ฤทธิ์ขจัดอนุมูลอิสระและยับยั้งการเพิ่มจำนวนเซลล์มะเร็งของโปรตีนไฮโดรไลเสตจากขนไก่ป่นที่เตรียมจากแอลคาไลน์โปรติเอส

นางสาวศศิประภา อยู่ขันสวัสดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

FREE RADICAL SCAVENGING AND ANTIPROLIFERATION OF PROTEIN HYDROLYSATE FROM CHICKEN FEATHER MEAL PREPARED BY ALKALINE PROTEASE

Miss Sasiprapa Yookhansawat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2014

Thesis Title	FREE RADICAL SCAVENGING AND ANTIPROLIFERATION OF PROTEIN HYDROLYSATE FROM CHICKEN FEATHER MEAL PREPARED BY ALKALINE PROTEASE
By	Miss Sasiprapa Yookhansawat
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Polkit Sangvanich, Ph.D.
Thesis Co-Advisor	Assistant Professor Aphichart Karnchanatat, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

.....Chairman
(Professor Sirirat Rengpipat, Ph.D.)

.....Thesis Advisor
(Associate Professor Polkit Sangvanich, Ph.D.)

.....Thesis Co-Advisor
(Assistant Professor Aphichart Karnchanatat, Ph.D.)

.....Examiner
(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

.....Examiner
(Associate Professor Chanpen Chanchao, Ph.D.)

.....External Examiner
(Chantragan Phiphobmongkol, Ph.D.)

ศศิประภา อยู่ขันสวัสดิ์ : ฤทธิ์ขจัดอนุมูลอิสระและยับยั้งการเพิ่มจำนวนเซลล์มะเร็งของโปรตีนไฮโดรไลเสตจากขนไก่ป่นที่เตรียมจากแอลคาไลน์โปรติเอส (FREE RADICAL SCAVENGING AND ANTIPROLIFERATION OF PROTEIN HYDROLYSATE FROM CHICKEN FEATHER MEAL PREPARED BY ALKALINE PROTEASE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.พลกฤษณ์ แสงวณิช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. อภิชาติ กาญจนทัต, 66 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาภาวะที่เหมาะสมในการเตรียมโปรตีนไฮโดรไลเสตจากขนไก่ป่นที่มีฤทธิ์ยับยั้งอนุมูลอิสระและการลดจำนวนของเซลล์มะเร็งโดยการย่อยสลายด้วยเอนไซม์ โปรตีนไฮโดรไลเสตจากขนไก่ป่น ในงานวิจัยนี้เตรียมได้จากการย่อยสลายด้วยโปรติเอสจีซิก และแยกตามขนาดโมเลกุลด้วยเทคนิคอัลตราฟิลเตรชันได้เป็น 5 ส่วนตามขนาดของเยื่อกรอง แล้วทดสอบกิจกรรมยับยั้งอนุมูลอิสระของเปปไทด์ผสมสามารถติดตามได้ด้วยฤทธิ์ยับยั้งอนุมูลอิสระด้วยสาร DPPH, ABTS และวิธีไนตริกออกไซด์ พบว่าเปปไทด์ผสมที่มีขนาดโมเลกุลต่ำกว่า 3 กิโลดาลตัน มีฤทธิ์ยับยั้งอนุมูลอิสระได้ดีที่สุด ในงานวิจัยได้ศึกษาฤทธิ์การลดจำนวนของเซลล์มะเร็ง พบว่า เปปไทด์ผสมที่มีขนาดโมเลกุลต่ำกว่า 3 กิโลดาลตัน มีฤทธิ์การลดจำนวนของเซลล์มะเร็งเต้านมได้ดีที่สุด เมื่อพิสูจน์เอกลักษณ์ของเปปไทด์ผสมที่มีขนาดโมเลกุลต่ำกว่า 3 กิโลดาลตัน พบสายของเปปไทด์ประมาณ 11 สาย จากการค้นหาฤทธิ์ยับยั้งอนุมูลอิสระที่มีศักยภาพสูงจากโปรตีน ไฮโดรไลเสตจากขนไก่ป่น พบว่าสามารถนำไปใช้เป็นส่วนผสมในผลิตภัณฑ์เพื่อสุขภาพเพื่อควบคุมภาวะความเครียดออกซิเดชันในร่างกายได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5572211823 : MAJOR BIOTECHNOLOGY
KEYWORDS: PROTEIN HYDROLYSATES / CHICKEN FEATHER MEAL / ANTIOXIDANT / ANTIPROLIFERATIVE / AMINO ACID SEQUENCES

SASIPRAPA YOOKHANSAWAT: FREE RADICAL SCAVENGING AND ANTIPROLIFERATION OF PROTEIN HYDROLYSATE FROM CHICKEN FEATHER MEAL PREPARED BY ALKALINE PROTEASE. ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., CO-ADVISOR: ASST. PROF. APHICHART KARNCHANATAT, Ph.D., 66 pp.

This research was aimed to determine the optimal conditions of enzymatic hydrolysis for the preparation of protein hydrolysates from chicken feather meal that possessed antioxidant and antiproliferative inhibition activity. Chicken feather meal hydrolysate was prepared by Protease G6 digestion and separated into five fractions by membrane ultrafiltration. Antioxidant peptide activities were analyzed using three techniques DPPH, ABTS, and NO. The low-molecular-weight fraction MW < 3 kDa showed the best antioxidant activity. In the study of the five malignant cell lines, the protein hydrolysate MW <3 kDa exhibited strongest antiproliferative activity for human malignant cell line BT474 (breast) cancer. The peptides were identified by mass spectrometry techniques, and amino acid sequences were characterized into eleven peptide chains. The search for more potent antioxidant properties of protein hydrolysates continues for ingredients in the formulation of functional food and also for nutraceutical uses in controlling of oxidative stress in the body.

Field of Study: Biotechnology
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express our sincere gratitude to Associate Professor Polkit Sangvanich, Ph.D., my advisor for him worthy help, excellent suggestion, guidance, encouragement and supportive throughout the entire period of conducting this work not only the research methodologies but also many other in life. This thesis would not have been completed without all support from him.

I would also like to extend my gratitude to Assistant Professor Dr. Aphichart Karnchanatat, Professor Sirirat Rengpipat, Associate Professor Dr. Chanpen Chanchao, Associate Professor Nattaya Ngamrojanavanich, Ph.D., and Dr. Chantragan Phiphobmongkol for serving as the committee and for their editorial assistance and comments.

The authors thank Program in Biotechnology, Faculty of Science, Department of Botany, Faculty of Science, and the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, are both acknowledged for support and facilities.

Finally, I would like to thank my family for their full support, encouragement, understanding and helpfulness

CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

%	Percentage
Ab	Absorbance
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BHA	Butylated hydroxyl anisole
BHT	Butylated hydroxyl toluene
BSA	Bovine serum albumin
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DU	Alpha- amylase Dextrinizing units
EDTA	Ethylenediamine tetraacetic acid
et al.	and others
g	Gram(s)
GPX	Glutathione peroxidase
GSH	Glutathione
h	Hour(s)
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
IC_{50}	Median inhibitory concentration 50%
l	Litre(s)
M	Molar
mg	Milligram(s)
mg/l	Milligram per litre
min	Minute(s)
ml	Millilitre(s)
mM	Millimolar
mmol	Millimole
MW	Molecular weight
NaCl	Sodium chloride

NED	N-(1-naphthyl) ethylene diamine
NO	Nitric oxide
$O_2^{\bullet-}$	Superoxide anion
rpm	Revolution per min
S.E.	Standard error
SOD	Superoxide dismutase
SODs	Superoxide dismutase enzymes
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
µ	Micro(s)

CHAPTER I
INTRODUCTION

People these days pay more attention to health care, especially in the prevention and treatment of disease. They understand the importance of antioxidants which bind the free radicals in the body. Humans have a complex antioxidant system which protects body cells from damage caused by free radicals such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). Antioxidants can directly interact against free radicals and inhibit oxidation (Diplock,1991). There are many antioxidant agents including ascorbic acid, α-tocopherol, flavoniods, and β-carotene (Amadou *et al.*,2009; Pérez *et al.*,2007; Zhang *et al.*,2009). Reactive oxygen species (ROS) affect many substances in the human body, including fatty acids, proteins, and DNA (Sivoňová *et al.*,2007). The generation of ROS or free radicals such as superoxide, hydroxyl radical, and hydrogen peroxide, during metabolism and other activities beyond the antioxidant capacity of a biological system, gives rise to oxidative stress (Krishnaiah *et al.*,2007; Zima *et al.*,2001). Oxidative stress plays a role in heart diseases, malaria, neurodegenerative diseases, AIDS, cancer, and the aging process (Ames,1983; Ames *et al.*,1993; Astley,2003; Epstein *et al.*,1997; Gey,1990; Harman,1995; Smith *et al.*,1996). All organisms have antioxidant systems that are able to control and counter the onslaught of free radical mediated oxidative damage. Therefore, dietary sources have been recognized as safer and effective antioxidants in the context of their efficiency and nontoxicity. The intake of fruits and vegetables containing high amounts of antioxidative nutraceuticals has been linked to the balance of free radicals or antioxidant status, and helps to minimize the oxidative stress in the body and reduce the risks of disease.

Protein hydrolysate is a sterile solution of amino acids and peptides prepared from a suitable protein by enzymatic hydrolysis (Taheri *et al.*,2012). Therefore, the antioxidant activities of protein hydrolysates as well as amino acids and peptides were investigated. Proteins and peptides obtained from natural sources such as rape

seed (He *et al.*,2013), foxtail millet (Mohamed *et al.*,2012), buckwheat (*Fagopyrum esculentum Moench*) (Tang *et al.*,2009), chicken breast (Sun *et al.*,2012), and round scad (*Decapterus maruadsi*) (Thiansilakul *et al.*,2007) all have antioxidant properties.

Bioactive proteins and peptides have physiological properties. Recently several studies identified and optimized the isolation of biopeptides from both plant and animal sources. These peptides are generated both *in vivo* and *in vitro* from the proteolytic hydrolysis of food proteins (Korhonen and Pihlanto,2003). The enzymatic hydrolysis of proteins is one approach used to release bioactive peptides, and is widely applied to improve functional and nutritional properties of protein sources (Je *et al.*,2005). The biological activity of a peptide is based on amino acid composition. Peptides could be used in the formulation of functional foods and nutraceuticals to reduce damage related to oxidative stress in human disease conditions. Moreover, natural antioxidants are preferable; they can be used at higher concentrations, without any toxic side effects that might result from the use of synthetic equivalents.

Chicken feathers are generated in large amounts as a waste by-product in commercial poultry-processing plants; representing 5-7 % by weight of mature chickens (Manczinger *et al.*,2003). The feathers are composed of over 90 % protein, fibrous insoluble, and highly cross-linked with disulphide and other bonds (Taskin *et al.*,2012). The main component is insoluble keratin with cysteine content (Bielorai *et al.*,1982). Chicken feathers have an adverse effect when discharged into the environment as they contain large amounts of amino acids such as cysteine, glycine, arginine and phenylalanine (Kumar *et al.*,2012). Therefore, the objective of this study was to determine the *in vitro* antioxidant activity of chicken feather meal protein hydrolysates of different molecular weights, and also evaluate the antiproliferative activity on the growth of human malignant cell lines.

CHAPTER II

LITERATURE REVIEW

2.1 Free radicals

Free radicals are highly reactive chemical species (atoms, ions or molecules) that have unpaired electrons in the outer (valance) shell of the molecule that can be considered fragments of molecules and which are generally very reactive. They are produced continuously in cells either as accidental by-products of metabolism or deliberately during, for example, phagocytosis (Cheeseman and Slater,1993). A free radical is generated during oxidation of protein, carbohydrates, and fats through both anaerobic and aerobic processes. The most important reactants in free radical biochemistry are aerobic cells and oxygen and its radical derivatives are superoxide and hydroxyl radicals (Neeraj *et al.*,2013). Reactive oxygen species (ROS) include free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (•OH) and those which are not free radicals but can form oxidizing properties or can be easily changed to free radicals such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3). Reactive nitrogen species (RNS) consist of nitric oxide radical ($NO^{\bullet}$) and nitrogen dioxide radical ($NO_2^{\bullet}$) (Halliwell,2001). Free radicals can be formed in three ways: (i) by the hemolytic cleavage of a covalent bond of a normal molecule, where each fragment retains one of the paired electrons (ii) by the loss of a single electron from a normal molecule or (iii) by the addition of a single electron to a normal molecule (Cheeseman and Slater,1993). Reactive free radicals formed within cells can oxidize biomolecules and lead to cell death and tissue injury. Establishing the involvement of free radicals in the pathogenesis of a disease is extremely difficult due to the short lifetimes of these species.

The free radicals can create their functions by losing or gaining electrons. Their effects can be deleterious or beneficial for the body. Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) are major components of the free radical system and derived either from normal essential metabolic processes in the human body or from external sources such as cigarette smoke, environmental pollutants,

ultraviolet light, radiation, certain drugs, pesticides, anaesthetics and industrial chemicals (Bagchi and Puri,1998). They are continuously produced as intermediate products in vary enzymatic and non-enzymatic pathways in the cell beside the internally generated industrial free radicals.

2.2 Antioxidants

Antioxidants are classified as primary, secondary and synergistic antioxidants. Primary antioxidants interrupt the free radical chain of oxidative reaction by contributing hydrogen from the phenolic hydroxyl groups, themselves forming stable free radicals which do not initiate or propagate further oxidation of lipids (Harborne,1995). Many of natural phenolic compounds like flavonoid and rosemary antioxidants also have chain breaking properties. Primary antioxidants are effective at very low concentration and at higher levels they may become pro-oxidants (Vinson *et al.*,1995). Secondary and synergistic antioxidants can be broadly classified as oxygen scavengers, chelators and also provide an acidic medium that improve the stability of primary antioxidants (Pszczola,2001). Oxygen scavengers such as ascorbic acid will react with free oxygen and remove it in a closed system. Chelators like Ethylene diaminetetracetic acid (EDTA) are highly effective as a synergist with both primary antioxidants and oxygen scavengers. An unshared pairs of electrons in their molecule structure promotes the chelate action. They form stable complexes with pro-oxidant metals which promote initiation reactions and raise the energy of activation of the initiation reactions considerably.

Antioxidants are manufactured within the body and can also be extracted from fruits, vegetables, nuts, meats, seeds, and oil. The example of antioxidant enzymes that are found to provide a protection against ROS are superoxide dismutases (SOD), catalases, and glutathione peroxidases ,which are primary antioxidants that react with ROS. The non-enzymatic antioxidants consist of vitamins C, E and A. Glutathione, ubiquinone, and phenolic compounds are the secondary antioxidants that react with ROS. A trace metal is also required for proper function of one of the body's antioxidant enzyme systems (Shalaby and Shanab,2013). The body cannot manufacture these micronutrients so they must be supplied in the diet.

There is no doubt that antioxidants are necessary components for our health but we do not forget that the body's finely tuned mechanisms are carefully balanced to withstand a variety of insults. Taking chemicals without a complete understanding of all of their effects may disrupt this balance. In order to take antioxidants, reducing externally free radical sources in our lives may be the better solution for our health (Pala and Tabakçıoğlu,2007). Types of antioxidants in the body can build up their own or from the various foods have 2 groups.

2.3 Type of antioxidants

2.3.1 Synthetic enzymatic antioxidants

2.3.1.1 Superoxide Dismutase (SODs)

Superoxide Dismutase (EC. 1.15.1.1) is enzymes that catalyze the dismutation of superoxide radical to oxygen and hydrogen peroxide, can be found in living organism. Depending on the type of organisms divided 3 kinds (Culotta *et al.*,2006).

