โปรตีนไฮโดรไลเสตถั่วเขียวงอก: ฤทธิ์ต้านออกซิเดชันและการป้องกันออกซิเดชันของไขมัน

นายเสกสรร วงศ์ศิริ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาเทคโนโลยีทางอาหาร ภาควิชาเทคโนโลยีทางอาหาร

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2554

GERMINATED MUNG BEAN PROTEIN HYDROLYSATE: ANTIOXIDANT ACTIVITIES AND PREVENTION OF LIPID OXIDATION

Mr. Seksan Wongsiri

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Food Technology
Department of Food Technology
Faculty of Science
Chulalongkorn University
Academic Year 2011

Thesis Title	GERMINATED MUNG BEAN PROTEIN HYDROLYSATE: ANTIOXIDANT ACTIVITIES AND PREVENTION OF LIPID OXIDATION
	Mr. Seksan Wongsiri
Field of Study	Food Technology
Thesis Advisor	Assistant Professor Kiattisak Duangmal, Ph.D.
Thesis Co-advisor	Professor Toshiaki Ohshima, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

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

THESIS COMMITTEE

…….. Chairman
(Assistant Professor Romanee Sanguandeekul, Ph.D.)

……. Thesis Advisor
(Assistant Professor Kiattisak Duangmal, Ph.D.)

…….. Thesis Co-advisor
(Professor Toshiaki Ohshima, Ph.D.)

…….. Member
(Professor Pranee Anprung, Ph.D.)

…….. Member
(Associate Professor Alisa Vangnai, Ph.D.)

…….. Member
(Thanachan Mahawanich, Ph.D.)

…….. External Member
(Associate Professor Prapan Pinsirodom, Ph.D.)

เสกสรร วงศ์ศิริ: โปรตีนไฮโดรไลเสตถั่วเขียวงอก: ฤทธิ์ต้านออกซิเดชันและการป้องกันออกซิเดชันของไขมัน (GERMINATED MUNG BEAN PROTEIN HYDROLYSATE: ANTIOXIDANT ACTIVITIES AND PREVENTION OF LIPID OXIDATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.เกียรติศักดิ์ ดวงมาลย์ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: Professor Toshiaki Ohshima, Ph.D., 114 หน้า.

เมื่อเพาะถั่วเขียวในที่มืดที่อุณหภูมิห้อง (30±2^{O}C) เป็นเวลา 0, 6, 12, 18 และ 24 ชั่วโมง พบว่าระยะเวลาในการงอกเพิ่มขึ้นส่งผลให้ปริมาณโปรตีนทั้งหมดในถั่วเขียวเพิ่มขึ้นอย่างมีนัยสำคัญ ($p<0.05$) ถั่วเขียวที่เพาะที่ 24 ชั่วโมงมีปริมาณโปรตีนทั้งหมดสูงที่สุดแต่ไม่แตกต่างอย่างมีนัยสำคัญเมื่อเทียบกับปริมาณโปรตีนทั้งหมดในถั่วเขียวที่เพาะที่ 18 ชั่วโมง อย่างไรก็ตามระยะเวลาในการเพาะไม่มีผลต่ออัตราส่วนของปริมาณกรดอะมิโนแต่ละชนิดต่อปริมาณกรดอะมิโนทั้งหมดในถั่วเขียวงอก ปริมาณสารประกอบฟีนอลิคทั้งหมดในถั่วเขียวเพิ่มขึ้นอย่างมีนัยสำคัญ ($p<0.05$) เมื่อระยะเวลาในการเพาะเพิ่มขึ้น ถั่วที่เพาะที่ 24 ชั่วโมงมีปริมาณสารประกอบฟีนอลิคทั้งหมดสูงที่สุด (5.78 mg gallic acid/g ตัวอย่างแห้ง) โดยปริมาณดังกล่าวเพิ่มขึ้นเป็นสองเท่าเมื่อเทียบกับถั่วที่เพาะที่ 0 ชั่วโมงซึ่งมีปริมาณสารประกอบฟีนอลิคทั้งหมด 2.81 mg gallic acid/g ตัวอย่างแห้ง ในการทดลองนี้เลือกถั่วที่เพาะที่ 18 ชั่วโมงเป็นวัตถุดิบในการผลิตโปรตีนไฮโดรไลเสต โดยให้ถั่วที่เพาะที่ 0 ชั่วโมงเป็นชุดการทดลองควบคุม ถั่วเขียวงอกถูกย่อยด้วยเอนไซม์ Flavourzyme® ความเข้มข้น 0, 1, 3 และ 5% w/w โดยน้ำหนักแห้งของถั่ว เป็นเวลา 0, 1, 2, 3, 4 และ 5 ชั่วโมง ที่อุณหภูมิ 50^{O}C pH 6.0 ผลการทดลองพบว่าเมื่อปริมาณเอนไซม์ที่ใช้และระยะเวลาในการย่อยเพิ่มขึ้นระดับการย่อยของถั่วเขียวที่เพาะที่ 0 และ 18 ชั่วโมงเพิ่มขึ้นอย่างมีนัยสำคัญ ($p<0.05$) ระดับการย่อยมีผลต่อปริมาณหมู่อะมิโนอิสระและปริมาณสารประกอบฟีนอลิคทั้งหมดในโปรตีนไฮโดรไลเสตที่ได้จากทุกๆ ภาวะการย่อยในถั่วที่เพาะที่ 0 และ 18 ชั่วโมงอย่างมีนัยสำคัญ ($p<0.05$) การย่อยถั่วที่เพาะที่ 0 และ 18 ชั่วโมงด้วยเอนไซม์ Flavouzyme®5% w/w โดยน้ำหนักแห้งของถั่ว เป็นเวลา 5 ชั่วโมงให้ปริมาณหมู่อะมิโนอิสระและปริมาณสารประกอบฟีนอลิคทั้งหมดสูงที่สุด เมื่อเทียบกับภาวะในการย่อยอื่นในถั่วที่เพาะในเวลาเดียวกัน ผลการทดลองแสดงให้เห็นว่ามีความสัมพันธ์เชิงเส้นที่สูงในทางบวกระหว่างระดับการย่อยและปริมาณหมู่อะมิโนทั้งหมดในโปรตีนไฮโดรไลเสตที่ได้จากถั่วที่เพาะที่ 0 และ 18 ชั่วโมง โดยมีค่า R^2 เท่ากับ 0.920 และ 0.997 ตามลำดับ นอกจากนี้ยังพบความสัมพันธ์เชิงเส้นที่สูงในทางบวกระหว่างระดับการย่อยกับปริมาณสารประกอบฟีนอลิคทั้งหมดในโปรตีนไฮโดรไลเสตที่ได้จากถั่วที่เพาะที่ 0 และ 18 ชั่วโมง โดยมีค่า R^2 เท่ากับ 0.971 และ 0.962 ตามลำดับ เมื่อวัดฤทธิ์ต้านออกซิเดชันของโปรตีน ไฮโดรไลเสตจากถั่วเขียวงอกที่ได้ด้วยวิธีทางเคมี โดยวัดการกำจัดอนุมูลอิสระของ 1,1-diphenyl-2-picrylhydrazyl (DPPH), การวัด ferric reducing antioxidant power (FRAP), และ การวัด metal chelating activity และวิธี electron spin resonance (ESR) โดยวัดการกำจัดอนุมูลอิสระของ DPPH, อนุมูลอิสระของ hydroxyl และอนุมูลอิสระของ carbon-centered พบว่า ฤทธิ์ต้านออกซิเดชันของโปรตีนไฮโดรไลเสตจากถั่วที่เพาะที่ 0 และ 18 ชั่วโมง ซึ่งวัดด้วยทั้งสองวิธีมีค่าเพิ่มขึ้นเมื่อระดับการย่อยเพิ่มขึ้น โดยโปรตีนไฮโดรไลเสตจากถั่วที่เพาะที่ 18 ชั่วโมงจะมีฤทธิ์ต้านออกซิเดชันสูงกว่าโปรตีนไฮโดรไลเสตที่ได้จากถั่วที่เพาะที่ 0 ชั่วโมง เมื่อวิเคราะห์กรดฟีนอลิคที่มีฤทธิ์ต้านออกซิเดชันที่พบในโปรตีนไฮโดรไลเสตจากถั่วเขียวงอกที่เพาะที่ 0 และ 18 ชั่วโมงด้วยวิธี HPLC-DPPH online พบกรดแกลลิค และสารประกอบอื่นที่ไม่สามารถระบุชนิดได้ จากผลการทดลองข้างต้นแสดงว่าฤทธิ์ต้านออกซิเดชันของโปรตีนไฮโดรไลเสตจากถั่วเขียวงอกเป็นผลมาจากหมู่อะมิโนอิสระและสารประกอบฟีนอลิคทั้งหมดในโปรตีนไฮโดรไลเสต

ภาควิชา เทคโนโลยีทางอาหาร ลายมือชื่อนิสิต.....

สาขาวิชา เทคโนโลยีทางอาหาร ลายมือชื่ออาจารย์ที่ปรึกษาหลัก.....

ปีการศึกษา 2554 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

5073893523: MAJOR FOOD TECHNOLOGY
KEYWORDS: GERMINATED MUNG BEAN / PROTEIN HYDROLYSATE / ANTIOXIDANT
SEKSAN WONGSIRI: GERMINATED MUNG BEAN PROTEIN HYDROLYSATE: ANTIOXIDANT ACTIVITIES AND PREVENTION OF LIPID OXIDATION.
ADVISOR: ASST. PROF. KIATTISAK DUANGMAL, Ph.D., CO–ADVISOR: PROF. TOSHIAKI OHSHIMA,Ph.D., 114 pp.

Mung bean seeds were germinated, at 30±2^{O}C, in the dark for 0, 6, 12, 18 and 24 h. Crude protein content of germinated mung bean significantly ($p<0.05$) increased during germination period. The 24 h-germinated mung bean (24 H-GMB) had the highest protein content but not significantly different compared to that of 18 h-germinated mung bean (18 H-GMB). However, this germination period did not affect the ratio of each amino acid to total amino acids in terms of content. Total phenolic content (TPC) of germinated mung bean significantly increased during germination period ($p<0.05$). The 24 H-GMB showed the highest TPC, amounting to 5.78 mg gallic acid g^{-1} db, the value doubled increased compared to 0 h-germinated mung bean (0 H-GMB), amounting to 2.81 mg gallic acid g^{-1} db. The 18 H-GMB was chosen as a raw material for protein hydrolysate production and 0 H-GMB was used as the control. These samples were hydrolyzed at 50^{O}C, pH 6.0. Four concentrations of Flavourzyme® (0, 1, 3, and 5% by dried weight of the germinated mung bean) were useds and hydrolysis periods were 0, 1, 2, 3, 4 and 5 h. The results showed that degree of hydrolysis (DH) increased with increasing in both Flavourzyme® concentration and hydrolysis period in both 0 H-GMB and 18 H-GMB. The DH significantly affected the amount of free amino group (FAG) and TPC in all hydrolysate samples obtained from both 0 H-GMB and 18 H-GMB ($p<0.05$). Hydrolysis of 0 H-GMB and 18 H-GMB with 5% Flavourzyme® for 5 h gave the highest FAG and TPC compared to other conditions in its group. There was a positive linear correlation between DH and FAG (R^2 = 0.920 for 0 H-GMB protein hydrolysate and R^2 = 0.997 for 18 H-GMB protein hydrolysate) and between DH and TPC (R^2 = 0.971 for 0 H-GMB protein hydrolysate and R^2 = 0.962 for 18 H-GMB protein hydrolysate). Antioxidant activities of the protein hydrolysate were measured using chemical methods including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP), and metal chelating activity, and using electron spin resonance (ESR) method for DPPH radical scavenging activity, hydroxyl radical scavenging activity and carbon-centered radical scavenging activity. It was found that antioxidant activities of the germinated mung bean protein hydrolysates, measured using both methods, increased when DH increased. This trend was observed in both of the 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. Higher antioxidant activity was found in the 18 H-GMB protein hydrolysate compared to that obtained from 0 H-GMB protein hydrolysate. Phenolic acid in the freeze dried germinated mung bean hydrolysate was determined by HPLC with an online DPPH radical scavenging activity system. The results showed that antioxidative phenolic acid found in the 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate was gallic acid and certain unidentified compounds. The above results showed that FAG and TPC contribute antioxidant activities of germinated mung bean protein hydrolysates.

Department: Food Technology Student's Signature……...…

Field of Study: Food Technology Advisor's Signature ……...

Academic Year: 2011 Co-advisor's Signature ……..……

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude my advisor, Assistant Professor Dr. Kiattisak Duangmal and my co-advisor Professor Dr. Toshiaki Ohshima for their valuable suggestion and great kindness throughout my doctoral degree pursuit.

I am deeply grateful to Assistant Professor Dr. Rommanee Sanguandeekul, Professor Dr. Pranee Arnpreang, Dr. Thanachan Mahawanich, Associate Professor Dr. Alisa Wangnai and Associate Dr. Prapan Pinsirodom for constructive comments as thesis committees.

Sincerest appreciation also extends to the Higher Education Commission for financial support of this research, and Chinat Field Crops Research for supplying the mung bean seeds.

A special thank you is extended to my best friends, all at Chulalongkorn University and Tokyo University of Marine Science and Technology who always give encouragements and invaluable helps, no matter I might be. Their friendship will be forever cherished. Various others friends are appreciated for their indispensable and crucial help. A hearty and special thank you also goes to them.

Finally, I wish to acknowledge the support, encouragement and love of my family and my friends throughout my Ph.D. program.

CONTENTS

PAGE

LIST OF TABLE

TABLE PAGE

LIST OF FIGURE

FIGURE PAGE

FIGURE PAGE

CHAPTER I
INTRODUCTION

Mung bean (*Vigna radiate*) is one of the important legumes in Thailand. Its seed has been used for making mung bean vermicelli, mung bean sprout and traditional Thai desserts. As mung bean contains high protein content, 26.75- 27.70% on a dry weight basis, it could be used as a protein source in human diets. Since mung bean seed contains some of anti-nutritional substances such as trypsin inhibitors, tannin and inosital phosphates, raw seeds are needed to be processed before consumption. It has been shown that germinated seeds are rich in nutritive values and the germination process is one of the methods to reduce anti-nutritional substances. In addition, germination causes an increase in protein content and bioactive compounds (i.e., phenolic compounds, vitamins C and E). Compared to the ungerminated mung bean seed proteins, the proteins of germinated seed are different in physical, chemical and functional properties.

To improve the functional properties and to increase alternative uses of protein, enzymatic modification of protein is an effective way. The peptides obtained from partial hydrolysis of proteins have smaller molecular size and simpler structure than the original ones. In addition, hydrolysis of proteins causes changes in protein structure such as an increase in the number of charged groups and exposure of reactive groups. These factors affect some physical, chemical and functional properties of proteins, including antioxidation properties. Some proteins have ability to inhibit lipid oxidation through multiple pathways including inactivation of reactive oxygen species, scavenging of free radicals, chelation to prooxidative transition metals, reduction of hydroperoxides, and alteration of physical properties of the food systems. However, the antioxidant activity of proteins is limited by the tertiary structure of the polypeptide, since many amino acids with antioxidation potentials are buried within the protein core where they are inaccessible to prooxidants. One approach to increase a protein's overall antioxidant activity is through tertiary structure disruption by enzymatic hydrolysis, which may potentially increase the

solvent accessibility of oxidatively labile amino acids. Protein hydrolysate has been reported to have good potential of being used as an antioxidant in food. The ability of proteins to inhibit lipid oxidation increases their uses as an endogenous antioxidant in foods. There has been some information on legume protein hydrolysate but no report on germinated legume protein hydrolysate. In this research, germinated mung bean seeds at different germination time will be hydrolyzed at different degree of hydrolysis. The properties of hydrolyzed protein will be characterized. The ability of mung bean protein hydrolysates to inhibit lipid oxidation in an oil-in-water emulsion model system will be studied. The knowledge on physical, chemical, functional properties and antioxidant activities of mung bean protein hydrolysates obtained from this research is essential for an assessment on the utilization of mung bean protein hydrolysate in food industry.

CHAPTER II
LITERATURE REVIEW

1. Mung bean

Mung bean (*Vigna radiate*) is one of the most important legumes in Thailand. Mung bean can be used in a wide variety of food preparations due to its good and inexpensive sources of protein. As mung bean contains high protein content, 26.75-27.70% db. (Mubarak, 2005; Lin and Lai, 2006; Ghavidel and Prakash, 2007), it can be considered as a potential protein source in human diets. The major mung bean storage proteins are the globulins legumin and vicilin (Bau *et al*., 1997).

Mung bean protein is rich in essential amino acids (such as aromatic amino acids, leucine, isoleucine and valine) and glutamic acid (Shehata and Thannoun, 1980; El-Moniem, 1999; Tang, Chen and Ma, 2009). However, threonine, tryptophan and sulfur-containing amino acids (methionine and cysteine) notably methionine which is the first limiting amino acids in legumes (Tang *et al*., 2009) are slightly deficient in mung bean protein (Mubarak, 2005). Moreover, low digestibility limits the utilization of mung bean. This has been attributed to many factors, including the presence of anti-nutritional factors such as protease inhibitors, lectins, phytates, polyphenols, tannins and inositol phosphate. Raw seeds are needed to be processed before consumption (Kuo et al., 2004; Tang *et al*, 2009).

It has been shown that the germination process is one of the methods to reduce anti-nutritional substances. Germination induces the formation of enzymes that eliminate or reduce the anti-nutritional and indigestible factors in legumes (Mubarak, 2005). Protease K1, B1 and B2 are essentially absent from dry soy bean seeds but increases greatly during germination. Their activity initiates the degradation of both Kunitz and Bowman-Birk soy bean trypsin inhibitors in germinated soy beans (Mc Grain *et al*., 1989; Papastoitsis and Wilson, 1991). The phytase activity of lupine seeds was reported to increase from 0.017 to 0.097 units per gram after germination for 4 days, a 5.7-fold increase in phytase activity compared with dry seed (Greiner, 2002). Mubarak (2005) found that germination at room temperature (25^{O}C) for 12 h can reduce many anti-nutritional factors of mung been seed e.g. trypsin inhibitors

(22.4% reduction), tannins (66.7% reduction) and phytic acid (30.5% reduction). Moreover, germination for 12 h was more effective in reducing tannins and phytic acid than boiling at 100^{O}C for 90 min (This boiling process can reduce only 45.5% of tannins and 25.8% of phytic acid). After germination in the dark at room temperature (25^{O}C), seed of *Erythrina americana* exhibited a decreased in trypsin inhibitors content from 85.18 to 1.18 TUI per mg (db.) and tannins from 0.14 to 0.03 g per 100 gram (db.) (Sotelo *et al*., 2003).

Germinated seeds are normally rich in nutritive values. Germination may cause changes in the nutrients, including secondary metabolites, through aerobic respiration and biochemical metabolism (Lin and Lai, 2006). It also causes an increase in protein content. This increase may be due to a synthesis of enzymes and proteins during germination or a compositional change following the degradation of other constituents (Bau *et al*., 1997). After germination at room temperature (25^{O}C) for 12 h, crude protein in mung bean seed increased from 27.5 to 30.0 g per 100 gram bean compared to ungerminated seed (Mubarak, 2005). Ghavidel and Prakash (2007) also found an increase in crude protein in mung bean from 27.7% in raw seed to 29.1% after germinated for 24 h.

In natural environment, seed-sprouting survives during germination by enhancing its defensive response through phenolic biosynthesis and phenolic compounds considering beneficial as antioxidants (Randhir, Lin and Shetty, 2004; Lin and Lai, 2006). Fernandez-Orozco *et al*. (2008) found that germination of *Vigna radiata cv. emerald* caused an increase in total phenolic compounds from 1.09 mg catechin per gram (db.) in raw seed to 3.46 mg catechin per gram (db.) after 7 days of germination. In addition, this 7 days of germination process caused a rise in vitamin C content from 1.86 mg per 100 gram (db.) in raw seed to 9.07 mg per 100 gram (db.) while α-tocopherol increased from 0.11 to 0.45 mg per 100 gram (db.).

2. Protein hydrolysate

Food proteins in their natural state do not necessarily possess optimal nutritional and functional properties. The modification of proteins by proteolysis might have a good chance of becoming attractive means of altering the structure and improving functional properties of proteins.

2.1 Definition of protein hydrolysate

Protein hydrolysate can be defined as protein that chemically or enzymatically broken down into peptides of varying sizes. The obtained peptides have smaller molecular size and simpler structure than the original proteins. Hydrolysis of proteins also causes many changes in protein structure such as an increase in the number of charged groups and exposure of reactive groups. These changes affect some functional properties of proteins such as antioxidant activities, solubility, foaming and emulsion properties. Protein hydrolysates are produced and widely used in the food industry, including milk replacers, protein supplements, and stabilizer in beverages (Kristinsson and Rosca, 2000).

To follow the reaction kinetics and measure the extent of the hydrolysis, a parameter named degree of hydrolysis is employed. It is most commonly used to describe hydrolysis of food proteins. The degree of hydrolysis (DH), defined as the ratio of the number of peptide bonds broken to the total number of bonds per unit weight (Shahidi, Han and Synowiecki, 1995). The advantage of using DH as a process parameter is that it appears to determine unambiguously the properties of a protein hydrolysate can be specified by DH while four process parameters a) protein substrate concentration, b) enzyme-substrate ratio, c) temperature and d) time can be left uncontrolled. From this, it is obvious that DH is a very simple and rapid method of measuring the extent of protein breakdown (Kristinsson and Rosca, 2000).

2.2 Protein hydrolysate preparation (Kristinsson and Rosca, 2000)

Both chemical and enzymatic methods are widely used for protein hydrolysate preparation. Chemical hydrolysis is more commonly used in industrial practices. Whilst biological processes, using added enzymes are employed because it results in products of high functionality and nutritive value.

2.2.1 Chemical methods for protein hydrolysis

Chemical hydrolysis of proteins is achieved by cleaving peptide bonds with either acid or base. This method has been a good choice in the past for the industry because it is relatively inexpensive and simple to conduct. However, there

are many limitations to food ingredients using this method. Protein hydrolysis with strong chemicals and solvents is performed at extreme temperature and pH and generally yield products with reduced nutritional qualities, poor functionality and restricted to use as flavor enhancers. Moreover, chemical hydrolysis tends to be a difficult process to control and almost invariably leads to products with variable composition and functional properties.

Acid hydrolysis: Acid hydrolysis of protein is used more commonly than hydrolysis under alkaline conditions. This process is still the preferred method for hydrolyzed vegetable proteins which are widely used for flavor and taste enhancement properties. Acid hydrolysis of protein has usually involved reacting proteins with hydrochloric acid, or in some case sulfuric acid; the proteins are completely hydrolyzed at high temperature, and sometimes high pressure. The hydrolysate is then neutralized to pH 6.0 to 7.0 and concentrated to either a paste or further dried. Due to extensively hydrolyzed, its primary functional property is high solubility. In addition, following the neutralization, the hydrolysate contains large amount of sodium chloride salt, which can make the product unpalatable and interferes with food functionality. Another drawback of acid hydrolysis is the destruction of tryptophan (Kristinsson and Rosca, 2000). Moreover, acid hydrolysis of protein with high concentration of hydrochloric acid and high temperature may produce 3-monochloropropane-1, 2-diol (3-MCPD) and 1-3-dichloro-2-propanol-2-dichlorohydrine (1-3-DCP) that are considered as the carcinogens (Light, 1974).

*Alkaline hydrolysis***:** The use of sodium hydroxide to hydrolyze protein often results in poor functionality and detrimental to the nutritive value of the hydrolysate. During alkaline hydrolysis of protein, rapid cleavage to large water-soluble polypeptides occurs, followed by further degradation at a slow rate. Draw backs of alkaline hydrolysis are 1) The production of D-amino acids, which are not absorbed by human. These are initiated by hydrogen abstraction from the alpha carbon of an amino acid and include racemization of L-amino acids, which produces D-amino acids. 2) Disulfide bonds are split with loss of cysteine, serine and threonine via β-elimination reactions and formations of lysinoalanine, ornithinoalanine, lanthionine and β-amino alanine can also occur. 3) Formation of toxic substances e.g.

lysinoalanine, that are highly undesirable in foods, may occur due to some of these elimination and addition reactions. 4) Alkaline hydrolysis reaction products have an inhibiting effect on proteolytic enzymes, reducing the rate of hydrolysis.

2.2.2 Enzymatic hydrolysis

Enzymes are introduced into this hydrolysis to improve or modify the physicochemical, functional and sensory properties of the native protein without decrease its nutritive value and protein absorption is usually improved. These enzymes based processes take place under mild conditions and do not produce hydrolytic degradation products via racemization reactions. This process does not result in formation of D-form amino acids and toxic substances. Under the reaction conditions employed and its substrate specificity, the process of using enzymes offers many advantages because it allows good control of the hydrolysis and the properties of the resulting products.

2.3 Factors affecting enzymatic hydrolysis

To produce protein hydrolysates using enzymatic hydrolysis of food protein, the hydrolytic process and reaction conditions vary depending on substrates and enzymes used and also the properties of desired protein hydrolysate.

2.3.1 Type of enzyme

To produce protein hydrolysates, enzymatic hydrolysis is performed by utilizing enzyme to hydrolyze peptide bonds. This results in a mixture of peptides of different molecular size and free amino acids. This process may occur via proteolytic enzymes already present in the raw material (endogenous proteases) or by adding enzyme from other sources (Kristinsson and Rosca, 2000). The ability of enzymes to hydrolyze a protein substrate is highly varied. Therefore, the selection of suitable enzymes to produce compounds with defined physicochemical and nutritional characteristics is essential. Proteolytic enzymes are classified by their hydrolysis mechanism into endopeptidases and exopeptidases. Endopeptidases hydrolyze the peptide bonds within protein molecules at random to produce relatively large peptides whilst exopeptidases systematically remove amino acids from either the N-terminus

or the C-terminus by hydrolyzing the terminal peptide bonds (Clemente, 2000). Although many factors affect the yields of the hydrolysates, the type of enzyme used had a marked effect on the yield and properties of the final product (Xie, Wang and Xu, 2007).

A. Endogenous enzyme

Enzymatic production of protein hydrolysates can be carried out by employing an autolysis process. This process depends on the action of the endogenous enzymes of the raw material itself. These enzymes in autolysis hydrolysis are a complex mixture of enzymes with different optimum activity requirements which result in end products of different molecular weight profiles. Wu, Chen and Shiau (2003) prepared mackerel hydrolysates from an autolysis process by mixing 250 g of mackerel meat with 500 g of distilled water and homogenized for about 2 min. Then, the autolysis process was performed at 50^{o}C for 0, 5, 10, 15 and 25 h. They found that the constituents of free amino acids in mackerel hydrolysates changed in levels over 25 hours of autolysis at 50^{o}C. Free amino acids increased gradually with autolysis time. The amount of total free amino acids in the hydrolysate after 25 h increased at least twice compared to that of the pre-hydrolyzed sample. However, histidine increased in the first 10 h, but decreased thereafter.

Capelin *(Mallotus villosus)* was hydrolysed using autolysis by endogenous enzymes in fish viscera. Whole fish were ground and homogenized then mixed with an equal amount of water and homogenized in a Waring blender for about 2 min. The pH of the mixture was adjusted to 3.0. The hydrolysis was performed at 25^{o}C for 2 h. The pH of the mixture was kept constant by continuous addition of a 4 M NaOH solution to the reaction mixture. The reaction was stopped by heating for 10 min at 70°C in order to inactivate the enzymes. The protein recovery from autolysis was only 22.9% compared to 70.6% protein recovery of the protein hydrolysate prepared by using Alcalase at the enzyme-substrate ratio ([E]/[S]) of 30 AU/kg, pH 8.5, 45-65^{o}C with the same hydrolysis period of autolysis (Shahidi *et al.*, 1995).

Autolysis methods and chemical methods often results in a final product with bad functionality. Despite these problems, endogenous proteolytic

enzymes are used to produce hydrolysed products, specifically fish sauces and fish silage (Kristinsson and Rosca, 2000).

B. Commercial enzyme

A wide variety of commercial enzymes have been used successfully to hydrolyse food proteins. Proteolytic enzymes from plants and microorganisms are always used to prepare protein hydrolysates. One of the common characteristics of enzymes using for protein hydrolysate is that they have to be food grade, and, if they are of microbial origin, the producing organism has to be non-pathogenic (Kristinsson and Rosca, 2000). Buckwheat (*Fagopyrum esculentum* Moench) protein isolate was enzymatic hydrolysed using Alcalase® 2.4 L. The hydrolysis resulted in remarkable decrease in the globulins or protein aggregates and concomitant increase in peptide fragments. The surface hydrophobicity of the hydrolysates decreased with increasing degree of hydrolysis (DH) and reached a minimum at DH 15%, but increased at further hydrolysis, whereas their amino acid compositions were unchanged. The hydrolysates exhibited excellent antioxidant activities, including DPPH radical scavenging ability, reducing power and ability to inhibit linoleic acid peroxidation (Tang, *et al*., 2009). Zhang, *et al*. (2011) prepared rice endosperm protein hydrolysate using five commercial protease (Alcalase®, Chymotrypsin®, Neutrase®, papain, and Flavorase®) to produce the antioxidative peptide. The DH of rice endosperm protein by Neutrase® was slightly lower than that of Chrymotrypsin®, but higher than those of other enzymes. The protein hydrolysate prepared using Neutrase® had DPPH radical scavenging activity similar to α-tocopherol and the reaction condition of Neutrase® hydrolysis was moderate (pH of 7.0 and temperature of 37 ^{o}C). Therefore, Neutrase® was chosen to be the suitable enzyme for producing the antioxidative peptide from rice endosperm protein.

2.3.2 Hydrolysis condition

The enzymatic hydrolysis processes and reaction conditions differ between different substrates and enzymes used and also depend on the properties desired for the hydrolysate. There are many process parameters affect the hydrolysis of food protein with enzyme.

2.3.2.1 pH

A change in pH affects both the substrate and enzyme by changing the charge distribution and conformation of the molecules. Most enzymes undergo irreversible denaturation in a high acid and alkaline solution, causing the loss of stability. The pH also affects the ionization of prototrophic groups which are involved in maintaining proper conformation of the active site of enzyme, binding of substrate to enzyme, and transforming substrate to product (Whitaker, 1994).

Diniz and Martin (1996) used the response surface methodology to describe the combined effects of pH, temperature and enzyme-substrate ratio on the hydrolysis of dogfish (*Squalus acanthias*) muscle with Alcalase®. They found that the pH was a one of hydrolysis factors that had a strong influence on degree of hydrolysis of dogfish muscle. The optimum pH that gave the maximum degree of hydrolysis (18.8%) was 8.3. In the hydrolysis of dogfish protein, an increase in degree of hydrolysis was achieved by increasign pH up to a certain level. Beyond this pH value, degree of hydrolysis slightly decreased. Such decrease in the percentage hydrolysis over the high and lower pH values is explained by increasing denaturation of the protease, reducing its biological activity. This result corroborate well with the results of Bhaskar *et al*. (2008) who studied on the optimization of enzymatic hydrolysis of visceral waste proteins of Catla (*Catla catla*) for preparing protein hydrolysate using Alcalase® by response surface methodology. They found that pH is the one of the independent variables that had relatively higher significant effect on degree of hydrolysis of visceral waste proteins of Catla. Degree of hydrolysis increased with an increase in substrate pH up to pH 8.5, beyond this point, degree of hydrolysis decreased.

