ฤทธิ์ยับยั้งไทโรซิเนสของโปรตีนไฮโดรไลเสตจากหอยหวาน *Babylonia areolata*
ที่เตรียมจากแอลคาไลน์โปรติเอส

นายภานุวัฒน์ ปรากฏ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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ANTI-TYROSINASE ACTIVITY OF PROTEIN HYDROLYSATE FROM SPOTTED BABYLON *Babylonia areolata* PREPARED BY ALKALINE PROTEASE

Mr. Phanuwat Prakot

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	ANTI-TYROSINASE ACTIVITY OF PROTEIN HYDROLYSATE FROM SPOTTED BABYLON *Babylonia areolata* PREPARED BY ALKALINE PROTEASE
By	Mr. Phanuwat Prakot
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Aphichart Karnchanatat, Ph.D.
Thesis Co-Advisor	Ninnaj Chaitanawisuti, 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
(Associate Professor Polkit Sangvanich, Ph.D.)

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

.....Thesis Co-Advisor
(Ninnaj Chaitanawisuti, Ph.D.)

.....Examiner
(Assistant Professor Sanit Piyapattanakorn, Ph.D.)

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

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

ภานุวัฒน์ ปรากฏ : ฤทธิ์ยับยั้งไทโรซิเนสของโปรตีนไฮโดรไลเสตจากหอยหวาน *Babylonia areolata* ที่เตรียมจากแอลคาไลน์โปรติเอส (ANTI-TYROSINASE ACTIVITY OF PROTEIN HYDROLYSATE FROM SPOTTED BABYLON *Babylonia areolata* PREPARED BY ALKALINE PROTEASE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.อภิชาติ กาญจนทัต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร. นิลนาจ ชัยธนาวิสุทธิ์, 95 หน้า.

งานวิจัยนี้เพื่อศึกษาฤทธิ์ยับยั้งไทโรซิเนสและฤทธิ์ต้านออกซิเดชันของโปรตีนไฮโดรไลเสตจากหอยหวานที่เตรียมจากแอลคาไลน์ โปรตีเอส (โปรติเอส จีซิก) พบว่าภาวะที่เหมาะสมในการเตรียมโปรตีนไฮโดรไลเสตคือ ที่ความเข้มข้นของโปรติเอส จีซิก ที่ไม่เจือจาง (5.8×10^5 ยูนิตต่อกรัม) เวลาในการย่อยสลาย 60 นาที สำหรับฤทธิ์ยับยั้งไทโรซิเนส และที่ความเข้มข้นของโปรตีเอส จีซิกเจือจาง 8 เท่า (7.25×10^4 ยูนิตต่อกรัม) เวลาในการย่อยสลาย 240 นาที สำหรับฤทธิ์ต้านออกซิเดชัน จากนั้นนำโปรตีนไฮโดรไลเสตทั้งสองภาวะนี้ไปคัดแยกขนาดโมเลกุลด้วยเทคนิคอัลตราฟิลเตรชัน โดยใช้แผ่นคัดแยกขนาดโมเลกุล 10 5 และ 3 กิโลดาลตันตามลำดับ และศึกษาฤทธิ์ชีวภาพของแต่ละขนาดของโปรตีนไฮโดรไลเสตที่ผ่านการคัดแยก ผลการศึกษาพบว่า โปรตีนไฮโดรไลเสตจากหอยหวานขนาดโมเลกุลน้อยกว่า 3 กิโลดาลตัน มีฤทธิ์ในการยับยั้งไทโรซิเนสดีที่สุดทั้งกิจกรรมโมโนฟีนอลเลสและไดฟีนอลเลส โดยแสดงค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งได้ร้อยละ 50 เท่ากับ 1.758 และ 8.995 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ และโปรตีนไฮโดรไลเสตจากหอยหวานขนาดน้อยกว่า 3 กิโลดาลตันแสดงฤทธิ์ต้านออกซิเดชันดีที่สุด โดยมีค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งอนุมูลอิสระได้ร้อยละ 50 ของฤทธิ์ยับยั้งอนุมูลอิสระด้วยสาร DPPH ABTS เท่ากับ 9.344 และ 5.689 ไมโครกรัมต่อมิลลิลิตร และฤทธิ์ยับยั้งอนุมูลอิสระด้วยวิธีไนตริกออกไซด์ เท่ากับ 10.708 ไมโครกรัมต่อมิลลิลิตรตามลำดับ จากนั้นศึกษาผลของโปรตีนไฮโดรไลเสตจากหอยหวานที่มีขนาดน้อยกว่า 3 กิโลดาลตัน ต่อการยับยั้งการสร้างเม็ดสีเมลานิน จากผลการศึกษาจลนพลศาสตร์ของกิจกรรมยับยั้งไทโรซิเนสพบว่ามีการยับยั้งแบบไม่แข่งขัน มีค่าสัมประสิทธิ์การยับยั้งเท่ากับ 2.21 และ 11.86 ไมโครกรัมต่อมิลลิลิตร สำหรับกิจกรรมโมโนฟีโนเลสและไดฟีโนเลสตามลำดับ และยังสามารถลดการการสร้างเมลานิน และลดกิจกรรมของไทโรซิเนสในเซลล์เมลาโนไซต์ของหนู B16F10 โดยไม่แสดงความเป็นพิษต่อเซลล์เมลาโนไซต์ B16F10 ดังนั้นโปรตีนไฮโดรไลเสตจากหอยหวานสามารถพัฒนาต่อเป็นตัวยับยั้งไทโรซิเนสและใช้เป็นส่วนประกอบในผลิตภัณฑ์ที่ทำให้ผิวขาวหรือใช้ในการรักษาความผิดปกติของสีผิว

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5572073523 : MAJOR BIOTECHNOLOGY
KEYWORDS: PROTEIN HYDROLYSATES / BABYLONIA AREOLATA / TYROSINASE INHIBITORY ACTIVITY

PHANUWAT PRAKOT: ANTI-TYROSINASE ACTIVITY OF PROTEIN HYDROLYSATE FROM SPOTTED BABYLON *Babylonia areolata* PREPARED BY ALKALINE PROTEASE. ADVISOR: ASST. PROF. APHICHART KARNCHANATAT, Ph.D., CO-ADVISOR: NINNAJ CHAITANAWISUTI, Ph.D., 95 pp.

In this research was designed to investigate the effect of protein hydrolysates from spotted babylon *Babylonia areolata* prepared by alkaline protease (Protease G6) on melanogenesis inhibition and antioxidant properties. The results revealed that the optimal condition of protein hydrolysates preparation for tyrosinase inhibitory activity was undiluted of Protease G6 (5.8×10^5 DU/g) at the hydrolysis time of 60 min with an IC_{50} value of 10.81 µg/ml and the optimal condition for antioxidant activity was 8-fold diluted of Protease G6 (7.25×10^4 DU/g) at 240 min hydrolysis time with an IC_{50} of 10.68 µg/ml. Spotted babylon hydrolysates of these two conditions were then fractioned using three different molecular weight cut-off membranes 10, 5, and 3 kDa, respectively. All fractions were evaluated for their antimelanogenesis effect by mushroom tyrosinase assay and antioxidant property by using DPPH, ABTS and NO radical scavenging. Spotted babylon hydrolysates MW < 3 kDa exhibited the highest as tyrosinase inhibition for monophenolase and diphenolase activity with IC_{50} values of 1.758 and 8.995 µg/ml ,respectively. Spotted babylon hydrolysates MW < 3 kDa also showed IC_{50}values of 9.344, 5.689 and 10.708 µg/ml for DPPH, ABTS and NO radical scavenging. Afterward, spotted babylon hydrolystes MW < 3 kDa were further assessed inhibition type on tyrosinase, cell viability assay, melanin content ,cellular tyrosinase activity assay in B16F10 melanoma cells. Kinetic studies determined that the spotted babylon hydrolysate MW < 3 kDa behaved as an uncompetitive inhibitor for monophenolase and diphenolase activity, showing kinetic inhibition constant values (K_i) of 2.21 and 11.86 µg/ml. The results demonstrated that spotted babylon hydrolysate MW < 3 kDa suppressed melanin content and decresed cellular tyrosinase activity, with no cytotoxicity to B16F10 murine melanoma cells. Therefore, the spotted babylon hydrolysates could be developed as tyrosinase inhibitor and formulated in skin-whitening products for cosmetic or therapeutic use.

Field of Study: Biotechnology
Academic Year: 2014

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

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Finally, I wish to thank my parent for supporting me all the time and has been an inspiration throughout my life and also my friends at the Institute of Biotechnology and Genetic Engineering for providing a lovely environment.

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LIST OF ABBREVIATION

ABTS	2,2'-azinobis-3-ethylbenzthiazoline-6-sulphonate
BHT	butylated hydroxytoluene
℃	degree Celsius
CO_2	carbon dioxide
DHI	5,6-dihydroxyindole
DHICA	indole-5,6-quinone-2-carboxylic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethysulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
DU	alpha-amylase Dextrinizing units
FBS	fetal bovine serum
g	gram
h	hour
HCl	hydrochloric acid
IC_{50}	half maximum inhibitory concentration
kDa	kilodalton
K_i	kinetic constant
K_m	apparent Michaelis constant
Min	minute
mm	millimeter
mM	millimolar

ml	milliliter
MW	molecular weight
MWCO	molecular weight cut-off
MTT	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	normalarity
NaCl	sodium chloride
NED	N-(1-Napthyl)- ethylenediamine dihydrochloride
nm	nanometer
NO	nitric oxide
L-DOPA	3,4-dihydroxyphenylalanine
LC-MS/MS	liquid chromatography-mass spectrometry
PBS	phosphate buffer saline
PI	propidium iodide
PMSF	phenylmethylsulfonyl fluoride
rpm	revolution per minute
RP-HPLC	reverse phase high performance liquid chromatography
SNP	sodium nitroprusside
TFA	trifluoroacetic acid
TRP-1	tyrosinase-related protein-1
TRP-2	tyrosinase-related protein-2
U	unit
UV	ultra violet

V_{max}	maximum velocity
w/v	weight by volume
µg	microgram
µl	microliter
%	percentage

CHAPTER I
INTRODUCTION

Melanin, the main component that determines the color of human skin, is produced by membrane-bound organelles called melanosome (Ubeid, Zhao, Wang, & Hantash, 2009). The major role of melanin is to protect the skin from ultraviolet radiation and other important function such as absorb toxic drugs and chemicals (Chan, Kim, & Cheah, 2011). Melanogenesis or melanin biosynthesis is a physiological response of human skin when expose to ultraviolet light or other stimuli. Melanogenesis is regulated by enzymes such as tyrosinase, tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2). Tyrosinase (EC 1.14.18.1), a copper-containing glycoprotein, is the rate-limiting enzyme for melanin biosynthesis involved in the first two steps of the melanogenesis which L-tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine or L-DOPA (monophenolase activity), followed by the oxidation of L-DOPA to dapaquinone (diphenolase activity) (Chiari, Vera, Palacios, & Carpinella, 2011). An overproduction and accumulation of melanin can lead to hyperpigmentation disorders such as melasma, freckles, and age spots. Undesired hyperpigmentation may impact on psychological stress and social well-being, leading to lower productivity, social functioning, and self-esteem (Ubeid et al., 2009). The regulation of tyrosinase activity has been considered as the most common strategy to achieve skin whiteness. Therefore, agents that are able to inhibit tyrosinase activity (tyrosinase inhibitors) may be beneficial as ingredients in therapeutic drugs or cosmetic preparations. There have been reported for tyrosinase inhibitors such as hydroquinone, kojic acid and cortical steroids but they show adverse side effects such as dermatitis, skin irritation and being cytotoxicity to melanocytes. Thus, the search for tyrosinase inhibitors that have potential on tyrosinase inhibitory activity and also show no cytotoxicity to melanocytes or other adverse side effects are still needed.

Protein hydrolysates are all products of protein hydrolysis which contain a mixture of peptides in different molecular size and free amino acids, typically small

fragments of peptides that contain 2-20 amino acids. They are sources of bioactive peptides holding several biological activities such as antioxidant, antihypertensive, and antifungal in which these properties based on the hydrolysis condition and the start material. Enzymatic hydrolysis has been widely used for protein hydrolysates preparation because enzymatic hydrolysis is performed under mild pH and temperature conditions which avoid side reactions and does not decrease the nutritional values of the protein source whereas chemical hydrolysis is conducted using strong chemicals and solvents with extreme conditions, affecting the nutritional value of the protein source (A. Clemente, 2000; Neklyudov, Ivankin, & Berdutina, 2000; Tavano, 2013). The biological activities of protein hydrolysates can be used in many fields such as cosmetics, health care, and the food industry (Haslaniza, Maskat, Wan Aida, & Mamot, 2010).

Spotted babylon *Babylonia areolata* Link1807 or in Thailand called Hoy Wan, is a carnivorous marine benthic gastropod which belongs to the family Buccinidae. It is the second most commercially important gastropod consumed in Thailand. Spotted babylon is rich in nutritional value with protein 20.01%, higher than other marine organisms such as oyster, mussel, and mackerel (Chaitanawisuti & Kritsanapuntu, 1999; Zhang, Zhou, & Cheng, 2009; นิลนาจ ชัยธนาวิสุทธิ์, ศิรุษา กฤษณะพันธุ์, แสนทวีสุข, & โพธิ์เพชร, 2556). The edible portion of spotted babylon contains 17 amino acids. Spotted babylon could be a good starting material for the production of protein hydrolysates due to their high nutritional value which may exhibit tyrosinase inhibitory activity and antioxidant activity. There have been rarely tyrosinase inhibitors from protein hydrolysates and none of tyrosinase inhibitors have been previously studied using protein hydrolysates from spotted babylon.

This study, therefore, examined the effect of protein hydrolysates from spotted babylon using alkaline protease (Protease G6) to discover whether spotted babylon hydrolysates possess antimelanogenetic property and antioxidant activity. This research reported the optimal condition in the preparation of spotted babylon hydrolysates that showed tyrosinase inhibitory and antioxidant activity. The ability of spotted babylon hydrolysates that behave as tyrosinase inhibitors was investigated including the inhibition on mushroom tyrosinase, kinetic inhibition, the ability to

inhibit cellular tyrosinase produced from B16F10 murine melanoma cells, and the ability to inhibit melanin production. The partial purification and identification of spotted babylon hydrolysates provided the best potency for tyrosinase inhibition and a preliminary studying the ability of spotted babylon hydrolysates to induce apoptosis of B16F10 melanoma cells were investigated.

CHAPTER II

LITERATURE REVIEW

2.1 Melanin and Melanogenesis

Human skin color is determined by the amount and type of pigment melanin (D'Orazio, Jarrett, Amaro-Ortiz, & Scott, 2013) which is produced by melanocytes through a melanin synthesis process called melanogenesis (Chang, 2012). Melanin plays a crucial role in protecting human skin from the harmful effects of ultraviolet radiation and other important functions such as scavenging chemicals or toxic drugs and removing reactive oxygen species to protect against oxidative injuries and antimicrobial activity (Solano, Briganti, Picardo, & Ghanem, 2006).

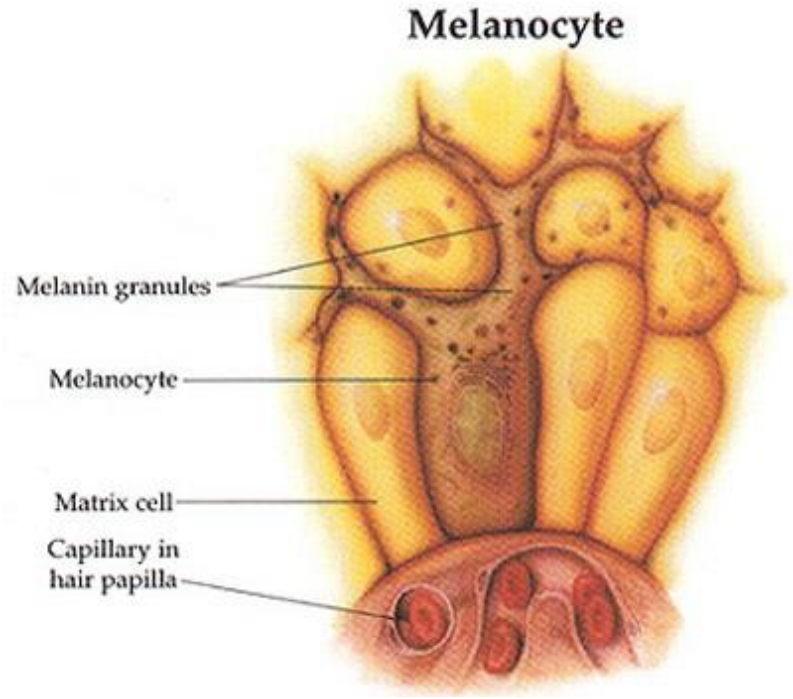


Figure 2.1 Melanocyte cell.

(source; http://www.dermweb.com/hairnailsmucousmembranes/figure1-7page.htm)

Melanin synthesis takes place in melanocyte cells which are derived from neural crest and are the second most abundant cell in the epidermis (Figure 2.1). Melanocytes have the unique lysosome-related organelles termed melanosomes

which contain several enzymes involved in melanin biosynthesis. Melanin is secreted from melanocyte cells and transferred to neighboring keratinocytes (Figure 2.2) where they produce visible color and prevent against ultraviolet radiation. Melanocytes generate two chemical forms of melanin: (1) eumelanin is a dark brown-black insoluble polymer expressed mostly in the skin of heavily pigmented persons and (2) pheomelanin is a light-colored sulfated pigment resulting from the incorporation of cysteines into melanin precursors (Ito, Wakamatsu, & Ozeki, 2000). Eumelanin shows more effective than pheomelanin in protecting from UV (D'Orazio et al., 2013). In contrast, Fair-skinned people are almost sensitive to UV and have a high risk of skin cancer. Moreover, some researches reported that pheomelanin may promote melanogenesis by generating free radicals in melanocyte cells even in the absence of UV and may cause oxidative DNA injury (Benedetto et al., 1981; Costantini, d'Ischia, Palumbo, & Prota, 1994; Sealy et al., 1982). Melanogenesis is a physiological response of human skin when exposed to UV radiation which plays an important role in protecting human skin from sun-related injury.

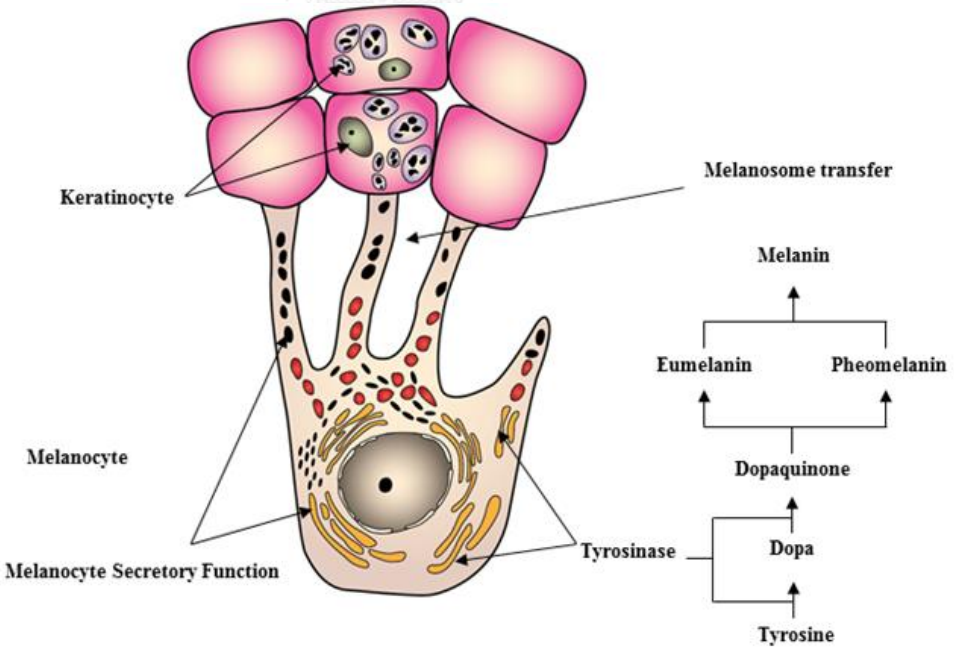


Figure 2.2 Diagram of epidermal melanin unit.

(source; http://www.dermamedics.com/hyperpigmentation_id60.html)

Melanogenesis is a complex process contained a series of pathways and occur inside a special organelle called melanosomes in melanocytes, causing both eumelanin and pheomelanin synthesized.

Melanogenesis is regulated by enzymes such as tyrosinase, tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2) but tyrosinase is the key enzyme in melanogenesis, which catalyzes the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) or monophenolase activity and subsequently the oxidation of L-DOPA to DOPAquinone or diphenolase activity. Monophenolase and diphenolase activity is a common step for both types of melelanin (Maeda & Fukuda, 1996a). Afterward, DOPAquinone is converted to DOPAchrome (Figure 2.3).

In the eumelanin synthesis pathway, DOPAchrome is either spontaneously decarboxylated to 5,6-dihydroxyindole (DHI) or TRP-2 catalyzes the conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) followed by the oxidation of DHICA to indole-5,6-quinone-2-carboxylic acid by TRP-1, which finally obtains eumelanin with further polymerization (Hu, Zheng, Zhang, Chen, & Wang, 2015). In the presence of cysteine, glutathione will react with L-DOPAquinone to form cysteinyldopa or glutathionyldopa. Subsequently, cysteinyldopa or glutathionyldopa is converted to alanine-hydroxyl dihydrobenzothazine and polymerized to pheomelanin, respectively. However, the excess accumulation of abnormal melanin amounts in different parts of the skin which can lead to the esthetic problems such as melasma, freckles and solar lentigines. In western countries, skin lighteners are utilized to prevent and treat irregular hyperpigmentation disorders whereas in Asia contries skin lighteners are used to make skin whiter because of traditional beliefs (Solano et al., 2006). The disruption of tyrosinase activity has been considered as a major target to search potential inhibitors for melanin synthesis. There are a number of tyrosinase inhibitors such as hydroquinone, kojic acid and arbutin but these agents have various side effects, for instance, adversely cutaneous toxicity of hydroquinone and tumor-promoting effects of kojic acid (Ishikawa, Kawase, & Ishii, 2007). Safety and effectiveness of tyrosinase inhibitors are considered as the vital things for the development of the tyrosinase inhibitors

which are important for human dermal applications. Therefore, agents that can inhibit tyrosinase may be useful in cosmetic products.