- Cu/Zn-SOD was the first enzyme to be characterized and is a copper and zinc-containing enzyme as a co-factor that commonly found in thylakoid of chloroplast, cytosol, lysosome, nucleus and intermembrane space of mitochondria in plants, animal and some bacteria (Bowler *et al.*,1992; Zelko *et al.*,2002).
- Mn-SOD was manganese (Mn) containing enzyme as a co-factor and exclusively to the mitochondrial spaces and may be found in cytosol of plants, animal and some bacteria (Bowler *et al.*,1992; Zelko *et al.*,2002).
- Fe-SOD was iron (Fe) containing enzyme as a co-factor that will be found in the area in stomach of chloroplast of plants, algae and bacteria (Muñoz *et al.*,2005).

The antioxidant mechanisms of SOD have 2 steps: the first step is group of oxidized metal co-factor donate electron to $O_2^{\cdot-}$ become to O_2 and the second step that reduced metal co-factor accept electron from $O_2^{\cdot-}$ and catalyze with 2 protons is H_2O_2 (Culotta *et al.*,2006).

$M^{(oxidized)}$-SOD + $O_2^{\cdot-}$ ⟶ $M^{(reduced)}$-SOD + O_2 equation (1)

$M^{(reduced)}$-SOD + $O_2^{\cdot-}$ + $2H^+$ ⟶ $M^{(oxidized)}$-SOD + H_2O_2 equation (2)

$2O_2^{\cdot-}$ + $2H^+$ ⟶ O_2+ H_2O_2 equation (3)

2.3.1.2 Catalase (CAT)

Catalase (EC.1.11.1.6) is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyze enzyme the decomposition of hydrogen peroxide (H_2O_2) to H_2O and O_2 (catalatic reaction) and can be found in glyoxysome or peroxisome, mitochondria, chloroplast, cytosol and endoplasmic recticulum (ER) of plants, algae and bacteria (Chelikani *et al.*,2004).

The antioxidant mechanism of CAT has 2 steps: the first step is co-factor of enzyme (Fe^{3+}-CAT) and accept two electron from hydrogen peroxide(H_2O_2) to water (H_2O)and compound I (O=Fe^{5+}-CAT) and the second step hydrogen peroxide donate 2 electron to compound I to obtain H_2O and O_2 (Chelikani *et al.*,2004).

H_2O_2 + Fe^{3+}-CAT ⟶ H_2O + compound I equation (4)

H_2O_2 + compound I ⟶ H_2O + O_2 + Fe^{3+}-CAT equation (5)

$2H_2O_2$ ⟶ $2H_2O$ + O_2 equation (6)

2.3.1.3 Glutathione reductase (GR)

Glutathione reductase (EC. 1.6.4.2) containing FAD as co-enzyme to convert oxidized form glutathione (GSSG) into reduced form (GSH), In order to change from dihydroascorbate (DHA) to ascorbate for utilizing as a precursor APX or directly eliminate other free radical. It can be found in chloroplast, mitochondria and cytosol of plants, animal and algae (Pai and Schulz,1983).

The antioxidant mechanism of GS has 2 steps: the first step NADPH+H^+ donate two electrons to FAD-GR enzyme as the compound I ($FADH_2$-GR) and the second step oxidized form glutathione (GSSG) accept two electron from compound I as the reduced form (GSH) (Pai and Schulz,1983).

FAD-GR + NADPH + H^+ ⟶ $NADP^+$ + compound I equation (7)

compound I + GSSG ⟶ 2GSH + FAD-GR equation (8)

GSSG + NADPH + H^+ ⟶ 2GSH + $NADP^+$ equation (9)

2.3.2 Synthetic non-enzymatic antioxidants

2.3.2.1 Phenolic compounds

Phenolic compounds are secondary metabolites. These compounds, one of the most widely occurring groups of photochemical, are of considerable physiological and morphological importance in plants. They play an important role in growth and reproduction, providing protection against pathogens and predators, besides contributing towards the color and sensory characteristics of fruits and vegetables. The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity. Phenolic compounds have been also widely known as a natural source of antioxidants (Balasundram *et al.*,2006).

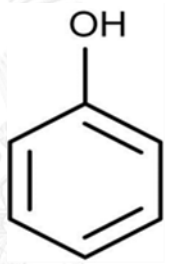


Figure 2.1 Structure of phenolic compounds

2.3.2.2 Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized according to chemical structure. They constitute the largest group of plant phenolics, accounting for over half of the eight thousands naturally occurring phenolic compounds. Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C_6–C_3–C_6 configuration. Essentially, the structure consists of two aromatic rings A and B, joined by a 3- carbon bridge, usually in the form of a heterocyclic ring C (Figure 2.2). Variations in substitution patterns to ring C result in the major flavonoid classes, i.e., flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols, and anthocyanidins (Figure 2.3), of which flavones and flavonols are the most widely occurring and structurally diverse. Substitutions to rings A and B give rise to the different compounds within each class of flavonoids (Balasundram *et al.*,2006).

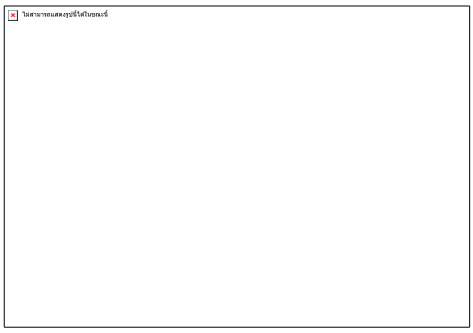
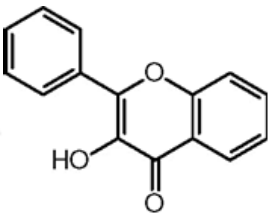
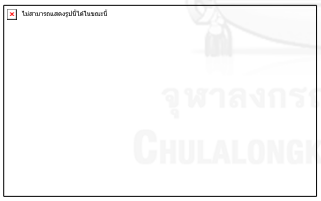


Figure 2.2 Generic structure of a flavonoid molecule

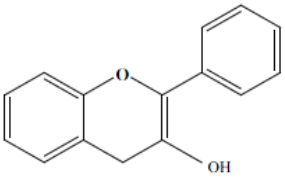
Flavones



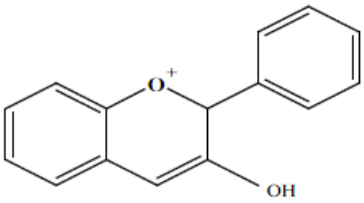
Flavonols



Flavanones



Flavanols



Anthocyanidines

Figure 2.3 Structure of major classes of flavonoids

2.3.2.3 Ascorbic acid

Ascorbic acid or vitamin C can be synthesized in plants and animals; it is soluble in water and is present in relatively high amounts in fruit and vegetables (Arrigoni and De Tullio,2002).

Ascorbic acid is a co-factor for several enzymes participating in the post-translational hydroxylation of collagen, carnitine synthesis as well as the facilitation of iron absorption and in the conversion of the neurotransmitter dopamine to norepinephrine in peptide amidation and in tyrosine metabolism (Hacisevkd,2009).

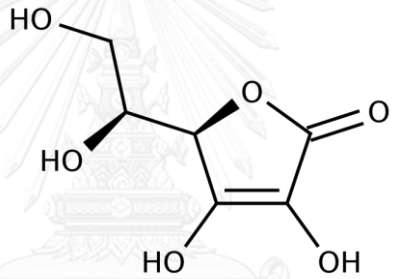


Figure 2.4 L-Ascorbic acid

2.3.2.4 Vitamin E

Vitamin E or tocopherols, are fat-soluble vitamins and lipophilic antioxidants are synthesized due to the hydroxyl group when vitamin E loss electrons can move into delocalization within the benzene ring of vitamin E where molecules are stabilized. Vitamin E functions as an efficient inhibitor of lipid peroxidation in vivo. Vitamin E helps the body to take vitamin A used for protection from toxic metals such as lead. Classes of tocopherols consist of alpha-tocopherol, beta-tocopherol, gamma-tocopherol and delta-tocopherol (Burton and Ingold,1981).

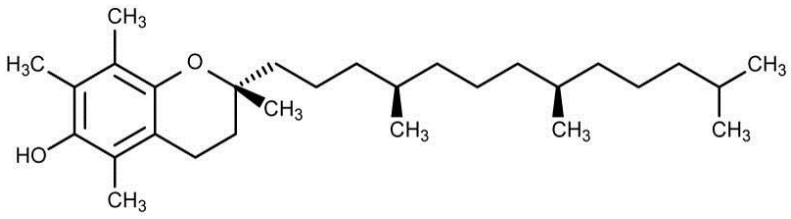


Figure 2.5 Alpha-tocopherol

2.3.2.5 Carotenoid

Carotenoid is a substance which can be converted into vitamin A and forms one of the most important classes of plant pigments and plays a crucial role in defining the quality parameters of fruits and vegetables such as carrots, tomatoes and oranges. The majority of carotenoids are derived from a 40-carbon polyene chain such as beta-Carotene, astaxanthin, diatoxanthin, lutein and lycopene (Eldahshan and Singab, 2013).

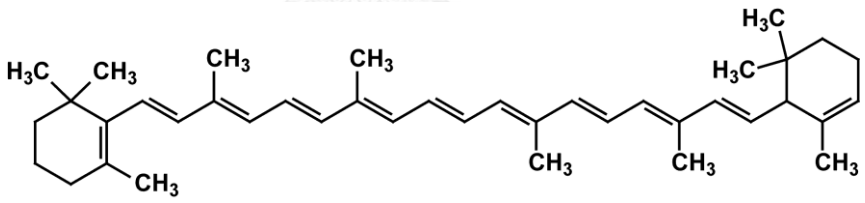


Figure 2.6 Beta-Carotene

2.3.2.6 Antioxidant peptide

Antioxidant peptide can be produced by enzymatic hydrolysis of various enzymes, that can be utilize free radicals (Boboev *et al.*,2012). Although, the mechanism of peptide represent to antioxidant activity that known some aromatic amino acid and hydrophobic amino acid are reported to plays a role for the activity (Chen *et al.*,1998). Generally, aromatic amino acid in peptide containing Phe, Tyr and Trp have very important to radical sacavenging activity of peptide due to their special

structure to scavenger unpaired electrons or radicals by donating protrons (Fan *et al.*,2012) Mendis *et al*. (Mendis *et al.*,2005) reported that peptide containing Trp, Tyr, Leu, Pro and Ala enhance the free radical scavenging activities. Histidine containing-peptides and some hydrophobic amino acids are related to the high effective antioxidant due to the proton-donation ability of the histidine imidazole group (Peña-Ramos *et al.*,2004). Met and Leu can be conduce to antioxidant activity (Park *et al.*,2001). Cysteine residue in the peptide can be expected to protect lipid and other biomolecules by donating protons to peroxyl radicals and other free radicals in the cell (Sheih *et al.*,2009).

Table 2.1 Protein hydrolysates from plant sources in antioxidant activity

Source of protein	**Enzyme hydrolysis**	**Antioxidant activity**	**Results**	**References**
Alfalfa leaf	Alcalase	DPPH, Hydroxyl radical, Superoxide anion, Metal ions	Protein hydrolysate MW <1 kDa is the most antioxidant activity	(Xie *et al.*,2008)
Buckwheat	Alcalase	DPPH, Reducing power	Protein hydrolysates exhibited excellent antioxidant activities	(Tang *et al.*,2009)
Hemp	Alcalase, Flavourzyme, Neutrase, Protamex, Pepsin, Trypsin	DPPH, Ferrous ion-chelating, Reducing power	Protein hydrolysate more antioxidant potency	(Tang *et al.*,2009)
Sunflower	Flavourzyme	Autoxidation pyrogallol	Peptide sequence is ACAHDKV	(Ren *et al.*,2010)
Rapeseed	Alcalase, Proteinase K, Pepsin, Pancreatin, Thermolysin, Flavourzyme,	DPPH, Metal ions, Ferric reducing power	Protein hydrolysate MW <1 kDa is the most antioxidant activity	(He *et al.*,2013)

Table 2. 2 Some Protein hydrolysates from other sources in antioxidant activity

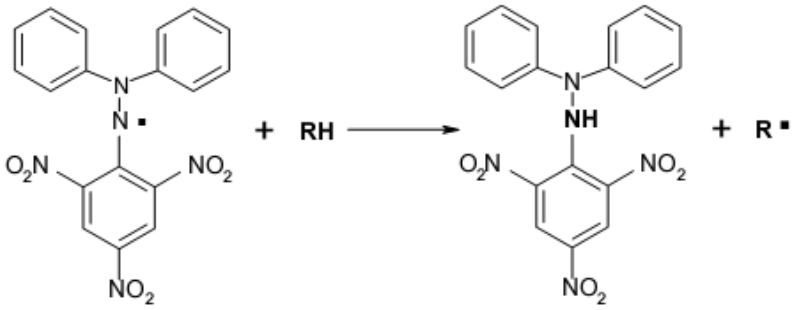
Source of protein	Enzyme hydrolysis	Antioxidant activity	Results	Referance
Chlorella ellipsoidea	papain, trypsin, pepsin, α-Chymotrypsin	DPPH, Hydroxyl radical, Peroxyl radical	Peptide sequence is LNGDVW	(Ko *et al.*,2012)
Flounder fish	Papain, Pepsin, Trypsin, Neutrase, Alcalase, Kojizyme, Protamex, α-Chymotrypsin.	DPPH, Hydroxyl radical, Peroxyl radical	Peptide sequence are VCSV and CAAP	(Ko *et al.*,2013)
Venison	Alcalase, Pepsin, Neutrase, Papain, Trypsin, α-Chymotrypsin	DPPH, Hydroxyl radical, Superoxide radical, Peroxyl radical	Peptide sequence are MQIFVKTLTG and DLSDGEQGVL	(Kim *et al.*,2009)
Sardinelle (*Sardinella aurita*)	Alcalase, Proteases from B. *licheniformis* NH1, Proteases from A. *clavatus* ES1	DPPH, Reducing power	Peptide sequence are LHY, LARL, GGEGAH, GAWA, PHYL, GALAAH	(Bougatef *et al.*,2010)
Tuna backbone	Alcalase, Neutrase, Papain, Pepsin, Trypsin, α-Chymotrypsin	DPPH, Hydroxyl radical, Superoxide radical, Lipid peroxidation	Peptide sequence is VKAGFAWTANQQLS	(Je *et al.*,2007)

2.4 Method for investigation of antioxidant activity

2.4.1 DPPH radical scavenging activity assay

The free radical, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) is widely used to test the ability of compounds to acts as free radical scavengers or hydrogen donors and to evaluate antioxidant activity. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applied to the overall antioxidant capacity of the sample.

DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radical. The delocalization also gives rise to deep purple color, characterized by an absorption band in ethanol solution centered at 517 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, this gives rise to the reduced form with the increase at yellow which is shown in Figure 2.7.



Diphenylpicrylhydrazyl (free radical) Diphenylpicrylhydrazine (nonradical)

Figure 2.7 Structure of DPPH and its reduction by antioxidant

(Huang *et al.*,2005)

Where the DPPH molecule is intended to represent the free radicals in the system whose activity is to be suppressed by antioxidant. DPPH-H is the reduced form and $R^{\bullet}$ is free radical produced in this first step. This latter radical will then undergo further reactions which control the overall stoichiometry that is the number of molecule of DPPH reduced by one molecule of the reagent. The reaction (Foti *et al.*,2004) is therefore intended to provide the link with the reactions taking place in oxidizing system such as the auto-oxidation of a lipid or other unsaturated substance.