Benjakul and Morrissey (1997) studied on effect of pH value on the activity of Alcalase® and Neutrase® to prepare Pacific whiting solid wastes protein hydrolysates. They found that the optimum pH values for Alcalase® and Neutrase® on Pacific whiting solid wastes protein hydrolysis were 9.5 and 7.0, respectively. Alcalase® showed a broad activity in alkaline pH range. However, a sharp decrease in activity was observed at pH 11.5 because this enzyme was more active at alkaline pH and remained active to pH 6.0. For Neutrase®, the activity reached the maximum at

pH 7.0. The activity was high in pH range of 6.5-8.5 but showed considerable loss of activity at pH 10.5.

2.3.2.2 Temperature

Nilsang *et al*. (2005) used Kojizyme^{TM} for a study on optimization of enzymatic hydrolysis of fish soluble concentrate regarding to the effect of temperatures (45, 50, 55 and 60^{o}C), time (1, 2, 3, 4, 5 and 6 h) and enzyme concentrations (1, 2, 3, 4 and 5% w w^{-1}) on the degree of hydrolysis of 20% w w^{-1} solid content fish soluble concentrate. The result found that hydrolysis using 5% Kojizyme^{TM} at 50^{o}C for 6 h gave the highest degree of hydrolysis (68%). All three variables had a linear effect on the degree of hydrolysis values. Temperature was the most important linear variable affecting the degree of hydrolysis values because it had the highest regression coefficient.

Xie *et al*. (2007) used response surface methodology to optimize *Bellamya purificata* snail foot protein hydrolysis using Proleather FG-F. Regression coefficient and the response surfaces were used to study the effect of pH, enzyme-substrate ratio and temperature on Proleather FG-F hydrolysis. The results showed that the temperature had both linear and quadratic effects to degree of hydrolysis and protein recover. This suggested that the amount of degree of hydrolysis and protein recovery increased until temperature reached an optimum point (58.5^{o}C) and then decreased. The protein recovery response was more significantly influenced by temperature than degree of hydrolysis. That was probably because temperature significantly affected the solubility of substrate.

2.3.2.3 Hydrolysis time

The enzymatic hydrolysis of food proteins is characterized by an initial rapid phase, during which a large number of peptide bonds are hydrolyzed. After this rate, enzymatic hydrolysis decreases and reaches a stationary phase. The shape of the hydrolysis curve has been associated with enzyme inactivation, product inhibition by hydrolysis products formed at high degree of hydrolysis, a low K_m value for the soluble peptides that act as effective substrate competitors to the unhydrolysed food protein and possibly auto digestion of the enzyme (Kristinsson and Rosca, 2000).

Nilsang *et al*. (2005) studied on optimization of enzymatic hydrolysis of fish soluble concentrate regarding to the effect of temperature, time and enzyme concentration on the degree of hydrolysis of 20% w w^{-1} solid content fish soluble concentrate. They found that the largest value of estimated regression coefficient for time indicated that it was the most important linear variable influencing the degree of hydrolysis values. The positive value pointed that the degree of hydrolysis values increased with increasing hydrolysis time. This result was similar to that reported by Kong, Zhou and Quain, 2007) who studied on enzymatic preparation and functional properties of wheat gluten hydrolysates. Wheat gluten was enzymatically hydrolyzed by several commercially available proteases (Pancreatin Trypsin 6.0S, Pancreatin and Alcalase® 2.4L) with the enzyme-substrate ratio of 1:100 w w^{-1} and 6 h hydrolysis time at the optimum pH and optimum temperature of each enzymes. The hydrolysis of wheat gluten with Alcalase, trypsin and Pancreatin proceeded at a rapid rate during the initial 30 minutes and then slowed down thereafter. Degree of hydrolysis values varied from 0% to 15.8% after 360 min of incubation, depending upon the enzyme used.

Moreover, Lin and Li (2006) found that the hydrolysis time of Jumbo flying squid (*Dosidicus eschrichitii* Steenstrup) skin gelatin with properase E (at pH 9.0, 45^{o}C and enzyme-substrate ratio of 1:50 for 6 h) affect the hydroxyl radical scavenging activity and superoxide radical scavenging activity of gelatin hydrolysates. The radical scavenging activity of gelatin hydrolysates gradually increased and reached a maximum in 3 hours; it was then kept at a constant value with increasing hydrolysis time.

2.3.3 Substrate

Enzymatic hydrolysis has been employed on a variety of different food proteins derived from livestock, poultry meat, milk and plant.

2.3.3.1 High protein content source

Animal and its products like milk and egg are the most important food protein source used in the production of protein hydrolysates because of their high protein content.

Herring (*Clupea harengus*) was hydrolysed using Alcalase® 2.4L, an endopeptidase preparation from *Bacillus licheniformi*, to produce fish protein hydrolysate. A 500 g portion of herring was mixed with an equal volume of distilled water, and homogenized in a blender for about 2 min. The mixture was stirred and adjusted to pH 8.0 and 50 °C for optimal enzyme activity. The enzyme was added at 0.5% w w^{-1} of the protein content in the mince. The mixture was continuously stirred for 60 min, and the enzyme activity was then terminated by increasing the temperature to above 85 °C for 15 min. The hydrolysed fish was centrifuged at 16,300 × *g* for 15 min. After decantation and removal of sludge, the soluble fraction was freeze-dried. The hydrolysis was yielding fish protein hydrolysate with 36% degree of hydrolysis. The herring hydrolysate presented good emulsifying stability (> 120 min) and an adequate foam expansion (142%), as compared to the soluble fraction from the unhydrolyzed control herring (Liceaga-Gesualdo and Li-Chan, 1999).

Egg-yolk protein hydrolysates were prepared by enzymatic hydrolysis of fat-free egg-yolk protein. The yolk-protein was dissolved in water at a concentration of around 20% and heat-treated at 90°C before enzyme digestion. Orientase (EC 3.4.21.62) and protease (EC 3.4.11.12) were sequentially used at pH 10 and 50°C. The hydrolysis reaction was stopped after 6 h by heating to 90°C for 5 min. The soluble fraction was then filtered and spray-dried. The main peak of the molecular mass distribution of egg-yolk protein hydrolysates was lower than 1,000 Da. The average chain length of the egg-yolk protein hydrolysates was 2.6 Da. Egg-yolk protein hydrolysates showed strong antioxidant activity in a linoleic acid oxidation system as compared with the egg-yolk protein or amino acids mixture in which egg-yolk protein hydrolysates were constituted (Sakanaka *et al*., 2004).

Mechanically deboned turkey residue (MDTR) was used to prepare protein hydrolysate by enzymatic hydrolysis. Five hundred grams of MDTR (19% protein) were added to 1 litre of water held at 60°C. The mixture was allowed to reach a temperature of 60°C then papain was added with the enzyme-substrate ratio 1:250 w w^{-1}. The mixture was continuously stirred with a mechanical stirrer for 120 min. At the end of the hydrolysis, the hydrolysate was filtered through a metal screen,

then through multiple layers of cheesecloth, and centrifuged at 3500 x *g* for 10 min to remove all solid particles and fat. The protein hydrolysate was heated to about 95°C for at least 15 min to inactivate the enzyme, and then allowed to cool to room temperature (20-22°C). Any fat layer on the surface of the liquid hydrolysate was siphoned off. The bulk of the hydrolysate was then freeze-dried. The degree of hydrolysis of the final protein hydrolysate was between 65-70%, with molecular masses lower than 6.5 kDa. The protein hydrolysate was very soluble at all pH range from 2-10, with the nitrogen solubility index values greater than 90%. The mean emulsion capacity (EC) of the protein hydrolysate at pH 4, 7 and 10 were 175.2, 172.1 and 183.7 ml oil per gram of hydrolysate, respectively (Fonkwe and Singh, 1996).

2.3.3.2 Low protein content source

Plant proteins are alternative to proteins from animal sources. Many studies have shown the interest in plant protein hydrolysates as functional foods and flavor enhancers. According to criteria of nutritional quality and cost, many plant sources have been investigated for the production of protein hydrolysates. Among plants, soybean is the most widely used. Other legumes such as peas and chickpeas are becoming increasingly important as a source of edible proteins with interesting functional and nutritional properties. Plant proteins need to be processed prior to enzymatic hydrolysis. The excellent properties of plant protein concentrates and isolates as substrates for proteolytic enzymes are well know. A high protein content and low level of polyphenols, sugars and protease inhibitors facilitate the control of the hydrolytic process, increasing the effectiveness of proteolytic enzymes and the yield of the process. The main drawback of plant protein hydrolysates with respect to animal protein hydrolysates is the low level of some essential amino acids, e.g. sulphur-containing amino acids in hydrolysates derived from legumes (Clemente, 2000).

Chickpea protein extract was hydrolyzed using pepsin. Enzyme hydrolysis increased soluble protein content (1.2 to 2-fold) and free radical scavenging activity (1.9 to 3-fold) of hydrolyzed chickpea protein extract. The hydrolysis almost unaffected protein hydrolysate potential in oil-in-water emulsion system and reduced its iron chelating capacity (1.3-fold). The majority of chickpea

proteins had pI between 4.5 and 5.5, and molecular weight (MW) between 15 and 40 kDa, while MW of their pepsin hydrolysis products ranged between 6.5 and 14.2 kDa (Arcan and Yemenicioglu, 2010). Wheat gluten protein hydrolysate was prepared using papain. The hydrolysate was separated based on the molecular weight of the peptides by membrane ultrafiltration (UF) with a molecular weight cut-off of 5 kDa into permeate (P) and retentate (5-K) fractions. These three fractions showed strong antioxidative activities in the linoleic acid oxidation system, and exhibited DPPH radical scavenging activity. The antioxidative activity of the P fraction was almost the same as that of vitamin E at pH 7.0. The molecular weight distribution of the P fraction was concentrated in 4.2 kDa (86.5%) after gel permeation chromatography fractionation using an HPLC system.The P and 5-K fractions had higher surface hydrophobicities at pH 7.0 compared with the hydrolysate. The resulting UF fractions were superior to the hydrolysate in terms of antioxidative activities (Wang *et al*., 2007).

3.Antioxidant activity of protein hydrolysate

The ability of proteins to inhibit lipid oxidation makes it a potential food antioxidant additive. Some proteins can inhibit lipid oxidation through multiple pathways including inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems. These proteins antioxidant mechanisms depend on amino acids composition. The most reactive amino acids tend to be those containing either nucleophilic sulfur-containing side chains (cysteine and methionine) or aromatic side chains (tryptophan, tyrosine, and phenylalanine) from which hydrogen is easily removed. Histidine's imidazole-containing side chain is also oxidatively labile. However, the antioxidant activity of these amino acids residues is limited by the tertiary structure of the polypeptide, since many amino acids with antioxidant potential do not expose to the surface (Elias, Kellerby and Deker, 2008). The hydrolysis is one of the possible ways to increase antioxidant activity of protein. Li *et al*. (2008) reported that chickpea protein hydrolyzed with Alcalase® (15% degree of hydrolysis) can inhibit the autoxidation of linoleic acid compared with untreated chickpea protein. In addition, it was found that the smaller size of chickpea

protein hydrolysate peptides, the greater the free radicals scarvenging effects. Tang *et al*. (2009) also found that after hydrolysis buckwheat protein isolate by Alcalase®, the antioxidant activities of buckwheat hydrolysate increase with increasing in degree of hydrolysis. The DPPH radical scavenging activity of buckwheat protein hydrolysate in term of IC_{50} values decreased from 0.78 to 0.65 mg ml-1 when degree of hydrolysis increased from 10 to 25%.

3.1 Lipid oxidation

Lipids in all food is triglycerides. It is a potential source of oxidative off-flavours in food. The spontaneous reaction of atmospheric oxygen with lipids, known as autoxidation, is the most common process leading to oxidative deterioration. Polyunsaturated fatty acids have the potential for decomposing by this process. When light and sensitizer such as chlorophyll are present, activation of oxygen to singlet oxygen may play a role in the initiation of oxidative deterioration. Alternatively, metals including iron or copper, or the enzyme lipoxygenase, may play a role in the process by with oxidative deterioration is initiated. The components formed in the initial stage of autoxidation are the hydroperoxide. Although hydroperoxide are involatile and odourless, they are relatively unstable compounds and they decompose either spontaneously or in catalysed reaction to form volatile aroma compounds, which are perceived as off-flavours. Free radicals are formed during lipid oxidation. These may also lead to a reduction of nutritional quality by react with vitamins, especially vitamin E, which is lost from food during its action as an antioxidant. The off-flavours that develop during lipid oxidation normally act as a warning that a food is no longer edible. Free radical produced by hydroperoxide decomposition many cause damage to protein, including enzyme, or to DNA and many also generate carcinogen (Gordon, 2001).

As a free radical reaction, autoxidation proceeds in three distinct steps (Figure 2.1). The first step is initiation in which lipid radical are formed from lipid molecules. Abstraction of a hydrogen atom by a reactive species such as a hydroxyl radical may lead to initiation of lipid oxidation. Secondary initiation by hemolytic cleavage of the hydroperxides is a relatively low energy reaction, and it is normally the main initiation reaction in edible oils. This reaction is commonly catalysed by

metal ions. After initiation, propagation reaction occurs in which one lipid radical is converted into a different lipid radical. These reactions commonly involve abstraction of a hydrogen atom from a lipid molecule or addition of oxygen to an alkyl radical. Propagation reactions occur rapidly compared with initiation reaction. At normal atmospheric pressure of oxygen, the reaction of alkyl radical with oxygen is very rapid, and the peroxy radicals are present at much higher concentration than the alkyl radicals.

Alkoxy radicals formed by hydroperoxide decomposition can decompose to release volatile hydrocarbons, alcohol or aldehydes that are no longer bound to the glycerol backbone when the fatty acid with present as a glyceride. Volatile aldehydes are particularly important as contributors to the aroma of oxidized oils.

3.2 Antioxidant

Antioxidant in food may be defined as any substance which is capable of delaying, retarding or preventing the development in food rancidity or other flavor deterioration due to oxidation. Antioxidants delay the development of off-flavours by extending the induction period. Addition of antioxidant after the end of this period tends to be ineffective in retarding rancidity development. Antioxidant can inhibit or retard oxidation in two ways: either by scavenging free radical, in which case the compound is described as a *primary antioxidant*, or by a mechanism that does not involve direct scavenging of free radical, in which case the compound is a *secondary antioxidant*. Primary antioxidant include phenolic compounds and vitamin E (α-tocopherol). These components are consumed during the induction period. Secondary antioxidant can operate by variety of mechanisms including binding of metal ions, scavenging oxygen, converting hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen. (Gordon, 2001)

Primary antioxidants terminate the free-radical chain reaction by donating hydrogen or electron to free radicals and converting them to more stable products. They may also function by addition in reactions with the lipid radicals, forming lipid antioxidant complexes. Many of the naturally occurring phenolic compounds like floavonoids, eugenol, vanillin and rosemary antioxidant also have

chain-breaking properties. Primary antioxidants are affective at very low concentration, and at higher levels they may become prooxidants.

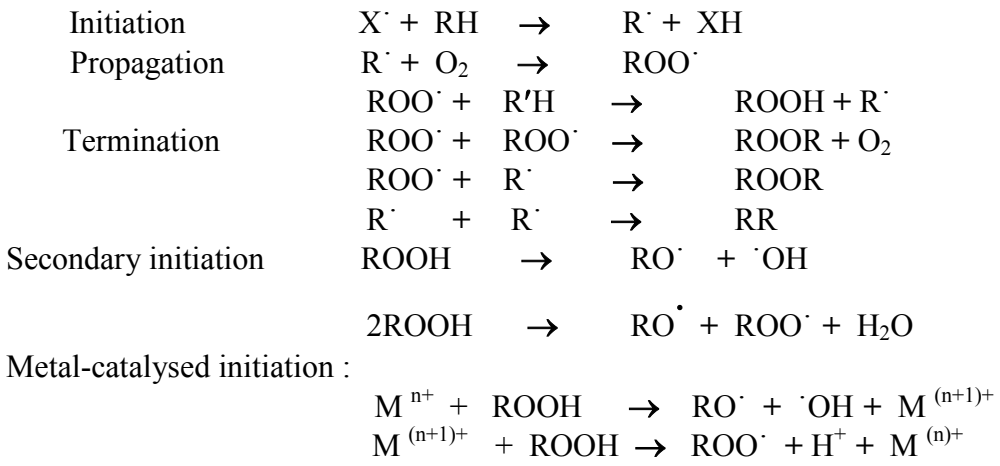
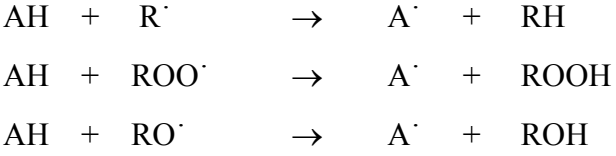


Figure 2.1 Mechanism of lipid oxidation
Source: Gordon (2001)

Primary antioxidants terminate the free-radical chain reaction by donating hydrogen or electron to free radicals and converting them to more stable products. They may also function by addition in reactions with the lipid radicals, forming lipid antioxidant complexes. Many of the naturally occurring phenolic compounds like floavonoids, eugenol, vanillin and rosemary antioxidant also have chain-breaking properties. Primary antioxidants are affective at very low concentration, and at higher levels they may become prooxidants.

Primary antioxidants may either delay or inhibit the initiation step by reacting with a free radical or inhibit the propagation step by reacting with the peroxy or alkoxy radicals (Rajalakshmi and Narasimhan, 1996).



The antioxidant free radical may further interfere with the chain-propagation reactions by forming peroxy antioxidant compounds.

$$A^{\cdot} + ROO^{\cdot} \rightarrow ROOA$$
$$A^{\cdot} + RO^{\cdot} \rightarrow ROA$$

Secondary antioxidants can be broadly classified as oxygen scavengers and chelators. Synergists function by various mechanisms. They may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant. Hence phenolic antioxidants can be used at lower levels if a synergist is added simultaneously to the stability of primary antioxidants (Rajalakshmi and Narasimhan, 1996).

3.3 Methods to determine antioxidant activity

Antioxidants are used in a wide variety of food products, and their activity may vary depending on the temperature, food composition, food structure and availability of oxygen. These can cause big changes in the activity of antioxidant in different food system. It is commonly observed that a non-polar antioxidant such as α-tocopherol is relatively ineffective in oil but is strongly effective in an oil-in-water emulsion. In contrast, a polar antioxidant such as ascorbic acid or trolox (a water-soluble derivative of α-tocopherol) is more effective in an oil than in an emulsion. This has been described as the polar paradox (Gordon, 2001).

Normally, a more rapid measurement of antioxidant activity is required than would be obtained by making the food product, storing it at ambient temperature and then measuring the oxidative state of the food.

3.3.1 Radical-scavenging methods

Radical-scavenging is the main mechanism by which antioxidants act in foods. Several methods have been developed in which the antioxidant activity is assessed by the scavenging of synthetic radicals in polar organic solvents, e.g. methanol, at room temperature.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical is a stable free radical that shows maximum absorbance at 515 nm in ethanol. The scavenging of DPPH radicals is followed by monitoring the decrease in absorbance at 515 nm which base on the reduction of $DPPH^{\cdot}$ solution in the presence of a hydrogen donation

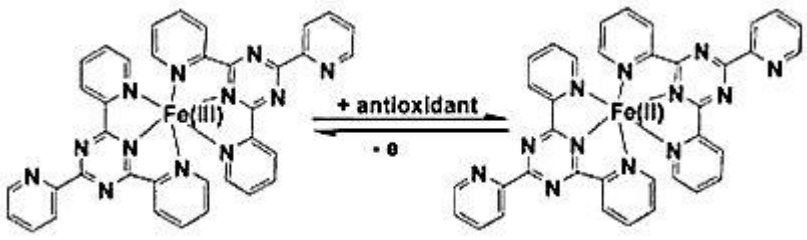
antioxidant, inducing the formation of non-radical form (DPPH-H) or reaction with a radical species ($R^{\cdot}$) to form DPPH-R.

$$DPPH^{\cdot} + AH \rightarrow DPPH\text{-}H + A^{\cdot}$$
$$DPPH^{\cdot} + R^{\cdot} \rightarrow DPPH\text{-}R$$

Fast reaction of DPPH radicals occurs with some phenols e.g. α-tocopherol, but slow secondary reaction may cause a progressive decrease in absorbance, so that the steady state may not be reached for several hours. Most papers in which the DPPH method has been used report the scavenging after 15 or 30 min reaction time. The data is commonly reported as EC_{50}, which is the concentration of antioxidant required for 50% scavenging of DPPH radicals in the specified time period (Gondor, 2001).

3.3.2 Ferric reducing antioxidant power

The mechanism of ferric reducing antioxidant power (FRAP) is based on single electron transfer between ferric tripyridyltriazine (Ferric-TPTZ) and single electron donor antioxidant to form blue color of ferrous tripyridyltriazine (ferrous-TPTZ) that shows maximum absorbance at 593 nm. This method is a reasonable screen for the ability of the sample to act as reducing agent (Prior, Wu, and Schaich ,2005)



Ferric $(TPTZ)_2]^{3+}$ $[Ferrous\ (TPTZ)_2]^{2+}$

(blue color)

Figure 2.2 Reaction of antioxidant and Ferric-TPTZ

Source: Prior, Wu, and Schaich (2005)

3.3.3 Metal chelating

Metal chelating shows the ability of antioxidant to chelate metal ion such as copper ion and ferrous ion (Rajalakshmi and Narasimhan, 1996). Chelating these metal ions by antioxidant can prevent oxidation reaction.

3.3.4 Electron spin resonance

Electron spin resonance (ESR) is used for investigation of paramagnetic substance. This technique is based on the measurement of transitions of unpaired electrons in a magnetic field, which can detect and identify molecules that have unpaired electrons, such as free radicals. ESR offers a sensitive, direct and accurate tool to monitor reactive species generated at room temperature. The high sensitivity of ESR allows the detection of low-concentration radicals (Rohn and Kroh, 2005; Peng, Xoing and Kong, 2009).

CHAPTER III
MATERIALS AND METHODS

Raw material

The same lot of dried mung bean seeds (*Vigna radiate* variety Kampangsean 2) obtained from Chainat Field Crops Research Center (Chainat, Thailand) in the harvest year of 2009 was used.

Enzymes

α-amylase (BAN® 480L) Food grade	East Asiatic	Thailand
Protease (Flavourzyme® 500L) Food grade	Novo Nordisk	Denmark

Chemicals

AccQ. Tag	Water	USA	HPLC grade
Acetic acid	Sigma	Germany	HPLC grade
Acetonitrile	Sigma	Germany	HPLC grade
Amino acid standard H	Pierce	USA	HPLC grade
2,2′-azobis(2-amidinopropane) dihydrochloride	Sigma	Germany	HPLC grade
Boric acid	Univar	Australia	A.R. grade
Bovine serum albumin (98%)	Sigma	Germany	A.R. grade
Copper sulphate	Carlo Erba	Italy	A.R. grade
diphenyl-1-pyrenylphosphate	Sigma	Germany	A.R. grade
5,5-dimethyl-1-pyroline-N-oxide	Sigma	Germany	A.R. grade
2, 2-diphenyl-1-picrylhydrazyl	Sigma	Germany	A.R. grade
Di-sodium hydrogen phosphate	Univar	Australia	A.R. grade
Ethylenediamine tetraacetic acid	Sigma	Germany	A.R. grade

Folin-Ciocalteu phenol reagent	Carlo Erba	Italy	A.R. grade
Hydrochloric acid	J.T. Baker	USA	A.R. grade
Iron (II) chloride tetrahydrate	Merck	Germany	A.R. grade
Iron (III) chloride	Sigma	Germany	A.R. grade
3-(2-pyridyl)-5, 6-diphenyl-1, 2, 4-triazine-4',4"-disulfonic acid sodium salt	Sigma	Germany	A.R. grade
Potassium cyanide	Merck	Germany	A.R. grade
Potassium hydrogen phthalate	Carlo Erba	Italy	A.R. grade
Picrylsulfonic acid	Sigma	Germany	A.R. grade
Sodium carbonate	Univar	Australia	A.R. grade
Sodium chloride	Univar	Australia	A.R. grade
Sodium citrate	Univar	Australia	A.R. grade
Sodium deoxycholate	Himedia	India	A.R. grade
Sodium dodecyl sulphate	Sigma	Germany	A.R. grade
Sodium hydroxide	Univar	Australia	A.R. grade
Sodium tetraborate	Univar	Australia	A.R. grade
Sulfanilic acid	Merck	Germany	A.R. grade
Trichloroacetic acid	Merck	Germany	A.R. grade
2,4,6-tris(2-pyridyl)-s-triazine	Sigma	Germany	A.R. grade

Equipments

Centrifuge (Hettich model Mikro 22 R, Tuttlingen, Germany)

Electron spin resonance spectrometer (JEOL Ltd., model JES-TE300 ESR spectrometry Tokyo, Japan)

Freeze dryer (Heto model DW8-85, hetoholten, Allerod, Denmark)

High Performance Liquid Chromatography (HPLC) system for amino acid analysis (Waters, Milford, MA)

- Auto sampler (Waters model 717, Milford, MA)

- Waters AccQ-Tag amino acid analysis column 3.9 mm i.d. x 150 mm (Waters, Milford, MA)

- UV/VIS detector (Waters model 2487, Milford, MA)

Hot air oven (Memmert model W350, Schwabach, Germany)

Matrix-assisted laser desorption/ionization-time of flight (Bruker Daltonics, model Autoflex II, Germany)

pH meter (Horiba model F-21, Kyoto, Japan)

Protein and nitrogen analyzer

- Digestion unit (BUCHI model K-424, Switzerland)

- Scrubber unit (BUCHI model B-414, Switzerland)

- Distillation unit (BUCHI model B-324, Switzerland)

Spectrophotometer (PerkinElmer model Lambda 25 UV/VIS Spectrometer, Shelton, MA)

Water bath (Gesells chaft für model GFL 1092, Burgwedel, Germany)

Methods

3.1 Effect of germination time on chemical composition of germinated mungbean

Dried mung bean seeds were soaked in excess tap water (1:10, w v^{-1}) at room temperature (30±2^{O}C) for 6 h. After being drained, the mung bean seeds were germinated, at 30±2^{O}C, in a basket lined with wet cheese cloth and covered with another piece of wet cheese cloth. The germinated seeds were randomly taken at 0, 6, 12, 18 and 24 h for chemical analyzes. They were analyzed as follows:

3.1.1 Moisture content

Moisture content of germinated mung beans was determined by drying the ground sample in an oven at 105^{O}C to constant weight (AOAC, 1995).

3.1.2 Total phenolics content

The germinated mung beans were blended with 95% ethanol at a ratio of 1:10 w v^{-1} and extracted at $60^{O}C$ in a water bath with continuous shaking for 2 h. The obtained slurry was centrifuged at 10,000 x *g* for 10 min and the supernatant was filtered through Whatman no. 1 filter paper. The filtrate was used for total phenolics content (TPC) analysis. TPC of the samples was expressed as gallic acid equivalents using Folin-Ciocalteu method according to Waterhouse (2005) as stated in Appendix A.1

3.1.3 Reducing sugar content

The germinated mung beans were blended with distilled water at a ratio of 1:10 w v^{-1} and extracted at $37^{O}C$ in a water bath with continuous shaking for 2 h. The obtained slurry was centrifuged at 10,000 x *g* for 10 min and the supernatant was filtered through Whatman no. 1 filter paper. The filtrate was used for reducing sugar content determination, expressed as glucose equivalents using Somogyi-Nelson method according to Fournier (2005) as stated in Appendix A.2

3.1.4 Crude protein content

Nitrogen content of germinated mung beans was analyzed using Kjeldahl method (AOAC, 1995). Crude protein was calculated by multiplying the percent Kjeldahl nitrogen by the factor 6.25.

3.1.5 Amino acid composition

Amino acid composition of germinated samples was analyzed using High Performance Liquid Chromatography (HPLC) according to AccQ. Tag method (Astephen, 1993). Hydrolysis of the germinated samples was performed in the presence of 6 N HCl at $110^{O}C$ for 24 h under a nitrogen atmosphere. Amino acids were derivatized at $55^{O}C$ with AccQ. Tag solution. A 10 µl of derivatized sample was injected to the column. The column used was Waters AccQ. Tag amino acid analysis column (3.9 mm i.d. x 150 mm), set at $37^{O}C$, (Waters, Milford, MA) equipped with UV detector (Waters Dual 2487 Absorbance detector, Milford, MA) set at 254 nm. Mobile phase was the gradient of 60% acetonitrile and AccQ. Tag Eluent A solution (Milli-Q water and AccQ. Tag Eluent A at the ratio of 1:10 v v^{-1}) with the flow rate 1.0 ml min^{-1} as shown in Table 3.1. The identification and

integration of peaks was performed by Empower software (Waters, Milford, MA), using an external amino acids standard ranging from 10 to 200 pmol (Amino Acid Standard H, Pierce, MA). Preparation of standard amino acids was stated in Appendix A.3.

For tryptophan, hydrolysis of the samples was performed in the presence of 4.5 N NaOH at $110^{O}C$ for 19 h under a nitrogen atmosphere. The analysis was performed using HPLC (Amino acid analyzer, LC-6A; Shimadzu, Kyoto, Japan) with Shim-pack ISC-07/S 1504Na column equipped with fluorescence detector (RF-20A/RF-20A, Shimadzu, Kyoto, Japan) set at $55^{O}C$, mobile phase was 0.4 ml min^{-1} isocratic of 0.2 N boric acid in 0.6 N sodium citrate.

All experiments in Section 3.1 were performed in triplicate. The data were analyzed using ANOVA. A multiple comparison procedure of means were performed by Duncan's new multiple range test.

Table 3.1 A ratio of 60% acetonitrile and AccQ. Tag Eluent A solution in the mobile phase gradient system.

Time (min)	**60% acetonitrile (v v^{-1})**	**AccQ. Tag Eluent A solution (Milli-Q water and AccQ. Tag Eluent A at the ratio of 1:10 v v^{-1})**
0	0	100
0.5	2	98
15	7	93
19	10	90
28	20	80
30	28	72
35	32	68
40	40	60
45	100	0
50	0	100

Protein content was selected as a criterion to determine the germination time. The germinated condition giving the highest amount of protein content was selected as source for protein hydrolysate in the next step while 0 h germinated mung bean (0 H-GMB) was used as the control.

