2005) and may help to protect tissues and organs from oxidative damage caused by ROS and RNS. (Wojcik 2010) In addition to these, amino acids, peptides and proteins also contribute to the overall anti-oxidative capacity of cells and towards maintaining the health of biological tissues. In the recent years, a considerable amount of researcher have also focused on the liberation of antioxidant peptides encrypted within food

protein, with a view to utilizing such peptides as functional food in gradients aimed at health maintenance. Interestingly, with the parent protein sequence, the peptides are inactive and thus must be released to exert an effect. These bioactive peptides are 2-20 amino acid residues in length, although some have been reported to have more bioactivity than amino acid residues. (Ryan 2011) Most researchers agree that free radicals are involved in the initiation stage and the promotion stage of cancer. At the initiation stage free radicals to reunite with various carcinogens may change the genetic of normal cells. In the promotion stage free radicals are affect to organic peroxides, which promoted cancer development. (Floyd 1990)

Advanced technologies such as surgery, chemotherapy and new medicines can be used to treat cancer, however incidence of cancer is increasing every day. Oncologists play their intention to prevent or slow down the growth of cancer cells with cure concurrently. Nowadays the treatment process for cancer patients are surgery to remove the tumor away, using high doses radiation to destroy the remaining of cancer cells, chemotherapy with chemical that are able to damage cancer cells directly or use a several methods to increase effectiveness. Developing a new medicines are necessary to cure cancer, for more effectively and with a minimum side effect to the patient. The using of bioactive compounds is called biotherapy which minimum side effect to the patient. Biotherapy is interesting new method for cancer treatment. This method is widely studied in a various countries. Extraction and purification methods were utilized to process improvement for support demand.

Protein hydrolysates have been reported to inhibit and be potential cancer chemopreventive agent for humans. Bowman-Brink protease inhibitor (BBI) is protein isolated from soybean, shown anticarcinogenic activity *in vitro* and *in vivo.* (Armstrong 2000) Reported Lunasin from soybean are a peptides that have an ovarian cancer by inhibit urokinase. More over, protein hydrolysates from soybean can inhibit growth of colon cancer cells by increasing cerebration of hormone somatostatin. (De Lumen 2005)The aims of this research were to purify and characterize protein hydrolysates from hairy basil (*Ocimum basilicum* L.) and to study their antioxidant and anticancer activities. The findings from this work will be helpful for undersatanding protein hydrolysates from hairy basil seeds and may well be significant for value-added economic development.

Objective

1. To determine the optimal conditions for the preparation of protein hydrolysates from the seeds of hairy basil with antioxidation activity and anticancer activity.
2. To purify and identify peptide from seeds of hairy basil.

CHAPTER II

LITERATURE REVIEWS

Free radicals

2.1 Oxidation and free radicals

Free radicals are atoms or molecules containing unpaired electrons, they may be generates by electron transfer reactions and energy transfer reactions (Erbas 2011) and usually unstable toward using or picking electron called oxidation reaction. Oxidation reaction causes free radicals which a reason of side chain reaction. Its can damage cell, despite oxidation reaction have an important to organism.

Oxidation reaction is one of main causative factor for many diseases such as cardiovascular disease, Alzheimer's disease, inflammation and certain cancer. They are 3 types of compounds, which can directly suppress free radical formation: antioxidants, free radical scavengers, and chelator. In addition to direct antioxidants, there 2 other important groups of free radical inhibitors antioxidant enzyme and the compounds possessing indirect antioxidant properties. Compounds having indirect antioxidative properties may affect the formation of free radicals by an indirect way, such as by inhibit pro-oxidant enzymes. (Erbas 2011)

2.2 Antioxidant

Antioxidant are substance that can reducing oxidative process or resist oxidation and can eliminate free radicals from the body. (Diplock 1991) (Asplund 2002) A biological antioxidant defined as "any substance that, when present at low

concentrations compared to those of an antioxidisble substrate, significantly delats or prevents oxidation of that substrate" (Halliwell 1995) Antioxidant system of the body is divided into 2 types are enzyme and antioxidants. The function is depend on different mechanism such as scavenge, inhibit and chelate.

2.3 Example of antioxidant

2.3.1 Natural antioxidant

2.3.1.2 Vitamin C

Vitamin C or ascorbic acid can be found in many fruit and vergetables, its solubility in water. (Buettner. 1993)

Ascorbate is an important dietary derived antioxidant, many research shows decreasing free radical in both plasma and pure aqueous solution (Benzie IFF 1999)

Ascorbic acid has a very active hydroxyl group and very efficient free radical scavenger. Oxidation and reduction reactions of vitamin C are widely studied. (Afanas'ev 1989) Its react with perhydroxyl radical quickly but slowly with superoxide. (Cabelli 1983)

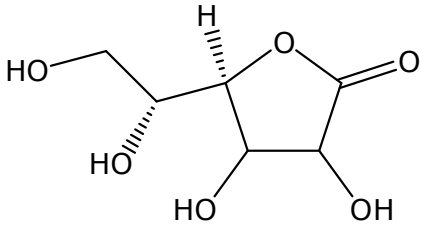


*Figure 1*Vitamin C

2.3.1.3 Vitamin E

α-Tocopherol is a lipid soluble, hydroxyl group of vitamin E is very active, which is responsibility for antioxidant. In contrast, superoxide scavenging cannot be inhibited by vitamin E but super oxide is able to drawn a hydrogen atom from vitamin E. (Afanas'ev 1989) Tocopherol acts as a hydrogen to peroxyl radical

α-tocopherol + LOO· ⟶ **α**-tocopherol· + LOOH

Figure 2 Tocopherol provide hydrogen atom to peroxyl radical

α-tocopherol· Can react with other peroxyl radicals are substances that are made stable (LOO-**α**-tocopherol) (Sodergren 2000)

α-tocopherol· **+** LOO· ⟶ LOO-**α**-tocopherol

Figure 3 **α***-tocopherol. can react with other peroxyl radicals are substances that made stable (LOO-***α***-tocopherol) There are many kind of vitamin E in natural which is separate into 2 groups ; tocopherol and tocotrienol.*

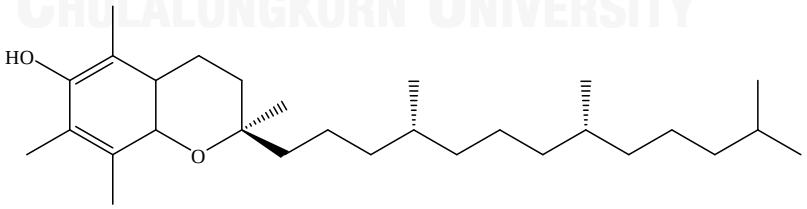
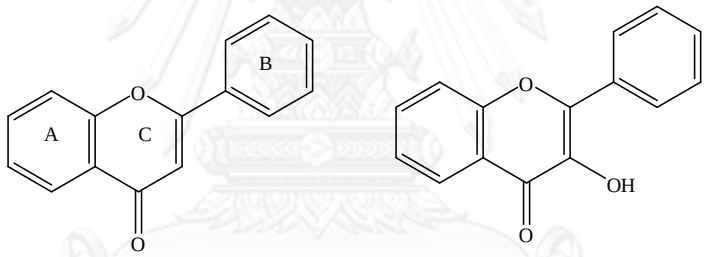


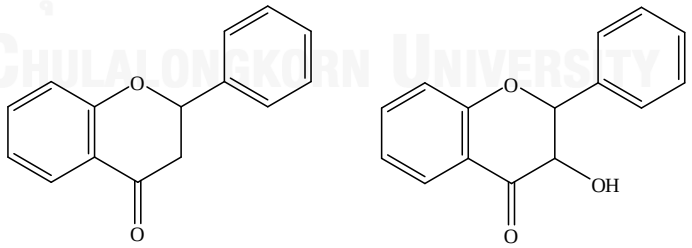
Figure 4 vitamin E

2.3.1.4 Flavonoid

Flavonoids are group of phenolic compound found on fruit or vegetable. The main sources of flavonoids are apples, onions, berries, tea, beer, and wine. Most of these compounds belong to four main groups: flavones, flavonols, flavanone, and flavanols. (Havesteen 1983) The functions of flavonoids are pigment, light filters with a specific wavelength and antioxidants, scavenging radical that occur in plant cells. Properties of flavonoid more over antioxidant its can decrease inflammation, conjugated the arteries for improve blood circulation system, against bacteria and virus and reduce cholesterol.