2.4.2 ABTS radical scavenging activity assay

This method involves the oxidation of 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid). The principle behind the technique involves the reaction between ABTS and potassium persulfate to produce the ABTS radical cation, a blue green chromogen. The presence of antioxidant, the colored radical is converted back to colorless ABTS, the absorbance of which is measured at 734 nm (Nenadis and Tsimidou,2002; Re *et al.*,1999).

The preformed radical mono-cation of 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen donating antioxidants which is shown in Figure 2.8.

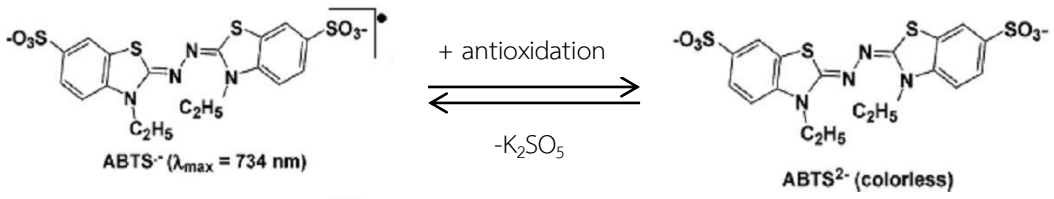


Figure 2.8 Structure of ABTS radical cation and its reduction by antioxidant.
(Huang *et al.*,2005)

2.4.3 Nitric oxide radical scavenging activity assay

Nitric oxide is classified as a free radical because of its unpaired electron and displays important reactivity with certain types of proteins and other free radicals such as superoxide (Amaeze *et al.*,2011). Nitric oxide (NO) is generated from amino acid L-arginine by the enzymes in the vascular endothelial cells, certain neuronal cells, and phagocytes. L-arginine is converted to nitric oxide in the body and can help relax the blood vessels and regulate blood vessel tone and flexibility. The result is reduced stress on the heart, improved circulation, and an increased ability to maintain blood pressure already in normal range (Nagmoti *et al.*,2012). NO is synthesized by three isoforms of the enzyme nitric oxide synthase (NOS), endothelial NOS, neuronal NOS, and inducible NOS (iNOS).

In vitro inhibition of nitric oxide radical is also a measure of antioxidant activity. This method is based on the inhibition of nitric oxide radical generated from sodium nitroprusside in buffer saline and measured by the Griess reagent. The nitric oxide generated from sodium nitroprusside at physiological pH (7.2) producing $NO^{\cdot}$. Under aerobic conditions, $NO^{\cdot}$ reacts with oxygen to produce stable products (nitrate and nitrite). The extract inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. In presence of scavengers, the absorbance of the chromophore is evaluated at 540 nm. The activity is expressed as % reduction of nitric oxide (Parul *et al.*,2008).

2.5 Cancer and free radical

Worldwide, the largest incidence of common cancer is lung cancer in men and breast cancer in women (Jema *et al.*,2011). For most of the people in the world today, Cancer or tumor malignancy is a major of diseases is the common cause of death worldwide, including Thailand; it is the third leading cause of death worldwide, only preceded by cardiovascular disease and infectious disease (Mathers *et al.*,2001). Cancer incidence in Thailand reported that cancer was the first cause of death in the

death rate for statistic of cancer in year 2001-2003 (Cancer in Thailand 2001-2003 Vol.V) from the ministry of health was 38.6, 24.9 per hundred thousand population per year to the first for liver and bile duct cancer and the second for trachea, bronchus and lung cancer in male, respectively and 20.9, 18.1 per hundred thousand population per year to the first for breast cancer and the second for cervix uteri cancer in female, respectively. Cancer-causing agents (carcinogens) can be present in water, food, in the air, chemicals, and sunlight. Currently, a high number of deaths in Thailand caused by diseases including cardiovascular disease, hypertension disease and diabetes mellitus. Moreover, the incidence of cancer in human as a contribution to death includes lung cancer, breast cancer, liver cancer and cervix uteri cancer. Cancer is most likely to be detected in the final period. The most common types of cancer can be detected in several ways such as MRI, X-rays (such as mammograms), CT, and ultrasound, including blood tests, and tissue tests where the presence of certain signs can indicate cancer. General rick factors for the incidence of cancer such as smoking, family history of cancer, and some types of viral infection (helicobacter pylori, hepatitis B virus (HBV), hepatitis C virus (HCV) and some types of human papilloma virus (HPV)), physical inactivity, older age, environmental and occupational risks including UV from sunlight, ionizing and non-ionizing radiation. Scientists interested in the role of free radicals in cancer cells report that overproduction of free radicals can stimulate cancer development at all three stages: (1) the induction of DNA mutation in a somatic cell (initiation), (2) the simulation of tumorigenic expansion of cell clone (promotion), and (3) the malignant conversion of the tumor into cancer (progression) (Dreher and Junod,1996). Scientists have studied the association between free radicals and cancer in order to develop the treatment of cancer patients for adjuvant with modern technology, surgery, radiotherapy and use of new medicines to treat cancer. The first choice for cancer treatment is surgery because it is the single most effective tool in the anticancer armamtarium (Allison,2001).

Free radicals can be produced during chronic inflammation that can induce a number of modifications, including gene mutations and post-translations of key cancers related to protein. The initiation point of cancer is caused by the reaction

between carcinogens and DNA in the body by free radicals inducing damages to DNA, protein, RNA and lipids, when the body has incurred damage of cells by dividing uncontrollably and forming lumps of tissues called tumors (Hussain *et al.*,2003).

2.6 Protein hydrolysates

Protein hydrolysates are known for the digestion of proteins that contain shot-chain peptides with characteristic free amino acid composition and defined molecular sizes are highly desirable for specific formulation. The hydrolysis of protein can be digested two ways: (1) enzymatic hydrolysis is a commonly used method in the modification of protein structure in order to enhance the functional properties of proteins (Corredig and Dalgleish,1997). (2) Chemical hydrolysis is an acid or alkaline substance that is predominantly applied in chemical hydrolyses such as sulfuric acid and hydrochloric acid and has also been used (Taherzadeh and Karimi,2007). Enzymatic hydrolysate of protein is an important bioprocess to improve the chemical, physical, functional and nutritional properties of original protein. Technological process of enzymatic hydrolysis is developed under mild conditions of pH 6-8 and temperature 40-60 °C, avoid the extremes usually required for chemical and physical treatments and minimizing side reactions (Clemente *et al.*,1999).

Sources of protein hydrolysate are mostly abundant in cheap materials or waste agricultural products made from animals or plants (Franěk *et al.*,2000), such as waste from plants, food by-products and seafood processing. Protein hydrolysates of plants protein source are corn (Li *et al.*,2010), soy (Park *et al.*,2010), rapeseed (He *et al.*,2013) and sunflower (Ren *et al.*,2010). Protein hydrolysates from animal sources are shrimp (Kleekayai *et al.*,2015), marine shellfish (*Mytilus coruscus*) (Kim *et al.*,2012), freshwater clams (Zeng *et al.*,2014), and yellowfin sole frame (Jun *et al.*,2004). The amino acids have an aromatic ring in the structure of amino acid; tyrosine, tryptophan and phenylalanine can be donated protons to free radicals. The peptides exhibiting several bioactivities have low molecular weight peptides that contain 2-20 amino acid residues that are present in amino acid composition and have great potency of antioxidant peptides. Generally, protein hydrolysate contains mostly short peptides (di and tripeptides) and normally the number peptides are

composed of 3-20 amino acid residues and are absorbed faster than free amino acids (Esan and Fasasi,2013).

2.7 Proteolytic enzyme

Proteolytic enzymes are also called protease or proteinase and are a group of enzymes where hydrolyse, the peptide linking amino acids of peptide in different sizes, are present in bacteria and plants and can be found in most animals. Proteolytic enzymes are classified by their hydrolysing mechanism into endopeptidase or exopeptidase with cleavage to the peptide bonds (Clemente,2000). Proteolytic enzymes are formed from the microorganisms are alcalase, neutase, protamex and protease N. Enzymes found in plants include pepsin, papain, and bromelain. Enzyme found in to other source includes trypsin and chymotrypsin, which trypsin found in the digestive system of many vertebrates system and chymotrypsin found in the acinar cells of the pancreas. Enzymatic hydrolysis using commercial proteases for modify proteins to peptides such as protein hydrolysate from Germinated Black Soybean (*Glycine max* L.) by pepsin and pancreatin digestion (Sefatie *et al.*,2013), protein hydrolysate from peanut using Alcalase 2.4L (Chen *et al.*,2007), and protein hydrolysate of Tea Seed (*Camellia oleifera* Abel.) prepared using alcalase (Li *et al.*,2014). The use of protein hydrolysates are used for the clinical treatment of patients with specific disorders of digestion, absorption and amino acid metabolism. Furthermore, depending on the amino acid sequence, they may be involved in various biological functions such as antioxidant, antithrombotic, anti-cancer, antimicrobial activities, opioid agonists or antagonists, immunomodulatory and antihypertension (Clare and Swaisgood,2000).

Alkaline protease is can be used to prepare peptides with characteristics that are a function of hydrolyzed protein, enzyme used, and conditions of hydrolysis. Alkaline proteases for industrial use can be obtained from different sources such as bacteria, fungi, or insects (Anwar and Saleemuddin,1998). Protease G6 enzyme (EC: 3.4.21.62) is a bacteria alkaline protease that enzyme type is alkaline serine endropeptidase derived from a selected strain of *Bacillus licheniformis*. Typical application areas for Protease G6 enzyme include baking, protein proceesing, silver

recovery from film material and pet food production. The pH range for the activity of Protease G6 is approximately 7.0 to 10.0, with an optimum performance at pH 9.5. The exact pH optimum will depend on process variables, including temperature and time substrate nature and condition. The activity of Protease G6 is effective in the temperature range from 25°C to 75°C, with an optimum performance at 60°C. The enzyme can be inactivated by holding for 5-10 minutes at operation pH, at a temperature of 80-85 °C or pH below 4 holding for 30 minutes at 50°C. Protease G6 is effective in hydrolyzing most proteins to lower molecular weight proteins such as hemogobin, casein, animal proteins, fish and other plant proteins.

2.8 Feather and chicken feather meal

The poultry processing industry have been by product as waste of feather, which occur from poultry processing by after choose bone, viscera, blood, feet and head. Chicken feathers produced discharged into the environment can be cause of pathogen and they adversely affect the environment. Processing of feather to feather meal can produce by hydrolysis the process using different high heat and pressure. The whole feathers are rich in protein rich more other parts of chicken and the waste by-product in the commercial production of poultry processing industries for human consumption is made up of 85-90% protein content (MacAlpine and Payne,1977; Taheri *et al.*,2012). Feather meals contain rich among of hydrophobic amino acids like glycine, arginine, cysteine, threonine and phenylalanine (Baker *et al.*,1981). At present, chicken feathers are used in the formulation of animal feed supplements such as fish feeds additives. It can also be used as an organic fertilizer (Nachman *et al.*,2012).

Table 2. 3 Research associated of feather and other part of chicken

Part of chicken	**Biological activity**	**Results**	**Reference**
Chicken feather	Antioxidant activities	Protein hydrolysate with high antioxidant activity	(Fakhfakh *et al.*,2011)
Chicken feather	Biochemical characterization and antioxidant activities	Protein hydrolysate possess good antioxidant potential	(Kumar *et al.*,2012)
Chicken feather	Antioxidant activities	Protein hydrolysate possess antioxidant potential	(Kumar *et al.*,2012)
Chicken feather	Carotenoid production	Chicken feathers have effectively used as a novel carotenoid production substrate for R. *glutinis*.	(Taskin *et al.*,2011)
Chicken feather	Fermentation for exopolysaccharide and mycelial biomass	Chicken feathers hydrolysate have effectively used as a novel EPS production substrate	(Taskin *et al.*,2012)
Chicken feather	Characterization of feather degrading	A new strain *Bacillus amyoliquefaciens* exhibited activity in base pH and 50°C	(Cortezi *et al.*,2008)
Feather meal	Biodiesel production	Feather meal biodiesel good quality more biodiesel from other common feedstocks	(Kondamudi *et al.*,2009)

Table 2.3 Research associated of feather and other part of chicken (continue)

Part of chicken	**Biological activity**	**Results**	**Reference**
Chicken feather	Antioxidant activities	Antioxidant activity of chicken feather lower than BHT	(Flaczyk *et al.*,2003)
Chicken skin	Antioxidant activities	Protein hydrolysate MW < 1 kDa showed better antioxidant activity	(Onuh *et al.*,2014)
Chicken breast	Antioxidant activities	Protein hydrolysate show strong reducing power	(Sun *et al.*,2012)
Chicken muscle	Antioxidant activities	Protein hydrlysate showed higher antioxidant potential	(Centenaro *et al.*,2014)

CHAPTER III

EXPERIMENTAL

3. Materials and methods

3.1 Biological materials

Chicken feather meal from Betagro Public Company (Thailand) was ground to a small size. Five human tumor cell lines, BT474 (breast), Chago-K1 (lung), Hep-G2 (hepatoma), KATO-III (gastric), and SW620 (colon) were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand.

3.2 Chemicals

L-ascorbic acid, bovine serum albumin (BSA), 2, 2-Diphenyl-1-picryhydrazyl (DPPH), ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate)), curcumin from *Curcuma longa* (Turmeric), napthylethylenediamine dihydrochloride (NED), sodium nitroprusside (SNP), BHT were purchased from Sigma Chemicals Co. (USA). Potassium dihydrogen phosphate (KH_2PO_4), potassium persulfate ($K_2S_2O_8$) and methanol were purchased from Merck KGaA Darmstadt (Germany). Biomax® 5 and Biomax® 10 were sourced from Amersham Biosciences (Sweden). Protease G6 was obtained from Siam Victory Chemicals Co., Ltd (Thailand). All other biochemicals and chemicals used in the investigation were of analytical grade.

3.3 Preparation of chicken feather meal

The chicken feather meal was dried at 60 ℃ overnight, and then filtered through a 150 micron sieve.

3.4 Amino acid composition analysis

The analysis method according to Li *et al* (Li *et al.*,2013) was followed, with slight modifications. One milligram of the chicken feather meal was dissolved in 5 ml

of 6 N HCl, and then placed in a test tube in a heating block at 110 ºC for 22 h. Then it was added to 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. Finally, it was 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 (Waters Alliance 2695 with heater Jasco FP2020 fluorescence detector at Ex 250 nm and Ex 395 nm) using a Hypersil GOLD column C18 (4.6 mm x 150 mm, 3µm) at 35 ±1 ºC. The injected sample volume was 5 µl. The elutes were sodium acetate buffer pH 4.90, and 60 % acetonitrile, at a flow rate of 0.3 ml/min. The run-time injection was 15 min.

3.5 Preparation of chicken feather meal protein hydrolysate by alkaline protease

The sieved chicken feather meal was hydrolysed using Protease G6 (alkaline serine protease) (2.9 x 10^5 DU/g) in 20 mM Tris-HCl buffer, containing 150 mM NaCl, pH 8.0 with a substrate: enzyme ratio of 0.5:10 (w/v) for five concentrations in ratio 1:1 (5.8 x 10^5 DU/g), 1:2 (2.9 x 10^5 DU/g), 1:4 (1.45 x 10^5 DU/g), 1:8 (7.25 x10^5 DU/g), and 1:16 (3.625 x 10^5 DU/g)(w/v). The hydrolysis reaction was conducted at 50 ºC in an incubator shaker at 125 rpm for 4 h. Samples were collected every 30 min from 0-240 min. The hydrolysis reaction was stopped by heating at 90 ºC for 10 min. Then the reaction mixture was centrifuged at 10,000 × g for 10 min at 4 ºC and the supernatant was collected. The optimized condition and time were monitored by DPPH assay. Protein content was determined by Bradford's procedure using bovine serum albumin (BSA) as a protein standard, and measuring the absorbance at 595 nm (Bradford,1976).

