ฤทธิ์ยับยั้งไทโรซิเนสของโปรตีนไฮโดรไลเสตจากขนไก่ป่นที่เตรียมจากเพปซิน-แพนครีเอตินและปาเปน

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

ANTI-TYROSINASE ACTIVITY OF PROTEIN HYDROLYSATE FROM CHICKEN FEATHER MEAL PREPARED BY PEPSIN-PANCREATIN AND PAPAIN

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Miss Puttaporn Pongkai

A Thesis Submitted in Partial Fulfillment of the Requirements
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By	Miss Puttaporn Pongkai
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.)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

พุทธพร ผ่องกาย : ฤทธิ์ยับยั้งไทโรซิเนสของโปรตีนไฮโดรไลเสตจากขนไก่ป่นที่เตรียมจากเพปซิน-แพนครีเอตินและปาเปน (ANTI-TYROSINASE ACTIVITY OF PROTEIN HYDROLYSATE FROM CHICKEN FEATHER MEAL PREPARED BY PEPSIN-PANCREATIN AND PAPAIN) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.พลกฤษณ์ แสงวณิช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.อภิชาติ กาญจนทัต, 73 หน้า.

ไทโรซิเนสเป็นเอนไซม์ที่มีคอปเปอร์เป็นองค์ประกอบ ซึ่งจะทำหน้าที่เร่งปฏิกิริยา 2 ขั้นตอน ซึ่งเกิดในช่วงเริ่มต้นของการสังเคราะห์เม็ดสีเมลานินสำหรับสัตว์เลี้ยงลูกด้วยน้ำนม งานวิจัยเกี่ยวกับสารยับยั้งไทโรซิเนสจึงกลายเป็นสิ่งที่มีความน่าสนใจเนื่องจากมีศักยภาพในการนำไปใช้ในผลิตภัณฑ์เพื่อผิวขาว ในงานวิจัยนี้ได้ศึกษาฤทธิ์ยับยั้งไทโรซิเนสของโปรตีนไฮโดรไลเสตที่เตรียมจากขนไก่ป่น โดยเตรียมจากเพปซิน-แพนครีเอตินและปาเปนในการย่อยสลาย คัดแยกโปรตีนไฮไดรไลเสตที่ได้ตามขนาดโมเลกุลด้วยเทคนิคอัลตราฟิลเตรชัน ผลการศึกษาพบว่าโปรตีนไฮไดรไลเสตที่เตรียมโดยใช้เพปซิน-แพนครีเอติน ที่มีขนาดโมเลกุลน้อยกว่า 3 กิโลดาลตัน มีฤทธิ์ในการยับยั้งไทโรซิเนสดีที่สุด โดยแสดงค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งได้ร้อยละ 50 (IC_{50}) ของกิจกรรมโมโนฟีโนเลส เท่ากับ 5.780 ± 0.188 ไมโครกรัมต่อมิลลิลิตร และกิจกรรมไดฟีโนเลส เท่ากับ IC_{50} 0.040 ± 0.024 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ผลการศึกษาจลนพลศาสตร์ของกิจกรรมยับยั้งไทโรซิเนสพบว่ามีการยับยั้งแบบไม่แข่งขัน มีค่าสัมประสิทธิ์การยับยั้ง (*Ki*) เท่ากับ 18.149 และ 27.189 ไมโครกรัมต่อมิลลิลิตร สำหรับกิจกรรมของโมโนฟีโนเลสและไดฟีโนเลส ตามลำดับ นอกจากนี้ผลจากการศึกษาในเซลล์เมลาโนไซต์พบว่าโปรตีนไฮไดรไลเสตที่เตรียมโดยใช้เพปซิน-แพนครีเอติน ที่มีขนาดโมเลกุลน้อยกว่า 3 กิโลดาลตันยังแสดงการยับยั้งดีที่สุดต่อการอยู่รอดของเซลล์ชนิด B16F10 โดยแสดงค่า IC_{50} เท่ากับ 1.124 ± 0.288 ไมโครกรัมต่อมิลลิลิตร และพบว่าการเติมโปรตีนไฮโดรไลเสตที่ความเข้มข้นเท่ากับ 0.210 ไมโครกรัมต่อมิลลิลิตรในเซลล์ สามารถยับยั้งกิจกรรมของไทโรซิเนสและยับยั้งการสร้างเมลานินได้เท่ากับ 50.493 เปอร์เซ็นต์ และ 14.680 เปอร์เซ็นต์ ตามลำดับ เมื่อเทียบกับเซลล์ปกติที่ไม่มีโปรตีนไฮโดรไลเสต นอกจากนี้โปรตีนไฮโดรไลเสตยังได้ถูกศึกษาผลต่อการตายแบบอะพอพโตซิสในเซลล์ และตรวจเอกลักษณ์ของโปรตีนไฮโดรไลเสตที่มีขนาดโมเลกุลน้อยกว่า 3 กิโลดาลตัน ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง และเทคนิคแมสสเปกโตรเมตรี ตามลำดับ การวิจัยเพื่อค้นหาฤทธิ์ยับยั้งไทโรซิเนสจากโปรตีนไฮไดรไลเสตที่เตรียมจากขนไก่ป่นนี้ มีความเป็นไปได้ที่จะนำไปประยุกต์ใช้เป็นส่วนประกอบสำหรับผลิตภัณฑ์เพื่อผิวขาวต่อไป

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ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

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CHULALONGKORN UNIVERSITY

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PUTTAPORN PONGKAI: ANTI-TYROSINASE ACTIVITY OF PROTEIN HYDROLYSATE FROM CHICKEN FEATHER MEAL PREPARED BY PEPSIN-PANCREATIN AND PAPAIN. ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., CO-ADVISOR: ASST. PROF. APHICHART KARNCHANATAT, Ph.D., 73 pp.

Tyrosinase is a copper containing enzyme which catalyzes the first two stages of mammalian melanogenesis. Tyrosinase inhibitors are important for their potential application in skin-whitening products. This study investigated the tyrosinase inhibitory properties of protein hydrolysates prepared from chicken feather meal using pepsin-pancreatin and papain hydrolysis. The protein hydrolysates were prepared and fractionated by ultrafiltration membrane. Protein hydrolysates prepared by pepsin-pancreatin with MW < 3 kDa exhibited strong tyrosinase inhibition activity for both monophenolase (IC_{50} 5.780 ± 0.188 µg/ml) and diphenolase (IC_{50} 0.040 ± 0.024 µg/ml) in a cell-free mushroom tyrosinase system and were found to be uncompetitive inhibitors with *Ki* values of 18.149 and 27.189 µg/ml in monophenolase and diphenolase activities, respectively. A cell culture model showed that protein hydrolysates prepared by pepsin-pancreatin with MW < 3 kDa had the strongest inhibition on the viability of B16F10 cells (IC_{50} 1.124 ± 0.288 µg/ml) and good inhibition on their tyrosinase activity. The addition of protein hydrolysates at a concentration of 0.210 µg/ml to B16F10 cells inhibited tyrosinase activity by 50.493 % and melanin synthesis by 14.680 % compared to the control without protein hydrolysates. Protein hydrolysates in the MW < 3 kDa fraction were selected for further study regarding apoptosis detection assays, and purification of tyrosinase inhibitor peptides by RP-HPLC; these peptides were identified by LC/MS/MS analysis. Tyrosinase inhibitors from protein hydrolysates have potential application as materials for skin whitening products.

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LIST OF ABBREVIATIONS

°C	degree Celsius
%	percentage
N	normal
M	molar
U	Unit activity
UV	ultraviolet
h	hour
l	litre
g	gram
min	minute
ml	millilitre
g/ml	gram per millilitre
mm	millimetre
nm	nanometre
µm	micrometre
µl	microlitre
µg	microgram
kg	kilogram
mg	milligram
mg/l	milligram per litre
mM	millimolar
w/v	weight by volume
rpm	revolution per min
MW	molecular weight
MWCO	molecular weight cut off
kDa	kilodalton
Abs	absorbance
IC_{50}	the half maximum inhibition

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K_i	the inhibitor constant
K_m	Michaelis-Menten constant
V_{max}	Maximum velocity
NaCl	Sodium chloride
NaOH	Sodium hydroxide
HCl	hydrochloric acid
TFA	Trifluoroacetic acid
Tris	tris (hydroxymethyl) aminomethane
PPO	polyphenol oxidase
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
PI	propidium iodide
FCS	fetal calf serum
PMSF	phenylmethanesulfonylfluoride
PBS	phosphate buffer saline solution
$NaHCO_3$	sodiumbicarbonate
CO_2	carbon dioxide
L-DOPA	3,4-dihydroxy-L-phenylalanine
TYR	tyrosinase
TRP	tyrosinase-related proteins
DCT	dopachrome tautomerase
DHI	5,6-dihydroxyindole
DHICA	5,6- dihydroxyindole-2-carboxylic acid
ICAQ	indole-2-carboxylic acid-5,6-quinone
IQ	indole-5,6-quinone
HBTA	5-hydroxy-1,4-benzothiazinylalanine
H_2O_2	Hydrogen peroxide
HPLC	High performance Liquid Chromatography
LC/MS	Liquid Chromatography- Mass spectrometry

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SD	standard deviation
et al.	and others

CHAPTER I
INTRODUCTION

Nowadays people care more about their health, and complexion. Many countries, including Thailand experience bright sunlight. Light rays from the sun stimulate melanin production which results in darker skin color. However a white skin is a major criterion of personal beauty in many countries, where women desire a white and fair complexion. Many skin whitening products have therefore been developed. The color of mammalian skin is controlled by many factors. One important factor is the degree and distribution of melanin pigmentation. Melanin is a widely distributed pigment, which plays an important role in protecting human skin from the harmful effects of ultraviolet (UV) radiation by absorbing UV sunlight, thereby removing reactive oxygen species. Melanin is secreted by melanocyte cells distributed in the basal layer of the dermis (Kim and Uyama, 2005, Momtaz *et al.*, 2008). Melanogenesis is the process of melanin production by melanocytes within the skin and hair follicles. Melanocytes have specialized lysosome-like organelles, termed melanosomes, which contain several enzymes that mediate the production of melanin (Curto *et al.*, 1999). Melanins can be of two basic types: eumelanins, which are brown or black, and pheomelanins, which are red or yellow (Parvez *et al.*, 2006). The type, amount and distribution of melanin in the surrounding keratinocytes determine the actual color of the skin (Kim and Uyama, 2005). The accumulation of an abnormal melanin amount in specific parts of the skin, as more pigmented patches (melasma, freckles, ephelide, and senile lentigines), can become an aesthetic problem (Solano *et al.*, 2006).

Melanin is formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of the enzyme tyrosinase. Tyrosinase (EC 1.14.18.1), a copper-containing polyphenol oxidase (PPO), is widely distributed in nature (Nerya *et al.*, 2003). Tyrosinase is found exclusively in melanocytes with a molecular weight of 60-70 kDa in mammals (Parvez *et al.*, 2006), and can accept a wide range of p-substituted monophenolic and diphenolic substrates. Among these, L-tyrosine and L-DOPA (3,4-dihydroxy-L-phenylalanine) are the natural precursors of melanins.

Tyrosinase is the key enzyme of melanogenesis that catalyzes two distinct reactions in melanin synthesis: the hydroxylation of tyrosine to DOPA (monophenolase activity), and the oxidation of DOPA to dopaquinone (diphenolase activity) (Lin *et al.*, 2007, Tripathi *et al.*, 1992). Quinones are highly reactive and can polymerize spontaneously to form high-molecular weight compounds or brown pigments, eumelanin or pheomelanin (Momtaz *et al.*, 2008). Tyrosinase-related proteins TRP-1, and DCT/TRP-2 catalyze distal melanin biosynthesis steps that control the type of melanin produced (Lin *et al.*, 2007).

In mammals, tyrosinase is not only responsible for hair color and skin pigmentation, but also for skin anomalies such as hypo (vitiligo) or hyper (flecks or freckles) pigmentation. Tyrosinase inhibitors have recently become increasingly important for medicinal and cosmetic products used to prevent or treat pigmentation disorders (Kim and Uyama, 2005). Tyrosinase inhibitors from natural sources have great potential; they are considered to be safe and largely free from adverse side effects. Therefore, peptides and protein hydrolysates are of great interest as natural sources of tyrosinase inhibitors. Proteins and peptides from milk, wheat, honey, and silk were investigated, and all appeared able to inhibit tyrosinase activity (Schurink *et al.*, 2007).

Protein hydrolysate is produced by protein digestion. Proteins are broken down into peptides of different sizes and free amino acids by enzymatic or chemical hydrolysis. Enzymatic hydrolysis is widely used because the enzymatic processes can be performed under mild conditions, which do not decrease the nutritional value of the protein source. Chemical processes, including alkaline or acid hydrolysis, are often difficult to control, and yield products with reduced nutritional qualities (Clemente, 2000, Tavano, 2013). Enzymatic hydrolysis is influenced by several factors, including pH, time, enzyme to substrate ratio, and temperature (Bhaskar and Mahendrakar, 2008). Protein hydrolysates have a variety of applications in industry, including pharmaceutical, human nutrition, and cosmetic (Bueno-Solano *et al.*, 2009) Protein hydrolysates have technological advantages such as improved solubility, heat stability, and a relatively high resistance to precipitation by many agents, such as pH or metal ions (Clemente, 2000). Enzymatic hydrolysis of the byproducts is effective

for protein recovery from animal and fish processing industries (Jamdar and Harikumar, 2008). The amino acid components of protein hydrolysate side chains are specific for each amino acid; they show the main characteristics of the protein, and biological antioxidation activity (Fakhfakh *et al.*, 2011).

Chicken feathers are a protein rich waste product of the poultry processing industry (Rani, 2012). Raw feathers contain a high proportion of keratin protein that has cysteine disulfide bonds. Chicken feathers discharged into the environment can result in environmental problems as they contain large amounts of cysteine, glycine, arginine, and phenylalanine (Kumar *et al.*, 2012). Chicken feather meal is interesting as a new source of protein hydrolysate that may have tyrosinase inhibition activity. The objective of this study was to determine the possibility of tyrosinase inhibitory activity of protein hydrolysates from chicken feather meal. The ability of protein hydrolysates to inhibit tyrosinase on melanin biosynthesis was investigated, using a cell-free mushroom tyrosinase system and a cell culture model. The results indicated the potential of this new source for skin-lightening applications.

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Chulalongkorn University

CHAPTER II
LITERATURE REVIEWS

2.1 Hyperpigmentation

Skin color is determined by various factors including melanin content, oxygenation state of hemoglobin in capillary vessels, carotenoid content, water content, and organization of collagen fibers in the dermis. Among these factors, melanin is the major determinant of skin color. Visible pigmentation in mammals results from the synthesis and distribution of melanin in the skin and hair bulbs (Schaffer and Bolognia, 2001, Seiberg *et al.*, 2000). Melanin plays an important role in protecting human skin from the harmful effects of UV radiation from the sun by the absorption of free radicals generated within the cytoplasm and in shielding the host from various types of ionizing radiations (Parvez *et al.*, 2007). Melanin also determines our phenotypic appearance. Although melanin has mainly a photoprotective function in human skin, the increased production and accumulation of an abnormal amount of melanins might become an esthetic problem and characterize a large number of skin diseases, which include acquired hyperpigmentation. Hyperpigmentation is darkening of the skin color due to excessive pigmentation. Usually, hyperpigmentation issues are major concerns for people of color. Hyperpigmentation related diseases include melasma, lentigines, nevus, ephelis, freckles, postinflammatory hyperpigmentation, and age spots (Briganti *et al.*, 2003, Chang, 2009, Kim, 2015).

2.2 Melanogenesis

The major constituent of pigment, melanin, is produced in melanosomes by the melanocyte through a complex process called melanogenesis. The melanocyte interacts with the endocrine, immune, inflammatory and central nervous systems, and its activity is also regulated by extrinsic factors, such as ultraviolet radiation and chemicals (Lee *et al.*, 2015). Melanocytes are specialized cells in the skin that find their embryonic origin at the neural crest. During embryonic development, melanoblasts migrate to reach the basal layer of the epidermis where they

differentiate into mature melanocytes. Melanins are synthesized in melanosomes that contain the specific enzymes required for proper melanin production. (Busca and Ballotti, 2000). Differences in skin color are related to the size, number, shape, and distribution of melanosomes (Kim, 2015). In general, melanosome structure correlates with the type of melanin produced, for example, eumelanosomes are elliptical and contain fibrillar matrix while the shape of pheomelanosomes is variable with predominantly rounded contours and they contain a vesiculoglobular matrix (Slominski *et al.*, 2004). Mature melanocytes form long dendritic processes that ramify among the neighboring keratinocytes. This association enables the melanocyte to transfer melanin into the keratinocytes (Tsatmali *et al.*, 2002). Confined mainly to the basal layer, melanocytes form a complex inter-relationship with proximal keratinocytes on which they depend for both differentiation and function. The epidermal melanin unit comprises one melanocyte and about 36 keratinocytes and one Langerhans cell (Agar and Young, 2005). During its development the melanosome acquires tyrosinase and tyrosinase-related proteins 1 and 2 (TRP1, TRP2). Tyrosinase is the rate-limiting enzyme for melanogenesis and catalyzes the conversion of l-tyrosine to dopaquinone, which is required for the synthesis of melanin (Tsatmali *et al.*, 2002). There are two main types of melanin: red/yellow pheomelanin and brown/black eumelanin. They differ not only in color but also in size, shape and granule packaging (Lee *et al.*, 2015).

2.2.1 Pathway of melanogenesis

The first two steps in the pathway are the hydroxylation of monophenol to o-diphenol (monophenolase or cresolase activity) and the oxidation of diphenol to o-quinones (diphenolase or catecholase activity), both using molecular oxygen followed by a series of nonenzymatic steps resulting in the formation of melanin (Seo *et al.*, 2003). Tyrosinase catalyzes three different reactions in the biosynthetic pathway of melanin in melanocytes: the hydroxylation of tyrosine to L-DOPA and the oxidation of the L-DOPA to dopaquinone. Furthermore, in humans dopaquinone is converted by a series of complex reactions involving cyclization and oxidative polymerizations which finally result in the formation of melanin (Olivares *et al.*, 2001, Raper, 1928). In the presence of cysteine or glutathione, dopaquinone is converted

to cysteinyldopa or glutathionyldopa. Subsequently, pheomelanin is formed (Chang, 2009). In the eumelanogenesis pathway, after the spontaneous conversion of dopaquinone to dopachrome, dopachrome tautomerase (tyrosinase-related protein-2, DCT/TRP-2) catalyzes the conversion of dopachrome to 5,6- dihydroxyindole-2-carboxylic acid (DHICA). Subsequently, DHICA is converted to indole–quinone–carboxylic acid by DHICA oxidase (tyrosinase-related protein-1, TRP-1) (Lin *et al.*, 2007).