3.2. Effect of Flavourzyme® concentration and hydrolysis time on free amino groups, degree of hydrolysis, and total phenolics content of mung bean protein hydrolysates

The germinated condition giving the highest amount of protein content selected from Section 3.1 was used as raw material while 0 h-germinated mung bean (0 H-GMB) was used as the control. The samples were homogenized with 0.1 M phosphate buffer, pH 6.0, containing 0.5 g l^{-1} $CaCl_2$ at a ratio of 1:3 w v^{-1}. In order to prevent gel formation of the slurries during heating, 1% (by dried weight of germinated mung bean) BAN® 480L was added into slurries. The slurries were then heated to 70^{O}C in a water bath and the hydrolysis was carried out for 1 h. Then, BAN® 480L activity was inactivated by heating the slurries at 85^{O}C for 20 min. After being cool down to 50^{O}C, Flavourzyme® at the level of 0, 1, 3 and 5% (by dried weight of germinated mung bean) was added into the slurries. The reactions were conducted at 50^{O}C for 0, 1, 2, 3, 4 and 5 h. Then, the slurries were heated at 85^{O}C for 10 min to inactivated Flavourzyme® activity. The samples were then cooled down and centrifuged at 6,000 x *g* for 20 min. Supernatants were analyzed as follows:

3.2.1 Free amino groups and degree of hydrolysis

Free amino group (FAG) was determined using the reaction with trinitrobenzensulphonic acid (TNBS) (Alder-Nissen, 1979) as stated in Appendix A.4.

Degree of hydrolysis (DH) of the samples based on an increase in free amino group during the enzymatic hydrolysis was determined.

The percent of DH was calculated according to equation 3.1

$$DH\ (\%) = \frac{(L_t - L_0)}{L_{max} - L_0} \times 100 \quad \text{.....(3.1)}$$

where L_0 = initial amount of free amino group in the solution

L_t = amount of free amino group in the solution at time t

L_{max} = amount of free amino group in the solution after acid hydrolysis

3.2.2 Total phenolics content

Total phenolics content (TPC) was analysed using Folin-Ciocalteu method (Waterhouse, 2005) as stated in Appendix A.1.

All experiments in Section 3.2 were performed in triplicate. The data were analyzed using ANOVA. A multiple comparison procedure of means were performed by Duncan's new multiple range test.

In order to study on the effect of DH on antioxidant activities and some properties of germinated mung bean protein hydrolysate, the supernatant of mung bean protein hydrolysates which obtained from 18 h germinated mung bean (18 H-GMB) with different DH, varying from 0 to 50%, were chosen for further study while 0 H-GMB with different DH, varying from 0 to 40%, were used for comparison.

3.3. Antioxidant activity of freeze dried mung bean protein hydrolysates

The supernatant of 11 samples of protein hydrolysate form 18 H-GMB with different DH level, varying from 0 to 50%, and supernatant of 11 samples of protein hydrolysate from 0 H-GMB with different DH level, varying from 0 to 40%, that also had high positive linear correlation between DH and FAG and high positive linear correlation between DH and TPC were selected for representative of the germinated mung bean protein hydrlysate. The samples were subjected to freeze drying under 0.05 h Pa vacuum for 15 h. The dried powder (1 g) was packed in aluminum foil laminated to polyethylene bag, 3X5 inch with the heat sealed. Sample were kept at -20^{O}C for further analysis.

The 22 freeze dried powder samples were analyzed for antioxidant activity as follows:

3.3.1 Activity measured by chemical method

3.3.1.1 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of germinated mung bean protein hydrolysates was measured using the method described by Park *et al*. (2008) with a slight modification. A 1.5 ml (1-20 mg ml^{-1}) protein hydrolysate solution (or phosphate buffer pH 7.0 itself as the control) was added to 1.5 ml DPPH (0.15 mM) in 85% ethanol solution. After mixing vigorously, the samples were allowed to stand at room temperature (30±2^{O}C) in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm. DPPH radical scavenging activity was calculated based on the equation 3.2.

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_0 - A}{A_0} \times 100 \quad \text{.....(3.2)}$$

Where A_0 = absorbance value of DPPH without sample (control)

A = absorbance value of sample and DPPH

3.3.1.2 Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) of germinated mung bean protein hydrolysate was measured using the method of Binsan *et al*. (2008). Stock solution including 300 mM acetate buffer (pH 3.7), 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ) solution in 40 mM HCl, and 20 mM ferric chloride ($FeCl_3$) solution. A working solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3$ solution. The mixed solution was incubated at 37^{O}C for 30 min and referred as FRAP solution. A 150 µl of germinated mung bean protein hydrolysates (1 mg ml^{-1}) was mixed with 2,850 µl of FRAP solution and kept in the dark at room temperature (30±2^{O}C) for 30 min. The ferrous tripyridyltriazine complex (colored product) was measured by reading absorbance at 593 nm. Trolox® (50-600 µM) was used to prepare standard curve. The FRAP activity was expressed as µmol Trolox® equivalent per gram sample.

3.3.1.3 Metal chelating activity

The ferrous ion (Fe^{2+}) metal chelating activity of germinated mung bean protein hydrolysate at 0.5-4 mg ml^{-1} concentration was measured by the method of Jamdar *et al*. (2010) with a slight modification. A test sample of 1 ml was premixed with 0.05 ml of 2 mM iron dichloride solution and 1.85 ml of double distilled water. Thereafter, 0.1 ml of 5 mM ferrozine solution was added and mixed vigorously. The absorbance was determined at 562 nm after the mixture was allowed to stand for 10 min at room temperature (30±2^{O}C). Double distilled water was used as the control. The Fe^{2+} chelating activity was calculated using the equation 3.3.

$$Fe^{2+} \text{ chelating activity (\%)} = \frac{A_0 - A}{A_0} \times 100 \quad \text{.....(3.3)}$$

Where A_0 = absorbance value of control

A = absorbance value of sample

3.3.2 Activity measured by electron spin resonance method

3.3.2.1 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical spin resonance was measured to follow up DPPH radical-scavenging activity as described by Giri *et al*. (2011) with some modifications. A 50 µl portion of 1 mg ml^{-1} crude mung bean protein hydrolysate solution was added to 50 µl of DPPH solution (0.15 mM in 85% ethanol) and thoroughly vortexed. After being kept in the dark at room temperature (30±2^{O}C) for 15 min, the mixture was transferred into a 20 µL Pyrex NMR capillary tube. The DPPH radical spin resonance was measured using JES-TE300 electron spin resonance (ESR) spectrometry (JEOL Ltd., Tokyo, Japan) set at microwave power 1.2 mW, microwave frequency 9149.3 MHz, magnetic field, 325.5±25 mT and sweep time 30 s. DPPH radical scavenging activity was calculated based on the equation 3.4.

$$\text{DPPH radical scavenging activity (\%)} = \frac{H_0 - H}{H_0} \times 100 \qquad \text{…….(3.4)}$$

Where H_0 = the height of the third resonance peak of sample without protein hydrolysate (control)

H = the height of the third resonance peak of sample with protein hydrolysate

3.3.2.2. Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by the Fenton reaction (Je, Kim and Kim, 2005) and measured for their spin resonance. A 20 µL portion of 1 mg ml^{-1} crude mung bean protein hydrolysate solution was added to 20 µL of 0.3 M 5,5-dimethyl-1-pyroline-N-oxide (DMPO) and 20 µL of 10 mM $FeSO_4$. While 20 µL of 10 mM phosphate buffer (pH 7.4) as the control. The reaction was initiated by adding 20 µL of 10 mM H_2O_2. The reaction mixture was then transferred to a 20 µL Pyrex NMR capillary tube; DMPO-OH adduct was recorded after 2.5 min using a JES-TE300 ESR spectrometer with the conditions described in 3.3.2.1. Hydroxyl radical scavenging activity was calculated based on the equation 3.5.

$$\text{Hydroxyl radical scavenging activity (\%)} = \frac{H_0 - H}{H_0} \times 100 \qquad \text{…….(3.5)}$$

where H_0 = the height of the second resonance peak of sample without protein hydrolysate (control)

H = the height of the second resonance peak of sample with protein hydrolysate

3.3.2.3. Carbon-centered radicals scavenging activity

Carbon-centered radicals were generated with 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) as described by Giri *et al*. (2011) with a slight modification. A 20 µl of 1 mg ml^{-1} crude mung bean protein hydrolysate solution, 20 µl of 40 mM α-(4-pirydryl-1-oxide)-N-tert-butylnitrone (4-POBN) and

20 µl of 40mM AAPH, followed by incubation at 37 °C for 30 min. The reaction mixture was transferred to a 20 µL PyrexNMR capillary tube; spin adduct was recorded as described in 3.3.2.1. Carbon-centered radicals scavenging activity was calculated based on the equation 3.6.

$$\text{Carbon-center radical scavenging activity (\%)} = \frac{H_0 - H}{H_0} \times 100 \quad \text{.....(3.6)}$$

where H_0 = the height of the first resonance peak of sample without protein hydrolysate (control)

H = the height of the first resonance peak of sample with protein hydrolysate

3.3.3 Activity of protein hydrolysate on inhibition of lipid oxidation

Germinated mung bean protein hydrolysate samples that gave high antioxidant activity from 0 H-GMB and 18 H-GMB were selected for the study on inhibition of linoleic acid oxidation in oil-in-water emulsion model system. The oxidation of linoleic acid was measured by follow-up the change in total lipid hydroperoxides content during storage described by Sohn *et al*. (2005) and the change in oxygen uptake described by Villiere *et al*. (2005) with a slight modification.

3.3.3.1 Total lipid hydroperoxides content

Linoleic acid was purified by open column chromatography (20 cm length x 2.4 cm i.d.) on spherical silica gel using *n*-hexane. A 10 ml of the emulsion containing protein hydrolysate was prepared in each Erlenmeyer flask. A 5 ml 99.5% ethanol containing 65 µl purified linoleic acid was mixed with 5 ml 0.2 M phosphate buffer, pH 7.0, containing crude mung bean protein hydrolysate at the concentration of 0, 0.15 or 0.30 mg ml^{-1} of emulsion. All Erlenmeyer flasks containing emulsion were closed with cotton stopper, and incubated at $40^{O}C$ in a shaking incubator in the dark for 8 days. Each flask was randomly taken every day, for 8 days, for total lipid hydroperoxides determination. A 10 ml of emulsion prepared by mixing 5 ml 99.5% ethanol containing 65 µl of purified linoleic acid and 750 µg of butylate

hydroxytoluen (BHT) and 5 ml 0.2 M phosphate buffer, pH 7.0, was used as the control.

Total lipid hydroperoxide was measured using a flow injection analysis (FIA) system as described by Sohn *et al*. (2005) with a slight modification. For lipid hydroperoxide extraction, 5 ml of mixture was mixed with 5 ml chloroform ($CHCl_3$), 9 ml methanol and 1 ml 4.82 nmol 1-Myristoyl-2-(12-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)dodecanoyl)-sn-glycero-3-phosphocholine (NBD-labeled PC) and homogenized at 13,000 rpm for 3 min. After being centrifuged at 3,000 x *g* for 10 min, the supernatant was decanted and the lower layer was dehydrated with sodium sulphate and made to 10 ml with $CHCl_3$ then filtered with 0.2 µm polytetrafluoroethylene (PTFE) filter. A filtrate was injected to FIA system. The mobile phase (a mixture of 1-butanol and methanol at a ratio of 2:1) was passed through a LC-9A HPLC pump (Shimadzu, Kyoto, Japan) at a flow rate 0.5 ml min^{-1} and subsequently transferred to a stainless steel reaction coil (40 m length x 0.25 mm i.d.) immersed in a water bath (NTT-1400, Eyela, Tokyo, Japan) controlled at $89^{O}C$. A diphenyl-1-pyrenylphosphate (DPPP) solution (5 mg DPPP and 200 ppm butylated hydroxytoluene (BHT) dissolved in 200 ml of 1-butanol and 100 ml of methanol) kept in brown bottle on ice in the dark was pumped with a LC-10AS HPLC pump (Shimadzu, Kyoto, Japan) at a flow rate 0.3 ml min^{-1} and mixed with the mobile phase in a T-connector before flowed through the reaction coil. At the inlet of the reaction coil, 20 µl of filtrate lipid hydroperoxide extract was injected. The nonfluorescent DPPP reagent reacted with lipid hydroperoxide to produce fluorescent DPPP oxide in the coil. After the reaction, the eluent from the coil was cooled down by passed through a short stainless steel coil (1 m length x 0.25 mm i.d.) immersed in an ice-bath. The fluorescence intensity of the DPPP oxide was mornitored at 352 nm of excitation wavelength and at 380 nm of emission wave length with a RF 535 fluorescence detector (Shimadzu, Ktoto, Japan). The NBD-labeled PC (50-2,000 nmol) was used as an internal standard.

3.3.3.2 Oxygen uptake

A 10 ml of emulsion was prepared as stated in Section 3.3.3.1. An aliquot (2 ml) of the emulsion was transferred into 20 ml vial with a 18 ml headspace volume. The vials were sealed tightly with a PTFE/silicone septum and aluminum crimp cap then incubated at 40^{O}C in shaking incubator in the dark for 8 days. Every day each vial was randomly taken, oxygen content in the headspace of the vial was measured using gas chromatography (GC-2014, Shimadzu, Kyoto, Japan) paired with a thermal conductivity detector (TCD) as described by Villiere *et al*. (2005) with a slight modification. A 1,000 µl portion of the headspace air of the vial was injected with a gastight syringe into the injector (injector temperature 50 ^{O}C) connected to a molecular sieve 13XS 60/80 column (3-m, 0.32-mm i.d). Analysis was performed in isothermal mode at 40 ^{O}C with TCD temperature set at 125 ^{O}C. The flow rate of helium carrier gas was 2 ml min^{-1}. The oxygen peak area to nitrogen peak area ratio in the vial headspace was compared to the surrounding air. The total amount of oxygen in the vial was then calculated from the theoretical concentration of oxygen and nitrogen in ambient air and the headspace volume then subtracted from the initial amount of oxygen in the headspace of the vial. The results were expressed in ml of consumed oxygen per ml of emulsion.

All experiments were performed in triplicate. In Section 3.3.1 and 3.3.2 data were analyzed in order to compare the effect of DH on antioxidant activity of germinated mung bean protein hydrolysate using ANOVA. A multiple comparison procedure of means were performed by Duncan's new multiple range test. Correlation between DH and antioxidant activity, correlation between free amino group and antioxidant activity, and correlation between total phenolics content and antioxidant activity were evaluated.

3.4. Properties of freeze dried germinated mung bean protein hydrolysate

The freeze dried powder samples of germinated mung bean protein hydrolysates were analyzed as follows:

3.4.1 Phenolic acids and flavonoids composition

Twenty-two freeze dried samples of crude germinated mung bean protein hydrolysates from Section 3.3 were used for analysis. The sample (0.5g) was dissolved in 1 ml of 95% ethanol and centrifuged at 10,000 x *g* for 20 min. The supernatant was filtered through 0.45 µm syringe filter and analyzed for phenolic acids and flavonoids composition using online HPLC according to Giri *et al*. (2011). The separation of phenolic acids and flavonoids components was carried out by LC-10AD HPLC pump (Shimadzu, Kyoto, Japan), DGU-4A degasser (Shimadzu, Kyoto, Japan), CTO-10AC column oven coupled (Shimadzu, Kyoto, Japan) set up at 37^{O}C, with a pre-reaction photodiode array detector SPD-M10A set at 280 nm (Shimadzu, Kyoto, Japan). The samples were loaded onto a L-column ODS (4.6 mm i.d. x 250 mm, Chemical Inspection & Testing Institute, Tokyo, Japan). Mobile phase comprising of methanol, water and acetic acid at the ratio of 30:69:1 was applied at the flow rate of 0.175 ml min^{-1}. Identification of phenolics was carried out by comparing the retention time spectra of those of authentic standards. The standard phenolic acids used were gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, syringic acid, *trans-p*- coumaric acid and *trans*-ferulic acid (Sosulski and Dabrowski, 1984).

The HPLC system for flavonoids analysis was same as that for phenolic acid analysis but mobile phase used comprising of methanol, water and acetic acid at the ratio of 40:59:1 were applied at the flow rate 0.4 ml min^{-1}. The standard flavonoids used were rutin, quercitrin, quercetin and kaem ferol (Liu *et al*., 2008)).

3.4.2 Online DPPH radical scavenging activity

Online DPPH radical scavenging activity using online HPLC-DPPH method according to Giri *et al*. (2011) was performed. The HPLC separated analytes from 3.4.1 was reacted with DPPH solution at a concentration of 0.15 mM in methanol, set to 0.2 ml min^{-1} (LC-10AD HPLC pump; Shimadzu, Kyoto, Japan) in the post-column. The length of the capillary used for the post-column reaction was adjusted to achieve

a reaction time of 180 seconds. The induced bleaching was detected as a negative peak-photo metrically at 517 nm.

3.4.3 Molecular weight distribution

The molecular weight distribution of germinated mung bean protein hydrolysates was determine by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) according to Sun *et al*. (2005) on an Autoflex II (Bruker Daltonics, Germany) operated in a linear and reflector, positive ion mode. The crude germinated mung bean protein hydrolysate was dissolved in 0.1% trifluoroacetic acid (TFA) aqueous solution with the concentration of 10 pmol. 3,5-dimethoxy-4-hydrocycinnamic acid dissolved in 0.1% TFA in 33% acetonitrile at saturation state were used as the matrix. A 1 µl of crude mung bean protein hydrolysate solution was added to 4 µl of the matrix solution and 1 µl of this mixture was spotted onto a stainless steel target and air-dried. An ionization of the dried mixture and sample free matrix (control) were induced by a nitrogen laser at 337 nm with a pulse width of 3 ns at an accelerating voltage of 25 kV.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Effect of germination time on chemical composition of germinated mung bean

Mung beans were germinated in the dark at room temperature ($30\pm2^{O}C$) for 0, 6, 12, 18 and 24 h then analyzed for moisture content, total phenolics content (TPC), reducing sugar content, protein content and amino acid composition. As shown in Table 4.1, the moisture contents of germinated mung beans significantly increased during 24 h of germination period ($p<0.05$). This may be due to water absorption during germination of the seeds. However, TPC, reducing sugar contents, protein contents and amino acid compositions of the germinated mung beans were calculated in dry basis (db.)

The TPC of germinated mung beans significantly increased during the germination period ($p<0.05$). As shown in Table 4.1 the 24 h-germinated mung beans (24 H-GMB) showed the highest TPC, the value doubled increased compared to that of the 0 h-germinated mung beans (0 H-GMB). This may be because in natural environment seed-sprouting survives during germination by enhancing its defensive response through phenolic biosynthesis (Randhir *et al*., 2004). Fernandez-Orozco *et al*. (2008) found that during germination of *Vigna radiata* cv. emmerald, the TPC increased from 1.09 to 3.46 mg g^{-1} (db) after 7 days of germination period. McCue and Shetty (2002) also reported that phenolics content of mung beans was increased during germination in the dark.

Folin-Ciocateau reaction, based on chemical reduction of phosphomolybdic/phosphotungstic acid complex to form chromogens that can be spectrophotometrically detected, was used to determine TPC. This reaction can be interfered by the presence of reducing sugar in the samples (Waterhouse, 2005). As shown in Table 4.1, the reducing sugar contents of germinated mung beans slightly increased during 24 h of germination period. Thus, the increase in the amount of TPC shown in this experiment should be mainly through phenolic biosynthesis during

Table 4.1 Effect of germination periods on moisture contents, total phenolics contents, reducing sugar and protein contents of germinated mung beans.

Germination period (h)	Moisture (%)	Total phenolics content (mg gallic acid g^{-1} db)	Reducing sugar (mg glucose g^{-1} db)	Protein content (%Nx6.25; % db)
0	53.93^{a}±0.82	2.81^{a}±0.12	0.60^{a}±0.03	25.58^{a}±0.65
6	56.02^{ab}±2.26	3.48^{b}±0.35	1.05^{b}±0.05	26.10^{ab}±0.60
12	58.24^{b}±3.46	3.73^{b}±0.46	1.51^{c}±0.07	26.72^{bc}±0.12
18	65.19^{c}±1.59	4.86^{c}±0.35	1.65^{c}±0.12	27.41^{cd}±0.41
24	67.80^{c}±1.22	5.78^{d}±0.04	1.95^{d}±0.19	28.03^{d}±0.72

^{a,b,c,d} Means in the same column with different letters are significantly different ($p<_{0.05}$).

db, drybasis

germination. Randhir *et al*. (2004) reported that during germination seeds mobilize storage starch in mung bean seed into simpler carbohydrates and monosaccharides via certain amylolytic enzymes. Venkateswaran and Vijayalakshmi (2010) reported that reducing sugar of finger millet (*Eleusine coracana*) increased from 5.3 g per 100 g in raw seed to 9.6 g per 100 g after 24 h of germination.

During seed germination the amount of enzymes, especially proteolytic enzyme, significantly increases. These enzymes catalyse hydrolysis of polymerized food forms such as protein stored in the cotyledon into peptides and free amino acids. However, there is synthesis of protein also occurs during germination (Kuo *et al*., 2004; Randhir, *et al*., 2004; Ghavidel and Prakash, 2007). The crude protein contents of the germinated mung beans increased during the germination period, as shown in Table 4.1. The 24 H-GMB had the highest amount of crude proteins but not being significant different ($p \geq 0.05$) compared to that of the 18 h-germinated mung beans (18 H-GMB). Many researchers have reported that germination of mung bean seeds resulted in an increase in crude protein content compared to the case of raw mung bean seeds (Mubarak, 2005; Ghavidel and Prakash, 2007). Raymundo, Quizon and Hurtada (1994) found that the crude protein contents of the germinated mung bean increased during 24 h of the germination process, from 2,117 mg per 100 g at 0 h-germination period to 2,463 mg per 100 g at 24 h-germination period, and then gradually decreased. Some reports showed that an increase in crude protein contents during seed germination may not be due to an increase in true protein but the result evaluated of true protein plus non-protein nitrogen (Sattar *et al*., 1989). El-Shimi, Damir and Ragab (1984) found that non-protein nitrogen of fenugreek seeds increased from 0.36% during soaking to 0.38% after germination for 2 days. Raymundo *et al*. (1994) germinated mung bean in the dark for 48 h and reported that purine and pyrimidine base nucleic acids increased 9 to 10 fold after germination compared to non-germinated mung bean. Synthesis of nucleic acid was accompanied by an increase in total protein.

Table 4.2 shows that the total essential amino acids contents (TEAA), total non-essential amino acids contents (TNAA) and total amino acid contents (TAA) of the germinated mung beans increased with increasing the germination period. It was

found that the 24 H-GMB had the highest contents of TEAA, TNAA and TAA but not being significantly different ($p \geq 0.05$) compared to those obtained from the 18 H-GMB. The results indicated that essential amino acids such as lysine, isoleucine, leucine, phenylalanine, threonine and non-essential amino acids such as aspartic acid, glutamic acid, glycine, arginine, alanine, proline, cysteine and tyrosine significantly increased with the germination period ($p < 0.05$). However, the increased amounts of valine, methionine, tryptophan, histidine, and serine content were not significant different ($p \geq 0.05$). Mubarak (2005) reported that mung bean protein was rich in essential amino acids such as total aromatic amino acids, leucine, isoleucine and valine; but threonine, total sulfur amino acids, lysine and tryptophan were slightly deficient in the mung bean protein. In addition, germination process caused an increase in TAA.

The ratio of each amino acids to TAA were calculated and shown in Table 4.3. The results showed that the germination period during 0 to 24 h did not affect the ratios of each amino acids to TAA. This may be because during germination of mung bean, unique protein with the specific amino acid profile has been synthesized.

Crude protein content was selected as a criterion to determine the germination period. As the protein contents of the 24 H-GMB and the 18 H-GMB were not significantly different ($p \geq 0.05$), the 18 H-GMB was selected as a source for protein hydrolysate for this study while 0 H-GMB was used as a control.

4.2 Effect of Flavourzyme® concentration and hydrolysis time on free amino groups, degree of hydrolysis, and total phenolics content of germinated mung bean protein hydrolysate

The 18 H-GMB and 0 H-GMB were hydrolyzed by using Flavourzyme® at the level of 0, 1, 3 and 5% (in dried weight of germinated mung bean) for 0, 1, 2, 3, 4 and 5 h as described in Section 3.2. The obtained germinated mung bean hydrolysates were analyzed for free amino groups (FAG), degree of hydrolysis (DH), and total phenolics content (TPC).

Table 4.2 Effects of germination periods on amino acid compositions of germinated mung beans

Amino acid (mg per 100 g db)	Germination period (h)				
	0	**6**	**12**	**18**	**24**
Essential amino acids					
Valine[ns]	176.67±24.80	190.93 ±5.47	207.29±8.77	195.71±43.80	179.60±14.72
Methionine[ns12]	35.07±1.22	41.80 ±5.37	37.71±3.77	43.23±0.76	38.61±5.27
Lysine[1]	37.62[a] ±2.60	43.62[b] ±4.60	51.30[c]±2.42	51.21[c]±0.99	40.94[ab]±3.49
Isoleucine	132.05[a] ±14.02	154.72[b] ±1.71	156.16[b]±5.96	175.31[c]±4.90	168.08[bc]±15.22
Leucine	278.80[a] ±29.04	315.69[b] ±7.71	327.61[bc]±10.58	379.38[d]±9.31	349.92[cd]±30.21
Phenylalanine[2]	446.02 [b]±33.74	360.01[a]±34.70	446.23[b]±6.58	506.26[c] ±55.78	521.78[c]±33.15
Tryptophan[ns12]	216.12±5.50	206.40±21.89	231.91±48.00	221.50±4.27	214.55±53.22
Histidine[ns 2]	150.08 ±19.91	171.85±30.68	154.81±7.48	156.32±14.55	179.40±17.87
Threonine[1]	130.26 [a]±13.18	149.78[ab]±21.54	189.04[cd]±4.32	168.11[bc]±19.43	215.73[d]±25.35
TEAA	*1,602.7 [a]±125.12*	*1,634.83 [a]±94.75*	*1,802.17 [b]±74.13*	*1,896.92 [b]±80.55*	*1,908.60[b] ±155.50*
Non-essential amino acids					
Aspartic acid	188.88[a] ±31.73	182.90[a] ±4.23	237.02 [b]±2.12	239.49 [b] ±29.94	254.49[b]±15.87
Serine[ns]	191.17±25.40	195.05±30.74	202.98±18.23	192.89±7.88	237.46±23.85
Glutamic acid	344.96 [a]±63.80	366.87[a]±14.06	427.82 [b] ±5.98	473.49 [b] ±54.18	432.95[b]±25.83
Glycine	166.50 [a]±19.82	184.78[ab]±12.74	180.62[ab]±3.93	222.82[c]±6.69	199.79 [b]±16.20
Arginine	277.14 [a]±13.94	329.04 [b]±29.85	333.00 [b]±16.51	339.36 [b]±21.53	348.58 [b] ±43.55
Alanine	65.64 [a]±10.93	94.95 [b]±11.08	91.72 [b] ±3.56	94.81 [b]±14.45	90.33 [b] ±7.64
Proline	85.40 [ab]±17.17	79.28[a]±1.77	1 04.65 [bc]±12.13	104.46[bc]±10.21	107.88 [c] ±19.83
Cysteine[2]	18.48[a]±1.95	26.73 [b]±3.57	17.41 [a] ±0.49	24.67 [b] ±2.28	20.37 [a] ±2.98
Tyrosine[2]	152.40 [a]±10.92	184.24 [b]±13.98	1 83.35 [b]±21.39	176.23[b]±7.75	185.19 [b] ±10.29
TNAA	*1,480.61[a]±188.60*	*1,643.81 [a]±144.62*	*1,778.6 [bc] ±68.22*	*1,868.27 [c]±86.74*	*1,877.04 [c]±152.94*
TAA	*3,083.37 [a]±312.54*	*3,278.70[ab]±207.19*	*3,580.60[bc]±128.33*	*3,765.18 [c]±98.59*	*3,785.67[c]±301.99*

[ns] Means in the same row are not different ($p \geq 0.05$)

[a,b,c,d] Means in the same row with different letters are significantly different ($p < 0.05$)

[1] Limited amino acid in mung bean

[2] Claimed as antioxidant amino acid

Table 4.3 Effects of germination period on molar ratios of each amino acid content to total amino acid content of the germinated mung beans

Amino acids	Germination period (h)				
	0	6	12	18	24
Essential amino acids					
Valine:TAA	1.19:1.00	1.21:1.00	1.20:1.00	1.08:1.00	0.98:1.00
Methionine[1 2]:TAA	0.19:1.00	0.21:1.00	0.17:1.00	0.19:1.00	0.17:1.00
Lysine[1]:TAA	0.20:1.00	0.22:1.00	0.24:1.00	0.23:1.00	0.18:1.00
Isoleucine:TAA	0.79:1.00	0.87:1.00	0.81:1.00	0.86:1.00	0.82:1.00
Leucine:TAA	1.68:1.00	1.79:1.00	1.69:1.00	1.87:1.00	1.71:1.00
Phenylalanine[2]:TAA	2.68:1.00	2.04:1.00	2.30:1.00	2.49:1.00	2.55:1.00
Tryptophan[1 2]:TAA	0.83:1.00	0.75:1.00	0.77:1.00	0.70:1.00	0.67:1.00
Histidine[2]:TAA	0.76:1.00	0.82:1.00	0.67:1.00	0.65:1.00	0.74:1.00
Threonine[1]:TAA	0.86:1.00	0.93:1.00	1.07:1.00	0.91:1.00	1.16:1.00
TEAA:TAA	*0.98:1.00*	*0.94:1.00*	*0.95:1.00*	*0.95:1.00*	*0.95:1.00*
Non-essential amino acids					
Aspartic acid:TAA	1.12:1.00	1.02:1.00	1.20:1.00	1.16:1.00	1.23:1.00
Serine:TAA	1.43:1.00	1.38:1.00	1.31:1.00	1.19:1.00	1.45:1.00
Glutamic acid:TAA	1.85:1.00	1.85:1.00	1.97:1.00	2.08:1.00	1.89:1.00
Glycine:TAA	1.75:1.00	1.82:1.00	1.63:1.00	1.92:1.00	1.71:1.00
Arginine:TAA	1.25:1.00	1.40:1.00	1.29:1.00	1.26:1.00	1.28:1.00
Alanine:TAA	0.58:1.00	0.79:1.00	0.70:1.00	0.69:1.00	0.65:1.00
Proline:TAA	0.58:1.00	0.51:1.00	0.61:1.00	0.59:1.00	0.60:1.00
Cysteine[2]:TAA	0.12:1.00	0.16:1.00	0.10:1.00	0.13:1.00	0.11:1.00
Tyrosine[2]:TAA	0.66:1.00	0.75:1.00	0.68:1.00	0.63:1.00	0.66:1.00
TNAA:TAA	*1.02:1.00*	*1.07:1.00*	*1.05:1.00*	*1.06:1.00*	*1.06:1.00*

[*] Mean from triplicate determinations
[1] Limited amino acid in mung bean
[2] Claimed as antioxidant amino acids

4.2.1 Free amino groups and degree of hydrolysis

Table 4.4 shows that the Flavourzyme® concentration and the hydrolysis period significantly affected the amount of FAG obtained from the hydrolysis of both 0 H-GMB and 18 H-GMB ($p<0.05$). There was an interaction between Flavourzyme® concentration and hydrolysis period on FAG in both 0 H-GMB and 18 H-GMB. For both 0 H-GMB and 18 H-GMB, hydrolysis with 5% Flavourzyme® for 5 h gave the highest amount of FAG compared to other conditions in the same group. The amount of FAG in 0 H-GMB and 18 H-GMB increased 7 and 3 folds, respectively, after hydrolyzed by 5% Flavourzyme® for 5 h compared to each germinated mung bean treated with 0% Flavourzyme® for 0 h . This was because during enzymatic hydrolysis proteolytic enzyme, Flavourzyme®, broke down the peptide bonds and generates amino acid groups. Achouri, Zhang and Shiying (1998) reported that an increase in number of FAG in soy protein hydrolysate was directly related to the extent of hydrolysis. The FAG in soy protein hydrolysate increased from 0.247 to 0.274 NH_2number of FAG in soy protein hydrolysate was directly related to the extent of hydrolysis. The FAG in soy protein hydrolysate increased from 0.247 to 0.274 NH_2 per 100 gram of protein when DH increased from 4 to 8%, respectively. Yee, Shipe and Kinsella (1980) also found that the FAG in the pepsin treated soy protein increased nearly double after 8 h hydrolysis compared to 0 h hydrolysis.