3.6 Molecular weight cut-off by ultrafiltration

The protein hydrolysates were fractionated through ultrafiltration membranes using a bioreactor system (Amersham Biosciences, Sweden). Chicken

feather meal peptide solution was pumped through a range of nominal molecular weight cut-off (MWCO) membranes of 10, 5, and 3 kDa in order of decreasing pore size. Five fractions were collected from the membrane filtration; retentate from 10 kDa membrane (MW > 10 kDa), retentate from 5 kDa membrane (MW 5 - 10 kDa), retentate from 3 kDa membrane (MW 3 - 5 kDa), permeate from 5 kDa membrane (MW < 5 kDa), and permeate from 3 kDa membrane (MW < 3 kDa).

3.7 DPPH radical scavenging activity

The antioxidant activity of each fraction was determined using DPPH with slight modifications from the method used by Aluko and Monu (Aluko and Monu, 2003). In a 96-well plate, 40 µl of each chicken feather meal protein hydrolysate was combined with 160 µl of 0.1 mM DPPH dissolved in 95 % methanol and kept in the dark to react for 30 min at room temperature. The absorbance was measured at 517 nm with a spectrophotometer. Higher DPPH radical scavenging activity shows lower absorbance. Ascorbic acid was used as the positive control. The percentage inhibition of radical scavenging activity was calculated from the following equation:

$$\% \text{ scavenging activity} = \frac{[Abs_{control} - (Abs_{sample} - Abs_{background})] \times 100}{Abs_{control} - Abs_{blank}}$$

where Ac is the absorbance of water plus DPPH (in methanol), Acb is the absorbance of the blank (water plus methanol without DPPH), As is the absorbance of the sample plus DPPH (in methanol), and Asb is the absorbance of the sample plus methanol without DPPH. Different sample concentrations were used to obtain a calibration curve and calculate the IC_{50} values.

3.8 ABTS scavenging activity

ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate)) radical scavenging activity of chicken feather meal protein hydrolysates was determined following the

method described by Tanzadehpanah *et al*. (Tanzadehpanah *et al.*,2012), with slight modifications for a 96-well plate. The stock solution was 7 mM $ABTS^{\bullet+}$ and 2.45 mM potassium persulfate. The solution mixture was kept in the dark at room temperature for 12-16 h before use. For the assay, 25 µl of sample was mixed with 300 µl of $ABTS^{\bullet+}$ solution, and incubated at room temperature for 10 min. The absorbance was measured at 734 nm with a spectrophotometer. Ascorbic acid was used as the positive control. The percentage inhibition of radical scavenging activity was calculated from the following equation:

$$\% \text{ scavenging activity} = \frac{[Abs_{control} - (Abs_{sample} - Abs_{background})] \times 100}{Abs_{control} - Abs_{blank}}$$

where Abs control is the absorbance of control (no sample), Abs sample is the absorbance of sample, Abs background is the absorbance of background (color of sample), and Abs blank is the absorbance of blank (water).

3.9 Nitric oxide scavenging activity

Nitric oxide radical scavenging activity was assayed following the method of (Garrat, 1964), with slight modifications for a 96-well plate. Each sample was mixed with 10 mM of sodium nitroprusside in a phosphate buffer saline pH 7.4, and incubated at room temperature for 150 min. Then Griess reagent (0.33 % sulfanilamide in 20 % acetic acid and 15 N-naphthyl-ethyldiamine (NED)) was added. The absorbance was measured at 540 nm with a spectrophotometer. The percentage of scavenging activity of the sample was compared with ascorbic acid as the positive control. The percentage inhibition of radical scavenging activity was calculated from the following equation:

$$\% \text{ scavenging activity} = \frac{[Abs_{control} - (Abs_{sample} - Abs_{background})] \times 100}{Abs_{control} - Abs_{blank}}$$

where Abs control is the absorbance of control (no sample), Abs sample is the absorbance of sample, Abs background is the absorbance of background (color of sample), and Abs blank is the absorbance of blank (water).

3.10 Measurement of protein content

The protein concentration of chicken feather meal protein hydrolysate was determined according to the method of Bradford (Bradford,1976), using bovine serum albumin (BSA) as the standard. Twenty microliters of each sample was mixed with 200 µl Bradford working buffer in a 96 well plate and then incubated for 20 min. The absorbance was determined at 595 nm with a spectrophotometer.

3.11 Cytotoxicity assay for human malignant cell lines

A cytotoxicity assay for the *in vitro* antiproliferative activity of five different human malignant cell lines BT474 (breast), Chago-K1 (lung), Hep-G2 (hepatoma), KATO-III (gastric), and SW620 (colon) was performed on tissue culture. The cells were kept in a complete medium composed of RPMI-1640 along with 2.0 mM L-glutamine and 10 % (v/v) FCS at 37 ºC under 5 % (v/v) CO_2 conditions. The cells were aspirated, trypsinized, and finally washed, prior to seeding at a density of $5x10^3$ cells/µl in 200 µl of complete medium in a 96-well culture plate. They were then cultured for 1 day. Serial dilutions of the samples were then added into each well and incubated for 3 days. Ten microliters of 3-[5,5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium] bromide solution (5 mg/ml) (MTT) was then poured into the wells and the samples incubated for 4 h prior to aspiration of the medium and washing with RPMI-1640 to remove any residual medium still left in the solution. Subsequently, 150 µl of DMSO was added into each well and then left for 30 min. The cell and its solution were then aspirated and the absorbance determined at 540 nm with a microplate reader. The percentage of anticancer activity was measured by the following equation:

$$\text{Cytotoxicity (\%)} = ((A_0 - A_1)/A_0) \times 100$$

where, A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the hydrolysates.

3.12 Flow cytometry

Apoptosis was assayed by Annexin V-FITC and propidium iodide (PI) reagent staining, followed by analysis with fluorescence-activated cell sorting (BioLegend Inc., Sandiego, CA, USA). The method described in the Annexin V-FITC/PI Detection Kit was followed. The BT474 cells were seeded in 25 cm^2 flasks, at $2x10^5$ cells per flask, in a complete medium composed of RPMI-1640 with 2.0 mM L-glutamine, and 10 % (v/v) fetal calf serum (FCS) at 37 ℃ under 5 % (v/v) CO_2 conditions. After incubation overnight at 37 ℃ in 5 % CO_2, the cells were treated with protein hydrolysate and then incubated for 8 h at 37 ℃ in 5 % CO_2. The cells were then harvested by trypsinization, and washed twice in cool phosphate buffer saline (PBS) pH 7.2 with 1 % fetal calf serum (FCS). Resuspended cell pellets in 100 µl Annexin V Binding Buffer and 100 µl cell suspension were transferred to a 1.5 ml micro centrifuge tube. Then 2.5 µl of FITC Annexin V and 5 µl of propidium iodide solution were added into each tube. The cell suspension was vortexed and incubated in the dark for 15 min at room temperature. Then 200 µl of Annexin V binding buffer was added and the apoptosis was immediately measured by flow cytometry.

3.13 Purification of antioxidant peptides

Protein hydrolysates MW <3 kDa were analyzed by RP-HPLC. The analyses were carried out on a Luna C18 (4.6 mm × 250 mm) column. The elution was performed using a mobile phase consisting of 70 % (v/v) acetonitrile (ACN) in deionized water, containing 0.05 % (v/v) trifluoroacetic acid (TFA) (elute A), and 0.1 % v/v) (TFA) (elute B). The linear gradient was 0 % to 70 % of elute A at a flow rate of 0.7 ml/min. Fifty microliters of fractionate sample MW <3 kDa were injected. The

elution peaks were detected at a wavelength of 280 nm. Individual fractions were lyophilized until used.

3.14 Identify of peptide sequences

The desirable fraction after RP-HPLC purification of protein and peptide analysis by mass spectrometry using the LC/MS/MS system consisted of a liquid chromatography (LC) part (Dionex Ultimate 3000, Thermo Scientific) in combination with an electrospray ionization (ESI)/ quadrupole ion trap mass spectrometer (Model Amazon SL, Bruker, Germany). The LC separation was performed on a reversed phase column (Hypersil GOLD 50 mm × 0.5 mm, 5 µm C18), protected by a guard column (Hypersil GOLD 30 mm × 0.5 mm, 5 µm C18), eluted at a flow rate of 100 µl/min under gradient conditions of 5-80 %B over 50 min. Mobile phase A consisted of water/formic acid (99.9:0.1, v/v), and B consisted of acetonitrile (100, v). Mass spectral data from 300 to 1500 m/z was collected in the positive ionization mode. All data were processed to the MASCOT database in matrix science to identify the peptides.

3.15 Statistical analysis

All experiments were performed in triplicate, based on the protein equivalent of each sample, and the results were expressed as mean±standard error (SEM). Regression analysis and IC_{50} values were calculated using GraphPad Prism Version 6.01 for Windows (GraphPad Software Inc.)

Statistical analysis for comparing the results were performed by one-way ANOVA, followed by Duncan's test at $P < 0.05$, was considered to represent statistical significance. All statistical analysis were performed according to the statistics program by SPSS version 22.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Amino acid content of chicken feather meal

The amino acid compositions were analyzed by RP-HPLC analysis as shown in Table 4.1. Chicken feather meal powder was rich in glutamic acid, proline, serine, glycine, arginine, leucine, valine, aspartic acid, phenylalanine, threonine, isoleucine, alanine, cysteine, lysine, tyrosine, methionine, histidine, and tryptophan. Peptides possessing antioxidant activity contain aromatic amino acids such as tyrosine and tryptophan (Wang *et al.*,2005). Aromatic amino acids can donate protons to the free radical for stabilization. Chicken feather meal contained 4.03 %, 1.10 %, and 0.28 % of phenylalanine, tyrosine and tryptophan, repectively and could therefore produce antioxidant activity. Saito *et al* (Saito *et al.*,2003) reported that several amino acids, tyrosine, methioine, histidine, lysine, and tryptophan are accepted to be antioxidative and exhibit higher antioxidant activities when incorporated into peptides. Peptides that contain leucine, arginine, aspartic acid, alanine, and methionine show strong antioxidant activity (Dong *et al.*,2008; Wang *et al.*,2007).

Table 4. 1 Total amino acid profiles of chicken feather meal

Amino acids	Results (%)
Alanine (Ala)	3.80
Arginine (Arg)	6.30
Glycine (Gly)	6.82
Aspartic acid (Asp)	5.70
Valine (Val)	5.85
Cystine (Cys)	2.90
Glutamic acid (Glu)	10.6
Leucine (Leu)	6.46
Isoleucine (Ile)	3.94
Histidine (His)	0.59
Threonine (Thr)	3.96
Proline (Pro)	8.37
Lysine (Lys)	1.45
Methionine (Met)	0.67
Hydroxyproline (Hyp)	Not detected
Serine (Ser)	7.84
Phenylalanine (Phe)	4.03
Hydroxylysine (Hyl)	Not detected
Tyrosine (Tyr)	1.10
Tryptophan (Trp)	0.28

4.2 Optimization of enzymatic hydrolysis conditions of chicken feathers by Protease G6

The optimization of enzymatic hydrolysis conditions of chicken feathers by Protease G6 was screened at different enzyme concentration ratios (1:1, 1:2, 1:4, 1:8, and 1:16) at various times (0, 15, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min). The protein hydrolysates were compared for DPPH radical scavenging inhibitory activity. The IC_{50} values of chicken feather meal protein hydrolysates produced by enzymatic hydrolysis with Protease G6 at difference concentrations and time intervals are presented in Table 4.2. The optimal condition of protein hydrolysate for the five different enzyme concentrations was screened. All protein hydrolysates were evaluated for antioxidant activity by DPPH radical scavenging. The twofold dilution assay gave good antioxidant activity and a low IC_{50} value of 34.48±0.597 μg/ml at 30 min hydrolysate time. The best condition of protein hydrolysate representd the IC_{50} value at concentration 1:2 (2.9×10^5 DU/g) (w/v) at 30 min and they were selected for further study.

Table 4. 2 The optimal condition of protein hydrolysate for the five different screened enzyme concentrations compared to their DPPH radical scavenging activity assay

Hydrolysate time (min)	Enzyme concentration (DU/g)				
	Undiluted (5.8×10^5 DU/g)	2-fold diluted (2.9×10^5 DU/g)	4-fold diluted (1.45×10^5 DU/g)	8-fold diluted (7.25×10^6 DU/g)	16-fold diluted (3.625×10^6 DU/g)
	IC_{50}(µg/ml)) ±SEM	IC_{50}(µg/ml)) ±SEM	IC_{50}(µg/ml)) ±SEM	IC_{50}(µg/ml))± SEM	IC_{50}(µg/ml))± SEM
0	62.08±3.457	62.35±2.227	78.61±2.998	56.97±5.183	50.375±2.494
15	54.19±3.273	51.63±1.237	77.26±3.068	82.52±0.756	96.29±8.499
30	51.02±4.907	34.48±0.597	78.65±0.089	>300±122.395	88.63±2.800
45	60.45±0.049	47.94±1.880	99.76±5.147	88.99±3.768	101.35±0.778
60	55.79±6.272	50.22±0.155	96.21±8.478	103.50±4.666	107.10±4.384
90	57.18±0.947	45.51±0.424	95.51±6.774	108.90±0.989	99.475±0.219
120	57.97±1.371	63.33±7.007	113.45±3.040	123.60±0.000	107.65±1.344
150	62.15±3.273	59.01±3.104	108.05±2.050	139.40±4.242	215.25±17.890
180	69.13±2.976	79.40±11.207	103.40±0.140	116.05±0.212	104.78±7.241
210	73.06±0.000	60.14±3.189	113.90±1.272	134.15±4.879	136.05±2.758
240	71.15±1.251	109.00±1.979	125.45±9.828	129.45±0.212	85.61±1.216

All data are shown as mean (±) standard deviation (SEM) obtained from three replicated determinations

4.3 Antioxidant activities of protein hydrolysate fraction

4.3.1 DPPH radical scavenging activity assay

The relatively stable DPPH radical has been widely used to assess the ability of compounds to act as free radical scavengers or hydrogen donors (Li *et al.*,2008). DPPH radicals confront a proton-donating substance such as a peptide, antioxidant substance, or phenolic compound. The reaction of DPPH radical scavenging activity assay leaded to a change in color from purple to yellow, and this absorbance was detected at 517 nm. The results from the chicken feather meal protein hydrolysates are shown in Table 4.3. The hydrolysates were further fractionated into peptide sizes

of MW >10 kDa, 5-10 kDa, <5 kDa, 3-5 kDa, and <3 kDa using membrance ultrafitration. The ultrafiltration fractions were each assayed for DPPH radical scavenging activity. From all the fractions of protein hydrolysate, MW <3 kDa showed the best IC_{50} value (8.94±0.515 µg/ml) which showed less potency than the positive control ascorbic acid (0.02±0.002 µg/ml) (Table 4.3). Similar findings, showing higher DPPH radical scavenging activity of low molecular weight peptides were reported for hemp seed protein hydrolysate (Girgih *et al.*,2011) and quinoa protein hydrolysate fractions (Aluko and Monu,2003).