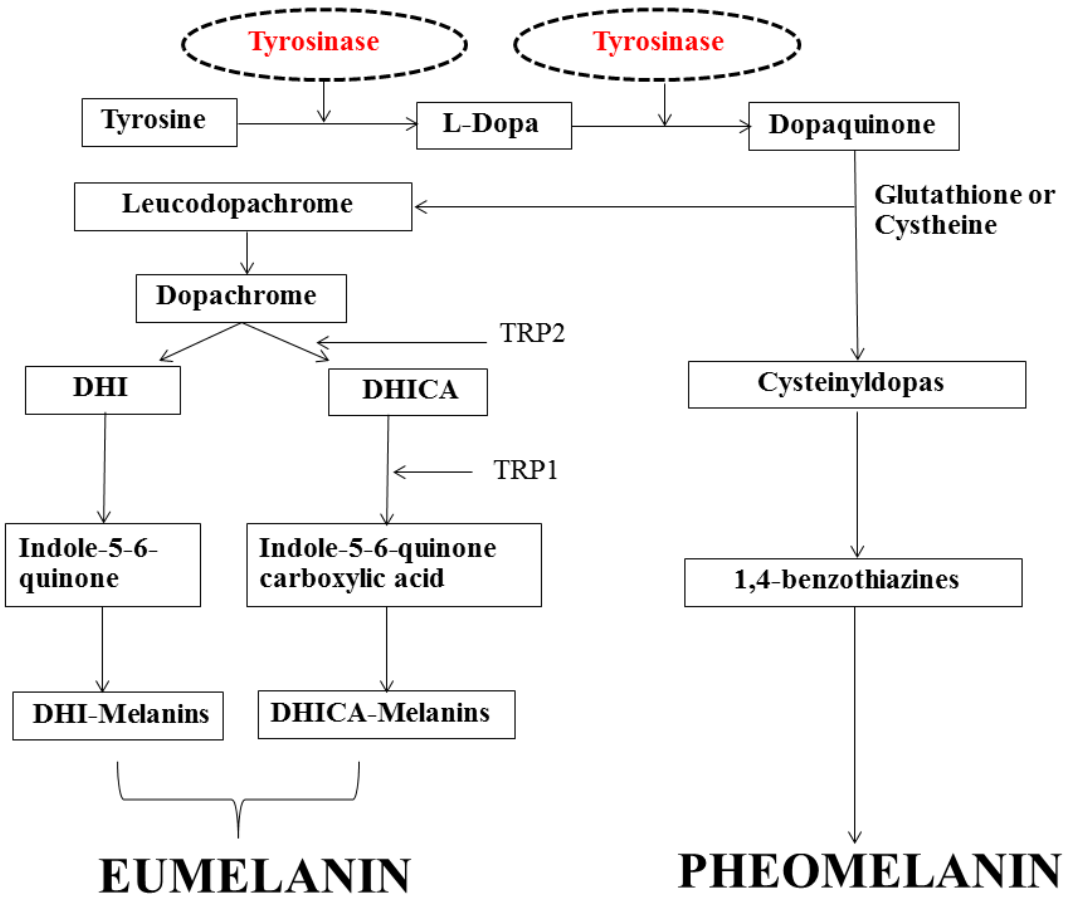


Figure 2.3 Melanin Biosynthetic pathway.

2.2 Tyrosinase

Tyrosinase (EC 1.14.18.1), a copper containing enzyme, is widely distributed in various organisms such as fungi, vertebrates and plant (Plonka & Grabacka, 2006; Takahashi et al., 2010). This enzyme is produced by melanocytes and trafficked to melanosome where melanin are synthesized (Chang, 2012). Tyrosinase catalyzes the first two step of melanin biosynthesis; (1) the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and (2) the oxidation of L-DOPA to DOPAquinone.

Tyrosinase has three domains where the central domain has two cupric ions individually connected with three histidine residues at the active site. There are three types of tyrosinase: *oxy*tyrosinase (E_{oxy}), *met*tyrosinase (E_{met}) and *deoxy*tyrosinase (E_{deoxy}). During the process of melanin synthesis there are different binuclear copper structures of the active site. In the mechanism of monophenolase activity, monophenol can react only with the oxy form of tyrosinase. In the diphenolase activity, o-diphenol can react with the oxy and met form and oxidized into o-quinone. Monophenolase activity shows a characteristic lag period before the maximum velocity of the hydroxylation step is reached. The lag period is the time required to reduce the met form to the deoxy form of tyrosinase. The lag period can be shortened or abolished by cofactors such as L-DOPA and catechin (Chen et al., 2014).

2.3 Targeting tyrosinase as the key enzyme of melanogenesis

The most common target for melanogenesis inhibition is to inhibit tyrosinase activity. Tyrosinase is produced only by melanocytes of which tyrosinase inhibitors are highly specific toward melanogenesis without other side effects. On the contrary, tyrosinase inhibitors that target to the tyrosinase gene expression or protein degradations are rarely utilized because of non-specific and global effects via intracellular signaling pathways. Thus, the search for tyrosinase inhibitors seems to be a major field of interesting for further study (Chang, 2012). Recently, scientific literatures have reported the inhibition of tyrosinase activity but much of this research use mushroom tyrosinase as a model. Mushroom tyrosinase plays an important role in tyrosinase inhibitor studies because mushroom tyrosinase is well studied and is easily purified from the mushroom *Agaricus bisporus*. However, using mushroom tyrosinase is problematic because the regulation of tyrosinase differs from mammalian tyrosinase in several respects. For example, the inhibition of mushroom tyrosinase is not well correlated with melanin production in melanocytes because mushroom tyrosinase is a cytosol enzyme whereas the human tyrosinase is membrane bound (Parvez et al., 2006). Some agents are active against mushroom

tyrosinase but do not affect mammalian tyrosinase (Ando, Kondoh, Ichihashi, & Hearing, 2007). The tyrosinase inhibitors can be classified as competitive, uncompetitive, mixed-type and non-competitive inhibitors. Tyrosinase inhibitors can reveal their inhibition types by measuring enzyme inhibition kinetics using Lineweaver-Bluk plots. Knowledge on inhibition types of tyrosinase inhibitors is important for designing skin-lightening products (Ando et al., 2007; Smit & Vicanova, 2009)

2.4 Tyrosinase inhibitors

2.4.1 Hydroquinone and its derivatives

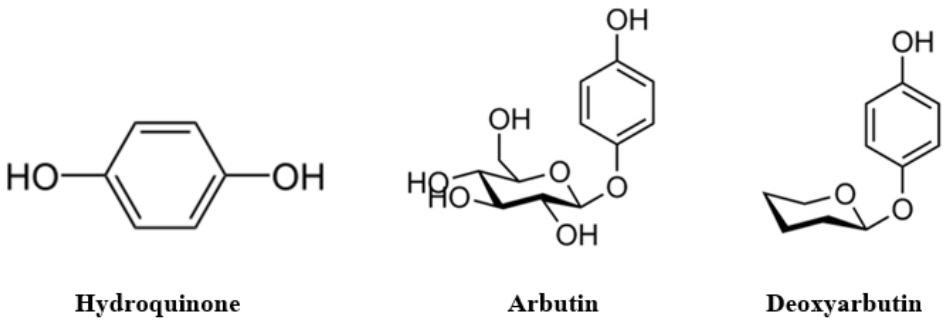


Figure 2.4 Hydroquinone, Arbutin and Deoxyarbutin

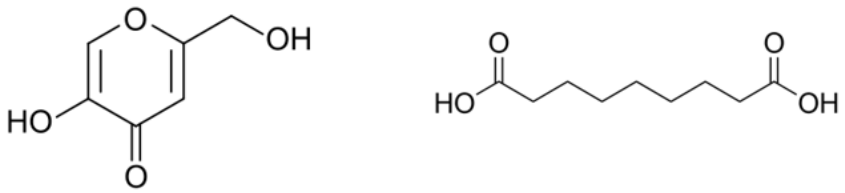
Hydroquinone is a well-known tyrosinase inhibitor via its interaction with copper at the active site. It was observed that the oxidation of hydroquinone leads to oxidative damage to membrane lipid and protein such as tyrosinase as well as glutathione depletion (H. Kim, Choi, Kim, & Park, 2012). However, hydroquinone in cosmetics has been banned by European Union because it can lead to permanent loss of melanocytes and an irreversible loss of inherited skin color (Arndt & Fitzpatrick, 1965).

Arbutin is a glycosylated form of hydroquinone found in blueberries, cranberries, wheat and pears and can be synthesized from hydroquinone by glucosidation. It is commonly utilized as an effective treatment of hyperpigmentation disorders and shows less cytotoxicity to melanocytes than hydroquinone. Moreover,

arbutin has been described as a tyrosinase inhibitor by competitive binding to tyrosinase without affecting mRNA transcription of tyrosinase (Maeda & Fukuda, 1996b). Although the safety of arbutin as a skin-lightening agent, some studies do not confirm its effect in clinical trial (Curto et al., 1999).

Deoxyarbutin, a new arbutin derivative, is synthesized by the removal of all hydroxyl groups from the glucose side chain. It shows much lower cytotoxicity than arbutin and has been identified as an excellent tyrosinase inhibitor (Boissy, Visscher, & DeLong, 2005). Deoxyarbutin shows more sustained skin-lightening effect than hydroquinone because deoxysugars increase the ability of skin penetration and binding affinity to tyrosinase (Solano et al., 2006).

2.4.2 Tyrosinase inhibitors originating from microorganism



Kojic acid **Azelaic acid**

Figure 2.5 kojic acid and Azelaic acid

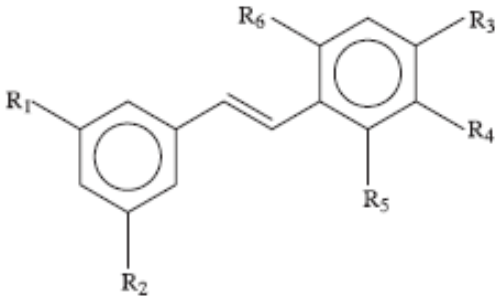
Kojic acid (5-hydroxy-2-hydroxy-methyl-4*H*-pyran-4-one) is a fungal metabolite obtained from the species of *Acetobacter*, *Aspergillus* and *Penicillium*. The action of kojic acid is its chelating ability with copper atoms in the active site of tyrosinase and suppressing the tautomerization of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (Cabanes, Chazarra, & Garcia-Carmona, 1994). Although kojic acid is a good tyrosinases inhibitor, it can lead to contact dermatitis, sensitization and erythema(Badreshia-Bansal & Draelos, 2007).

Azelaic acid (1,7-heptanedicarboxyilic acis) is a saturated dicarboxylic acid produced by *Pityrosporum ovale*. It is utilized as an effective agent in the treatment of skin pigmentation, freckles and lentigines. The mechanism of azelaic acid may

prevent the interaction of tyrosine with tyrosinase or act as a competitive inhibitor (Gillbro & Olsson, 2011).

2.4.3 The families of flavonoids-like agents

There are more than 4,000 members of flavonoids have been identified and widely distributed in leaves, bark and flowers of plant polyphenols. All have phenolic and pyrane ring and classified into six major group; flavonols, flavones, chalcones, flavanones, isoflavones and antocyanidins, that is different in the conjugation of rings and the position of hydroxyl, methoxyl and glycosidic group (Y. M. Kim et al., 2002). Flavonoids are reported to possess a variety of beneficial effects such as being anti-imflammatory, antioxidant, anticarcinogenic, and provides protection against UV. Several flavonoids are frequently utilized in skin-lightening preparation as they can inhibit tyrosinase and also act on the distal part of the melanogenesis oxidative pathway. The main flavonoids with skin-lightening properties



include hydroxystibene derivatives, hydroxycoumarin, and aloesin etc.

Figure 2.6 Hydroxystilbenes, Resveratrol $R_1 = R_2 = R_3 = -OH$, Oxyresveratrol $R_1 = R_2 = R_3 = R_5 = -OH$, Gnetol $R_1 = R_2 = R_3 = R_6 = -OH$.

Hydroxystilbene derivatives appear as being among the most efficient tyrosinase inhibitor because they have high affinity for tyrosinase (Y. M. Kim et al., 2002). Hydroxystilbene derivatives include resveratrol and other isomers such as oxyresveratrol, gnetol and methoxylated. Oxyresveratrol and gnetal show more potential as tyrosinase inhibitors than resveratrol because the hydroxyl groups are important for inhibition (Ohguchi, Akao, & Nozawa, 2005). The extract from *Morus*

alba L. which contains 2-oxyresveratol showed remarkable inhibition on tyrosinase activity with no toxicity and passed several tests such as human skin irritation and eyes irritation. Besides natural extracts, many of the chemically resveratrol derived compounds are reported as being good tyrosinase inhibitor. In the group of resveratrol structure analogs, 4,4'-dihydroxybiphenyl has shown to be a new potent tyrosinase inhibitor in B16 murine melanoma (Y. J. Kim, No, Lee, & Chung, 2005).

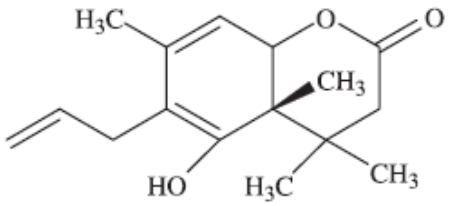


Figure 2.7 7-allyl-6-hydroxy-4,4,5,8-tetramethyl-hydrocumarin.

Hydroxycoumarins and coumarins are lactones of phenylpropanoid acid with an H-benzopyranone nucleus. Some hydroxylated derivatives show melanogenesis inhibition property which these compounds directly interact with tyrosinase. The 7-allyl-6-hydroxy-4,4,5,8-tetramethyl-hydrocumarin is the best of these compounds. It can activate glutathione synthesis and pheomelanogenesis (Okombi et al., 2006).

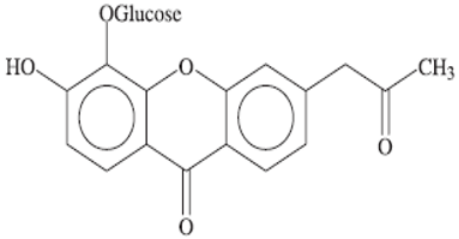


Figure 2.8 Aloesin.

Aloesin is a C-glycosylated chromone which is isolated from aloe plants (Piao et al., 2002). The tyrosinase inhibitory activity of aloesin is stronger than arbutin and

kojic acid, particularly 5-methyl-7-methoxy-2-(2'-benzyl-3'-oxobutyl)-chromone. Further study revealed that aloesin is a competitive inhibitor of tyrosinase and can inhibit melanin synthesis in cultured normal melanocytes (Jones, Hughes, Hong, Jia, & Orndorff, 2002). Recent studies demonstrated that aloesin extracts contains the compounds isorabaichromone, feruloylaloesine and *p*-cumaroylaloesine which have influential antioxidant activity in cellular organelles such as mitochondria, melanosomes and microsomes. Moreover, the combined treatment of aloesin and arbutin show synergistic effects as being non-competitive and competitive tyrosinase inhibitors (Choi, Lee, Kim, Chung, & Park, 2002).

2.5 Disorders of pigmentation

Disorders of pigmentation present skin as being discolored, blotchy, or darker or lighter than the normal skin color. These disorders of pigmentation are involved in melanin synthesis or distribution. They occur when the body produces too much melanin content or hyperpigmentation such as melasma, freckles and lentigines and produce too little melanin content or hypopigmentation such as vitiligo and albinism.

2.5.1 Hyperpigmentation disorder

Hyperpigmentation refers to the darkening of skin caused by an overproduction of melanin. Hyperpigmentation can affect the skin pigmentation of any race. The major cause of hyperpigmentation is overexposure to sunlight and also caused by drugs such as anti-seizure, antibiotics and hormone treatment.

Melasma (also known as cloasma) is a common disorder of hyperpigmentation. This condition is characterized by tan or brown patches and is found most often on the facial areas of women who are using anovulation drugs or during pregnancy (A.-Y. Lee, 2014; Sheth & Pandya, 2011).

Freckles are small brown patches on the skin which do not increase the number of melanocytes, but have cells that overproduce melanin pigment. Freckles occur only from exposure to sunlight and tend to darken in summer and fade in winter (Lorincz, 1959).

Lentigines are small, rounded areas that are brown to black color. Lentigines differ from freckles in that they do not darken with exposure to sunlight, but tend to increase in number with age.

2.5.2 Hypopigmentation disorder

Hypopigmentation in skin is caused by a reduction in melanin production such as vitiligo and albinism.

Vitiligo is a chronic skin disease in which the pigment-producing cells are damaged or unable to function leading to white patches on the skin or all over the body in some people. The cause of this disease is unknown. Some researches suggest that it occurs from autoimmune, oxidative stress and viral causes. Until recently there was no treatment for vitiligo, but now there are several treatments available such as using corticosteroid creams, ultraviolet light treatments and cosmetic cover-ups (Essien & Harris, 2014).

Albinism is a rare inherited disorder that leads the skin, eyes or hair to have little or no color. The cause of albinism is associated with the absence of an enzyme producing melanin pigment leading to a complete lack of melanin pigment which can occur in all races. Therefore, people who have albinism should protect their skin by using sunscreen at all times because they can be especially susceptible to sun damage and skin cancer (Bakos et al., 2009).

2.6 Tyrosinase inhibitors with antioxidant property

It is reported that UV radiation generates reactive oxygen species (ROS) in the skin. ROS plays an important role in the regulation of melanin biosynthesis that may induce melanogenesis by activating tyrosinase activity. Additionally, even melanogenesis produces ROS which exposes human melanocyte cells to high levels of oxidative stress. Therefore, the use of tyrosinase inhibitors with antioxidant properties is a new trend in dermatology and skin products fields because it can make skin lighter, protect skin from the harmful effect of UV radiation and reduce the ROS level in melanocytes which may prevent the activation of melanogenesis. The B16F10 murine melanoma cell line was a good model in the study of human

melanoma to evaluate the effectiveness of inhibitors. Huang and coworkers (Huang, Hsieh, Niu, & Chang, 2014) revealed that the Adlay extract inhibited intracellular tyrosinase activity and reduced the amount of melanin in B16F10 murine melanoma cells. Moreover, this extract showed antioxidant properties such as free radical scavenging capacity and reducing power. Wu and coworkers (L.-c. Wu, Chang, Chen, Fan, & Ho, 2009) investigated the antioxidant activity and melanogenesis inhibitory effect of the acetonic extract of *Osmanthus fragrans*. They reported that the acetonic extract of *O. fragrans* showed mushroom tyrosinase inhibitory activity with IC_{50} values of 2.314 mg/ml and 44.20 mg/ml using L-tyrosine and L-DOPA as a substrate, a kinetic study indicated an uncompetitive inhibitor. They were also tested with B16F10 murine melanoma cells and found that the acetonic extract of *O. fragrans* was able to modulate cellular tyrosinase activity and reduced melanin content. Moreover, the results exhibited that this extract provided antioxidant properties by the scavenging activity against ABTS and DPPH radicals. Hyun-Sun Lee and his coworkers evaluated the effectiveness of *Terminalia chebula* as a whithening agent. They examined the antioxidant activity and depigmenting activity. The results indicated that *T. chebula* possessed antioxidant properties in comparison to ascorbic acid. Additionally, *T. chebula* was able to reduce melanin content through tyrosinase inhibition in B16F10 cells. However, in order to investigate the potential of newly discovered tyrosinase inhibitors as therapeutic drugs need to be further evaluated *in vivo* antimelanogenesis activity. Recently, the animal model to evaluate antimelanogenesis agents is zebra fish because of its easy maintenance and handling as well as the high efficiency in drug penetration through the skin (Chang, 2012).

2.7 Apoptotic induction in B16F10 murine melanoma cells

In this study, we investigated the effect of spotted babylon hydrolysates on apoptosis in B16F10 melanoma cells to examine in the possibility of spotted babylon hydrolysates as anticancer agents. Melanoma is a skin cancer that occurs from the malignant transformation of melanocytes at a rate faster than other cancers (Looi et al., 2013). The characteristic of melanoma is resistant to cytotoxic agents. Apoptosis

or programmed cell death is an evolutionarily conserved process which plays an important role in the regulation of cell numbers during embryonic development and tissue homeostasis. Induction of apoptosis is considered as an ideal strategy for cancer chemotherapy because cancer can inhibit cell apoptosis (Rajasekar et al., 2012). Apoptosis cells lose phosphatidylserine (PS) asymmetry of plasma membrane. The exposure of PS of the plasma membrane to the outer layer of membrane indicates early apoptosis which can be strained by annexin V (Olszewska-Slonina, Styczynski, Czajkowski, Drewa, & Musialkiewicz, 2007). Therefore, the search for agents that are able to induce apoptosis have the effectiveness to be utilized for cancer therapy. Azevedo and his coworkers (Azevedo et al., 2012) studied the antitumor effect of cationic INKKI peptide from bovine β-casein on melanoma B16F10. The results found that INKKI peptide was able to induce apoptosis detected by using annexin V/PI assays, INKKI peptide increased the percentage of cells in sub-G1 and increased of caspase-3 cleavage. Rajasekar and his coworkers (Rajasekar et al., 2012) studied the anticancer effects of the *Lithospermum erythrorhizon* (LEH) extract on B16F10 murine melanoma and found that LEH induced apoptosis in B16F10 cells increased the percentage of cells in the sub-G1 phase. LEH slowed down regulation of anti-apoptosis Bcl-2 family protein and upped the regulation of apoptotic Bax protein expression.

2.8 Protein hydrolysates

Protein plays a crucial role in the human diet which provides essential amino acids. The hydrolysis process has been utilized to enhance the biological value of proteins such as improving their digestibility and reducing allergenicity (Li, Youravong, & H-Kittikun, 2010). Proteins are the greatest sources of bioactive peptides which are inactive and become active during the hydrolysis protein process. Protein hydrolysates are defined as all the products of protein hydrolysis such as polypeptides, oligopeptides and amino acids that are obtained after hydrolysis protein sources using hydrolysis by chemical or enzymatic hydrolysis (McCarthy, O'Callaghan, & O'Brien, 2013).

Protein hydrolysates are typically small fragments of peptides that contain 2-20 amino acids, particularly di- and tripeptides showing high nutritional and therapeutic values. Bioactive peptides are short chain amino acid sequences which exert various potential physiological functions such as antioxidant, antihypertensive and anticancer (McCarthy et al., 2013; Zambrowicz, Timmer, Polanowski, Lubec, & Trziszka, 2013).

2.8.1 Preparation of protein hydrolysates

Protein hydrolysis is associated with the cleavage of peptide bonds to yield peptides in varying sizes and amino acid composition. The production of protein hydrolysates can be carried out by enzymatic hydrolysis and chemical hydrolysis. Chemical hydrolysis including acid or alkaline hydrolysis is difficult to control and provides products with reduced nutritional valued products. Acid hydrolysis is conducted under extreme conditions using 6 M HCl at 110°C for more than 24 h, and can destroy tryptophan. Alkaline hydrolysis can reduce many amino acid contents such as cysteine, arginine, threonine, serine, isoleucine and/or lysine content. Additionally, chemical hydrolysis can produce toxic substances such as lysine-alanine and D-form amino acids. Enzymatic hydrolysis is performed under mild conditions of pH (6-8) and temperature (40-60°C). Enzymatic hydrolysis can avoid side reaction and do not decrease the nutritional properties of the protein source (Tavano, 2013). After hydrolysis, bioactive peptides which are inactive in the intact protein become active when exposed to proteolytic enzymes (Schaafsma, 2009).

2.8.2 Post-hydrolysis treatment

The crude hydrolysates obtained from hydrolysis protein may be further processed which typically utilizes post-hydrolysis treatment including heat inactivation and ultrafiltration. Ultrafiltration (UF) has been reported to be the most efficient post-hydrolysis method to remove high molecular weight peptides and proteins (Alfonso Clemente, 2000).