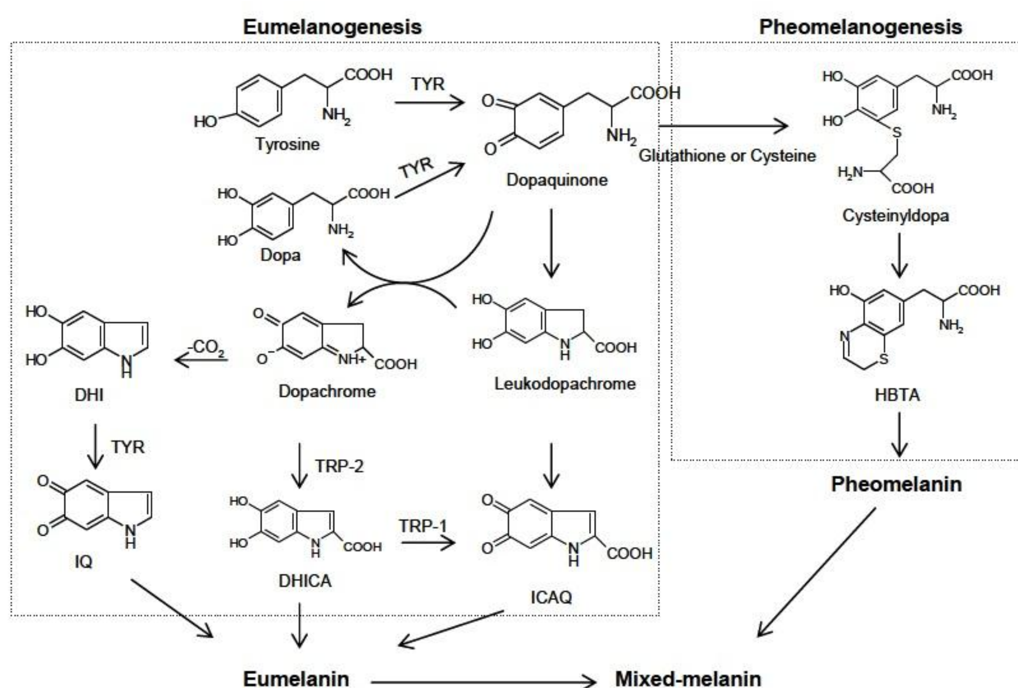

Figure 1. Biosynthetic pathway of melanin; TYR, tyrosinase; TRP, tyrosinase related protein; dopa, 3,4-dihydroxyphenylalanine; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHI, 5,6-dihydroxyindole; ICAQ, indole-2-carboxylic acid-5,6-quinone; IQ, indole-5,6-quinone; HBTA, 5-hydroxy-1,4-benzothiazinylalanine.

2.3 Tyrosinase

2.3.1 Classification and properties of tyrosinase

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase catalysing the o-hydroxylation of monophenols to the corresponding catechols (monophenolase or cresolase activity), and the oxidation of catechols to the corresponding o-quinones (diphenolase or catecholase activity). Tyrosinases are widespread in the living world, where they are often referred to as phenolases, phenol oxidases, polyphenol

oxidases, catechol oxidases, depending on the particular source or on the authors who have described any particular enzyme (Rescigno *et al.*, 2002). This enzyme was first isolated from the mushroom *Russula nigricans* in 1895, whose cut flesh turns red and then black upon exposure to air (Bourquelot and Bertrand, 1895). A number of studies have been made to identify the main cause behind the color change. Since its initial isolation, the enzyme has been found widely throughout the phylogenetic scale from bacteria to mammals. The term tyrosinase refers to its typical substrate, tyrosine. Both tyrosinase activities appear to have broad substrate specificities, although the enzyme has a higher affinity for the L-isomers of the substrates than for the corresponding D-isomers (Chang, 2009). Tyrosinases contain a dinuclear copper center, which is able to insert oxygen in an ortho position to an existing hydroxyl group in an aromatic ring, followed by the oxidation of the diphenol to the corresponding quinone. Molecular oxygen is incorporated into the dinuclear center to form an active oxytyrosinase. The structure of the active site of the enzyme, in which copper is bound by six or seven histidine residues and a single cysteine residue, is highly conserved (Lee *et al.*, 2015). The best-characterized tyrosinases are derived from *Streptomyces glausescens*, the fungi *Neurospora crassa* and *Agaricus bisporus* (Parvez *et al.*, 2007). Tyrosinase extracted from the champignon mushroom *A. bisporus* is highly homologous with the mammalian ones, and this makes it well suited as a model for studies on melanogenesis (Rescigno *et al.*, 2002). In fungi and vertebrates, tyrosinase catalyzes the initial step in the formation of the pigment melanin from tyrosine. In plants, the physiological substrates are a variety of phenolics. Tyrosinase oxidizes them in the browning pathway observed when tissues are injured (Chang, 2009).

2.3.2 Mechanism of tyrosinase action

The mechanism of tyrosinase action includes the molecular mechanism for the monophenolase and diphenolase activities of tyrosinase. In the monophenolase cycle, the monophenol can react only with the oxy form, which binds to the axial position of one of the coppers of this oxy form. A coordinated o-diphenol is produced and oxidized to the o-quinone. Oxytyrosinase is then regenerated after the binding of the molecular oxygen to deoxytyrosinase. This results in a deoxy form

ready for further dioxygen binding. If only o-diphenol is present (the diphenolase cycle), both the oxy and met forms react with o-diphenol, oxidizing it to the o-quinone. o-Diphenol binds to the oxy form and is oxidized to o-quinone, yielding the met form of the enzyme. The latter form transforms another o-diphenol molecule into o-quinone and is reduced to the bicuprous deoxy form (Chang, 2009, Kim and Uyama, 2005).

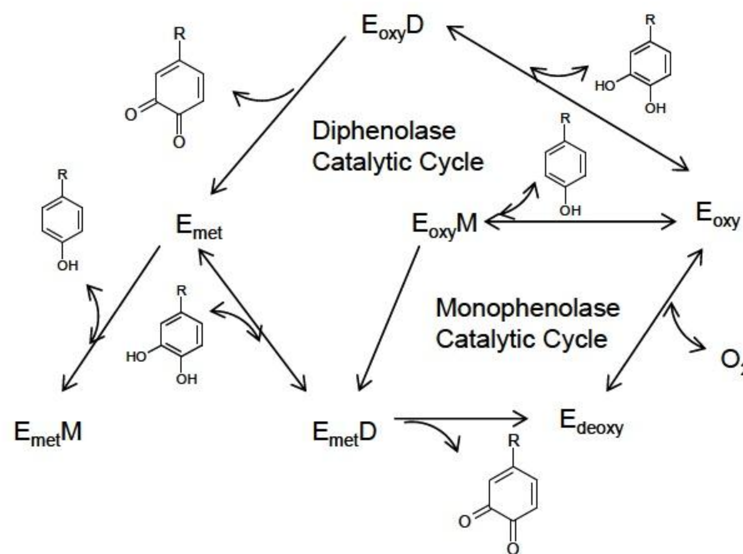

Figure 2. Cytalytic cycles of the hydroxylation of monophenol and oxidation of o-diphenol to o-quinone by tyrosinase. E_{oxy}, E_{met} and E_{deoxy} are the three types of tyrosinase respectively. $E_{oxy}D$, $E_{oxy}M$ and $E_{met}M$ are E_{oxy}-Diphenol, E_{oxy}-Monophenol and E_{met}-Monophenol complexes respectively (Chang, 2009).

2.4 Tyrosinase inhibitors

2.4.1 Important of tyrosinase inhibitors

The production of abnormal melanin pigmentation (melasma, freckles, ephelide, senile lentigines, etc.) is a serious esthetic problem in human beings. Tyrosinase inhibitors may be clinically used for the treatment of some skin disorders associated with melanin hyperpigmentation and are also important in cosmetics for skin whitening effects (Maeda and Fukuda, 1991, Seiberg *et al.*, 2000). In addition, tyrosinase is responsible for the undesired enzymatic browning of fruits and vegetables that takes place during senescence or damage at the time of postharvest handling (Seo *et al.*, 2003). The browning phenomenon in fruit and fungi is also

usually related to oxidative polymerization, conceptually similar to melanogenesis. Hyperpigmentation in human skin and enzymatic browning in fruits are not desirable (Chang, 2009). This makes the identification of novel tyrosinase inhibitors extremely important.

A number of researchers have dedicated themselves to identifying inhibitors from plants, fungal metabolites and marine algae and to establishing their structure-activity relationship. Tyrosinase inhibitors from natural sources usually attract more attention compared to chemically synthesized compounds due to the demand for cosmetics (Lee *et al.*, 2015). Many putative inhibitors are examined in the presence of tyrosine or dopa as the enzyme substrate, and activity is assessed in terms of dopachrome formation (Chang, 2009).

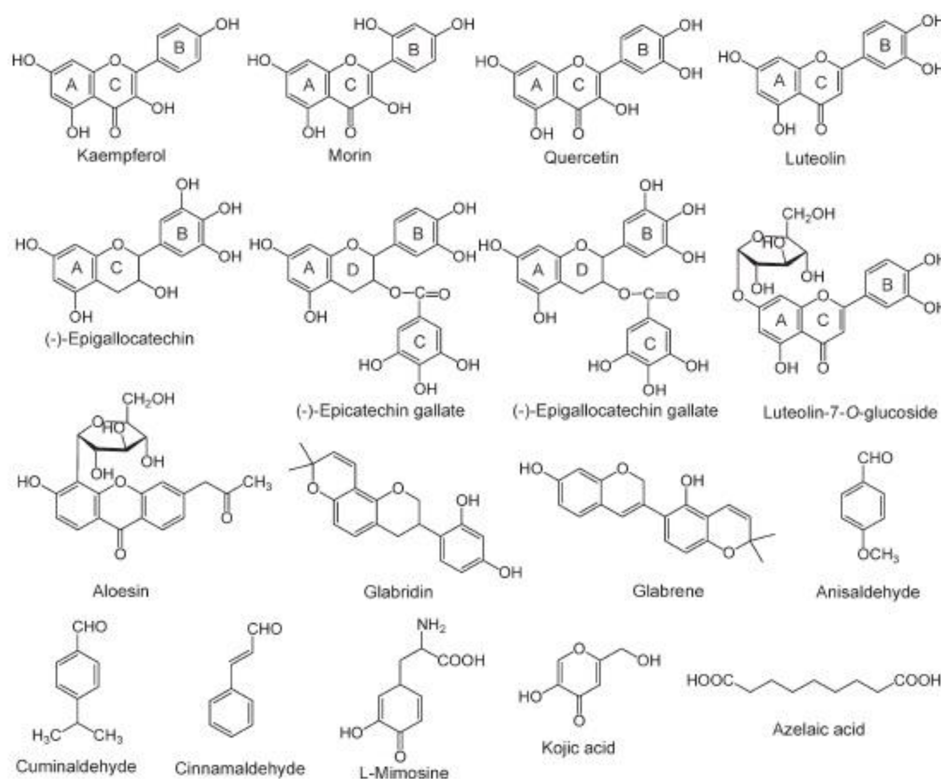

Figure 3. Structures of tyrosinase inhibitors from natural sources.

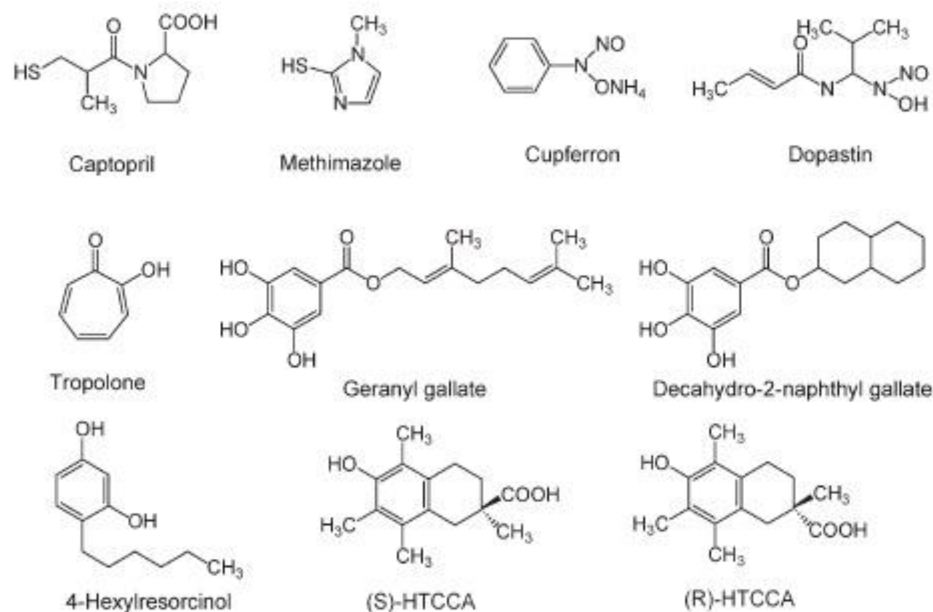

Figure 4. Structures of tyrosinase inhibitors from synthetic sources.

2.4.2 Type of tyrosinase inhibitors

Previous studies have shown the tyrosinase inhibitors to display many types of inhibition. The types of tyrosinase inhibitors include competitive inhibitors, uncompetitive inhibitors (Xie *et al.*, 2003), mixed type (competitive/uncompetitive) inhibitors (Huang *et al.*, 2006) and non-competitive inhibitors (Wu *et al.*, 2012). A competitive inhibitor is an inhibitor that can combine with a free enzyme, which prevents substrate binding because of true competition for the same site. A competitive inhibitor might be a copper chelator, non-metabolizable analogs, or derivatives of the true substrate. In contrast, an uncompetitive inhibitor is an inhibitor that can bind only to the enzyme-substrate complex and not with the free enzyme. A mixed (competitive and uncompetitive mixed) type inhibitor is a substance that can bind with both a free enzyme and an enzyme-substrate complex, which differ in their equilibrium binding constants. A non-competitive inhibitor can bind to a free enzyme and an enzyme-substrate complex with the same equilibrium constant. The inhibitor strength of tyrosinase inhibitor is usually expressed as the concentration of an inhibitor where 50% of the enzyme activity is inhibited (IC_{50} value) (Chang, 2009).

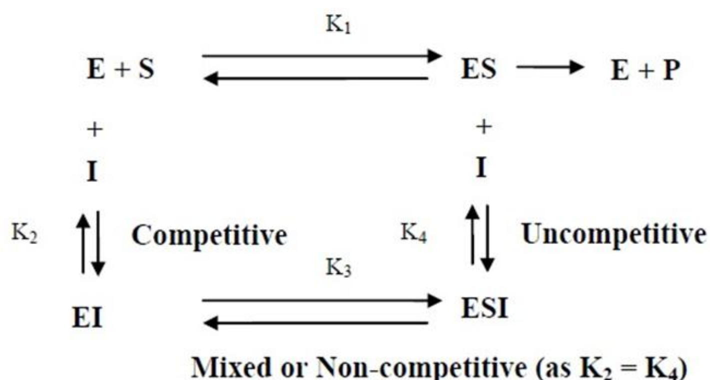

Figure 5. Action mechanism of reversible inhibitors; E, S, I, and P are the enzyme, substrate, inhibitor and product respectively; ES, EI and ESI are the enzyme-substrate complex, the enzyme-inhibitor and enzyme-substrate-inhibitor complexs respectively (Chang, 2009).

2.4.3 Sources of tyrosinase inhibitors

2.4.3.1 Inhibitors from natural sources

A broad spectrum of compounds has been obtained from the natural products and investigated for mushroom tyrosinase inhibitory activity. Plants are one important natural source for tyrosinase inhibitors as they are a rich source of bioactive chemicals and are mostly free from harmful side effects, so, there is an ongoing effort to search for tyrosinase inhibitors from them. A review of the literature indicates that inhibitors from higher plants are categorized into two main subgroups: polyphenols and aldehydes and other derivatives (Seo *et al.*, 2003).

2.4.3.1.1 Tyrosinase inhibitor from plants polyphenol

Polyphenols are a group of chemical compounds that have numerous biological activities. These compounds are produced as secondary metabolites by higher plants and are widely distributed throughout nature. The complex compounds of polyphenols are found in the bark, root, and leaves of plants, whereas others are simple compounds present in most fresh fruits, vegetables, and tea (Kim and Uyama, 2005, Seo *et al.*, 2003).

Flavonoids are one group of plant polyphenols that have been investigated by a number of studies. Flavonoids are benzo-*g*-pyrone derivatives consisting of phenolic and pyrane rings. These compounds can protect plants from UV radiation,

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pathogens and herbivores (Harborne and Williams, 2000). Some flavonoids have been reported to exhibit tyrosinase inhibitory activity, such as kaempferol (Kubo and Kinst-Hori, 1999, Kubo and Yokokawa, 1992), quercetin (Chen and Kubo, 2002, Kubo *et al.*, 1994), kurarinone, kushnol F and morin. Kubo *et al.* (2000) reported that, flavanoides inhibit the tyrosinase owing to their ability to chelate copper at the active site of the enzyme. In addition, Badria and Elgayyar (2000) reported that the R-keto group of flavonoids is important for their tyrosinase inhibitory activity due to the similarity between the dihydroxyphenyl group in L-dopa and the R-keto group in flavonoids. Another important compound of this group is gallic acid. Gallic acid and its esters are widely used as additives in the food industry. Some gallic acid derivatives from green tea (No *et al.*, 1999) and *Galla rhois* (Kim *et al*., 1998) were identified as strong tyrosinase inhibitors.

2.4.3.1.2 Tyrosinase inhibitor from aldehydes and other derivatives

Aldehydes are compounds that are also isolated and characterized as tyrosinase inhibitors. A large number of these compounds and other derivatives were studied, such as cuminaldehyde and cumic acid (Kubo and Kinst-Hori, 1998), anisaldehyde (Kubo and Kinst-Hori, 1998), 2-hydroxy-4-methoxybenzaldehyde (Kubo and Kinst-Hori, 1999), (2E)-alkenals (Kubo and Kinst-Hori, 1999), *trans*-cinnamaldehyde (Lee *et al.*, 2000), and 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid (Lee, 2002).