4.3.2 ABTS radical scavenging activity assay

ABTS radical scavenging activity assays measure the antioxidant activity observed as discolorations of blue-green color at absorbance of 734 nm (Arts *et al.*,2004). Table 4.3 shows IC_{50} values (µg/ml) of chicken feather meal protein hydrolysate. The IC_{50} value for MW <3 kDa, 2.50±0.047 µg/ml showed the highest scavenging activity (Table 4.3). BHT as positive control showed the lowest scavenging activity (56.80±1.678 µg/ml) for ABTS. (Siswoyo *et al.*,2011) stated that ABTS can be dissolved in aqueous as well as organic media. In contrast, DPPH can be dissolved only in organic media (especially alcoholic), not in aqueous solution. $ABTS^{\bullet+}$ can also be evaluated for both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids (Awika *et al.*,2003).

4.3.3 Nitric oxide radical scavenging activity assay

The fractions of chicken feather meal were subjected to an ultrafiltration technique. The protein hydrolysates were incubated with a solution of SNP in phosphate buffer saline for 150 min at 25 ºC. This resulted in the generation of nitric oxide, and they were then incubated with Griess reagent. Protein hydrolysate fractions can effectively reduce the generation of nitric oxide. Table 4.3 shows the IC_{50} values of the seven fractions. The fraction MW <3 kDa gave the best IC_{50} value for nitric oxide radical scavenging activity (0.07±0.027 µg/ml), 22 times more active than positive control (curcumin IC_{50} = 31.18±8.226 µg/ml). The reactive oxygen species of nitric oxide (NO) is an important chemical mediator, generated by

macrophages, endothetial cells, and neurons (Parul *et al.*,2008). The NO radical can play a multiple role in various biological processes, serving as an effector molecular, vasodilator, antimicrobial agent, and neuronal messenger (Hagerman *et al.*,1998).

Table 4.3 IC_{50} value of *in vitro* antioxidant activities of chicken feather meal protein hydrolysate fraction

Fractions	Antioxidation activity IC_{50} value (µg/ml)		
	$DPPH^{\cdot}$ scavenging	$ABTS^{+\cdot}$ scavenging	NO^{-} scavenging
Crude protein	272.27±1.414^{e}	120.00±5.936^{e}	133.10±3.916^{d}
Protein hydrolysate	37.99±0.260^{b}	15.38±1.002^{b}	11.18±1.228^{b}
MW > 10 kDa	116.50±3.912^{d}	61.25±0.871^{d}	31.30±1.124^{c}
MW 5-10 kDa	63.03±0.355^{c}	28.04±1.548^{c}	14.84±1.226^{b}
MW < 5 kDa	9.46±0.422^{a}	5.31±0.151^{a,b}	3.54±0.708^{a}
MW 3-5 kDa	10.30±0.218^{a}	8.69±0.128^{a}	2.70±0.297^{a}
MW < 3 kDa	8.94±0.515^{a}	2.50±0.047^{a}	0.07±0.027^{a}
L- ascorbic acid	0.02±0.002^{a}	-	-
BHT	-	57.26±1.678^{b}	-
Curcumin	-	-	31.18±8.226^{c}

All data are shown as mean values (±) standard deviation (SD) of mean and are obtained from three replicated determination. IC_{50} values of various concentrations of substrate digested by enzyme hydrolysis of protease G6. Value are expressed as mean ± SD and a, b, c, d and e show significant value at p > .05.

4.5 Cytotoxicity assay

The chicken feather meal protein hydrolysate was applied in a cytotoxicity assay of human malignant cell lines. The efficiency of antioxidant activity by radical scavenging was evaluated. Five human malignant cell lines were chosen for the investigation of cancer cells consisting of BT474 (breast), Chago-K1 (lung), Hep-G2 (hepatoma), KATO-III (gastric), and SW620 (colon). Table 4.4, shows the in vitro antioxidant activity of all fractions, reported as the IC_{50} value of sufficient ability to inhibit the human cancer cell. The CFMPH fraction shows significant anticancer activity against the five human malignant cell lines, BT474 (0.027±0.040 µg/ml), Chago-K1 (1.29±0.294 µg/ml), Hep-G2 (1.91±0.125 µg/ml), KATO-III (1.67±0.263 µg/ml),

and SW620 (1.95±0.468 μg/ml). These results show that BT474 cells had the best anticancer activity. Kannan *et al*. (Kannan *et al.*,2008) reported that several bioactive peptides demonstrated antioxidant, antihypertensive, antiangiogenic, and antiobesity activity. However, there have been few studies to assess the ability of proteins or peptides from animals to suppress human malignant cell lines.

Table 4. 4 The results of five different human malignant cell lines treated with chicken feather meal

IC_{50} values (μg/ml)	**Human malignant cell line**				
	BT474	Chago-K1	Hep-G2	KATO-III	SW620
Crude protein	0.03±0.004^{a}	1.87±0.305^{a}	20.81±0.800^{c}	6.68±0.477^{b}	0.36±0.147^{a}
Protein hydrolysate	1.03±0.121^{b,c}	5.14±0.362^{b}	18.38±0.591^{c}	17.89±1.316^{c}	17.33±1.191^{d}
MW >10kDa	3.77±0.418^{e}	6.25±0.382^{b}	26.94±1.199^{b}	23.90±2.629^{d}	23.06±1.814^{e}
MW5-10kDa	7.49±0.475^{f}	13.29±1.158^{c}	30.45±1.409^{e}	25.99±2.194^{d}	22.35±2.345^{e}
MW<5 kDa	1.37±0.309^{c}	2.78±0.354^{a}	3.64±0.356^{a}	4.01±0.583^{a,b}	4.50±0.705^{b,c}
MW3-5 kDa	2.77±0.239^{d}	2.97±0.413^{a}	11.79±0.937^{b}	7.58±0.859^{a}	6.63±0.502^{c}
MW <3 kDa	0.27±0.040^{a,b}	1.29±0.274^{a}	1.91±0.125^{a}	1.67±0.263^{a}	1.95±0.468^{a,b}
Doxorubicin	0.89±0.056^{a}	0.29±0.030^{b}	0.13±0.012^{a,b}	1.04±0.113^{c}	0.02±0.004^{a}

All data are shown as mean values (±) standard deviation (SD) of mean and are obtained from three replicated determination. IC_{50} values of various concentrations of substrate digested by enzyme hydrolysis of protease G6. Value are expressed as mean ± SD and a, b, c, d, e and f show significant value at p > .05.

4.6 Apoptosis

Apoptosis is a cell death process which is also called programmed cell death. The results of MTT assay suggested that protein hydrolysates prepared from alkaline protease affected BT474 cell viability. The effect of protein hydrolysates on the promotion of apoptosis in BT474 cells was also investigated. To study the effect of protein hydrolysates on early apoptosis in breast cancer cells (BT474), apoptosis cells were detected by flow cytometry using Annexin-V FITC and propidium iodide

(PI) staining. The BT474 cells were cultured in RPMI-1640 medium for 24 h, and then treated with protein hydrolysate MWCO <3 kDa (0.27 µg/ml) for 8 h. The percentage of cell death at the early apoptotic stage is shown in Figure 4.1. Results showed that the populations of BT474 cells treated with protein hydrolysate induced apoptosis. The early and late stages of apoptotic cells detected were 1.71 % and 1.00 %, respectively (Figure 4.1 (B)), compared with the population of control (Figure 4.1 (A)).

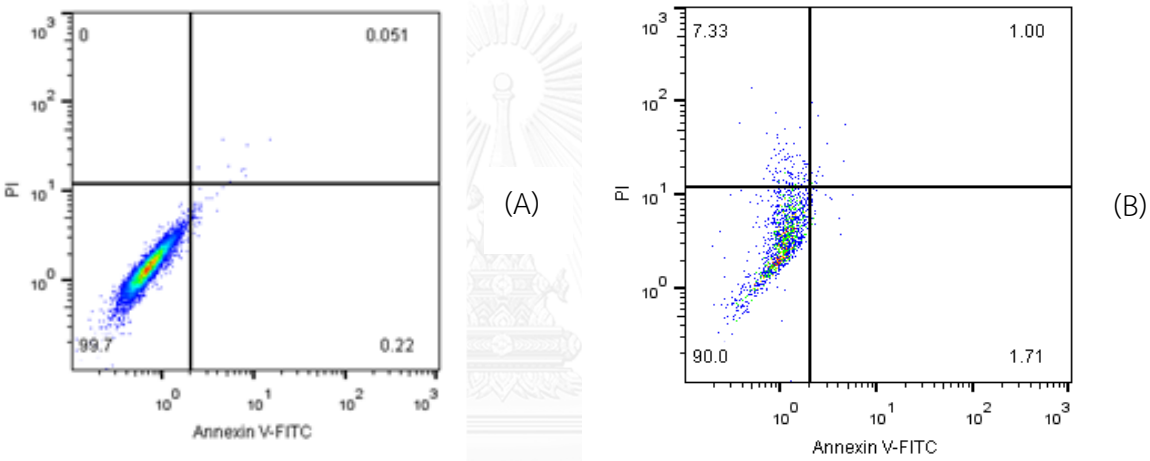


Figure 4. 1 Effect of protein hydrolysates on apoptosis of BT474 breast cancer cells after Annexin V FITC/PI staining, (A) Control, and (B) BT474 cells treated for 8 h with protein hydrolysates

4.7 Purification of antioxidative peptide

The fraction after purification with the ultrafiltration membrane of MW <3 kDa was CFMPH. This fraction was further analyzed by RP-HPLC on a Luna 5u C18 (4.6 mm × 250 mm) column at 280 nm, using a linear gradient of acetonitrile solvent containing 0.1 % trifluoroacetic acid (TFA) at a flow rate 0.7 ml/min. The results are shown in Figure 4.2. Four fractions were collected and named as CFMPH1-4. Fractions of protein hydrolysates were collected at retention time 0-10 (CFMPH1), 10-20 (CFMPH2), 20-30 (CFMPH3), and 30-40 min (CFMPH4). All fractions were assessed their

antioxidant activity using DPPH, ABTS and nitric oxide radical scavenging activity. The results percentage maximal inhibition of DPPH radical scavenging activity were showed that protein hydrolysate CFMPH1 exhibited the highest of maximal inhibition at 45.70 % followed by CFMPH3, CFMPH2 and CFMPH4 at 14.57 %, 13.60 % and 3.328 %, respectively. The results of four fractions were examined ABTS radical scavenging activity could not be detected. The percentage maximal inhibitions of nitric oxide radical scavenging activity were CFMPH1, CFMPH4, CFMPH2 and CFMPH3 at 34.50 %, 34.41 %, 33.70 % and 33.17 %, respectively. All of these fractions were subjected to further identification of the amino acid sequences of the peptide by mass spectrometry (LC/MS/MS).

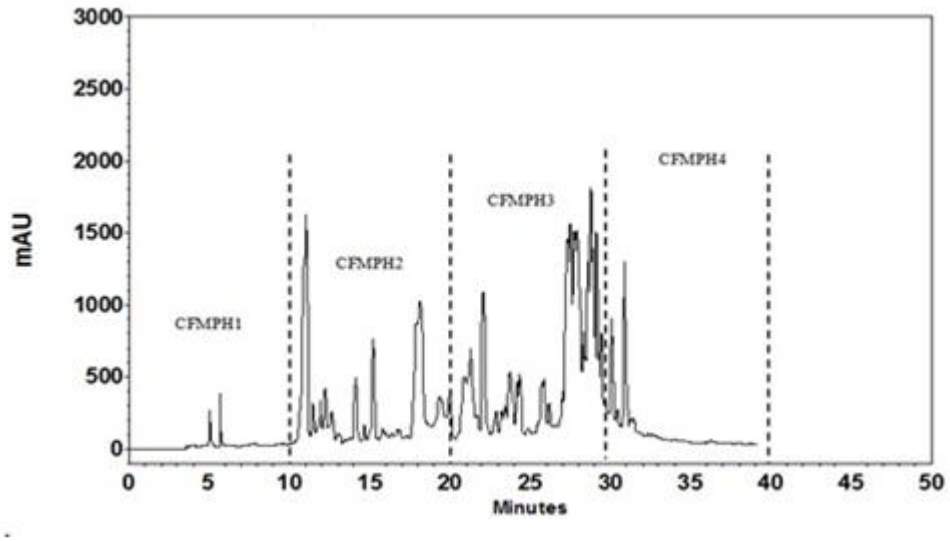


Figure 4. 2 RP-HPLC chromatogram of MW <3 kDa protein hydrolysates from chicken feather meal prepared by Protease G6 digestion on a Luna C18 (4.6 mm × 250 mm) column

4.8 Identification of antioxidant peptides by LC/MS/MS

The identification of antioxidant peptides by mass spectrometry has been widely used to analyze the amino acid sequences of peptides and proteins due to high throughput and sensitivity (Li *et al.*,2007). Purified peptide from chicken feather meal was subjected to matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy and the results analyzed by the MASCOT database

program. Chicken feather hydrolysate MW <3 kDa purified by RP-HPLC gave four fractions of protein hydrolysate for analysis by LC/MS/MS. Table 4.5 shows the four fractions (CFMPH1-4). The amino acid sequence and molecular mass identified eleven peptides. Results showed 7-22 amino acid residues in the four fractions. Fraction 1 (CFMPH1) consisted of FGTYGHPRPRFYYNP (P1) and FVGSLPF (P2). Fraction 2 (CFMPH2) consisted of GDLQCPALCPPGPGGVGEHSGS (P3), LGVISIERA (P4), and LADVDGCHIVGYFGK (P5). Fraction 3 (CFMPH3) consisted of SVEAVSPEVFCHWLIG (P6), and APEAQVSVQPLFGQ (P7). Fraction 4 (CFMPH4) consisted of ETNNGGWTL (P8), GLLIGDDLSAVV (P9), GPDGLSEPPGV (P10), and LCASSLDGGYNSPLYFAAG (P11).

In previous research Try, Typ, Phe, His, Cys, and Met showed the highest antioxidant activity (Zhang *et al.*,2014; Zhou *et al.*,2012). Generally, aromatic amino acids in peptides consisting of Phe, Tyr, and Trp have very good radical scavenging activity due to their special structure which allows the scavenging of unpaired electrons or radicals by donating protons (Fan *et al.*,2012; Zhang *et al.*,2014), the imidazole group in His has proton-donation ability (Ren *et al.*,2008), Cys donates the sulfur hydrogen (Rajapakse *et al.*,2005). The combination of the Gly-Pro and Leu-Gly sequences plays an important role in radical scavenging potency (Byun *et al.*,2009). Moreover, positioning of hydrophobic amino acid residues, Val or Leu at the N-terminus end, and Pro, Asp, His or Tyr in the sequence of antioxidative peptides is important in antioxidant activity (Ren *et al.*,2010). Bioactive peptides usually contain 2-20 amino acid residues per molecule and the lower their molecular weight, then the higher their chance to cross the intestinal barrier and exert a biological effect (Pihlanto-Leppälä,2000).