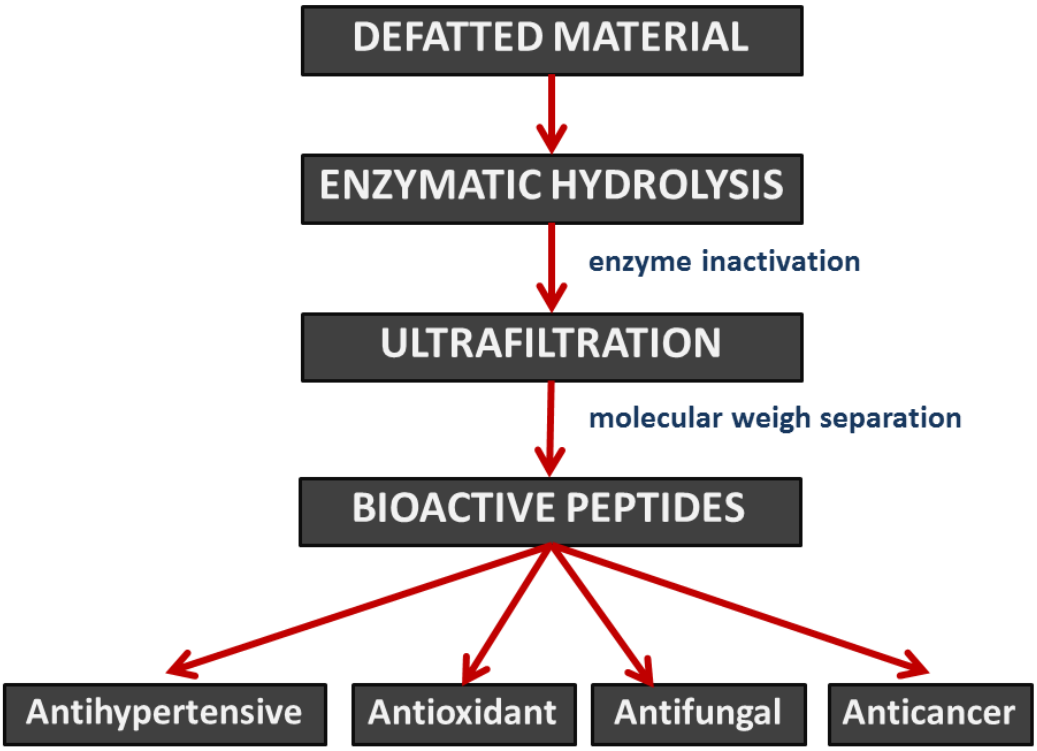


Figure 2.9 Flow diagram for the development of enzymatic protein hydrolysates.

2.9 Proteolytic enzyme

Proteolytic enzymes hydrolyse proteins more gently than chemicals hydrolysis which do not require high temperature and specific to peptide bonds. It hydrolyses the peptide bond between amino acid, generating a mixture of peptides in different size and free amino acid. Proteolytic enzymes can be classified by their hydrolysis mechanism into endopeptidases or exoproteases. Endopeptidase hydrolyse the peptide bond in the middle of protein molecules. On the other hand, exoprotease hydrolyse the terminal peptide bond from either N terminus or C terminus (Tavano, 2013). The specificity of proteases will determine the position which the protease catalyze peptide bond hydrolysis and provide information leading to a better selection to act on specific substrates. The source of proteolytic enzyme is highly variable such as microorganisms, plants and animals. For example,

pepsin will cleave in which there is phenylalanine or leucine bond or papain will cut the chain adjacent to arginine, lysine, tyrosine and phenylalanine. Therefore, the selection of proper proteolytic enzymes to produce bioactive peptides is important in the preparation of protein hydrolysates.

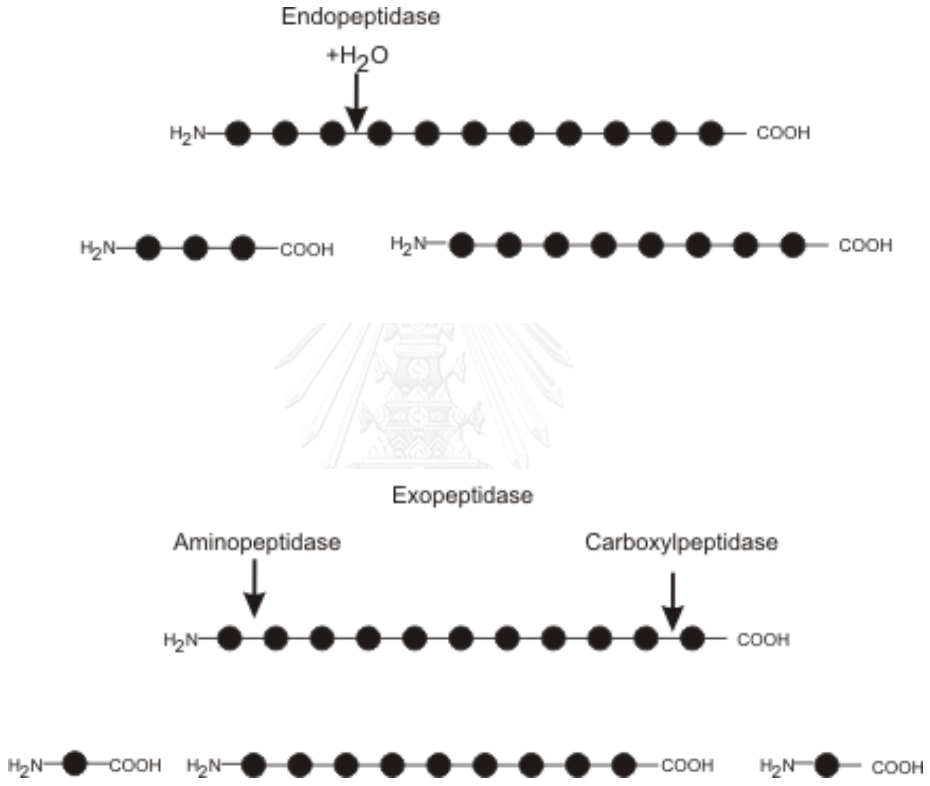


Figure 2.10 The hydrolysis mechanism of endopeptidase and exopeptidase.

Table 2.1 Enzymes commonly used in protein hydrolysis.

Protease	Hydrolysis reaction
Aminopeptidase A	Release of N-terminal Leu, but also may be other amino acids including Pro but not Arg or Lys.
Carboxypeptidase A	Release of C-terminal amino acid, but little or no action with -Asp, -Glu, -Arg, -Lys or -Pro.
Papain	Preferential for an amino acid bearing a large hydrophobic side chain at the P2 position.
Pepsin	Preferential cleavage hydrophobic, aromatic residues.
Subtilisin	Hyrolysis protein with board specificity for peptide bonds, preference for a large uncharge residue in P1.
Trypsin	Preferential release of N-terminal Arg and Lys at P1

Modified from (Tavano, 2013).

2.9.1 Alkaline protease (Protease G6)

Protease G6 is produced from *Bacillus licheniformis*. The application of Protease G6 include baking, protein processing, silver recoveryfrom film material and pet food production.

2.9.1.1 Biochemical parameters

Protease G6 (3.4.21.62) was alkaline serine endopeptidase. None of activators or cofactors are required for maximum activity.

2.9.1.2 pH dependency

The pH range for the activity of this enzyme is approximately from 7.0 to 10.0, an optimum pH at 9.5. However, the exact pH optimum will be depended on process variables such as temperature, time, nature of substrate and concentration.

2.9.1.3 Temperature dependency

The activity of this enzyme is effective in the temperature range from 25 ºC to 70 ºC with an optimum temperature at 60 ºC. However, the exact temperature

optimum will be depended on many process variables such as pH, time, nature of substrate and concentration.

2.9.1.4 Application recommendations

The effectiveness of Protease G6 in hydrolyzing is utilized to hydrolyze most proteins such as hemoglobin, casein, egg yolk, gelatin, fish, plant and animal proteins to yield lower molecular weight peptides. This enzyme will express more effective in hydrolytic activity when the substrate is unfolded or denatured protein.

There are several factors that directly affect the production of bioactive peptides such as proteolytic enzymes, hydrolysis time and protein sources. Hydrolysis time is also important factor in the production of bioactive peptides. A longer time for protein hydrolysis can influence the effectiveness of proteases and has a limitation when the substrate has no site to cleave (Tavano, 2013).

2.10 Marine-derived bioactive peptides

The marine world presents a tremendous resource in the discovery of potential therapeutic compounds. Several novel therapeutic compounds have been found in marine organism (Ngo, Vo, Ngo, Wijesekara, & Kim, 2012). Marine organisms are considered as a potential source to produce novel bioactive compounds for the development of pharmaceuticals. Much attention has been paid to marine peptides because they show the potential to promote health and are able to decrease the risk of disease. These peptides can be obtained from fish, mollusk, algae etc. Based on the amino acid sequence of bioactive peptides, they have been shown to possess various physiological functions such as antioxidant, antihypertensive, anticoagulant and antimicrobial activities (Erdmann, Cheung, & Schroder, 2008).

Bioactive peptides are typically 3-20 amino acids residues which their activities depend on amino acid composition and sequence (S.-K. Kim & Wijesekara, 2010). The enzymatic hydrolysis method is preferred over other methods in the production of bioactive peptides, particularly in the pharmaceutical and food industries due to the lack of residual organic solvents and toxic chemicals. The

hydrolysis condition such as the pH of the protein solution and temperature must be adjusted to optimize the activity of enzymes. Additionally, the molecular weight of peptides is an important factor in the production of bioactive peptide. Therefore, ultrafiltration is considered as an effective method in order to gain bioactive peptides with a desired molecular size and biological property.

Table 2.2 Bioactive peptides from marine animals.

Reference	Source	Biological activity	Amino acid sequence
(Qian, Je, & Kim, 2007)	Big eye tuna (muscle)	Antihypertensive activity	WPEAAELMMEVDP
(Zhao et al., 2009)	Sea cucumber	Antihypertensive activity	MEGAQAGD
(J. K. Lee, Hong, Jeon, Kim, & Byun, 2009)	Rotifer	Antihypertensive activity	DDTGHDFEDTGEAM
(Fahmi et al., 2004)	Sea bream	Antihypertensive activity	VIY
(Sheih, Wu, & Fang, 2009)	Microalga	Antioxidant activity	VECYGPNRPQF
(Qian, Jung, Byun, & Kim, 2008)	Oyster	Antioxidant activity	LKQELEDLLEKQE

Reference	Source	Biological activity	Amino acid sequence
(Niranjan Rajapakse, Mendis, Jung, Je, & Kim, 2005)	Blue mussle	Antioxidant activity	HFGDPFH
(Jun, Park, Jung, & Kim, 2004)	Yellowfin sole	Antioxidant activity	RPDFDLEPPY
(Liu et al., 2008)	Oyster	Antimicrobial activity	CLEDFYIG
(Battison, Summerfield, & Patrzykat, 2008)	American lobster	Antimicrobial activity	QYGNLLSLLNGYR
(Bartlett et al., 2002)	Shrimp	Antimicrobial activity	PRP
(Jung & Kim, 2009)	Blue mussle	Anti-coagulant	EADIDGDGQVNYEEVAM MTSK
(N. Rajapakse, Jung, Mendis, Moon, & Kim, 2005)	Yellowfin sole	Anti-coagulant	TDGSEDYGILEIDSR

2.11 Spotted babylon ***Babylonia areolata***

Figure 2.11 Spotted babylon *Babylonia areolata*.
(source; http://www.aquatic-blue.com/english/index-en.html)

Spotted babylon *Babylonia areolata* Link1807, or in Thailand called Hoy Wan, is a carnivorous marine benthic gastropod belongs to the family Buccinidae (Chaitanawisuti & Kritsanapuntu, 1999) which is the second most commercially important gastropod consumed in Thailand (Zhang et al., 2009). It is one type of gastropod which has high nutritional value in comparison to other mollusk and has the same nutritional value compared to economic importance of marine organisms such as coral, mackerel, mussel, oyster, shrimp and so on. The nutritional value of the edible portion of natural spotted babylon is nearly the same as the cultivated spotted babylon and the components of proteins of both types of spotted babylon show higher than oysters and abalones. Moreover, protein components are comparable with white perch and tiger prawns as shown in Table 2.3. Spotted babylon consists of protein 18.78%, fat 2.86%, carbohydrate 5.18%, ash 5.27% and moisture 67.91%. It contains 17 amino acids in different volume and amino acids, in small size of spotted babylon (3-4 of shell-length) and big size (5-7 cm of shell-length) as shown in Table 2.4 translated from (นิลนาจ ชัยธนาวิสุทธิ์ et al., 2556).

Table 2.3 Comparison of nutritional values of spotted babylon with other economically important marine organisms

Organism	Composition of nutritional value (%)				
	Protein	Fat	Ash	Carbohydrate	Moisture
Natural spotted babylon	18.03	2.39	2.38	3.33	73.87
Cultivated spotted babylon	20.01	6.33	2.04	0.79	70.83
Mussel	11.90	2.24	1.59	3.69	80.58
Oyster	9.45	2.30	1.23	4.95	82.06
Abalones	17.10	0.76	1.57	6.01	74.56
Tiger prawn	20.31	1.73	1.20	0.90	75.86

Modified from (นิลนาจ ชัยธนาวิสุทธิ์ et al., 2556).

Table 2.4 Amino acid content (mg/100mg) in small and big size of spotted babylon.

Amino acid	Big size of spotted babylon	Small size of spotted babylon
Threonine	731.55	502.07
Isoleucine	1,071.44	767.10
Leucine	2,211.60	1,948.33
Lysine	2,792.39	2,714.73
Methionine	300.32	303.42
Cystine	35.54	18.55
Phenylalanine	1,171.00	1,851.35
Tyrosine	1,624.58	1,321.69
Valine	1,031.18	725.92
Arginine	229.81	131.89
Histidine	1,540.16	1,212.00
Alanine	1,149.63	788.54
Aspartic acid	1,006.29	477.89
Glutamic acid	1,133.58	1,433.07
Glycine	1,274.81	874.41
Proline	1,749.84	1,147.63
Serine	22.06	17.31

Translated from (นิลนาจ ชัยธนาวิสุทธิ์ et al., 2556).

CHAPTER III

MATERIALS AND METHODOLOGY

3.1 Spotted babylon materials

Spotted babylon were obtained from Sunset farm, Chonburi province, Thailand. Spotted babylon were washed thoroughly in running tap water. They were then anaesthetized on ice for 15 min and was separated the flesh from the shell. The flesh was chopped and homogenized with a mixer. Fat was removed from the spotted babylon homogenated following the method described by Wang (Wang et al., 2013), using isopropanol at a ratio of 1:4 (w/v) (isopropanol : homogenate), continuously stirred at room temperature for 60 min. Isopropanol was removed from the homogenate by vacuum filtration. Afterward, the homogenated was dried at 60 ℃ in a drying oven. The dried homogenate was ground into a powder using a mixer, and referred as spotted babylon powder. Spotted babylon powder was kept in a desiccator until required for using.

3.2 Chemical and biological materials

Mushroom tyrosinase, bovine serum albumin (BSA), L-tyrosine, L-3,4-dihydroxylphenylalanine (L-DOPA), kojic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS), potassium persulfate, acetic acid, ascorbic acid, sodium nitroprusside (SNP), sulfanilamide, phosphoric acid, phenylmethylsulfonyl fluoride (PMSF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), and trypsin were purchased from Sigma-Aldrich (USA). Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). The B16F10 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The FITC Annexin V/PI Apoptosis Detection Kit was purchased from BD Bioscience. Alkaline protease was purchased from Siam Victory Chemicals Co., Ltd. (Thailand). All the other chemicals and reagents purchased were of research grade.

3.3 Spotted babylon hydrolysates preparation

The spotted babylon powder was hydrolyzed to prepare protein hydrolysates using alkaline serine endopeptidase (Protease G6). The hydrolysis ratio was 0.5:10 (w/v) (substrate: enzyme). In this experiment, Protease G6 was varied into five concentrations; (1) undiluted (5.80×10^5 DU/g), (2) 2-fold diluted (2.90×10^5 DU/g), (3) 4-fold diluted (1.45×10^5 DU/g), (4) 8-fold diluted (7.25×10^4 DU/g), and (5) 16-fold diluted (3.625×10^4 DU/g), in 20 mM Tris-HCl buffer containing 150 mM NaCl pH 8.0. The hydrolysis time was varied into 11 times; 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 360, and 480 min. The hydrolysis reaction was performed at 50 °C in a shaker at 125 r min^{-1}, and terminated by heating at 90 °C for 10 min. Finally, the spotted babylon hydrolysates was centrifuged at 10000 rpm for 15 min. The supernatant was collected and kept at -4 °C until required for using.

3.4 Determination of amino acid composition of spotted babylon

3.4.1 Acid hydrolysis

Five milliliters of HCl 6 N were added (5 mg protein/ml HCl) and mixed. The tube was flushed with nitrogen for 1 min to remove air. Hydrolysis reaction was then carried out at 110 °C for 22 h. The internal standard (10 ml of 2.5 mM L-**α**-amino-*n*-butyric acid in HCl 0.1 M) was added and diluted with water to 250 ml. The solution was filtered through a 0.20 μm filter and followed by derivatized with 6 aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Flour reagent). It was then heated in a heating block at 55 °C for 10 min. Heating converts the minor tyrosine side product to a major mono-derivative compound. Total amino acid content was determined by high performance liquid chromatography.

3.4.2 Chromatographic conditions

Liquid chromatography was performed using a Waters Alliance 2695 separation model (Milford, MA, USA). Analytes were separated on a Hypersil GOLD C18 column water elute isocratically with sodium acetate buffer pH 4.90 and 60%

acetonitrile, delivered at a flow rate of 0.3 ml/min. The injection volume was 5 µl and the total run time of the method was 15 min.

3.5 Molecular weight cut-off by ultrafiltration

The spotted babylon hydrolysates were fractionated by ultrafiltration membranes using a bioreactor system (Amersham Biosciences, Sweden). They were pumped through a range of nominal molecular weight cut-off (MWCO) membranes of 10, 5, and 3 kDa, in order of decreasing pore size. All fractions were collected from the membrane filtration including retentated from 10 kDa membrane (MW >10 kDa), retentated from 5 kDa membrane (5 kDa< MW <10 kDa), permeated from 5 kDa membrane (MW < 5 kDa), retentated from 3 kDa membrane (3 kDa < MW < 5 kDa), and permeated from 3 kDa membrane (MW < 3 kDa).

3.6 Tyrosinase inhibitory activity assay

The tyrosinase inhibitory activity assay was slightly modified from the method of Batubara (Batubara, Darusman, Mitsunaga, Rahminiwati, & Djauhari, 2010) using L-tyrosine and L-DOPA as substrates. Mushroom tyrosinase was used due to its commercial availability. The serial dilutions of spotted babylon hydrolysates were prepared prior to the test. Roughtly, 35 µl of each protein hydrolysates fraction was combined with 15 µl of tyrosinase (333 U/ml) and incubated at 25 °C for 5 min. Next, 55 µl of substrate (12 mM L-DOPA and 2 mM L-tyrosine) was added and incubated at 25 °C for 30 min (L-DOPA), and for 120 min (L-tyrosine). Tyrosinase inhibitory activity was monitored by measuring the absorbance at 510 nm with a microplate reader. Kojic acid was used as the positive control.

3.7 Evaluation of antioxidant activity

3.7.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method described by Wu (L. C. Wu, Ho, Shieh, & Lu, 2005) with slightly modifications. The

serial dilutions of spotted babylon hydrolysates were prepared prior to the test. DPPH solution (0.004%) was then added to each dilution of spotted babylon hydrolysates and incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a microplate reader. L-ascorbic acid was used as the positive control.

3.7.2 ABTS radical scavenging activity

The $ABTS^{\bullet+}$ solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate and left in the dark for 12-16 h at room temperature to generate radical cation ($ABTS^{\bullet+}$). After incubation for 12-16 h, the $ABTS^{\cdot+}$ solution was diluted with deionized water to obtain an absorbance of 0.70 ± 0.02 at 734 nm prior to use. Then 900 µl of the diluted $ABTS^{\cdot+}$ solution was pipetted into each 30 µl dilution of spotted babylon hydrolysates. The mixture was then incubated at room temperature for 10 min. The absorbance was measured at 734 nm using a microplate reader (Wu et al., 2009). Butylated hydroxytoluene (BHT) was used as the positive control.

3.7.3 Nitric oxide radical scavenging

Nitric oxide scavenging activity was determined according to the method described by Nitha (Nitha, De, Adhikari, Devasagayam, & Janardhanan, 2010) with slight modifications. Nitric oxide (NO) is generated from an aqueous SNP solution that interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Ilosvay reaction (Govindarajan et al., 2003). The reaction mixture contained 10 mM SNP, phosphate buffered saline pH 7.4 (PBS) and serial dilutions of spotted babylon hydrolysate. After incubation for 150 min 0.1% (w/v) N - (1-Napthyl) ethylenediamine dihydrochloride (NED) solution was added and incubated for 30 min at room temperature. The pink chromophore generated during the diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured by absorbance at 540 nm. Curcumin was used as the positive control.

3.8 The calculation of percentage inhibition on tyrosinase activity and percentage of radical scavenging

The percentage inhibition of tyrosinase and the percentage of radical scavenging was calculated as follows:

$$\% \text{ inhibition, radical scavenging} = [((A - B) - (C - D))/(A - B)] \times 100$$

Where A is the absorbance of control (no sample), B is the absorbance of blank (deionized water), C is the absorbance of sample (spotted babylon hydrolysates). D is the absorbance of background (the color absorbance of the sample) The IC_{50} values (the concentration of spotted babylon hydrolysate which produced 50% inhibition of tyrosinase or 50% radical scavenging) were calculated using GraphPad Prism version 6. All tests were performed in triplicate. The values were expressed as mean ± standard deviation of the triplicate data.

3.9 Cell line and cell culture

The B16F10 murine melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2mM glutamine, 100 mg/ml streptomycin, and 100 U/ml in humidified 5% CO_2 at 37°C. The media was changed every 3 days to maintain logarithmic growth. The cells were harvested by trypsinization when they reached 70%. The cells were counted with a haematocytometer and seeded with the appropriate number into a wells of cell culture plate for further investigation of cell viability assay, determination of melanin content, determination of cellular tyrosinase activity, and apoptotic induction assay.

3.9.1 Cell viability assay

B16F10 cells were seeded in a 96-well plate at a density of 5×10^3 cells/well and left to adhere overnight. They were then treated with serial concentrations of

spotted babylon hydrolysates for 72 h. MTT was prepared at 2.5 mg/ml in PBS. Ten microliters of MTT solution was pipetted into each well. The plate was incubated at 37°C for 4 h in a humidified 5% CO_2 incubator. The medium was then removed and 200 µl DMSO was added to each well. The absorbance was measured at 540 nm with a microplate reader. All tests were performed in triplicate. The values are expressed as percentage of cell viability of the triplicate data. The percentage of cell viability was calculated as follow;

$$\% \text{ cell viability} = (A/B) \times 100$$

Where A is the absorbance of sample (spotted babylon hydrolysates), B is the absorbance of control (no sample), The IC_{50} values (the concentration of spotted babylon hydrolysate which produced 50% inhibition of cell proliferation) were calculated using GraphPad Prism version 6. All tests were performed in triplicate. The values are expressed as mean ± standard deviation of the triplicate data.