In order to compare DH among the hydrolysis conditions used, the amount of FAG obtained from each of the conditions was converted to DH using the equation 3.1 in Section 3.2.1. As shown in Table 4.5 the results showed that the DH increased with increasing both Flavourzyme® concentration and hydrolysis period in both 0 H-GMB and 18 H-GMB. As DH is defined as the ratio of the number of peptide bonds broken down to the total number of peptide bonds in the sample (Shahidi *et al*, 1995), the higher enzyme concentration used and hydrolysis period caused an increase in the amount of free amino group leading to an increase in DH. Lamsal, Jung and Johnson (2007) reported that during hydrolysis of soy protein isolate using bromelain, DH increased from 2 to 4% after increasing the enzyme to substrate ratio used from 0.14 to 0.61%. Chabanon *et al*. (2007) also found that an increase in Alcalase 2.4L®

Table 4.4 Effects of Flavourzyme® concentrations and hydrolysis period on free amino groups of the 0 H-GMB and 18 H-GMB.

Flavourzyme® concentration (% db)	Free amino group (µmole g^{-1} protein)					
	Hydrolysis period (h)					
	0	1	2	3	4	5
0 H-GMB						
0	75.79 ^{a} ±1.73	77.99^{ab} ±6.53	79.41^{ab}±5.61	77.57^{ab}±4.06	80.44^{ab}±7.46	81.63 ^{abc}±3.98
1	82.84 ^{abc}±3.04	93.20^{bcd}±4.39	98.91^{bcd}±5.00	104.76^{cd}±5.33	130.90^{d}±7.77	147.53 ^{e} ±6.84
3	112.14^{d}±12.14	184.04^{f}±13.51	256.40 ^{g}±12.36	356.74^{i}±24.12	408.86^{k}±14.09	446.55 ^{l} ±16.57
5	113.52^{d}±6.33	202.45 ^{f}±10.29	285.54 ^{h}±12.36	381.75^{j}±25.57	468.79^{l}±12.60	537.41 ^{m}±0.18
18 H-GMB						
0	217.35^{a}±7.39	222.53^{a} ±4.21	225.69^{a}±1.20	227.29^{a}±1.42	227.52^{ab}±0.79	228.20^{a}±0.78
1	255.25^{b}±4.40	325.94^{d}±10.08	379.13^{e}±8.91	429.13^{f}±14.05	488.23^{h}±6.14	546.87^{i}±10.57
3	300.48^{c}±9.86	432.54^{f}±10.57	491.86^{h}±20.41	542.80^{i}±7.06	595.97^{k}±23.90	647.79^{m}±10.97
5	300.03^{c}±9.86	458.23^{g}±8.29	568.46 ^{j}±10.12	623.02^{l}±21.91	668.24^{n}±6.81	711.13^{o}±19.68

$^{a,b,c,\cdots}$Means among the same group of germination period with different letters are significantly different ($p<0.05$).

db, dry basis

concentration to hydrolyse rapeseed globulins isolate from 2.27 to 11.3 AU per litter led to an increase in DH from 14.0 to 20.4% after 3 h of hydrolysis. In this experiment the result showed that at same condition of hydrolysis with the same amount enzyme and hydrolysis time, DH of 18 H-GMB was higher than that obtained from 0 H-GMB. It was because germination induces the synthesis of proteolytic enzymes that leading to partial hydrolysis of some proteins into peptides and amino acids. This new structure of protein in 18 H-GMB was in the form which easy to be hydrolyzed by Flavourzyme® compared to native protein in 0 H-GMB.

The enzymatic hydrolysis of food proteins is characterized by an initial phase with a rapid hydrolysis rate, which a large number of peptide bonds are hydrolyzed. After this rapid stage, rate of enzymatic hydrolysis decreases and finally reaches a stationary phase where no apparent increase in hydrolysis takes place. The shape of the hydrolysis curve is associated with enzyme inactivation, product inhibition by hydrolysis products formed at high degree of hydrolysis, a low K_m value for the soluble peptides that acts as effective substrate competitors to the unhydrolyzed foodprotein and possibly auto- digestion of the enzyme (Kristinsson and Rasca, 2000). In this experiment, the results show that the enzymatic hydrolysis of the germinated mungs bean was remarkably fast in the first 3 h and then slowly increased for all concentration of the enzyme added. This pattern occurred in both 0 H-GMB and 18 H-GMB. Clemente *et al*. (1999) reported that the enzymatic hydrolysis of chickpea protein isolate using Flavourzyme® was quits fast in the initial stage (0 to 120 min) and a decrease of the reaction rate was found in the final stage (120 to 180 min). Tang *et al*. (2009) also found that DH of buckwheat protein isolate during hydrolysis using Alcalase® was dependent on the enzyme concentration and hydrolysis time. The rate of the hydrolysis was fast during the initial 30 min and gradually decreased with the hydrolysis time increasing (180 to 360 min).

Table 4.5 Effects of Flavourzyme® concentrations and hydrolysis period on degree of hydrolysis of the 0 H-GMB and 18 H-GMB.

Flavourzyme® concentration (% db)	Degree of hydrolysis (%)					
	Hydrolysis period (h)					
	0	1	2	3	4	5
0 H-GMB						
0	0.00 ^{a}±0.00	0.49 ^{a}±0.05	0.73^{a}±0.06	0.72 ^{a}±0.02	0.72^{a}±0.01	0.73^{a}±0.04
1	1.36 ^{a}±0.15	4.55^{b}±0.47	5.98^{c}±0.11	8.17 ^{d}±0.25	11.62^{e}±2.36	14.64^{g}±0.54
3	3.61 ^{b}±0.68	12.76^{f}±0.57	16.21^{h}±0.23	19.50 ^{j}±0.81	23.60^{k}±0.39	27.54 ^{l}±0.53
5	6.55 ^{c}±0.67	18.08^{i}±0.33	23.25^{k}±0.67	32.53^{m}±0.70	34.64^{n}±0.57	40.42^{o}±0.59
18 H-GMB						
0	0.00 ^{a}±0.00	0.30 ^{a}±0.04	0.64^{a}±0.12	0.80^{a}±0.14	0.83 ^{a}±0.08	0.90^{a}±0.08
1	3.70 ^{b}±0.46	11.06^{d}±0.77	16.59^{e}±0.92	21.79^{f}±1.45	27.94 ^{g}±0.63	34.04^{h}±1.10
3	8.42 ^{c}±0.86	22.13^{f}±1.30	28.32^{g}±2.11	33.62^{h}±0.74	39.15 ^{j}±1.48	44.54^{l}±1.14
5	8.37 ^{c}±0.54	28.82^{g}±0.86	36.29^{i}±1.06	41.96^{k}±2.27	46.66^{m}±0.71	50.79^{n}±1.92

$^{a,b,c,\cdots}$Means among the same group of germination period with different letters are significantly different ($p<0.05$) .

db, dry basis

A mathematical model was applied to predict the equation for DH for the hydrolysis process of 0 H-GMB and 18 H-GMB. As the relationship between hydrolysis time and DH is rectangular hyperbola, the data of hydrolysis period and DH of each sample set were plotted and fitted to rectangular hyperbola equation as shown in Figure 4.1. DH at 0 h was ignored because it is an inheritance from the substrates. Least square analysis was used to calculate the constant using the Solver software in Microsoft Excel 2007™. The equation, coefficient of determination (*r*), Root Mean Square Error (*RMSE*) and Mean Rasidue Least Square (*MRS*) of predicted equation are shown in Table 4.6. It was found that, at all of the enzyme concentrations used, the relationships between hydrolysis time and DH showed a good fit in the form of rectangular hyperbola because it gave a higher *r* value and low *RMSE* and *MRS* compared to those of linear relationship. The above results showed that rectangular hyperbola equation was applicable for the prediction of DH in both of the 0 H-GMB and 18 H-GMB at the concentrations of 1 to 5% of Flavourzyme®.

In order to determine the relation between DH and FAG, the data of DH and FAG of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate were plotted, Figure 4.2. The result showed that the amount of FAG in germinated mung bean protein hydrolysates increased when DH increased. This trend was observed in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. The amount of FAG obtained from 18 H-GMB was higher than that obtained from 0 H-GMB at the similar level of DH. There was a strong positive linear correlation between DH and FAG for 0 H-GMB protein hydrolysate and also for 18 H-GMB protein hydrolysate with $r = 0.959$ and 0.998, respectively. It can be concluded that DH is the main factor that affects the amount of FAG in the germinated mung beans protein hydrolysate.

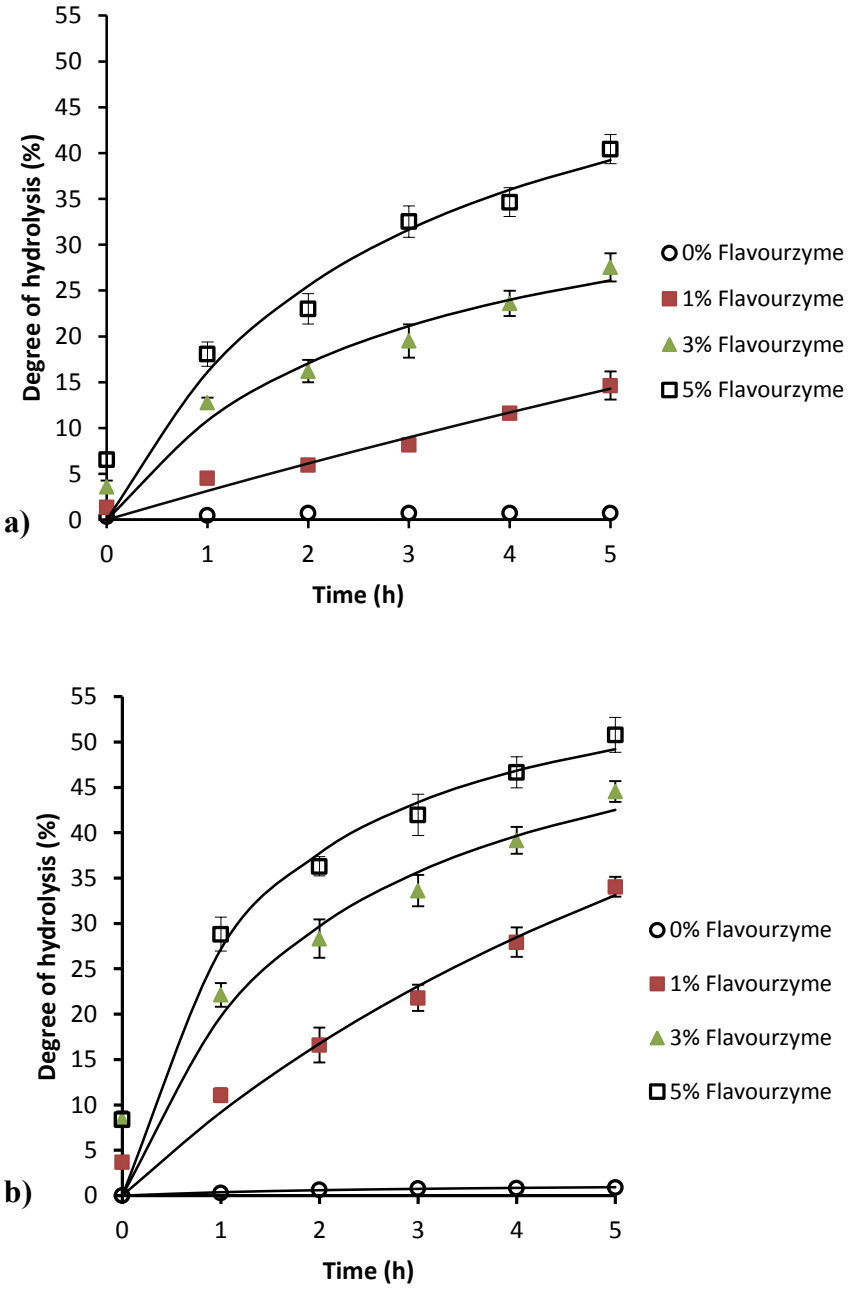


Figure 4.1 Effect of Flavourzyme® concentration and hydrolysis period on degree of hydrolysis of a) 0 H-GMB and b) 18 H-GMB.

Table 4.6 Equation and statistical coefficient parameters for regtangular hyperbola model to the experimentally derived degree of hydrolysis data of 0 H-GMB and 18 H-GMB.

Sample	Linear correlation				Rectangular hyperbola			
	Equation	***r***	***RMSE***	***MRS***	**Equation**	***r***	***RMSE***	***MRS***
0 H-GMB								
0% Flavourzyme®	y = 0.0737x + 0.439	0.843	0.0186	0.0372	$y = \frac{0.85x}{x+0.59}$	0.896	0.0007	0.0018
1% Flavourzyme®	y = 2.5657x + 1.3057	0.993	0.1353	0.2707	$y = \frac{126.57x}{x+39.26}$	0.983	0.2325	0.5579
3% Flavourzyme®	y = 4.4417x + 6.099	0.981	1.1077	2.2154	$y = \frac{40.42x}{x+2.74}$	0.968	0.7800	1.8721
5% Flavourzyme®	y = 6.53x + 9.546	0.981	2.4277	4.8554	$y = \frac{61.19x}{x+2.80}$	0.981	1.0826	2.5983
18 H-GMB								
0% Flavourzyme®	y = 0.1786x + 0.1319	0.940	0.0061	0.0122	$y = \frac{1.47x}{x+2.94}$	0.977	0.0001	0.0023
1% Flavourzyme®	y = 5.93x + 4.3633	0.998	0.1096	0.2192	$y = \frac{95.15x}{x+9.36}$	0.992	0.5312	1.2748
3% Flavourzyme®	y = 6.7703x + 12.438	0.979	2.8432	5.6864	$y = \frac{59.75x}{x+2.03}$	0.975	1.3384	3.2121
5% Flavourzyme®	y = 7.7497x + 16.106	0.943	10.8282	21.6564	$y = \frac{61.75x}{x+1.27}$	0.985	0.7716	1.8518

r, *correlation analysis*; *RMSE*, Root Mean Square Error and *MRS*, Mean Residue Least Square

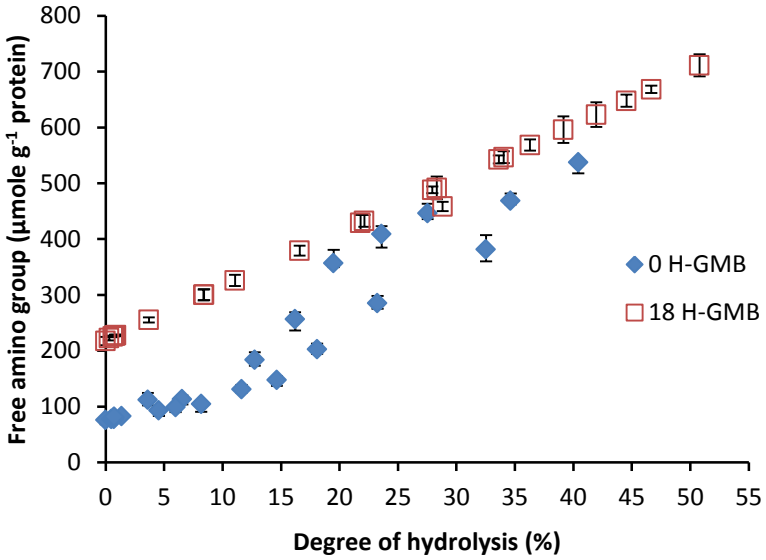


Figure 4.2 Effect of degree of hydrolysis on free amino group of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate.

4.2.2 Total phenolics content

As shown in Table 4.7, Flavourzyme® concentration and hydrolysis period significantly affected the total phenolics content (TPC) in all samples from 0 H-GMB to 18 H-GMB ($p<0.05$). The higher enzyme concentration and hydrolysis time resulted in the higher amount of TPC. There was an interaction between Flavourzyme® concentration and hydrolysis time on TPC in both 0 H-GMB and 18 H-GMB. For both 0 H-GMB and 18 H-GMB, hydrolysis of sample with 5% Flavourzyme® for 5 h gave the significantly highest TPC compared to other conditions ($p<0.05$). The amount of TPC in the raw germinated mung bean was 5.78 mg gallic acid g^{-1} (db) in the 18 H-GMB whilst the amount of TPC was 2.81 mg gallic acid g^{-1} (db) in 0 H-GMB. At all hydrolysis conditions, the amount of TPC in the 18 H-GMB protein hydrolysate was higher than that obtained from the 0 H-GMB protein hydrolysate. Higher amount of TPC was found when both the hydrolysis period increased and the enzyme concentration increased. The increase in the phenolics

Table 4.7 Effects of Flavourzyme® concentrations and the hydrolysis period on the total phenolics contents of the0 H-GMB and 18 H-GMB.

Flavourzyme® concentration (% db)	Total phenolics content (µg gallic acid ml^{-1} protein hydrolysate)					
	Hydrolysis period (h)					
	0	1	2	3	4	5
0 H-GMB						
0	320.68^{a} ±0.15	348.20^{a} ±0.08	362.73^{a} ±0.11	360.21^{a} ±0.12	359.44^{a} ±0.04	361.80^{a} ±0.17
1	390.66^{ab} ±0.12	426.32^{bc} ±0.16	466.93^{c} ±0.11	559.98^{d} ±0.12	584.91^{de} ±0.11	611.24^{e} ±0.13
3	467.44^{c} ±0.11	671.83^{f} ±0.16	702.52^{f} ±0.08	813.81^{g} ±0.17	863.18^{h} ±0.15	927.70^{ij} ±0.07
5	471.97^{c} ±0.23	713.54^{f} ±0.09	846.53^{gh} ±0.02	933.42^{i} ±0.22	1006.23^{jk} ±0.01	1040.92^{k} ±0.07
18 H-GMB						
0	496.67^{a} ±0.11	509.83^{ab} ±0.06	514.00^{ab} ±0.14	521.09^{ab} ±0.07	523.12^{ab} ±0.06	523.67^{ab} ±0.04
1	538.09^{ab} ±0.09	564.08^{b} ±0.19	621.50^{c} ±0.16	651.00^{cd} ±0.54	734.67^{de} ±0.42	838.48^{fg} ±0.59
3	554.33^{ab} ±0.29	697.50^{de} ±0.12	753.58^{ef} ±0.13	878.50^{h} ±0.28	920.38^{ij} ±0.61	1007.04^{ij} ±0.24
5	555.83^{ab} ±0.03	847.83^{gh} ±0.13	958.67^{i} ±0.02	1003.83^{ij} ±0.12	1026.38^{j} ±0.21	1106.14^{k} ±0.09

$^{a,b,c,\cdots}$Means among the same group of germination period with different letters are significantly different ($p<0.05$).
db, dry basis

content may be because phenolics in the seed are in the protein-bound form. They are released during hydrolysis. Wang and Tang (2012) hydrolyzed buckwheat protein isolate using trypsin and found that the amount of free phenolic content, expressed as rutin equivalent, in buckwheat protein hydrolysate increased from 0.38 to 0.97 % with an increasing of hydrolysis time from 0 to 2 h.

In order to determine the relation between DH and TPC, the data of DH and TPC of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate were plotted and presented in Figure 4.3. The results showed that the amount of TPC in the germinated mung bean protein hydrolysates increased when the DH increased. This trend was observed for both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysates. Tang *et al*. (2009) reported that during the hydrolysis of buckwheat protein isolate using Alcalase®, the hydrolysate resulted in the amount of TPC to a variable extent, depending upon the DH. With the DH increasing from 5 to 15%, TPC of the hydrolysates gradually decreased from 2.4 g to 1.1 g of rutin equivalent per 100 g of the sample, but the amount of TPC distinctly increased upon further hydrolysis obtaining 2.6 g of rutin equivalent 100 g^{-1} of the sample at DH of 20–25%. There was a strong positive linear correlation between DH and TPC for the 0 H-GMB protein hydrolysates and also for the 18 H-GMB protein hydrolysates with r = 0.985 and 0.980, respectively. It can be concluded that in the range of 0-50% DH of the germinated mung bean protein hydrolysates, higher DH leads to higher amount of TPC.

To study on the effects of DH on antioxidant ativities of the germinated mung bean protein hydrolysates in the next step, 11 conditions of the 0 H-GMB protein hydrolysates with different DH level ranges from 0 to 40% and another 11 conditions of the 18 H-GMB protein hydrolysates with different DH levels range from 0 to 50% were selected as representatives for the study. The DH levels of the selected germinated mung bean protein hydrolysate from each of the germinated groups were

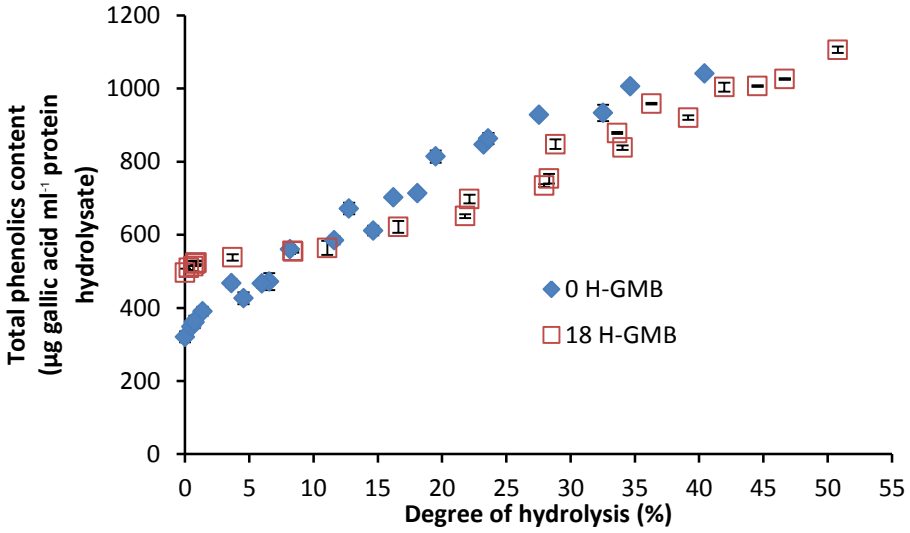


Figure 4.3 Effects of degree on total phenolics contents of the protein hydrolysates from the 0 H-GMB protein hydrolysates and the 18 H-GMB protein hydrolysates.

spread throughout the range of the DH level obtained from Section 4.2.1. (Table 4.5). The relationship between DH and FAG of 11 samples of the protein hydrolysates from each germinated group was plotted and presented in Figure 4.4 a. It was found that there was also a high positive linear correlation between DH and FAG in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with r = 0.979 and 0.997, respectively.

The relationship between DH and TPC of 11 samples from the 0 H-GMB protein hydrolysates and another 11 samples from the 18 H-GMB protein hydrolysates were also plotted and presented in Figure 4.4 b. It was also found that there was also a high positive linear correlation between DH and TPC in both of the 0 H-GMB protein hydrolysates and the 18 H-GMB protein hydrolysates with r = 0.988 and 0.983, respectively.

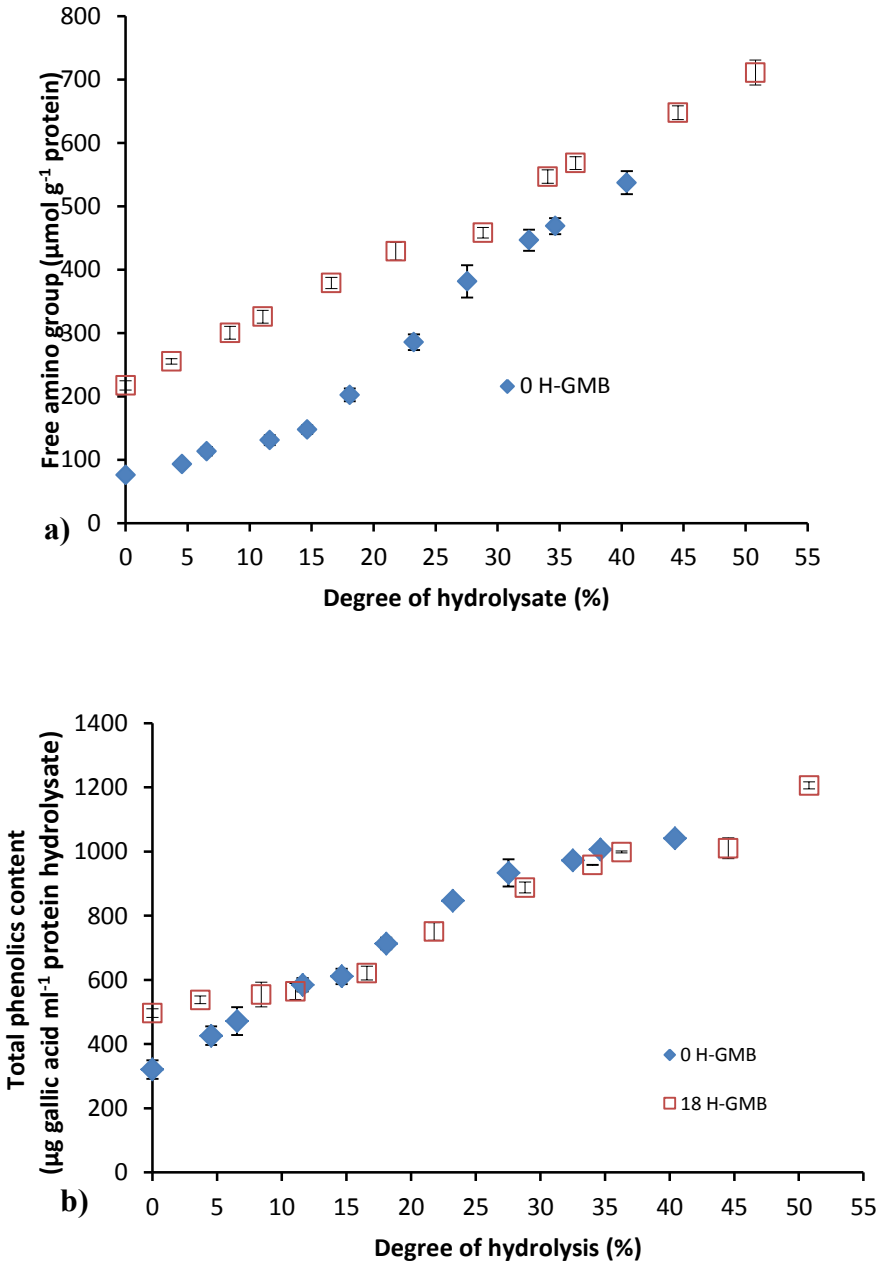


Figure 4.4 a) Relationship between degree of hydrolysis and free amino group and b) relationship between degree of hydrolysis and total phenolics content of hydrolysates obtained from the 0 h germinated mung bean (0 H-GMB) and the 18 h germinated mung beans (18 H-GMB).

From higher correlation of DH and FAG and higher correlation of DH and TPC of the selected samples from both of the 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysates, it can be concluded that these selected 11 samples of each germinated mung beans protein hydrolysates were good representatives for the whole samples from each of the germinated groups. These samples were selected for further study classified in the next section.

4.3 Antioxidant activities of freeze dried mung beans protein hydrolysates

The 11 samples of the 0 H-GMB protein hydrolysates and another 11 samples of the 18 H-GMB protein hydrolysates, with different levels of DH ranging from 0 to 50%, selected from Section 4.2 were subjected to freeze drying. The freeze-dried samples were kept in aluminum foil laminated-polyethylene bags at -20^{O}C and determined for antioxidant activities.

4.3.1 Antioxidant activity measured by chemical method

4.3.1.1 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable synthetic radical which is usully used as a substrate to evaluate the radical scavenging activity of antioxidative compounds (Sanchez-Moreno, 2002). Table 4.8 shows that the values in EC_{50} significantly decreased when DH increased. This trend can be observed for both 0 H-GMB protein hydrolysates and 18 H-GMB protein hydrolysates. It showed that DPPH radical scavenging activity of germinated mung bean protein hydrolysates significantly increased when DH increased (p<0.05). Thus, the results showed that germinated mung beans protein hydrolysates at relatively higher DH potentially contained certain substances which serve as hydrogen atom donors and could react with free radicals to convert them to more stable products and terminated the radical chain reaction. In order to make it easy for comparison with other standard antioxidant compounds, EC_{50} was converted to ascorbic acid equivalent antioxidant capacity. From Table 4.8, it can be seen that the DPPH radical scavenging activity of the 0 H-GMB protein hydrolysate at 40% DH and the 18 H-GMB protein hydrolysates at 50% DH were 557.65 and 540.50 mg ascorbic acid equivalent per

100g protein hydrolysate, respectively. Chan, Lim, and Chew (2007) evaluated DPPH radical scavenging activity of the methanol extracts of fresh tea leaves and also express as ascorbic acid equivalent antioxidant capacity. They found that mature tea leaves contained DPPH radical scavenging activity of 10,219 mg ascorbic acid equivalent per 100g.

Table 4.8 Effects of the degree of hydrolysis of the 0 H-GMB protein hydrolysates and the 18 H-GMB protein hydrolysates on DPPH radical scavenging activities expressed as EC_{50} and ascorbic acid equivalent antioxidant capacities.