Kubo and Kinst-Hori (1999) reported that, except for 2-hydroxy-4-methoxybenzaldehyde, the above-indicated aromatic aldehydes were described as noncompetitive tyrosinase inhibitors. In 2001, Jimenez *et al.* (2001) reported that all of the 4-substituted benzaldehyde derivatives behave as competitive inhibitors of L-DOPA oxidation. In addition, (2E)-alkenals inhibited the oxidation of L-DOPA by tyrosinase as noncompetitive inhibitors. It has been proposed that the tyrosinase inhibitory activity of aldehyde is due to its ability to form a Schiff base with a primary amino group in the enzyme (Kubo and Kinst-Hori, 1998). Moreover, an electron-donating group (isopropyl and methoxy) at the para position in cuminaldehyde and benzaldehyde, is important for their stability to the Schiff base and inhibitory activity.

Therefore, the binding affinity of the hydrophobic electron-donating groups such as methoxy and isopropyl to the enzyme may also be related to the inhibitory effect (Seo *et al.*, 2003).

2.4.3.1.3 Tyrosinase inhibitor from fungal metabolites

Besides higher plants, fungal sources have also been reported to have compounds with tyrosinase inhibitory activity (Schallreuter and Wood, 1990). Kojic acid (5-hydroxy-2-(hydroxymethyl)-gamma-pyrone) is a fungal metabolite derived from various fungal species such as *Aspergillus* and *Penicillium* (Burdock *et al.*, 2001, Parrish *et al.*, 1966). It is a slow-binding tyrosinase inhibitor which is used in the cosmetic and food industries as a skin-whitening agent and as a food additive for preventing enzymatic browning respectively. Kojic acid effectively inhibits the formation of pigment products, which is a competitive inhibitor of monophenolase activity and a mixed type of inhibitor of the diphenolase activity of mushroom tyrosinase. Kojic acid functions by chelating copper at the active site of the tyrosinase enzyme and inhibits oxygen uptake when catecholamines such as L-DOPA, norepinephrine and dopamine are oxidized by tyrosinase. Therefore, kojic acid is able to reduce o-quinone to o-diphenol to prevent the final pigment forming. Moon *et al.* (2001) reported that melanocytes that were treated with kojic acid become nondendritic and have decreased melanin content. Moreover, kojic acid also acts as a free radical scavenger which scavenges reactive oxygen species that are released excessively from cells or generated in tissue or blood (Cabanes *et al.*, 1994). However, its usage in cosmetics is limited due to its instability under light and an aerobic environment (Lee *et al.*, 2015, Parvez *et al.*, 2006).

Azelaic acid (1, 7-heptanedicarboxylic acid) is an organic compound and naturally occurring straight-chain, saturated dicarboxylic acid. It is a tyrosinase inhibitor produced by the lipoperoxidation of free and esterified *cis*-polyunsaturated fatty acids by yeast, *Pityrosporum ovale*, which acts as a rather weak competitive and reversible inhibitor of tyrosinase in vitro (Kim and Uyama, 2005). The depigmenting activity of azelaic acid also appears to be mediated by the inhibition of mitochondrial oxidoreductase activation and DNA synthesis (Parvez *et al.*, 2006). This dicarboxylic acid does not have a cytotoxic effect on normal melanocytes, but

appears to affect the malignant melanocytes of primary cutaneous melanoma (Parvez *et al.*, 2007).

The yeast metallothioneins are widespread cytosolic proteins normally characterized by the selective binding of a large amount of heavy metal ions (Zn^{2+}, Cu^{2+} and Cd^{2+}) and high cysteine content (Byrd *et al.*, 1988, Lerch, 1980). Metallothioneins from fungal sources have been reported as tyrosinase inhibitors; for example, *Neurospora crassa* copper-metallothionein was reported as a metal donor for apotyrosinase (Lerch, 1980). In addition, metallothionein from *Aspergillus niger* (Goetghebeur and Kermasha, 1996) was found to be an inhibitor for a commercially purified mushroom tyrosinase, which has strong avidity to chelate copper at the active site of mushroom tyrosinase (Seo *et al.*, 2003) and exhibit strong reduction activity (Bremner, 1991). Therefore, it can be used as an inhibitor for tyrosinase by a decrease in oxygen consumption and the formation of colorless compounds. Moreover, metallothionein also exhibited a higher inhibitory effect on the oxidation of catechin compared with that of chlorogenic acid (Goetghebeur and Kermasha, 1996).

Tyrosinase inhibitors form other fungal extracts such as agaritine from *Agaricus* species were also isolated, purified and characterized (Espin *et al.*, 1998, Madhosingh and Sundberg, 1974). Agaritine exhibited a depigmenting effect that prevented melanin biosynthesis by inhibiting mushroom tyrosinase in vitro. The inhibition type of agaritine was uncompetitive when L-DOPA was used as the substrate and partially competitive when tyrosine was used as the substrate (Espin *et al.*, 1998).

2.4.3.2 Tyrosinas inhibitor from synthetic origins

A variety of tyrosinase inhibitors from synthetic origin have been reported. These inhibitors are derived from drugs and simple chemicals.

1) Drugs: Captopril ((2S)-1-(3-mercapto-2-methylpropionyl)-L-proline) is an antihypertensive drug which has an inhibitory effect on the angiotensin-converting enzyme (Cleland, 1994, Cleland *et al.*, 1984). Captopril is also able to prevent melanin formation (Espin and Wichers, 2001) by inhibiting mushroom tyrosinase activity. The types of inhibition in mono and diphenolase activities are noncompetitive and competitive, respectively. Moreover, captopril is known as a

copper chelator (Bartosz *et al.*, 1996, Jay *et al.*, 1991), therefore, it is reasonable to suppose that captopril mainly exerts its inhibitory effect by chelating copper ions at the active site of tyrosinase. Other drugs that have an inhibitory effect on tyrosinase activity include penicillamine and methimazole. Penicillamine is used in therapy for Wilson's disease (Lovstad, 1976) and methimazole is an antithyroid drug (Andrawis and Kahn, 1986).

Methimazole (1-methyl-2-mercaptoimidazole) is a mixed-type inhibitor, which inhibits both the monophenolase and diphenolase activities of mushroom tyrosinase. This inhibitor inhibits mushroom tyrosinase activity by chelating copper at the active site of the enzyme and conjugating with o-quinones causing inhibition in pigmented product formation (Kim and Uyama, 2005, Seo *et al.*, 2003).

2) Chemicals: From the review of the literature, many chemicals have been reported for their tyrosinase inhibitory activity, such as hydrogen peroxide, hydroxylamine, tiron, thiols, and aromatic carboxylic acids (Seo *et al.*, 2003). Hydrogen peroxide is a chemical with the ability to inactivate several copper-containing enzymes, such as dopamine β-monooxygenase and mushroom tyrosinase (Andrawis and Kahn, 1985, Skotland and Ljones, 1980). Hydrogen peroxide inhibits tyrosinase in both monophenolase and diphenolase activities. The rate of inactivation of the enzyme is dependent on the concentration of H_2O_2 and is faster under anaerobic conditions than aerobic conditions.

Among all the inhibitors studied, tropolone (2-hydroxy-2,4,6-cycloheptatriene) is one of the most potent tyrosinase inhibitors. Tropolone is an effective copper chelator (Kahn and Andrawis, 1985) and is structurally analogous to the o-diphenolic substrates of tyrosinase, which show slow-binding inhibition on tyrosinase and can only bind the oxy form (Espin and Wichers, 1999). The equilibria between enzyme, inhibitor and enzyme-inhibitor complexes occur slowly in the steady-state time scale (Kim and Uyama, 2005).

2.4.4 Application of tyrosinase inhibitors

2.4.4.1 Agricultural and food fields

Enzymatic browning results from the action of polyphenol oxidase (PPO) (EC1.14.18.1), which is also known as tyrosinase, phenol oxidase, monophenol

oxidase or cresolase (Mcevily *et al.*, 1992). The enzymatic browning of fruits, vegetables, and beverages takes place in the presence of oxygen when tyrosinase and its polyphenolic substrates are mixed after brushing, peeling, and crushing operations, which lead to the rupture of the cell structure (Hurrell and Finot, 1984). The rate of enzymatic browning depends on the concentration of active tyrosinase and phenolic substrates, oxygen availability, pH and temperature (Zheng *et al.*, 2008).

Enzymatic browning is one major problem of the agricultural and food industries as it has a major impact on the quality of fruits, vegetables, and seafood products. Enzymatic browning can produce undesirable changes in flavour, texture and nutritional values of the product during handling, processing and storage results in a shorter shelf life and reduced market value (Fu *et al.*, 2005). Thus, it is necessary to identify various methods to stop the enzymatic browning caused by tyrosinase. Current conventional techniques to avoid browning include the use of autoclave and blanching methods. Tyrosinase is inactivated through immersion in a liquid at 80–90 °C for 10–12 min or passes through a forced steam flow. These processes cause important weight and nutrient losses in the product (Konanayakam and Sastry, 1988). Many compounds have been identified as having the ability to inhibit the enzymatic browning in food products through the interference of tyrosinase-mediated reactions or through the reduction of o-quinones to o-diphenols, but only few enzyme inhibitors are used in the industry due to off-flavors, food safety, and economic feasibility (Eskin *et al.*, 1971). Various chemicals such as halide salts, aromatic carboxylic acids (Rouet-Mayer and Philippon, 1986) and other compounds with reducing properties such as citric acid, ascorbic acid and its derivatives (Hsu *et al.*, 1988, Santerre *et al.*, 1988) are known to inhibit tyrosinase. Ascorbic acid and citric acid are used as a reducing agent that can inhibit enzymatic browning in various vegetables, fruits and many products in the food industry (Iyengar and Mcevily, 1992).

Among the compounds that inhibit browning, sulphite is effectively used for the prevention of the enzymatic browning reaction and has been widely employed in agricultural and seafood products for many years. The primary mode of sulphite in the browning prevention is to react with the o-quinones formed by PPO catalysis to

produce stable, colorless adducts (Iyengar and Mcevily, 1992), and it also directly reacts with the enzyme structure leading to the inactivation of PPO (Duangmal and Apenten, 1999). However, sulfite-containing compounds can create undesirable flavor and a problem for human health (Mcevily *et al.*, 1992, Zhu *et al.*, 2007). Therefore, the application of sulphite on fresh-cut food products has been banned by the U.S. Food and Drug Administration. The use of 4-hexylresorcinol is considered to be safe in the food industry and is quite effective in the prevention of shrimp melanosis (Iyengar *et al.*, 1991, Mcevily *et al.*, 1991) and for browning control in fresh and dried fruit slices (Frankos *et al.*, 1991). Safety is very important in the food industry. In addition, there is increasing consumer demand for substituting synthetic compounds with natural substances (Jang *et al.*, 2002). Therefore, better natural antibrowning agents free of any harmful side effects have been widely investigated (Kubglomsong and Theerakulkait, 2014, Loizzo *et al.*, 2012).

2.4.4.2 Cosmetic and medicinal fields

Tyrosinase inhibitors are important in the cosmetic and medicinal industries for their potential applications in skin-whitening products and their preventive effect on pigmentation disorders. The inhibitory effects of tyrosinase inhibitors are causes of reducing melanin production. Therefore, they are possible targets for developing medicines to treat hypopigmentation-related problems and are used in cosmetic products for hyperpigmentation-related concerns, including the formation of freckles. A number of tyrosinase inhibitors from both natural and synthetic sources have been reported, but only a few of them can be used as skin-whitening agents due to safety concerns (Kim and Uyama, 2005). For example, linoleic acid, hinokitiol, kojic acid, arbutin, naturally occurring hydroquinones, and catechols were reported to inhibit enzyme activity but also exhibited side effects (Maeda and Fukuda, 1991).

Arbutin (hydroquinone-O-beta-D-glucopyranoside) is one tyrosinase inhibitor which is used in the cosmetic industry as a whitening agent as it shows strong tyrosinase inhibition activity. Arbutin is isolated from *Aesculus californica*, the fruit of the California buckeye. Various researchers have reported the inhibitory effects of arbutin on tyrosinase activity, which inhibits the oxidation of L-DOPA catalysed by mushroom tyrosinase (Kubo and Bai-Ping, 1992, Seo *et al.*, 2003). A recent study

indicated that the inhibitory effects of arbutin are due to the inhibition of melanosomal tyrosinase activity rather than the suppression of this enzyme's synthesis and expression (Yang *et al.*, 1999). Moreover, arbutin is effective in the topical treatment of various cutaneous hyperpigmentations characterized by hyperactive melanocyte function (Parvez *et al.*, 2006).

Another tyrosinase inhibitor studied for application in the medicinal field is azelaic acid. Sarkar *et al.* (2001) reported that 132 Asian women with melasma underwent treatment with azelaic acid. The results showed that azelaic acid caused both greater lightening of pigmented lesions and a reduction in lesion size. Additionally, in 1991, Fitton and Goa (1991) applied azelaic acid to produce clinical and histological resolution in facial lentigo maligna and were successful in treating rosacea, solar keratosis and hyperpigmentation associated with burns and herpes labialis (Parvez *et al.*, 2006).

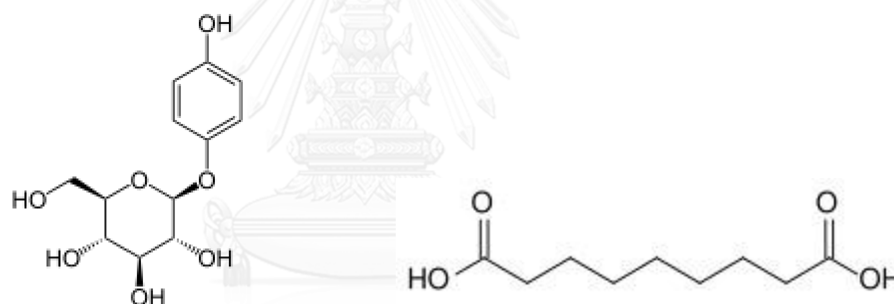

Figure 6. Chemical structures of Arbutin and Azelaic acid respectively.

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2.5 Protein hydrolysate

Protein hydrolysates are products of protein digestion. Proteins are broken down into smaller peptides of different sizes and free amino acids by enzymatic or chemical hydrolysis. Chemical processes, including alkaline or acid hydrolysis, tend to be difficult to control, and almost invariably lead to products with variable chemical composition and functional properties. Moreover, chemical processes yield products with reduced nutritional qualities and poor functionality (Kristinsson and Rasco, 2000, Tavano, 2013). Enzymatic proteolysis unlike acid hydrolysis is mild and controllable, which helps in improving the quality and functional properties of protein (Jamdar and Harikumar, 2008).

The cleavage of peptide bonds make three major changes in the properties of proteins: (1) increases in the amounts of ionized groups (NH_4 and COO^-) associated with an increase in hydrophilicity and total charge, (2) decreases in the molecular sizes of polypeptide chains, and (3) molecular structural changes and exposure of residues of hydrophobic amino acids to the surface of oligopeptides (Neklyudov *et al.*, 2000).

Generally, protein hydrolysates are small fragments of peptides that contain 2–20 amino acids (Chalamaiah *et al.*, 2012). Peptides present in enzymatically digested protein hydrolysates have exhibited different physicochemical properties and biological activities depending on their molecular weights and amino acid sequences (Kim and Mendis, 2006). The physiological function of protein hydrolysates is also related to various other factors, including the protein source, enzyme used, enzyme-to-substrate ratio, pH, time, and temperature (Bhaskar and Mahendrakar, 2008, Xia *et al.*, 2007).

2.6 Proteolytic enzyme

Proteolytic enzyme, also called protease, proteinase, or peptidase, is a group of enzymes that cleavage peptide bonds and break the long chain of proteins into shorter peptides and amino acids. Proteolytic enzymes are present in bacteria, archaea, certain types of algae, some viruses, animals and plants. Proteolytic enzymes produce from microbial such as alcalase, neutrase and flavourzyme. Proteolytic enzymes produce from animals and plants such as pepsin, pancreatin and papain respectively. There are different types of proteolytic enzymes, which are classified according to their hydrolyzing mechanism into endopeptidases or exoproteases. Endopeptidases hydrolyse the peptide bonds within protein molecules at random to produce relatively large peptides. Exoproteases systematically remove amino acids from either the N terminus or C terminus by hydrolyzing the terminal ends of proteins (Clemente, 2000)

Proteolytic enzyme from animals and plants were reported to use in the production of peptides which have biological activity. Luo *et al.* (2013) reported that papain can hydrolyse *Sphyrna lewini* muscle protein to antioxidant peptide. Papain hydrolysate from black-bone silky fowl (*Gallus gallus domesticus Brisson*) muscle exhibited strong antioxidant activity (Liu *et al.*, 2011). Papain hydrolysates from pea protein can inhibit DPPH scavenging activity and also have ACE-inhibitory activity (Humiski and Aluko, 2007). Hydrolysates of sunflower proteins, produced by pepsin and pancreatin can inhibit beta-carotene oxidation (Megias *et al.*, 2008). Marczak *et al.* (2003) reported that pepsin and pancreatin can use to produce antihypertensive peptides from rapeseed. Moreover, pepsin and pancreatin can also use to produce chelating peptides from chickpea protein (Torres-Fuentes *et al.*, 2011).

Papain (E.C. 3.4.22.2) is one of the proteolytic enzymes found in the latex of the leaves and of the green fruit of the papaya tree (*Carica papaya L., Caricaceae*). Papain consists of a single polypeptide chain with three disulfide bridges and a sulfhydryl group necessary for activity of the enzyme. Optimal pH for activity of papain is 6.0-7.0 and temperature optimum for activity is 65 °C. Papain will digest most protein substrates more extensively than the pancreatic proteases. Papain exhibits broad specificity, cleaving peptide bonds of basic amino acids, leucine, or glycine. It also hydrolyzes esters and amides.

Pepsin (E.C. 3.4.23.1) is the predominant digestive protease in the gastric juice of vertebrates. Optimal pH for activity of pepsin is 1.0-4.0. Pepsin can hydrolyzes only peptide bonds. It does not hydrolyze non-peptide amide or ester linkages. Pepsin will preferentially cleave at the carboxyl side of phenylalanine and leucine and to a lesser extent at the carboxyl side of glutamic acid residues.

Pancreatin is a mixture of several digestive enzymes produced by the exocrine cells of the pancreas. Pancreatin contains enzymatic components including trypsin, amylase and lipase. The trypsin found in pancreatin works to hydrolyze proteins into oligopeptides; amylase hydrolyzes starches into oligosaccharides and the disaccharide maltose; and lipase hydrolyzes triglycerides into fatty acids and glycerols (http://www.sigmaaldrich.com).

2.7 Feather and Feather meal

The production and consumption of poultry products have been on the increase globally. The poultry processing industry produces a lot of byproducts as waste, which include blood, head, viscera, feet, bones and feathers. Feathers are a protein rich waste, which have higher protein contents than the others (Table 1). The feather constitutes about 5–7% of live bird weight (Grazziotin *et al.*, 2008). A whole feather is almost a pure protein material with a crude protein content of over 90% (Taskin and Kurbanoglu, 2011). Raw feathers contain a high proportion of keratin protein that has cystine disulfide bonds. Furthermore, feathers contain large amounts of cystine, glycine, arginine and phenylalanine (Kumar *et al.*, 2012).

Some industries produce feather meal by steam pressure cooking, which requires high-energy input. This feather meal is used as organic fertilizers and animal feed supplements. However, the production of feather meal makes it deficient in some amino acids such as methionine and histidine, which limits its use in animal feed (Fakhfakh *et al.*, 2011, Rani, 2012). Feather by-products can be converted to hydrolysates with functional, bioactive and nutritional benefits (Lasekan *et al.*, 2013).

The hydrolysed proteins from many sources have been found to exhibit antioxidant activity, such as milk casein, soybean, rice bran, quinoa seed protein, canola, egg yolk protein and porcine myofibrillar proteins. Hydrolysed proteins from feathers were also reported to have antioxidant activity (Fakhfakh *et al.*, 2011, Kumar *et al.*, 2012). Moreover, proteins and peptides from egg, milk, honey, houseflies and silk appear to be able to inhibit tyrosinase activity. Li *et al.* (2006) reported that hen egg white lysozyme (HEWL) had an inhibitory effect on mushroom tyrosinase, with a mode of inhibition that was mixed type. Milk kefir whey components (kefir whey, peptides and lactic acid) had inhibitory ability against melanin synthesis (peptides showed a concentration-dependent reduction in tyrosinase activity with IC_{50} value at 4.23 mg/ml and the IC_{50} value for kefir whey at 15 mg/ml) (Chen *et al.*, 2006). A small peptide from honey with an approximate molecular weight of 0.6 kDa is responsible for the noncompetitive inhibition of tyrosinase (Oszmianski and Lee, 1990). Peptide from the hemolymph of the housefly *Musca domestica* has been

determined to competitively inhibit phenol oxidase with a *Ki* of around 10 nM (Daquinag *et al.*, 1995), and silk protein (sericin) was found to inhibit lipid peroxidation and tyrosinase activities (Kato *et al.*, 1998). However, no tyrosinase inhibitory activity has been reported in the poultry feather protein hydrolysate. Chicken feather meal is interesting as a new source of protein hydrolysate that may have tyrosinase inhibition activity.

Table 1 Protein content of different chicken by-products.

Chicken by-product	Protein (%)	References
Feather and feather meal	85–99	Sangali and Brandelli (2000) and Grazziotin *et al.* (2007)
Blood meal	60–80	Huang and Liu (2010) and Sant'Anna *et al.* (2010)
Heads and feet	16	Okanovic *et al.* (2009)
Bone	23–24	Cheng *et al.* (2008) and Zhang *et al.* (2010)
Viscera	11–12	Jamdar and Harikumar (2008) and Rivera *et al.* (2000)
Chicken intestine	53–60	Jamdar and Harikumar (2005) and Muthukumar and Kandeepan (2009)
Offal (heads, feet, viscera)	12–15	Cai *et al.* (1995) and Russell *et al.* (1992)

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CHAPTER III
EXPERIMENTS

3.1 Biological and chemical materials

Chicken feather meal from Betagro Public Company (Thailand) was ground to a small size and dried at 60 °C overnight. The feather meal was then filtered through a 150 micron sieve. Bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), pancreatin from porcine pancreas, pepsin from porcine gastric mucosa, L-DOPA, L-tyrosine, and tyrosinase from mushroom were purchased from Sigma Chemicals Co. (USA). All other chemicals used in the investigation were of analytical grade.

3.2 Amino acid content analysis

The amino acid content of chicken feather meal was determined based on the standard AOAC 994.12 acid hydrolysis method. The samples were mixed with 5 ml of 6 N HCl and placed in a heating block at 110 °C for 24 h. to liberate the individual amino acids, before reversed-phase high performance liquid chromatography (RP-HPLC) analysis. The analyses were carried out on a C18 (4.6 mm × 250 mm, 5 μm) column (Waters, Ireland), using a gradient of Buffer A (12.5 mM phosphate buffer pH 7.2) and Buffer B (Buffer A containing 50 % acetonitrile), with a flow rate of 1 ml/min (Jamdar and Harikumar, 2008).

3.3 Protein hydrolysate preparation

Chicken feather meal was mixed with 20 mM potassium phosphate buffer at pH 7.2 at 0.0125 g/ml. The suspension was stirred overnight at 4 °C. The protein hydrolysate was then prepared following the method of Torres-Fuentes *et al.* (2011).

3.3.1 Pepsin-pancreatin

Crude protein was adjusted to pH 2.5 with addition of 1 M HCl and then mixed with pepsin, using an enzyme: substrate ratio of 1: 20 (w/v). The hydrolysis was carried out for 180 min at 37 °C with shaking (180 rpm), and then inactivated by adding 1 M NaOH to pH 7.5. Pancreatin was then added to a 1: 20 (w/v) enzyme: substrate ratio and shaken (180 rpm) for 180 min at 37 °C. The hydrolysis (enzyme

reaction) was stopped by heating at 80 °C for 20 min. Hydrolysates were clarified by centrifugation at 10,000 rpm for 10 min at 4 °C and kept at 4 °C until use.

3.3.2 Papain

Crude protein was adjusted to pH 6.5 and mixed with papain, using an enzyme: substrate ratio of 1: 20 (w/v). The hydrolysis was carried out for 240 min at 65 °C with shaking (180 rpm). The hydrolysis (enzyme reaction) was stopped by heating at 80 °C for 20 min. Hydrolysates were clarified by centrifugation at 10,000 rpm for 10 min at 4 °C and kept at 4 °C until use.

3.4 Molecular weight cut-off by ultrafiltration membrane

The protein hydrolysates were fractionated by ultrafiltration membranes using a bioreactor system (Amersham Biosciences, Sweden). The suspension of protein hydrolysate prepared from chicken feather meal 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).

3.5 Tyrosinase inhibition assay

The tyrosinase inhibition assay was modified from the method used by Batubara *et al.* (2010). The protein hydrolysate (35 μl) was mixed with 15 μl of tyrosinase (333 U/ml in phosphate buffer 50 mM pH 6.5), and incubated at room temperature for 5 min. Then, 55 μl of substrate (L-DOPA 12 mM) was added to each tube and incubated for 30 min. The absorbance was determined at 510 nm using a microplate reader. Kojic acid was used as the positive control. The percentage inhibition of tyrosinase activity was calculated using the following equation.

$$\% \text{ Inhibition} = \frac{[(\text{Abs control} - \text{Abs blank}) - (\text{Abs sample} - \text{Abs background})] \times 100}{(\text{Abs control} - \text{Abs blank})}$$

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 (deionized water). IC_{50} is the

concentration of protein hydrolysate where 50 % of enzyme activity is inhibited. Alls investigations were performed in triplicate.

3.6 Cell culture

Cell culture was performed based on the method used by Momtaz *et al.* (2008). The mouse melanocyte cell line B16F10, was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum, 1.5 g/l $NaHCO_3$, 2mM L-glutamine, 10 μg/ml penicillin, 10 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, then incubated at 37 °C with 5 % CO_2 in a humidified atmosphere. Cells were sub-cultured at a ratio of 1: 3 on every third or fourth day.

3.7 Determination of protein hydrolysate toxicity in melanocytes (MTT assay)

B16F10 cells in complete DMEM were added into the wells of a 96 well plate (5×10^3 cell /well /200 μl), and incubated overnight in a 37 °C incubator with 5 % CO_2. A test sample was then added to each well and incubated for another 3 days. The treated cells were then labeled with MTT dye reagent concentration 5 mg/ml (10 μl /well) and incubated at 37 °C for 4 h. The formazan precipitates were dissolved in DMSO (150 μl/ well) and after addition of 25 μl of 0.1 M glycine, the absorbance values of the supernatant were measured at 540 nm. Alls investigations were performed in triplicate.

3.8 Determination of melanin content in melanocytes

Melanin content determination was performed based on the method used by Si *et al.* (2014), with some modifications. B16F10 cells in complete DMEM were added to the cell culture flasks (1×10^5 cell /flask/5 ml), and incubated overnight at 37 °C with 5 % CO_2. A test sample and the positive control kojic acid were then added to each flask and incubated for another 3 days. The treated cells were then harvested and washed twice with phosphate buffered saline (PBS) pH 7.4. Finally, all cells were lysed with 1 N NaOH 500 μl. After 1 h of incubation at 90 °C, the lysates were centrifuged at 3,000 g for 10 min, and the absorbance values measured at 405 nm. Alls investigations were performed in triplicate.

3.9 Cellular tyrosinase inhibition assay

Cellular tyrosinase inhibition assay measurement was modified from the method used by Si *et al.* (2014). B16F10 cells in complete DMEM were added to the

cell culture flasks (1×10^5 cell /flask/5 ml), and incubated overnight at 37 °C with 5 % CO_2. The cells were then treated with a test sample and positive control kojic acid. After 3 days of incubation, the treated cells were harvested and washed twice with cold PBS pH 7.4. Finally, all cells were lysed with PBS (pH 6.8) containing 1 % (w/v) Triton X-100 and 1 mM phenylmethanesulfonylfluoride (PMSF). The enzymatic assay was commenced by mixed 100 µl of cell extract with 100 µl of L-DOPA (2 mM) in a 96-well plate. After 1 h of incubation at 37 °C the absorbance values were measured at 490 nm. Alls investigations were performed in triplicate.

3.10 Apoptosis detection assays

B16F10 cells in complete DMEM were seeded into cell culture flasks at a density of 2×10^5 cells / flask / 5ml. After incubation overnight at 37 °C in 5 % CO_2, a test sample was added to each flask and incubated for a further 8 h. The treated cells were then identified as apoptotic and necrotic cells, using the FITC Annexin V Apoptosis Detection Kit with propidium iodide (PI) (BioLegend Inc., Sandiego, CA, USA). The treated cells were harvested by trypsinization and washed twice with 20 mM cold PBS with 1 % fetal calf serum (FCS). Then, resuspended cell pellets in 100 µl Annexin V binding buffer and cells suspension 100 µl were transferred to 1.5 ml micro centrifuge tubes. Then 2.5 µl of FITC Annexin V and 5 µl of PI solution were added to each tube. The cells were gently vortexed and incubated for 15 min at room temperature in the dark. Then, 200 µl of Annexin V binding buffer was added to each tube and the cells were analyzed by flow cytometer. Alls investigations were performed in triplicate.

3.11 Isolation of peptides with tyrosinase inhibitory activity

Protein hydrolysates with MW < 3 kDa were analyzed by HPLC. The analyses were conducted on a Luna C18 (4.6 mm × 250 mm) column. The mobile phases consisted of solvent A (0.1 (w/v) trifluoroacetic acid (TFA)), and solvent B (70 % (v/v) acetonitrile in water containing 0.05 % (w/v) TFA). The flow rate was set at 0.7 ml/min and the detection system monitored absorbance at 280 nm. The sample injection volume was 50 µl.

3.12 Identification of tyrosinase inhibitor peptides

The LC/MS/MS method was used to identify the amino acid sequence of each internal fragment of protein hydrolysate. The LC/MS/MS system consists 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). Mobile phase A consisted of water/formic acid (99.9:0.1, v/v), and B acetonitrile (100, v). Analyte separation was performed using gradient conditions of 5-80 % B over 50 min at a flow rate of 100 μl/min. Mass spectral data from 300 to 1500 *m/z* was collected in the positive ionization mode. All data were processed and submitted to a MASCOT search of the NCBI database (http://www.matrixscience.com, http://blast.ncbi.nlm.nih.gov).

3.13 Kinetic analysis of tyrosinase inhibition

The assay was performed using the same protocol as the measurement for tyrosinase inhibition activity, apart from changes in the concentrations of the substrate. The reaction mixture consisted of different concentrations of substrate (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, 1.8, and 2.0 mM for L-tyrosine and 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 mM for L-DOPA), and mushroom tyrosinase (333 U/ml in 50 mM phosphate buffer pH 6.5). Three different concentrations of inhibitors were added to each reaction mixture and incubated at 37 °C. The Lineweaver-Burk plot method was used to determine the reaction kinetics (Lineweaver and Burk, 1934).

3.14 Protein content determination

The protein content was determined by Bradford's procedure (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard with nine concentrations (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 μg/ml) to generate the standard curve.

3.15 Statistical analysis

The results of all measurements were expressed as the mean ± standard deviation. All investigations were performed in triplicate. IC_{50} values were calculated

using GraphPad Prism (Version 6.00, GraphPad Software Inc., La Jolla, CA, USA) for Windows.

Statistical analysis for comparing the results was performed by Student's t-test or a one-way ANOVA, followed by Duncan's test. $p < .05$ was considered to represent statistical significance. All statistical analyses were performed according to the statistics program, SPSS version 22.

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CHAPTER IV
RESULTS AND DISSCUSSION

4.1 Amino acid content of chicken feather meal

Amino acid compositions of chicken feather meal are shown in Table 2. Amino acids detected included alanine, arginine, glycine, aspartic acid, valine, cystine, glutamic acid, leucine, isoleucine, histidine, threonine, proline, lysine, methionine, serine, phenylalanine, tyrosine and tryptophan in difference amounts. Arginine, phenylalanine, valine, alanine, and leucine described by Schurink *et al.* (2007) consisted of good tyrosinase inhibitory peptides, considered important in the inhibition of tyrosinase. Apart from the hydrophobic residues, peptides containing the polar, uncharged residues cysteine and serine, also showed significant inhibitory activity. Ishikawa *et al.* (2007) noted that some amino acids such as L-alanine, glycine, L-isoleucine, and L-leucine possessed beneficial effects towards the disruption of melanogenesis. The chicken feather meal powder contained small amounts of tyrosine. Tyrosine is a substrate of tyrosinase in melanogenesis, therefore there should not be a large amount of tyrosine in a tyrosinase inhibitor because it might increase the concentration of substrate and reduce inhibitory activity. Chicken feather meal powder contained a large quantity of serine, considered as a tyrosinase inhibitor in previous studies. Sericin (silk protein) is composed of high levels of serine (30-33 % of total amino acid), and phosvitin (phosphoglycoprotein present in egg yolk) has a specific amino acid composition comprising 50 % serine. Both have an ability to inhibit tyrosinase activity (Jung *et al.*, 2012, Kato *et al.*, 1998).

Table 2 Total amino acid profile of chicken feather meal

Amino acids	mg/100 mg
Alanine	3.80
Arginine	6.30
Glycine	6.82
Aspartic acid	5.70
Valine	5.85
Cystine	2.90
Glutamic acid	10.6
Leucine	6.46
Isoleucine	3.94
Histidine	0.59
Threonine	3.96
Proline	8.37
Lysine	1.45
Methionine	0.67
Hydroxyproline	Not Detected
Serine	7.84
Phenylalanine	4.03
Hydroxylysine	Not Detected
Tyrosine	1.1
Tryptophan	0.28

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4.2 Optimization of enzymatic hydrolysis condition of feather meal (by pepsin-pancreatin and papain)

The optimization of the enzymatic hydrolysis condition of feather meal was screened from seven different concentrations of substrates (0.00625, 0.0125, 0.0250, 0.0500, 0.1000, 0.2500, and 0.5000 g/ml). The protein hydrolysate was prepared following the procedure described in the experimental section (3.3) using two types of enzymes (pepsin-pancreatin and papain) and all were compared for their

tyrosinase inhibitory activity. The IC_{50} values of protein hydrolysates prepared from enzymatic hydrolysis by pepsin-pancreatin and papain at difference substrate concentrations are presented in Figure 7. The protein hydrolysate from substrate concentration 0.0125 g/ml provided the best IC_{50} value 5.369 ± 2.361 μg/ml of protein hydrolysates from pepsin-pancreatin hydrolysis and 17.220 ± 7.618 μg/ml from papain hydrolysis. The protein hydrolysate at this condition was selected for further study.

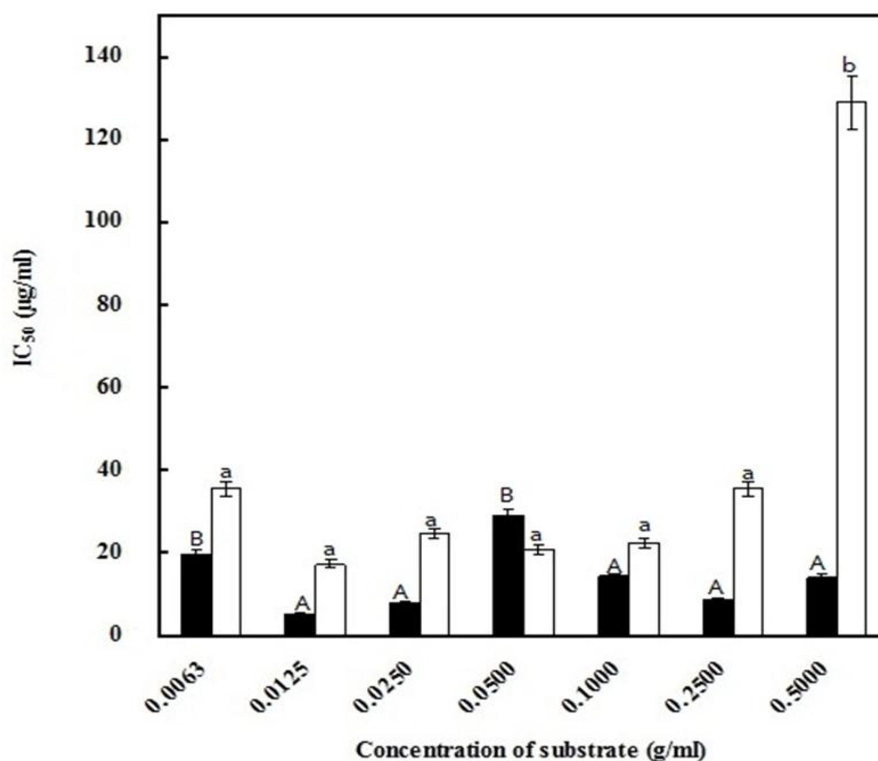

Figure 7. The IC_{50} values of protein hydrolysates at difference substrate concentration. The protein hydrolysate from enzymatic hydrolysis by pepsin-pancreatin (black), and protein hydrolysate from enzymatic hydrolysis by papain (white) are shown. Each value is expressed as mean ± SD (n=3) and a, b, A and B show significant difference value at $p < .05$

4.3 Tyrosinase inhibition activity of protein hydrolysate fraction

The protein hydrolysate in this optimal condition was fractionated into different sizes of peptide, MW x > 10 KDa, 5 < x < 10 KDa, x < 5 kDa, 3 < x < 5 kDa, and x < 3kDa using ultrafiltration membranes and a Microsep Advance Centrifugal Device. All fractions were assayed for *in vitro* tyrosinase inhibitory activities to test for

their ability as tyrosinase inhibitors in both monophenolase and diphenolase activities, according to the procedure described in the experimental section. The results are shown in Table 3. The protein hydrolysate prepared by pepsin-pancreatin with MW < 3 kDa showed the lowest IC_{50} value in both monophenolase (5.780 ± 0.188 μg/ml) and diphenolase activities (0.040 ± 0.024 μg/ml). In monophenolase activities, protein hydrolysates with MW < 3 kDa showed stronger tyrosinase inhibitory activity than the positive control kojic acid (6.076 ± 0.001 μg/ml). The IC_{50} value of protein hydrolysates with MW < 3 kDa (0.040 ± 0.024) in diphenolase activities was less potent than kojic acid (0.034 ± 0.000 μg/ml) but showed no statistically significant difference. These results indicated that protein hydrolysates prepared by pepsin-pancreatin with MW < 3 kDa have good ability as tyrosinase inhibitors as they can inhibit tyrosinase using the lowest concentration of sample. Tyrosinase inhibition in diphenolase activity was stronger than in monophenolase activity. Therefore, protein hydrolysates prepared by pepsin-pancreatin were selected for further investigation.

These results compared well with previously reported data for inhibition of tyrosinase activity. Wu *et al.* (2008) found that sericin hydrolysate exhibited tyrosinase-inhibitory effect in a dose-dependent manner with IC_{50} value 10 mg/ml. Manosroi *et al.* (2010) reported that silk protein (sericin) from native Thai silkworms showed tyrosinase inhibition activity with IC_{50} value 1.2 - 18.76 mg/ml. Zhuang *et al.* (2009) noted that the tyrosinase inhibition activity of hydrolysates of jellyfish umbrella collagen (HF-2) was higher than 50 % at 5 mg/ml which showed lower tyrosinase inhibition activity than protein hydrolysates in this experiment. Their studies indicated that HF-2 with MW 1000 Da < HF-2 < 3000 Da exhibited the best tyrosinase-inhibitory activity, and these have similar size to protein hydrolysates tested here.

Table 3 Fractions of protein hydrolysates prepared from chicken feather meal using pepsin-pancreatin and papain hydrolysis and their tyrosinase inhibition activity

Molecular weight (kDa)	Tyrosinase inhibition IC_{50} (µg/ml)*			
	Monophenolase activity		Diphenolase activity	
	Pepsin-pancreatin	papain	Pepsin-pancreatin	papain
crude protein	22.697 ± 4.177^{b}	22.697 ± 4.177^{d}	$21.363 \pm 5.940^{b,c}$	21.363 ± 5.940^{c}
crude hydrolysate	34.627 ± 1.845^{d}	8.617 ± 0.203^{a}	5.369 ± 2.361^{a}	$17.220 \pm 7.618^{b,c}$
> 10 kDa	63.277 ± 6.046^{e}	8.546 ± 0.167^{a}	26.753 ± 19.388^{c}	35.583 ± 9.694^{d}
5-10 kDa	28.713 ± 1.320^{c}	15.873 ± 0.160^{b}	$10.561 \pm 3.689^{a,b}$	40.053 ± 5.871^{d}
< 5 kDa	19.857 ± 0.075^{b}	18.927 ± 0.370^{c}	3.927 ± 1.713^{a}	$5.908 \pm 4.812^{a,b}$
3-5 kDa	19.637 ± 0.635^{b}	27.930 ± 1.400^{e}	1.360 ± 1.370^{a}	0.997 ± 0.278^{a}
< 3 kDa	5.780 ± 0.188^{a}	31.190 ± 1.771^{f}	0.040 ± 0.024^{a}	$10.776 \pm 13.412^{a,b,c}$
Kojic acid	6.076 ± 0.001^{a}	6.076 ± 0.001^{a}	0.034 ± 0.000^{a}	0.034 ± 0.000^{a}

* All data are shown as the mean ± standard deviation, obtained from three repeated determinations (a, b, c, d, e and f show significant difference value at $p < .05$)

4.4 Inhibitory effect on B16F10 cells of protein hydrolysates fraction

4.4.1 The effect of protein hydrolysates on cell viability

Results suggest that protein hydrolysates prepared from chicken feather meal may be useful tyrosinase inhibitors due to their potent enzymatic inhibitory activity, but the effect on cells is also a highly important factor. All fractions of protein hydrolysates prepared from chicken feather meal were determined for their cytotoxicity using melanoma B16F10 cells as a model system. The B16F10 murine melanoma cell line was used in this study because they were reported to be a good model for studying human melanoma which has a short population doubling time. They are easy to culture and have better survival rates than human melanocyte cells (Huang *et al.*, 2014, Moleephan, 2012). Moreover, research using B16 melanoma cells

has suggested that the action mechanism for melanogenesis is similar to that in normal human melanocytes (Huang *et al.*, 2012).

B16F10 cells were treated with six concentrations of samples in seven fractions and tested by MTT cell viability assay. The IC_{50} values of all fractions are shown in Table 4. The protein hydrolysate with MW < 3 kDa showed the lowest IC_{50} value (1.124 ± 0.288 μg/ml). The results showed that all fractions of protein hydrolysates have a cytotoxic activity and the cytotoxic effect was related to size (molecular weight) of the protein hydrolysates.

Table 4 Fractions of protein hydrolysates prepared from chicken feather meal using pepsin-pancreatin hydrolysis and their inhibitory effect on B16F10 cells

Molecular weight (kDa)	Melanocyte cells viability IC_{50} (μg/ml)*
crude protein	26.083 ± 0.876[d]
crude hydrolysate	17.370 ± 2.258[c]
> 10 kDa	24.900 ± 3.330[d]
5 - 10 kDa	14.640 ± 1.906[c]
< 5 kDa	3.583 ± 0.413[a,b]
3 - 5 kDa	4.786 ± 0.841[b]
< 3 kDa	1.124 ± 0.288[a]
Kojic acid	1,219.000 ± 0.000

* All data are shown as the mean ± standard deviation, obtained from three repeated determinations (a, b, c and d show significant difference value at $p < .05$)

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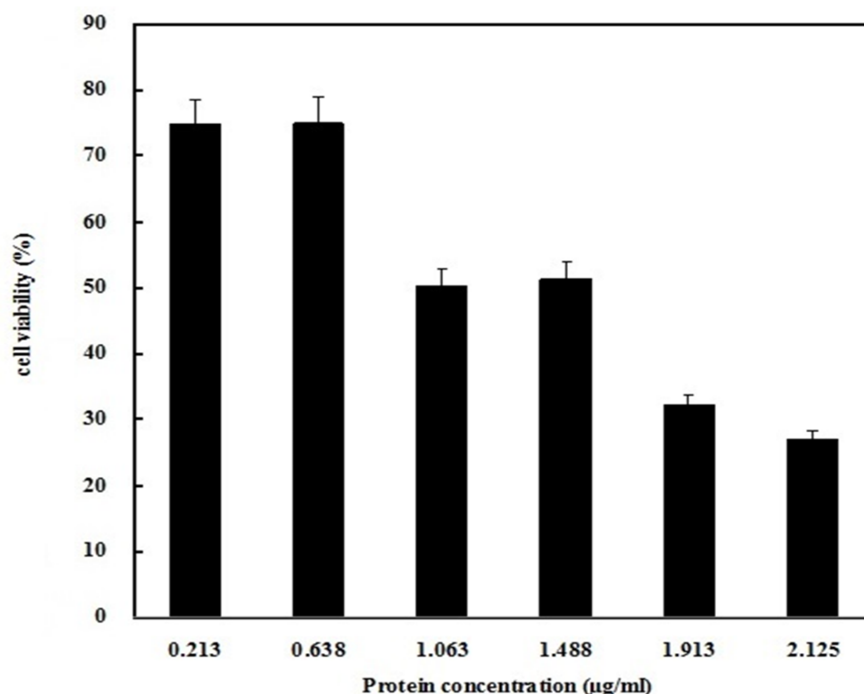

Figure 8. Viability of B16F10 cells after treatment with various concentrations of protein hydrolysates prepared from chicken feather meal (MW < 3 kDa) for 72 h

From the results of the MTT cell viability assay shown in Figure 8, the majority of cells were still viable at sample concentrations of 0.638 and 0.213 µg/ml. Samples in these concentrations were further used to test for cellular tyrosinase inhibitory activities as a desirable skin-whitening agent should inhibit melanin synthesis in melanosomes, by acting specifically to reduce the synthesis or activity of tyrosinase with little or no cytotoxicity (Chan *et al.*, 2011, Dooley, 1997). The protein hydrolysate with MW < 3 kDa at higher concentration levels (1.063, 1.488, 1.913, and 2.125 µg/ml) was not further studied due to its greater cytotoxicity on the melanoma cells.

4.4.2 The effect of protein hydrolysates on cellular tyrosinase activity

Results indicated that among all sample fractions, protein hydrolysates with MW < 3 kDa showed the best activity for mushroom tyrosinase inhibition. Therefore, this fraction was selected for further investigation of its ability to inhibit tyrosinase activity in B16F10 cells. Samples with concentrations that had low toxicity to cells were selected to test for cellular tyrosinase inhibition assay. The protein hydrolysate MW < 3 kDa at a concentration 0.638 µg/ml was prepared into three dilutions and

tested by cellular tyrosinase inhibition assay according to the procedure described in the experimental section. Figure 9 demonstrates that the samples reduced cellular tyrosinase in B16F10 cells. The inhibition was dose-dependent: protein hydrolysates at 0.050, 0.100, and 0.210 μg/ml induced inhibition on cellular tyrosinase in B16F10 cells by 13.260, 45.295, and 50.493 %, respectively. The results at 0.100 and 0.210 μg/ml had statistically significant difference when compared to the control, but showed no statistically significant difference between each other. The sample showed higher tyrosinase inhibitory activity than kojic acid used as the positive control (data not shown).

4.4.3 The effect of protein hydrolysates on melanin synthesis in B16F10 cells

The protein hydrolysate prepared from chicken feather meal with MW < 3 kDa was investigated for its ability to inhibit melanogenesis in B16F10 cells. Melanoma cells were treated with three concentrations of samples (0.210, 0.100, and 0.050 μg/ml). Then, the melanin content was measured according to the procedure described in the experimental section (3.8). The melanin content (% of control) is shown in Figure 10. The control readings were set as 100 %. The effect of protein hydrolysates on B16F10 cells was expressed as percentage of control. The results showed that melanin synthesis was not reduced at a concentration of 0.050 μg/ml, while at concentrations 0.100 and 0.210 μg/ml the inhibition on melanin synthesis in B16F10 cells was 21.601 and 14.680 %, respectively. The results showed no statistically significant difference when compared to the control, and the results from 0.100 and 0.210 μg/ml also showed no statistically significant differences. The ability of samples to inhibit melanogenesis in B16F10 cells was lower than kojic acid (data not shown).

The effect of protein hydrolysates on melanin reduction did not correlate with tyrosinase inhibition. These results were inconsistent with previous findings. When melanogenesis is regulated such as in tyrosinase inhibition, these parameters as well as melanin content, are either increased or decreased together (Pinon *et al.*, 2011). This observation may be due to the possibility that melanogenesis in B16F10 cells occurs via multiple steps. Thus, the reduction in the melanin formation by

protein hydrolysates may not be due to direct tyrosinase inhibition. These results compared with other studies, Jung *et al.* (2012) reported that phosvitin (phosphoglycoprotein present in egg yolk) at a concentration of 50 μg/ml inhibited the tyrosinase activity of B16F10 melanoma cells by 42 %, and inhibited melanin synthesis by 17 %, lower tyrosinase inhibition activity than this study.

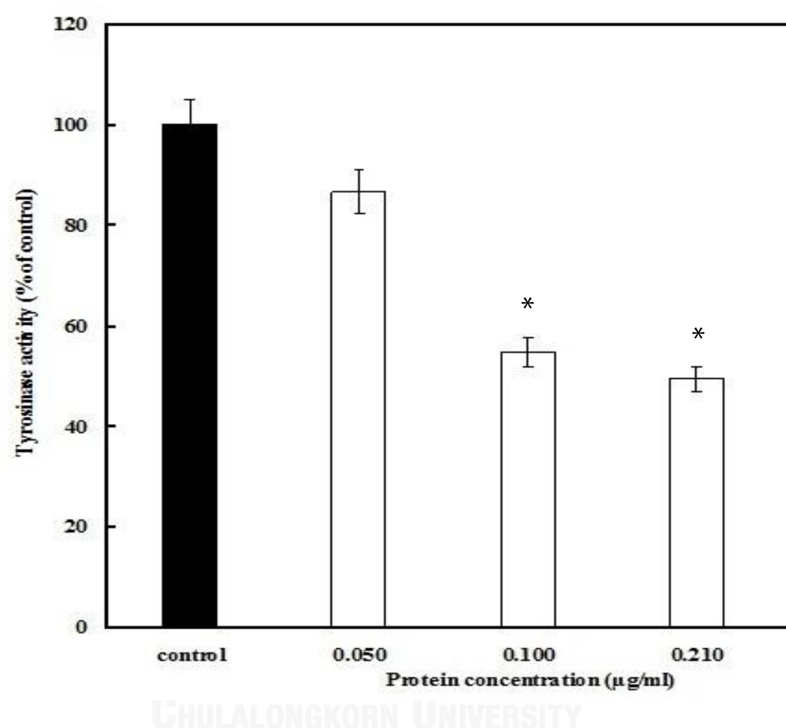

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Figure 9. Effect of protein hydrolysates on tyrosinase activity (% of control) in B16F10 cells. *$p < .05$ indicated a significant difference when compared to the control

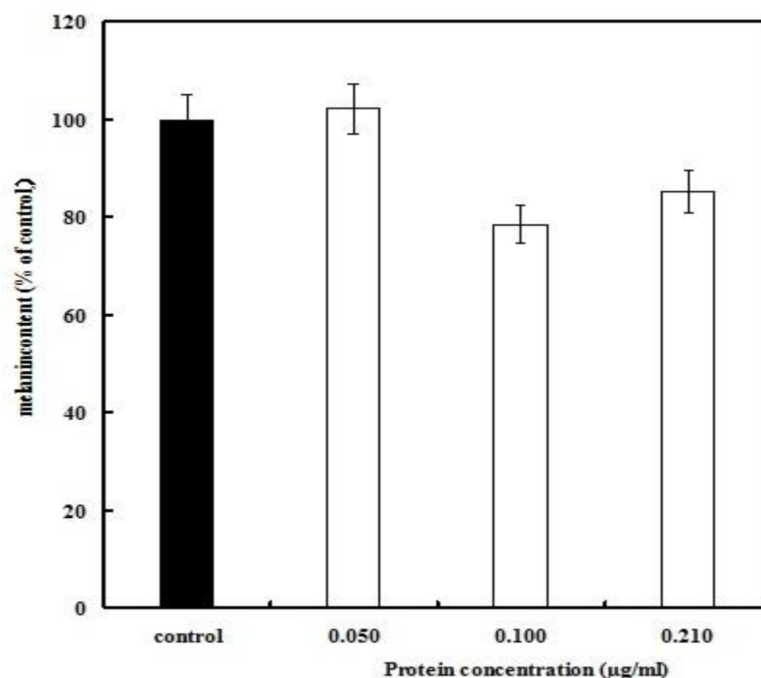

Figure 10. Effect of protein hydrolysates on melanogenesis (% of control) in B16F10 cells

4.5 Apoptosis detection assays

Apoptosis is a programmed form of cell death, essential for the development and maintenance of tissue homeostasis and the elimination of unwanted or damaged cells from multicellular organisms (Liu *et al.*, 2011, Yang *et al.*, 2015). The results of MTT assay (3.7), suggest that protein hydrolysates prepared from chicken feather meal in high concentrations affected B16F10 cell viability. The effect of protein hydrolysates on the promotion of apoptosis in B16F10 cells was investigated.

Cell apoptosis was determined by flow cytometer. B16F10 cells were incubated with protein hydrolysates (2.223 µg/ml) for 8 h., and then apoptotic and necrotic cells were determined by Annexin-V and PI double staining. The results are shown in Figure 11. Compared to the control, cells treated with protein hydrolysate increased necrotic (Q1) and late stage apoptotic cells (Q2). Results showed that protein hydrolysate treatment did not increase early stage (Q4) apoptotic cells.

In the process of apoptosis, cells commonly lose their phospholipid membrane asymmetry and expose phosphatidylserine at the cell surface. Annexin V is useful for identifying apoptotic cells with exposed phosphatidylserine because it is

a phospholipid-binding protein with high affinity for phosphatidylserine. Necrotic cells are readily stained with PI as well as with Annexin V, whereas apoptotic cells only stain with Annexin V (Castro *et al.*, 2009, Ito *et al.*, 2002). Kluza *et al.* (2002) reported that B16 melanoma cells were notoriously resistant to most chemotherapeutic agents and their evidence for apoptosis in B16 cells was weaker than in HL-60 cells. In addition, they reported that flow cytometry analysis was less appropriate to study B16 cells because membrane damage can occur during harvesting. Moreover, trypsin can remove bound Annexin V from the surface by proteolysis and chelation of Ca^{2+}. Therefore, harvesting with trypsin can also influence the measurable amount of bound Annexin V. To validate the conclusion that protein hydrolysates can induces apoptosis in B16F10 cells or not, more experimental work, such as microscopic examination to observe morphology of cells is required.

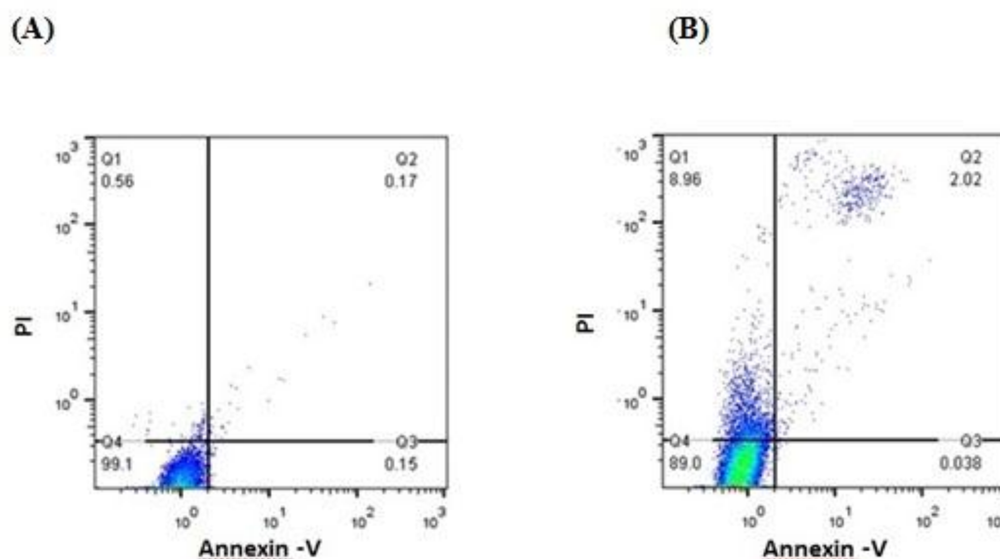

Figure 11. Effects of the protein hydrolysate on apoptosis on B16F10 cells. Control (A), B16F10 cells were incubated with protein hydrolysates (B)

4.6 Mechanism of inhibition

The inhibition modes of the tyrosinase inhibitor from protein hydrolysates of chicken feather meal prepared by pepsin-pancreatin, with MW < 3 kDa in monophenolase (substrate: L-tyrosine) and diphenolase (substrate: L-DOPA) activities were investigated, based on Lineweaver-Burk plot analysis. Lineweaver-Burk plots

show that 1/v versus 1/[S] give a family of parallel straight lines (data not shown). This means that inhibitors in different concentrations can change the apparent value of V_{max} but the ratio of K_m/V_{max} remained unchanged. The results showed that all types of inhibition in both monophenolase and diphenolase activities of protein hydrolysates from chicken feather meal were uncompetitive inhibitors. The inhibition constant (K_i value) were 18.149 and 27.189 μg protein/ml, respectively (Table 5). An uncompetitive inhibitor is an inhibitor that can bind only to the enzyme-substrate complex and not with the free enzyme (Kubo *et al.*, 2003).

The tyrosinase inhibitors previously studied showed many types of inhibition. Uncompetitive inhibitor is one inhibitor type that showed potent tyrosinase inhibitory activity. Chai *et al.* (2013) reported that furoic acid had the ability to inhibit tyrosinase activities with a mode of inhibition that was uncompetitive. 2-chlorocinnamic acid and 2,4-dichlorocinnamic acid displayed a reversible and uncompetitive mechanism for tyrosinase inhibition (Hu *et al.*, 2014). Other types of tyrosinase inhibitor such as *Betula pendula* leaves ethanolic extract exhibited a noncompetitive inhibition on tyrosinase activities (Germanò *et al.*, 2012), and 3',5'-di-C-b-glucopyranosylphloretin demonstrated good tyrosinase inhibitory activity in the competitive mode (Lou *et al.*, 2012). Among tyrosinase inhibitors, inhibitors from natural sources were interesting, but the tyrosinase inhibitors from proteins and peptides are rare. The tyrosinase inhibitors kinetic of protein hydrolysates of chicken feather meal found in the present study indicated a putative uncompetitive mechanism that may have potential as a new source for skin-lightening applications.

Table 5 Mode of tyrosinase inhibition from protein hydrolysates in 2 types of activity

Type of activity	K_i (μg protein/ml)	Type of Inhibition
Monophenolase activity	18.149	Uncompetitive inhibitor
Diphenolase activity	27.189	Uncompetitive inhibitor

4.7 Purification of tyrosinase inhibitor peptides by RP-HPLC

The protein hydrolysate of chicken feather meal prepared by pepsin-pancreatin with MW < 3 kDa showed the best result for tyrosinase inhibition. Thus,

protein hydrolysates MW < 3 kDa were subjected to peptide separation by RP-HPLC on a Shimpack C18 column using a trifluoroacetic acid/acetonitrile solvent system, and detected at UV-280 nm. The chromatography profile was shown in Figure 12. Fractions of protein hydrolysates were collected at retention time 0-10 min (fraction 1), 10-20 min (fraction 2), 20-30 min (fraction 3), and 30-40 min (fraction 4). All fractions were assayed for *in vitro* tyrosinase inhibitory activity to screen the maximal inhibition in diphenolase activities, according to the procedure described in the experimental section. The results were showed that protein hydrolysates fraction 1 exhibited the highest of maximal inhibition at 49.655 % followed by fraction 3, fraction 2 and fraction 4 at 31.766, 22.447 and 7.589 %, respectively. Next, all fractions were further identified for tyrosinase inhibitor peptides.

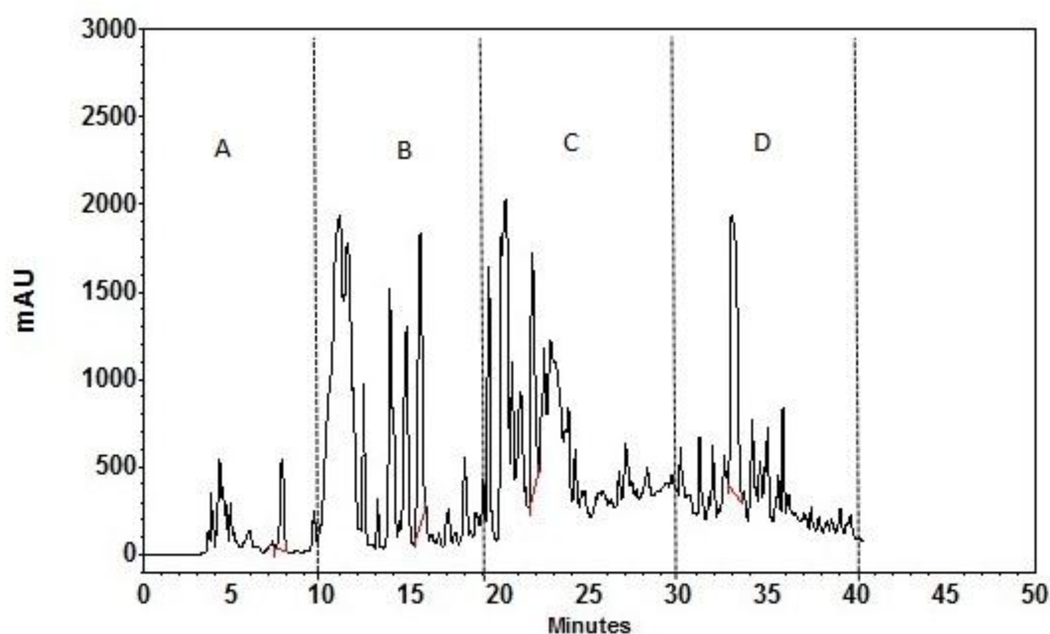

Figure 12. RP-HPLC chromatogram of a fraction with MW < 3 kDa of protein hydrolysates from chicken feather meal, prepared by pepsin-pancreatin digestion on a Luna C18 (4.6 mm × 250 mm) column

4.8 Identification tyrosinase inhibitor peptides

The relationship between tyrosinase inhibition activity and the amino acid constituents has been previously reported (Ishikawa *et al.*, 2007, Schurink *et al.*, 2007). The presence of R, F, V, A, L, G, S, C or I (arginine, phenylalanine, valine, alanine, leucine, glycine, serine, cysteine, and isoleucine) could enhance the

tyrosinase inhibitory activity in peptides. Schurink *et al.* (2007) suggested that the peptide should contain one or more arginine residues for strong tyrosinase inhibitory and binding activity. Their studies also indicated that tyrosinase inhibition is optimal when arginine and/or phenylalanine is/are combined with hydrophobic aliphatic residues such as valine, alanine, or leucine. (Ubeid *et al.* (2009)) reported that oligopeptides P3 and P4 (RADSRADC and YRSRKYSSWY) are highly active inhibitory sequences for mushroom and human tyrosinase which contains the key residues. Their results showed that oligopeptide P3 contains the combination Arg–Ala in positions 1/2 and 5/6, as well as the amino acids serine and cysteine at positions 4 and 8, respectively. For P4, the presence of arginine at positions 2 and 4, and serine at 3, 7, and 8 may explain the strong inhibitory activity observed for this oligopeptide.

In this study, the protein hydrolysate with MW < 3 kDa had peptide sequences identified by LC/MS/MS analysis. Eighteen peptide sequences were identified in four fractions. The results are shown in Table 6. Fractions 1, 2, 3, and 4 showed 5, 5, 5, and 3 peptide sequences, respectively. Fraction 1 had combinations Arg-Val in positions 15/16 of peptide GCGYKPCDPQVIRDRVA and Arg-Leu in position 4/5 of peptide CSARLVNYGYTFGSG. The combination Phe-Ala was present in position 8/9 of peptide GCYIEGFFATLGGEIALW from fraction 2 and Arg-Val was present in position 2/3 of peptide NRVYVHPF from fraction 4. Moreover, all sequences of identified peptides showed at least one R, F, V, A, L, G, S, C or I, which may contribute to the observed tyrosinase inhibition activity. Thus the identified peptides are suitable as tyrosinase inhibitors.

Table 6 Tyrosinase inhibitor peptides of protein hydrolysate with MW < 3 kDa from chicken feather meal prepared by pepsin-pancreatin.

Fraction	Sequence	Protein name	Accession number	Organism
1	GAGESKC	transthyretin	1666482	*Rattus norvegicus*
	SIFNKGKSIVHKDAW	pkhd1	303225819	*Didelphis virginiana*
	GCGYKPCDPQVIRDRVA	beta spectrin1 protein	218331463	*Sicista betulina*
	AKEKEVTFQSGGPT	Scolopendra 5885.28 Da toxin, partial	212288535	*Scolopendra viridicornis nigra*
	CSARLVNYGYTFGSG	TCR V beta 2-J beta 1.2	1477978	*Human*
2	LYFCASSDGLPQDTQYF	T cell receptor beta chain, partial	2894971	*Mus musculus*
	GCYIEGFFATLGGEIALW	rhodopsin, partial	194240743	*Grus Canadensis*
	GNRWLRQAKNG	follistatin	108679	*Bovine*
	M.AAACRCLSLLLLSTCVALLL	Putative pancreatic polypeptide 2	74719120	*Homo sapiens*
	TSMYLCASSSGDREAFFG	T-cell receptor beta chain	5882081	*Homo sapiens*
3	HDDKAAVDAR	Fibrinogen beta chain	120108	*Ceratotherium simum*
	M.SGYGRFHFDQLCHCSFSK	rCG38921	149031319	*Rattus norvegicus*
	NSTMDSLLQLGR	Uncharacterized protein IMPPll, partial	229890311	*Nautilus macromphalus*
	LVNISFGGFIICVFCISIV	Short wavelength sensitive type 1 opsin	226374739	*Larus argentatus*
	ALPGYLK	Glutathione S – transferase P	2143764	*Rat*
4	VATVSLPR	Sarcoplasmic calcium-binding protein, partial	338819392	*Chionoecetes opilio*
	NYMKPKLLYYSNGGH	Fibroblast growth factor 1	122735	*Canis familiaris*
	NRVYVHPF	[Asn1,Val5] angiotensin II	998865	*Amphiuma tridactylum*

CHAPTER V
CONCLUSION

This study investigated the possibility of tyrosinase inhibitory activity of protein hydrolysates from chicken feather meal. Protein hydrolysates prepared from chicken feather meal were hydrolyzed by pepsin-pancreatin and papain. The optimal hydrolysis condition (substrate concentration 0.0125 g/ml) was obtained, in which the highest tyrosinase inhibition activity was achieved. The protein hydrolysates were fractionated by ultrafiltration membrane and their ability to inhibit tyrosinase on melanin biosynthesis was investigated, using a cell-free mushroom tyrosinase system and a cell culture model. The protein hydrolysate prepared by pepsin-pancreatin with MW < 3 kDa exhibited the best tyrosinase inhibition activity in a cell-free mushroom tyrosinase system, and was found to be an uncompetitive inhibitor. For a cell culture model, protein hydrolysates in this fraction showed the strongest inhibition on the viability of B16F10 cells and exhibited good inhibition on the tyrosinase activity of B16F10 cells. Tyrosinase inhibitor peptides were purified by RP-HPLC and peptides identified by LC/MS/MS analysis. To determine the effect of protein hydrolysates on the promotion of apoptosis in B16F10 cells, apoptosis detection assays were investigated. Results suggested that protein hydrolysates from chicken feather meal could be a potential new source for skin-lightening applications. However, additional studies are required to clarify the mechanisms and safety of protein hydrolysates.

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APPENDICES

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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 acid	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.

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APPENDIX B

Preparation of 20 mM potassium phosphate buffer (pH 7.2)

20 mM KH_2PO_4

Potassium dihydrogen phosphate 2.72 g/l

20 mM K_2HPO_4

Dipotassium phosphate 3.49 g/l

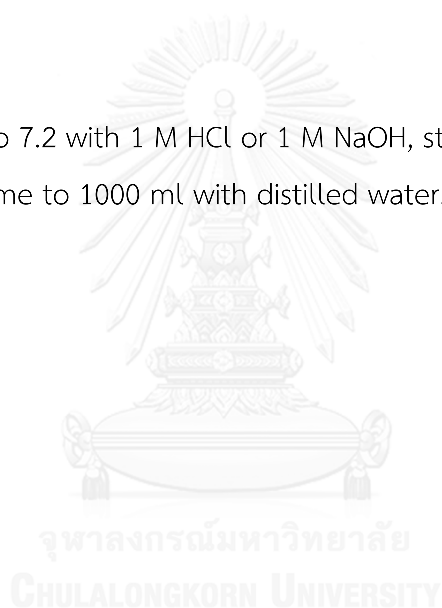
0.15 M NaCl

Sodium chloride 8.77 g/l

0.00015 M NaN_3

Sodium azide 0.01 g/l

Adjusted pH to 7.2 with 1 M HCl or 1 M NaOH, stirred until dissolved and adjusted volume to 1000 ml with distilled water.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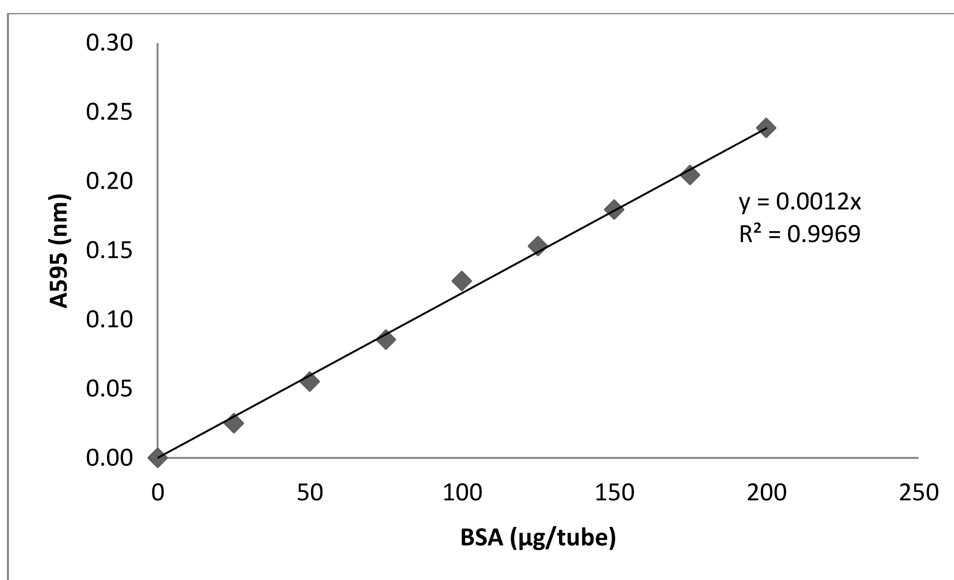
APPENDIX C

Figure 13. Calibratioin curve for protein determination by Bradford method



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APPENDIX D

Mass spectrum

Figure 14. Peptide; GAGESKC

Observed; 651.2000, Mr(expt); 650.1927, Mr(calc); 650.2694

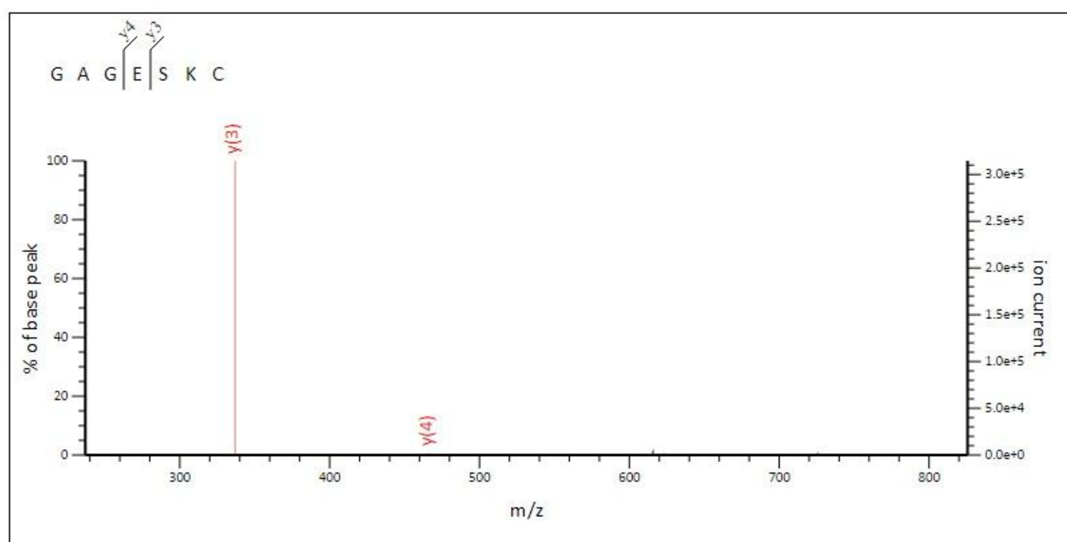


Figure 15. Peptide; SIFNKGKSIVHKDAW

Observed; 577.4900, Mr(expt); 1729.4482, Mr(calc); 1728.9311

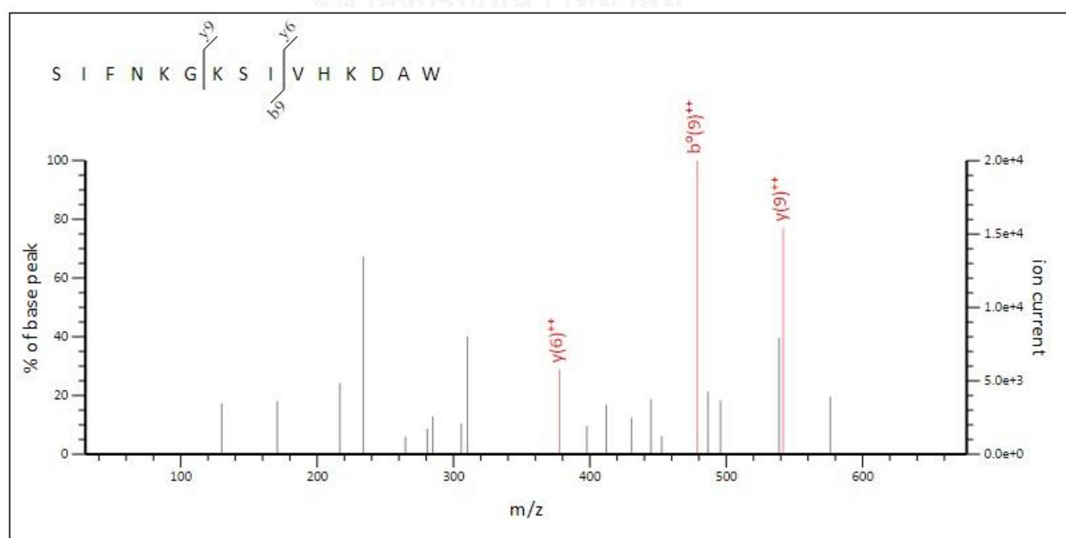


Figure 16. Peptide; GCGYKPCDPQVIRDRVA

Observed; 626.2400, Mr(expt); 1875.6982, Mr(calc); 1875.9084

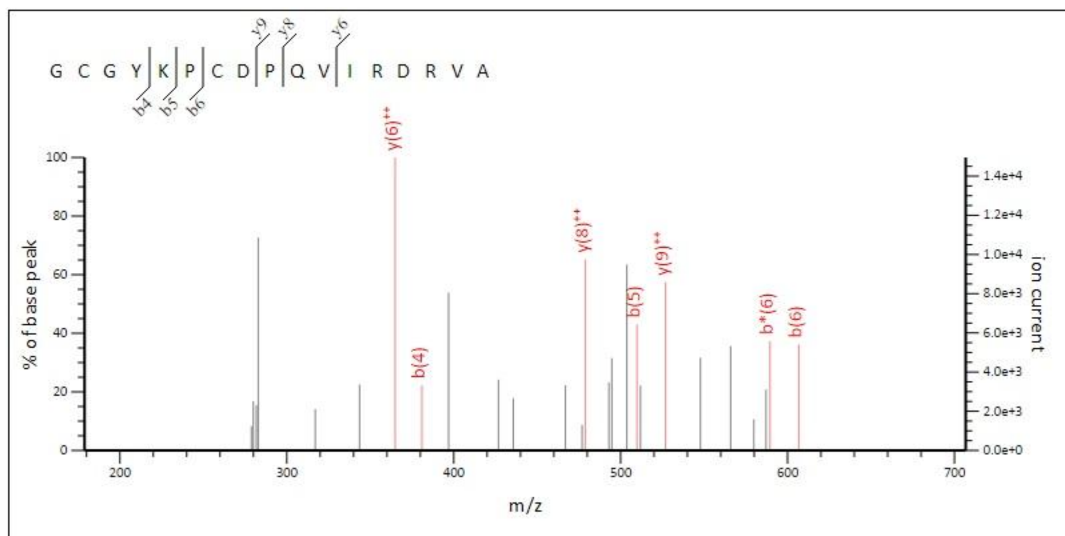


Figure 17. Peptide; AKEKEVTFQSGGPT

Observed; 739.9300, Mr(expt); 1477.8454, Mr(calc); 1477.7413

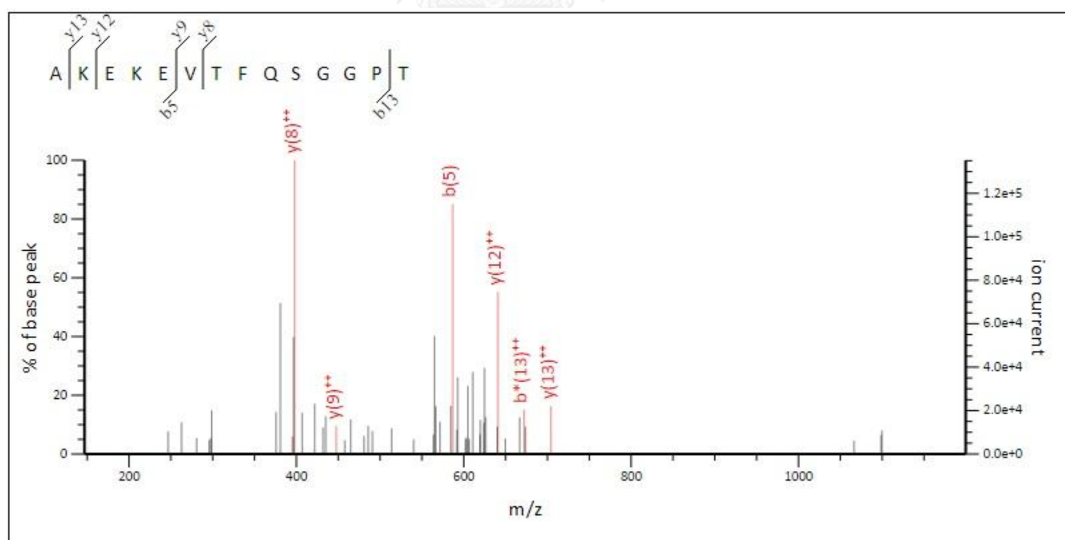


Figure 18. Peptide; CSARLVNYGYTFGSG

Observed; 797.9700, Mr(expt); 1593.9254, Mr(calc); 1593.7246

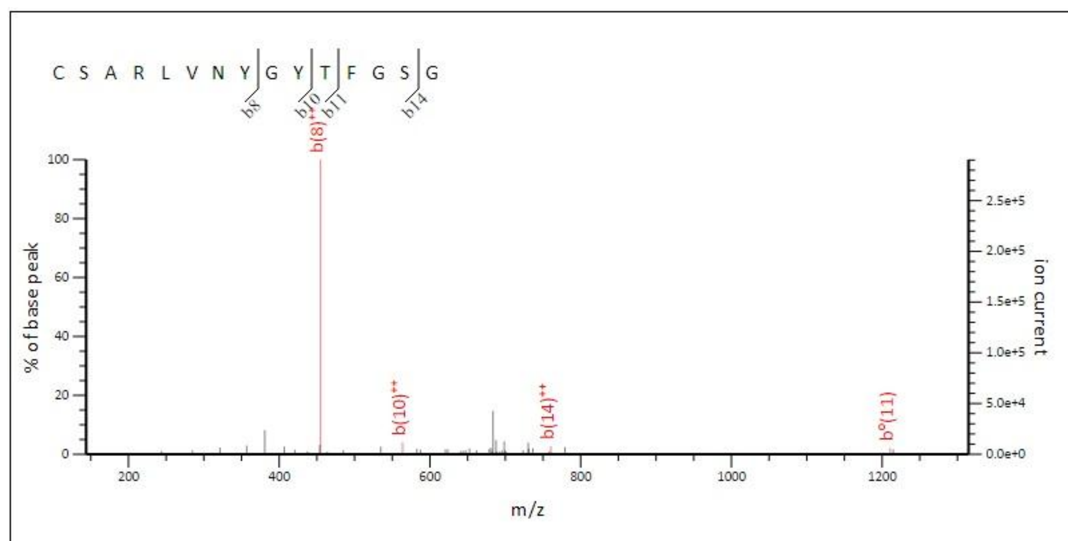


Figure 19. Peptide; LYFCASSDGLPQDTQYF

Observed; 651.9400, Mr(expt); 1952.7982, Mr(calc); 1953.8455

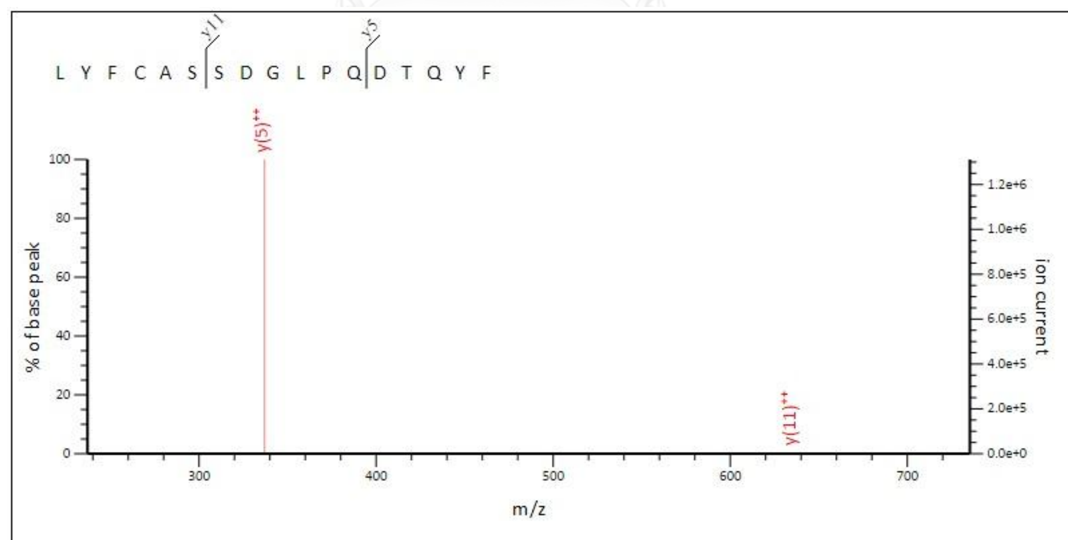


Figure 20. Peptide; GCYIEGFFATLGGEIALW

Observed; 649.3500, Mr(expt); 1945.0282, Mr(calc); 1945.9284

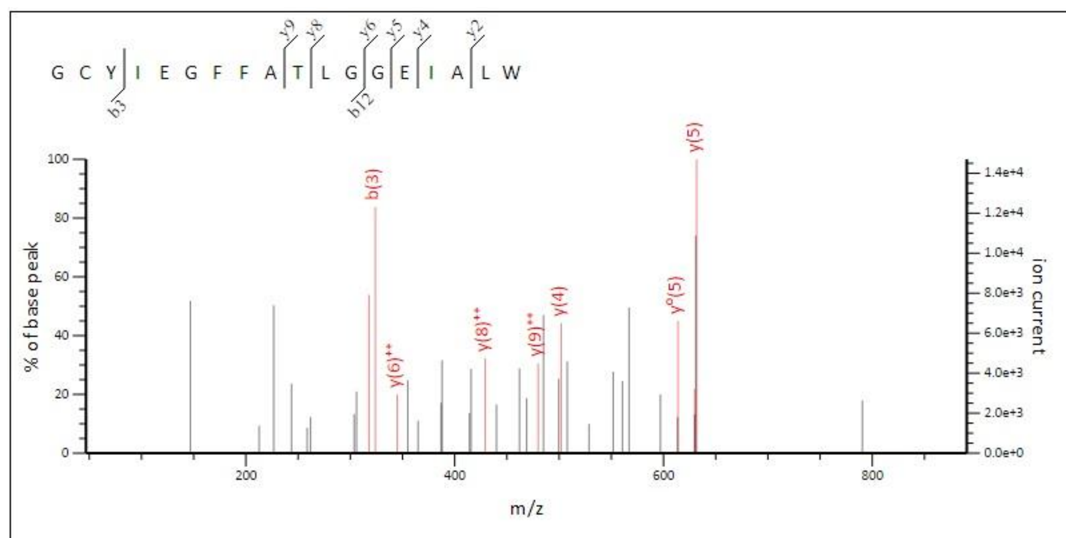


Figure 21. Peptide; GNRWLRQAKNG

Observed; 650.9300, Mr(expt); 1299.8454, Mr(calc); 1298.6956

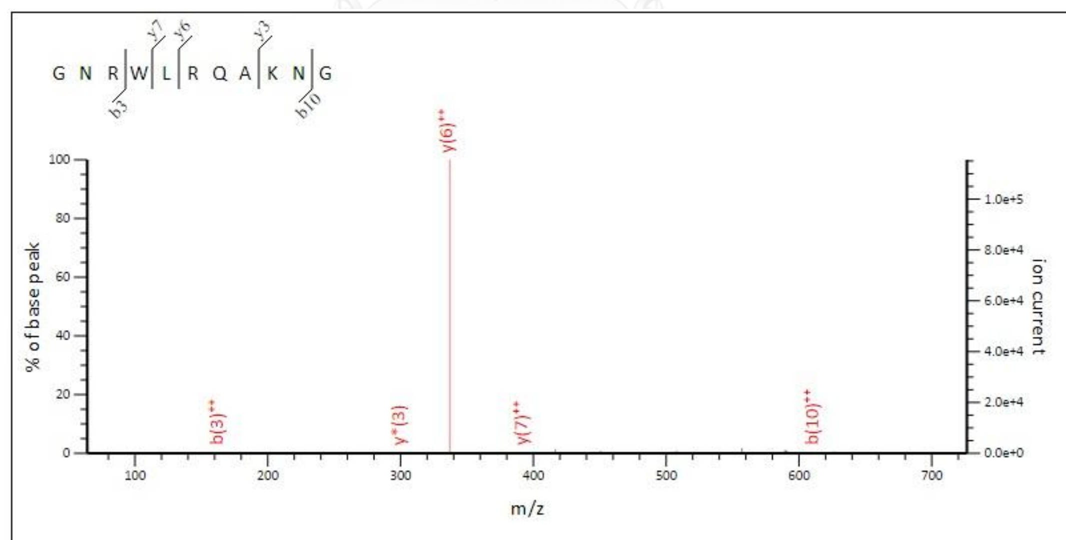


Figure 22. Peptide; M.AAACRCLSLLLLSTCVALLL

Observed; 683.1500, Mr(expt); 2046.4282, Mr(calc); 2046.1403

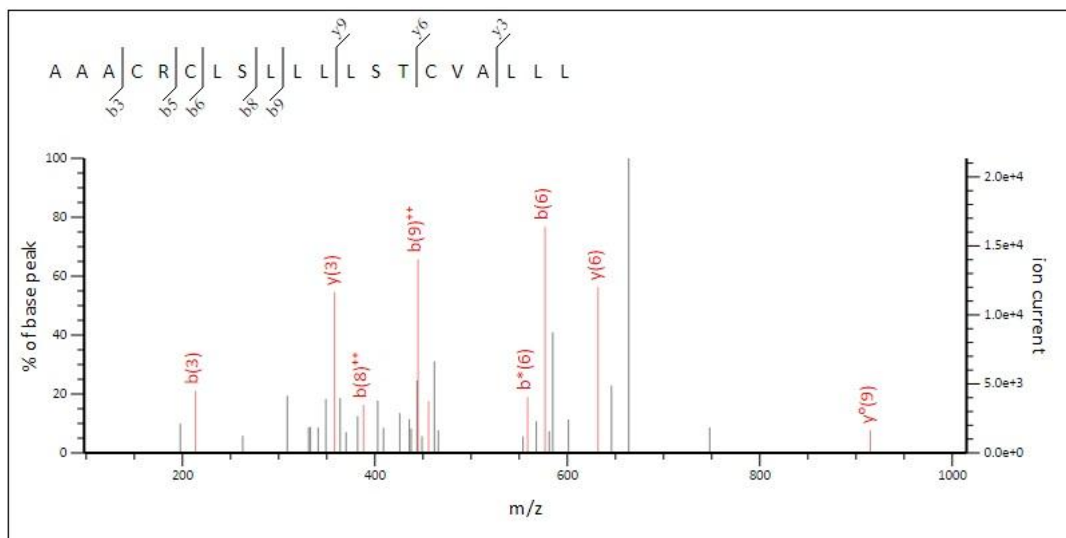


Figure 23. Peptide; TSMYLCASSSGDREAFFG

Observed; 643.3000, Mr(expt); 1926.8782, Mr(calc); 1927.8080

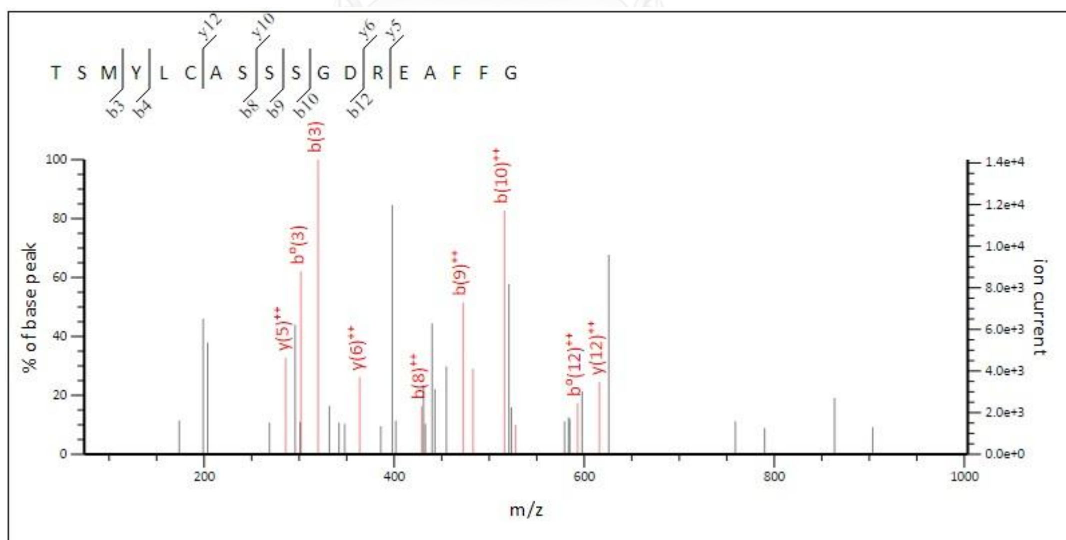


Figure 24. Peptide; HDDKAAVDAR

Observed; 549.2500, Mr(expt); 1096.4854, Mr(calc); 1096.5261

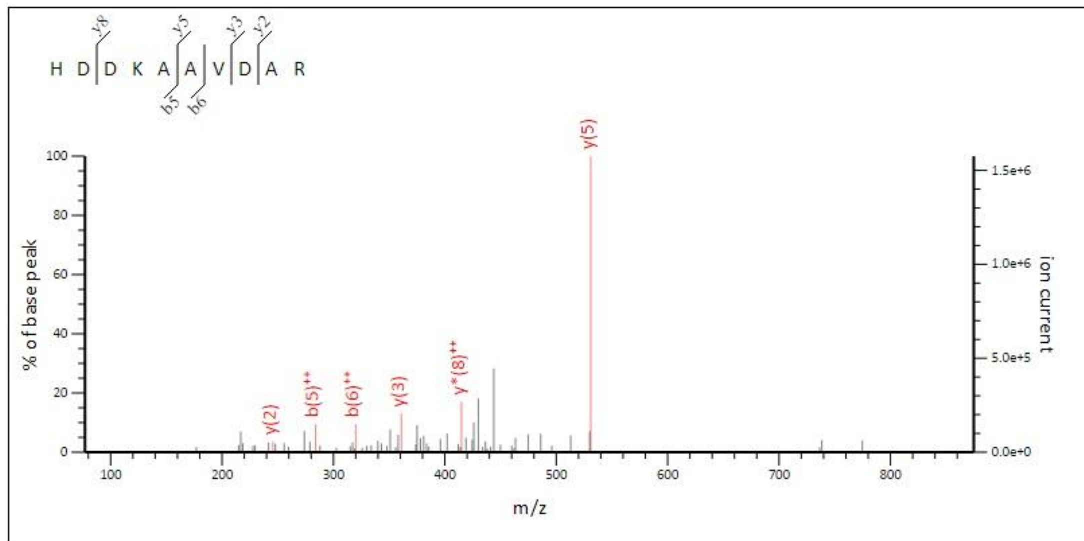


Figure 25. Peptide; M.SGYGRFHFDQLCHCSFSK

Observed; 706.6700, Mr(expt); 2116.9882, Mr(calc); 2117.9200

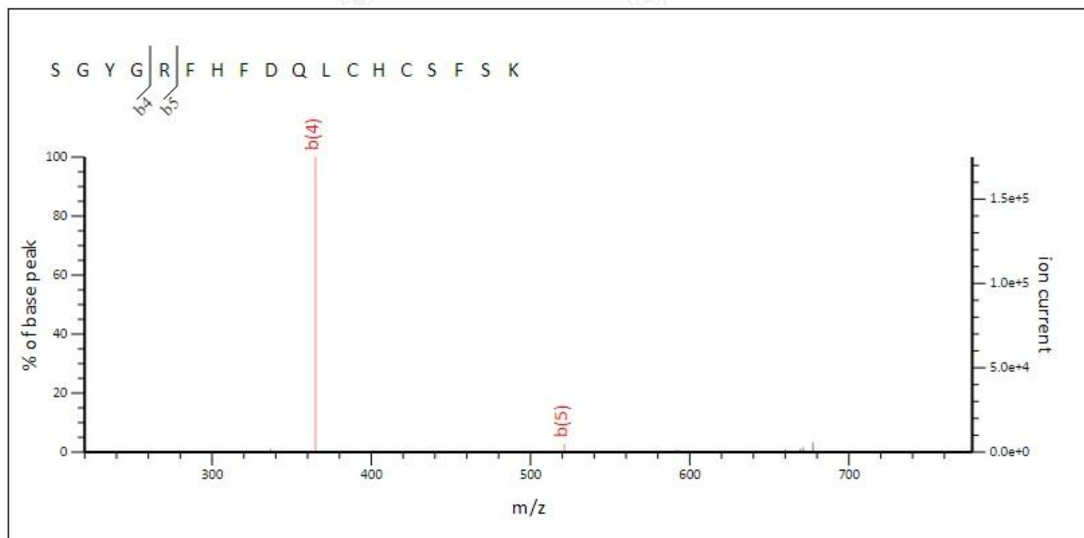


Figure 26. Peptide; NSTMDSLLQLGR

Observed; 334.5000, Mr(expt); 1333.9709, Mr(calc); 1333.6660

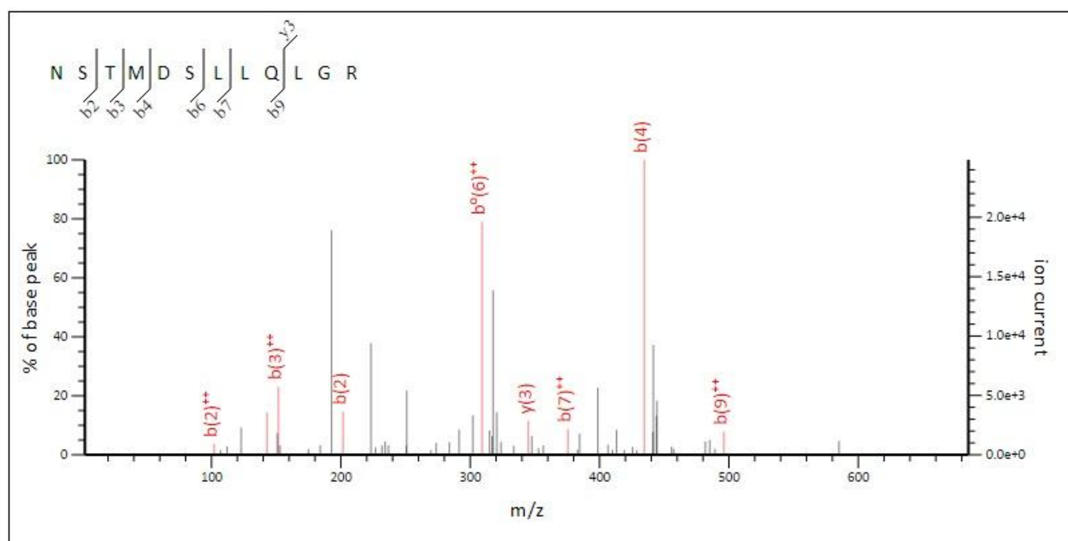


Figure 27. Peptide; LVNISFGGFIICVFCISIV

Observed; 682.3300, Mr(expt); 2043.9682, Mr(calc); 2043.0937

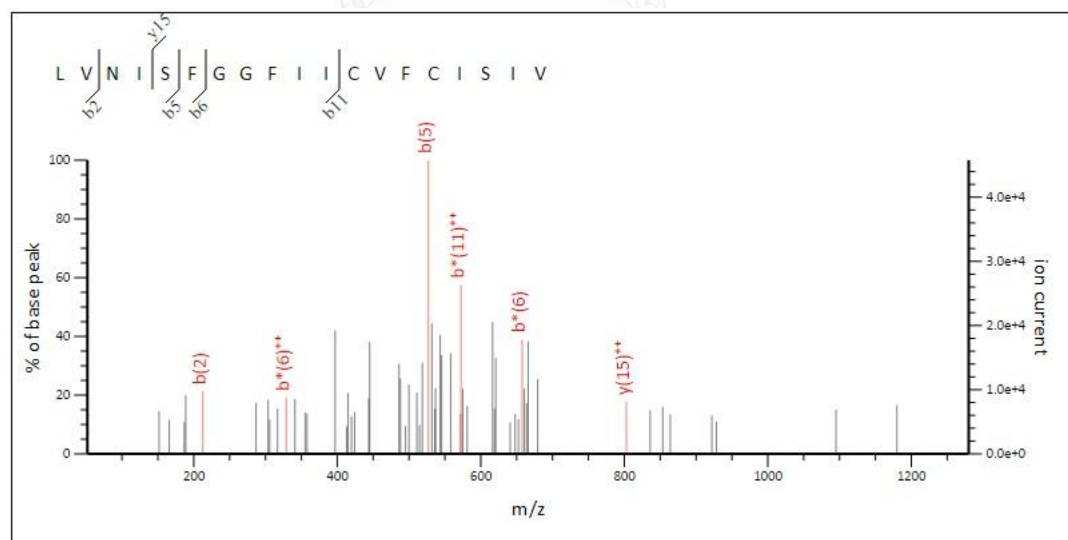


Figure 28. Peptide; ALPGYLK

Observed; 762.3900, Mr(expt); 761.3827, Mr(calc); 760.4483

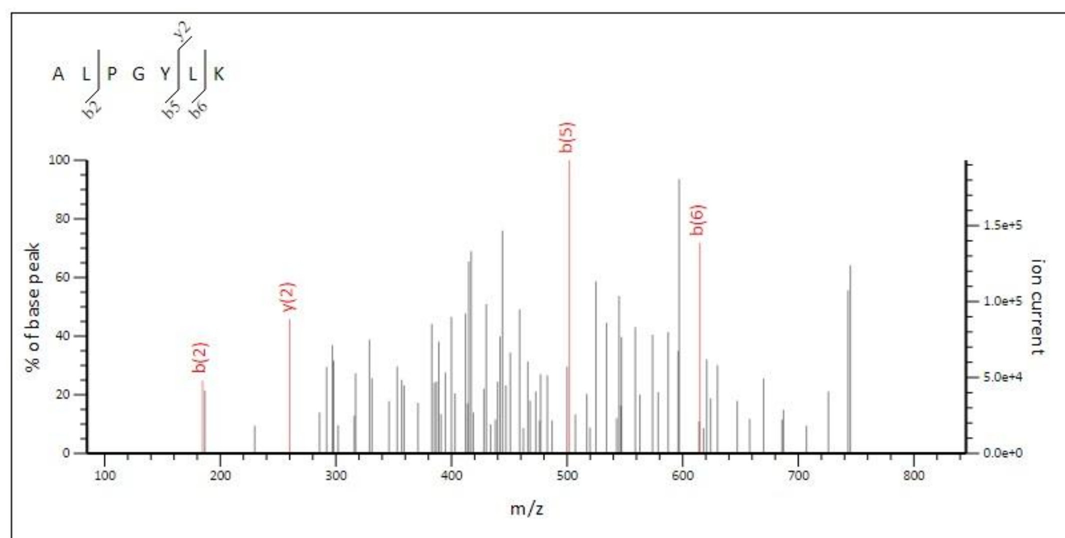


Figure 29. Peptide; VATVSLPR

Observed; 421.7700, Mr(expt); 841.5254, Mr(calc); 841.5022

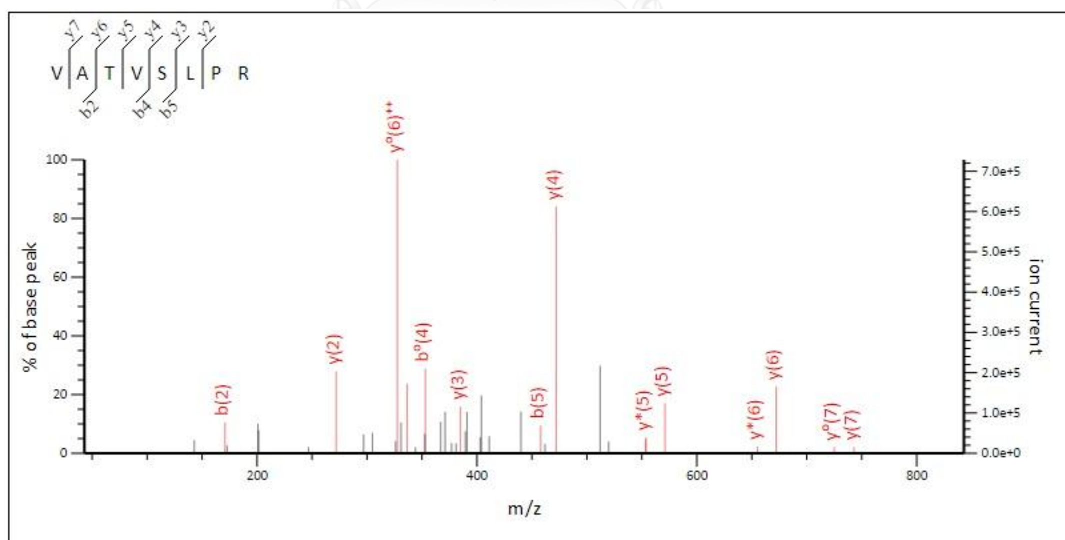


Figure 30. Peptide; NYMKPKLLYYSNGGH

Observed; 595.2900, Mr(expt); 1782.8482, Mr(calc); 1783.8715

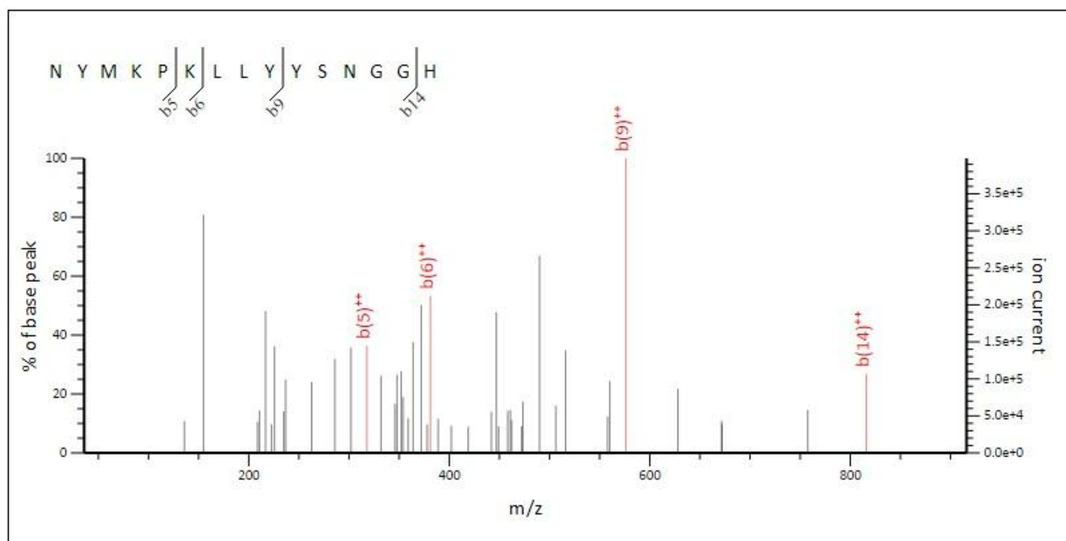
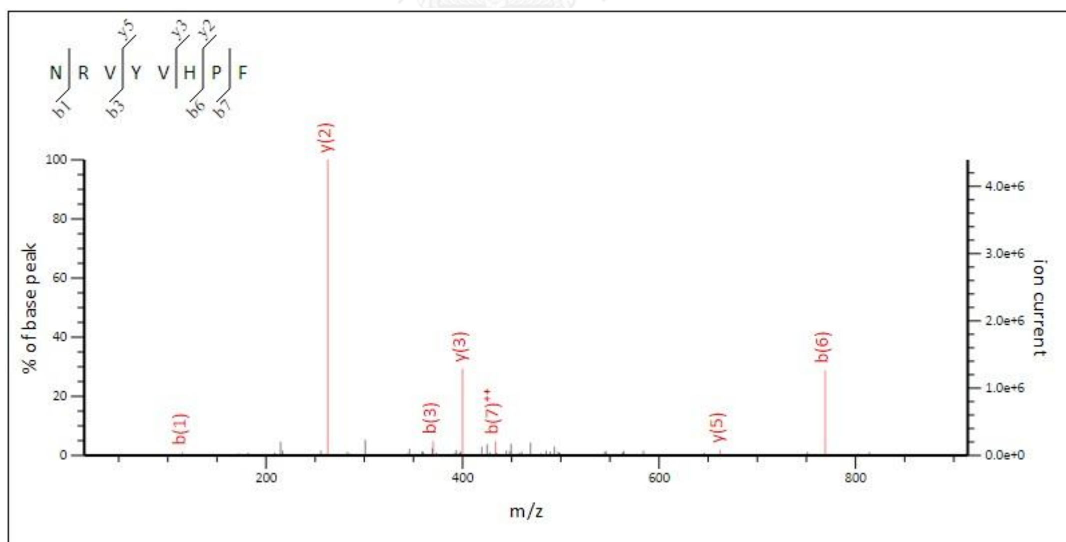


Figure 31. Peptide; NRVYVHPF

Observed; 516.2600, Mr(expt); 1030.5054, Mr(calc); 1030.5349



VITA

Miss Puttaporn Pongkai was born on December 4, 1989 in Bangkok, Thailand. She received Bachelor Degree of Science from Department of Biology, Faculty of Science, Chulalongkorn University in 2012. She has further studied to the Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2012.

Academic presentation;

1. Pongkai, P., Puthong, S., Sangvanich, P., and Karnchanatat, A. (2014) Inhibitory effects of protein hydrolysate from chicken feather meal on tyrosinase activity and melanin formation in B16F10 murine melanoma cells, TSB International forum 2014 “Green Bioprocess Engineering”, 16-19 September 2014, BITEC Bang Na Hall EH 101-102, Conference 101 B, Bangkok, Thailand.

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