3.9.2 Melanin content assay

B16F10 cells (1×10^5 cells/flask) were grown in cultured flasks for 24 h to measure melanin content. After 24 h incubation, the cells were treated with serial dilutions of spotted babylon hydrolysates for 72 h. The cells were then harvested by trypsinization and washed twice with cool PBS. Next, 0.3 ml of 1 N NaOH was added and incubated at 90 ºC for 1 hour to lyse cells. Afterward, the cell lysates were centrifuged at 3000 g for 10 min. The amount of melanin was measured at 405 nm with a microplate reader. The protein content in the supernatant was determined by Bradford's procedure. The results were expressed as a percentage of control which controls was set as 100% melanin content. All tests were performed in triplicate. The values are expressed as mean ± standard deviation of the triplicate data.

3.9.3 Cellular tyrosinase assay

B16F10 cells (1×10^5 cells/flask) were grown in culture flasks for 24 h to measure the rate of oxidation of L-DOPA. After 24 h incubation, the cells were

treated with serial dilutions of spotted babylon hydrolysates. Next, the cells were harvested by trypsinization and washed twice with cool PBS. Then, 0.2 ml of PBS buffer (pH 6.8), containing 1% (w/v) Triton X-100 and 1 mM phenylmethanesulfonylfluoride (PMSF) was added to lyse cell. Then, 100 µl of the cell lysates were pipetted into a 96-well plate followed by adding 100 µl of 2 mM L-DOPA and incubating at 37°C for 1 h. The dopachrome formation in the reaction mixture was detected at 490 nm with the microplate reader. The results were expressed as a percentage of control which control was set as 100% cellular tyrosinase activity. All tests were performed in triplicate. The values are expressed as mean ± standard deviation of the triplicate data.

3.10 Enzyme inhibition assay

This experiment was conducted similarly to the tyrosinase inhibitory activity assay by slightly modifications from Darusman (Darusman et al., 2012). Tyrosine and L-DOPA were used as the substrate. The concentration of substrates were varied; 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2 mM for L-tyrosine and 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 mM for L-DOPA. Spotted babylon hydrolysates were varied into three concentrations; undiluted, 2-fold diluted, and 4-fold diluted, respectively. The Lineweaver-Burk plots were used to analyze the apparent Michaelis constant (K_m), maximum velocity (V_{max}), and the type of inhibition. The secondary plot were performed to determine kinetic inhibition constant (K*i*).

3.11 RP-HPLC analysis

The most potent of the spotted babylon hydrolysates was purified by reverse phase high performance liquid chromatography (RP-HPLC) using Luna 5U C18 column (250 mm × 4.6 mm). The solvent system were consisted of eluents A; 0.1% trifluoroacetic acid (TFA) and eluent B; acetonitrile containing 0.05% TFA in water, respectively. Fractions were eluted from the column at a flow rate of 0.7 ml/min, with a gradient from 0% - 100% of eluent B. The separation was performed using a gradient elution of 8% B at 0 min, increasing to 40% B at 40 min, and 100% B at 50

min, then maintained at 100% B from 50-55 min, and returned to 8% B at 60 min. The injection of spotted babylon hydrolysates were 20 µl. Elusion was monitored by a UV detector at 220, 254, and 280 nm, respectively.

3.12 Identification of peptides by liquid chromatography-mass spectrometry

The LC-MS/MS system consisted of a liquid chromatography part (Dionex Ultimate 3000, thermo scientific) in the combination with an electrospray ionization (ESI)/ quadrupole ion trap mass spectrometer (Model Amazon SL, Bruker, Germany). The separation was carried out using a reverse 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). The separation was performed at a flow rate of 100 µl/min under gradient conditions of 5-80% B over 50 min. Solvent systems consisted of solvent A (water with 0.1% formic acid) and solvent B (100% acetonitrile). Mass spectral data from 300 to 1500 *m/z* were collected in the positive ionization mode. All data obtained from ion trap LC/MS/MS were analyzed using the MASCOT database.

3.13 Detection of apoptotic cells

Apoptotic cells were examined using a double-staining method with an FITC-labeled Annexin V/PI Apoptosis Induction Detection Kit. B16F10 cells (1×10^5 cells/flask) were treated with spotted babylon hydrolysate at the concentration of IC_{50} value and untreated cells were considered as control for 8 h at 37 °C. Then, the cells were harvested by trypsinization and washed twice with cool PBS. Next, the cells were stained with FITC-labeled Annexin V and PI (BD Bioscience). Stained cells were analyzed by the flow cytometric system. Data acquisition and analysis were performed using Flowjo software.

3.14 Protein content determination

The protein content was determined by Bradford's procedure (Bradford, 1976). Bovine serum albumin (BSA) was used as the protein 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

Each experiment was performed in triplicate. The data were expressed as the mean value ± standard deviation. GraphPad Prism (version 6.00, GraphPad software Inc, La Jolla, CA, USA) for Windows was used to calculate IC_{50} values. Statistical analysis was carried out by SPSS variance (ANOVA) with post hoc comparison (one-way) using Duncan's Multiple Range test. In the Student's *t*-test analysis, $p < .05$ was considered statistically significant.

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Spotted babylon protein hydrolysates production

Spotted babylon was selected as a substrate to prepare protein hydrolysates which have tyrosinase inhibitory activity and antioxidant activity. The principal composition of spotted babylon is 20.01% protein, higher than other marine organisms such as oyster (9.45%), mussels (11.90%), and mackerel (19.29%). Spotted babylon are consisted of 17 amino acids reported by (นิลนาจ ชัยธนาวิสุทธิ์ et al., 2556) such as arginine, alanine, and leucine. Spotted babylon, therefore, was considered as a potential protein source for the preparation of protein hydrolysates. The other important factor in the preparation of the protein hydrolysates is protease. The selected protease in this study was alkaline serine endopeptidase (Protease G6, EC 3.4.21.62) from *Bacillus licheniformis*, because of its ability in hydrolyzing proteins such as egg yolk, fish, and animal proteins. For the experiment, hydrolysis time and protease concentration were varied. Protease G6 was varied into five concentrations; (1) undiluted (5.8×10^5 DU/g), (2) 2-fold diluted (2.9×10^5 DU/g), (3) 4-fold diluted (1.45×10^5 DU/g), (4) 8-fold diluted (7.25×10^4 DU/g), and (5) 16-fold diluted (3.625×10^4 DU/g). The hydrolysis time was varied into eleven times; 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 360, and 480 min. To determine the optimal condition for tyrosinase inhibitory activity, L-DOPA was used as substrate of tyrosinase for avoiding a lag period that occurred in monophenolase activity when L-tyrosine is used as the substrate. The results indicated that the optimal condition for tyrosinase inhibitory activity was undiluted protease G6 (5.8×10^5 DU/g) with a hydrolysis time of 60 min, showing an IC_{50} value of 10.81± 0.003 µg/ml and 8-fold diluted protease G6 (7.25×10^4 DU/g) at a hydrolysis time of 240 min for the antioxidant activity using DPPH radical scavenging assay to screen, showing an IC_{50} of 10.68 ± 0.003 µg/ml. The spotted babylon hydrolysates of these two conditions were further fractioned using three different molecular weight cut-off membranes; 10, 5, and 3 kDa to evaluate their

ability for tyrosinase inhibition and antioxidant activity. Data are shown in table 4.1 and 4.2.

Table 4.1 IC_{50} values of spotted babylon hydrolysates on tyrosinase inhibitory activity using L-DOPA as substrate.

Time (min)	Enzyme concentration							
	Undiluted 5.8×10^5 DU/g		2-fold diluted 2.9×10^5 DU/g		4-fold diluted 1.45×10^5 DU/g		8-fold diluted 7.25×10^4 DU/g	16-fold diluted 3.625×10^4 DU/g
	IC_{50} (µg/ml ± SD)		IC_{50} (µg/ml ± SD)		IC_{50} (µg/ml ± SD)		IC_{50} (µg/ml ± SD)	IC_{50} (µg/ml ± SD)
0	32.92 ± 0.002		>200 ± 0.003		>200 ± 0.005		>500 ± 0.006	>400 ± 0.006
15	35.23 ± 0.002		>200 ± 0.001		>100 ± 0.003		>400 ± 0.004	>300 ± 0.003
30	27.71 ± 0.003		>200 ± 0.003		>100 ± 0.004		>500 ± 0.002	>300 ± 0.005
45	40.19 ± 0.004		>100 ± 0.002		>200 ± 0.004		>400 ± 0.006	>300 ± 0.004
60	10.81 ± 0.003		>100 ± 0.002		>200 ± 0.003		>500 ± 0.001	>400 ± 0.007
90	16.78 ± 0.002		79.91 ± 0.001		>100 ± 0.007		>500 ± 0.005	>400 ± 0.004
120	28.07 ± 0.003		43.00 ± 0.005		>100 ± 0.003		>400 ± 0.004	>400 ± 0.007
150	35.45 ± 0.002		54.18 ± 0.005		>100 ± 0.006		>400 ± 0.004	>400 ± 0.007
180	29.81 ± 0.003		33.65 ± 0.004		>100 ± 0.003		>200 ± 0.003	>300 ± 0.004
210	28.18 ± 0.001		57.99 ± 0.001		>100 ± 0.007		>300 ± 0.004	>200 ± 0.006
240	18.67 ± 0.003		98.95 ± 0.005		>100 ± 0.003		>500 ± 0.005	>400 ± 0.005
360	54.10 ± 0.005		>100 ± 0.006		>100 ± 0.004		>500 ± 0.006	>400 ± 0.005
480	39.89 ± 0.003		>100 ± 0.005		>100 ± 0.004		>100 ± 0.007	>100 ± 0.005

Table 4.2 IC_{50} values of spotted babylon hydrolysates on antioxidant activity using DPPH radical scavenging.

Time (min)	Enzyme concentration				
	Undiluted 5.8×10^5 DU/g	2-fold diluted 2.9×10^5 DU/g	4-fold diluted 1.45×10^5 DU/g	8-fold diluted 7.25×10^4 DU/g	16-fold diluted 3.625×10^4 DU/g
	IC_{50} (μg/ml ± SD)	IC_{50} (μg/ml ± SD)	IC_{50} (μg/ml ± SD)	IC_{50} (μg/ml ± SD)	IC_{50} (μg/ml ± SD)
0	29.41 ± 5.64	39.53 ± 1.824	35.88 ± 0.007	19.43 ± 0.026	24.34 ± 0.009
15	21.91 ± 0.100	29.86 ± 1.407	27.56 ± 0.002	13.89 ± 0.007	15.99 ± 0.003
30	33.68 ± 7.361	29.33 ± 0.233	21.91 ± 0.014	31.02 ± 0.005	12.38 ± 0.005
45	19.83 ± 0.240	17.03 ± 0.275	18.92 ± 0.014	31.06 ± 0.003	12.63 ± 0.004
60	15.98 ± 0.912	16.86 ± 4.426	20.29 ± 0.002	25.19 ± 0.003	28.46 ± 0.014
90	16.39 ± 0.651	16.56 ± 1.146	15.25 ± 0.003	19.45 ± 0.001	24.79 ± 0.003
120	20.81 ± 0.032	12.91 ± 0.403	13.61 ± 0.004	18.45 ± 0.007	20.52 ± 0.001
150	35.97 ± 6.102	16.85 ± 4.426	13.59 ± 0.001	14.81 ± 0.002	17.74 ± 0.006
180	19.13 ± 0.467	13.01 ± 1.908	14.35 ± 0.004	14.41 ± 0.020	18.80 ± 0.001
210	18.04 ± 0.629	11.97 ± 0.191	11.63 ± 0.003	18.67 ± 0.014	17.32 ± 0.001
240	17.43 ± 0.714	12.28 ± 0.283	23.91 ± 0.002	10.69 ± 0.003	26.47 ± 0.001
360	27.48 ± 3.154	12.56 ± 0.056	21.91 ± 0.042	19.67 ± 0.034	25.90 ± 0.035
480	26.69 ± 2.065	16.56 ± 0.042	25.56 ± 0.035	23.65 ± 0.027	27.67 ± 0.029

4.2 Amino acid composition of spotted babylon

Table 4.3 Amino acid composition of spotted babylon *Babylonia areolata* (mg/100mg).

Amino acid profile	Results (%)
Alanine	4.36
Arginine	5.85
Glycine	4.30
Aspartic Acid	7.09
Valine	3.03
Cystine	0.64
Glutamic Acid	11.70
Leucine	5.27
Isoleucine	2.36
Histidine	1.20
Threonine	3.15
Proline	3.12
Lysine	4.42
Methionine	1.65
Hydroxyproline	Not Detected
Serine	3.12
Phenylalanine	2.43
Hydroxylysine	Not Detected
Tyrosine	2.37
Tryptophan	0.40

The amino acid composition of materials used in the preparation of the protein hydrolysates was considered as one of important factors which influenced their biological activities. The functional amino acids in peptides are associated with the R-group differences in each amino acid type. The amino acid compositions of

spotted babylon is shown in Table 4.3, and are comprised of 18 amino acid; alanine, arginine, glycine, aspartic acid, valine, cystine, glutamic acid, leucine, isoleucine, histidine, threonine, proline, lysine, methionine, serine, phenylalanine, tyrosine and tryptophan. Alanine, glycine, isoleucine, and leucine are considered important for tyrosinase inhibitory activity and the disruption of melanogenesis (Ishikawa et al., 2007). Schurink and coworkers (Schurink, van Berkel, Wichers, & Boeriu, 2007) proposed that peptides should contained one or more arginine residues. Phenylalanine is also considered very important, with a structure similar to tyrosine the natural substrate of tyrosinase. Additionally, the combination between arginine and/or phenylalanine with hydrophobic aliphatic residues such as valine, alanine, or leucine indicated significant tyrosinase inhibition. Moreover, peptides containing polar, uncharged residues, cysteine and serine, and/or threonine exhibited good tyrosinase inhibitory activity. The results from the amino acids composition analysis showed that spotted babylon contains all amino acids involved in tyrosinase inhibitory activity mentioned above. For antioxidant activity, the most reactive amino acids contain nucleophilic sulfur-containing side chains, aromatic side chains, or imidazole-containing side chains. Amino acids with aromatic residues such as tryptophan, phenylalanine, and tyrosine are considered important because they are able to donate electrons to convert radicals into stable molecules (Shi, Kovacs-Nolan, Jiang, Tsao, & Mine, 2014).

4.3 *In vitro* tyrosinase inhibitory activity

The optimal condition of spotted babylon hydrolysates preparation for tyrosinase inhibitory activity was shown by the undiluted protease G6 enzyme concentration with 60 min hydrolysis time. Spotted babylon hydrolysates at this condition was further fractioned using three different molecular weight cut-off membranes 10, 5, and 3 kDa. Six fractions of spotted babylon hydrolysate were separated; (1) MW > 10 kDa, (2) MW < 10 kDa, (3) MW 5-10 kDa, (4) MW < 5 kDa, (5) MW 3-5 kDa, and (6) MW < 3 kDa. Of these MW < 3 kDa indicated the best potential on tyrosinase inhibition activity using both tyrosine and L-DOPA as the substrate. With

L-tyrosine as the substrate MW < 3 kDa showed an IC_{50} value of 1.758±0.038 μg/ml which the result was similar to kojic acid on tyrosinase inhibition for monophenolase activity. With L-DOPA, MW < 3 kDa showed an IC_{50} value of 8.995 ± 2.033 μg/ml which the result was also similar to kojic acid for diphenolase activity, as shown in Table 4.4. These IC_{50} values indicated that spotted babylon hydrolysate MW < 3 kDa had the best potential on tyrosinase inhibition of both the first stage of oxidation (monophenolase activity) and the second stage of enzyme inhibition (diphenolase activity). Thus, spotted babylon hyrolysate MW < 3 kDa has the potential for further development as a therapeutic agent.

Table 4.4 Inhibitory effect of spotted babylon hydrolysates on mushroom tyrosinase using L-tyrosine and L-DOPA as substrate. Each experiment was conducted in triplicate. The data were expressed as the mean ± standard deviation.

Molecular weight (kDa)	Monophenolase activity IC_{50} (μg/ml) L-tyrosine as substrate	Diphenolase activity IC_{50} (μg/ml) L-DOPA as substrate
MW > 10 kDa	21.207 ± 0.905^{d}	43.630 ± 1.924^{b}
MW < 10 kDa	26.373 ± 0.375^{e}	42.486 ± 6.950^{c}
MW 5-10 kDa	25.837 ± 1.773^{e}	32.896 ± 0.200^{b,c}
MW < 5 kDa	5.826 ± 0.321^{b}	23.846 ± 0.488^{b}
MW 3-5 kDa	8.452 ± 0.152^{c}	22.620 ± 3.726^{c}
MW < 3 kDa	1.758 ± 0.038^{a}	8.995 ± 2.033^{a}
positive control	0.701 ± 0.016^{a}	5.588 ± 0.220^{a}

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

4.4 Antioxidant activity

The optimal condition of spotted babylon hydrolysates preparation for antioxidant activity was shown by the 8-fold diluted protease G6 enzyme concentration with 240 min hydrolysis time. Spotted babylon hydrolysate at this condition was further fractioned using three different molecular weight cut-off membranes 10, 5, and 3 kDa. Six fractions were separated namely; MW > 10 kDa, MW < 10 kDa, MW 5-10 kDa, MW < 5 kDa, MW 3-5 kDa, and MW < 3 kDa. To determine the antioxidant activity of the hydrolysates, DPPH radical scavenging, ABTS radical scavenging, and nitric oxide radical scavenging were carried out and compared with the positive control.

4.4.1 DPPH radical scavenging activity

The radical DPPH has been widely used to evaluate the ability of compounds that act as free radical scavenging or hydrogen donors. The DPPH radical is a stable free radical that shows a maximum absorbance at 517 nm (Kedare & Singh, 2011). This method is based on the reduction of the stable DPPH radical to diphenyl-picrylhydrazine by donating hydrogen from antioxidants to free radicals that leads to non-toxic species. The color of DPPH solution change from deep violet to yellow occurs when the free radicals are scavenged by accepting an electron or hydrogen radical. The results are shown in Table 2. MW < 3 kDa exhibited the strongest DPPH radical scavenging activity with an IC_{50} value of 9.344 µg/ml compared to the other fractions. Moreover, MW < 3 kDa was more efficient than ascorbic acid (IC_{50} = 20.57 µg/ml).

4.4.2 ABTS radical scavenging activity

The oxidation of ABTS by potassium persulfate generates a colored-cation radical ($ABTS^{\cdot +}$) that shows the maximum absorbance at 734 nm (Re et al., 1999). This cationic ABTS radical is able to be scavenged by antioxidants that act as electron donors or hydrogen donors in free radical reaction (Tanzadehpanah, Asoodeh, & Chamani, 2012). Table 2 shows that MW < 3 kDa exhibited the greatest $ABTS^{\cdot +}$ scavenging activity (IC_{50}= 5.689 µg/ml) and this was higher than BHT (IC_{50} =

57.750 μg/ml). Therefore, MW < 3 kDa probably acted as electron donors, transforming radical cations into the non-radical ABTS.

4.4.3 Nitric oxide radical scavenging activity

Nitric oxide (NO) is a free radical which is an effective inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, and inhibition of platelet aggregation. NO is also involved in inflammation, cancer, and other pathological conditions (Singh et al., 2012; Xu et al., 2014). NO becomes toxic after reaction with oxygen and the superoxide radicals which lead to cellular damage. Table 2 shows that MW < 3 kDa exhibited the best potential for decreasing NO radicals because of its scavenging ability. MW < 3 kDa had an IC_{50} value of 10.708 μg/ml whereas IC_{50} value of curcumin was 45.64 μg/ml.

The antioxidant activity of protein hydrolysate is based on the protease and hydrolysis condition employed (Jun et al., 2004). A variety of smaller peptides and free amino acids are generated during the hydrolysis reaction. The antioxidant activity results demonstrated that spotted babylon hydrolysates MW < 3 kDa showed the strongest antioxidant activity for three assay assessments compared to the other fractions. Therefore, mw < 3 kDa can be further improved and used in many applications such as functional food ingredients and cosmetic additives.

Table 4.5 Antioxidant activities of spotted babylon hydrolysates.

Molecular weight (kDa)	DPPH radical scavenging activity IC_{50} (μg/ml)	ABTS radical scavenging activity IC_{50} (μg/ml)	Nitric oxide radical scavenging activity IC_{50} (μg/ml)
MW > 10 kDa	15.717 ± 0.901^{b}	19.133 ± 0.711^{e}	Not Detected
MW < 10 kDa	$19.277 \pm 1.427^{c,d}$	20.903 ± 0.979^{f}	38.523 ± 4.298^{d}
MW 5-10 kDa	20.657 ± 0.936^{d}	9.027 ± 0.502^{b}	19.527 ± 1.167^{b}
MW < 5 kDa	$16.773 \pm 0.966^{b,c}$	15.627 ± 1.246^{d}	70.120 ± 0.871^{e}
MW 3-5 kDa	29.447 ± 1.174^{f}	11.223 ± 0.370^{c}	25.927 ± 1.979^{c}
MW < 3 kDa	9.344 ± 0.645^{a}	5.689 ± 0.114^{a}	10.708 ± 1.339^{a}
positive control	24.847 ± 3.929^{e}	15.700 ± 0.334^{d}	31.760 ± 1.833^{c}

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

4.5 Effect of spotted babylon hydrolysates on B16F10 murine melanoma

The cytotoxic effects of serial concentrations of spotted babylon hydrolysates towards B16F10 cell proliferation were investigated using the MTT method. This method is based on the reduction of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a blue formazan by mitochondria enzymes in viable cells (Chan et al., 2011). The blue formazan product occurs in proportion to the number of viable cells. Spotted babylon hydrolysates MW < 3 kDa were selected to test the cytotoxic effect on B16F10 cells as it exhibited the greatest potential for mushroom tyrosinase inhibitory activity. The B16F10 cells were exposed to the serial concentrations of spotted babylon hydrolysate MW < 3 kDa for a 72 h treatment period. The result indicated that spotted babylon hydrolysates MW < 3 kDa inhibited cell proliferation in a dose dependent manner, showing an IC_{50} value of 3.97 μg/ml. At the concentration of 8 μg/ml had the greatest cytotoxic effect on B16F10 cells, with percentage of cell viability 17.79%. At the concentration of 4 μg/ml showed a minor cytotoxic effect, with percentage of cell viability at 53.69%. At lower

concentrations ranging from 2, 1, 0.5, and 0.25 µg/ml showed no cytotoxic effect with percentages of cell viability at 78.12, 90.44, 88.87, and 91.51%, respectively (Figure 4.1). Therefore, the cytotoxic effect of spotted babylon hydrolysates MW > 3 kDa on B16F10 cell proliferation were dose-dependent at lower concentrations (2-0.25 µg/ml) and did not have any cytotoxic effect on B16F10 murine melanoma. Kojic acid was used as the positive control to study the cytotoxic effect, showed a dose dependent manner similar to spotted babylon hydrolysates MW < 3 kDa (data not shown). Moreover, the cytotoxic effects of spotted babylon hydrolysate MW < 3 kDa on fibroblast normal cells were also investigated. The result revealed that there were no cytotoxic activity on fibroblast normal cells (data not shown).