Samples*	Degree of hydrolysis (%)	EC_{50} (mg ml^{-1})	Ascorbic acid equivalent antioxidant capacity (mg per100 g protein hydrolysate)
0 H-GMB			
0_{00}	0.00±0.00	4.75^{a} ±0.29	177.69^{a}±10.50
0_{11}	4.55±0.47	4.39^{a} ±0.76	195.44^{a}±31.56
0_{50}	6.55±0.67	4.69^{a} ±0.12	179.26^{a}±5.44
0_{14}	11.62±2.36	4.59^{a} ±0.28	183.77^{a}±11.38
0_{15}	14.64±0.54	3.09^{b}±0.28	273.97^{b}±24.26
0_{51}	18.08±0.33	2.57^{bc}±0.30	330.57^{bc}±38.43
0_{52}	23.25±0.67	2.71^{bc}±0.26	312.28^{bc}±30.62
0_{53}	27.54±0.53	2.85^{bc}±0.10	298.30^{bc}±14.42
0_{35}	32.53±0.70	2.33^{cd}±0.13	358.69^{c}±13.90
0_{54}	34.64±0.57	1.99^{de}±0.22	427.68^{d}±51.33
0_{55}	40.42±0.59	1.53^{e}±0.20	557.65^{e}±72.05
18 H-GMB			
18_{00}	0.00±0.00	4.21^{a}±0.46	201.48^{a}±22.35
18_{10}	3.70±0.46	3.63^{b}±0.55	236.16^{ab}±39.37
18_{30}	8.42±0.86	3.46^{b}±0.06	243.40^{bc}±4.22
18_{11}	11.06±0.77	3.40^{bc}±0.13	247.67^{bcd}±9.98
18_{12}	16.59±0.92	3.42^{bc}±0.27	247.19^{bcd}±18.78
18_{13}	21.79±1.45	2.81^{d}±0.29	301.74^{e}±29.92
18_{51}	28.82±0.86	2.91^{d}±0.05	289.09^{de}±5.52
18_{15}	34.04±1.10	2.97^{cd}±0.13	283.87^{cde}±12.38
18_{52}	36.29±1.06	2.03^{e}±0.19	416.34^{f}±36.49
18_{35}	44.54±1.14	1.99^{ef}±0.09	422.94^{f}±18.53
18_{55}	50.79±1.92	1.56^{f}±0.07	540.50^{g}±24.56

*Number is a germination period (h), first subscript is Flavourzyme® concentration (%) and second subscript is hydrolysis period (h)

$^{a,b,c,\cdots}$ Means in the same column among the same group of germination period with different letters are significantly different ($p<0.05$).

The increase in DPPH radical scavenging activity resulted from the increase in the amount of FAG and TPC as discussed in Sections 4.2.1 and 4.2.2. Jamdar *et al*. (2010) reported that the DPPH radical scavenging activity of the pea nut protein hydrolysates increased from 21 to 51% when the DH increased from 10 to 20% at a concentration of the protein hydrolysates at 2.0 mg ml^{-1}. Yang *et al*. (2011) also found that DPPH radical scavenging activity of soy sauce lees, hydrolyzed with Alcalase®2.4L, increased when hydrolysis time increased from 10 to 60 min.

The increase in antioxidant activity come from the hydrolysis of protein into small peptides and amino acids. The smaller peptide has the higher antioxidant activity than the large protein molecule. Moreover, during hydrolysis of mung bean released more bound-form phenolic compounds that also containing antioxidant activity.

From these results, it can be concluded that the 0 H-GMB protein hydrolysates and the 18 H-GMB protein hydrolysates could act as hydrogen atom donors to convert DPPH radial into stable products. Thus, it can be correlated that ability to scavenge free radical in food system.

4.3.1.2 Ferric reducing antioxidant power

As shown in Table 4.9 ferric reducing antioxidant power (FRAP) expressed as Trolox® equivalent of the germinated mung beans protein hydrolysates significantly increased when DH increased (p<0.05). This trend observed for both the 0 H-GMB protein hydrolysate and 18 the H-GMB protein hydrolysate. The values found in the 0 H-GMB protein hydroysates were similar to those in the 18 H-GMB. It means that at higher DH, the germinated mung beans protein hydrolysates had more reducing ability. Thus, these protein hydrolysates can react with reactive oxygen to prevent lipid oxidation in food systems. Zhang *et al*. (2011) prepared peanut peptides with different DH via fermentation of the peanut meals with *Bacillus subtilis* for 48, 72 and 96 h. It was found that the reducing power ability of peanut peptides increased from 25.33 to 33.17 and 36.80% when DH increased from 11.18, 16.20 and 21.41%, respectively.

As shown in Table 4.9, FRAP of 0 the H-GMB protein hydrolysates at 40% DH and the 18 H-GMB protein hydrolysate at 50% DH were 1.56 and 1.71 μmol Trolox® equivalent per g protein hydrolysate, respectively. Phanturat *et al*.(2010) evaluated FRAP of the skin gelatin hydrolysates prepared using Neutrase® with DH 5, 10 and 15%. The result showed that the skin gelatin hydrolysates with DH 5, 10 and 15% contained FRAP of 0.28, 0.32 and 0.59 μmol Trolox® equivalent per g protein, respectively.

Table 4.9 Effects of the degree of hydrolysis of the 0 H-GMB protein hydrolysates and the 18 H-GMB protein hydrolysates on ferric reducing antioxidant powers.

Samples*	Degree of hydrolysis (%)	FRAP (μmol Trolox® equivalent per g protein hydrolysate)
0 H-GMB		
0_{00}	0.00±0.00	0.67^{a}±0.08
0_{11}	4.55±0.47	0.79^{bc}±0.06
0_{50}	6.55±0.67	0.74^{ab}±0.04
0_{14}	11.62±2.36	0.90^{c}±0.02
0_{15}	14.64±0.54	1.16^{de}±0.01
0_{51}	18.08±0.33	1.20^{de}±0.06
0_{52}	23.25±0.67	1.11^{d}±0.11
0_{53}	27.54±0.53	1.16^{de}±0.11
0_{35}	32.53±0.70	1.26^{ef}±0.02
0_{54}	34.64±0.57	1.32^{f}±0.03
0_{55}	40.42±0.59	1.56^{g}±0.02
18 H-GMB		
18_{00}	0.00±0.00	0.88^{a}±0.02
18_{10}	3.70±0.46	0.89^{a}±0.05
18_{30}	8.42±0.86	1.00^{ab}±0.01
18_{11}	11.06±0.77	1.07^{b}±0.01
18_{12}	16.59±0.92	1.22^{c}±0.08
18_{13}	21.7±1.45	1.23^{c}±0.05
18_{51}	28.82±0.86	1.27^{cd}±0.02
18_{15}	34.04±1.10	1.33^{cd}±0.04
18_{52}	36.29±1.06	1.32^{cd}±0.10
18_{35}	44.54±1.14	1.40^{d}±0.04
18_{55}	50.79±1.92	1.71^{e}±0.21

*Number is a germination period (h), first subscript is Flavourzyme® concentration (%) and second subscript is hydrolysis period (h)

$^{a,b,c,\cdots}$ Means in the same column among the same group of germination period with different letters are significantly different ($p<0.05$).

4.3.1.3 Ferrous ion metal chelating activity

Table 4.10 shows that the value of EC_{50} significantly decreased when DN increased. This trend was observed for both of the 0 H-GMB protein hydrolysates and the 18 H-GMB protein hydrolysates. The results showed that the ferrous ion metal chelating activity of the germinated mung beans protein hydrolysates significantly increased when DH increased ($p<0.05$). This work suggested that higher degree of cleavage of peptide bonds renders a hydrolysate with higher metal chelating activities. In order to make it easy for comparison with other standard antioxidant compounds, EC_{50} of protein hydrolysate was converted to EDTA equivalent chelating capacity. It can be concluded that ferrous ion metal chelating activity of 18 H-GMB protein hydrolysate was higher than that of 0 H-GMB protein hydrolysate at similar DH level. The results showed that germinated mung bean protein hydrolysate possess high activity to chelate metal ion such as ferrous ion and copper ion. Since these metal ions can promote lipid oxidation as they catalyse the decomposition of lipid hydroperoxide into free radicals, thus protein hydrolysate containing chelating metal ion can also prevent lipid oxidation in food system. Jamdar *et al*. (2010) studied the influence of DH on antioxidant activity of peanut protein hydrolysate and found that ferrous ion metal chelating activity of hydrolysates were 62, 66, 78 and 85% when DH of peanut protein hydrolysates were 10, 20, 30, and 40%, respectively.

Thus, it can be inferred that germinated mung bean protein hydrolysate possess antioxidant activity because it has ability to scavenge free radical, react with reactive oxygen species and chelate metal ion. These properties of protein hydrolysate were dependent on DH of germinated mung bean protein hydrolysate. The higher DH led to an increase in antioxidant activity.

Table 4.10 Effects of the degree of hydrolysis of the 0 H-GMB protein hydrolysates and the 18 H-GMB protein hydrolysates on ferrous ion metal chelating activity expressed as EC_{50} and EDTA equivalent.

Samples*	Degree of hydrolysis (%)	EC_{50} (mg ml^{-1})	EDTA equivalent Fe^{2+} ion chelating capacity (mg per 100 g protein hydrolysate)
0 H-GMB			
0_{00}	0.00±0.00	2.55^{a}±0.05	330.19^{a}±6.34
0_{11}	4.55±0.47	2.15^{b}±0.10	403.98^{b}±18.58
0_{50}	6.55±0.67	1.99^{c}±0.06	434.67^{c}±11.98
0_{14}	11.62±2.36	1.82^{d}±0.05	476.02^{d}±11.90
0_{15}	14.64±0.54	1.74^{de}±0.03	497.78^{de}±7.51
0_{51}	18.08±0.33	1.69^{e}±0.04	511.59^{e}±11.58
0_{52}	23.25±0.67	1.60^{f}±0.09	543.44^{f}±29.75
0_{53}	27.54±0.53	1.45^{g}±0.04	596.19^{g}±16.87
0_{35}	32.53±0.70	1.37^{gh}±0.05	632.59^{h}±21.39
0_{54}	34.64±0.57	1.30^{h}±0.01	666.18^{i}±5.21
0_{55}	40.42±0.59	1.16^{i}±0.05	749.63^{j}±32.74
18 H-GMB			
18_{00}	0.00±0.00	1.89^{a}±0.04	457.54^{a}±9.97
18_{10}	3.70±0.46	1.75^{b}±0.03	494.02^{ab}±8.66
18_{30}	8.42±0.86	1.65^{c}±0.03	524.9^{6b}±9.55
18_{11}	11.06±0.77	1.50^{d}±0.02	577.40^{c}±7.70
18_{12}	16.59±0.92	1.39^{e}±0.05	624.96^{d}±20.41
18_{13}	21.7±1.45	1.33^{e}±0.06	650.32^{d}±27.97
18_{51}	28.82±0.86	1.21^{f}±0.04	716.12^{e}±21.10
18_{15}	34.04±1.10	1.13^{g}±0.01	766.41^{f}±6.78
18_{52}	36.29±1.06	1.06^{h}±0.02	814.53^{g}±11.65
18_{35}	44.54±1.14	0.91^{i}±0.03	948.65^{h}±26.01
18_{55}	50.79±1.92	0.75^{j}±0.04	1161.76^{i}±57.37

*Number is a germination period (h), first subscript is Flavourzyme® concentration (%) and second subscript is hydrolysis period (h).

$^{a,b,c,\cdots}$ Means in the same column among the same group of germination period with different letters are significantly different ($p<0.05$).

4.3.2 Antioxidant activity measured by electron spin resonance method

Electron spin resonance (ESR) method is used for the investigation of paramagnetic of a chemical compounds that given unpaired electron (Rohn and Kroh, 2005). This method was introduced to determine the radical scavenging activity of freeze-dried mung bean protein hydrolysates. Three types of radical, 1,1-diphynyl-2-

picrylhydrazyl (DPPH) radical, hydroxyl radical, and carbon center radical, were used as free radical species in this study.

4.3.2.1 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical is usually used as a substrate to evaluate the radical scavenging activity of antioxidants. To compare the radical scavenging activity with the chemical method, this radical was also used as free radical to determine the radical scavenging activity of the germinated mung beans protein hydrolysates. In this ESR technique to scavenge DPPH radical an antioxidant should have ability to be a hydrogen donor. After hydrogen atom is transferred from antioxidant, DPPH was converted to a stable product containing no lone pair electron. This stable product gave no signal in ESR spectroscopy. An example of ESR spectrum of DPPH radical was shown in Appendix B.1. The height of the third resonance peak was evaluated. As shown in Table 4.11 DPPH radical scavenging activity of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate significantly increased when DH increased ($p<0.05$). It can be concluded that after hydrolysis germinated mung bean with Flavourzyme®, germinated mung bean protein hydrolysate had higher ability to act as hydrogen donor. This ability of germinated mung bean protein hydrolysate changed DPPH radial to become a stable diamagnetic molecule. This action was a result from an increase in the amount FAG and TPC as discussed in Section 4.2.1 and 4.2.2. DPPH radical scavenging activity of 0 H-GMB protein hydrolysate increased in double (from 17.98 to 35.11%) when DH increased from 0 to 40%. DPPH radical scavenging activity of 18 H-GMB protein hydrolysate also increased in double (from 20.76 to 39.33 %) when DH increased from 0 to 50%. This increase in DPPH radical scavenging actitities was from hydrolysis of protein into small peptides and amino acids, and the release of TPC during hydrolysis. These compounds contribute antioxidant activity of germinated mung bean as discussed before. Peng *et al.* (2009) hydrolyzed whey protein isolate using Alcalase®2.4L for 5 h. After subjected to gel filtration chromatography, the whey protein hydrolysate was evaluated for DPPH radical scavenging activity by ESR method. It was found that whey protein hydrolysate that composed of peptides with a molecular weight ranging

Table 4.11 Effect of degree of hydrolysis on radical scavenging activity of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolyate measured using electron spin resonance at the concentration 1 mg $ml^{-1}$of crude protein.

Samples*	Degree of hydrolysis (%)	DPPH radical scavenging activity (%)	Hydroxyl radical scavenging activity (%)	Carbon center radical scavenging activity (%)
0 H-GMB				
0_{00}	0.00±0.00	17.98^{a}±1.80	20.61^{a}±2.23	29.99^{a}±1.89
0_{11}	4.55±0.47	18.12^{a}±1.11	27.59^{b}±0.02	35.08^{b}±1.69
0_{50}	6.55±0.67	18.67^{a}±1.43	33.95^{c}±0.13	37.89^{b}±0.68
0_{14}	11.62±2.36	20.67^{a}±1.41	37.67^{d}±0.31	47.75^{c}±2.73
0_{15}	14.64±0.54	28.59^{b}±1.49	41.31^{e}±0.02	51.31^{d}±1.57
0_{51}	18.08±0.33	28.60^{b}±0.05	43.36^{f}±0.28	55.74^{e}±0.28
0_{52}	23.25±0.67	29.14^{b}±1.39	44.37^{fg}±0.30	58.10^{ef}±0.32
0_{35}	27.54±0.53	29.24^{b}±1.59	45.76^{g}±0.18	59.84^{f}±0.84
0_{53}	32.53±0.70	29.75^{b}±0.72	45.86^{g}±0.03	65.63^{g}±1.49
0_{54}	34.64±0.57	30.88^{b}±0.90	48.06^{h}±0.16	70.13^{h}±1.00
0_{55}	40.42±0.59	35.11^{c}±0.13	52.57^{i}±1.08	72.09^{h}±0.40
18 H-GMB				
18_{00}	0.00±0.00	20.76^{a}±0.33	29.54^{a}±2.45	42.64^{a}±0.88
18_{10}	3.70±0.46	21.98^{b}±0.19	31.27^{a}±0.57	47.21^{b}±0.60
18_{30}	8.42±0.86	23.85^{c}±0.20	35.34^{b}±2.22	50.20^{c}±0.88
18_{11}	11.06±0.77	25.86^{d}±0.19	38.61^{c}±2.19	52.39^{d}±0.52
18_{12}	16.59±0.92	27.81^{e}±0.33	41.51^{c}±0.42	54.15^{d}±0.60
18_{13}	21.79±1.45	29.67^{f}±0.49	44.54^{d}±0.69	56.25^{e}±1.33
18_{51}	28.82±0.86	30.61^{f}±0.42	47.50^{e}±0.51	58.10^{e}±0.88
18_{15}	34.04±1.10	32.17^{g}±1.06	50.26^{e}±0.39	64.30^{f}±1.05
18_{52}	36.29±1.06	32.98^{g}±0.13	53.42^{f}±1.12	67.77^{g}±1.13
18_{35}	44.54±1.14	35.30^{h}±0.74	56.29^{fg}±1.03	74.56^{h}±0.76
18_{55}	50.79±1.92	39.33^{i}±0.56	58.55^{g}±0.63	77.29^{i}±0.12

*Number is a germination period (h), first subscript is Flavourzyme® concentration (%) and second subscript is hydrolysis period (h).

$^{a,b,c,\cdots}$ Means in the same column among the same group of germination period with different letters are significantly different ($p<0.05$).

from 0.1 to 2.8 kDa exhibited the strongest DPPH radical scavenging activity (58.67%).

4.3.2.2 Hydroxyl radical scavenging activity

Hydroxyl radical is one of the free radicals products occurred in lipid oxidation. In this experiment, hydroxyl radical was generated by Fenton's reaction (Je *et al.*, 2005). Since half-life of hydroxyl radical is very short, 5,5-dimethyl-1-pyroline-N-oxide (DMPO) spin trap was used to trap this radical. The adduct DMPO–OH radical exhibits a characteristic ESR response.

With ESR technique, to scavenging hydroxyl radical an antioxidant should have ability to be hydrogen donor to fulfill the lone pair electron in hydroxyl radical. This obtained stable product shows no signal in ESR spectroscopy. An example of ESR spectrum of hydroxyl radical was shown in Appendix B.2. The height of the second resonance peak was evaluated. Table 4.11 shows that hydroxyl radical scavenging activity of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate significantly increased when DH increased ($p<0.05$). From this result, it can be seen that after hydrolysis germinated mung bean with Flavourzyme®, germinated mung bean protein hydrolysate had higher ability to act as hydrogen donor and stabilized hydroxyl radical into stable product. Hydroxyl radical scavenging activity of 0 H-GMB protein hydrolysate increased from 20.61 to 52.57% when DH increased from 0 to 40%. Hydroxyl radical scavenging activity of 18 H-GMB protein hydrolysate also increased from 29.54 to 58.55% when DH increased from 0 to 50%. The data showed that with higher DH, germinated mung bean protein hydrolysate had higher ability to prevent lipid oxidation via scavenge hydroxyl radical. Peng *et al*. (2009) fractionated whey protein into four fractions (I, II, III and IV) that were composed of peptides of ≥ 40 kDa, 2.8-40kDa, 0.1-2.8 kDa, and <0.1 kDa. These fractions were evaluated for hydroxyl radical scavenging activity. The result showed that hydroxyl radical scavenging activity of whey protein hydrolysate depended on peptides size. Fraction III obtained the highest hydroxyl radical scavenging activity (65.67%) follow by fraction II (47.33%), fraction I (35.00%) and fraction IV (23.33%), respectively.

4.3.2.3 Carbon-centerd radical scavenging activity

Carbon-centered radical can react with molecular oxygen to give peroxyl radical. Peroxyl radical is a radical produced from lipid oxidation. In this study carbon-centered radical, generated from 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and α-(4-pirydryl-1-oxide)-N-tret-butylnitrone (4-POBN) was used as spin-trapping agent. With this ESR technique, to scavenge carbon-centered radical an antioxidant should have ability to be hydrogen donor. After hydrogen atom is transferred from antioxidant, carbon-centered radical was converted to a stable product. This stable product shows no signal in ESR spectroscopy. An example of ESR spectrum of carbon center radical was shown in Appendix B.3. The height of the first resonance peak was evaluated. Table 4.11 shows that carbon center radical scavenging activity of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate significantly increased when DH increased ($p<0.05$). Carbon center radical scavenging activity of 0 H-GMB protein hydrolysate increased from 29.99 to 72.09% when DH increased from 0 to 40. Carbon-centered radical scavenging activity of 18 H-GMB protein hydrolysate also increased from 42.64 to 77.29% when DH increased from 0 to 50%. The results showed that germinated mung bean protein hydrolysate can prevent lipid oxidation via scavenge peroxyl radical scavenging. This scavenging ability depended on DH of germinated mung bean protein hydrolysate. Je *et al.* (2005) hydrolyzed hoki frame protein with pepsin. Hoki frame protein hydrolysate (HPH) was fractionated into four major types, HPH I (5-10 kDa), HPH II (3-5 kDa), HPH III (1-3 kDa), and HPH IV (below 1 kDa), using an ultrafiltration membrane. HPH were determined for carbon-centered radical scavenging activity. It was found that carbon-centered radical scavenging activity of HPH depended on molecular weight. HPH III obtained the highest carbon-centered radical scavenging activity follow by HPH II, HPH I, and HPH IV, respectively.

4.3.3 Correlation between degree of hydrolysis and antioxidant activities of germinated mung bean protein hydrolysate

Germinated mung bean protein hydrolysate at different level of degree of hydrolysis (DH) contained different amount of free amino group (FAG) and total phenolics content (TPC). Antioxidant activity of germinated mung bean protein hydrolysates depended on DH, FAG and TPC. Thus, the correlation between DH and antioxidant activities, the correlation between FAG and antioxidant activities, and the correlation between DH and antioxidant activities were determined.

4.3.3.1 Correlation between degree of hydrolysis and antioxidant activities measured by chemical method

Figure 4.5 (a) shows the effect of DH on DPPH radical scavenging activity of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong negative linear correlation between DH and DPPH radical scavenging activity expressed as EC_{50} in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with r = 0.937 and 0.950, respectively (Table 4.12). Higher correlation between DH and DPPH radical scavenging activity was found in 18 H-GMB protein hydrolysate compared to that obtained from 0 H-GMB protein hydrolysate. Correlation between DH and DPPH radical scavenging activity of 22 samples, 11 samples of 0 H-GMB protein hydrolysate and 11 samples from18 H-GMB protein hydrolysate, also gave a high r value (0.901). It can be seen that there was a linear correlation between DH and DPPH radical scavenging activity in germinated mung bean hydrolysate. Thus, DPPH radical scavenging activity can be predicted using linear equation.

Figure 4.5 (b) shows the effect of DH on ferric reducing antioxidant power (FRAP) of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was also a strong positive linear correlation between DH and FRAP in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with r = 0.942 and 0.958, respectively (Table 4.12). Lowerer correlation between DH and FRAP was found in 0 H-GMB protein hydrolysate compared to that obtained from 18 H-GMB protein hydrolysate. Correlation between DH and FRAP of 22 samples, obtained from both 0

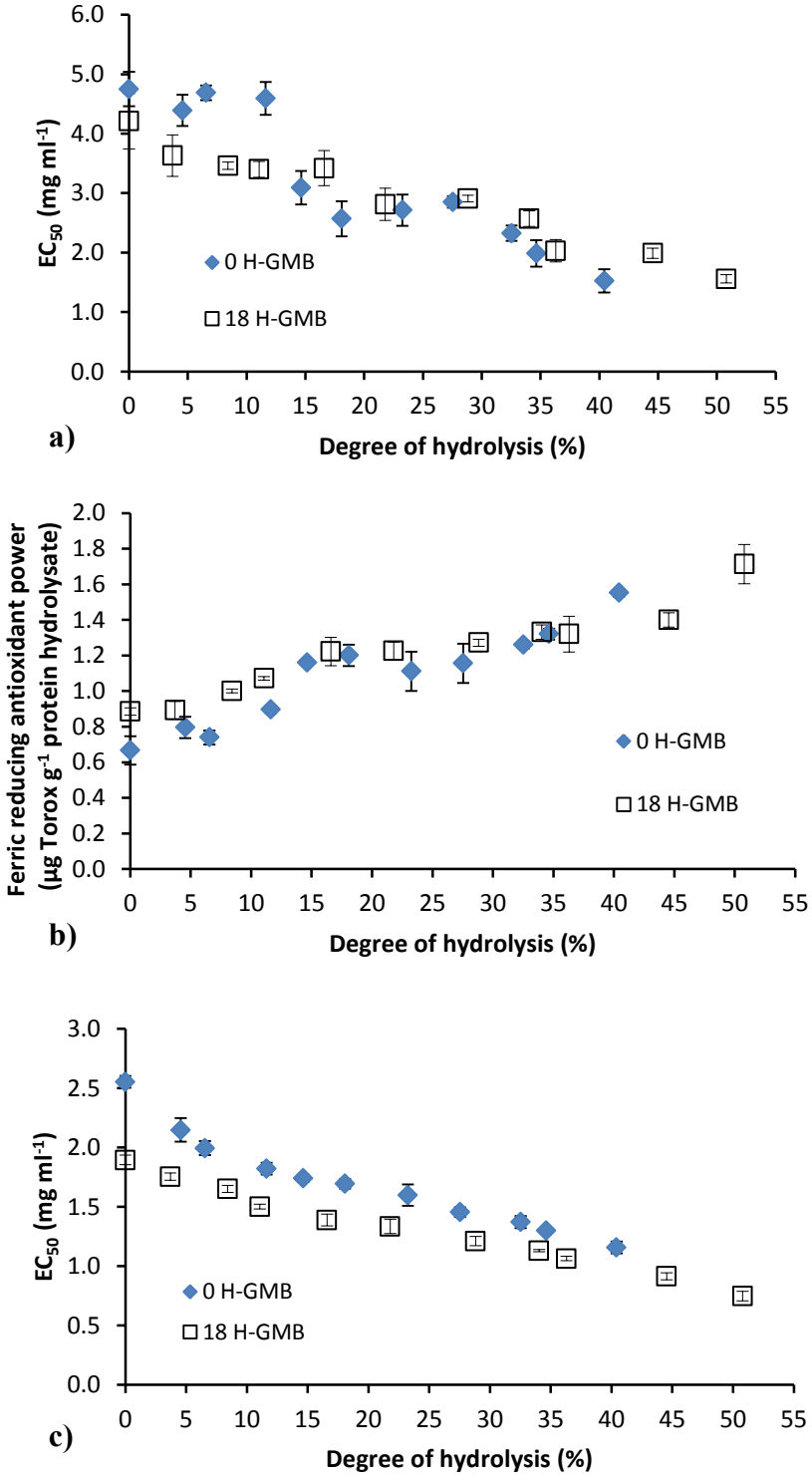


Figure 4.5 a) Effect of degree of hydrolysis on DPPH radical scavenging activity expressed as EC_{50}, b) effect of degree of hydrolysis on ferric reducing antioxidant power, and c) effect of degree of hydrolysis on ferrous ion metal chelating activity expressed as EC_{50}

Table 4.12 Correlation between degree of hydrolysis and antioxidant activities of germinated mung bean protein hydrolysates.

Correlation	Sample	Equation	*r*
DH and DPPH radical scavenging activity	0 H-GMB	y = -0.082x + 4.83	0.935
	18 H-GMB	y = -0.045x + 3.99	0.950
	0 and 18 H-GMB*	y = -0.059x + 4.44	0.901
DH and FRAP	0 H-GMB	y = 0.019x + 0.703	0.942
	18 H-GMB	y = 0.013x + 0.891	0.958
	0 and 18 H-GMB*	y = 0.016x + 0.799	0.930
DH and metal chelating activity	0 H-GMB	y = -0.029x + 2.283	0.963
	18 H-GMB	y = -0.020x + 1.811	0.990
	0 and 18 H-GMB*	y = -0.025x +2.062	0.930

* 22 samples that came from 11 samples of 0 H-GMB protein hydrolysate and 11 samples of 18 H-GMB protein hydrolysate.

H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate, also gave a high *r* value (0.930). It can be seen that there was a linear correlation between DH and FRAP in germinated mung bean hydrolysate. Thus, FRAP can be predicted using linear equation.

Figure 4.5 (c) shows the effect of DH on ferrous ion metal chelating activity of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong negative linear correlation between DH and ferrous ion metal chelating activity expressed as EC_{50} in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with r = 0.963 and 0.990, respectively (Table 4.12). Higher correlation between DH and ferrous ion metal chelating activity was found in 18 H-GMB protein hydrolysate compared to that obtained from 0 H-GMB protein hydrolysate. Correlation between DH and ferrous ion metal chelating activity of 22 samples, selected from Section 4.2, also gave a high *r* value (0.930). It can be seen that there was a linear correlation between DH and ferrous ion metal chelating activity in germinated mung bean hydrolysate. Thus ferrous ion metal chelating activity can be predicted using linear equation.

High *r* value gave a high correlation between two variables. In all sample groups, *r* value calculated from the correlation between DH and metal

chelating activity was higher than that obtained from the correlation between DH and DPPH radical scavenging activity and the correlation between DH and FRAP. From the *r* value depicted in Table 4.12, it may be concluded that DH had more effect on ferrous ion metal chelating activity than DPPH radical scavenging activity and FRAP.

4.3.3.2 Correlation between free amino group and antioxidant activities measured by chemical method

Figure 4.6 (a) shows the correlation between FAG and DPPH radical scavenging activity of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong negative linear correlation between FAG and DPPH radical scavenging activity expressed as EC_{50} in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with r = 0.890 and 0.980, respectively (Table 4.13). Higher correlation between FAG and DPPH radical scavenging activity was found in 18 H-GMB protein hydrolysate compared to that obtained from 0 H-GMB protein hydrolysate. Correlation between FAG and DPPH radical scavenging activity of 22 samples, 11 samples of 0 H-GMB protein hydrolysate and 11 samples from18 H-GMB protein hydrolysate, also gave a high *r* value (0.868). It can be seen that there was a linear correlation between FAG and DPPH radical scavenging activity in germinated mung bean hydrolysate.

Figure 4.6 (b) shows the effect of FAG and FRAP of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong positive linear correlation between free amino group and FRAP in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with r = 0.868 and 0.959, respectively (Table 4.13). Lower correlation between FAG and FRAP was found in 0 H-GMB protein hydrolysate compared to that obtained from 18 H-GMB protein hydrolysate. Correlation between FAG and FRAP of 22 samples, obtained from both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate, also gave a high *r* value (0.899). It can be seen that there was a linear correlation between FAG and FRAP in germinated mung bean hydrolysate.

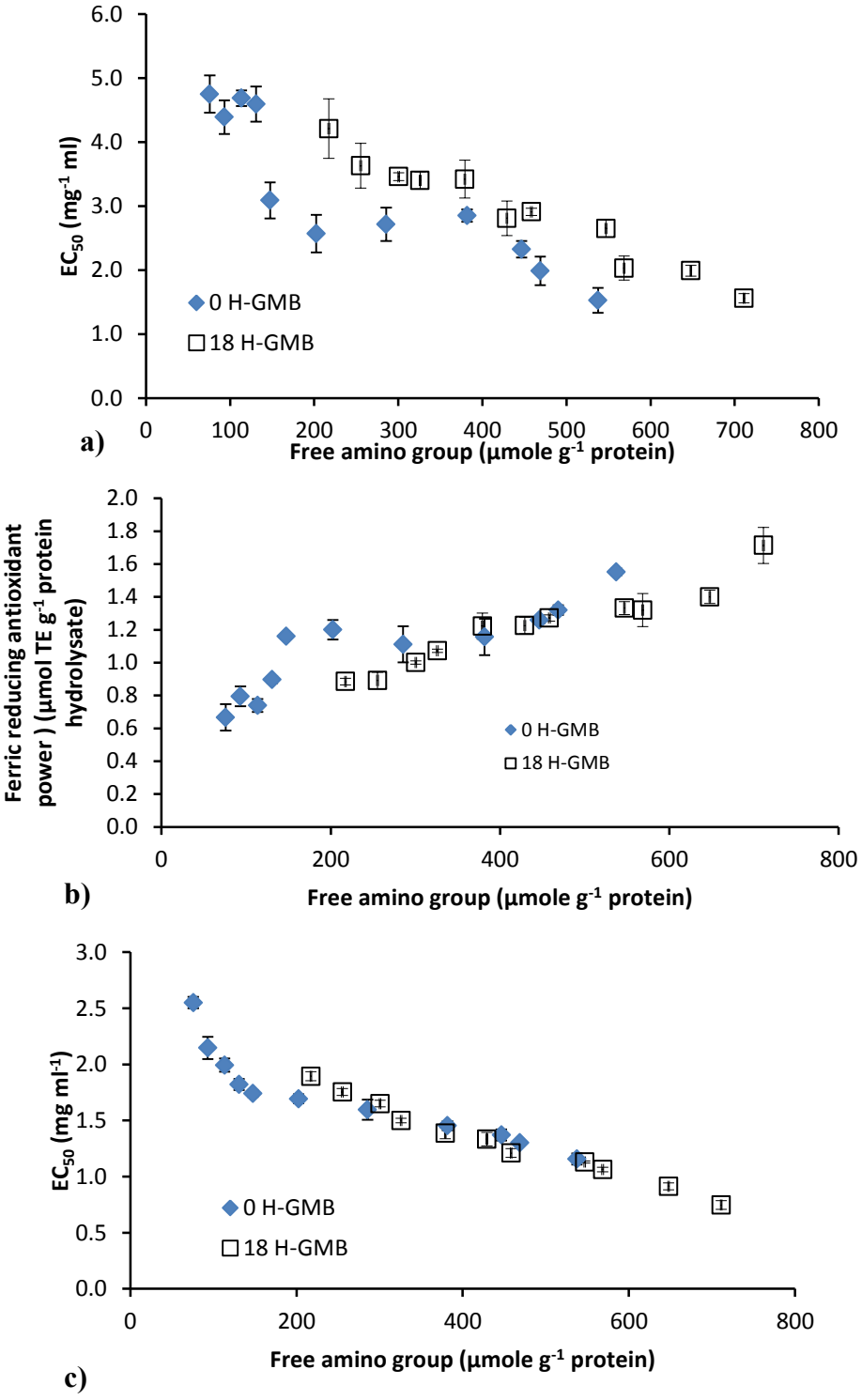


Figure 4.6 a) Correlation between free amino group and DPPH radical scavenging activity expressed as EC_{50}, b) correlation between free amino group and ferric reducing antioxidant power, and c) correlation between free amino group and ferrous ion metal chelating activity expressed as EC_{50}.