Table 4. 5 Amino acid sequence of protein hydrolysates from chicken feather meal with molecular weight less than 3 kDa by LC/MS/MS

Fractions	Sequence	Protein name	Accession number	Organism
1	(P1)FGTYGHPRPRFYYNP	BPTI-like protein (fragment)	363805369	*Daboia siamensis*
	(P2)FVGSLPF	Hormone receptor-like in 38	115501314	*Apis mellifera*
2	(P3)GDLQCPALCPPGPGGVGEHSGS	Collagen alpha 3(IX) chain	2134932	*Human (fragment)*
	(P4)LGVISIERA	Na+- phosphate cotransporter type II	1470056	*Homo sapiens*
	(P5)LADVDGCHIVGYFGK	CHM	167784173	*Drosophila melanogaster*
3	(P6)SVEAVSPEVFCHWLIG	cytochrome c oxidase subunit II, partial (mitochondrion)	671706335	*Arcidens confragosus*
	(P7)APEAQVSVQPLFGQ	cerebrin 30	542782	*Human (fragment)*
4	(P8)ETNNGGWTL	beta-fibrinogen	156753235	*Ixonotus guttatus*
	(P9)GLLIGDDLSAVV	Cytochrome P450 UT-7b	543379	*Rat (fragment)*
	(P10)GPDGLSEPPGV	arginosuccinate synthetase, partial	154184403	*Homo sapiens*
	(P11)LCASSLDGGYNSPLYFAAG	T cell receptor beta-chain CDR3	6960042	*Mus musculus*

CHAPTER V
CONCLUSION

Results from this study show that the protein hydrolysates prepared from chicken feather meal using protease G6, could produce peptides with antioxidative activities. The activity assessment for fractions separated by membrance ultrafiltration showed that low molecular weight peptides (MW < 3 kDa) were important for their antioxidant activities. The fractions of protein hydrolysate were applied to cytotoxicity assay in five different human malignant cell lines. The results showed that the best inhibition antiproliferation cancer cell was the breast cancer cell (BT474). MW < 3 kDa was further separated using RP-HPLC into four fractions which were collected and identified by LC/MS/MS. The obtained antioxidant peptide comprised 2-22 amino acids in peptide chains. The antioxidant amino acids present in the identified peptides might be responsible for their antioxidant activities, especially Trp, Tyr, Phe, Cys, Met, and His. Based on these results, the peptides obtained from chicken feather meal by enzymatic hydrolysis could be used as potential natural antioxidants to enrich the antioxidant properties of functional and fresh foods, and neutraceuticals.

REFERENCES

Allison, M.R. (2001). Cancer. In Encyclopedia of life science. Nature Publishing Group, 1-8.

Aluko, R., and Monu, E. (2003). Functional and bioactive properties of quinoa seed protein hydrolysates. *Journal of Food Science* 68, 1254-1258.

Amadou, I., Yong-Hui, S., Sun, J., and Guo-Wei, L. (2009). Fermented soybean products: some methods, antioxidants compound extraction and their scavenging activity. *Asian Journal of Biochemistry* 4, 68-76.

Amaeze, O., Ayoola, G., Sofidiya, M., Adepoju-Bello, A., Adegoke, A., and Coker, H. (2011). Evaluation of antioxidant activity of *Tetracarpidium conophorum* (Müll. Arg) Hutch & Dalziel leaves. *Oxidative Medicine and Cellular Longevity* 2011.

Ames, B.N. (1983). Dietary carcinogens and anticarcinogens oxygen radicals and degenerative diseases. *Science* 221, 1256-1264.

Ames, B.N., Shigenaga, M.K., and Hagen, T.M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences* 90, 7915-7922.

Anwar, A., and Saleemuddin, M. (1998). Alkaline proteases: a review. *Bioresource Technology* 64, 175-183.

Arrigoni, O., and De Tullio, M.C. (2002). Ascorbic acid: much more than just an antioxidant. *Biochimica et Biophysica Acta (BBA)-General Subjects* 1569, 1-9.

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

Astley, S.B. (2003). Dietary antioxidants—past, present and future? *Trends in Food Science and Technology* 14, 93-98.

Awika, J.M., Rooney, L.W., Wu, X., Prior, R.L., and Cisneros-Zevallos, L. (2003). Screening methods to measure antioxidant activity of sorghum (*Sorghum*

bicolor) and sorghum products. *Journal of Agricultural and Food Chemistry* 51, 6657-6662.

Bagchi, K., and Puri, S. (1998). Free radicals and antioxidants in health and disease. *Eastern Mediterranean Health Journal* 4, 350-360.

Baker, D.H., Blitenthal, R.C., Boebel, K.P., Czarnecki, G.L., Southern, L., and Willis, G.M. (1981). Protein-amino acid evaluation of steam-processed feather meal. *Poultry Science* 60, 1865-1872.

Balasundram, N., Sundram, K., and Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry* 99, 191-203.

Bielorai, R., Iosif, B., Neumaek, H., and Alumot, E. (1982). Low Nutritional Value of Feather-Meal Protein for Chicks. *The Journal of Nutrition* 112, 249-254.

Boboev, A., Hasanov, A., Yotova, L., and Hasanov, H. (2012). Antioxidant activity of peptides obtained from wheat and cottonseed proteins. *Bulgarian Journal of Agricultural Science* 18, 103-111.

Bougatef, A., Nedjar-Arroume, N., Manni, L., Ravallec, R., Barkia, A., Guillochon, D., and Nasri, M. (2010). Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinellaaurita*) by-products proteins. *Food Chemistry* 118, 559-565.

Bowler, C., Montagu, M.v., and Inze, D. (1992). Superoxide dismutase and stress tolerance. *Annual Review of Plant Biology* 43, 83-116.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.

Burton, G., and Ingold, K. (1981). Autoxidation of biological molecules. 1. Antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants *in vitro*. *Journal of the American Chemical Society* 103, 6472-6477.

Byun, H.-G., Lee, J.K., Park, H.G., Jeon, J.-K., and Kim, S.-K. (2009). Antioxidant peptides isolated from the marine rotifer, *Brachionus rotundiformis*. *Process Biochemistry* 44, 842-846.

Centenaro, G.S., Salas-Mellado, M., Pires, C., Batista, I., Nunes, M.L., and Prentice, C. (2014). Fractionation of protein hydrolysates of fish and chicken using membrane ultrafiltration: investigation of antioxidant activity. *Applied Biochemistry and Biotechnology* 172, 2877-2893.

Cheeseman, K., and Slater, T. (1993). An introduction to free radical biochemistry. *British Medical Bulletin* 49, 481-493.

Chelikani, P., Fita, I., and Loewen, P. (2004). Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences CMLS* 61, 192-208.

Chen, G.t., Zhao, L., Zhao, L.y., Cong, T., and Bao, S.f. (2007). *In vitro* study on antioxidant activities of peanut protein hydrolysate. *Journal of the Science of Food and Agriculture* 87, 357-362.

Chen, H.-M., Muramoto, K., Yamauchi, F., Fujimoto, K., and Nokihara, K. (1998). Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *Journal of Agricultural and Food Chemistry* 46, 49-53.

Clare, D., and Swaisgood, H. (2000). Bioactive milk peptides: a prospectus. *Journal of Dairy Science* 83, 1187-1195.

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science and Technology* 11, 254-262.

Clemente, A., Vioque, J., and Millan, F. (1999). Vegetable protein hydrolysates. *Department of Physiology and Technology of Vegetable Products Institute of the Fat* 2, 25-32.

Corredig, M., and Dalgleish, D.G. (1997). Studies on the susceptibility of membrane-derived proteins to proteolysis as related to changes in their emulsifying properties. *Food Research International* 30, 689-697.

Cortezi, M., Contiero, J., Lima, C., Lovaglio, R.B., and Monti, R. (2008). Characterization of a feather degrading by *Bacillus amyloliquefaciens* protease: A new strain. *World Journal of Agricultural Sciences* 4, 648-656.

Culotta, V.C., Yang, M., and O'Halloran, T.V. (2006). Activation of superoxide dismutases: putting the metal to the pedal. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1763, 747-758.

Diplock, A.T. (1991). Antioxidant nutrients and disease prevention: an overview. *The American Journal of Clinical Nutrition* 53, 189S-193S.

Dong, S., Zeng, M., Wang, D., Liu, Z., Zhao, Y., and Yang, H. (2008). Antioxidant and biochemical properties of protein hydrolysates prepared from Silver carp (*Hypophthalmichthys molitrix*). *Food Chemistry* 107, 1485-1493.

Dreher, D., and Junod, A.F. (1996). Role of oxygen free radicals in cancer development. *European Journal of Cancer* 32, 30-38.

Eldahshan, O.A., and Singab, A.N.B. (2013). Carotenoids. *Journal of Pharmacognosy and Phytochemistry* 2, 225–234.

Epstein, F.H., Diaz, M.N., Frei, B., Vita, J.A., and Keaney Jr, J.F. (1997). Antioxidants and atherosclerotic heart disease. *New England Journal of Medicine* 337, 408-416.

Esan, Y.O., and Fasasi, O.S. (2013). Amino acid composition and antioxidant properties of African yam bean (*Spenostylis stenocarpa*) protein hydrolysates. . *African Journal of Food Science and Technology* 4, 100-105.

Fakhfakh, N., Ktari, N., Haddar, A., Mnif, I.H., Dahmen, I., and Nasri, M. (2011). Total solubilisation of the chicken feathers by fermentation with a keratinolytic bacterium, *Bacillus pumilus* A1, and the production of protein hydrolysate with high antioxidative activity. *Process Biochemistry* 46, 1731-1737.

Fan, J., He, J., Zhuang, Y., and Sun, L. (2012). Purification and identification of antioxidant peptides from enzymatic hydrolysates of tilapia (*Oreochromis niloticus*) frame protein. *Molecules* 17, 12836-12850.

Flaczyk, E., Amarowicz, R., and Korczak, J. (2003). Antioxidant activity of protein hydrolysate from By-products of food industry. *Journal of Food Lipids* 10, 129-140.

Foti, M.C., Daquino, C., and Geraci, C. (2004). Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH radical in alcoholic solutions. *The Journal of Organic Chemistry* 69, 2309-2314.

Franek, F., Hohenwarter, O., and Katinger, H. (2000). Plant protein hydrolysates: preparation of defined peptide fractions promoting growth and production in animal cells cultures. *Biotechnology Progress* 16, 688-692.

Garrat, D.C. (1964). The quantitative analysis of drugs. *Biochemistry Analytical Chemistry* 3, 456-458.

Gey, K. (1990). The antioxidant hypothesis of cardiovascular disease: epidemiology and mechanisms. *Biochemical Society Transactions* 18, 1041-1045.

Girgih, A.T., Udenigwe, C.C., and Aluko, R.E. (2011). *In vitro* antioxidant properties of hemp seed (*Cannabis sativa* L.) protein hydrolysate fractions. *Journal of the American Oil Chemists' Society* 88, 381-389.

Hacisevkd, A. (2009). An overview of ascorbic acid biochemistry. *Journal of Faculty of Pharmacy of Ankara* 38, 233-255.

Hagerman, A.E., Riedl, K.M., Jones, G.A., Sovik, K.N., Ritchard, N.T., Hartzfeld, P.W., and Riechel, T.L. (1998). High molecular weight plant polyphenolics (tannins) as biological antioxidants. *Journal of Agricultural and Food Chemistry* 46, 1887-1892.

Halliwell, B. (2001). Role of free radicals in the neurodegenerative diseases. *Drugs and Aging* 18, 685-716.

Harborne, J.B. (1995). Phytochemical methods.(New York: Marcel Dekker, Inc.).

Harman, D. (1995). Role of antioxidant nutrients in aging: overview. *Age* 18, 51-62.

He, R., Girgih, A.T., Malomo, S.A., Ju, X., and Aluko, R.E. (2013). Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *Journal of Functional Foods* 5, 219-227.

Huang, D., Ou, B., and Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry* 53, 1841-1856.

Hussain, S.P., Hofseth, L.J., and Harris, C.C. (2003). Radical causes of cancer. *Nature Reviews Cancer* 3, 276-285.

Je, J.-Y., Park, P.-J., and Kim, S.-K. (2005). Antioxidant activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Food Research International* 38, 45-50.

Je, J.-Y., Qian, Z.-J., Byun, H.-G., and Kim, S.-K. (2007). Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochemistry* 42, 840-846.

Jema, A., Bray, F., and Center, M.M. (2011). Global cancer statistics. *CA A Cancer Journal for Clinicians* 61, 69-90.

Jun, S.-Y., Park, P.-J., Jung, W.-K., and Kim, S.-K. (2004). Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *European Food Research and Technology* 219, 20-26.

Kannan, A., Hettiarachchy, N., Johnson, M.G., and Nannapaneni, R. (2008). Human colon and liver cancer cell proliferation inhibition by peptide hydrolysates derived from heat-stabilized defatted rice bran. *Journal of Agricultural and Food Chemistry* 56, 11643-11647.

Kim, E.-K., Lee, S.-J., Jeon, B.-T., Moon, S.-H., Kim, B., Park, T.-K., Han, J.-S., and Park, P.-J. (2009). Purification and characterisation of antioxidative peptides from enzymatic hydrolysates of venison protein. *Food Chemistry* 114, 1365-1370.

Kim, E.K., Kim, Y.S., Ahn, C.B., Moon, S.H., and Park, P.J. (2012). Purification of a novel anticancer peptide from enzymatic hydrolysate of *Mytilus coruscus*. *Journal of Microbiology and Biotechnology* 22, 1381-1387.

Kleekayai, T., Harnedy, P.A., O'Keeffe, M.B., Poyarkov, A.A., CunhaNeves, A., Suntornsuk, W., and FitzGerald, R.J. (2015). Extraction of antioxidant and ACE inhibitory peptides from Thai traditional fermented shrimp pastes. *Food Chemistry* 176, 441-447.

Ko, J.-Y., Lee, J.-H., Samarakoon, K., Kim, J.-S., and Jeon, Y.-J. (2013). Purification and determination of two novel antioxidant peptides from flounder fish (*Paralichthys olivaceus*) using digestive proteases. *Food and Chemical Toxicology* 52, 113-120.

Ko, S.-C., Kim, D., and Jeon, Y.-J. (2012). Protective effect of a novel antioxidative peptide purified from a marine *Chlorella ellipsoidea* protein against free radical-induced oxidative stress. *Food and Chemical Toxicology* 50, 2294-2302.

Kondamudi, N., Strull, J., Misra, M., and Mohapatra, S.K. (2009). A green process for producing biodiesel from feather meal. *Journal of Agricultural and Food Chemistry* 57, 6163-6166.

Korhonen, H., and Pihlanto, A. (2003). Food-derived bioactive peptides-opportunities for designing future foods. *Current Pharmaceutical Design* 9, 1297-1308.

Krishnaiah, D., Sarbatly, R., and Bono, A. (2007). Phytochemical antioxidants for health and medicine–A move towards nature. *Biotechnology and Molecular Biology Reviews* 1, 97-104.

Kumar, D.M., Priya, P., Balasundari, S.N., Devi, G., Rebecca, A.I.N., and Kalaichelvan, P. (2012). Production and optimization of feather protein hydrolysate from *Bacillus* sp. MPTK6 and its antioxidant potential. *Middle-East Journal of Scientific Research* 11, 900-907.

Kumar, D.M., Lavanya, S., Priya, P., Rebecca, A.I.N., Balakumaran, M., and Kalaichelvan, P. (2012). Production of Feather Protein Concentrate from Feathers by *In vitro* Enzymatic Treatment, its Biochemical Characterization and Antioxidant Nature. *Middle-East Journal of Scientific Research* 11, 881-886.

Li, B., Chen, F., Wang, X., Ji, B., and Wu, Y. (2007). Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. *Food Chemistry* 102, 1135-1143.

Li, B., Wang, C., Chen, X., Lyu, J., and Guo, S. (2013). Highly specific separation for antitumor Spiropreussione A from endophytic fungal [*Preussia* sp.] fermentation broth by one-step macroporous resins AB-8 treatment. *Journal of Chromatography B* 938, 1-7.

Li, H.M., Hu, X., Guo, P., Fu, P., Xu, L., and Zhang, X.Z. (2010). Antioxidant properties and possible mode of action of corn protein peptides and zein peptides. *Journal of Food Biochemistry* 34, 44-60.

Li, X., Shen, S., Deng, J., Li, T., and Ding, C. (2014). Antioxidant activities and functional properties of tea seed protein hydrolysates (*Camellia oleifera* Abel.) influenced by the degree of enzymatic hydrolysis. *Food Science and Biotechnology* 23, 2075-2082.

Li, Y., Jiang, B., Zhang, T., Mu, W., and Liu, J. (2008). Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). *Food Chemistry* 106, 444-450.

MacAlpine, R., and Payne, C. (1977). Hydrolysed feather protein as a source of amino acids for broilers. *British Poultry Science* 18, 265-273.

Manczinger, L., Rozs, M., Vagvolgyi, C., and Kevei, F. (2003). Isolation and characterization of a new karatinolytic *Bacillus licheniformis* strain. *Journal of Microbiology and Biotechnology* 19, 35-39.

Mathers, C.D., Boschi-Pinto, C., Lopez, A.D., and Murray, C.J. (2001). Cancer incidence, mortality and survival by site for 14 regions of the world. *Geneva: World Health Organization* 8.

Mendis, E., Rajapakse, N., and Kim, S.-K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *Journal of Agricultural and Food Chemistry* 53, 581-587.

Mohamed, T., Issoufou, A., and Zhou, H. (2012). Antioxidant activity of fractionated foxtail millet protein hydrolysate. *International Food Research Journal* 19, 207-213.

Muñoz, I.G., Moran, J.F., Becana, M., and Montoya, G. (2005). The crystal structure of an eukaryotic iron superoxide dismutase suggests intersubunit cooperation during catalysis. *Protein Science* 14, 387-394.

Nachman, K., Raber, G., Francesconi, K., Navas-Acien, A., and Love, D. (2012). Arsenic species in poultry feather meal. *Science of the Total Environment* 417, 183-188.

Nagmoti, D.M., Khatri, D.K., Juvekar, P.R., and Juvekar, A.R. (2012). Antioxidant activity free radical-scavenging potential of *Pithecellobium dulce* Benth seed extracts. *Free Radicals and Antioxidants* 2, 37-43.

Neeraj, J.P., Singh, S., and Singh, J. (2013). Role of free radicals and antioxidants in human health and disease. *International Journal of Current Research and Review* 5, 14-22.

Nenadis, N., and Tsimidou, M. (2002). Observations on the estimation of scavenging activity of phenolic compounds using rapid 1, 1-diphenyl-2-picrylhydrazyl (DPPH•) tests. *Journal of the American Oil Chemists' Society* 79, 1191-1195.

Onuh, J.O., Girgih, A.T., Aluko, R.E., and Aliani, M. (2014). *In vitro* antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions. *Food Chemistry* 150, 366-373.

Pai, E., and Schulz, G.E. (1983). The catalytic mechanism of glutathione reductase as derived from x-ray diffraction analyses of reaction intermediates. *Journal of Biological Chemistry* 258, 1752-1757.

Pala, F.S., and Tabakçıoğlu, K. (2007). Free radicals: Our enemies or friends?

Park, P.-J., Jung, W.-K., Nam, K.-S., Shahidi, F., and Kim, S.-K. (2001). Purification and characterization of antioxidative peptides from protein hydrolysate of lecithin-free egg yolk. *Journal of the American Oil Chemists' Society* 78, 651-656.

Park, S.Y., Lee, J.S., Baek, H.H., and Lee, H.G. (2010). Purification and characterization of antioxidant peptides from soy protein hydrolysate. *Journal of Food Biochemistry* 34, 120-132.

Parul, R., Kundu, S.K., and Saha, P. (2008). *In vitro* nitric oxide scavenging activity of methanol extract of three Bangladeshi medicinal plants. *The Pharma Innovation - Journal* 1, 83-88.

Peña-Ramos, E.A., Xiong, Y.L., and Arteaga, G.E. (2004). Fractionation and characterisation for antioxidant activity of hydrolysed whey protein. *Journal of the Science of Food and Agriculture* 84, 1908-1918.

Pérez, R.A., Iglesias, M.T., Pueyo, E., González, M., and de Lorenzo, C. (2007). Amino acid composition and antioxidant capacity of Spanish honeys. *Journal of Agricultural and Food Chemistry* 55, 360-365.

Pihlanto-Leppälä, A. (2000). Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends in Food Science and Technology* 11, 347-356.

Pszczola, D.E. (2001). Antioxidants: From preserving food quality to quality of life. *Food Technology* 55, 51-59.

Rajapakse, N., Mendis, E., Byun, H.-G., and Kim, S.-K. (2005). Purification and *in vitro* antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *The Journal of Nutritional Biochemistry* 16, 562-569.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* 26, 1231-1237.

Ren, J., Zheng, X.-Q., Liu, X.-L., and Liu, H. (2010). Purification and characterization of antioxidant peptide from sunflower protein hydrolysate. *Food Technology and Biotechnology* 48, 519-523.

Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., Kakuda, Y., and Xue, S.J. (2008). Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chemistry* 108, 727-736.

Saito, K., Jin, D.-H., Ogawa, T., Muramoto, K., Hatakeyama, E., Yasuhara, T., and Nokihara, K. (2003). Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry. *Journal of Agricultural and Food Chemistry* 51, 3668-3674.

Sefatie, R.S., Fatoumata, T., Eric, K., Shi, Y.H., and Guo-Wei, L. (2013). *In vitro* antioxidant activities of protein hydrolysate from germinated black soybean (*Glycine max* L.). *Advance Journal of Food Science and Technology 5(4): 453-459* 5, 453-459.

Shalaby, E.A., and Shanab, S.M. (2013). Antioxidant compounds, assays of determination and mode of action. *African Journal of Pharmacy and Pharmacolgy* 7, 528-539.

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

Siswoyo, T.A., Mardiana, E., Lee, K.O., and Hoshokawa, K. (2011). Isolation and characterization of antioxidant protein fractions from melinjo (*Gnetum gnemon*) seeds. *Journal of Agricultural and Food Chemistry* 59, 5648-5656.

Sivoňová, M., Tatarkova, Z., Duračková, Z., Dobrota, D., Lehotský, J., Matakova, T., and Kaplan, P. (2007). Relationship between antioxidant potential and oxidative damage to lipids, proteins and DNA in aged rats. *Physiological Research* 56, 757-764.

Smith, M., Richey, G., Sayre, L., Anderson, V., Beal, M., and Kowall, N. (1996). Test for oxidative damage in Alzheimer's. *Nature* 382, 120-121.

Sun, Y., Pan, D., Guo, Y., and Li, J. (2012). Purification of chicken breast protein hydrolysate and analysis of its antioxidant activity. *Food and Chemical Toxicology* 50, 3397-3404.

Taheri, A., Anvar, S., Ahari, H., and Fogliano, V. (2012). Comparison the functional properties of protein Hydrolysates from poultry byproducts and rainbow trout (*Onchorhynchus mykiss*) viscera. *Iranian Journal of Fisheries Sciences* 12, 154-169.

Taherzadeh, M.J., and Karimi, K. (2007). Acid-based hydrolysis processes for ethanol from lignocellulosic materials: a review. *BioResources* 2, 472-499.

Tang, C.-H., Wang, X.-S., and Yang, X.-Q. (2009). Enzymatic hydrolysis of hemp (*Cannabis sativa* L.) protein isolate by various proteases and antioxidant properties of the resulting hydrolysates. *Food Chemistry* 114, 1484-1490.

Tang, C.-H., Peng, J., Zhen, D.-W., and Chen, Z. (2009). Physicochemical and antioxidant properties of buckwheat (*Fagopyrum esculentum Moench*) protein hydrolysates. *Food Chemistry* 115, 672-678.

Tanzadehpanah, H., Asoodeh, A., and Chamani, J. (2012). An antioxidant peptide derived from Ostrich (*Struthio camelus*) egg white protein hydrolysates. *Food Research International* 49, 105-111.

Taskin, M., Sisman, T., Erdal, S., and Kurbanoglu, E.B. (2011). Use of waste chicken feathers as peptone for production of carotenoids in submerged culture of *Rhodotorula glutinis* MT-5. *European Food Research and Technology* 233, 657-665.

Taskin, M., Ozkan, B., Atici, O., and Aydogan, M.N. (2012). Utilization of chicken feather hydrolysate as a novel fermentation substrate for production of exopolysaccharide and mycelial biomass from edible mushroom *Morchella esculenta*. *International Journal of Food Sciences and Nutrition* 63, 597-602.

Thiansilakul, Y., Benjakul, S., and Shahidi, F. (2007). Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). *Food Chemistry* 103, 1385-1394.

Vinson, J.A., Dabbagh, Y.A., Serry, M.M., and Jang, J. (1995). Plant flavonoids, especially tea flavonols, are powerful antioxidants using an *in vitro* oxidation model for heart disease. *Journal of Agricultural and Food Chemistry* 43, 2800-2802.

Wang, J.-s., Zhao, M.-m., Zhao, Q.-z., and Jiang, Y.-m. (2007). Antioxidant properties of papain hydrolysates of wheat gluten in different oxidation systems. *Food Chemistry* 101, 1658-1663.

Wang, W., Mejia, D., and Gonzalez, E. (2005). A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. *Comprehensive Reviews in Food Science and Food Safety* 4, 63-78.

Xie, Z., Huang, J., Xu, X., and Jin, Z. (2008). Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. *Food Chemistry* 111, 370-376.

Zelko, I.N., Mariani, T.J., and Folz, R.J. (2002). Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radical Biology and Medicine* 33, 337-349.

Zeng, Y., Guan, Y., Han, W., and Sun, Y. (2014). Antioxidant peptides from freshwater clam extract using enzymatic hydrolysis. *African Journal of Food Science* 8, 148-154.

Zhang, J., Zhang, H., Wang, L., Guo, X., Wang, X., and Yao, H. (2009). Antioxidant activities of the rice endosperm protein hydrolysate: identification of the active peptide. *European Food Research and Technology* 229, 709-719.

Zhang, M., Mu, T.-H., and Sun, M.-J. (2014). Purification and identification of antioxidant peptides from sweet potato protein hydrolysates by Alcalase. *Journal of Functional Foods* 7, 191-200.

Zhou, X., Wang, C., and Jiang, A. (2012). Antioxidant peptides isolated from sea cucumber *Stichopus Japonicus*. *European Food Research and Technology* 234, 441-447.

Zima, T., Fialová, L., Mestek, O., Janebová, M., Crkovská, J., Malbohan, I., Štípek, S., Mikulíková, L., and Popov, P. (2001). Oxidative stress, metabolism of ethanol and alcohol-related diseases. *Journal of Biomedical Science* 8, 59-70.

APPENDIXES

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

Preparation for buffer

1. 50 mM Potassium phosphate buffer, pH 7.0 (Extraction buffer)

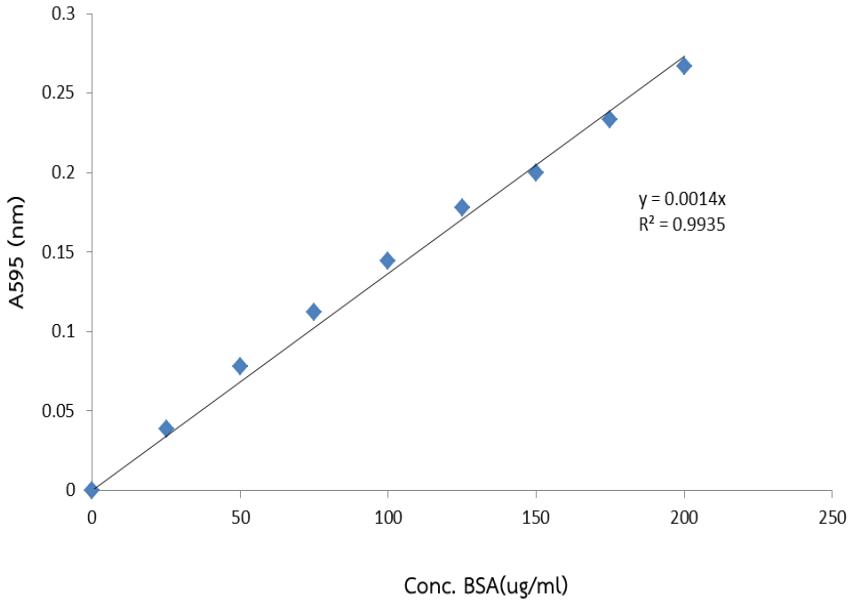
KH_2PO_4	3.4023 gL^{-1}
K_2HPO_4	4.3545 gL^{-1}
EDTA	0.0372 gL^{-1}
Triton X	3 ml
Polyvinylpyrrolidone (PVP-40)	10 gL-1

2. Phosphate buffer, pH 7.2

KH_2PO_4	1.361 gL^{-1}
K_2HPO_4	1.742 gL^{-1}

APPENDIX C

Standard curve for protein determination by Bradford method



APPENDIX D

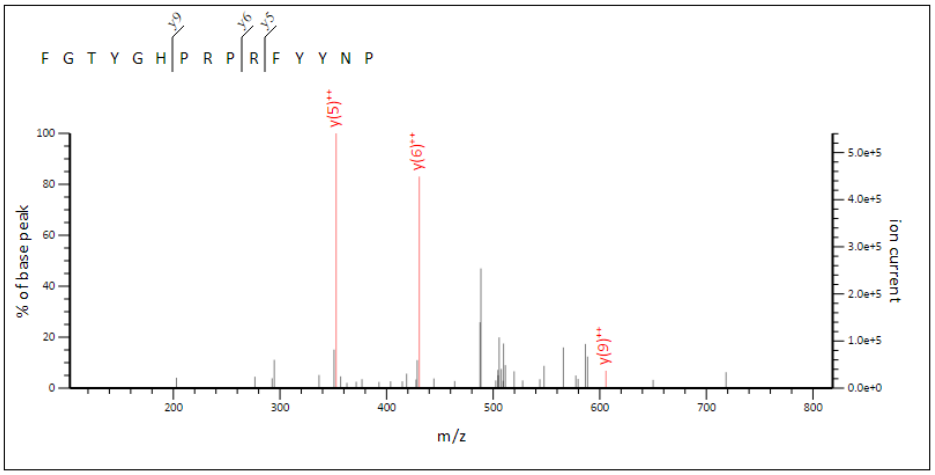
Amino acid abbreviation

Amino acid	3 Letter	1 Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
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
Asn or Asp	Asx	B
Gln or Glu	Glx	Z
Leu or Ile	Xle	J
Selenocysteine (UGA)	Sec	U
Pyrrolysine (UAG)	Pyl	O
Unknown	Unk	X

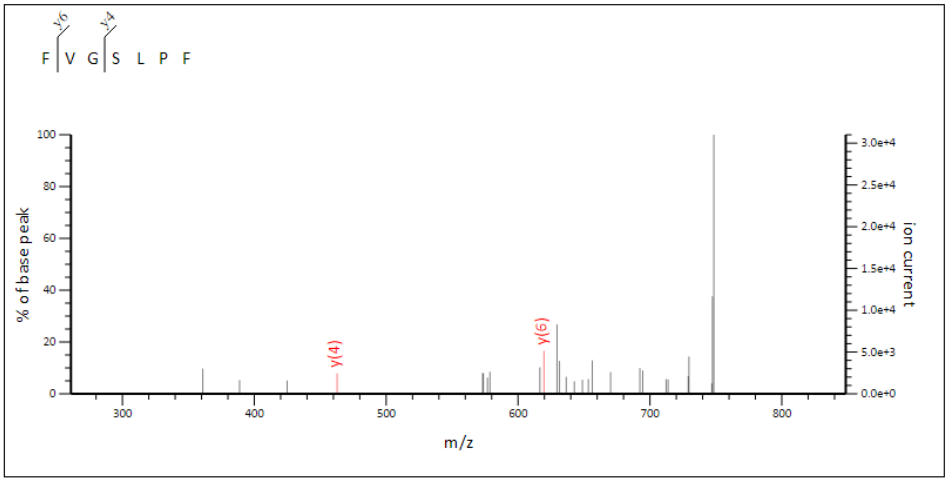
APPENDIX E

Fraction 1 (0-10 min)

P1 observed; 624.890, Mr (expt); 1871.648, Mr (cala); 1870.890

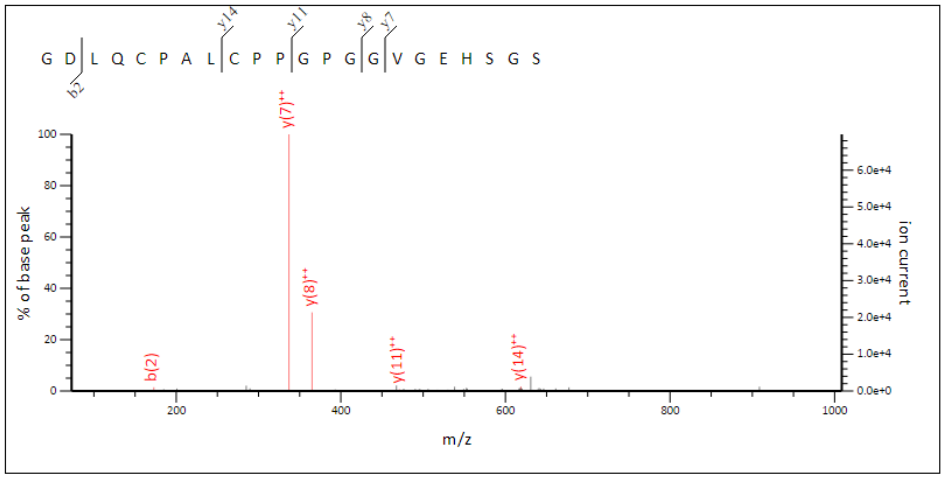


P2 observed; 766.850, Mr (expt); 765.842, Mr (cala); 765.406

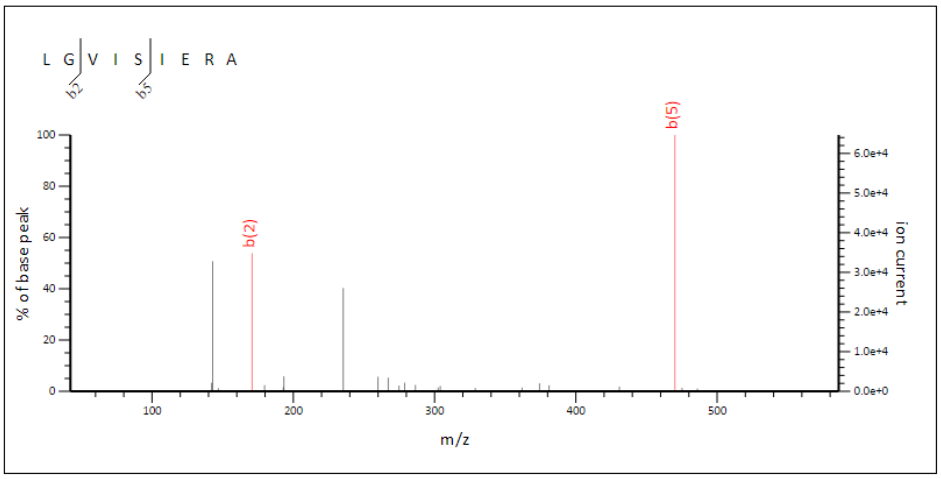


Fraction 2 (10-20 min)

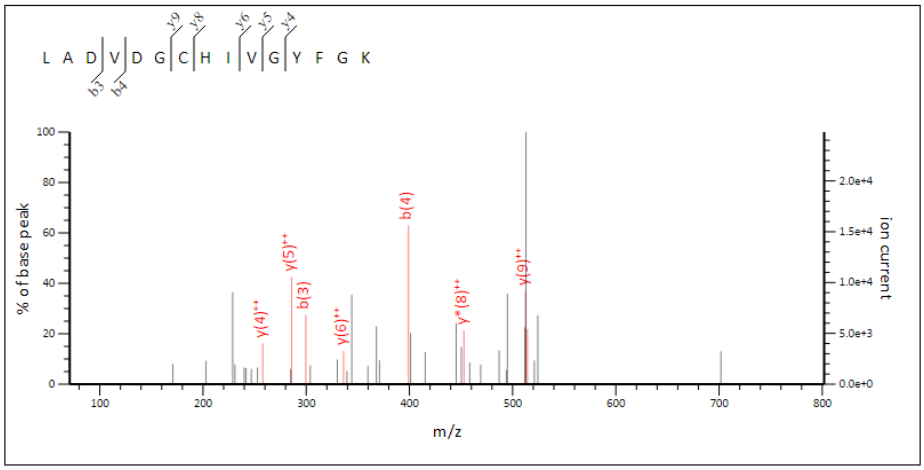
P3 observed; 678.950, Mr (expt); 2033.828, Mr (cala); 2033.893



P4 observed; 319.520, Mr (expt); 955.538, Mr (cala); 956.565

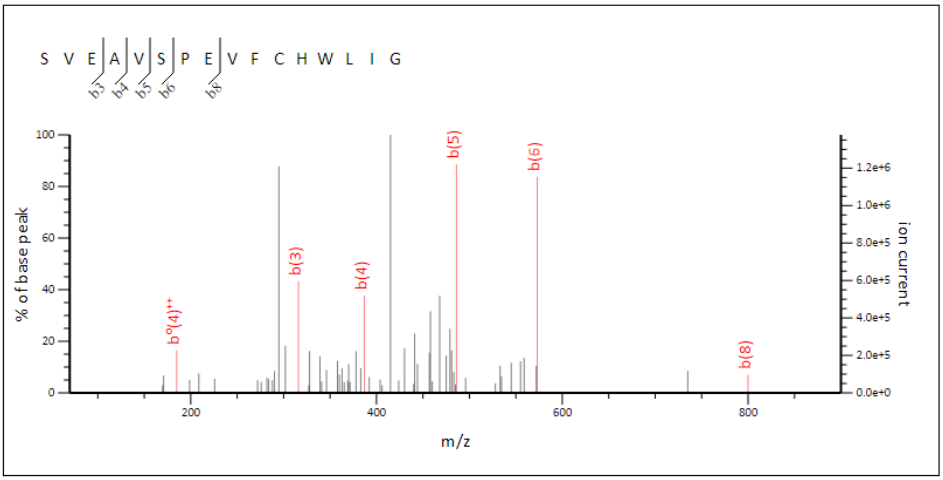


P5 observed; 531.890, Mr (expt); 1592.648, Mr (cala); 1592.765

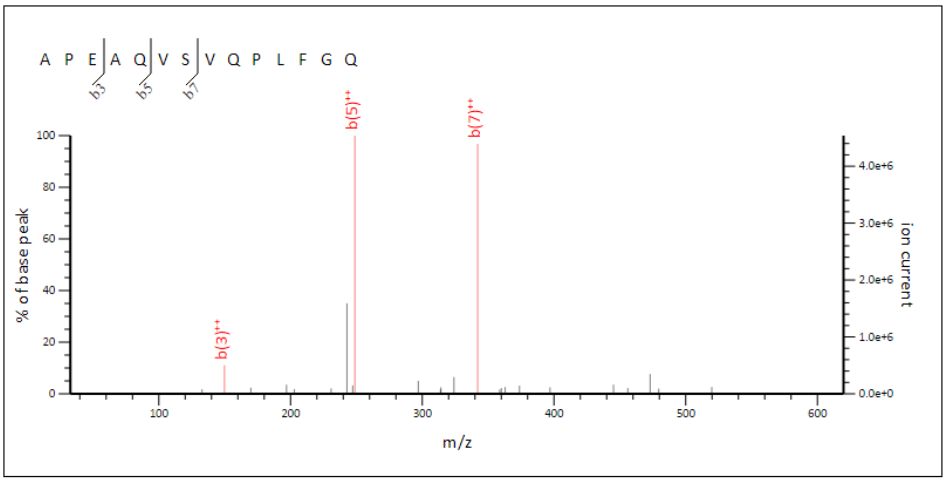


Fraction 3 (20-30 min)

P6 observed; 591.330, Mr (expt); 1770.968, Mr (cala); 1771.860

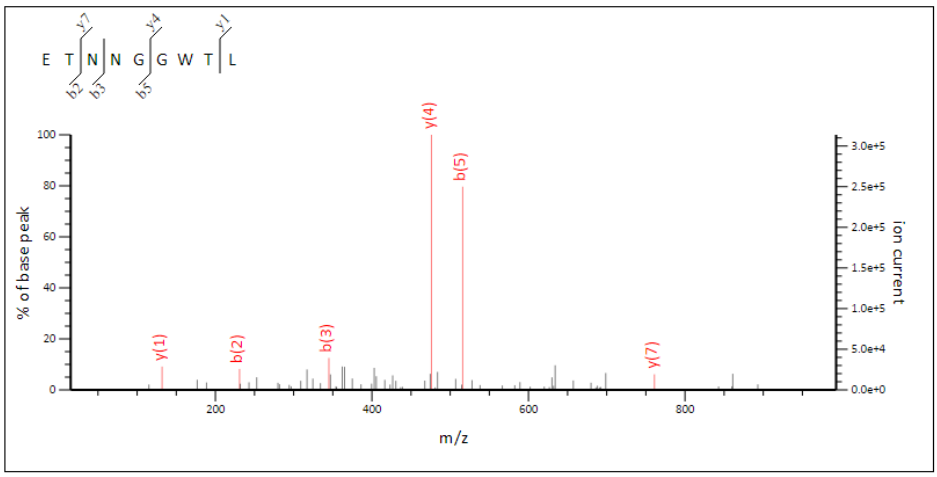


P7 observed; 491.250, Mr (expt); 1470.728, Mr (cala); 1469.751

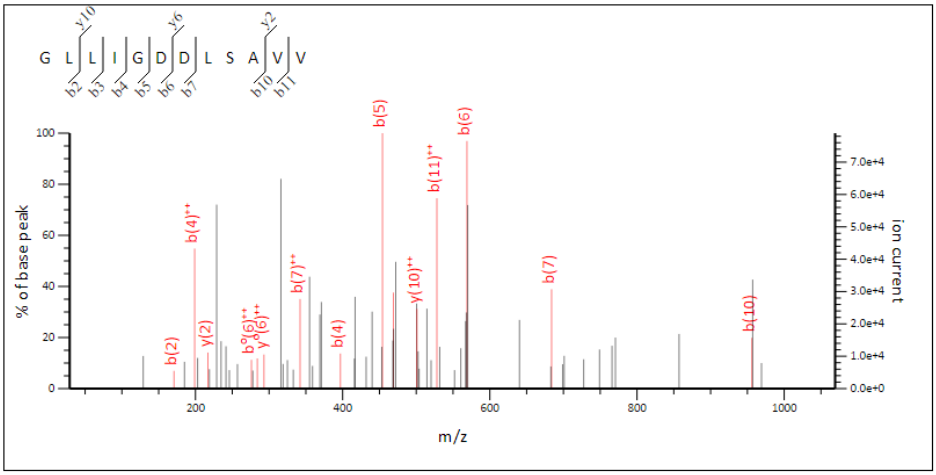


Fraction 4 (30-40 min)

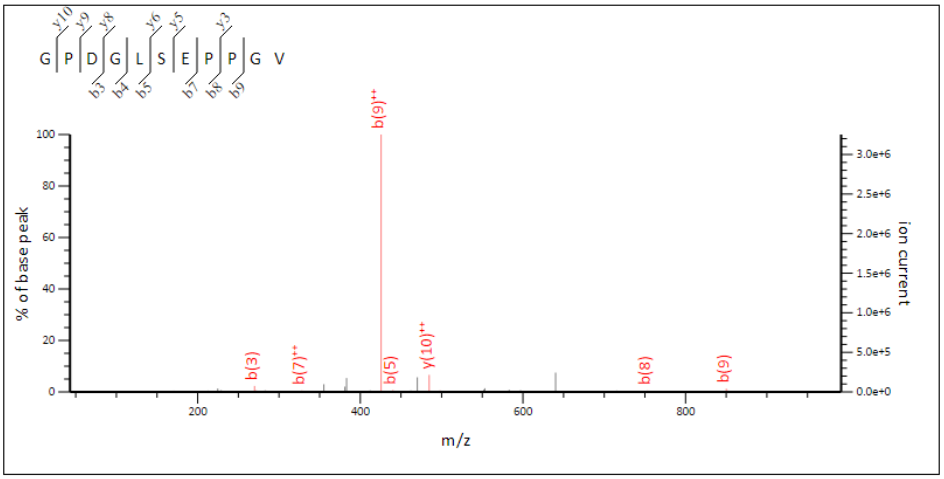
P8 observed; 496.250, Mr (expt); 990.485, Mr (cala); 990.440



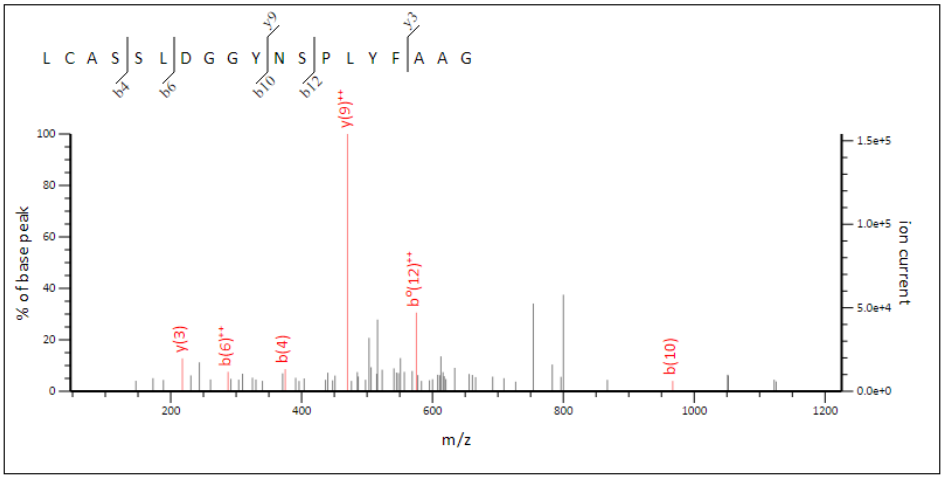
P9 observed; 586.800, Mr (expt); 1171.585, Mr (cala); 1170.649



P10 observed; 512.370, Mr (expt); 1022.725, Mr (cala); 1023.487



P11 observed; 635.830, Mr (expt); 1904.468, Mr (cala); 1904.861



VITA

Miss Sasiprapa Yookhansawat was born on May 26, 1989 in Sukhothai, Thailand. She graduated with a Bachelor's Degree of Science from Department of Cosmetic Science, Faculty of Cosmetic Science, Mae Fah Luang University, Thailand in 2011. She has further studied to the Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University in 2012.

Academic presentation;

1) Yookhansawat, S., Puthong, S., Sangvanich, P., and Karnchanatat, A. Antioxidation and antiproliferative activities of protein hydrolysates from chicken feather meal. In "TSB International forum 2014 (Green Bioprocess Engineering)" 16-19 September 2014, BITEC Bang Na Hall EH 101-102, Conference 101 B, Bangkok, THAILAND (Proceeding book)