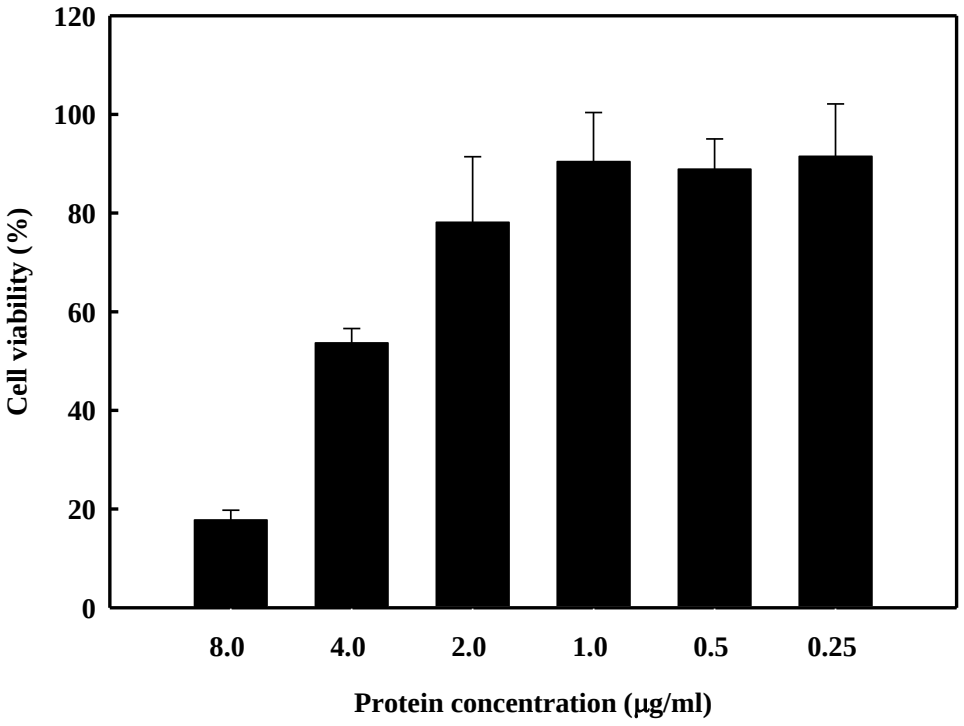


Figure 4.1 The effect of spotted babylon hydrolysates MW < 3 kDa on B16F10 cell proliferation. Each experiment was conducted in triplicate.

4.6 Effect of spotted babylon hydrolysates on melanin synthesis in B16F10 melanoma cells

To determine whether spotted babylon hydrolysates affect melanogenesis, melanin synthesis in B16F10 cells was examined. The B16F10 cells line were used because B16F10 cells shared melanogenic mechanisms with normal human melanocytes (Chang, 2012) and also easy to culture *in vitro*. The B16F10 cells were exposed to the serial concentrations of spotted babylon hydrolysates ranging from 4-0.25 μg/ml, except the concentration of 8 μg/ml. The inhibitory efficiency of spotted babylon hydrolysate on melanin production is shown in Figure 4.2. In this experiment, control was untreated with spotted babylon hydrolysates which was set as 100% melanin content. The data demonstrated that high concentrations of 2 and 1 μg/ml showed no inhibitory activity on melanin production at 75.37% and 94.10% of control, whereas 4 μg/ml showed significant inhibitory activity at 44.07%. The test dose of 4 μg/ml showed the greatest cytotoxic effect on B16F10 cell proliferation with the percentage of cell viability 53.69%. Surprisingly, at low concentrations of 0.5 and 0.25 μg/ml exhibited significant inhibitory activity on melanin production, 65.09% and 67.46% of control, respectively. Therefore, spotted babylon hydrolysates MW < 3 kDa inhibited melanin production at concentrations that did not affect cell viability. This result may suggest that the optimal concentrations of 0.5 and 0.25 μg/ml are able to inhibit melanin production.

4.7 Inhibition of tyrosinase activity in B16F10 melanoma cells by spotted babylon hydrolysates

The effect of spotted babylon hydrolysates on cellular tyrosinase activity was investigated to determine antimelanogenic properties. B16F10 cells were treated with serial concentrations of spotted babylon hydrolysates, ranging from 4-0.25 μg/ml. Results were in agreement with the melanin content results. Figure 4.3 showed the tyrosinase activity of B16F10 cells. In this experiment, control was untreated with spotted babylon hydrolysates which was set as 100% cellular tyrosinase activity. At high concentrations of 2 and 1 μg/ml cellular tyrosinase activity

increased to 100.59% and 111.41% of control, while at concentration of 4 μg/ml significantly reduced activity to 37.54%, but with the greatest cytotoxic activity. Surprisingly, at low concentrations of 0.50 and 0.25 μg/ml significantly decreased cellular tyrosinase activity with 71 and 74% of control, respectively. Dooley (Dooley, 1997) suggested that a skin-whitening agent should inhibit melanin production in melanosomes by decreasing melanin synthesis or reducing the activity of tyrosinase and show little and no cytotoxicity. According to the result, spotted babylon hydrolysates inhibited cellular tyrosinase activity at concentrations that did not affect cytotoxicity at concentrations of 0.5 and 0.25 μg/ml. Thus, spotted babylon hydrolysate can be developed and safety formulated in the skin-whitening cosmetic field or for therapeutic uses. However, these two concentrations must be further carefully calibrated before formulation or treatment.

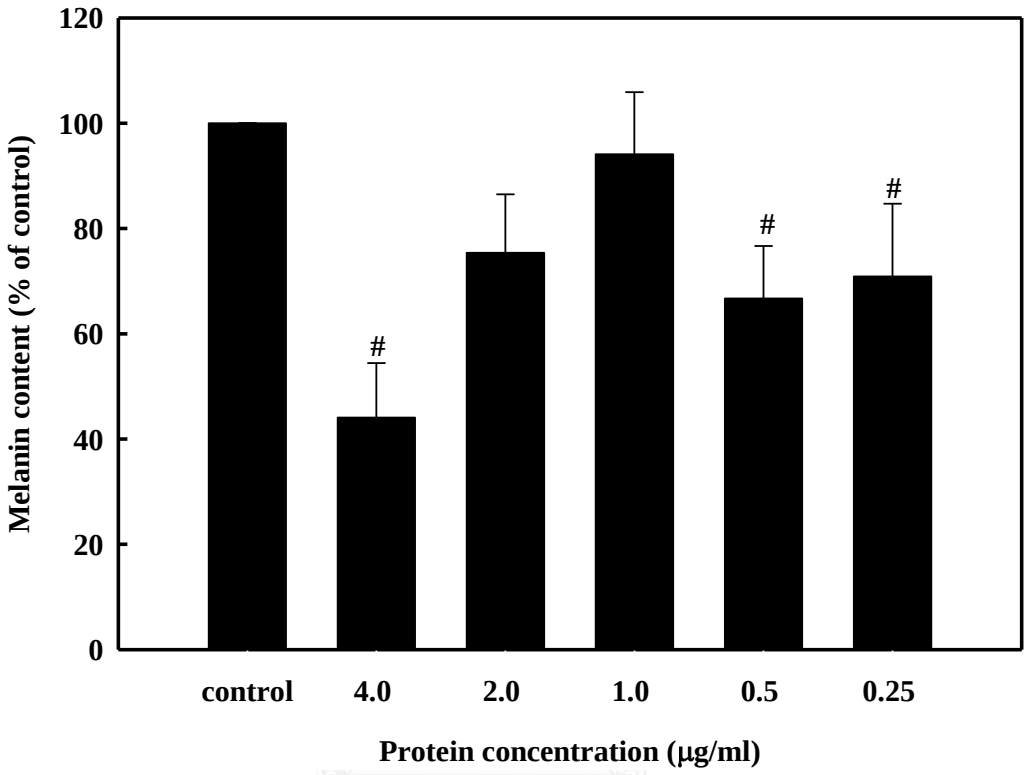


Figure 4.2 The effect of spotted babylon hydrolysates MW < 3 kDa on melanin production. Statistical analysis was assessed by Students *t*-test which was used to compare differences between spotted babylon hydrolysates MW < 3 kDa to control (100% melanin content). Bars with # have significantly different mean values ($p < 0.05$).

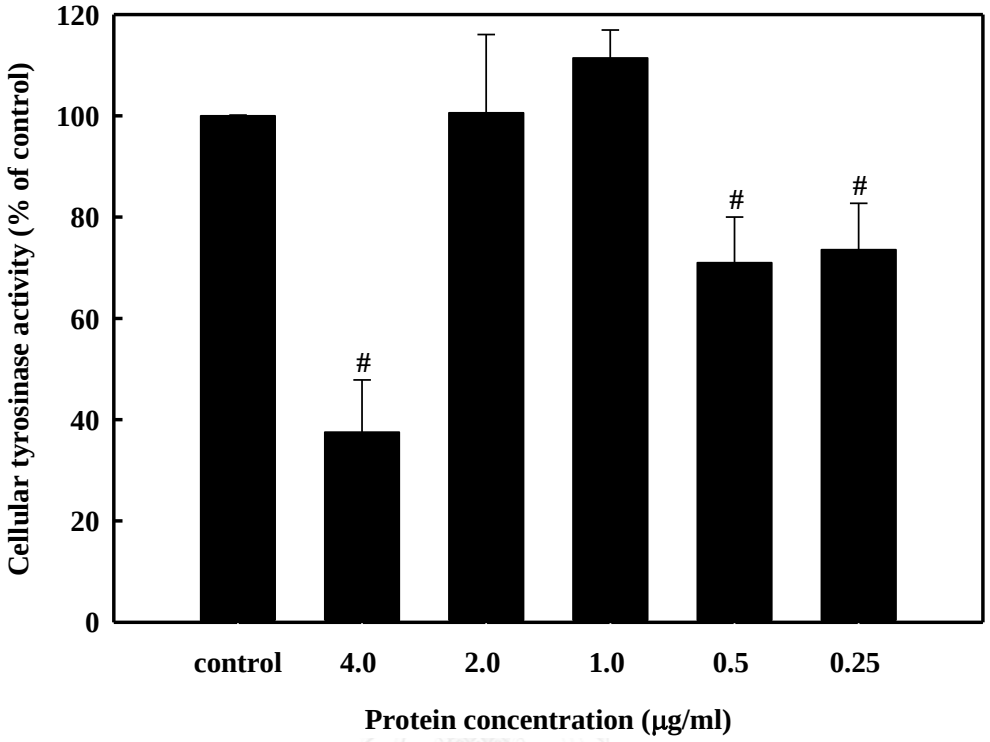


Figure 4.3 The effect of spotted babylon hydrolysate MW < 3 kDa on cellular tyrosinase activity. Statistical analysis was assessed by Students *t*-test which was used to compare differences between spotted babylon hydrolysates MW < 3 kDa to control (100% cellular tyrosinase activity). Bars with # have significantly different mean values ($p < 0.05$).

4.8 Kinetic of mushroom tyrosinase inhibition by spotted babylon hydrolysates

To examine the inhibitory mechanism of spotted babylon hydrolysates, a kinetic study on mushroom tyrosinase was conducted under two conditions: the first using L-tyrosine as the substrate (monophenolase activity), and the second using L-DOPA as the substrate (diphenolase activity). The mode of enzyme inhibition was determined by Lineweaver-Burk double reciprocal method as shown in Figure 4.4 (A) and (B). Increasing concentrations of L-tyrosine gave a series of parallel lines of almost equal slope. When the concentration of spotted babylon hydrolysates increased, the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) decreased. The kinetic inhibition constant (K_i) was calculated as 2.21 µg/ml. The results indicated that the mode of inhibition was an uncompetitive inhibitor of tyrosinase. With L-DOPA as substrate, the double reciprocal plots gave a series of parallel lines. When the concentration of spotted babylon hydrolysates MW < 3 kDa increased, K_m and V_{max} decreased indicating that it was an uncompetitive inhibitor of tyrosinase. The inhibition constant (K_i) for spotted babylon hydrolysates was calculated as 11.86 µg/ml. Spotted babylon hydrolysatses MW < 3 kDa acted as an uncompetitive inhibitor for both monophenolase and diphenolase activities which uncompetitive inhibitors can only bind the enzyme-substrate complex. The interaction between spotted babylon hydrolysates MW < 3 kDa and tyrosinase indicated that when using L-tyrosine as substrate, the binding capacity to tyrosinase (K_i = 2.21 µg/ml) was stronger than when using L-DOPA as substrate (K*i* = 11.86 µg/ml). The kinetic parameters determined by the Lineweaver-Burk plot are shown in Table 4.6.

Table 4.6 Kinetic parameters for monophenolase and diphenolase activities.

Substrate	Concentration of spotted babylon hydrolysates	K_m (µg/ml)	V_{max} (µg/ml)	K_i (µg/ml)	Inhibition type
L-tyrosine	Undiluted	0.051	0.140	2.21	uncompetitive
	2-fold diluted	0.076	0.248		
	4-fold diluted	0.175	0.385		
L-tyrosine	Undiluted	0.359	0.274	11.86	Uncompetitive
	2-fold diluted	0.393	0.351		
	4-fold diluted	0.510	0.459		

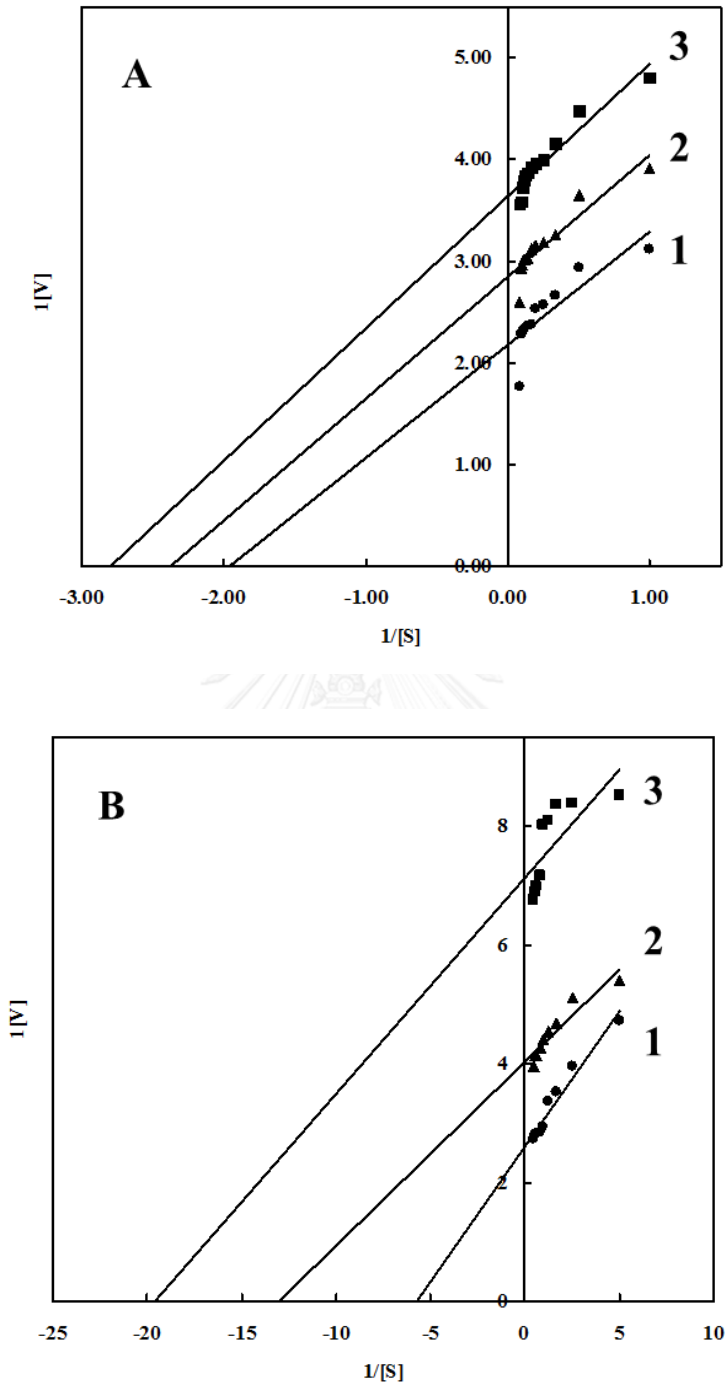


Figure 4.4 Lineweaver-Burke plots for inhibition on mushroom tyrosinase in the presence of spotted babylon hydrolysates. The concentrations of spotted babylon hydrolysates MW < 3 kDa for curves 1-3 were undiluted, 2-fold diluted, and 4-fold diluted respectively using L-tyrosine (A) and L-DOPA as the substrate (B).

4.9 Purification of tyrosinase inhibitory peptide by RP-HPLC

According to the results of mushroom tyrosinase inhibitory activity, the cytotoxic effect on B16F10 cells, melanin content, and cellular tyrosinase activity of spotted babylon hydrolysates MW < 3 kDa was selected for further purification as it exhibited the highest effectiveness. Figure 4.5 shows a chromatogram of the separation of spotted babylon hydrolysates MW < 3 kDa on an analytical Luna 5u C18 column (250 × 46 mm) using trifluoroacetic acid/acetonitrile solvent system. The separation was performed using a gradient elution of 8% B at 0 min, increased to 40% B at 40 min, and 100% B at 50 min, then maintained at 100% B from 50-55 min and returned to 8% B at 60 min. Elutions were monitored at 220, 254, and 280 nm. Peptide fractions were collected and pooled into four components where each fraction was collected at 10 min intervals and named as SBH1, SBH2, SBH3, and SBH4 (Figure 4.5). All of these fractions were evaporated to remove solvent before the evaluation their ability of tyrosinase inhibitory activity by using mushroom tyrosinase. According to the results (figure 4.6), SBH 2 showed the highest effectiveness on tyrosinase inhibitory activity with the percentage maximal inhibition of 59.63% and followed by SBH 3, SBH 1 and SBH 4 showing the percentage maximal inhibition of 58.93%, 57.95% and 57.81%, respectively. When looking at the result, we found that the percentage inhibition of all fractions was barely difference when compared to each other. Moreover, the result also revealed that it may be synergistic effect within the SBH because unfractioned (SBH) showed the percentage maximal inhibition of 88.02 % higher than peptides which were separated into SBH1-SBH4.

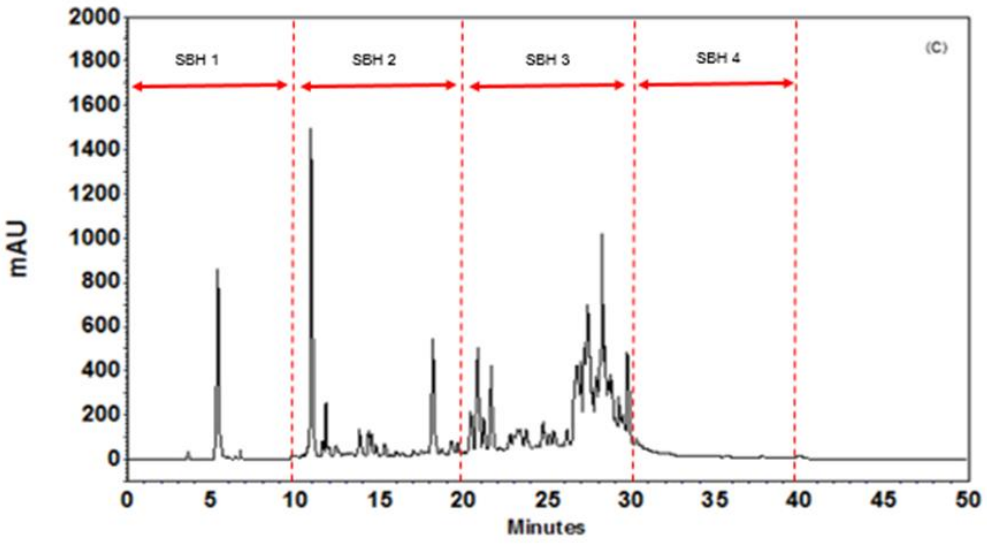


Figure 4.5 RP-HPLC chromatogram of spotted babylon hydrolysate MW < 3 kDa at 280 nm.

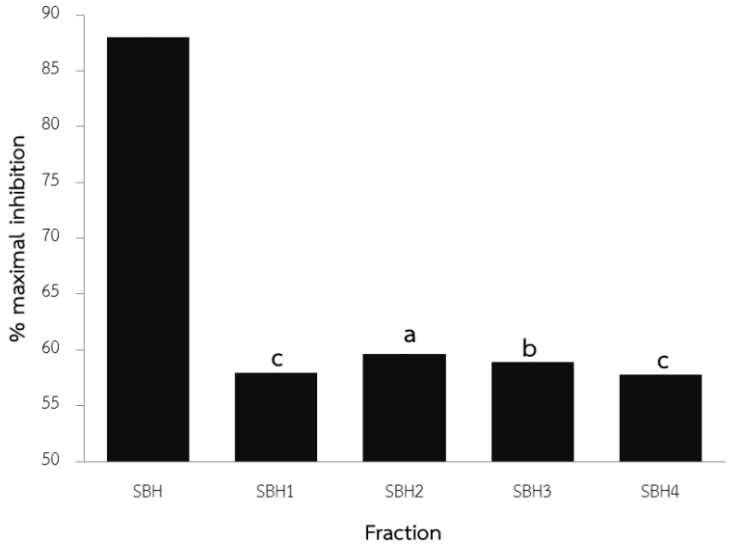


Figure 4.6 Tyrosinase inhibitory activity of spotted babylon hydrolysates MW < 3 kDa and RP-HPLC separated peptide fractions. [a, b, c, d, e, f] Statistic analysis by SPSS variance (ANOVA) with post hoc comparison (one-way) by using Duncan's multiple Range Test. The same of superscripts alphabet are not significantly different at ($p < 0.05$).