I Flavones II Flavonols



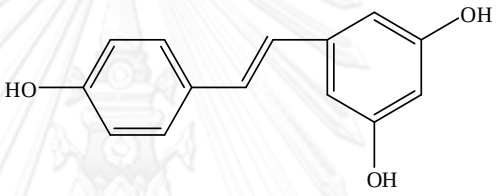
III Flavanones IV Flavanols

Figure 5 *4 main group of Flavonoids*

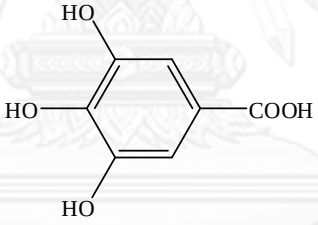
2.3.1.5 Phenolic compounds other than flavonoids

There are various polyphenolic compounds activating in antioxidant one of nonflavoniods is reaveratrol (3,5,4'- trihydroxy-*trans*-stilbene) which can inhibit free radical-mediated cellular process. (Stojanovic 2001)

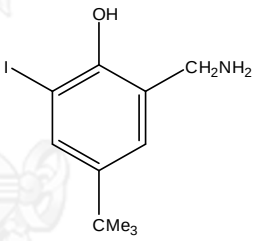
Same as flavonoids reaveratrol may exhibit prooxidant activity, such as promote DNA fracmentations despite, its prooxidant properties is unnecessary under physiological conditions. (Burkitt 2000)



Resveratrol



Gallic acid



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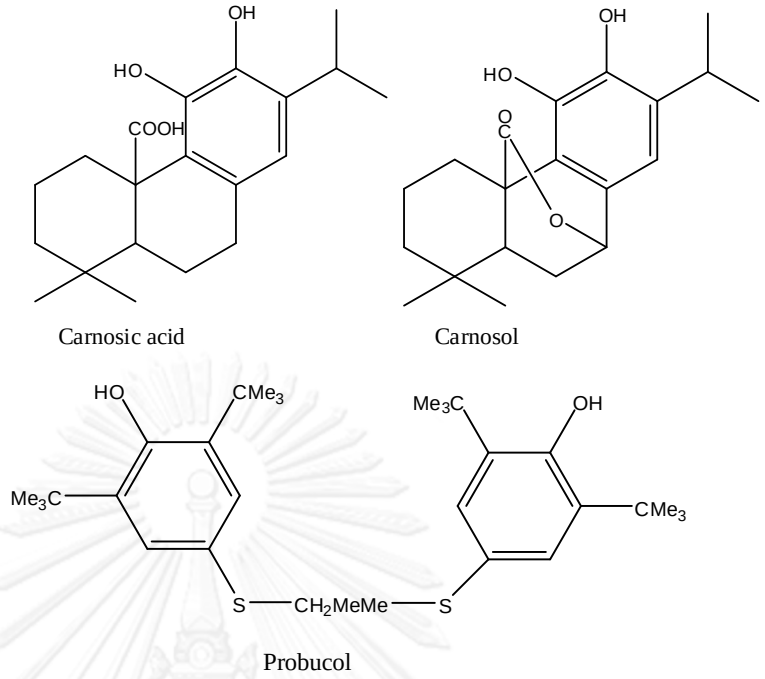

Figure 6 *Phenolic compounds other than flavonoids.*

2.3.1.6 Thiols

Thiols are contain with very active SH group thus they are potentially efficient antioxidants. Endogenous and synthetic thiols, have been studied and applied as antioxidative drugs and food supplements, but the most important antioxidant thiols are undoubtedly lipoic and glutathione.

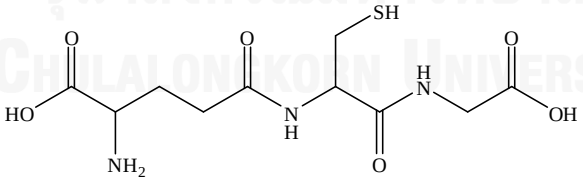

Figure 7 *glutathione*

2.3.1.7 Carotenoid

ß- Carotene is a nutrient that found in fruits and vergetables. Carotenoids were considered to be the antioxidant and free radical scavengers. ß-carotene chemical structure very active in free radical reactions. (Everett 1996)Tetraterpene skeleton of carotenoids are may be a ring at the end of one or both of side chain. Single oxygen of free radical can repressed by carotenoids and another function of carotenoids is to shield cells by absorbing excess excitation energy from chlorophyll (Arora 2002)

2.3.1.8 Vitamin B6

Vitamin B6 (pyridoxine) can inhibit superoxide production, reduce lipid preroxidation and glycosylation in high glucose-exposed erythrocytes. (Jain 2001)
The suppression of oxidative stress in erythrocytes may be a natural compound which is the development of complication in diabetes (Evgeny 2005)

2.3.1.9 Amino acid

Amino acid such as arginine do not have free radical scavenging substituents, but it has been proposed can inhibits myocardial contractility in buffer-perfused rat hearts by suppressing oxygen radical generation by xanthine oxidase. Taurine is an effective scavenger of hypochlorous acid, which is known to participate in tissue damage associated with reperfusion injury mediated by neutrophils. Thus, taurine protected against the cytotoxic action oh HOCl in neuronal cells (Kearns 2000) Futhermore, taurine was found to reduce hydroxyl radical-induced damage to DNA. (Messina 2000)

2.3.2 Synthesis antioxidant

2.3.2.1 BHT

Butylate hydroxyl toluene is a phenolic compounds commonly used as a food stabilizer. BHT can biding free radicals, to reduce oxidation reaction and inhibit chain reaction. (Maria 1985)

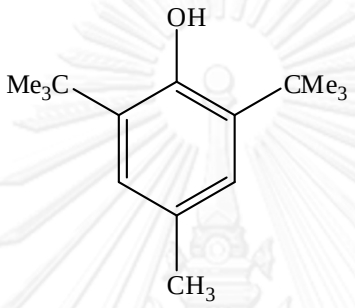

Figure 8 *Butylate hydroxyl toluene*

2.3.2.2 BHA

Synthetic phenolic antioxidants butylated hydroxyl anisole is the most common used of antioxidants in food product. (USDA, 2000) BHA also used in medicines, such as isotretinoin, lovastatin and simvastatin. Conjugated aromatic ring of BHA is able to delocalized free radicals to inhibit oxidation reduction. (Lam 1979)

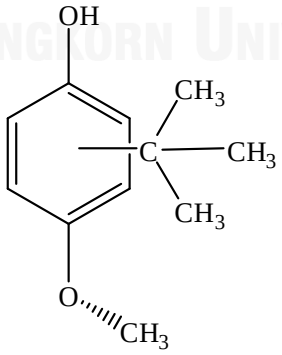

Figure 9 *butylated hydroxyl anisole*

2.3.2.3 Trolox

Trolox or 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid is a derivative of vitamin E. It is a popular free radical scavenger and the carboxyl group which is a water soluble in the chemical structure of trolox made its more advantage than vitamin E in antioxidant activity. (Lam 1979)

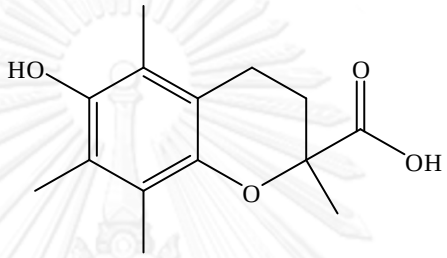

Figure 10 *Trolox*

2.3.2.4 TBHQ

Tert-Butylhydroquinone is a hydroquinone derivative which is an aromatic ring. TBHQ is an effective radicals inhibitor in food and popular in industry as a stabilizer of organic peroxidation. (Almeida 2011)

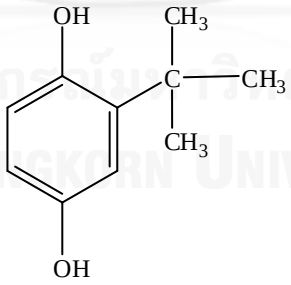

Figure 11 *TBHQ*

2.3.2.5 EDTA

Ethylenediaminetetraacetic acid is an aminopolycarboxylic acid that is a powerful antioxidant activity. EDTA can biding to metals such as iron, zinc, lead and

cadmium, can used as a metal removaland to prevent an injuring blood vessel walls from free radical. (Martin-Sanchez 2014)