Table 4.13 Correlation between free amino group and antioxidant activities of germinated mung bean protein hydrolysates.

Correlation	Sample	Equation	*r*
FAG and DPPH radical scavenging activity	0 H-GMB	y = -0.006x + 4.843	0.890
	18 H-GMB	y = -0.005x + 5.071	0.980
	0 and 18 H-GMB*	y = -0.004x + 4.720	0.868
FAG and FRAP	0 H-GMB	y = 0.001x + 0.706	0.880
	18 H-GMB	y = 0.001x + 0.582	0.959
	0 and 18 H-GMB*	y = 0.001x + 0.701	0.899
FAG and metal chelating activity	0 H-GMB	y = -0.002x + 2.280	0.902
	18 H-GMB	y = -0.002x + 2.278	0.988
	0 and 18 H-GMB*	y = -0.002x +2.279	0.953

* 22 samples that came from 11 samples of 0 H-GMB protein hydrolysate and 11 samples of 18 H-GMB protein hydrolysate.

Figure 4.5 (c) shows the effect of FAG and ferrous ion metal chelating activity of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong negative linear correlation between FAG and ferrous ion metal chelating activity expressed as EC_{50} in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with r = 0.902 and 0.988, respectively (Table 4.13). Higher correlation between FAG and ferrous ion metal chelating activity was found in 18 H-GMB protein hydrolysate compared to that obtained from 0 H-GMB protein hydrolysate. Correlation between FAG and ferrous ion metal chelating activity of 22 samples, selected from Section 4.2, also gave a high r value (0.953). It can be seen that there was a linear correlation between FAG and ferrous ion metal chelating activity in germinated mung bean hydrolysate.

High r value gave a high correlation between two variables. In all sample groups, r value calculated from the correlation between FAG and metal chelating activity was higher than that obtained from the correlation between FAG and DPPH radical scavenging activity and the correlation between FAG and FRAP. From the r value depicted in Table 4.13 it may be concluded that FAG had more effect on ferrous ion metal chelating activity than DPPH radical scavenging activity and FRAP.

4.3.3.3 Correlation between total phenolics content and antioxidant activities measured by chemical method

Figure 4.7 (a) shows the correlation between TPC and DPPH radical scavenging activity of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong negative linear correlation between TPC and DPPH radical scavenging activity expressed as EC_{50} in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with r = 0.920 and 0.963, respectively (Table 4.14). Higher correlation between TPC and DPPH radical scavenging activity was found in 18 H-GMB protein hydrolysate compared to that obtained from 0 H-GMB protein hydrolysate. Correlation between TPC and DPPH radical scavenging activity of 22 samples, 11 samples of 0 H-GMB protein hydrolysate and 11 samples from18 H-GMB protein hydrolysate, also gave a high r value (0.934). It can be seen that there was a linear correlation between TPC and DPPH radical scavenging activity in germinated mung bean hydrolysate. Marathe *et al*. (2011) reported that there was a positive linear correlation between TPC and DPPH radical scavenging activity in thirty commonly consumed legumes in India with r = 0.975.

Figure 4.7 (b) shows the effect of TPC and FRAP of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong positive linear correlation between TPC and FRAP in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with r = 0.914 and 0.966, respectively (Table 4.14). Lower correlation between TPC and FRAP was found in 0 H-GMB protein hydrolysate compared to that obtained from 18 H-GMB protein hydrolysate. Correlation between TPC and FRAP of 22 samples, from both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate, also gave a high r value (0.935). It can be seen that there was a linear correlation between TPC and FRAP in germinated mung bean hydrolysate. Marathe *et al*. (2011) reported that there was a positive linear correlation between TPC and FRAP in thirty commonly consumed legumes in India with r = 0.997.

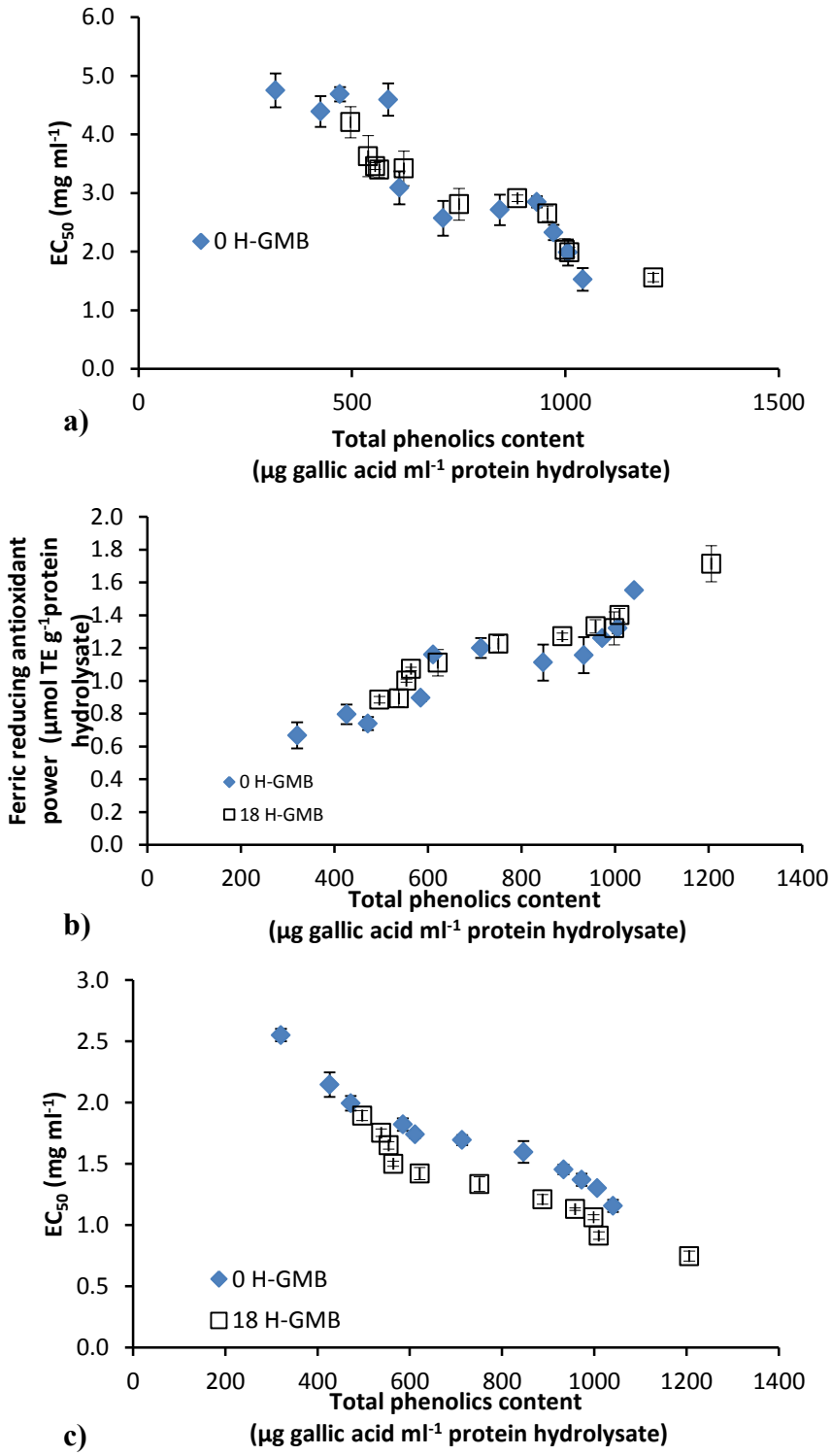


Figure 4.7 a) Correlation between total phenolics content and DPPH radical scavenging activity expressed as EC_{50}, b) correlation between total phenolics content and ferric reducing antioxidant power, and c) correlation between total phenolics content and ferrous ion metal chelating activity expressed as EC_{50}.

Table 4.14 Correlation between total phenolics content and antioxidant activities of germinated mung bean protein hydrolysates.

Correlation	Sample	Equation	*r*
TPC and DPPH radical scavenging activity	0 H-GMB	y = -0.004x + 6.187	0.920
	18 H-GMB	y = -0.003x + 5.453	0.963
	0 and 18 H-GMB*	y = -0.004x + 5.996	0.934
TPC and FRAP	0 H-GMB	y = 0.001x + 0.371	0.914
	18 H-GMB	y = 0.001x + 0.420	0.966
	0 and 18 H-GMB*	y = 0.001x + 0.381	0.935
TPC and metal chelating activity	0 H-GMB	y = -0.001x + 2.862	0.966
	18 H-GMB	y = -0.001x + 2.486	0.974
	0 and 18 H-GMB*	y = -0.001x +2.733	0.920

* 22 samples that came from the similar DH level of 11 samples of 0 H-GMB protein hydrolysate and 11 samples of 18 H-GMB protein hydrolysate.

Figure 4.7 (c) shows the effect of TPC and ferrous ion metal chelating activity of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong negative linear correlation between TPC and ferrous ion metal chelating activity expressed as EC_{50} in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with r = 0.966 and 0.974, respectively (Table 4.14). Higher correlation between TPC and ferrous ion metal chelating activity was found in 18 H-GMB protein hydrolysate compared to that obtained from 0 H-GMB protein hydrolysate. Correlation between TPC and ferrous ion metal chelating activity of 22 samples, selected from Section 4.2, also gave a high r value (0.920). It can be seen that there was a linear correlation between TPC and ferrous ion metal chelating activity in germinated mung bean hydrolysate. However, Marathe *et al*. (2011) reported a poor correlation between TPC and ferrous ion metal chelating activity in thirty commonly consumed legumes in India with r = 0.296.

High r value gave a high correlation between two variables. In all sample groups, r value calculated from the correlation between TPC and metal chelating activity was higher than that obtained from the correlation between TPC and DPPH radical scavenging activity and the correlation between TPC and FRAP. From the r value depicted in Table 4.14 it may be pointed that TPC had more effect on ferrous ion metal chelating activity than DPPH radical scavenging activity and FRAP.

However, Marathe *et al*. (2011) also reported that TPC in thirty commonly consumed legumes in India had more effect on DPPH radical scavenging activity and FRAP than ferrous ion metal chelating activity.

4.3.3.4 Correlation between degree of hydrolysis and radical scavenging activities measured by electron spin resonance method

Figure 4.8 shows the effect of DH on radical scavenging activity, measured by electron spin resonance method, of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong positive linear correlation between DH and radical scavenging activity in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with high *r* value (Table 4.15). Higher correlation between DH and radical scavenging activity was found in 18 H-GMB protein hydrolysate, compared to 0 H-GMB protein hydrolysate. Correlation between DH and radical scavenging activity of 22 samples, 11 samples of 0 H-GMB protein hydrolysate and 11 samples from18 H-GMB protein hydrolysate, also gave a high *r* value. At all DH levels, the highest scavenging activity was found on carbon-centered radical in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate followed by scavenging activity on hydroxyl radical and scavenging activity on DPPH radical, respectively. Data also showed that there was a linear correlation between DH radical scavenging activities measured by electron spin resonance method in germinated mung bean hydrolysate, Table 4.15. Qian, Jung and Kim (2008) hydrolyzed bullfrog skin with Alcalase® and Neutrase® for 8 h. The hydrolysates were subjected to evaluate scavenging activity on DPPH radical, hydroxyl radical and peroxyl (carbon-centered) radical using ESR method. It was found that bullfrog skin protein hydrolysate prepared by Alcalase® (DH 58.7%) gave the highest scavenging activity on hydroxyl radical (63.8%) followed by scavenging activity on carbon-centered radical (58.4%) and scavenging activity on DPPH radical (56.3%), respectively. However, bullfrog skin protein hydrolysate prepared by Neutrase® (DH 37.2%) gave the highest scavenging activity on carbon-centered radical (47.6%) followed by scavenging activity on hydroxyl radical (42.3%) and scavenging activity on DPPH radical (30.5%), respectively.

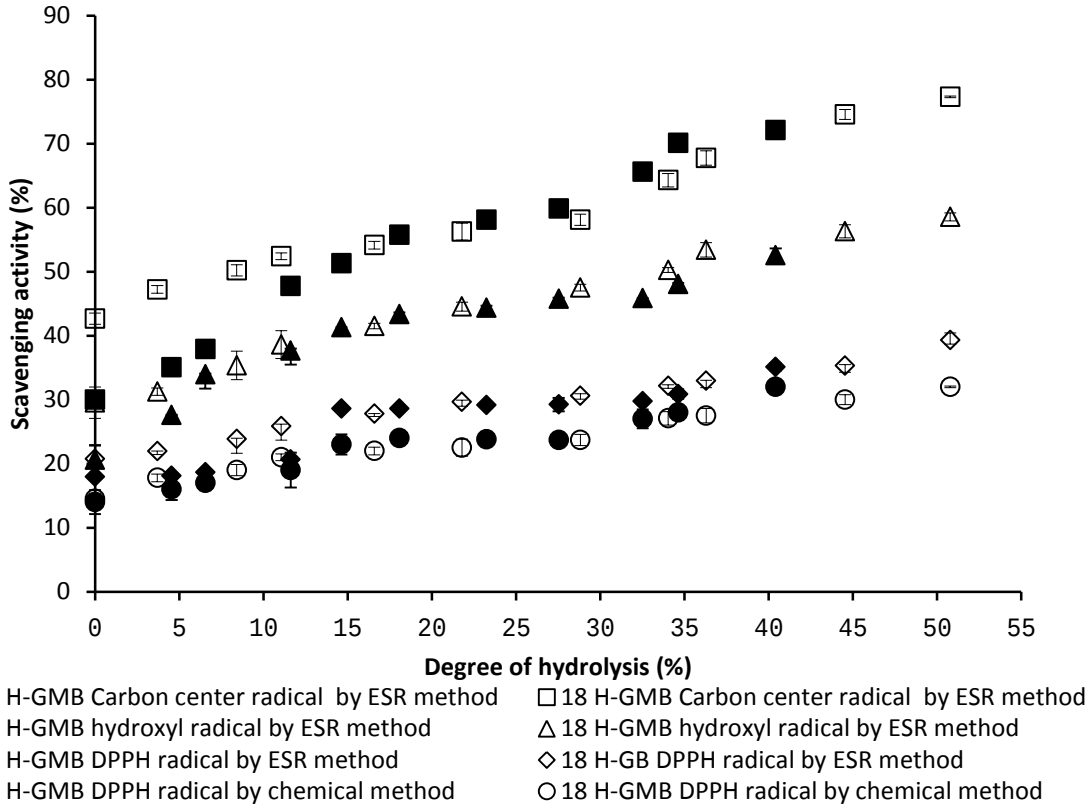


Figure 4.8 Effect of degree of hydrolysis and radical scavenging activities measured by electron spin resonance method.

Table 4.15 Correlation between degree of hydrolysis and radical scavenging activities of germinated mung bean protein hydrolysates measured by ESR.

Correlation	Sample	Equation	*r*
DH and carbon-centered radical	0 H-GMB	y = 1.045x + 32.72	0.982
	18 H-GMB	y = 0.648x + 43.52	0.986
	0 and 18 H-GMB*	y = 0.811x +38.50	0.953
DH and hydroxyl radical	0 H-GMB	y = 0.661x + 27.24	0.931
	18 H-GMB	y = 0.581x + 30.72	0.992
	0 and 18 H-GMB*	y = 0.620x + 28.93	0.959
DH and DPPH radical	0 H-GMB	y = 0.420x + 17.89	0.928
	18 H-GMB	y = 0.336x + 21.29	0.990
	0 and 18 H-GMB*	y = 0.375x + 19.56	0.946
DH and DPPH radical (by chemical method)	0 H-GMB	y = 0.401x + 14.70	0.971
	18 H-GMB	y = 0.312x + 16.11	0.986
	0 and 18 H-GMB*	y = 0.344x + 15.59	0.970

* 22 samples that came from the similar DH level of 11 samples of 0 H-GMB protein hydrolysate and 11 samples of 18 H-GMB protein hydrolysate.

Higher scavenging activity of DPPH radical was found with measurement using ESR compared chemical method in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. This may be because these two methods have different mechanism. ERS method directly measures on unpaired electron in the reaction under magnetic field. The ESR signal decrease when antioxidant transfers hydrogen atom or electron to unpaired electron in the reaction (Rohn and Kroh, 2005). Chemical method measures the decreasing in absorbance of DPPH chromagen by spectrophotometer at 515 nm when DPPH radical receives hydrogen atom from antioxidant (Brand-Williams, Cuvelier and Berset, 1995).

4.3.3.5 Correlation between free amino group and radical scavenging activities measured by electron spin resonance method

Figure 4.9 shows the correlation between FAG and radical scavenging activity, measured by electron spin resonance method, of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong positive linear correlation between FAG and radical scavenging activity in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate with high *r* value (Table 4.16). Lower correlation between FAG and radical scavenging activity was found in 0 H-GMB protein hydrolysate compared to that obtained from 18 H-GMB protein hydrolysate. Correlation between FAG and radical scavenging activity of 22 samples, from both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate, also gave a high *r* value. At all FAG contents, the highest scavenging activity was found on carbon-centered radical in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate followed by scavenging activity on hydroxyl radical and scavenging activity on DPPH radical, respectively. It can be seen that there was a linear correlation between FAG and radical scavenging activities measured by electron spin resonance method in germinated mung bean hydrolysate.

Higher scavenging activity of DPPH radical was also found with measurement by ESR compared to chemical method in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. The ESR signal decrease when antioxidant transfers hydrogen atom or electron to unpaired electron in the reaction

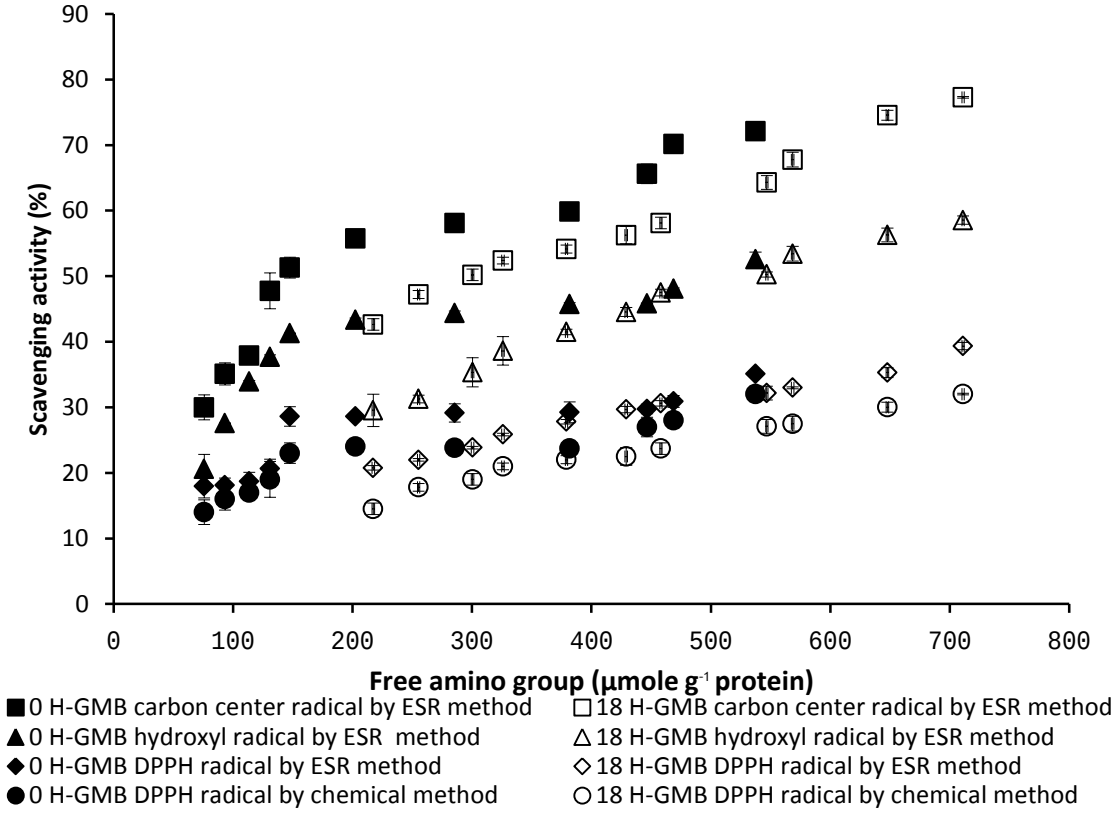


Figure 4.9 Correlation between free amino group and radical scavenging activities of germinated mung bean protein hydrolysate measured by ERS.

Table 4.16 Correlation between free amino group and radical scavenging activities of germinated mung bean protein hydrolysates by ESR.

Correlation	Sample	Equation	*r*
FAG and carbon-centered radical	0 H-GMB	y = 0.077x + 32.62	0.925
	18 H-GMB	y = 0.068x + 28.74	0.992
	0 and 18 H-GMB*	y = 0.063x +33.68	0.931
FAG and hydroxyl radical	0 H-GMB	y = 0.047x + 27.69	0.847
	18 H-GMB	y = 0.060x + 17.71	0.989
	0 and 18 H-GMB*	y = 0.046x + 25.91	0.888
FAG and DPPH radical	0 H-GMB	y = 0.030x + 17.99	0.865
	18 H-GMB	y = 0.035x + 13.73	0.989
	0 and 18 H-GMB*	y = 0.029x + 17.39	0.906
FAG and DPPH radical (by chemical method)	0 H-GMB	y = 0.030x + 14.66	0.918
	18 H-GMB	y = 0.326x + 9.04	0.990
	0 and 18 H-GMB*	y = 0.25x + 14.20	0.869

* 22 samples that came from the similar DH level of 11 samples of 0 H-GMB protein hydrolysate and 11 samples of 18 H-GMB protein hydrolysate.

(Rohn and Kroh, 2005). Chemical method measures the decreasing in absorbance of DPPH chromagen by spectrophotometer at 515 nm when DPPH radical receives hydrogen atom from antioxidant (Brand-Williams *et al*., 1995).

4.3.3.6 Correlation between total phenolics content and radical scavenging activities measured by electron spin resonance method

Figure 4.10 shows the correlation between total phenolic content (TPC) and radical scavenging activities measured by electron spin resonance method of 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. There was a strong positive linear correlation between TPC and radical scavenging activity in both 0 H- GMB protein hydrolysate and 18 H-GMB protein hydrolysate with high *r* (Table 4.17). Higher correlation between TPC and radical scavenging activity was found in 18 H-GMB protein hydrolysate compared to that obtained from 0 H-GMB protein hydrolysate. Correlation between TPC and radical scavenging activity of 22 samples, selected from Section 4.2, also gave a high *r* value. At all total phenolics content, the highest scavenging activity was found on carbon-centered radical in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate followed by scavenging activity on hydroxyl radical and scavenging activity on DPPH radical, respectively. It can be seen that there was a linear correlation between TPC and radical scavenging activities measured by electron spin resonance method in germinated mung bean hydrolysate (Table 4.17).

Higher scavenging activity of DPPH radical was also found when measured by ESR than measured by chemical method in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. The ESR signal decrease when antioxidant transfers hydrogen atom or electron to unpaired electron in the reaction (Rohn and Kroh, 2005). Chemical method measures the decreasing in absorbance of DPPH chromagen by spectrophotometer at 515 nm when DPPH radical receives hydrogen atom from antioxidant (Brand-Williams *et al*., 1995).

4.3.4 Activity of protein hydrolysate on inhibition of lipid oxidation

Germinated mung bean protein hydrolysate that gave the highest antioxidant activity from the 0 H-GMB (sample 0_{55}) and the 18 H-GMB (sample 18_{55})

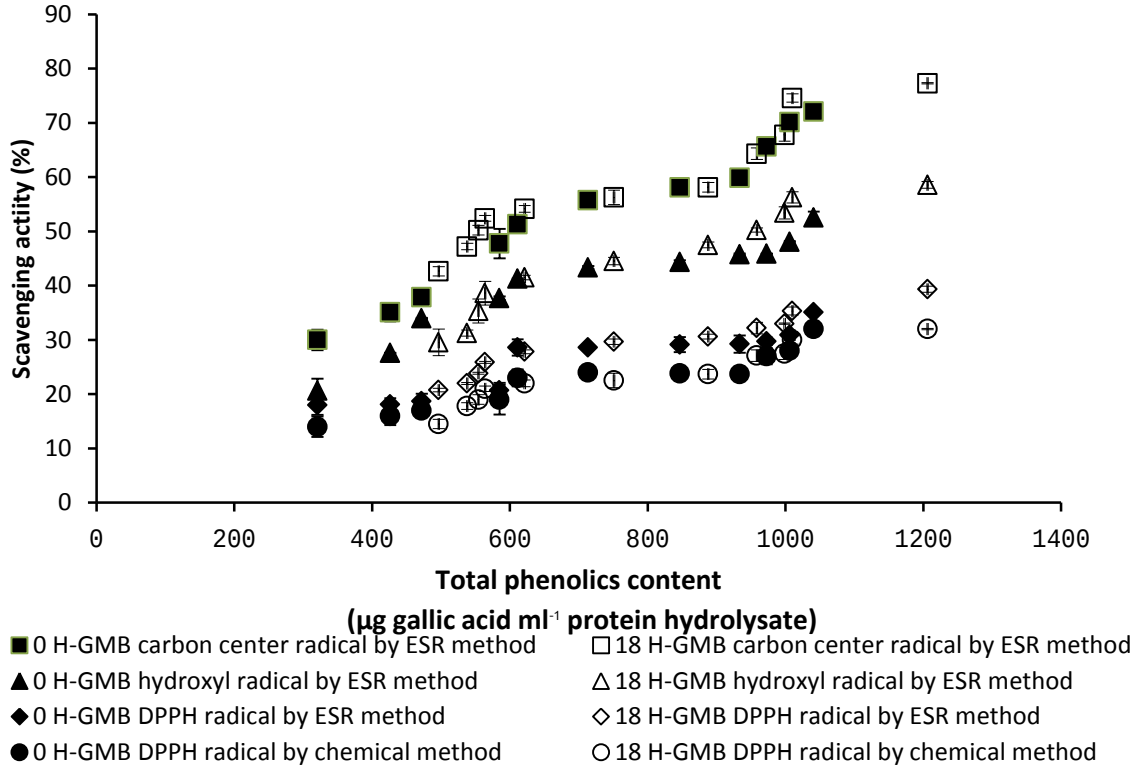


Figure 4.10 Correlation between total phenolics content and radical scavenging activities of germinated mung bean protein hydrolysates.

Table 4.17 Correlation between total phenolics content and radical scavenging activities of germinated mung bean protein hydrolysates by ESR.

Correlation	Sample	Equation	*r*
TPC and carbon-centered radical	0 H-GMB	y = 0.054x + 13.65	0.981
	18 H-GMB	y = 0.044x + 24.26	0.958
	0 and 18 H-GMB*	y = 0.050x +18.09	0.963
TPC and hydroxyl radical	0 H-GMB	y = 0.035x + 14.90	0.940
	18 H-GMB	y = 0.039x + 13.39	0.966
	0 and 18 H-GMB*	y = 0.037x + 13.91	0.949
TPC and DPPH radical	0 H-GMB	y = 0.021x + 10.36	0.919
	18 H-GMB	y = 0.023x + 11.19	0.969
	0 and 18 H-GMB*	y = 0.022x + 10.48	0.935
TPC and DPPH radical (by chemical method)	0 H-GMB	y = 0.020x + 7.75	0.945
	18 H-GMB	y = 0.021x + 6.90	0.956
	0 and 18 H-GMB*	y = 0.020x + 7.42	0.949

* 22 samples that came from the similar DH level of 11 samples of 0 H-GMB protein hydrolysate and 11 samples of 18 H-GMB protein hydrolysate.

were selected for the study of activity on inhibition of linoleic acid oxidation in oil-in-water emulsion model system as detail in Section 3.3.3. To compare the activity on inhibition of lipid oxidation with other standard antioxidant compounds, butylated hydroxyl toluene (BHT) was used as positive control.

4.3.4.1 Total lipid hydroperoxides conthent

Since hydroperoxides are primary intermediate product of lipid oxidation, in this study linoleic oxidation was evaluated by determination of the total lipid hydroperoxides content in the emulsion model system. Total lipid

hydroperoxides content in the emulsion was determined using flow injection analysis (FIA) system. Activity on inhibition of lipid oxidation of the sample was evaluated by the lower amount of total lipid hydroperoxides content in an emulsion with sample compared to that obtained from an emulsion without antioxidant (control).

As shown in Figure 4.11, both of the 0 H-GMB protein hydrolysate and the 18 H-GMB protein hydrolysate with the concentration of 0.15 and 0.30 mg ml^{-1} emulsion had ability to inhibit linoleic acid oxidation. With high concentration (0.30 mg ml^{-1} emulsion) both germinated mung bean gave higher activity on inhibition of lipid oxidation than lower concentration (0.15 mg ml^{-1} emulsion). At the same concentration, sample 18_{55} gave higher activity on inhibition of lipid oxidation than sample 0_{55}. This was because sample 18_{55} had higher antioxidant activity than sample 0_{55} as discussed in Section 4.3.1 and 4.3.2. During the first 4 days, both of the 0 H-GMB protein hydrolysate and the 18 H-GMB protein hydrolysate at both concentrations showed activity on inhibition of lipid oxidation equivalent to BHT. Moreover, the 18 H-GMB protein hydrolysate at concentration of 0.30 mg ml^{-1} emulsion, maintained the ability to control total lipid hydroperoxides and inhibit lipid oxidation equivalent to action of BHT until 6 days. Therefore, it can be concluded that germinated mung bean protein hydrolysate had high antioxidant activity and could be used to prevent lipid oxidation in food. The 18 H-GMB protein hydrolysate had higher ability on inhibit lipid oxidation than the 0 H-GMB protein hydrolysate. Ajibola *et al*. (2011) reported that African yam bean protein hydroysate, with

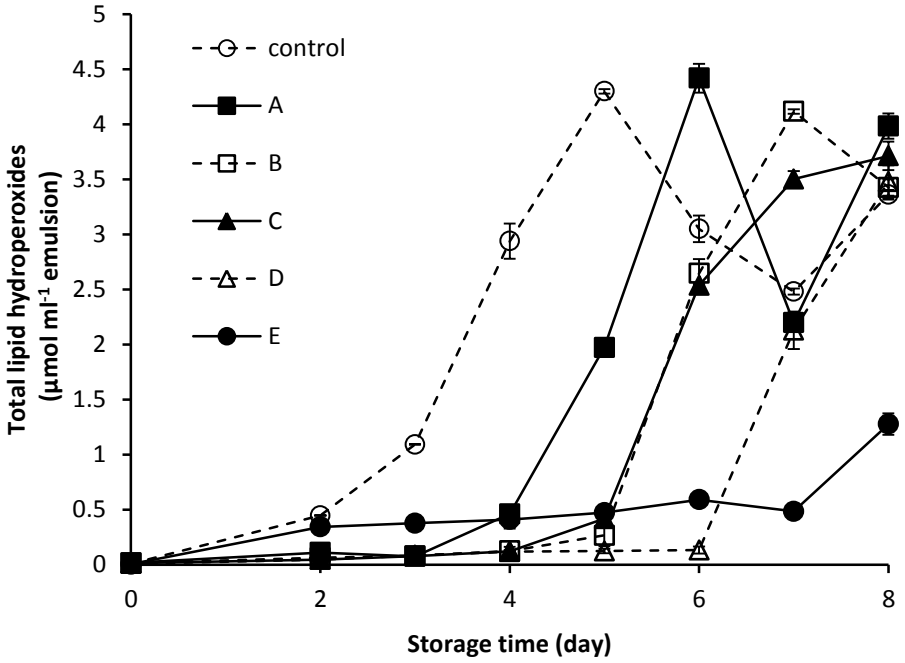


Figure 4.11 Changes in total lipid hydroperoxides determined by the flow injection analysis system in linoleic acid oil-in-water emulsion model system without antioxidant (control), with sample 0_{55} 0.15 mg ml^{-1} emulsion (A), with sample 0_{55} 0.30 mg ml^{-1} emulsion (B), with sample 18_{55} 0.15 mg ml^{-1} emulsion (C), with sample 18_{55} 0.30 mg ml^{-1} emulsion (D), and with BHT 7.5 µg ml^{-1} emulsion (E) during storage in the dark at 40^{O}C.

concentration of 0.25-1.0 mg per ml of linoleic emulsion system, could inhibit linoleic acid oxidation. They found that inhibition of linoleic acid oxidation by this protein hydrolysate was concentration dependent. High concentration of protein hydrolysate gave higher activity on inhibition of linoleic acid oxidation than low concentration of protein hydrolysate. However, inhibitory activity of African yam bean protein hydroysate was lower than BHT at the same concentration.

As shown in Figure 4.11, the amount of total hydroperoxides content in control emulsion and emulsion with the sample 0_{00} at both concentrations could be controlled during the first 4 days and then fluctuated. This may be because hydroperoxides,

primary intermediate products of lipid oxidation, are not stable. They are decomposed into secondary oxidation products such as aldehydes, ketones, alcohols, small acids and alkane.

4.3.4.2 Oxygen uptake

Since hydroperoxides can be decomposed into secondary oxidation products as discussed in Section 4.3.4.1, oxygen uptake in the emulsion system during storage was measured using gas chromatography (GC) to confirm the ability of germinated mung bean protein hydrolysate to prevent lipid oxidation. The lower amount of oxygen uptake in an emulsion with germinated mung bean protein hydroysate compared to that obtained from an emulsion without antioxidant (control) indicated the ability of the hydrolysate samples to inhibit lipid oxidation. The use of BHT in an emulsion system was set as the reference.

Figure 4.11 shows that The oxygen uptake in all samples increased during storage. The amount of oxygen uptake in the control was thhighest. Both of the 0 H-GMB protein hydrolysate and the 18 H-GMB protein hydrolysate with the concentration of 0.15 and 0.3 mg ml^{-1} emulsion had ability to inhibit linoleic oxidation compared to the control. This result supported the increase in the amount of hydroperoxide measured using FIA system (Figure 4.10) in Section 4.3.4.1. At the same protein hydrolysate concentration, the sample 18_{55} had a higher ability to inhibit lipid oxidation than the sample 0_{55}. A high concentration of protein hydrolysate showed higher activity on inhibition of linoleic acid oxidation compared to a lower concentration of protein hydrolysate. Villiere *et al*. (2005) prepared 30% sunflower oil emulsions stabilized by bovine serum albumin or sodium caseinate and measured oxygen uptake during aging in the dark at $50^{O}C$. They found that oxygen uptake increased during aging. Type of emulsifying protein affected amount of oxygen uptake during storage; the emulsion stabilized by sodium caseinate gave the higher oxygen uptake compared to emulsion stabilized by bovine serum album.

During storage of this emulsion containing germinated mung bean protein hydrolysate, it was found that linoleic acid oil-in-water emulsion with both

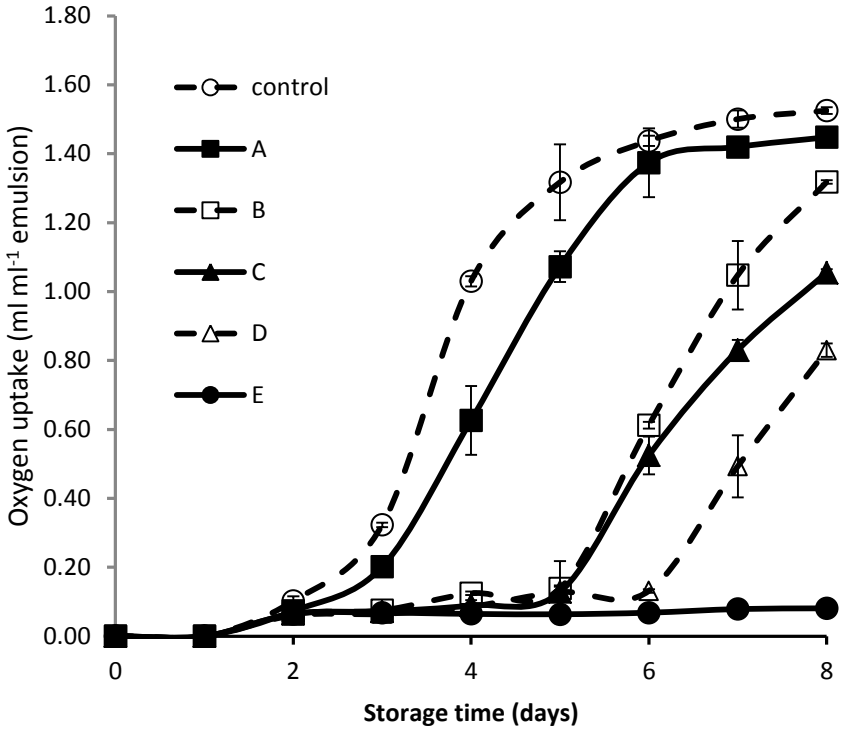


Figure 4.12 Changes in oxygen uptake determined by the gas chromatography in linoleic acid oil-in-water emulsion model system without antioxidant (control), with sample 0_{55} 0.15 mg ml^{-1} emulsion (A), with sample 0_{55} 0.30 mg ml^{-1} emulsion (B), with sample 18_{55} 0.15 mg ml^{-1} emulsion (C), with sample 18_{55} 0.30 mg ml^{-1} emulsion (D), and with BHT 7.5 µg ml^{-1} emulsion (E) during storage in the dark at $40^{O}C$.

germinated mung bean protein hydrolysate at both concentration was stable; the separation could not be observed between oil phase and water phase during 8 days of storage. However, separation of oil phase and water phase in the control emulsion and the emulsion with BHT was observed due to the absence of emulsifying agent. The above results showed that this germinated mung bean protein hydrolysate possessed a potential emulsifying property. This protein hydrolysate could be used as an emulsifier in certain emulsion products due to its ability to act as emulsifier and to inhibit lipid oxidation.

4.4. Properties of freeze dried mung bean protein hydrolysates

4.4.1 Phenolic acids and flavonoids composition

Twenty-two freeze dried samples of germinated mung bean protein hydrolysates were determined for phenolic acids composition using online HPLC-DPPH method. The standard phenolic acids used in this study were gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, syringic acid, *trans*-*p*- coumaric acid and *trans*-ferulic acid. To determine scavenging activity, DPPH was used as free radical. As shown in Table 4.18, phenolic acid found in 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate was only gallic acid while other phenolic acids were not found. The amount of gallic acid significantly increased with higher DH ($p<0.05$). This trend was found in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. Gallic acid approximately increased 2 fold in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysates after hydrolysed with 5% Flavourzyme® for 5 h compared to protein hydrolyssate treated with 0% Flavourzyme® for 0 h. At all DH levels, amount of phenolic acids in 18 H-GMB protein hydrolysate was found to be higher than that obtained from 0 H-GMB protein hydrolysate. This result was agreed with the amount of TPC in hydrolysate (Section 4.2.2). As found in the previous experiment, DH significantly affected the amount of phenolic acid content in both 0 H-GMB and 18 H-GMB protein hydrolysates ($p<0.05$).

According to Figure 4.13a the DPPH radical scavenging activity of each compound is confirm by the presence of negative peak opposite the peak of phenolic compound. Among the standard phenolic acids, gallic acid had the highest DPPH radical scavenging activity compared to other compounds while trans-*p*-coumaric acid could not scavenge DPPH radical because negative peak was not observed. In sample 0_{55} (Figure 4.13b) and sample 18_{55} (Figure 4.13c), it was shown that only gallic acid had DPPH radical scavenging activity but the other peaks showed not DPPH radical scavenging activity due to no negative peak. Sosulski and Dabrowski (1984) reported the presence of *trans*-*p*-coumaric in mung bean flour and presence of *p*-hydroxybenzoic, protocatechuic, syringic, gallic, *trans*-*p*-coumaric, and *trans*-ferulic

Table 4.18 Effect of degree of hydrolysis on phenolic acids composition of freeze dried 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate (mg g^{-1} protein hydrolysate).

Sample	GA	PCCA	HBA	TPCA	TFA
0 H-GMB					
0_{00}	1.04±0.06^{a}	nd	nd	nd	nd
0_{11}	1.42±0.03^{b}	nd	nd	nd	nd
0_{50}	1.61±0.03^{c}	nd	nd	nd	nd
0_{14}	1.75±0.07^{d}	nd	nd	nd	nd
0_{15}	1.87±0.04^{e}	nd	nd	nd	nd
0_{51}	1.99±0.02^{f}	nd	nd	nd	nd
0_{52}	2.28±0.07^{g}	nd	nd	nd	nd
0_{35}	2.35±0.06^{gh}	nd	nd	nd	nd
0_{53}	2.40±0.02^{hi}	nd	nd	nd	nd
0_{54}	2.47±0.05^{i}	nd	nd	nd	nd
0_{55}	2.58±0.07^{j}	nd	nd	nd	nd
18 H-GMB					
18_{00}	2.16±0.05^{a}	nd	nd	nd	nd
18_{10}	2.35±0.04^{b}	nd	nd	nd	nd
18_{30}	2.48±0.02^{c}	nd	nd	nd	nd
18_{11}	2.49±0.06^{c}	nd	nd	nd	nd
18_{12}	2.64±0.05^{d}	nd	nd	nd	nd
18_{13}	2.89±0.08^{e}	nd	nd	nd	nd
18_{51}	3.51±0.11^{f}	nd	nd	nd	nd
18_{15}	3.43±0.08^{f}	nd	nd	nd	nd
18_{52}	3.75±0.07^{g}	nd	nd	nd	nd
18_{35}	3.68±0.06^{g}	nd	nd	nd	nd
18_{55}	4.03±0.12^{h}	nd	nd	nd	nd

$^{a,b,c,\cdots}$ Means in the same column with different letters are significantly different ($p \leq 0.05$) among the same group of germination period. GA, gallic acid; PCCA, protocatechuic acid; HBA, *p*-hydroxybenzoic acid; TPCA, trans-*p*-coumaric acid; TFA, trans-ferulic acid; nd, not detected

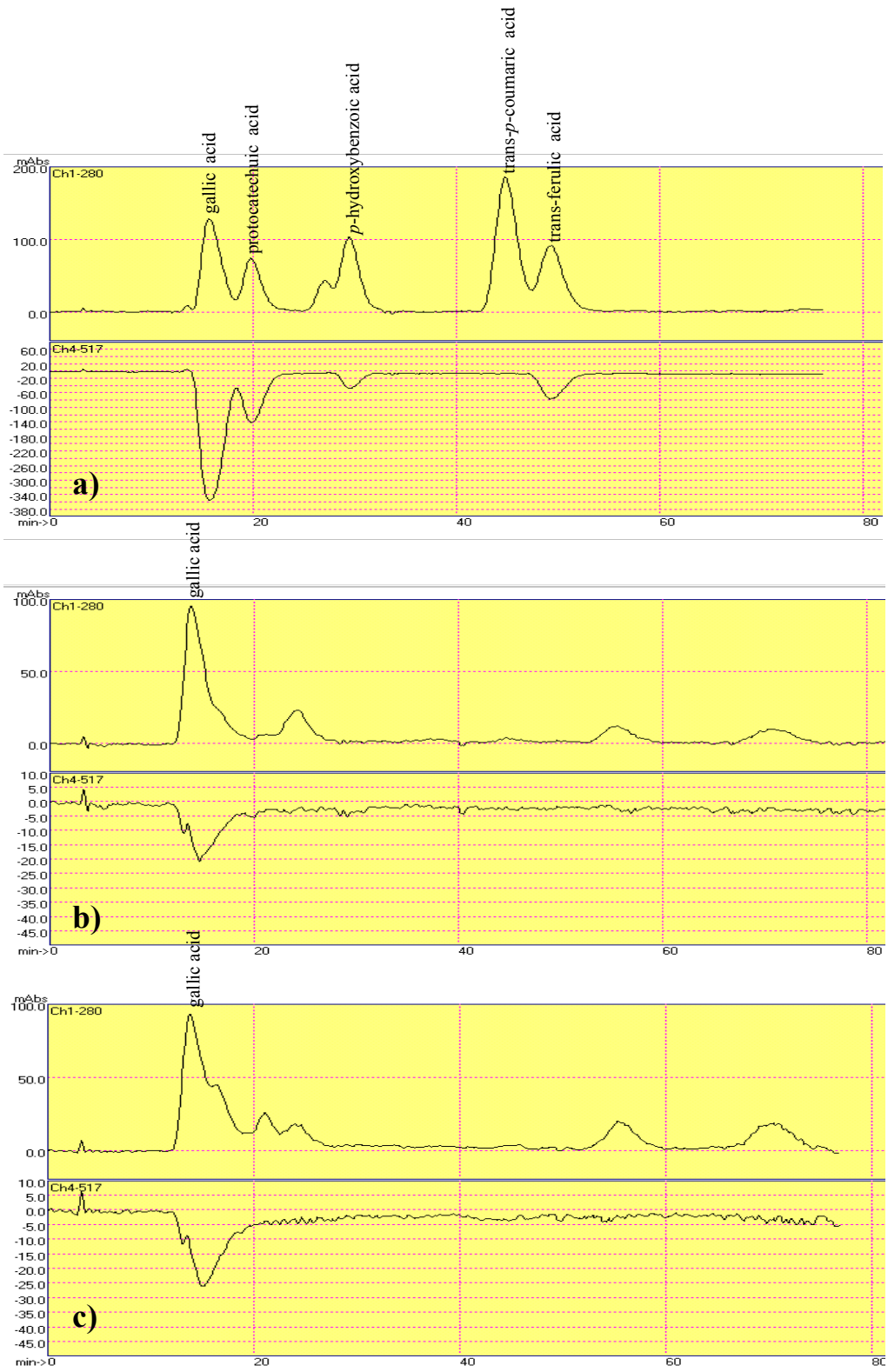


Figure 4.13 HPLC chromatogram of phenolic acids and their DPPH radical scavenging capacity measured through online HPLC a) standard phenolic acids, b)sample $0_{55,}$ c) sample 18_{55}.

in mung bean hull. However, in our experiment, the sample was germinated mung bean protein hydrolysate. It was possible that these phenolic compounds were not present in hydrolysate or decomposed changed into another form due to heating during enzyme inactivation. Thus, only gallic acid was detected.

To determine flavonoids composition, in hydrolysate sample, in this study rutin, quacitrin, quacetin and kaemferol were used as standard. DPPH was also used as free radical to evaluate radical scavenging activity. Table 4.19 shows that there was no flavonoids in all samples from both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysates.

As shown in Figure 4.14a, among standard flavornoids quercetin has the highest DPPH radical scavenging activity compared to other compounds. In sample 0_{55} (Figure 4.14b) and sample 18_{55} (Figure 4.14c) only the first peak shows DPPH radical scavenging activity. However, this peak could not be confirmed to be rutin due to the different in eluted retention time. This peak may be present the total phenolic acids that eluted from the column due to low polarity of eluted solvent. The other peak in the hydrolysate sample did not show DPPH radical scavenging activity due to no negative peak. Liu *et al*. (2008) found rutin, isoorientin, vitexin, isovitexin, quercitrin and quercetin in buckwheat sprout. However, in this experiment, flavonoids composition of mung bean hydrolysate was studied. It was possible that there was not flavonoids in the mung bean protein hydrolysate or decomposed changed into another form due to heating during enzyme inactivation.

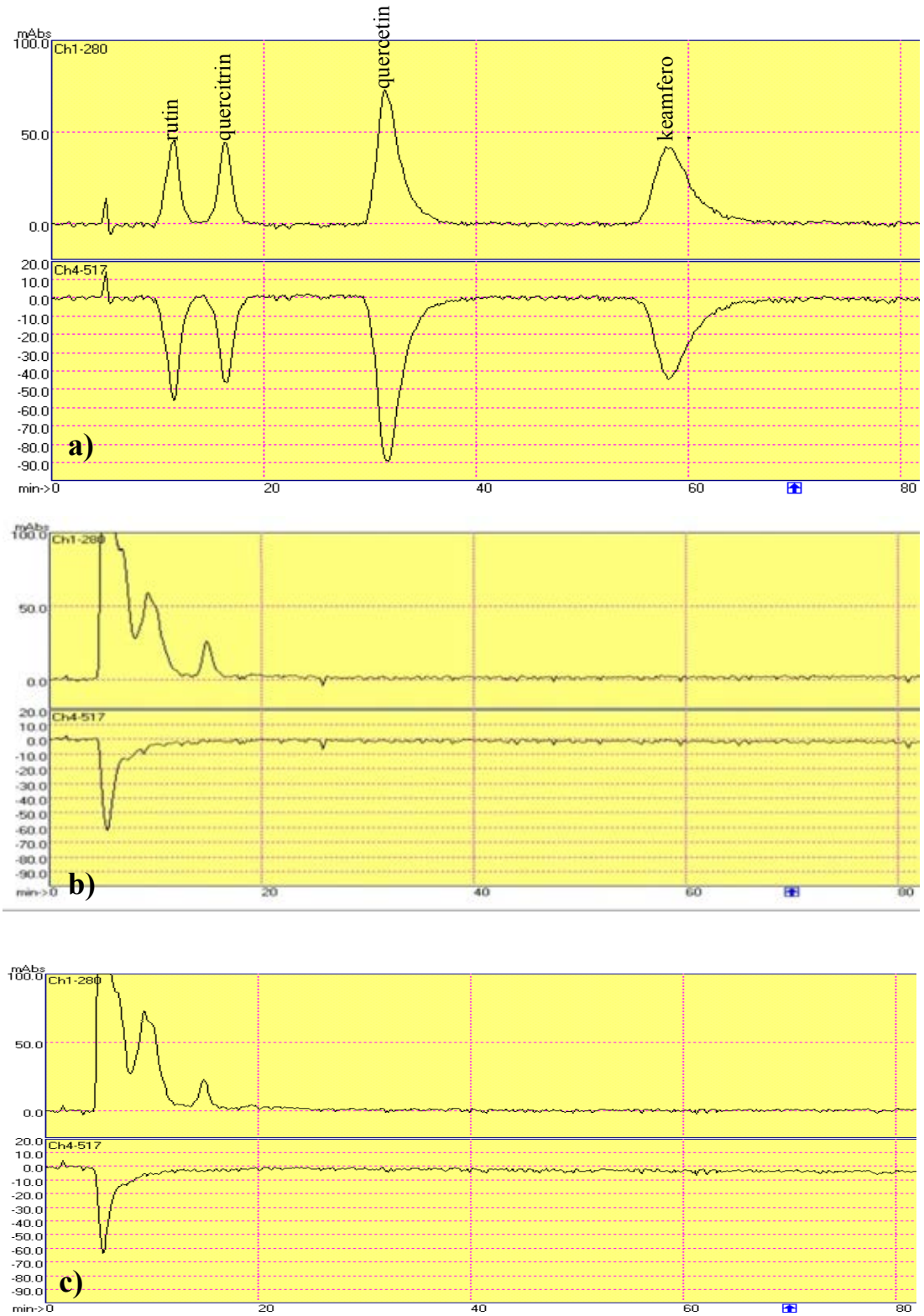
4.4.2 Molecular weight distribution

Twenty-two freeze dried germinated mung bean protein hydrolysates were determined for molecular weight distribution using MALDI-TOF. Figure 4.15 shows the molecular distribution of 0 H-GMB protein hydrolysates (sample 0_{00} and sample 0_{54}) and 18 H-GMB protein hydrolysate (sample 18_{00} and sample 18_{35}). The results, Figure 4.15, show that hydrolysis germinated mung bean using Flavourzyme® resulted in the smaller molecular weight peptides as can be seen in the pattern of molecular weight distribution between sample 0_{00} and sample 0_{54} and also in a pattern of molecular weight distribution between sample 18_{00} and sample 18_{35}. This is

Table 4.19 Effect of degree of hydrolysis on Flavonoids composition of freeze dried 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate (mg g^{-1} protein hydrolysate).

Sample	Rutin	Quercitrin	Quercetin	Keamferol
0 H-GMB				
0_{00}	nd	nd	nd	nd
0_{11}	nd	nd	nd	nd
0_{50}	nd	nd	nd	nd
0_{14}	nd	nd	nd	nd
0_{15}	nd	nd	nd	nd
0_{51}	nd	nd	nd	nd
0_{52}	nd	nd	nd	nd
0_{35}	nd	nd	nd	nd
0_{53}	nd	nd	nd	nd
0_{54}	nd	nd	nd	nd
0_{55}	nd	nd	nd	nd
18 H-GMB				
18_{00}	nd	nd	nd	nd
18_{10}	nd	nd	nd	nd
18_{30}	nd	nd	nd	nd
18_{11}	nd	nd	nd	nd
18_{12}	nd	nd	nd	nd
18_{13}	nd	nd	nd	nd
18_{51}	nd	nd	nd	nd
18_{15}	nd	nd	nd	nd
18_{52}	nd	nd	nd	nd
18_{35}	nd	nd	nd	nd
18_{55}	nd	nd	nd	nd

nd, not detected



Fgure 4.14 HPLC chromatogram of flavonoids and their DPPH radical scavenging capacity measured through online HPLC a) standard flavonoids b) sample0_{55} c) sample18_{55}.

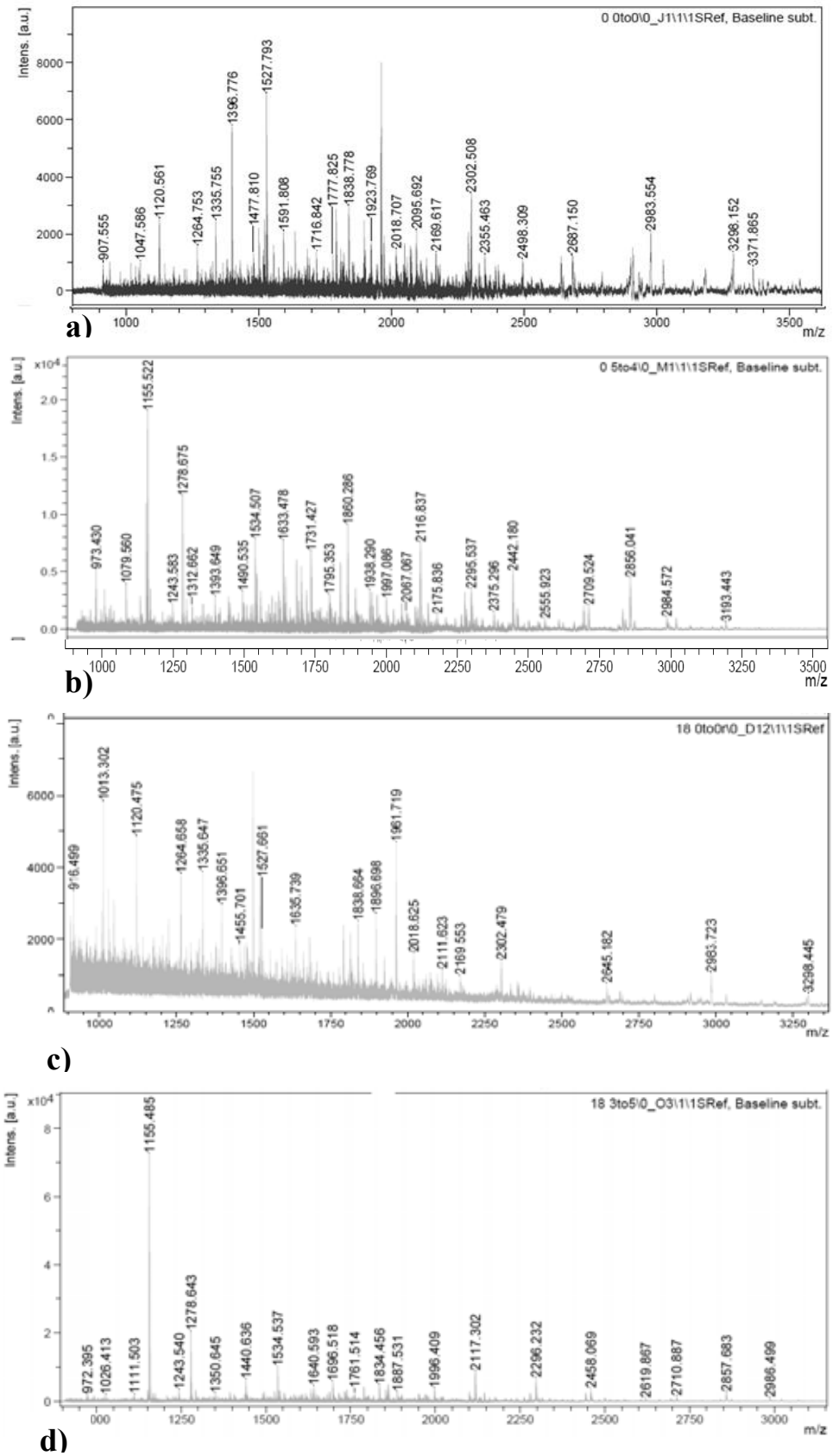


Figure 4.15 MALDI-TOF spectrum of a) sample0_{00} , b) sample 0_{54}, c) sample 18_{00} and d) sample 18_{35}

because during enzymatic hydrolysis, proteolytic enzyme breaks down the peptide bonds in the germinated sample and generates small peptides and amino acids. Looking at molecular weight distribution of peptide between 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysates, it can be found that 18 H-GMB protein hydrolysate contains a smaller peptide than 0 H-GMB protein hydrolysate. The reason is because germination induces the synthesis of proteolytic enzyme leading to partial hydrolysis of some protein into peptides and amino acids. This new structure of protein in 18 H-GMB was in the form which easy to be hydrolyzed by Flavourzyme® compared to native protein in 0 H-GMB as earlier discussed in Section 4.2. Jeong and Hur (2010) hydrolyzed silk fibroin using papain and determined molecular weight distribution by MALDI-TOF. They reported that with higher DH, silk fibroin hydrolysate was composed of smaller peptides compared to those peptides found in the lower DH silk fibroin hydrolysate.

CHAPTER V
CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

Crude protein content of germinated mung bean significantly increased during germination period ($p \leq 0.05$). The 24 H-GMB had the highest protein content but not significant different compared to that of 18 H-GMB. However, germination period, during 0 to 24 h, did not affect the ratio of each amino acid to total amino acid content. Total phenolic content (TPC) of germinated mung bean significantly increased during germination period ($p \leq 0.05$).

The18 H-GMB was selected to hydrolyze using Flavourzyme® while 0H-GMB 0 H-GMB was used as the control. Degree of hydrolysis (DH) increased with increasing in both Flavourzyme® concentration and hydrolysis time this trend was observed in both 0 H-GMB and 18 H-GMB. It was found that relationships between hydrolysis period and DH showed a good fit in the form of rectangular hyperbola. DH significantly affected the amount of free amino group (FAG) and TPC in all hydrolysate samples obtained from both 0 H-GMB and 18 H-GMB ($p \leq 0.05$). Hydrolysis of 0 H-GMB and 18 H-GMB with 5% Flavourzyme® for 5 h gave the significantly highest FAG and TPC compared to other conditions in its group. There were strong positive linear correlation between DH and FAG (R^2 = 0.920 for 0 H-GMB protein hydrolysate and R^2 = 0.997 for 18 H-GMB protein hydrolysate) and between DH and TPC (R^2 = 0.971 for 0 H-GMB protein hydrolysate and R^2 = 0.962 for 18 H-GMB protein hydrolysate).

Antioxidant activities, measured using both chemical method and electron spin resonance (ESR) method, of germinated mung bean protein hydrolysates increased when DH increased. Lower DPPH radical scavenging activity was found when measurement using chemical method compared to that measurement using ESR method. This trend was observed in both 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate. Higher antioxidant activity was found in 18 H-GMB protein hydrolysate compared to 0 H-GMB protein hydrolysate. Smaller peptides

were found in both germinated mung bean protein hydrolesate when DH increased Antioxidative phenolic acid found in 0 H-GMB protein hydrolysate and 18 H-GMB protein hydrolysate was gallic acid. No antioxidative flavonoid was found in both germinated mung bean protein hydrolysates.

5.2 Suggestions

Antioxidant activities of germinated mung bean protein hydrolysate in this study were from both peptides and total phenolics content (TPC). For further study, TPC should be separated from germinated mung bean protein hydrolysate. Antioxidant activities of hydrolyzed peptide and TPC separately studied in depth.

REFERENCES

Achouri, A., Zhang, W. and Shiying, X. 1998. Enzymatic hydrolysis of soy protein isolate and effect of succinylation on the functional properties of resulting protein hydrolysates. Food Research International. 31(9): 617-623.

Ajibola, C.F., Fashakin, J.B., Fagbemi, T.N. and Aluko, R. E. 2011. Effect of peptide size on antioxidant properties of African yam bean seed (*Sphenostylis stenocarpa*) protein hydrolysate fractions. International Journal of Molecular Sciences. 12: 6685-6702.

Bhaskar, N., Benila, T., Radha, C. and Lalitha, R.G. 2008. Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (*Catla catla*) for preparing protein hydrolysate using a commercial protease. Bioresource Technology. 99: 335–343.

Binsan, W., Benjakul, S., Visessanguan, W., Roytrakul, S., Tanaka, M. and Kishimura, H. 2008. Antioxidative activity of Mungoong, an extract paste, from the cephalothorax of white shimp (*Litopenaeus vannamei*). Food Chemistry. 106: 185-193.

Brand-Williams, W., Cuvelier, M.E. and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. LWT-Food Science and Technology. 28: 25-30.

Chabanon, G., Chevalot ,I., Framboisier, X., Chenu, S. and Marc, I. 2007. Hydrolysis of rapeseed protein isolates: Kinetics, characterization and functional properties of hydrolysates. Process Biochemistry. 42: 1419–1428.

Chan, E.W.C., Lim, Y.Y. and Chew, Y.L. 2007. Antioxidant activity of *Camellia sinensis* leaves and tea from a lowland plantation in Malaysia. Food Chemistry. 102: 1214-1222.

Clemente, A., Vioque, J., Sanchez-Vioque, R., Pedroche, J. and Millan, F. 1999. Production of extensive chickpea (*Cicer arietinum* L.) protein hydrolysates with reduced antigenic activity. Journal of Agricultural and Food Chemistry. 47: 3776-3781.

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

Diniz, F.M. and Martin, A.M. 1996. Use of response surface methodology to describe the combined effects of pH, temperature and E/S ratio on the hydrolysis of dogfish (*Squalus acanthias*) muscle. International Journal of Food Science and Technology. 31: 419-426.

Elias, R.J., Kellerby, S.S. and Decker, E.A. 2008. Antioxidant activity of proteins and peptides. Critical Reviews in Food Science and Nutrition. 48: 430-441.

El-Moniem, G.A. 1999. Sensory evaluation and in vitro protein digestibility of mung bean as effected by cooking time. Journal of the Science of Food and Agriculture. 79: 2025-2028.

E1-Shimi, N.M., Damir, A.A. and Ragab, M. 1984. Changes in some nutrients of fenugreek seeds during germination. Food Chemistry. 14: 11-19.

Fernandez-Orozco, R., Frias, J., Zielinski, H., Piskula, M.K., Kozlowska, H. and Vidal-Valverde, C. 2008. Kinetic study of the antioxidant compounds and antioxidant capacity during germination of *Vigna radiata cv.* emmerald, *Glycine max cv.* jutro and *Glycine max cv.* merit. Food Chemistry. 111: 622–630.

Fonkwe, L.G. and Singh, R. K. 1996. Protein recovery from mechanically deboned turkey residue by enzymic hydrolysis. Process Biochemistry. 31(6): 605-616.

Fournier, E. 2005. Mono- and oligosaccharides. In Wrolstad, R.E., et al. (ed.), Handbook of Food Analytical Chemistry. pp. 651-660. London: Wiley Interscience.

Ghavidel, R.A. and Prakash, J. 2007. The impact of germination and dehulling on nutrients, antinutrients, in vitro iron and calcium bioavailability and in vitro starch and protein digestibility of some legume seeds. LWT-Food Science and Technology. 40: 292-1299.

Giri, A. Osako, K., Okamoto, A., Okazaki, E. and Oshima, T. 2011. Antioxidant properties of aqueous and aroma extracts of squid miso prepared with *Aspergillus oryzae*-inoculated koji. Food Research International. 44: 317-325.

Gordon, M. 2001. Measuring antioxidant activity. In Pokorny, J., Yanishlieva, N. and Gordon, M. (ed.), Antioxidants in Food Practical Applications. pp. 71-84. New York: CRC Press.

Greiner, R. 2002. Purification and characterization of three phytases from germinated lupine seeds (*Lupinus albus* Var. Amiga). Journal of Agricultural and Food Chemistry. 50: 6858-6864.

Jamdar, S.N., Rajalakshmi, V., Pednekar, M.D., Juan, F., Yardi, V. and Sharma, A. 2010. Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. Food Chemistry. 121: 178–184.

Je, J.Y., Kim, S.Y. and Kim, S.K. 2005. Preparation and antioxidative activity of hoki frame protein hydrolysate using ultrafiltration membranes. European Food Research and Technology. 221: 157-162.

Jeong, J. and Hur, W. 2010. Even-numbered peptides from papain hydrolysate of silk fibroin. Journal of Chromatography B. 878: 836-840.

Kong, X., Zhou, H. and Quian, H. 2007. Enzymatic preparation and functional properties of wheat gluten hydrolysates. Food Chemistry. 101: 615-620.

Kristinsson, H.G. and Rosca, B.A. 2000. Fish protein hydrolysates: production, biochemical, and functional properties. Critical Reviews in Food Science and Nutrition. 40(1): 43-81.

Kuo., Y.H., Rozan., P., Lambein., F. and Frias., J. 2004. Effect of different germination conditions on the contents of free protein and non-protein amino acid of commercial legumes. Food Chemistry. 86: 537-545.

Lamsal, B.P., Jung, S. and Johnson, L.A. 2007. Rheological properties of soy protein hydrolysates obtained from limited enzymatic hydrolysis. LWT-Food Science and Technology. 40: 1215–1223.

Liceaga-Gesualdo, A.M. and Li-Chan, E.C.Y. 1999. Functional properties of fish protein hydrolysate from herring (*Clupea harengus*). Journal of Food Science. 64(6): 1000-1004.

Li, Y., Jiang, B., Zhang, T, Mu, Z. and Lui, J. 2008. Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CHP). Food Chemistry. 106: 444-450.

Lin, P.Y. and Lai, H.M. 2006. Bioactive compounds in legumes and their germinated products. Journal of Agricultural and Food Chemistry. 54: 3807-3814.

Lin, L. and Li, B. 2006. Radical scavenging properties of protein hydrolysates from Jumbo flying squid (*Dosidicus eschrichitii* Steenstrup) skin gelatin. Journal of the Science of Food and Agriculture. 86: 2290–2295.

Liu, C.L., Chen, Y.S., Yang, J.H. and Chiang, B.H. 2008. Antioxidant activity of tartary (*Fagopyrum tataricum* (L.) Gaertn.) and common (*Fagopyrum esculentum* Moench) buckwheat. Journal of Agricultural and Food Chemistry. 56: 173-178.

Marathe, S.A., Rajalakshmi, V., Jamdar, S.N. and Sharma, A. 2011. Comparative study on antioxidant activity of different varieties of commonly consumed legumes in India. Food and Chemistry Toxicology. 49: 2005-2012.

McCue, P. and Shetty, K. 2002. A biochemical analysis of mung bean (*Vigna radiata*) response to microbial polysaccharides and potential phenolic-enhancing effects for nutraceutical applications. Food Biotechnology. 16: 57-79.

Mc Grain, A.K., Chen, J.C., Wilson, K.A. and Tan-Wilson, A.L. 1989. Degradation of trypsin inhibitors during soybean germination. Phytochemistry. 28: 1013-1017.

Mubarak, A.E. 2005. Nutritional composition and antinutritional factors of mung bean seeds (*Phaseolus aureus*) as effected by some home traditional processes. Food Chemistry. 89: 489-495.

Nilsang, S., Lertsiri, S., Suphantharika, M. and Assavanig, A. 2005. Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. Journal of Food Engineering. 70: 571–578

Papastoitsis, G. and Wilson, K.A. 1991. Initiation of the degradation of the soybean Kunitz and Bowman-Birk trypsin inhibitors by a cysteine protease. Plant Physiology. 96: 1086-1092.

Park, E.Y., Morimae, M., Matsumura, Y., Nakamura, Y. and Sato, K. 2008. Antioxidant activity of some protein hydrolysates and their fraction with different isoelectric points. Journal of Agricultural and Food Chemistry. 56: 9246-9251.

Peng, X., Xiong, Y.L., and Kong, B. 2009. Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance. Food Chemistry. 113: 196-201.

Phanturat, P., Benjakul, S., Visessanguan, W. and Roytrakul, S. 2010. Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the production of gelatin hydrolysate with antioxidative activity. LWT-Food Science and Technology. 43: 86-97.

Prior, R.L., Wu, X., and Schaich, K. 2005. Standard methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry. 53(10): 4290-4302.

Qian, Z.J., Jung, W.K. and Kim, S.K. 2008. Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, *Rana catesbeiana* Shaw. Bioresource Technology. 99: 1690–1698.

Rajalakshmi, D. and Narasimhan, S. 1996. Food antioxidants: Sources and methods of evaluation. In D.L. Madhavi, S.S. Deshpande, and D.K. Salunkhe (eds.), Food Antioxidants Technological, Toxicological, and Health Perspectives; pp.65-157. New York: Marcel Dekker.

Randhir, R., Lin, Y.T. and Shetty, K. 2004. Stimulation of phenolics, antioxidant and antimicrobial activities in dark germinated mung bean sprouts in response to peptide and phytochemical elicitors. Process Biochemistry. 39: 637–646.

Raymundo, L.C., Quizon, M.L.B. and Hurtada, W.A. 1994. Biochemical and nutritional implications of nucleic acids in germinated seeds of mungbean (*Vigna radiate* L.). The Philippine Agriculturist. 70(2): 243-250.

Rohn, S. and Kroh, L.W. 2005. Electron spin resonance - A spectroscopic method for determining the antioxidative activity . Molecular Nutrition and Food Research. 49: 898-907.

Sakanaka, S., Tachibana, Y., Ishihara, N. and Juneja, L.R. 2004. Antioxidant activity of egg-yolk protein hydrolysates in a linoleic acid oxidation system. Food Chemistry. 86: 99-103.

Sattar, A., Durrani, S.K., Mahmood, F., Ahmad, A. and Khan, I. 1989. Effect of soaking and germination temperatures on selected nutrients and antinutrients of mungbean. Food Chemistry. 34: 111-120.

Sanchez-Moreno, C. 2002. Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Science and Technology International. 8: 121-137.

Shahidi, F., Han, X.Q. and Synowiecki, J. 1995. Production and characteristics of protein hydrolysates from capelin *(Mallotus villosus)*. Food Chemistry. 53: 285-293.

Shehata, A.A.Y. and Thannoun, A.M. 1980. Chemical and amino acid composition of Iraqi mung beans. Zeitschrift for Lebensmittel Untersuchung und Forschung. 171: 360-362.

Sohn., J.H., Taki., Y., Ushio., H. and Oshoma., T. 2005. Quantitative determination of total lipid hydroperoxide by a flow injection analysis system. Lipids. 40: 203-209.

Sosulski., F.W. and Dabrowski., K.J. 1984. Composition of free and hydrolysable phenolic acids in the flours and hulls of ten legume species. Journal of Agricultural and Food Chemistry. 32: 131-133.

Sotelo, A., Argote, R.M., Moreno, R.I., Flores, N.I. and Diaz, M. 2003. Nutritive evaluation of the seed, germinated seed, and string bean of *Erythrina americana* and the detoxification of the material by boiling. Journal of Agricultural and Food Chemistry. 51: 2821-2825.

Sun, Y., Hayakawa, S., Ogawa, M. and Izumori, K. 2005. Evaluation of the site specific protein glycation and antioxidant capacity of rare sugar-protein/peptide conjugates. Journal of Agricultural and Food Chemistry. 53: 10205-10212.

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

Tang, C.H., Chen, L. and Ma, C.Y. 2009. Thermal aggregation, amino acid composition and in vitro digestibility of vicilin-rich protein isolates from three Phaseolus legumes: A comparative study. Food Chemistry. 113: 957–963.

Venkateswaran, V. and Vijayalakshmi, G. 2010. Value addition to finger millet (*Eleusine coracana*) by germination and fermentation with *Monascus purpureus*. International Journal of Food Sciences and Nutrition. 61(7): 722–727.

Villiere, A., Viau, M., Bronnec, I., Moreau, N. and Genot, C. 2005. Oxidative stability of bovine serum albumin and sodium caseinate stabilized emulsions depends on metal availability. Journal of Agricultural and Food Chemistry. 53: 1514-1520.

Wang., J.S., Zhao., W.W., Zhao., O.Z. and Jiang., Y.M. 2007. Antioxidant properties of papain hydrolysates of wheat gluten in different oxidation syetems. Food Chemistry. 101: 1658-1663.

Wang, X.Y. and Tang, C.H. 2012. Physicochemical and antioxidant properties of buckwheat protein isolates with different polyphenolic content modified by limited hydrolysis with trypsin. Food Technology and Biochemistry. 50(1): 17-24.

Waterhouse, A.L. 2005. Polyphenolics. In Wrolstad, R.E., et al. (ed.), Handbook of Food Analytical Chemistry. pp. 461-470. London: Wiley Interscience.

Whitaker, J.R. 1994. The proteolytic enzyme. In Principles of Enzymology for The Food Sciences. Marcel Dekker, Inc., New York, USA. pp. 469-498.

Wu, H.C., Chen, H.M. and Shiau, C.Y. 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). Food Research International. 36: 949–957.

Xie, S.H., Wang, Z. and Xu, S.Y. 2007. Characteristics of *Bellamya purificata* snail foot protein and enzymatic hydrolysates. Food Chemistry. 101: 1188–1196

Yang, B., Yang, H., Li, J., Li, Z. and Jiang, Y. 2011. Amino acid composition, molecular weight distribution and antioxidant activity of protein hydrolysates of soy sauce lees. Food Chemistry. 124: 551–555.

Yee, J.J., Shipe, W.F. and Kinsella, J.E. 1980. Antioxidant effect of soy protein hydrolysates on copper-catalyzed methyl linoleate oxidation. Journal of Food Science. 45: 1802-1803.

Zhang, Y., Zhang, H., Wang, L., Guo, X., Qi, X. and Qian, H. 2011. Influence of the degree of hydrolysis (DH) on antioxidant properties and radical-scavenging activities of peanut peptides prepared from fermented peanut meal. European Food Research Technology. 232: 941–950.

APPENDICES

APPENDIX A

ASSAY TECHNIQUES

Appendix A.1 Total phenolics content (Waterhouse, 2005)

1. Prepared sodium carbonate solution by dissolved 200 g of sodium carbonate in 800 ml of water and boiled. After the solution was cooled down, a few crystals of sodium carbonate was added. After 24 h, the solution was filtered with Whatman no. 1 filter paper and the volume of the solution was made up to 1000 ml in a volumetric flask.

2. Placed 1 ml sample, a gallic acid calibration standard, or blank (deionized or distilled water) in a 100 ml volumetric flask.

3. Added 70 ml water, followed by 5 ml Folin-Ciocalteau reagent. Swirled to mix and incubate 1 to 8 min at room temperature.

4. Added 15 ml sodium carbonates solution.

5. Added water to the 100 ml line, mix, and incubate 2 h at room temperature.

6. Transferred 2 ml to a plastic or glass cuvette and measure its absorbance at 765 nm in a spectrophotometer.

7. Subtracted the absorbance of the blank from all reading and create a calibration curve from the standard. The standard curve was shown is Figure A.1.

8. Used this curve to determine the corresponding gallic acid concentration of the sample. Report values in gallic acid equivalents.

Appendix A.1 Reducing sugar content (Fournier, 2005)

1. Prepared low-alkalinity copper reagent by dissolved 12 g of sodium potassium tartrate, 24 g of anhydrous sodium carbonate, 4 g of copper sulfate, and 16 g of sodium hydrogen carbonate in 450 ml of water. Separately, prepare a solution of 180 g anhydrous sodium sulfate in 500 ml of boiling water. Combined the two solutions in a 1000 ml volumetric flask and made up the final solution to 1000 ml with water.

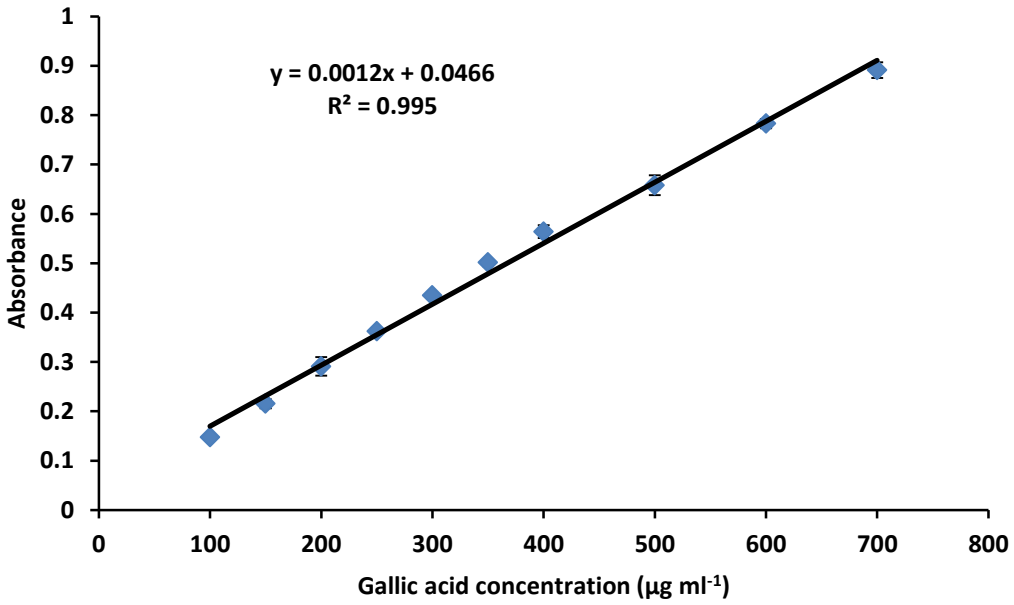


Figure A.1 Gallic acid standard curve for total phenolics content determination

2. Prepared arsenomolybdate reagent by dissolved 25 g of ammonium molybdate in 450 ml water. Added with stirring, 21 ml concentrated sulfuric acid and 25 ml water containing 3 g of disodium hydrogen arsenate heptahydrate. Continued stirring 24 h at room temperature, stored the solution in a brown bottle.

3. Prepared standard glucose solution with the concentration ranging from 5 to 100 µg ml^{-1}.

4. Placed 1 ml of sample, standard glucose solution, or blank in a test tube.

5. Adde 1 ml of low-alkalinity copper reagent and heated in boiling water for 10 min.

6. Added 1 ml of arsenomolybdate reagent

7. Transferred the solution from the tube to cuvette and measured its absorbance at 500 nm in a spectrophotometer.

8. Created a calibration curve from the standards, Figure A.2, and used this curve to determine the corresponding reducing sugar concentration of the samples.

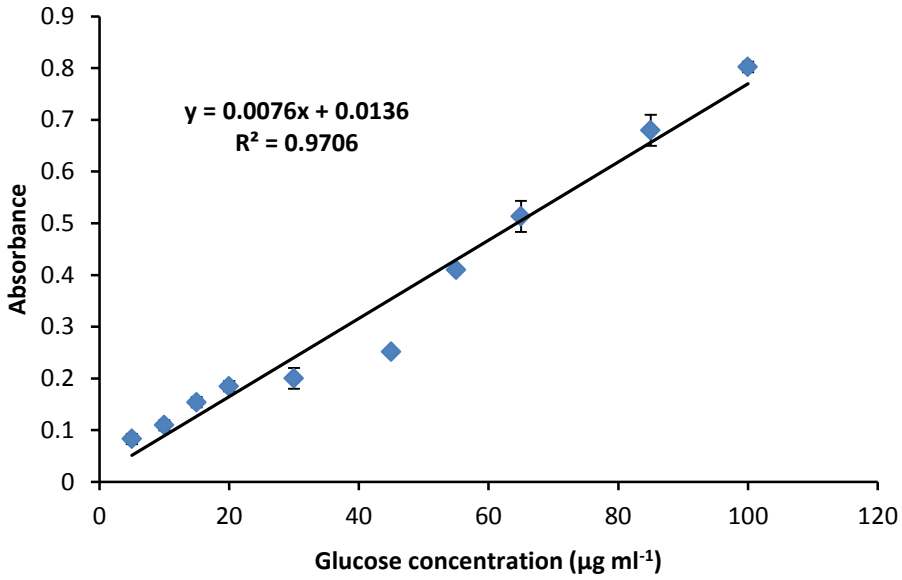


Figure A.2 Glucose standard curve for reducing sugar content determination

Appendix A.3 Preparation of standard amino acids

1. Prepared Waters AccQ. reagent by added 1 ml of Waters AccQ. fluor reagent diluent into Waters AccQ. fluor reagent powder then sealed and mixed. The reagent was incubated at 55^{O}C for 10 min.

2 .Prepared standard amino acid by pipetted 40 µl of mixed 17 amino acid stock solution (Amino acid standard H, Pierce,MA) into 6x50 mm test tube and added 960 µl of Milli-Q water then mixed. This solution contained 100 pmol ml^{-1} of each amino acid. Then pipetted this standard amino acid 1 to 20 µl into 6x50 mm test tube and made up to 1 ml with Waters AccQ. fluor borate buffer. These standard amino acid solutions contained 10 to 200 pmol of each amino acid.

3. Derivertized the standard amino acid by added 20 µl of Wters AccQ. reagent into 1 ml of standard amino acid solution and mixed. Then the solution was incubated 55^{O}C for 10 min.

Appendix A.4 Free amino group (Alder-Nissen, 1979)

1. Placed 0.125 ml of protein hydrolyate solution into test tube then added 2 ml of 0.2 M phosphate buffer pH 8.2 and 2 ml of 0.05% trinitrobenzensluphonic acid (TNBS) and mixed.

2. Incubated the solution $50^{O}C$ for 60 min then added 4 ml of 0.1N hydrochloric acid.

3. After the solution was cooled down at room temperature for 30 min, transferred the solution from the tube to cuvette and measured its absorbance at 420 nm in a spectrophotometer.

The amount of free amino group was calculated by:

$$\text{Free amino group} = \frac{Abobance\ 420\ nm\ X\ ml\ of total\ reactnt\ solution}{\varepsilon\ x\ 1\ cm\ x\ g\ of\ protein\ in\ the\ sample}\ X\ 10^{6}$$

Where ε = molar extinction coeffienct (20300 $M^{-1}\ cm^{-1}$)

APPENDIX B

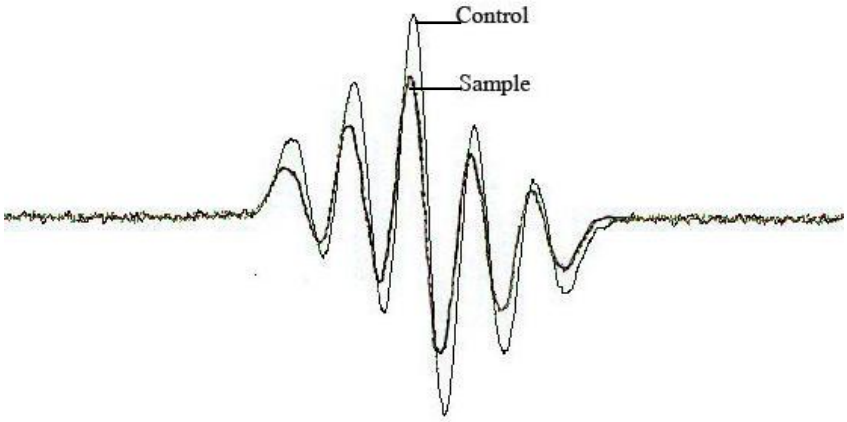


Figure B.1 ESR spectrum of DPPH radical signal.

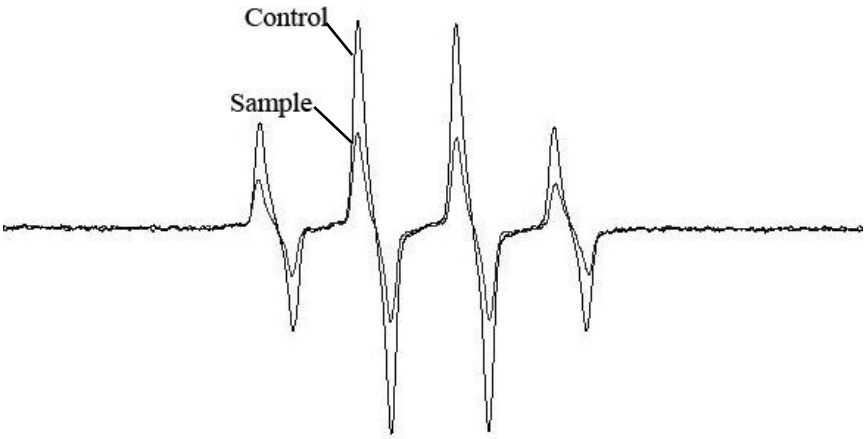


Figure B.2 ESR spectrum of hydroxyl radical signal.

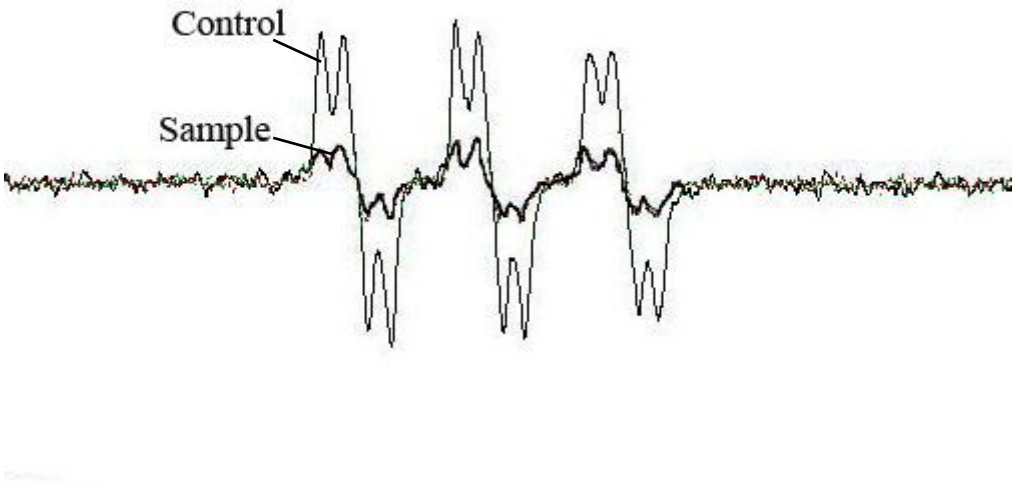


Figure B.3 ESR spectrum of carbon-centered radical signal

APPENDIX C

STATISTIC ANALYSIS

Table C.1 The ANOVA table showing the effect of germination time on chemical composition of germinated mung bean

SOV		df	MS
Moisture Content	trt	4	115.673*
	error	10	3.689
Protein Content	trt	4	4.139*
	error	10	0.263
Total Phenolic Content	trt	4	4207852.868*
	error	10	110690.095
Reducing Sugar Content	trt	4	0.840*
	error	10	0.013

* significantly different

Table C.2 The ANOVA table showing the effect of germination time on amino acid content of germinated mung bean.

SOV		df	MS
Aspartic acid	trt	4	4915.996*
	error	15	435.530
Serine	trt	4	1488.850
	error	15	511.412
Glutamic acid	trt	4	10962.729*
	error	15	1581.231
Glycine	trt	4	1836.428*
	error	15	175.596
Histidine	trt	4	624.840
	error	15	384.976
Arginine	trt	4	3132.143*
	error	15	743.571
Threonine	trt	4	4443.943*
	error	15	335.319
Alanine	trt	4	612.503*
	error	15	104.365
Proline	trt	4	678.723*
	error	15	188.507
Cysteine	trt	4	64.495*
	error	15	6.171
Tyrosine	trt	4	762.904*
	error	15	187.562
Valine	trt	4	618.394
	error	15	571.405
Methionine	trt	4	42.619
	error	15	14.576
Lysine	trt	4	151.005*
	error	15	9.404

* Significantly different

Table C.2 (Continue) The ANOVA table showing the effect of germination time on amino acid content of germinated mung bean.

SOV		df	MS
Isoleucine	trt	4	1079.770*
	error	15	98.168
Leucine	trt	4	5667.411*
	error	15	402.869
Phenylalanine	trt	4	16261.882*
	error	15	1319.267
Tryptophan	trt	4	355.592
	error	15	1132.811
Total essential amino acids	trt	4	82595.718*
	error	15	12159.354
Total non-essential amino acid	trt	4	112719.911*
	error	15	16845.808
Total amino acid	trt	4	381013.215*
	error	15	

* Significantly different

Table C.3 The ANOVA table showing the effect of enzyme concentration and hydrolysis time on free amino group, degree of hydrolysis and total phenolics content of 0 h germinated mung bean.

SOV		df	MS
Enzyme concentration			
Free amino group	trt	3	290827.929*
	error	48	167.263
Degree of hydrolysis	trt	3	2192.370*
	error	48	0.428
Total phenolics content	trt	3	19.773*
	error	48	0.016
Hydrolysis time			
Free amino group	trt	5	73287.898*
	error	48	167.263
Degree of hydrolysis	trt	5	496.397*
	error	48	0.428
Total phenolics content	trt	5	3.899*
	error	48	0.016
*Enzyme concentration * hydrolysis time*			
Free amino group	trt	15	18335.459*
	error	48	167.263
Degree of hydrolysis	trt	15	84.381*
	error	48	0.428
Total phenolics content	trt	15	0.632*
	error	48	0.016

* significantly different

Table C.4 The ANOVA table showing the effect of enzyme concentration and hydrolysis time on free amino group, degree of hydrolysis and total phenolics content of 18 h germinated mung bean.

SOV		df	MS
Enzyme concentration			
Free amino group	trt	3	379542.726*
	error	48	149.260
Degree of hydrolysis	trt	3	4086.741*
	error	48	1.569
Total phenolics content	trt	3	547568.841*
	error	48	707.863
Hydrolysis time			
Free amino group	trt	5	111395.219*
	error	48	149.260
Degree of hydrolysis	trt	5	1196.211*
	error	48	1.569
Total phenolics content	trt	5	164818.422*
	error	48	707.863
*Enzyme concentration * hydrolysis time*			
Free amino group	trt	15	13015.080*
	error	48	149.260
Degree of hydrolysis	trt	15	141.584*
	error	48	1.569
Total phenolics content	trt	15	23526.296*
	error	48	707.863

* Significantly different

VITA

Mr. Seksan Wongsiri was born on September 28th, 1973, in Lamphun, Thaailand. He attended Rajamongkgala Institute of Technology, Lampang Campus in 1991 and received Bachelor of Food Science and Technology in 1995. After graduating in 1995 he worked as an instructor for Department of Food Science, Faculty of Natural Resources, Rajamangala University of Technology Isan, Sakonnakorn Campus. In 1998, he went to study at Department of Food Technology, Faculty of Science, Chulalongkorn University and received Master of Science in 2002. In 2007, he enrolled in the doctoral degree program in Food Technology at Department of Food Technology, Faculty of Science, Chulalongkorn University.