4.10 Identification of tyrosinase inhibitor peptides from spotted babylon hydrolysate MW < 3 kDa by an ion trap mass spectrometer

Spotted babylon hydrolysate MW < 3 kDa was further identified using ion trap LC/MS-MS analysis. Four fractions of spotted babylon hydrolysate were identified with amino acid sequences by comparing data with the MASCOT data base (Table 4.7). Little is known about peptides involved in melanogenesis and tyrosinase inhibition. Peptides however have a great potential to replace toxic drugs such as hydroquinone. This is because peptides are composed of amino acids which are easily metabolized in the skin and devoid from risks of systemic absorption and liver-related mutagenesis during the chronic treatment of hyperpigmentation disorders (Abu Ubeid, Zhao, Wang, & Hantash, 2009). Schurink and coworkers (Schurink et al., 2007) proposed that for strong tyrosinase inhibitory and binding activity, peptides should contain one or more arginine residues. These are very beneficial because the interaction between tyrosinase and arginine-containing peptides is rather specific. Phenylalanine-containing peptides are also considered to be important as their structure is similar to tyrosine, the natural substrate of tyrosinase. Peptides with arginine and/or phenylalanine combined with hydrophobic aliphatic residues such as valine, alanine, or leucine showed significant tyrosinase inhibition. Moreover, peptides containing polar, uncharged residues, cysteine, serine, and/or threonine showed potential for tyrosinase inhibitory activity. Tyrosine-containing peptides did not bind very well to tyrosinase. Ishikawa and coworkwers (Ishikawa et al., 2007) reported the effect of amino acids on the disruption of melanogenesis. L-alanine, glycine, L-isoleucine, and L-leucine have all been suggested as potential melanogenesis inhibition which have no adverse effects. The combination of these four amino acids showed an additive effect on hypopigmentation similar to that of kojic acid, and they may be good candidates for safe effective melanogenesis inhibitors. The result in table 4 showed that all 23 peptides contained one or more important amino acid residues involved in melanogenesis inhibition. In fraction 1 (0-10 min), peptides P1, P2, and P3 (ARSHGYPA, ARSHCYPA and TARSSTGGKA) contain the combination arginine-alanine in the position 1/2, 1/2, and 2/3. P4 and P6

(MSAISSGLESLLSTHWLFNHG, YFCASGHRLGGEDTQYF) contain the combination phenylalanine-leucine and arginine-leucine in the position 17/18 and 8/9. In fraction 2 (10-20 min), P7 and P11 (LKNASDKRNQQ, MQLSRCF) contain arginine in positions 8 and 5. In fraction 3 (20-30 min), P13 (KIMVYFGK) contains the combination phenylalanine-glycine in position 6/7. P15 and P16 (RCTFGQTKVEIK, RMTFGQGTKVEIK) contain arginine residue and the combination phenylalanine-glycine in position 1, and 4/5. P17 (MEKARRGG) contains the combination arginine-alanine and arginine-glycine in positions 4/5 and 6/7. In fraction 4 (30-40 min), P19 (MKLFVPALLPLGA) contains the combination phenylalanine-leucine in position 3/4. P20 (RMYQIKRLLSE) contains the combination arginine-leucine in position 7/8. The presence of these important amino acid residues in the peptides show strong tyrosinase inhibition. Besides the combination of amino acid mentioned above, all 23 peptides also contain other important amino acids involved in melanogenesis inhibition such as cysteine, serine and threonine.

Table 4.7 Peptide sequence of spotted babylon hydrolysate MW < 3 kDa identified by ion trap LC/MS-MS.

Fraction	Amino acid sequence	Sciencetific name	Organism
SBH 1	P.1 ARSHGYPA	*Homo sapiens*	Human
	P.2 ARSHCYPA	*Homo sapiens*	Human
	P.3 TARSSTGGKA	*Rattus norvegicus*	Rat
	P.4 MSAISSGLESLLSTHWLF NHG	*Solenopsis invicta*	Red imported fire ant
	P.5 MWSLGALPDG	*Mus musculus*	House mouse
	P.6 YFCASGHRLGGEDTQYF	*Mus musculus*	House mouse
SBH 2	P.7 LKNASDKRNQQ	*Homo sapiens*	Human
	P.8 MEHGLSL	*Mus musculus*	House mouse
	P.9 AAAAGKQ	*Tursiops truncates*	Bottlenose dolphin
	P.10 AATLQVPVNDLNA	*Homo sapiens*	Human
	P.11 MQLSRCF	*Patiria miniata*	Bat star
SBH 3	P.12 KIPVYFGK	*Rana catesbeiana*	American bullfrog
	P.13 KIMVYFGK	*Rana catesbeiana*	American bullfrog
	P.14 AMVVLLGPGT	*Homo sapiens*	Human
	P.15 RCTFGQTKVEIK	*Homo sapiens*	Human
	P.16 RMTFGQGTKVEIK	*Homo sapiens*	Human
	P.17 MEKARRGG	*Homo sapiens*	Human
	P.18 MRIHVLENVD	*Sus scrofa*	Wild pig
SBH 4	P.19 MKLFVPALLPLGA	*Bubalus bubalus*	Swamp buffalo
	P.20 RMYQIKRLLSE	*Homo sapiens*	Human
	P.21 STASTVEWSTVVHSG	*Mus musculus*	House mouse
	P.22 MLTDPDLPQEGE	*Gallus gallus*	Chicken
	P.23 ETNNGGWTL	*Ixonotus guttatus*	Spotted guttatus

4.11 Preliminary study of the effect of spotted babylon hydrolysates on apoptosis induction in B16F10 murine melanoma cell

The apoptotic induction of spotted babylon hydrolysates on B16F10 cells was studied. Apoptosis plays a crucial role in the regulation of cell numbers during development and tissue homeostasis. The dysregulation of apoptosis can increase or decrease cell activity, which is related to a variety of clinical disorders such as autoimmunity, neurodegenerative diseases, and cancer. In cancer therapy apoptosis is used to eliminate tumor cells (Kiechle & Zhang, 2002). Induction of apoptosis has been recognized as an ideal strategy for cancer therapy. Agents that can induce apoptosis in tumors have potential to use in antitumor therapy.

This experiment aimed to determine whether spotted babylon hydrolysate induced apoptosis. B16F10 cells treated with spotted babylon hydrolysates MW < 3 kDa and untreated (control) were stained with fluorescein isothiocyanate (FITC)-labeled annexin V/propidium iodide (PI) double staining and then analyzed by flow cytometer. The exposure of phosphatidylinositol of the plasma membrane (PS) indicates early apoptosis which can be stained by annexin V. The results are shown in Figure 4.6 A and B. Increased early apoptosis cell (annexin V+, PI-) and late apoptosis cell (annexin V+, PI+) were detected. In the untreated B16F10 cells (control) showed more than 90 % of the cells are viable, with only 0.051 % in early apoptosis and 0.070 % in late apoptosis. After the B16F10 cells were treated with spotted babylon hydrolysate 4 μg/ml (IC_{50} value) for 8 h, the percentage of apoptosis cells in early stage increased to 7.12 % and 4.37 % in late apoptosis stage. These results indicated that spotted babylon hydrolysates induced apoptosis in B16F10 cells. Azevedo and coworkers (Azevedo et al., 2012) investigated the effect of cationic INKKI peptide and β-casein hydrolyzed with trypsin, on B16F10 cells. INKKI peptide induced apoptosis in B16F10 cells up to 82.82 % compared with the control of 3.99 %. Tumor cells show many modifications on the cell membrane such as glycoproteins, glycolipids, and also increasing in sialylation. These modifications give the tumor membrane as negative charges that increases the binding of the cationic peptides. They also investigated the apoptotic mechanism and suggested that it

occurred via a mitochondrial pathway. Results from this experiment showed a gradual induction of apoptosis compared to Azevedo study (Azevedo et al., 2012).

This may be because the spotted babylon hydrolysate MW < 3 kDa was a peptide mixture and not being isolated as a single peptide. The charge of peptide mixture MW < 3 KDa might not be appropriate to bind with the tumor membrane due to the improper charge of the peptide mixture. The induction apoptotic mechanism of spotted babylon hydrolysates MW < 3 kDa is required for further investigation. However, the most important concern for anticancer agents is safety. If anticancer agents can induce apoptosis in tumor cells but not in normal cells, they can be considered as harmless and safe.

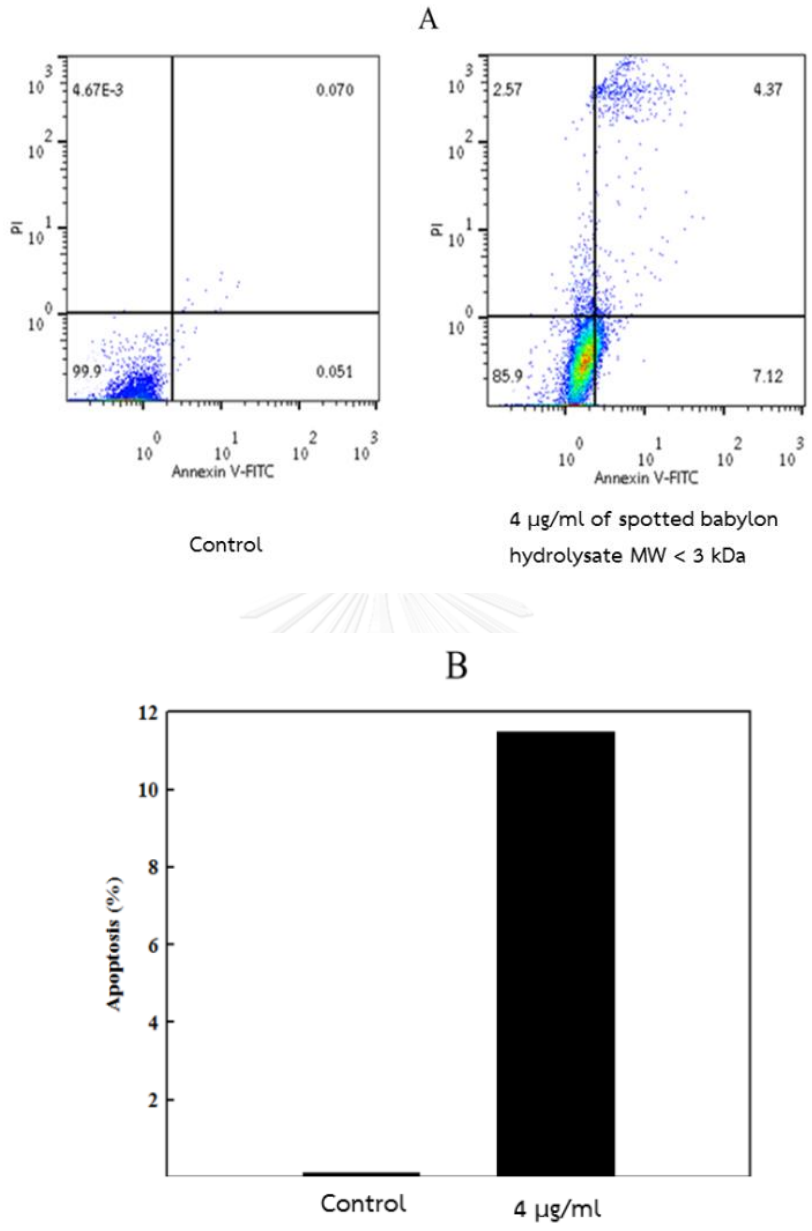


Figure 4.7 Effect of spotted babylon hydrolysate on apoptosis induction in B16F10 cells. (A) Dot plots representative of the apoptotic effect of spotted babylon hydrolysates MW < 3 kDa with annexin V/PI staining by flow cytometry (B) Quantification of the data presented in dot plots for cell undergoing apoptosis.

CHAPTER V

CONCLUSION

These results suggest that the spotted babylon hydrolysates MW < 3 kDa may be used as a tyrosinase inhibitor because it showed effective both monophenolase and diphenolase activity. Results obtained from cytotoxic effect, cellular tyrosinase activity, and melanin content assay, using B16F10 murine melanoma cells indicated that the spotted babylon hydrolysates MW < 3 kDa has potential to further study and develop as a therapeutic agent and for using in the cosmetics field. Because it showed strong inhibition of tyrosinase activity and reduced the production of melanin and cellular tyrosinase activity with minimal melanocyte toxicity. However, further studies are required to investigate the inhibition mechanisms of the spotted babylon hydrolysates MW < 3 kDa involved in melanogenesis. Moreover, the preliminary induction apoptosis experiment indicated that the spotted babylon hydrolysates can induce apoptosis of B16F10 murine melanoma cells, and may have an important role as a possible future anticancer agent.

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APPENDIX

APPENDIX A

Preparation of buffer and reagents

1. Bradford solution and assay

1.1 Bradford stock solution

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

1.2 Bradford working buffer

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

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

1.3 Bradford's assay

1.3.1 Pipet protein solution into tube (maximum 100 µl).

1.3.2 Add Bradford working buffer 1 ml and vortex.

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

2. 100 mM Phosphate buffer pH 6.5 and pH 7.4

KH_2PO_4	6.80 g
K_2HPO_4	8.71 g
Deionized water	1000 ml

Protocal: start with 800 ml deionized water to dissolve all reagents. Adjust the pH to 6.5 and 7.4 with KOH. Add distilled water to a final volume of 1 liter.

3. 20 mM Tris–HCl buffer pH 8.0

Tris base 2.43 g

Distilled water 1000 ml

Protocal: start with 800 ml deionized water to dissolve reagent. Adjust the pH to 8.0 with HCl. Add deionized water to a final volume of 1 liter.

4. Mushroom tyrosinase 333 U from 50 KU

4.1 0.8 KU mushroom tyrosinase 30 ml

$$C_1V_1 = C_2V_2$$

$$(50\times10^3\ U) \times V_1 = (0.8 \times 10^3\ U) \times 30\ ml$$

$$V_1 = 0.48\ ml$$

Protocal: start with dissolve 0.48 ml (480 µl) of 50 KU mushroom tyrosinase with 29.52 ml phosphate buffer pH 6.5.

4.2 333 U mushroom tyrosinase 50 ml

$$C_1V_1 = C_2V_2$$

$$(0.8 \times10^3\ U) \times V_1 = (333\ U) \times 50\ ml$$

$$V_1 = 20.81\ ml$$

Protocal: start with dissolve 20.81 ml of 0.8 KU mushroom tyrosinase with 29.19 ml phosphate buffer pH 6.5.

5. 2 mM L-tyrosine 50 ml

1 M L-tyrosine 181.19 g

2×10^{-3} M L-tyrosine 0.36 g

Protocal: Distilled water 1000 ml L-tyrosine 0.36 g

Distilled water 50 ml L-tyrosine 0.018 g

Thus, dissolve 0.018 g L-tyrosine with deionized water 50 ml and vortex.

6. 12 mM L-DOPA 50 ml

1 M L-DOPA 197.19 g

12×10^{-3} M L-DOPA 2.37 g

Protocal: Deionized water 1000 ml L-DOPA 2.37 g

Deionized water 50 ml L-DOPA 0.12 g

Thus, dissolve 0.12 g L-DOPA with deionized water 50 ml and vortex.

7. 0.004% DPPH solution 100 ml

DPPH 0.004 mg

Methanol 100 ml

Protocal: start with dissolve 0.004 mg DPPH with 100 ml methanol. Adjust volume by using volumetric flask.

8. 7 mM ABTS radical cation stock solution

8.1 dissolve 0.384 g in 100 ml deionized water to prepare 7 mM ABTS (solution A).

8.2 dissolve 0.066 g in 100 ml deionized water to prepare 2.45 mM potassium persulphate (solution B).

8.3 Mix 50 ml solution A and solution B above. Then, allow to stand in the dark room at room temperature for 12 – 16 hours before using. The ABTS radical cation is stable for at least two days.

8.4 Prepare fresh ABTS radical cation working solution: dilute 7 mM ABTS radical cation stock solution with distilled water to obtain an absorbance value of 0.7 ± 0.02 at 734 nm by using microplate reader.

9. **Nitric oxide assay's reagents**

9.1 10 mM sodium nitroprusside (SND), 100 ml

1 M sodium nitroprusside 297.95 g

10×10^{-3} M sodium nitroprusside 0.2980 g

Protocal: start with dissolve 0.2980 g sodium nitroprusside with 100 ml phosphate buffer pH 7.4.

9.2 0.33% (w/v) sulfanilamide in 20% acetic acid, 100 ml

Sulfanilamide	0.33 g
Acetic	20%

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

9.3 0.1% (w/v) N- (1-Napthyl) ethylenediamine dihydrochloride (NED)

NED	0.1 g
Distilled water	100 ml

Protocal: start with dissolve 0.1 g NED in 100ml deionized water.

10. Mobile phase in RP-HPLC analysis

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

$$C_1V_1 = C_2V_2$$

$$(100\%) \times V_1 = (0.1\%) \times 1000 \text{ ml}$$

$$V_1 = 1 \text{ ml}$$

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

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

(1) 70% acetonitrile, 1000 ml

$$C_1V_1 = C_2V_2$$

$$(100\%) \times V_1 = (70\%) \times 1000 \text{ ml}$$

$$V_1 = 700 \text{ ml}$$

(2) 0.05% TFA in double deionized water, 400 ml

$$C_1V_1 = C_2V_2$$

$$(100\%) \times V_1 = (0.05\%) \times 400 \text{ ml}$$

$$V_1 = 0.2 \text{ ml}$$

Then, add 0.2 ml TFA into 399.8 ml double deionized water

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

11. 5 mg/ml MTT solution

MTT	5 mg
Deionized water	1 ml

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

APPENDIX B

Caribration curve for protein determination by Bradford's procedure

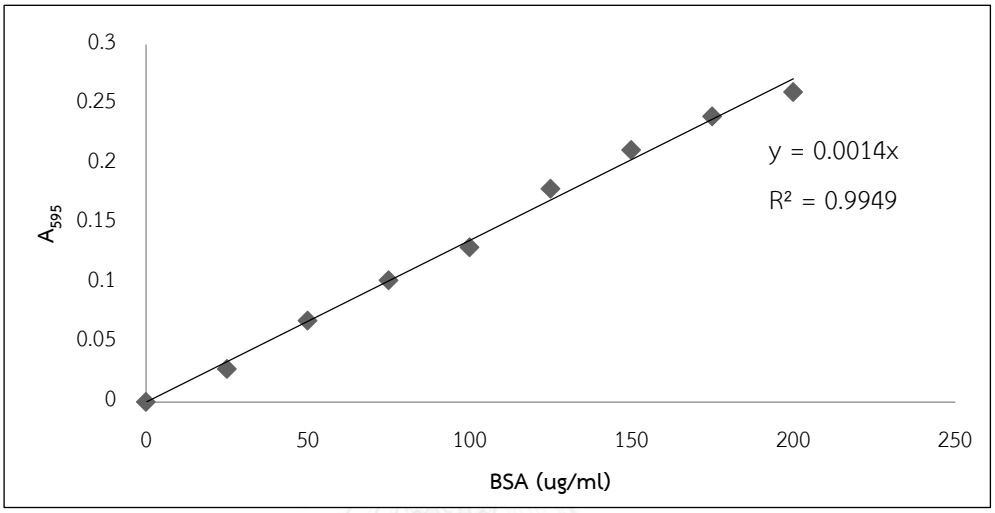


Figure B1 A standard curve of BSA for protein determination obtained from Bradford assay which measured at 595 nm by microplate spectrophotometer.

The equation obtained from the standard curve was used to calculate total protein as following:

$$y = 0.0014x$$

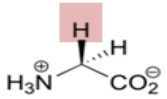
where y value is the absorbance value of each sample at 595 nm and x value is the total protein of each sample.

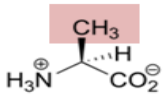
APPENDIX C

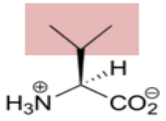
Amino acid abbreviations and structures

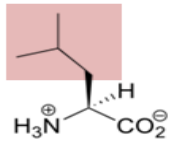
Amino acid	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine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

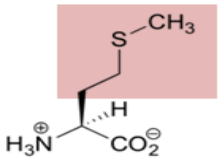
Nonpolar, aliphatic side groups

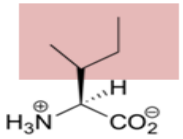

Glycine
Gly, G


Alanine
Ala, A

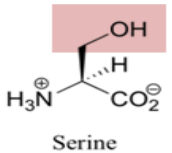

Valine
Val, V

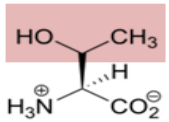

Leucine
Leu, L

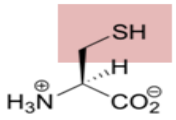

Methionine
Met, M

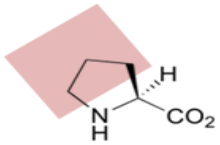

Isoleucine
Ile, I

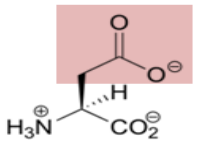
Polar, uncharged side groups

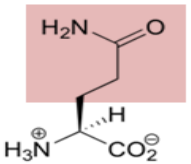

Serine
Ser, S


Threonine
Thr, T

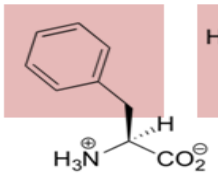

Cysteine
Cys, C

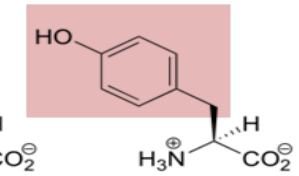

Proline
Pro, P

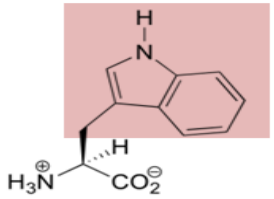

Aspartate
Asp, D


Glutamine
Gln, Q

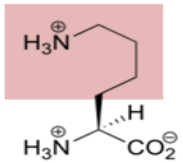
Aromatic side groups

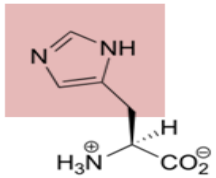

Phenylalanine
Phe, F

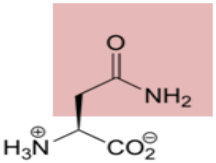

Tyrosine
Tyr, Y


Tryptophan
Trp, W

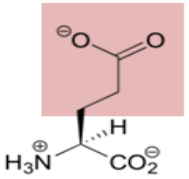
Positively charged side groups

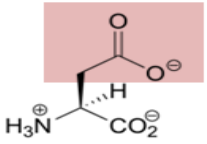

Lysine
Lys, K


Histidine
His, H


Asparagine
Asn, N

Negatively charged side groups


Glutamate
Glu, E


Aspartate
Asp, D

APPENDIX D

RP-HPLC chromatograms of spotted babylon hydrolysates MW < 3 kDa

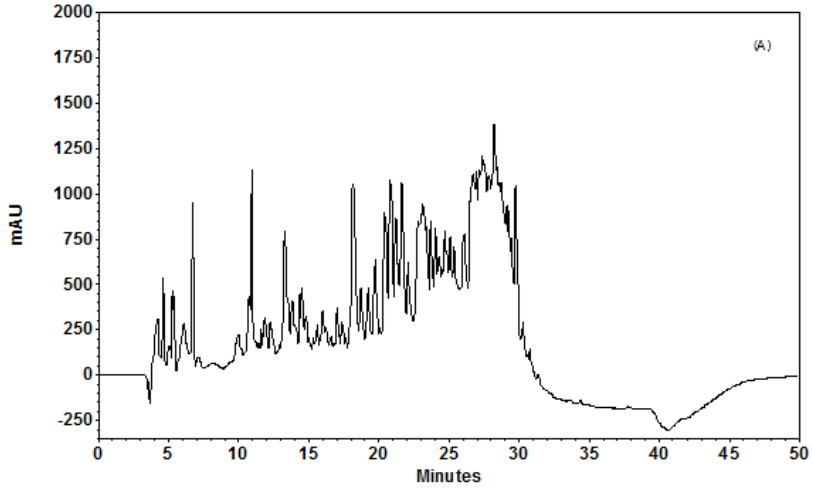


Figure D1 A RP-HPLC chromatogram of spotted babylon hydrolysate MW < 3 kDa detected at 220 nm

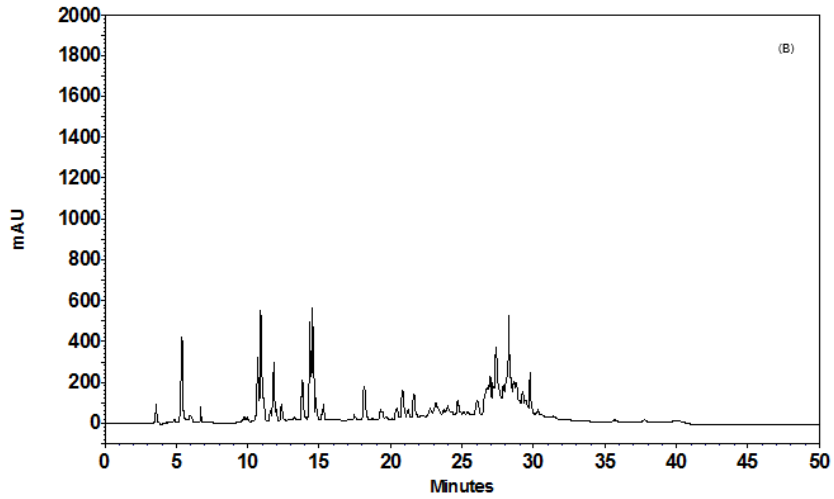


Figure D2 A RP-HPLC chromatogram of spotted babylon hydrolysate MW < 3 kDa detected at 254nm.