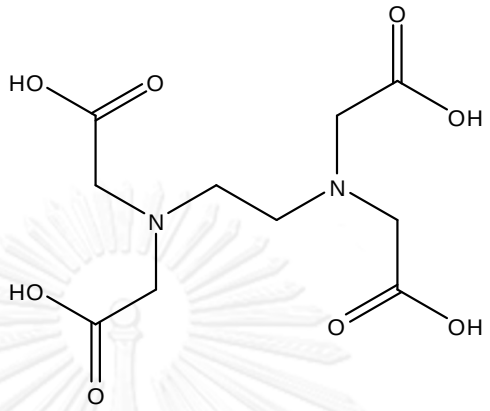

Figure 12 *EDTA*

2.4 cancer and free radical

Thailand is undergoing an increasing rates of cancer. Worldwide cancer incidence is projected to rise 70% by the year 2030. (Ferlat 1993)

Cancer is the majority cause of death of the population in most countries, and a commonly disease in world population, especially in developing countries from industrial pollution. Countryman lake of attention to their health, lack of exercise, junk food these cause cancer increase. Currently, the number of deaths from cancer in Thailand is much more than any disease in the past, such as infectious disease, cardiovascular disease, malnutrition or accident. Almost cancer patients known that cancer once logged to the last period. Nowadays cancer can be detected in several way including the presence of certain signs and cancer screening. Scientist interested in the role of free radicals in cancer cells reported the excessive production of free radicals in tumor cells. So, scientists have studied the association between cancer and

free radical in order to developing the treatment of cancer patients. Reported on the possible role of radicals in carcinogenesis. (Wei 1993)

Free radicals are able to increase or decrease the risk of cancer base on diverse situations and can act dual roles as cancer inhibitors or accelerators under different conditions. (Abdollahi 2012)

Modern technology such as surgery, radiation and new medicines are the way to treat cancer patients but the procreation of cancer are increasing. Treatment and prevention of cancer are developed along the way. Surgery is a treatment to remove the tumor and used a radiation to destroy cancer cells it remain or not removed and with the powerful of chemicals may be damaged a normal cells. So to develop a new medicines is a necessary to treat cancer. Bioactive compounds are more effectively and more safety to patients, the biological treatment is called biotherapy. Reported soy protein which is an antioxidant chemical structure can inhibit the growth of cancer cells, polypeptides from digested soy protein can inhibit ovarian cancer cells by hinder the work of urokinase. (De Lumen 2005)

2.5 protein hydrolysates

Protein hydrolysates commonly known as peptides, are used in a variety of product in biotechnology industry, it has been used for the production of value added products such as food, supplements, agriculture and pharmaceutical. The screening and manufacturing of protein hydrolysates are more complicated, with the source of material, analytical instrument, screening techniques, digested condition and

a novel enzyme. Enzymatic digestion is a good choice to produce protein hydrolysates it easier than chemical digestion to control product quality not only a mild condition but also high specific. Protease enzyme react with a molecule of water when peptide bond are split among carboxylic group and amino group is more independent, the ability and structure of protein were change. Digested protein were short and may have a biological activity because the side chain of short peptide is specific for amino acid by enzymatic digestion. Antioxidant activity is an important biological activity of protein hydrolysates. (Je 2007)

2.5.1 Source of protein hydrolysates

Sources of protein hydrolysates are mostly cheap or waste material from plant or animals. (Quitain 2011) such as waste from seafood processing, oil plan processing and slaughterhouse. Protein hydrolysates from plant is the product of the hydrolyzed material that is high in protein to amino acids, peptide and other compounds. Which is aromatic ring amino acid type; phenylalanine, tyrosine and tryptophan can donated proton to free radicals resulting material properties that can fight free radicals. Reported low molecular weight peptide comprised of histidine tryptophan and tyrosine that is effective in antioxidant activity.

2.5.2 Protein hydrolysates in antioxidant

Source of protein	enzyme	activity	results	Reference
peanut	alcalase	DPPH, Reducing chelating, ß-carotene bleaching power, metal	Protein hydrolystates more effective than protein isolate	(Jamdar 2009)
Plam kernals	Esperase, neutrase, pepsin, protease A, protease N	DPPH, Metal chelation, reducing power	Protein hydrolysates which molecular weight less than 3.5 kDa higher than ascorbic acid	(Hwang 2010)
alfalfa	alcalase	DPPH	Peptide which has molecular weight less than 1 kDa is the most antioxidant activity	Xie 2008
Lima bean and Jamapa beam	alcalase	ABTS	Protein hydrolysates consist of hydrophobic amino acid.	(Juan Torruco-Uco. 2009)
Peanut kernels	esperase	DPPH, reducing power	Protein hydrolysates which is molecular weight between 3-5 kDa is the most antioxidant activity	(Hwang 2010)

Source of protein	enzyme	activity	results	Reference
Rice endosperm	Neutrase	DPPH, hydroxyl and superoxide radical-scavenging	Peptides sequence are KHDRGDEF and FRDEHKK	(Zhang 2010)
Corn gluten	alcalase	Reducing power, lipid peroxidation and scavenging activity	Protein hydrolysates which is molecular weight between 0.5-1.5 kDa is the most antioxidant activity	(Zhang 2013)
Zein	Pepsin-pancreatin	Radical chelating and scavenging activity	Protein hydrolysates which is molecular weight less than 0.5 kDa is the most antioxidant activity	(Lijuan 2008)

2.6 Hairy basil

Hairy basil (*Ocimum basilicam* L.) is a biennial plant that can be grown easily all over Thiland. It can took an advantage of both of leaves and seeds. Fresh leaves often used for cooking, the seeds are eaten as a dessert and used as a mild laxative herbal texts. In addition, it can reduce blood sugar levels in diabetes patient. Reported oils from hairy basil seeds are composed of essential fatty acid in a high proportion (Suksai 2007) and also offer a benefit of the pharmaceutical and prevention. As the results of oil extraction, hairy seeds waste can added a value of this residue by generate

to protein hydrolysates with is enzymatic process. Bioactive peptides can be used in a preventing degenerative disease, more over may be used for applications in medical, pharmaceutical or research industry ;cosmetic and food industry.

CHAPTER III
EXPERIMENTS

3. Materials and methods

3.1 Plant materials

The dry hairy basil seeds (*Ocimum basilicum* L.) were provided from Yoawaraj market, Bangkok, Thailand. The sample was quickly taken to laboratory and kept in dark room at 4 ^{o}C until used.

3.2 Chemical and biological materials

Ascorbic acid, bovine serum albumin (BSA), α-tocopheral, sodium pyruvate, curcumin, 2,2-Diphenyl-1-picryhydrazyl (DPPH), ABTS, were purchased from sigma chemicals Co. (USA). Protease G6 (alkali serine protease) was purchased from Siam Victory Chemicals Co., Ltd (Thailand), Pancreatin from porcine pancreas, pepsin from porcinegastric mucosa, papain from sigma. Other biochemical reagents and general chemicals used were analytical grade.

3.3 Preparation of hairy basil seeds

3.3.1 Defatted hairy basil seed preparation

Raw hairy basil seeds were selected and cleaned to remove contaminants, crushed in a blender and was defatted by petroleum ether soxhlet for 10-12 hour at 65 ^{o}C. After that defatted hairy basil seeds were dried in hot air oven at

60 °C then the defatted Hairy basil seeds were sieved by using laboratory hammer mill through 90 micrometer and dry sample was kept in the desiccator until used.

3.4 Total amino acid analysis

3.4.1 Acid hydrolysis

Five milliliters of 6N HCl was added (5mg protein/ml HCl) and mixed together with dry sample. The tube was flushed with nitrogen for 1 min to remove air. Hydrolysis was then carried out at 110 °C for 22 hours. The internal standard (10 ml of 2.5 mM L-α-amino-*n*-butyric acid in HCl 0.1M) was added and diluted with water to 250 ml. The solution was filtered with 0.2 μm filter and was then derivatized with 6 aminoquinolyl-N-hydroxysuccinimidyl carbamate. (AccQ-Flour reagent) It was heated in a heating block at 55°C, for 10 min. Heating converts a minor side product of tyrosine to a major mono-derivatized compound. Total amino acid content was determined by high performance liquid chromatography. (Spackman 1958)

3.4.2 Chromatographic conditions

Chromatographic separation was carried out in a Waters Alliance 2695 with heater amino acid analysis Hypersil Gold column C18. The column was thermostat at 35 ± 1 °C and the flow rate was 1.0 ml/min. The injection volume was 5 μl, eluted isocratically with sodium acetate buffer pH 4.90 and 60% acetonitrile. (Spackman 1958)

3.5 Preparation of hairy basil seed protein hydrolysates with protease G6

The sieved hairy basil seeds were hydrolyzed by using protease G6 (alkali serine protease) according to the method described by Vastag. (Vastag 2001)

2 conditions of enzymatic digestion, time and concentration of enzyme were adjusted. Hydrolyze ratio substrate: enzyme was 0.5: 10 (g/ml) 6 concentration in a ratio 1:1 (5.8×10^5 DU/g), 1:2 (2.9×10^5 DU/g), 1:4 (1.45×10^5 DU/g), 1:8 (7.25×10^5 DU/g), 1:16 (3.625×10^5 DU/g)(w/v) and substrate without enzyme in 20 mM Tris-HCl buffer containing 150 mM NaCl pH 8.0 were conducted at 50 °C with shaking 150 rpm. Samples were collected every 30 min for 240 min. The reaction was stopped by heating at 90 °C for 10 min. After that, centrifuge at 15,000 x g, for 15 min, at 4 °C to kept supernatant. The optimized condition and time were monitoring by DPPH assay and the protein content was determined by Bradford's procedure. (Bradford 1976)

3.6 Preparation Hairy basil seeds protein with pepsin and pancreatin

The sieved hairy basil seeds 0.5 g/ml was stirred overnight in a cold room at 4 °C with phosphate buffer saline (PBS; 20 mM phosphate buffer pH 7.2 with 0.15 M NaCl) adjusted to pH 2.5 with 1 N HCl. The solution was mixed with pepsin at 3 various concentrations. The ratios of enzyme/substrate are 0.125, 0.25 and 0.5 (g/ml) (w/v). The hydrolysates were carried out for 180 min at 37 °C with shaking 180 rpm. Then, adjusted to pH 7.5 using 1 N NaOH. Pancreatin was added to a 20: 1 (ml/g) substrate per enzyme, shaken 180 rpm for 180 min at 37 °C. The hydrolysis reactioin was stoped by heating at 90 °C for 10 min. (Gallegos-Tintore 2011)

After that, centrifugation was used to keep supernatant at 15,000 x g, 15 min, 4 °C. The optimized condition was monitoring by DPPH assay and the protein content was determined by Bradford's procedure. (Bradford 1976)

3.7 Preparation hairy basil seeds protein with papain

The sieved hairy basil seeds were stirred overnight in a cold room 4 °C with phosphate buffer saline (PBS; 20 mM phosphate buffer pH 7.2 with 0.15 M NaCl), adjusted to pH 6.5 with 1 N KOH. The solution was mixed with papain concentration ratio of substrate/enzyme 20 : 1 (ml/g), the hydrolysates was carried out until 0- 240 min at 60 °C with shaking 180 rpm samples were collected every 30 min. Stop reaction by heating at 90 °C for 10 min. (Naqash 2013) After that, centrifugation was used to keep supernatant at 15,000 x g, 15 min, 4 °C. The best condition was determine by DPPH assay and the protein content was determined by Bradford's procedure. (Bradford 1976)

3.8 Determine of degree of hydrolysis (DH)

Degree of hydrolysis was determined by TCA procedure modulated from Galla. (Galla 2012) The protein hydrolysates from various enzyme; proteaseG6, pepsin-pancreatin, papain with alter concentration of enzyme and time were treated with 10% trichloroaceticacid (TCA) and centrifuge 15,000 x g for 30 min at 4 °C. Total nitrogen in protein hydrolysates and soluble nitrogen in sample was analyzed by Kjeldahl method. Samples were digested with 98% sulfuric acid using copper sulphate as a catalyst, then digested samples was reacted with 33% sodium hydroxide,

ammonia gas from this reaction was reacted with 4% boric acid. Titration boric acid with 0.1N hydrochloric acid and then exacted concentration of HCl with 0.1 N sodium hydroxide.

3.9 Molecular weight cut off by ultrafiltration

Obtained protein hydrolysates from the best enzymatic digestion with optimized condition was classified by size using ultrafiltration fiber membrane. Three protein fractions with different size were separated into X<5,000 kDa, 5,000<X<10,000 kDa and X>10,000 kDa.

3.10 DPPH radical scavenging activity

The DPPH radical scavenging activity was modulated from Mohsen (Mohsen 2009) protein hydrolysates at 10 different concentrations were mixed with 0.1 M DPPH in ethanol for 30 min in the dark room measured by using spectrophotometer at 517 nm. The IC_{50} value (the concentration that cause a decrease in initial DPPH concentration 50%) was determined from the linear regression of the DPPH inhibition against the concentration of protein. The negative control used water while the positive control was ascorbic acid. The percentage of radical was calculated as follows.

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

Where AC is the absorbance of water plus DPPH in ethanol, ACB is the absorbance of the blank (water plus ethanol without DPPH), AS is the absorbance of the sample plus

DPPH in ethanol and ASB is the absorbance of the sample plus ethanol without DPPH. Dilution of samples were used in order to obtain calibration curves and to calculate the IC_{50} values (IC_{50}; concentration required to obtain a 50% radical scavenging activity).

3.11 ABTS scavenging scavenging

The ABTS scavenging activity was modulated from method of Cai. (Cai 2004) Sulphonic acid-6-ethylbenzthiazoline-3(bis-azino-2' ,2) (ABTS) 7 mM in water was incubated with potassium persulfate 2.45 mM in the dark for 12 hour. The solution was diluted until the absorbance 734 nm was exactly 0.7 ± 0.02 nm. Working solution ABTS was mixed with various concentration of samples stand for 10 min, measured by using spectrophotometer **α**-tocopherol was prepared as a positive control as the same condition. The percentage of ABTS radical was calculated using the following formula

ABTS scavenging effect% = $\{[(A_0 - A_1)]/ A_0\} \times 100$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample

3.12 Analysis of nitric oxide scavenging activity

Nitric oxide scavenging activity was modulated from method of Govindarajan. (Govindarajan 2003) Sodium nitroprusside 10 mM in phosphate buffer saline (Griess solution) was mixed with different concentration of samples, incubated for 150 min, after 0.5 ml of incubated solution was added by 1 ml of 0.33% sulfanilamide in 20% acetic acid stand for 5 min and then added 1 ml of N-(1-naphthyl) ethylene diamine dichloride (NED) matured for 30 min. Nitric oxide scavenging activity was measured by

spectrophotometer at 540 nm. alongwith a curcumin as a standard. The percentage of NO radical was calculated using the following formula

NO scavenging effect% = $\{[(A_0 - A_1)]/ A_0\} \times 100$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample

3.13 Analysis of hydrogen peroxide decomposition

The hydrogen peroxide decomposition was belonged to Soares. (Soares 2009) The various concentration of sample was mixed with 50 mM H_2O_2 ratio 1:1 (v/v) and incubated for 30 min. Aliquots mixture 90 μl then added 10 μl of methanol and FOX reagent (prepared by dissolving butylated hydroxyl toluene (BHT) 3.96 mM and xylenol orange 0.1 mM in methanol with ammonium ferrous sulfate 0.256 mM in H_2SO_4 0.025 M). Samples were incubated for 30 min and absorbance was read at 560 nm. Using a spectrophotometer. Sodium pyruvate was used as a positive control. The percentage of H_2O_2 radical was calculated using the following formula

H_2O_2 scavenging effect% = $\{[(A_0 - A_1)]/ A_0\} \times 100$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample

3.14 Protein content determination

Protein hydrolysates were determined by Bradford's procedure. (Bradford 1976) Serial dilution of BSA was us as standard. 20 μl of samples were transfered to 96 well plate and 200 μl of Bradford working solution was added and mixed well then incubated for 10 min before calibration with spectrophotometer at 595 nm.

3.15 Antiproliferation of cancer cell by MTT method

Five different human cancer cell lines; Human breast carcinoma cell line (BT4740, Human liver carcinoma cell line (HEP-G2), Human lung bronchus carcinoma cell line (CHAGO), Human colon carcinoma cell line (SW620) and Human stomach carcinoma cell line (KATO-III) were cultivated in media, after that $5x10^4$ cell/mg were growth in 96-well culture plate incubated in CO_2 incubator at 37 ^oC for 24 hour. 10 various concentrations of samples were prepared and drop into 96-well plate. Culture cell lines were incubated in 5% CO_2 condition at 37 ^oC 24 hour then rinsed samples away DMEM (Dulbecco's Modified Eagle Medium) without FBS, gentamycin and 50 μl MTT (3,[4,4-dimethylthiazl-2-yl]-2, 5-diphynyl-tetrazolium bromide) were added to cell lines incubated for 180 min in dark room. After pouring out MTT solution, DMSO in ethanol was added and shacked for 10 min. Absorbance was read with microplate reader at 540 nm. Percentage of cell viability was calculated using doxorubicin as a positive control.

3.16 Purification antioxidant peptides by HPLC

The active protein hydrolysates which is more effective antioxidant activity after partial purify with ultrafiltration techniques were fractionated by using reverse-phase high performance chromatography (RP-HPLC) on a C-18 (Shim-pack column 250 x 46 mm). And eluted with 0.1% Trifluoroacetic acid (TFA) and linear gradient of acetonitrile (0 – 70%) containing 0.05 TFA in water at flow rate 0.7 ml/min. The injection volume

was 50 µL. The elution peaks were detected at 280 nm and fractions of peptides were speed vacuum to remove solvent before peptides identification.

3.17 identification antioxidant peptides

In this investigation the molecular weight and amino acid sequence of active protein hydrolysates were identified by using direct infusion MS/MS (Bruker daltonics) . The collected peak from RP-HPLC fractions were resuspended with 50% acetonitrile containing 0.1% formic acid and direct subjected to MS/MS with 180 µl/min, dilute in 0.05% formic acid and 2% acetonitrile. The spectra were recorded by the mass/charge (m/z) ranges of 100 – 1200 in both MS and MS/MS modes. All data were processed to MASCOT database.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Amino acid content of hairy basil seeds

Amino acid content of hairy basil was analyzed by acid hydrolysis method and HPLC column which was shown in table 1. Detected amino acids were glutamic acid and aspartic acid, the major non-essential amino acids. Results indicated that all essential amino acids, except S-containing types and tryptophan were presented in high amounts in this species. Moreover, protein showed the presence of phenylalanine, tyrosine and histidine, these amino acids have also been reported to show antioxidant activity as well as high nutritive value because of presence of important amino acids. Therefore, protein hydrolysates have antioxidant activity as well as high nutritive value because of the presence of important amino acid. (Chen 1998) The relationship between hydrophobic amino acid and reducing power of hydrolysates have been previously reported. (Bernardini 2011)

Table 1 Total amino acid of hairy basil

Amino acids	mg/100mg
Aspartic acid	4.61
Serine	3.58
Glutamic acid	10.55
Glycine	3.12
Histidine	1.70
Arginine	8.48
Threonine	2.16
Alanine	2.65
Proline	2.25
Tyrosine	2.08
Valine	2.63
Lysine	1.56
Isoleucine	1.91
Leucine	4.02
Phenylalanine	3.49

4.2 Degree of hydrolysis

Degree of hydrolysis (DH) is a percentage of peptides which is cut between protein digestion. Free amino acids were calculated compare with total amino acids in protein sample. (Adler-Nissen 1986)

4.2.1 Degree hydrolysis of proteaseG6

The maximum DH exhibited by hairy basil seeds protein hydrolysates in the current study (55.134 ± 0.1 %) for 270 min as shown in figure. It was greater than the DH values reported in protease hydrolyzed proteins from other oilseeds such as soy, which had 39.5% upon 8 hour of hydrolysis (HRČKOVÁ 2002) sunflower 42.2% upon 3 hour. (Villanueva 1999) These findings were in agreement with the results of enzymatic hydrolysis of other protein reported by other workers. The DH of rice endosperm protein(Zhang 2010) and wheat germ protein hydrolysates (Zhu 2006) were reported to be 11.7% and 25% after 6 hour of hydrolysis respectively; the DH increase with increase in time of hydrolysis. The maximum DH value of protein hydrolysates from hairy basil obtained by proteaseG6 hydrolysis only was also lower than the recently reported values for Alcalase-Flavourzyme hydrolysates of yellow pea protein hydrolysates (DH: 58.89%), Kabuli (DH:77.58%) and Desi (DH: 77.53%) chickpea protein hydrolysates were prepared by the protease enzymes. (Barbana 2010)

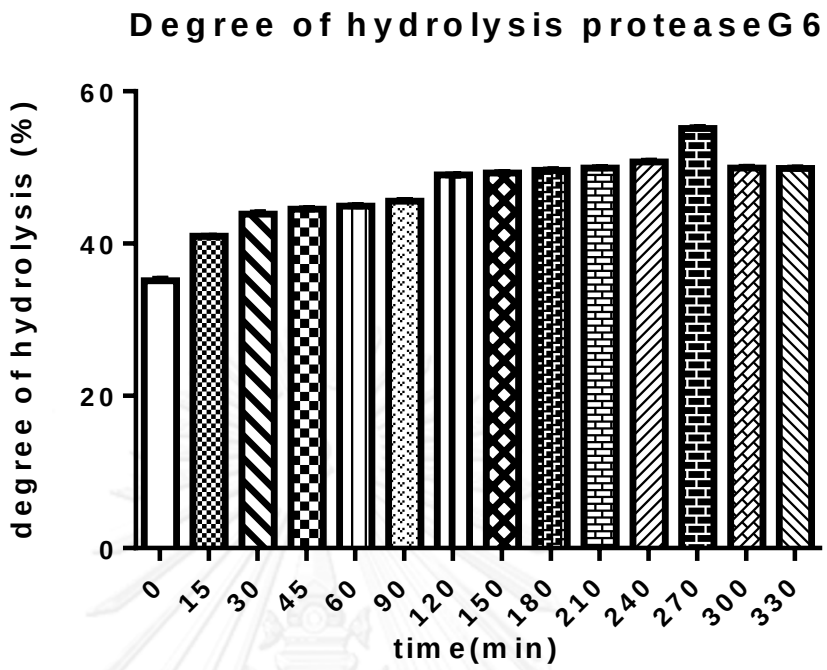

Figure 13 Degree of hydrolysis from protein hydrolysates hairy basil digested by proteaseG6

4.2.2 Degree hydrolysis of pepsin-pancreatin

Protein hydrolysates obtain from hairy basil by enzyme pepsin-pancreatin had 65.512 %DH highest at 3 hour as show in figure. It was greater than ornate threadfin (20%), which had high ability in antioxidant in DPPH. (Nalinanon 2008) By product of threadfin bream had reported maximum 30%DH at 15 min, however 5%DH was greater antioxidant activity than 30% tested with ABTS and DPPH. (Wiriyaphan 2012) 34% was found in rohu (Labeo rohita) at 3 hour followed by time. (Chalamaiah 2013)

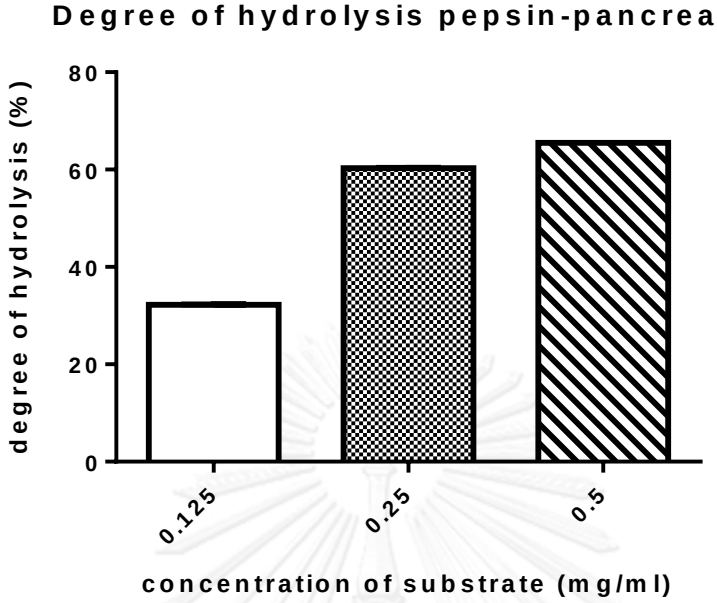

Figure 14 degree of hydrolysis from protein hydrolysates hairy basil digested by pepsin-pancreatin

4.2.3 Degree hydrolysis of papain

Papain is a cysteine protease from a plant source maximum DH of hairy basil was higher than older reported kabuli pea, desi chickpea and yellow pea were found at 40.36±0.11, 44.34±0.11 and 31.18±0.03 (Barbana 2010)

Protein hydrolysates from Green lentil and red lentil were found at 29.07±1.78 and 27.08±1.91. (Barbana 2011) The highest degree hydrolysates of Meriga egg was 17.1 at 90 min.

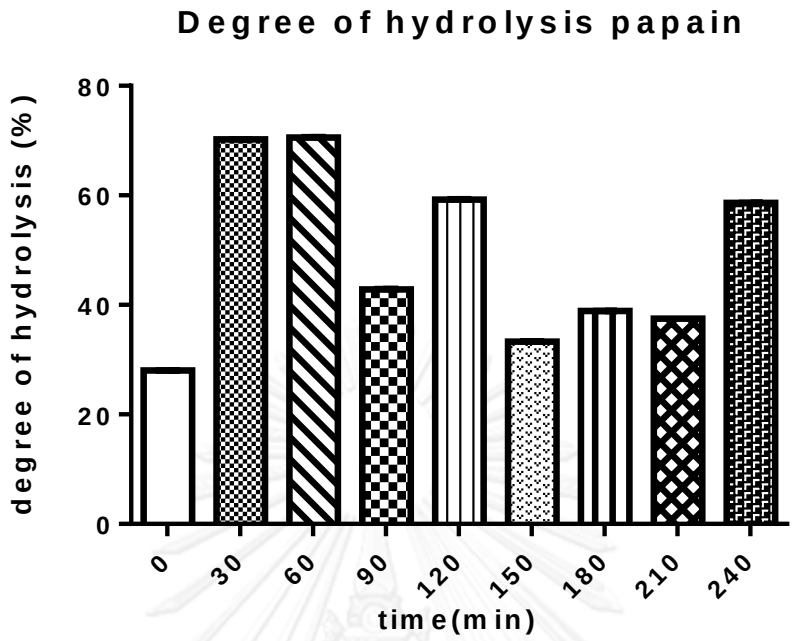

Figure 15 Degree of hydrolysis from protein hydrolysates hairy basil digested by papain

4.3 antioxidant activity

Free radicals are molecules or ion that have a lone pair electron which is unstable and highly reactive with bimolecular and can damage organ tissue such as DNA damage, denature of protein and lipid, covalent bond pairs with protein or enzyme caused their dysfunction. (Ames 1993) Free radical can be eliminated or decrease their harmful by antioxidations, which is binding the free radicals and then became to a more stable that can stop the chain reaction of free radicals. (Denisov 2005)

Antioxidants such as enzyme, bioactive compound and some protein may control the balance of free radicals and antioxidant. Thus their balance were lost the oxidative stress be destroy to organ and tissue of human body. The accumulation of

free radical lead to various pathological lesion including cancer, arthritis, Parkinson, Alzheimer and coronary thrombosis. (Ames 1993) So to get antioxidants from outside of body is an interested way to permit and prevent the harmful effect of free radicals.

Nowadays the analysis of the antioxidant activity of protein hydrolysates was no exact and specific. The examination of antioxidant activity only way was not enough to explain their mechanism. So, it should analyzed the properties of antioxidant activity with various method. In this paper 4 methods were chosen to analyze DPPH, ABTS, NO and H_2O_2 which had a different analysis depending on the mechanism of each types, to determine their ability to inhibit free radical.

4.3.1 DPPH radical scavenging activity

DPPH assay is a method to determine antioxidant activity generally used in protein hydrolysates. (Wu 2003) Free radicals were transfer to DPPH·(purple) stand for 30 min in dark room DPPH· were reduced by providing hydrogen atom or electron donating via free radical attacked to DPPHn (yellow). (Yang 2009) Monitoring %inhibit by spectrophotometer at 517 nm. In this research used DPPH method to review the most suitable type of enzyme digestion from hairy basil. The 50% inhibition value (IC_{50}) of proteaseG6 (19.6±0.0650 µg/ml) is higher than pepsin-pancreatin (81.3 ± 0.0080 µg/ml) and papain (203.3 ± 0.0210 µg/ml) because proteaseG6 is an alkali serine protease an endoprotein are having broad and high specificity toward aromatic

(Phe, Trp and Tyr), acidic (Glu), sulphur-containing (Met), aliphatic (Lue and Ala), hydroxyl (Ser), and basic (Lys) residues. (Doucet 2003)

In this research protein hydrolysates from hairy basil seed contained amino acids or peptides that could reacted with scavengers of aqueous phase radical.

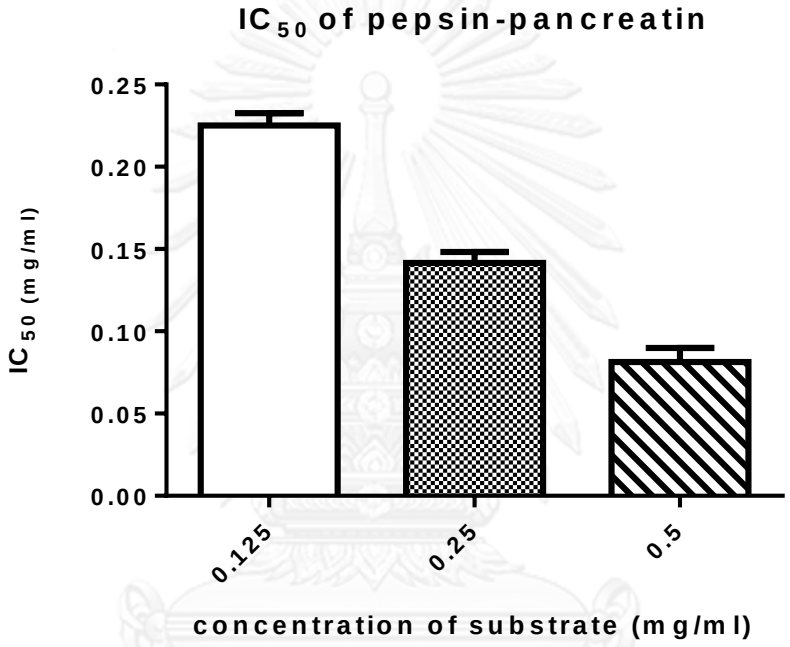

Figure 16 IC_{50} of various concentration of substrate digest by papsin-pancreatin

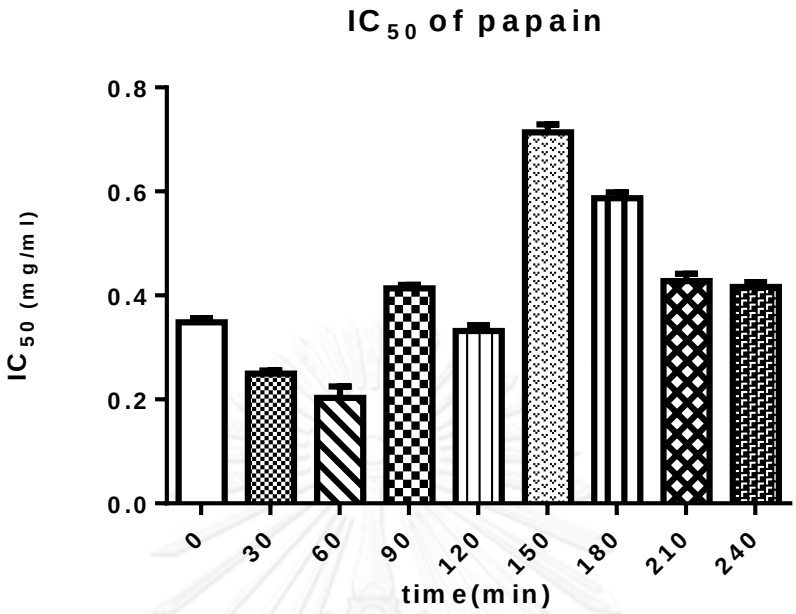

Figure 17 IC_{50} of various time digested by papain

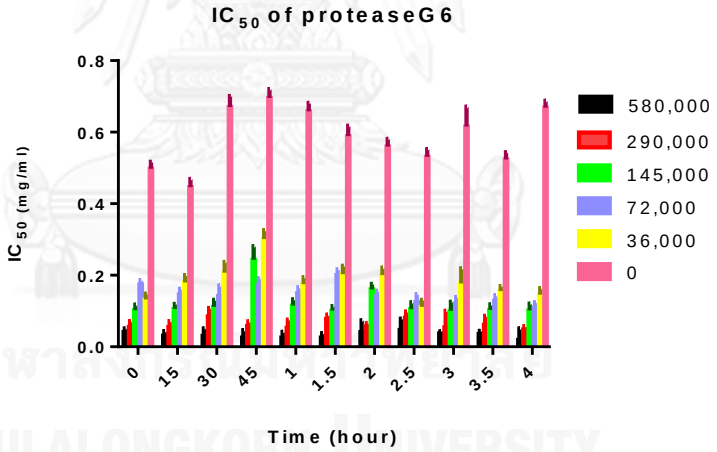

Figure 18 IC_{50} of various time and concentration unit enzyme digested by proteaseG6

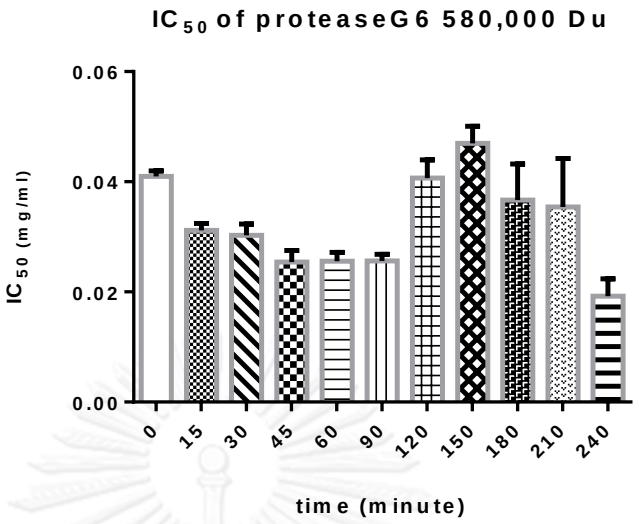

Figure 19 IC_{50} of various time digested by proteaseG6 5.8 x 10^5 DU/g

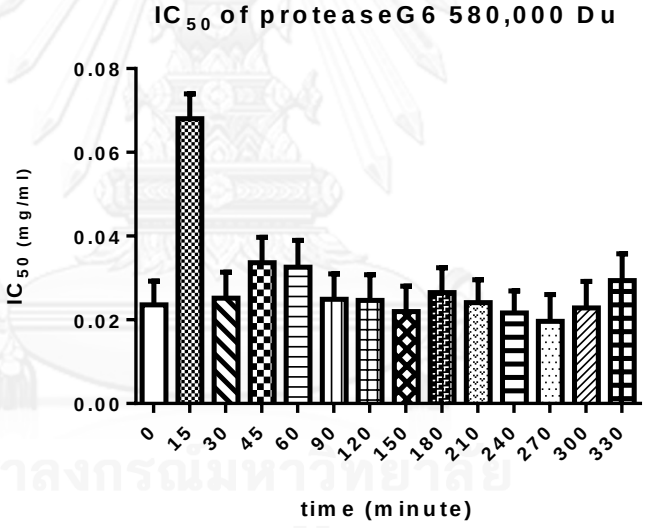

Figure 20 IC_{50} of various time digested by proteaseG6 5.8 x 10^5 DU/g (develop time to 330 min)

4.3.2 ABTS radical scavenging activity

ABTS radical cation decolorization assay is an antioxidant of hydrogen-donating compound (aqueous phase radical) and chain breaking (scavenger of lipid peroxyl radicals). (Binsan 2008)

The single electron transfer of blue green radical cation of ABTS+• was fades or disappear when an antioxidant is present to ABTS, the color of this reaction was measured by spectrophotometer at 734 nm **α** – tocopherol was used as a positive control. The scavenging ability of protein hydrolysates from hairy basil in the order of decreasing pore size on the ABTS free radical was observed in a dose-dependent manner, but with a lower activity than the reference standards (vitamin E). Thus, the IC_{50} value for permeate from 5 kDa membrane in the ABTS radical scavenging assay was 44.50 ± 2.00 μg/mL. ABTS+• reacts rapidly with antioxidants, and it can be used over a wide pH range to study the effects of pH on antioxidant mechanisms. Also, ABTS+• is soluble in both aqueous and organic solvents and is not affected by the solvent ionic strength, and thus can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids.

4.3.3 NO radical scavenging

Nitric oxide (NO) radicals are formed during the oxidation of L-arginine by NO synthases. NO is a gaseous neutral free radical with relatively long lifetime and at the same time is an active species capable of participating in many chemical reactions.

(Palmer 1987) Now researcher found that nitric oxide is synthesis by cell organism such as neurons, neutrophils, macrophages, hepatocytes and others. (Le Cras 2001)

This result was shown to be involved many diseases caused by physiological functions, including blood pressure, platelet non-aggregation, neurotransmission and toxicity in cell. (Moncada 1991) Maximum scavenging effect of nitric oxide from hairy basil permeated from 5,000 Dalton membrane is showed IC_{50} 212.1 ± 7.00 µg/ml which was lower than curcumin with IC_{50} 54.0 ± 4.90 µg/ml.

4.3.4 H_2O_2 radical scavenging

Hydrogenperoxide is a strong oxidizing agent directly active with few enzyme, more over its can reacted with cell membranes, in addition H_2O_2 radicals probably with Fe^{2+} and Cu^{2+} ions caused many toxicity effects. (Govindarajan 2003) Hydrogenperoxide scavenging activity is one of the useful method for measure antioxidant capacity of hydrogenperoxide. (Pa**Ź**dzioch-Czochra 2002)

Inhibition activity of an antioxidant can be determined their electron donating ability. Protein hydrolysates from hairy basil permeated from 5,000 dalton membrane caused a maximum antioxidant activity with IC_{50} 798.3 ± 3.00 µg/ml compared to sodium pyruvate as a positive control (IC_{50} = 15.53 ± 9.10 µg/ml)

Table 2 IC_{50} of Antioxidant activity with DPPH, ABTS, NO, H_2O_2 after permeated with 5,000 and 10,000 Dalton

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

4.4 Antiproliferation of cancer cell by MTT method

Free radicals participated with initiation and promotion state of cancer. At the ignition state radicals and various carcinogen may change genetic of normal cell. At promotion state free radicals promoted cancer development. Antioxidants are able to act with cancer suppressing or stimulating cancer growth, suggest that antioxidants may be applied for cancer treatment and prevention. Active protein hydrolysis fraction were

applied to cytotoxicity assay with human malignant cell lines. In this research 5 different human cell lines were chosen BT474, CHAGO, HEP-G2, KATO-III and SW620.

Permeated 5,000 Dalton membrane protein hydrolysates from hairy basil shown a high activity with cancer cell lines : gastric (KATO-III), hepatoma (Hep-G2), colon (SW620) and lung (Chago) with IC_{50} 19.25 ± 0.1971, 23.73 ± 0.05713, 24.88 ± 0.05785 and 28.76 ± 0.05630 µg/ml. However breast cancer cell lines (BT474) was maximum inhibit activity from 5,000-10,000 dalton membrane which IC_{50} 30.10 ± 0.0266 µg/ml.

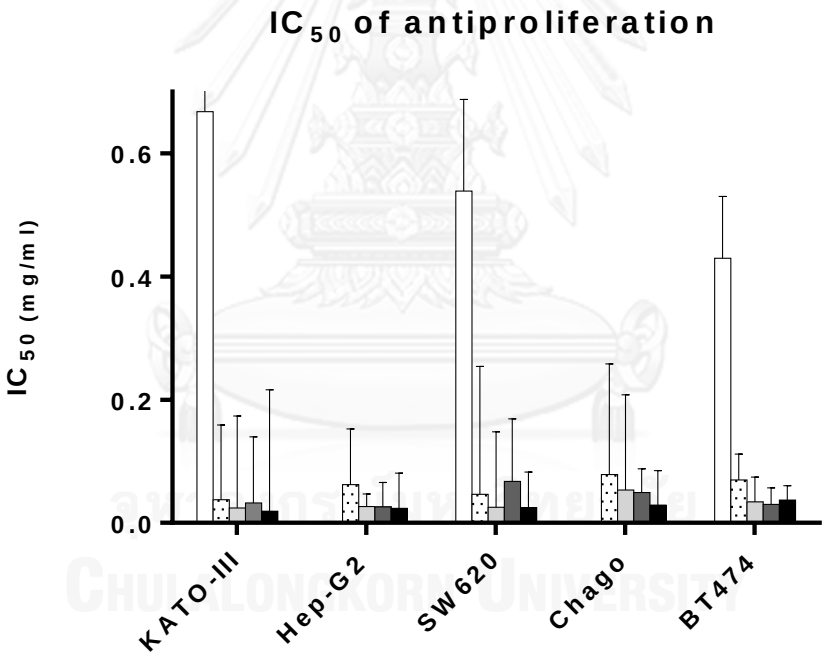

Figure 21 IC_{50} of antiproliferation activity

4.5 Purification antioxidative peptides by HPLC

After ultrafiltration process, peptides which lower than 5,000 Dalton were fractionated by reverse-phase high performance liquid chromatography on Shimpak C-18 column using TFA and CH_3CN solvent system and detected with UV 280 nm. 8 fraction were collected as the chromatographic profile

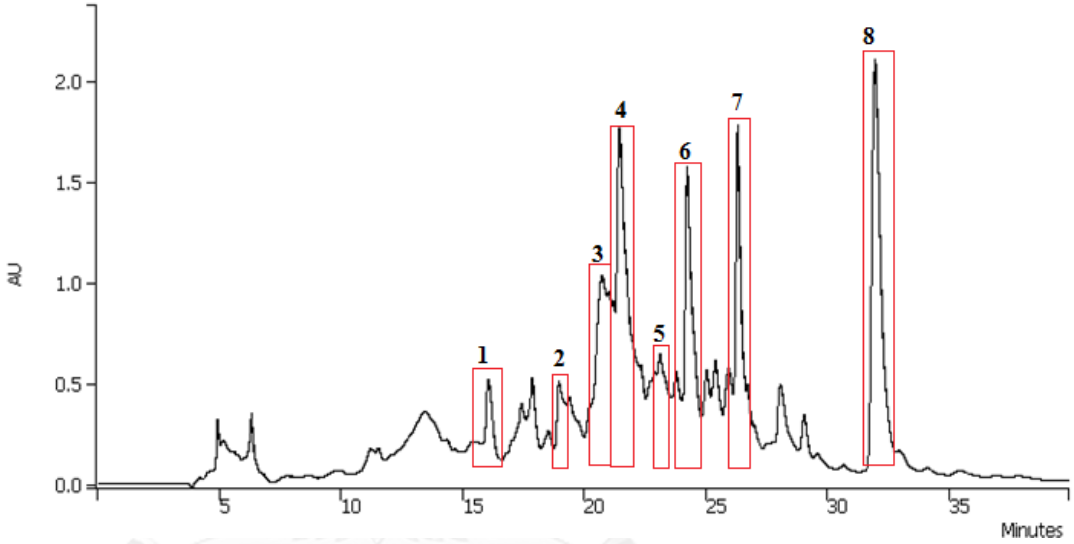

Figure 22 Chromatographic profile of protein hydrolysates from hairy basil with molecular weight less than 5,000 Dalton detected with UV 280 nm

4.6 identification of antioxidant peptides

Collected peptide fractions from RP-HPLC were indicated and speed vacuum for remove solvent. All of fractions were analyzed by LC-MS/MS to identified peptides sequences. Antioxidant peptides were processed to MASCOT database, and their amino sequence were shown in the table.

Table 3 amino acid sequence of protein hydrolysates from Hairy basil molecular weight less than 5,000 Dalton by LC-MS/MS

Fraction	sequence	Protein name	Accession number	organism	score
1	SKVIETKQQVVTEIADKLRA	50S ribosomal protein L10	P83063	*Bacillus cereus*	21
2	MYEVPIWVYAAQI	Unknown protein CP 4 from 2D-PAGE	P81349	*Clostridium pasteurianum*	41
3	QTFQYSRGWTN	Corazonin	B3A0J2	*Tyrannophasma gladiator (Gladiator) (Heel-walker)*	22
4	MYEVVIWVYAAQI	Unknown protein CP 4 from 2D-PAGE	P81349	*Clostridium pasteurianum*	20
5	QPDIMIFTIGPA	Unknown protein CP 10 from 2D-PAGE	P81345	*Clostridium pasteurianum*	17

Fraction	sequence	Protein name	Assesion number	organism	score
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

CHAPTER V

CONCLUSION

In this study, hairy basil was extracted by various enzyme alkali serine protease (proteaseG6), pepsin-pancreatin and papain. Antioxidant activity via DPPH method was chosen for investigate enzyme this method always used in protein hydrolysates antioxidant capacity, easily to observed. ProteaseG6 was successively shown higher antioxidant activity than pepsin-pancreatin and papain. 580,000 DU enzyme at 270 min was suitably condition of digestion after screening of DPPH method (IC_{50} 19.60 ± 64.20 µg/ml). Permeated protein hydrolysates which 3 fractions were tested with various antioxidant method ABTS, NO and H_2O_2. DPPH assay was highest IC_{50} value at 25.20 ± 143.70 µg/ml with molecular < 5 kDa. ABTS assay showed greatest antioxidant activity with molecular weight < 5 kDa (IC_{50} 44.50 ± 2.60 µg/ml). NO assay was IC_{50} 212.10 ± 7.00 µg/ml with molecular weight < 5 kDa. And H_2O_2 assay showed 798.30 ± 3.00 µg/ml with molecular weight < 5 kDa too. In this research can be concluded that protein hydrolysates from hairy basil which is molecular weight lower than 5 kDa had a good ability in inhibit free radicals by providing hydrogen atom or electron donating ,but not good ability in anti-strong oxidizing agent like NO and H_2O_2. Moreover protein hydrolysates was tested in cytotoxicity assay with 5 different cancer cell lines. The results showed permeated protein with 5 kDa membrane can control the growth of cancer cell : gastric (KATO-III), hepatoma (Hep-G2), colon (SW620) and lung (Chago) with IC_{50} 19.25 ± 197.10, 23.73 ± 571.30, 24.88 ± 578.50 and 28.76 ± 563.00 µg/ml and

protein hydrolysates molecular weight between 5-10 kDa was greatest IC_{50} value (30.10 ± 26.60 µg/ml) in breast cell lines(BT474). After, protein hydrolysates with lower 5,000 Dalton was purified by RP-HPLC. The collected fractions were speed vacuum and identified by mass spectrometer. Antioxidant peptides were processed to database and its amino sequence that have an antioxidant activities were MYEVTIVVVYAAQI, QTFQYSRGWTN, MYEVGIVVVYAAQI, QPDIMIFTIGPA, SSAAAAFAPSTPLA and SESYTPISGPNGYEVDVK

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APPENDIX

APPENDIX A

Preparation for Bradford assay

1. Bradford stock solution

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

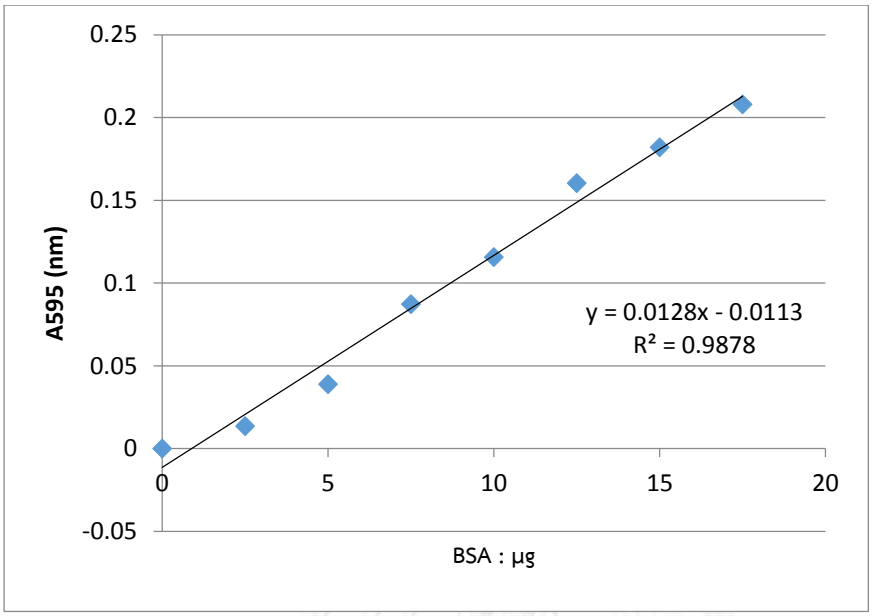
2. Bradford working buffer

Distilled water	425 ml
95 %Ethanol	15 ml
88% phosphoric	30 ml
Bradford stock solution	30 ml

Filter through Whatman No.1 paper, store at room temperature in brown glass bottle. Usable for several weeks, but may need to be refiltered.

APPENDIX B

Calibratioin curve for protein determination by Bradford method



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Academic presentation;

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