APPENDIX E

Secondary plot for determination of kinetic inhibition constant values

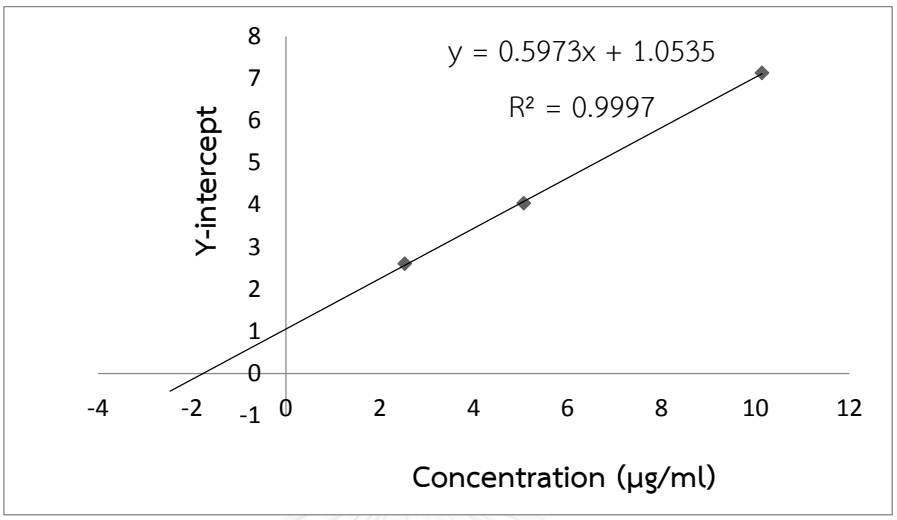


Figure E1 A secondary plot for determination of kinetic inhibition constant value (K*i*) using L-Tyrosine as substrate.

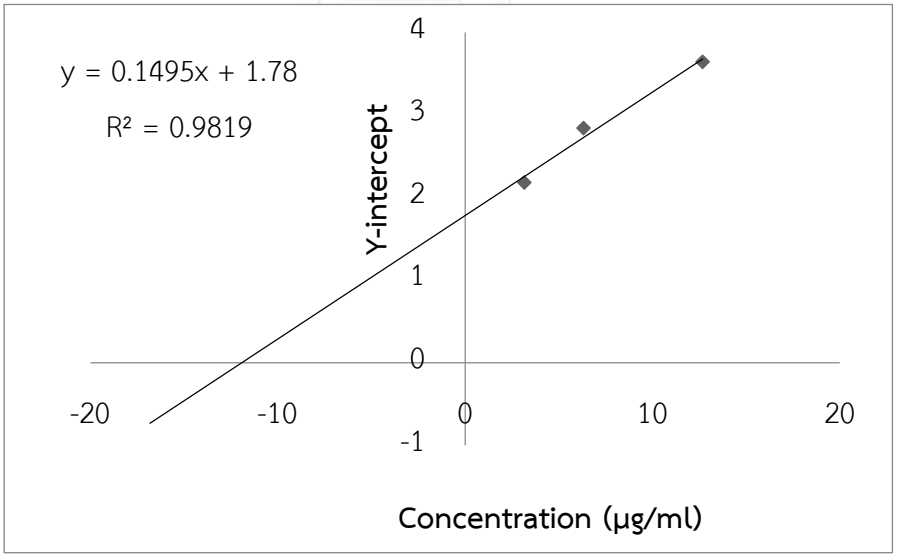


Figure E2 A secondary plot for determination of kinetic inhibition constant value (K*i*) using L-DOPA as substrate.

A secondary plot can be performed by two method; (1) plot a curve between concentration of inhibitor and Y-intercept obtained from the primary plot. (2) plot a curve between concentration of inhibitor and slope value obtained from the primary plot. Then, x-intercept value in the secondary plot is kinetic inhibition constant (K*i*)

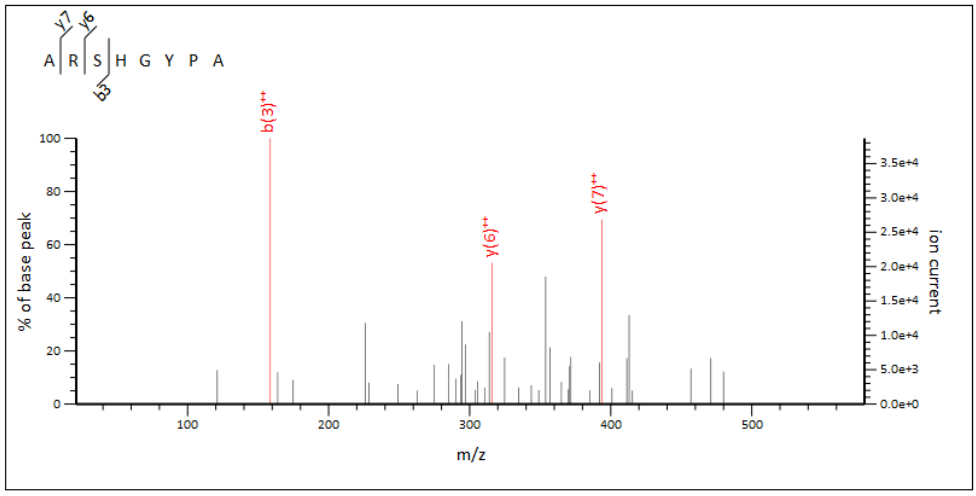
APPENDIX F

Mass spectrum and peptide sequences

Fraction 1 (0-10 min)

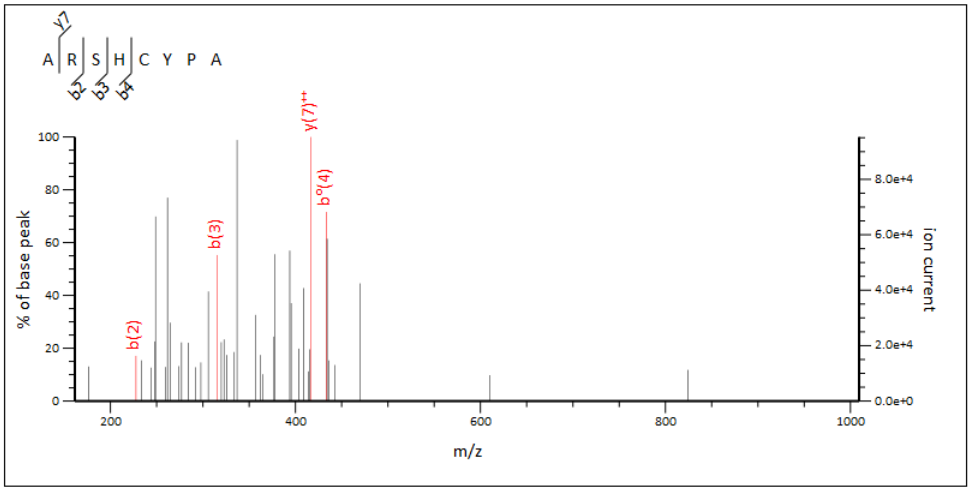
P.1 Observed; 430.0700**,** Mr(expt); 858.1254**,** Mr(calc); 857.4144

Peptide; **ARSHGYPA**



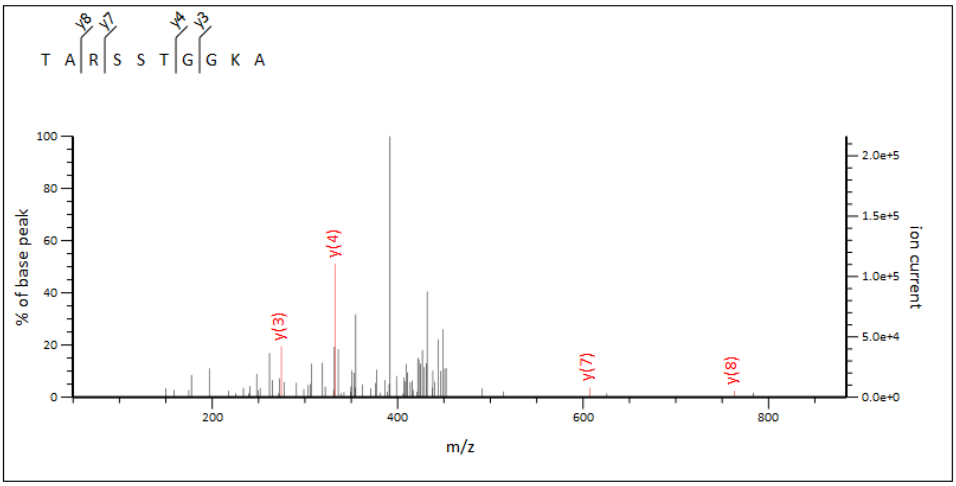
P.2 Observed; 453.2400, Mr(expt); 904.4654, Mr(calc); 903.4021

Peptide; **ARSHCYPA**



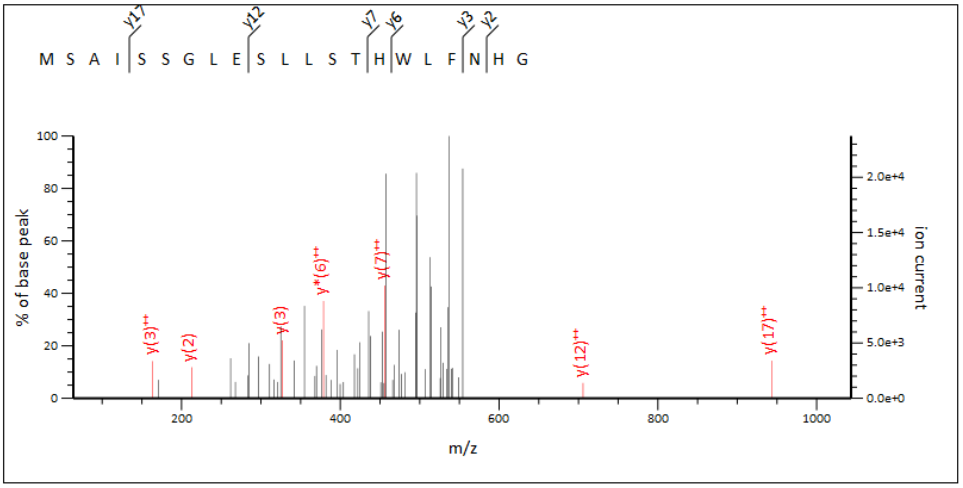
P.3 Observed; 467.8600, Mr(expt); 933.7054, Mr(calc); 934.4832

Peptide; **TARSSTGGKA**



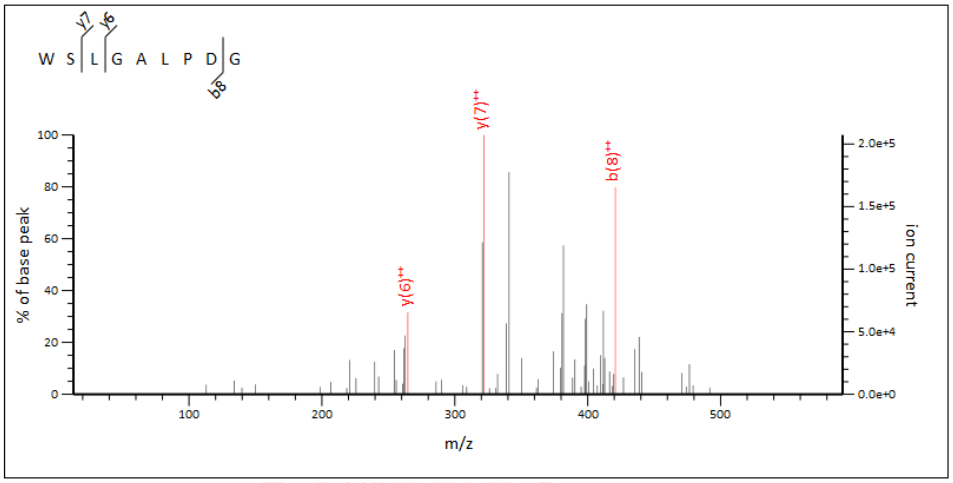
P.4 Observed; 572.7900, Mr(expt); 2287.1390, Mr(calc); 2286.1103

Peptide; **MSAISSGLESLLSTHWLFNHG**



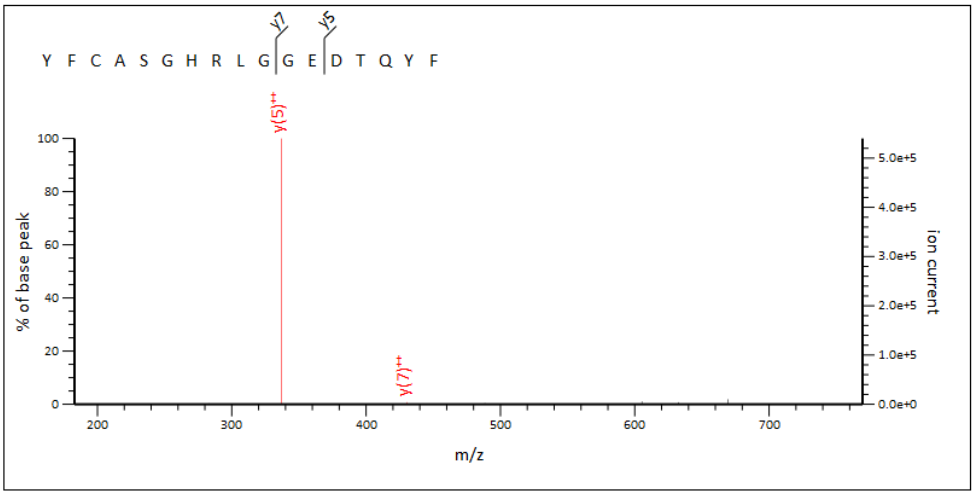
P.5 Observed; 572.7900, Mr(expt); 2287.1390, Mr(calc); 2286.1103

Peptide; **WSLGALPDG**



P.6 Observed; 650.9900, Mr(expt); 1949.9482, Mr(calc); 1949.8367

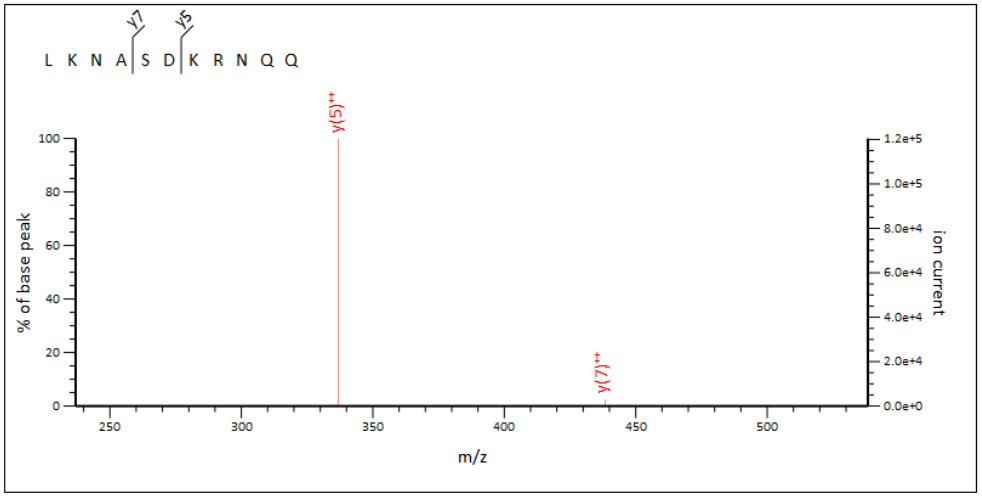
Peptide; **YFCASGHRLGGEDTQYF**



Fraction 2 (10-20 min)

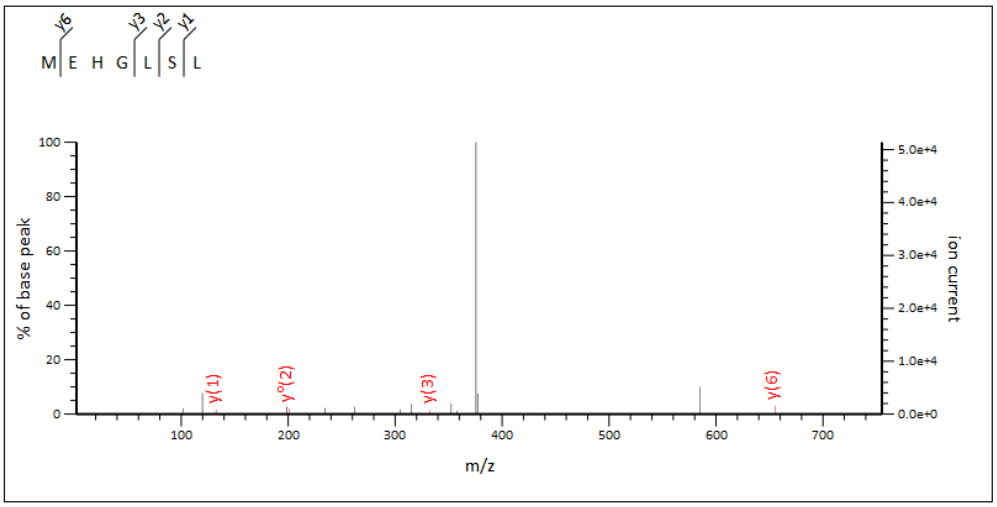
P.7 Observed; 651.2500, Mr(expt); 1300.4854, Mr(calc); 1300.6847

Peptide; **LKNASDKRNQQ**



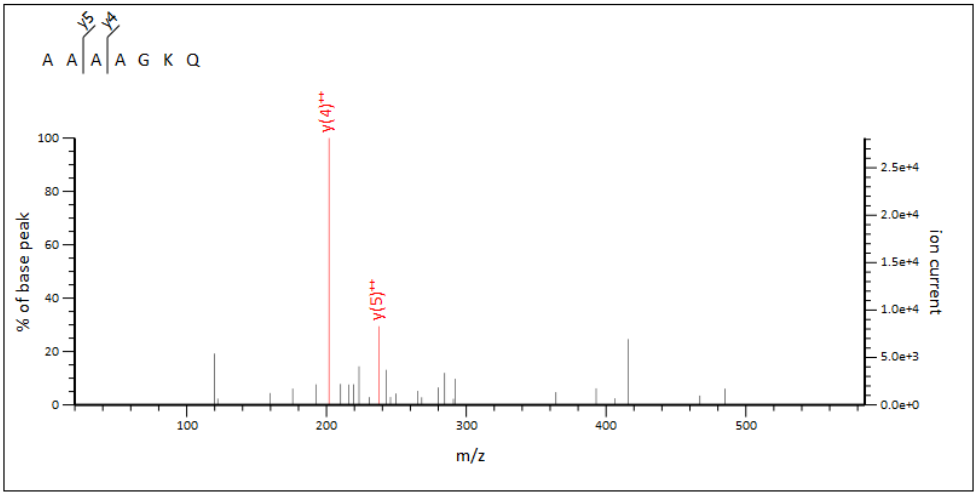
P.8 Observed; 394.2400, Mr(expt); 786.4654, Mr(calc); 785.3742

Peptide; **MEHGLSL**



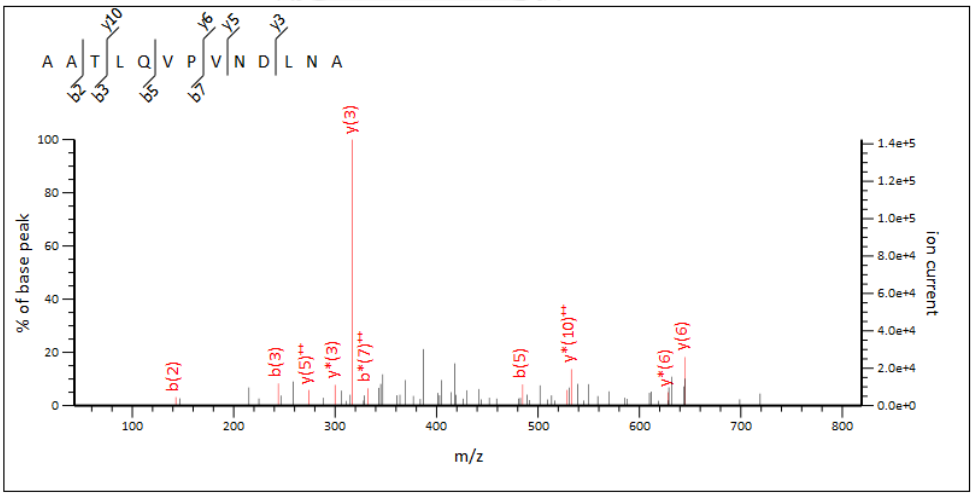
P9. Observed; 309.1600 Mr(expt); 616.3054 , Mr(calc); 615.334

Peptide; **AAAAGLKQ**



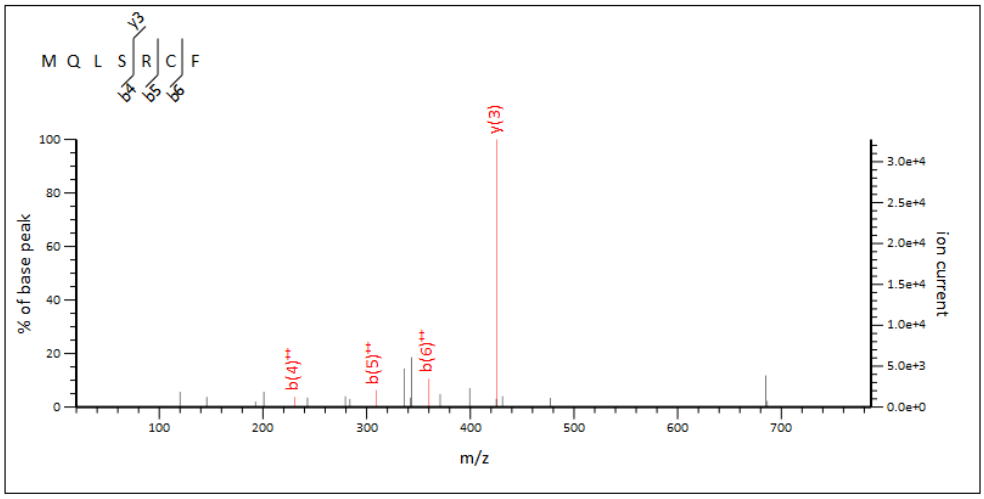
P.10 Observed; 663.6500, Mr(expt); 1325.2854, Mr(calc); 1324.6987

Peptide; **AATLQVPVNDLNA**



P.11 Observed; 443.220, Mr(expt); 884.4254, Mr(calc); 883.4044

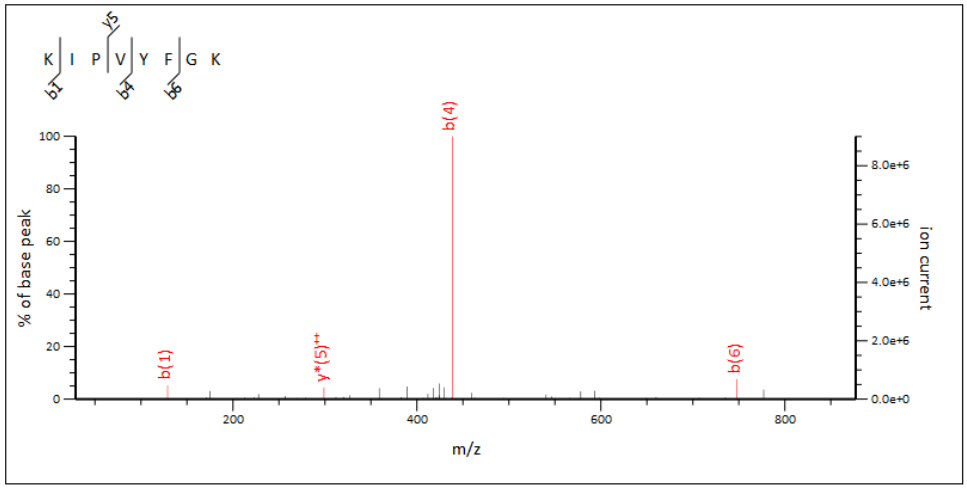
Peptide; **MQLSRCF**



Fraction 3 (20-30 min)

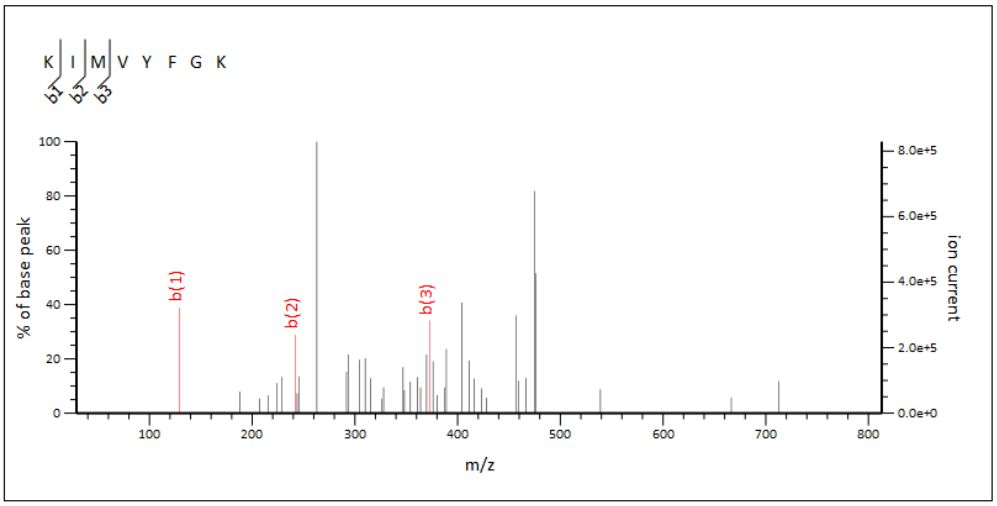
P.12 Observed; 476.2500, Mr(expt); 950.4854, Mr(calc); 950.5589

Peptide**; KIPVYFG**



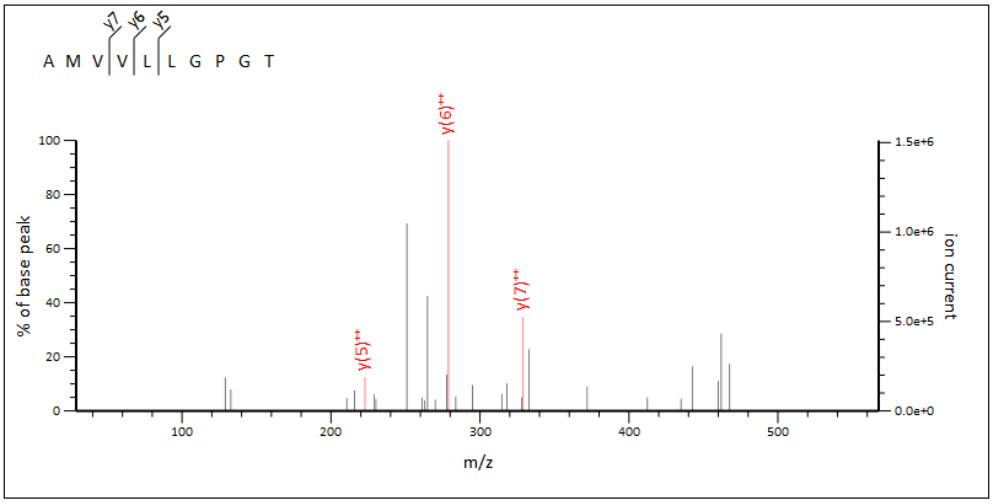
P.13 Observed; 493.1700, Mr(expt); 984.3254, Mr(calc); 984.5466

Peptide; **KIMVYFGK**



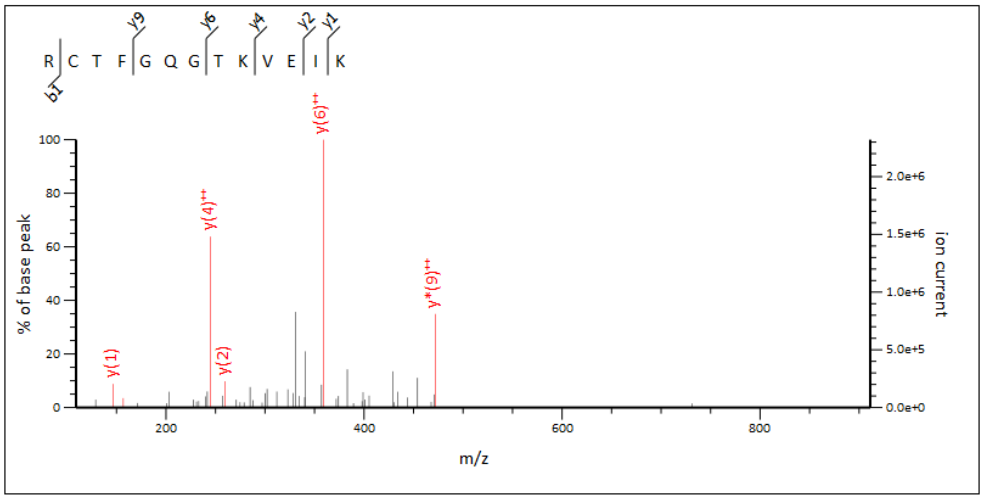
P.14 Observed; 479.2500, Mr(expt); 956.4854, Mr(calc); 956.5365

Peptide; **AMVVLLGPGT**



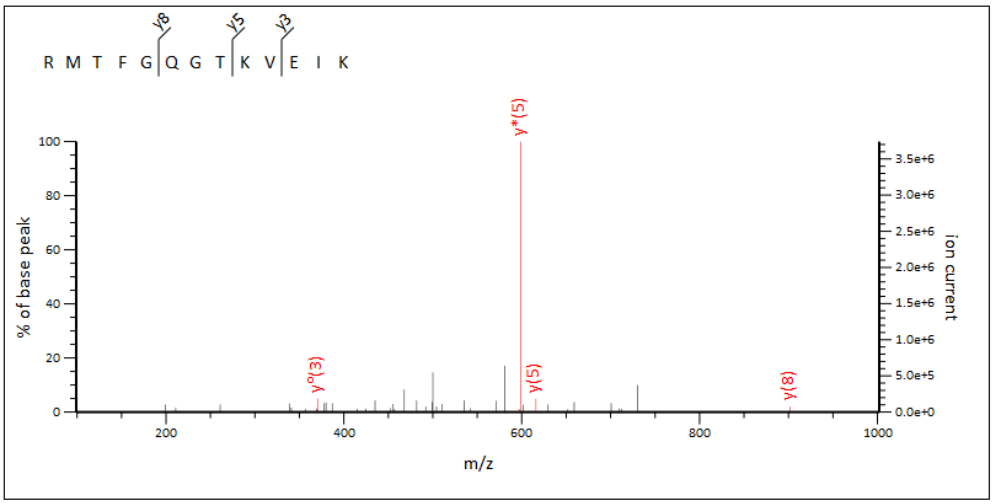
P.15 Observed; 489.9300, Mr(expt); 1466.7682, Mr(calc); 1465.7711

Peptide; **RCTFGQGTKVEIK**



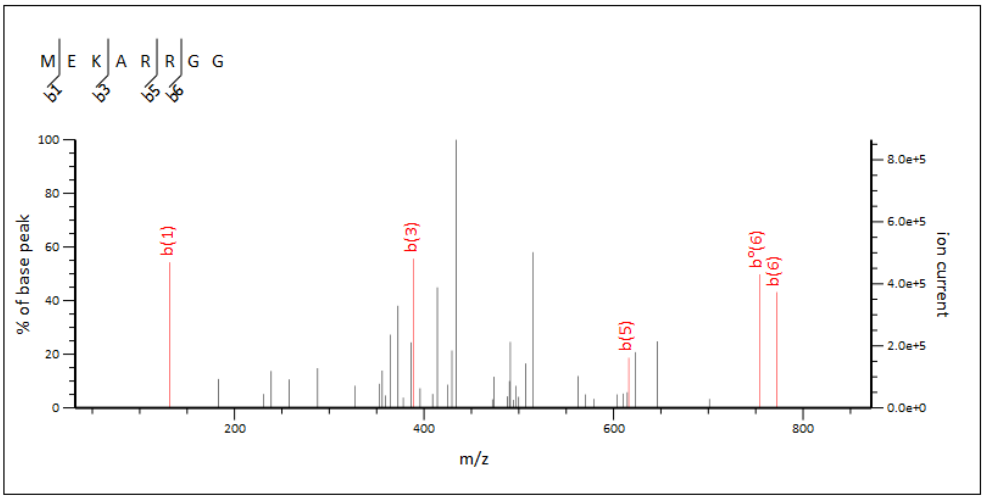
P.16 Observed; 748.3500, Mr(expt); 1494.6854, Mr(calc); 1493.8024

Peptide; **RMTFGQGTKVEIK**



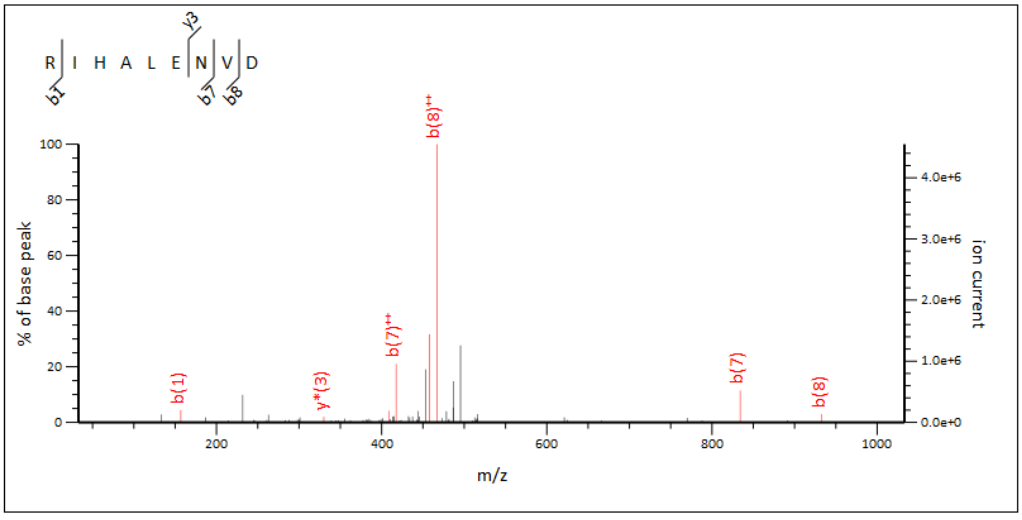
P.17 Observed; 452.2300, Mr(expt); 902.4454, Mr(calc); 903.4708

Peptide; **MEKARRGG**



P.18 Observed; 533.2600, Mr(expt); 1064.5054, Mr(calc); 1065.5567

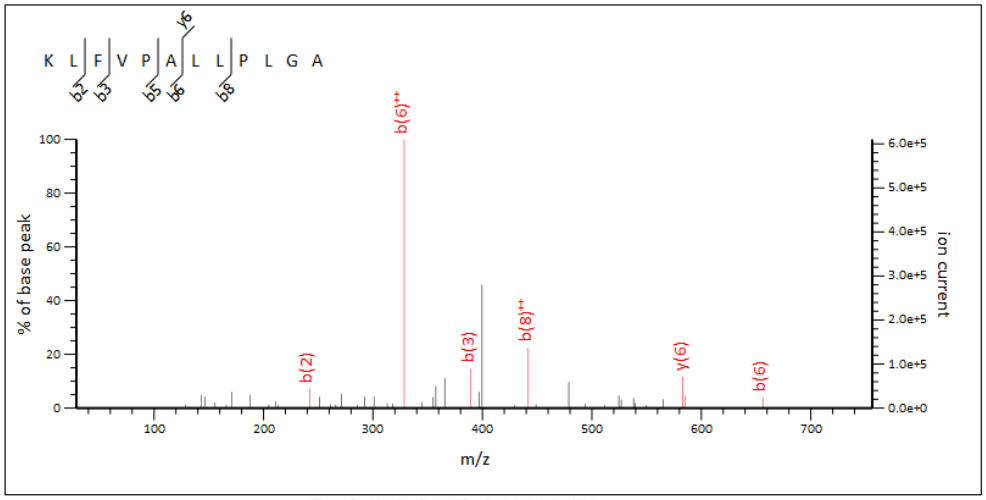
Peptide; **MRIHALENVD**



Fraction 4 (30-40 min)

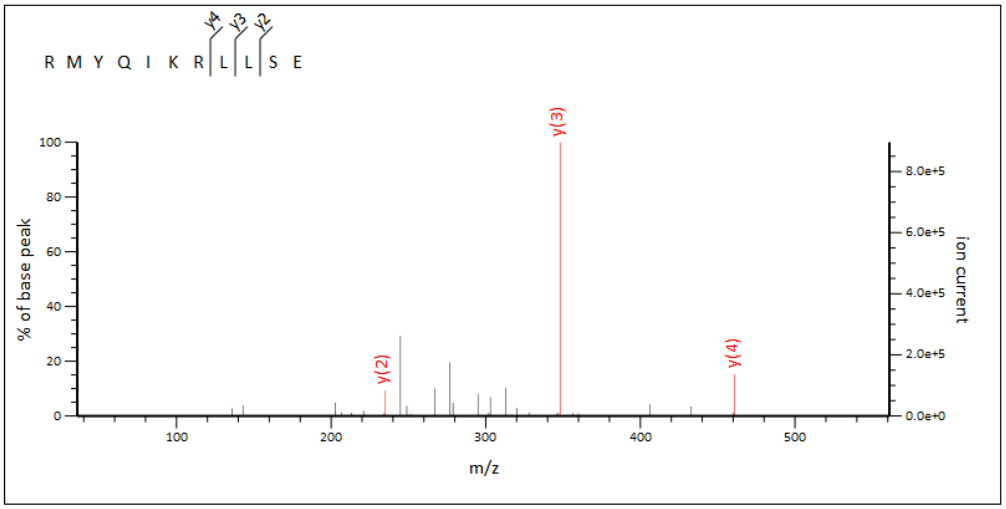
P.19 Observed; **413.7600**, Mr(expt); 1238.2582, Mr(calc); 1237.7798

Peptide; **KLFVPALLPLGA**



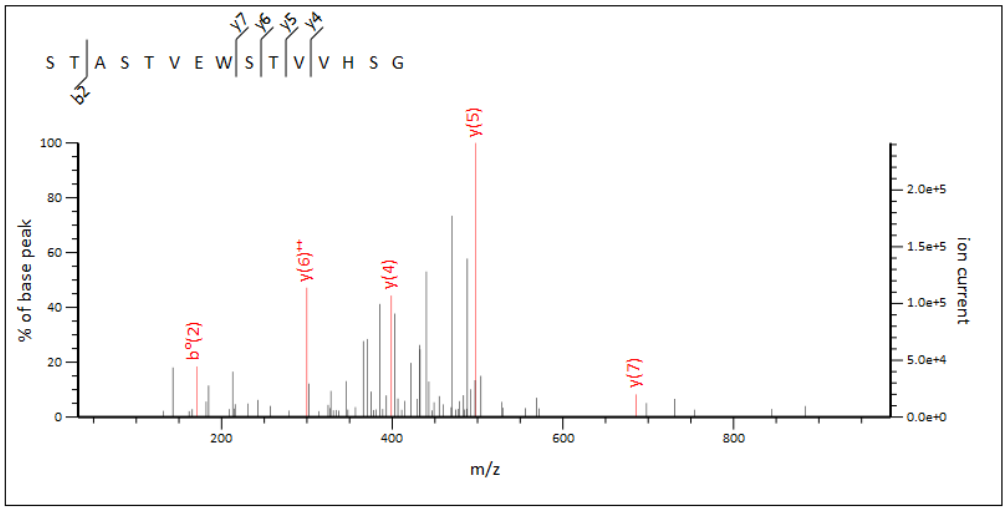
P.20 Observed; 479.2100, Mr(expt); 1434.6082, Mr(calc); 1435.7969

Peptide; **RMYQIKRLLSE**



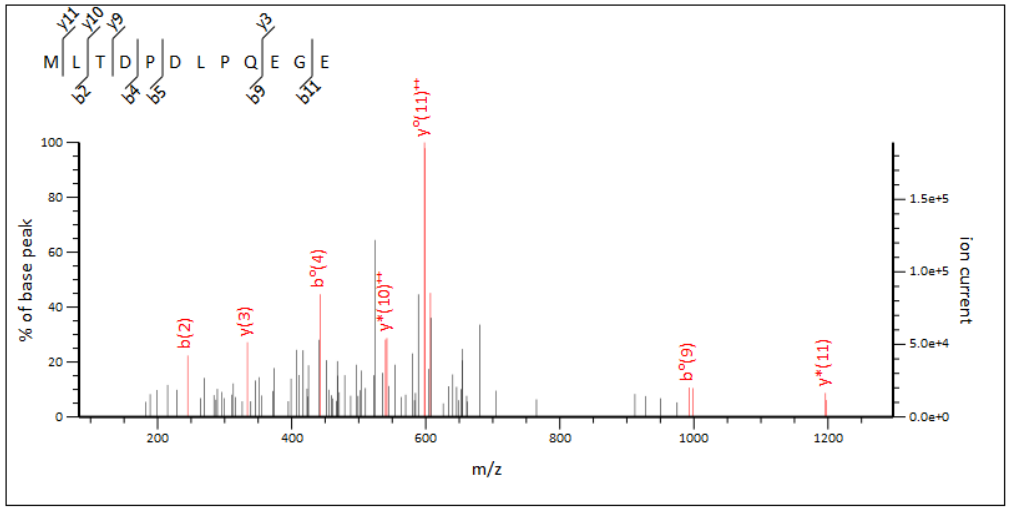
P.21 Observed; 516.311, Mr(expt); 1545.9082, Mr(calc); 1546.7264

Peptide; **STASTVEWSTVVHSG**



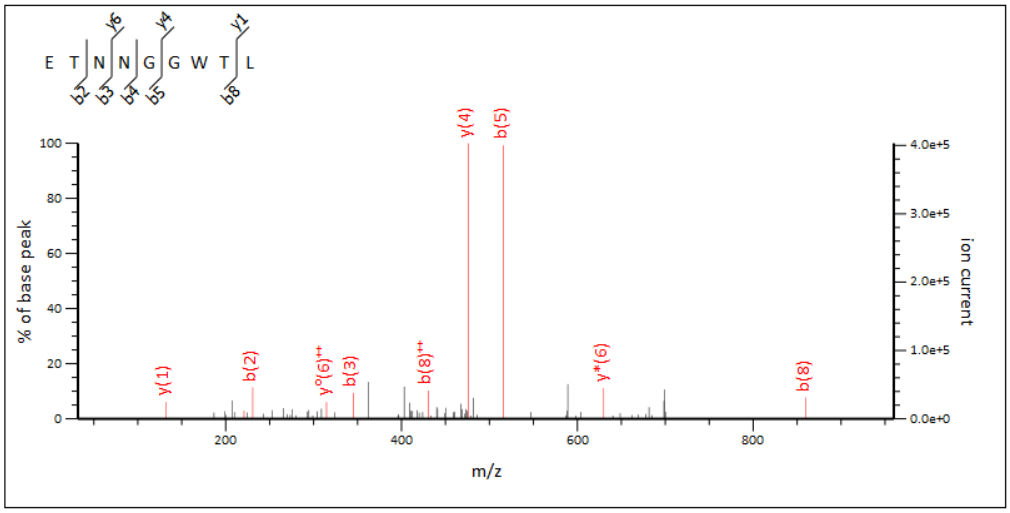
P.22 Observed; 672.8400, Mr(expt); 1343.6654, Mr(calc); 1343.5915

Peptide; **MLTDPDLPQEGE**



P.23 Observed; 496.2700, Mr(expt); 990.5254, Mr(calc); 990.4427

Peptide; **ETNNGGWTL**



VITA

Mister Phanuwat Prakot was born on April 23rd, 1988 in Yasothon, Thailand. He graduated with a Bachelor's degree of Science from Department of Biology, Faculty of Science, Chulalongkorn University in 2011. He had further studied to the Master Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University in 2012.

Academic presentations;

1. Prakot, P., Chaitanavisutti, N. and Karnchanatat, A. Effect of protein hydrolysate from spotted babylon Babylonia areolata on melanogenesis inhibition in B16F10 cell. 19th Biological Sciences Graduate Congress (BSGC), 12-14th December 2014, National University of Singapore, Singapore.

2. Prakot, P., Chaitanavisutti, N. and Karnchanatat, A. Melanogenesis and antityrosinase activity of protein hydrolysate from spotted babylon Babylonia areolata. The Joint 7th AOHUPO Congress and 9th International Symposium of the Protein Society of Thailand (7th AOHUPO/9th PST), 6-8th August 2014, Bangkok, Thailand.

3. Prakot, P., Chaitanavisutti, N. and Karnchanatat, A. In vitro anti-tyrosinase activity of protein hydrolysate from spotted babylon Babylonia areolata. The 2nd International conference on Food and Applied Bioscience, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand.