IN VITRO ANTIOXIDANT AND CYTOPROTECTIVE ACTIVITIES OF PALM KERNEL MEAL PROTEIN HYDROLYSATE

BY

MISS KAVISARA SURANGKULWATTANA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
THE MASTER OF SCIENCE (BIOMEDICAL SCIENCES)
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ENTITLED

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ABSTRACT

The reactive oxygen species (ROS) induces an oxidative stress which contributes to series of diseases, such as cardiovascular disease, Alzheimer's disease and carcinogenesis. Moreover, ROS could cause damage to cells and DNAs as they oxidize and modify some intracellular components and influence on mitochondria by damaging its membrane integrity and membrane potential. Antioxidant compounds play a key role in fighting against oxidative stress and could protect the cells against free radicals and inhibit the oxidation. There has been a major interest in searching for natural, efficient, and nontoxic antioxidants to replace the widely used chemical compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butyl hydroquinone (TBHQ). Protein hydrolysate serves as a valuable source of bioactivity as it consists of complex mixtures of peptides and free amino acids exerting several biological activities such as antioxidant activity, antimicrobial activity, and cytoprotective activity. In the palm oil industry, palm kernel meal (PKM) is a main by-product and there were around million tons of palm kernel meal produced each year in Thailand. It contains a high amount of protein (16 -18%)

which could be serve as a source of protein hydrolysate. In this study, protein from palm kernel meal was isolated and hydrolyzed with alcalase enzyme to obtain palm kernel meal protein hydrolysate (PKMPH). The antioxidant activity of PKMPH was evaluated *in vitro* using DPPH and ABTS assay. The results showed that PKMPH at hydrolysis time of 60 minutes had a strong antioxidant activity with IC_{50} values of 5.73±0.23 and 7.84±0.89 µg/mL as determined by DPPH and ABTS assay, respectively. At concentration less than or equal to 125 µg/mL, PKMPH showed low toxicity toward L929 mouse fibroblast cell. Furthermore, its cytoprotective effect on H_2O_2 oxidative damaged L929 cells were determined. Results revealed that PKMPH at concentrations ranging from 500-0.97 µg/mL could protect L929 cells from oxidative damage. Thereafter, the bioactive peptide exerting antioxidant activity was separated by Fast protein liquid chromatography and identified by mass spectrometry. Results demonstrated nine peptide sequences with molecular weights ranging from 1084 and 1291 Da and composed of hydrophobic and negatively charged amino acids. In conclusion, PKMPH or antioxidant peptide from palm kernel meal might be exploited as functional feed for animal in the future.

Keywords: Palm kernel meal, Protein hydrolysate, Antioxidant activity, Cytoprotective effect, Peptide

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Kavisara Surangkulwattana

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
%	Percent
°C	Degree(s) Celsius
μ	Micro
ABTS	The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	Analysis of variance
CAT	Catalase
Da	Dalton
DH	Degree of hydrolysia
DI	Deionized water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl (DPPH)-2,2-diphenyl-1-picrylhydrazyl
DW	Distilled water
FBS	Fetal bovine serum
GSH	Glutathione
H_2O_2	Hydrogen peroxide
IC_{50}	Half maximal inhibitory concentration
LC MS/MS	Liquid Chromatography with tandem mass spectrometry
mL	Milliliter
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
OD	Optical density
OPA	o-phthalaldehyde

LIST OF ABBREVIATIONS (Cont.)

Symbols/Abbreviations	**Terms**
OPA	O-phthalaldehyde
PBS	Phosphate-buffered saline
pI	Isoelectric point
PKMP	Palm kernel meal protein
PKMPH	Palm kernel meal protein hydrolysate
ROS	Reactive oxygen species
RT	Room temperature

CHAPTER 1

INTRODUCTION

Reactive oxygen species (ROS) and free radicals such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (•OH) are by product of mitochondrial oxidative metabolism.[1] They emerged as unstable molecule and highly reacted with other molecules affecting human health and leading to various diseases including cardiovascular disease, carcinogenesis and immune system.[2, 3] The overproduction of ROS and deficiency in the ability of antioxidant to defense resulted in an imbalance leading to oxidative stress in the body.[4] Oxidative stress can cause toxic effects through by products of several metabolic pathways leading to major oxidative damages at cellular level such as apoptosis, cellular signaling and gene expression. These will stimulate lipid peroxidation, DNA and protein damage and deleteriously effect on the living cell.[5] Oxidative stress is also associated with vascular disease, Alzheimer disease (AD), aging process or involved in the dopamine cell degeneration in Parkinson's disease (PD).[6-9] There were numerous studies linking oxidative stress with various diseases, where increased oxidative stress is clearly associated with the development of these diseases. In our body, antioxidative compounds including enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), vitamin A, C, E, glutathione and flavonoids were found. They are efficient in stabilizing or inactivating free radicals mediated oxidative damage before they damaged the intracellular components resulting in the balancing of harmful radicals which will maintain the human healthiness.[10] In present, there were two sources of antioxidants that can contribute to the prevention and inhibition of free radicals and reactive oxygen species (ROS) which are synthetic and natural antioxidant. The synthetic antioxidant such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ) has been widely used in food industry as food preservatives and for packaging purpose.[11, 12] Unfortunately, there were several studies reported the significant adverse effects of synthetic antioxidant to human health. Natural antioxidant compound from animal or plant extract has gained an interest and could serve as an alternative natural antioxidant with more efficiency and low toxicity.

Protein hydrolysate is a complex mixture of different molecular sizes of peptides and free amino acids obtained by degradation of peptide bond in native proteins. It possesses beneficial biological and pharmacological properties. For example, alfalfa leaf and sunflower protein hydrolysate were reported to exhibit free radical scavenging ability. In addition, bioactive peptide released by enzymatic procedure from whole protein molecule from alfalfa leaf, soybean and sunflower retains multifunctional properties such as antioxidant and antimicrobial activities.[13-15] Moreover, hemp seed and red seaweed protein hydrolysate also could aspects in human well-being by affecting antihypertensive, cardioprotective and anti-diabetes.[16, 17] Marcone et al., 2015 and Tonolo et al., 2018 demonstrated milk-derived bioactive peptide could prevent atherogenesis effects by inhibiting the NF-κB pathway and could protect against oxidative stress in Caco-2 cells.[18, 19] In conclusion, protein hydrolysate could serve as a valuable source of antioxidant peptides due to its bioactivity and low toxicity.

Palm kernel meal (PKM) is a main by-product from palm oil industry with millions of tons produced each year. Palm or *Elaeis guineensis* belongs to Arecaceae family. PKM has high content of protein (16-19%), fat (12-20%) and fiber (16.7%) and can be included in the diets of beef cattle, dairy cows, sheep, poultry and pigs with different level of inclusion rate. In order to develop a value-added functional feed from PKM, protein hydrolysate of PKM (PKMPH) could serve as a valuable source of bioactive peptide, notably, antioxidant peptide. In this study, the PKMPH were prepared and tested for its antioxidant activity and cytoprotective effect. In addition, bioactive peptide exerting antioxidant activity were purified and identified. PKMPH or antioxidant peptide from palm kernel meal might be exploited as functional feed for animal in the future.

Objective

1. To evaluate the antioxidant activity and cytoprotective effect of palm kernel meal protein hydrolysate.
2. To identify the antioxidant peptide from palm kernel meal protein hydrolysate.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Oxidative stress

Oxidative stress refers to the disruption of the equilibrium causing the imbalance between reactive oxygen molecules or radicals and antioxidants.[20] This situation related to various human health problem and pathologies such as oxidative stress can cause cellular inflammation and cardiovascular disease.[21] NADPH oxidase is a member of transmembrane protein responsible for transferring of an unpaired electron from NADPH to the oxygen atom thus resulting in superoxide formation. The reactive oxygen species (ROS) generation often occurs as a by-product.[22] Adrenoleukodystrophy, a genetic disease, is also caused by oxidative stress within mitochondria. The excess amount of ROS generated via metabolic process leads to the expression of hypoxia-inducible factor-1 alpha which increases TNF secretion and stimulates the liver damaged through immune response.[23] Moreover, the oxidative stress and mitochondria dysfunction can damage the cellular level of DNA and proteins leading to aging.[24] There is an increasing evidence of human disease conditions caused by the production of ROS which is the results from oxidative stress such as diabetes, cancer, and neurodegenerative diseases such as Alzheimer's.[25] [26, 27] In nature, our body could produce antioxidants such as glutathione to fight against these free radicals or slow down the damaged effect from these dangerous molecules.[10] However, the rate of antioxidant production in our body was decreased when we are older. Therefore, it has to rely on an external source of antioxidants.

Lipid peroxidation is a deterioration of the lipid because of oxidation process. It caused by the free radicals abstract an electron from the lipids of the cell membrane. This further generates an unstable lipid radical, which can react with oxygen atom forming a fatty acid peroxyl radical and eventually causes cell damaged. It often occurs on polyunsaturated fatty acids (PUFA) located on the cell membrane because of the double bonds containing methylene bridges (-CH_2-) and the presence of hydrogen atom which is highly reactive. There are three phases of this process: initiation, diffusion(propagation), and termination. The product of this reaction is called lipid peroxide or lipid oxidation product (LOP).[28] Oxidative stress caused by the accumulation of free radicals could destroy protein structure by three ways including (i) protein oxidative modifications, especially specific amino acid, (ii) free radical-mediated cleavage of the polypeptide and (iii) formation of protein cross-linkage due to reaction with lipid peroxidation products.[10] Moreover, the reactive oxygen species such as hydroxyl radical (•OH) can damage the DNA or breakdown the DNA strand by (i) addition to double bonds of DNA bases leads to single strand break and by (ii) deoxyribose hydrogen atom abstraction from methyl group of thymine and each of the C-H bonds of 2′-deoxyribose.[29]

2.2 Free radical

The free radicals are chemical molecules belonging to an unpaired electron with at least one or more electron. It can be positive, negative or neutral (uncharged). The unpaired electron can be regarded as part of chemical molecule which are easily reacts with other molecule and eventually becomes an unstable molecule that can damage the living cells in organism.[30] Free radical damage is one of the major cause of human diseases such as cancer, cardiovascular disease, hearth attacks, and etc.[31] Free radicals can occur when the bonds between atoms are broken down. They are unstable molecule and highly sensitive to react with nearby molecules in order to stabilize themselves. As a result, the surrounding molecules received or lost the possessing electrons, they become a new free radical which will continue to interact with other stable molecules as a chain reaction.[32]

2.2.1 Free radical formation

Free radicals are formed by homolysis cleavage of chemical bond named covalent, with each separated segment of normal molecule conserving one of the paired electrons (**Figure 2.1**).The covalent bond can be broken down into two ways.

2.2.1.1 Homolysis or homolytic cleavage

The molecules typically required strong energy intake from high temperature, UV light or ionizing radiation. When the bond was broken down, each fragment is called radicals or free radical which is electrically neutral charged.

2.2.1.2 Heterolysis or heterolytic cleavage

It is a bond cleavage where the paired electron splits to a particular atom while the other atom receives no electron. As the results, electrons will receive an anion and another side will lose the cation. This called “Ionic reaction”

The difference between homolytic cleavage and heterolytic cleavage, homolytic cleavage results in unpaired electrons or free radicals whereas heterolytic cleavage leads to ion pairs.

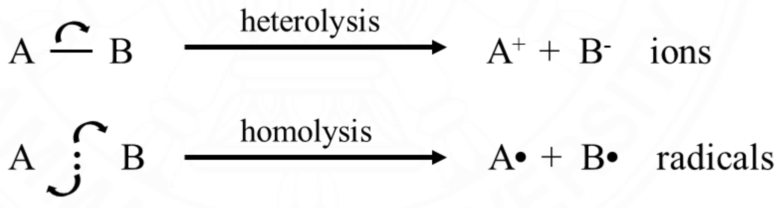


Figure 2.1 The formation of free radical

Free radical could be generated by reduction reaction of an atom or molecule. Moreover, the oxidation reaction can also cause the formation of free radical, in which they lose their unpaired electron and resulting in the generation of an electrically current or electron transfer (**Figure 2.2**).[33]

$$A + e- \longrightarrow A \bullet -$$

Figure 2.2 The generation of free radical by electron transfer

2.2.2 Sources of free radicals

There are two sources of free radical which are endogenous and exogenous sources.[10]

2.2.2.1 Endogenous sources

In the body, free radicals are formed from metabolic processes. When the oxygen consumption is high, it will convert into single atom with unpaired electron. In addition, ROS can be generated in immune cell signaling via the NOX pathway.[34] Host defense mechanism was also associated in the increasing of reactive oxygen species. When the body was invaded with pathogen, the ROS called "oxidative burst" was generated in the cell when macrophage and leukocytes were triggered by various stimuli. NADPH oxidase produced oxygen molecule (O_2) and this oxygen molecule will be converted to hydrogen peroxide (H_2O_2) and eventually form hypochlorite (HOCl) and caused damage to living cells.[35] Therefore, endogenous free radicals are generated from immune cell activation, inflammation, ischemia, infection and cancer.[36]

2.2.2.2 Exogenous sources

Environmental factors play a key part in the activation of reactive oxygen species. They can rapidly generate molecule such as hydroxyl radical ($\bullet OH$) and ionized water (H_2O^+). Besides, chemical substances also affect the intracellular cell damage. For example, metal catalysis by intracellular Fe^{2+} and cuprous oxide converted superoxide ($O2\bullet-$) and hydrogen peroxide (H_2O_2) to additional amount of hydroxyl radical ($\bullet OH$) leading to various responses of an organism such as DNA damage, bystander apathy and tissue injury.[37] The examples of exogenous sources of free radicals are smoking/alcohol, chemical, photo oxidation and UV irradiation.

2.2.3 Type of reactive species

Reactive species (RS) are produced during normal metabolism of cells.[38] They can be divided into two groups, (i) reactive oxygen species (ROS) and (ii) reactive nitrogen species (RNS).

(i) Reactive oxygen species (ROS)

Reactive Oxygen Species (ROS) are by product derived from oxygen molecules during mitochondrial electron transport. ROS also referred to a number of oxidants and reactive molecule in eukaryotic cells. In the oxidation reaction, ROS are essential intermediates in which the oxygen atom contains single or unpaired electrons in its outer shell of electrons. This structure leads to an oxygen atom which are more responsive to the formation of radical. The addition of unpaired electron decreased the number of oxygen atom leading to the increasing of ROS level formation in the cells including, hydrogen peroxide, superoxide anion radical (O_2-)and hydroxyl radical (HO) etc.[39] The example of reactive oxygen species (ROS) was shown in **Table 2.1**.

Table 2.1 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS)			
Radicals		Non-Radicals	
$O_2^{\bullet -}$	Superoxide	H_2O_2	Hydrogen peroxide
$OH\bullet$	Hydroxyl	$HOCl^-$	Hypochlorous acid
$RO_2\bullet$	Peroxly	O_3	Ozone
$RO\bullet$	Alkoxyl	$^1O_2$	Singlet oxygen
$HO_2\bullet$	Hydroperoxyl	$ONOO^-$	Peroxnynitrate

(ii) Reactive nitrogen species (RNS)

The emergence of reactive nitrogen species (RNS) has been conducted from nitric oxide (•NO) and superoxide (O2•−) associated with oxygen molecule. Nitric oxide radical were generated through enzymatic activity of inducible nitric oxide synthase (iNOS) and NADPH oxidase.[40] They worked together with reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) causing the destruction of cellular proteins, lipids, RNA and DNA. The increased production of RNS eventually caused the nitrosative stress.[41] RNS such as nitric oxide, peroxynitrite and nitrogen dioxide are reported to associate in various disease pathogenesis such as cardiovascular and neurodegenerative diseases.[42] The example of reactive nitrogen species (RNS) was shown in **Table 2.2**.

Table 2.2 Reactive Nitrogen Species (RNS)

Reactive nitrogen species (RNS)			
Radicals		Non-Radicals	
NO•	Nitric Oxide	$ONOO^-$	Peroxynitrite
NO_2•	Nitrogen dioxide	ROONO	Alkyl peroxynitrites
		N_2O_3	Dinitrogen trioxide
		N_2O_4	Dinitrigen tetroixide
		HNO_2	Nitous acid
		NO_2^+	Nitronium anion
		NO^-	Nitroxyl anion
		NO^+	Nitroxyl cation
		NO_2Cl	Nitryl chloride

ROS/RNS were mainly originated from mitochondria. They are by product of normal cellular metabolism. [43] The excess amount of reactive oxygen and nitrogen species (ROS and RNS) have been associated with the development of several diseases such as cancer, respiratory, neurodegenerative, and digestive diseases.[44] Interestingly, ROS and RNS did not exhibit only damaged effects but also showed some beneficial effects to the cells. For example, the role of ROS signaling involved in counteraction of microbial invasion.[45] Other reactive oxygen species such as lipoperoxyl radical and lipid hydroperoxide were found and caused harmful effects to the body.

2.3 Antioxidant

An antioxidant definition has been defined in various terms including chemical, biological and biochemistry and medical definition.

Chemical definition

Antioxidant is defined as "any substance that, when present at low concentration compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate". This substance will contribute to inhibit or slow down a chemical reaction leading to the formation of free radicals or reactive oxygen species (ROS), called oxidation and reduction reaction promoted by oxygen or peroxide radicals.[46, 47]

Biological definition

Antioxidant is defined as any natural or synthetic substance, which can inhibit or slow the damage or extend shelf life of foods or supplement products induced by oxygen molecules.[48]

Biochemistry and medical definition

Antioxidant is defined as an organic biomolecule or any enzyme, which can effectively oppose the harmful effects of oxidative stress when electrons were lost during a reaction by an oxidation reaction occurring in eukaryotic tissues.[48-50]

2.3.1 Type of antioxidant

The antioxidant molecule is responded to prevent or inhibit the human metabolism damage caused by free radicals or reactive oxygen species. The antioxidant can be classified into two types based on its activity as shown in **Figure 2.3**, which are enzymatic and nonenzymatic antioxidant.[51]

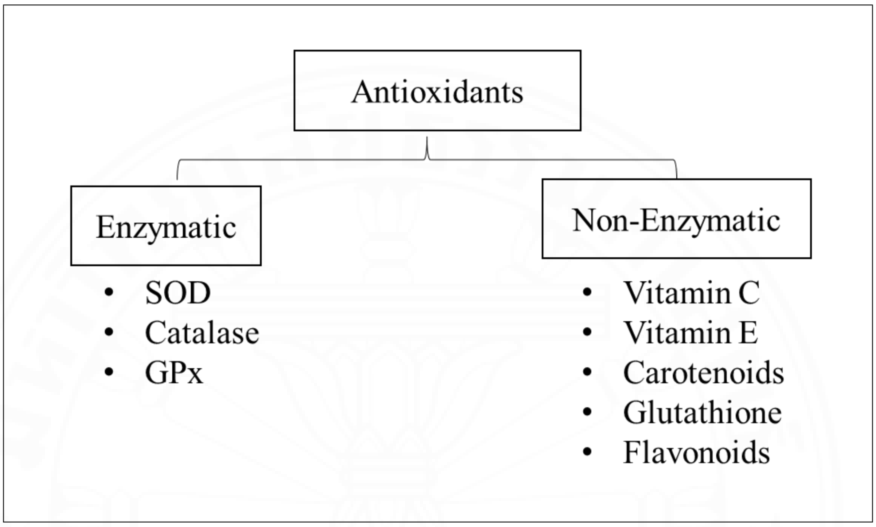


Figure 2.3 Type of antioxidant

2.3.1.1 Enzymatic antioxidant

The enzymatic antioxidant such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) operated by collapsing and removing free radicals.[52] The antioxidant enzymes can eliminate the hazard oxidative products, such as superoxide anions into hydrogen peroxide (H_2O_2) and are subsequently converted into water (H_2O) and oxygen molecule (O_2).[53, 54]

(i) Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is antioxidant enzyme that function to catalytically convert superoxide ($O_2^{\bullet -}$) radicals to oxygen (O_2) and hydrogen peroxide (H_2O_2).[55] Superoxide ($O_2^{\bullet -}$), a member of reactive oxygen species, consists of two oxygen atom.[56] The catalytic reaction of superoxide dismutase can be demonstrated in the following equation (**Figure 2.4**)

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

Figure 2.4 The catalytic reaction of superoxide dismutase

SOD needs cofactor or catalytic metal ions and accordingly, there are four different groups of SOD including iron SOD (FeSOD), manganese SOD (MnSOD), copper-zinc SOD (CuZnSOD) and nikel SOD (NiSOD) (**Figure 2.5**). Iron SOD (FeSOD) is an enzyme found in chloroplast of plants and mitochondria of human. FeSOD was also found in some bacterial cells such as *E.coli*.[57] Manganese-containing superoxide dismutase (MnSOD) typically presented in the peroxisomes and can also be found in mitochondria.[58] Copper-zinc SOD (CuZnSOD) are usually found in chloroplast and cytoplasm.[59] The nikel SOD (NiSOD) was originally found in bacteria, *Streptomyces* [60] and cyanobacteria.[61]

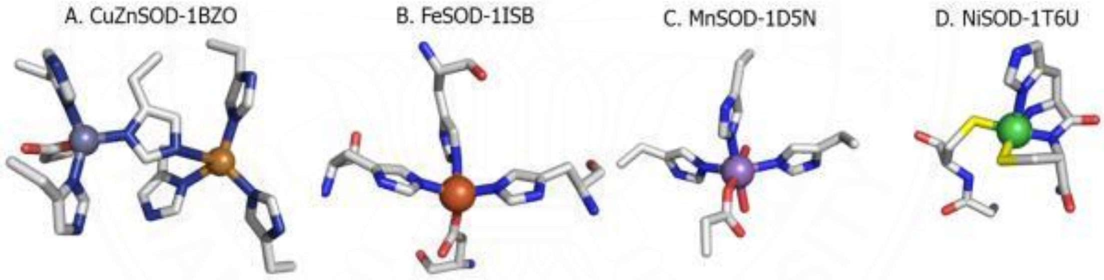


Figure 2.5 The active sites of (A) Cu/Zn-, (B) Fe-, (C) Mn-, and (D) NiSOD.[62]

(ii) Catalase (CAT)

Catalase is responsible for catalyzing the H_2O_2 separation into water and O_2 by using 2 molecules of H_2O_2 as substrate.[63] It was mostly found in plant and animal cells or the living organism. The catalytic reaction of catalase can be demonstrated in the following equation (**Figure 2.6**).[64, 65]

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Figure 2.6 The catalytic reaction of catalase

(iii) Glutathione peroxidase (GPx)

Glutathione peroxidase is one of major antioxidant enzyme found in cytosol and mitochondria. Glutathione is a tripeptide existing in two forms which are reduced (GSH) oxidized (GSSG) form. Normally, glutathione exists in reduced form.[66] Glutathione peroxidase consists of selenium in its active center and involved in the antioxidant defense mechanism of several human diseases.[67] In order to balance the oxidative stress and radicals in living organism, the reduced glutathione will serve as electron donor and donate the unpaired electron to others unstable molecule. In the reaction, hydrogen peroxide (H_2O_2) and organic peroxide (ROOH) were converted to water and alcohol, respectively (**Figure 2.7**).[68]

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

or

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

Figure 2.7 The catalytic reaction of glutathione peroxidase

SODs converted superoxide radical into hydrogen peroxide and molecular oxygen, whereas the catalase and peroxidases convert hydrogen peroxide into water.[69] A summary of enzymatic antioxidants and reaction was shown in **Table 2.3**.

Table 2.3 The reaction and substrate of enzymatic antioxidant[70]

Antioxidant	Substrate	Reaction
Fe/Mn/Cu/Zn SOD	$2O_2^{\bullet -}$	$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	H_2O_2	$2H_2O_2 \rightarrow 2H_2O + O_2$
Glutathione peroxidase	H_2O_2	$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ or $2GSH + ROOH \rightarrow GSSG + ROH + H_2O$

1. Non-enzymatic antioxidant

The defense mechanism of a non-enzymatic antioxidant such as vitamin C, E and carotenoids is responsible for removal of radicals or oxidants which are hazard molecules and by products from the exceed metabolism in cellular causing various diseases in human.[71]

(i) Vitamin C

The ascorbic acid or known as vitamin C is water-soluble antioxidant in human and act as an electron donor and can stimulate collagen production in the skin (**Figure 2.8A**).[72,73]

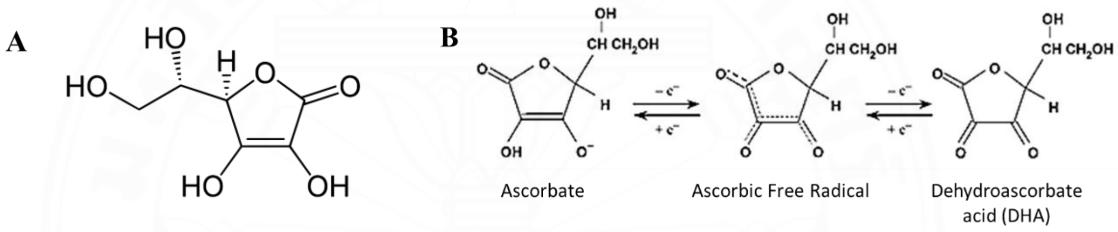


Fig. 2.8 (A) Structure of vitamin C and (B) Redox metabolism of vitamin C

The ascorbate free radicals caused by the generation of the oxidizable electron of ascorbate resulted in dehydroascorbic acid (DHA). Dehydroascorbic acid (DHA) is an unstable molecule represented in oxidized form of ascorbic acid.[74, 75] Moreover, the pro-oxidant or vitamin C can reduce Fe^{3+} into ascorbate-Fe^{2+} (**Figure 2.8B**).[76]

(ii) Vitamin E

Vitamin E or α-tocopherol is a lipid soluble antioxidants which can promote the defense mechanism against free radicals.[77] There were eight structures of vitamin E including four tocopherols (A) and four tocotrienols (B) as shown in **Figure 2.9**. Tocopherols consisted of saturated phytyl side chain while tocotrienols consisted of unsaturated isoprenoid tail.[78]

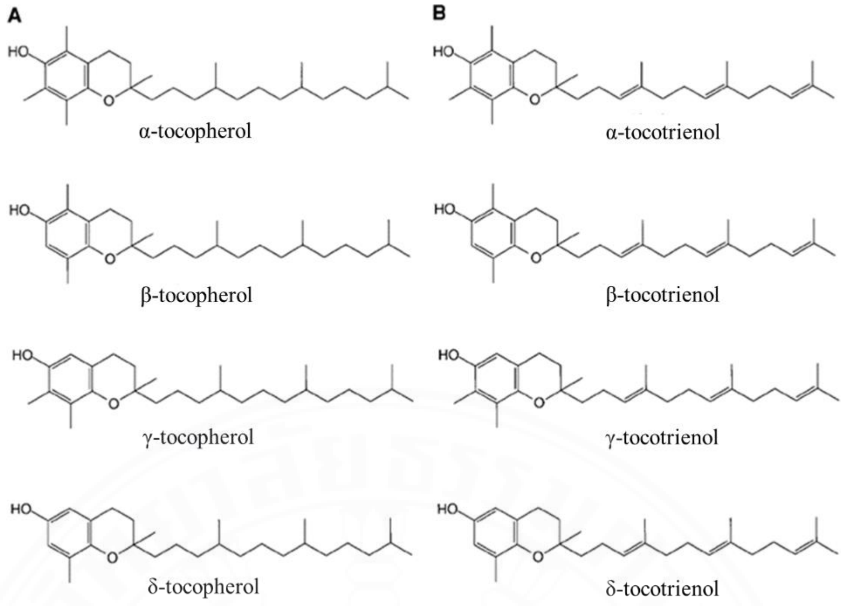


Figure 2.9 Structure of the naturally occurring forms of vitamin E[79]

Vitamin E can protect the cells from lipid peroxidation caused by radicals in biological membrane via the oxidization of α-tocopherol which is a stable form to tocopherol radical (**Figure 2.10**).[80] By the presence of antioxidant such as vitamin C or glutathione peroxidase, these radicals can be converted back to a stable molecule.[81]

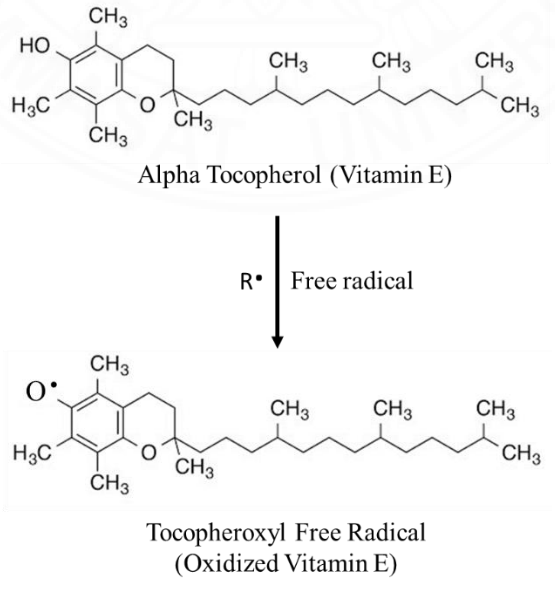


Figure 2.10 Antioxidant activity of vitamin E

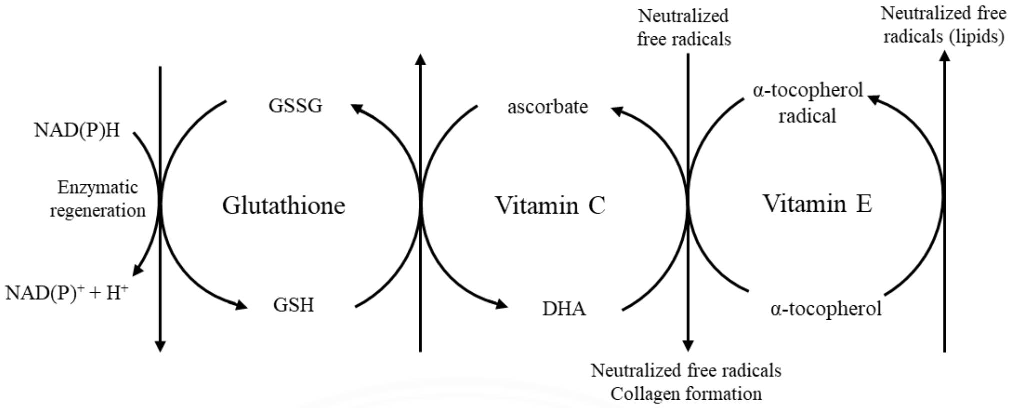


Figure 2.11 Mechanism of antioxidant action of vitamin C, E and glutathione[82]

Figure 2.11 showed the free radicals defensing and the reduced antioxidant regeneration of vitamins C and E, and glutathione.[82] In general, vitamin E is a lipid soluble and can function at the lipid membrane.[83] Lipid peroxidation occurred at the membrane resulted in the emerging of free radicals at lipid bilayer area. Then, vitamin E (α-tocopherol) which are stable molecule will receive the free radicals and convert into vitamin E radical. Vitamin C (ascorbic acid) is coworking with vitamin E and will further accept free radicals leading to the regeneration of the stable molecule of vitamin E.[84, 85] Glutathione (GSH) also play a crucial role in this cascade reaction. The reduced form of glutathione subsequently accepted the free radicals from ascorbic acid radical (dehydroascorbic acid) then coupling with another glutathione molecule and become to the oxidized formed (GSSG). For converting of oxidized glutathione (GSSG) into stable formed (GSH), vitamin C and dihydrolipoic acid helped to convert GSSG back to GSH.[84, 86]

(iii) Carotenoids

Carotenoids are lipid-soluble component found in plants, fruits and vegetables. There are over 750 structurally types of carotenoids found in nature. **Figure 2.12** presented four six of carotenoids including alpha-carotene, beta-carotene, beta-cryptosanthin, lutein, lycopene, and zexanthin which can be converted to retinol (vitamin A).[87] The most potent antioxidant among six forms of carotenoids are lycopene and beta-carotene. These carotenoids have the ability to bind more peroxyl radical than other ROSs because of their oil solubility property. The peroxyl radical is caused by lipid peroxidation in the cell membrane. Therefore, carotenoids play a major role in protecting the lipoprotein from ROS damage. When the carotenoid binds to free radicals, it can delocalize free radicals formed through long conjugated double bonds and thus the molecules are more stable.[88, 89]

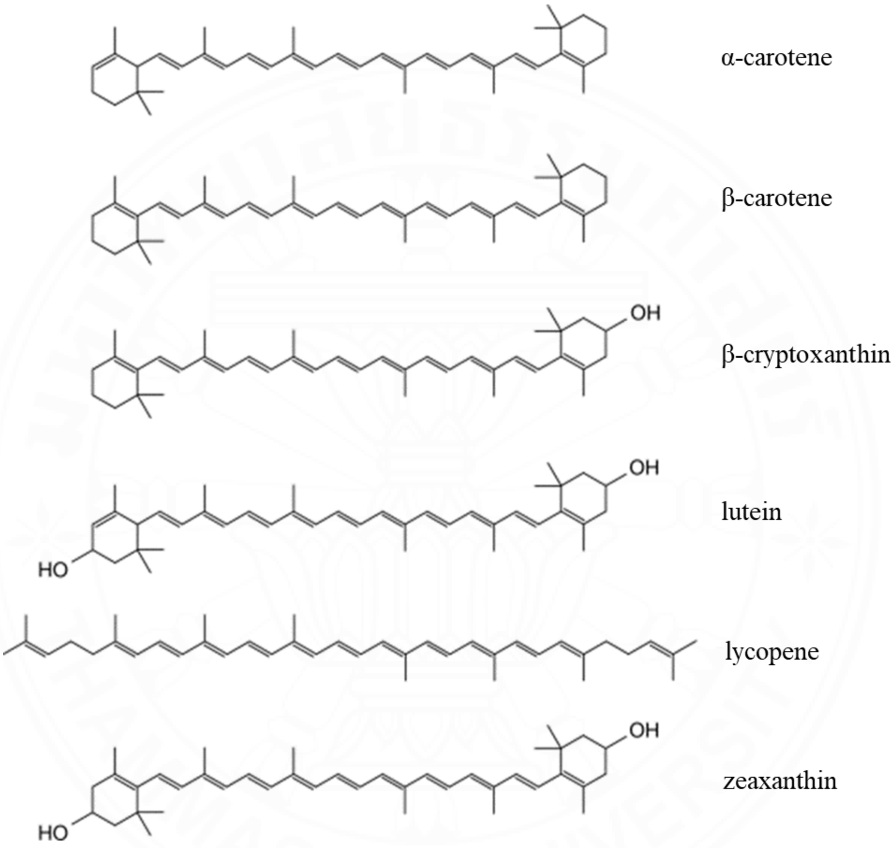


Figure 2.12 Structure of carotenoid

2.3.2 Sources of antioxidant

2.3.2.1 Synthetic antioxidant

Synthetic antioxidant is chemically synthesized and widely used in food industry as a preservative to help the prevention of lipid oxidation.[11] The example of synthetic antioxidant are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ) as shown in Fig 2.13.[90] Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were developed to protect flaxseed oil from oxidative and extension of its shelf life.[91] Synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have recently been reported to be a considerable cause of human disease such as cardiovascular disease, carcinogenesis and liver damaged etc.[10]

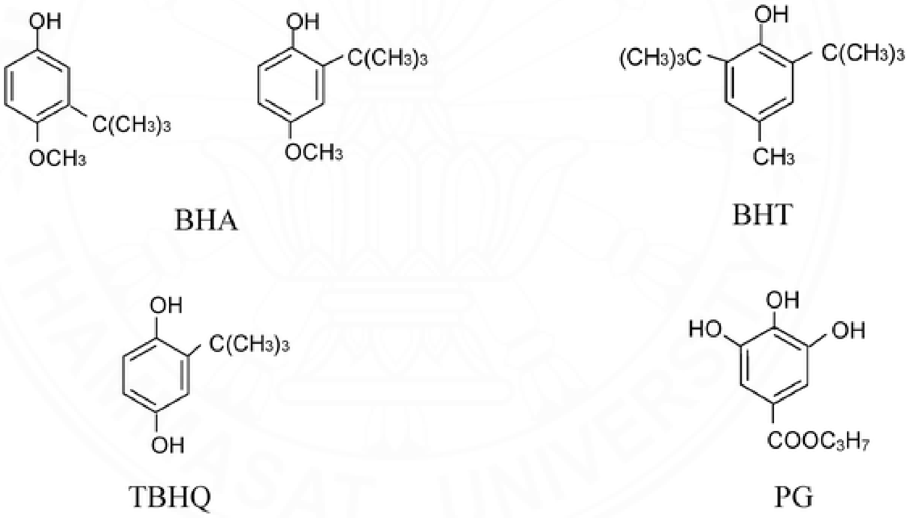
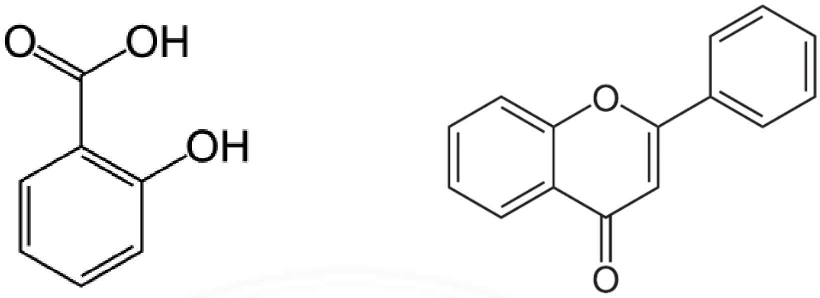


Figure 2.13 Structure of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ).

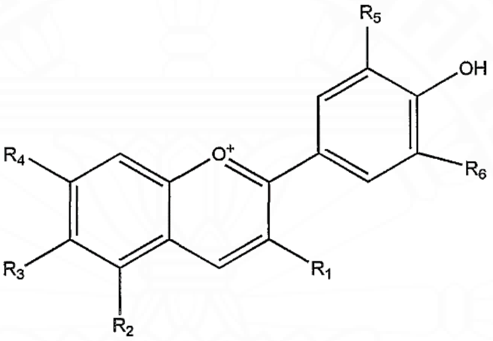
2.3.2.2 Natural antioxidant

The examples of natural antioxidant are α-tocopherol, vitamin A and C. The natural antioxidants from fruits and vegetables are mainly polyphenol group such as phenolic acids, flavonoids and anthocyanins (Fig. 2.14). They provided a wide range of biological effects against various diseases in human such as anti-inflammatory, anti-aging, and anticancer.[92-95]



Phenolic acid

Flavonoids



Antocyanin

Figure 2.14 Structure of phenolic acids, flavonoids and anthocyanins.

2.3.3 Classification of antioxidant

There were two types of antioxidants which are primary antioxidant, also known as endogenous antioxidant, and secondary antioxidant. From *in vitro* study, primary antioxidant produced via metabolic mechanism including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are strongest antioxidant with high protection effect. Primary antioxidant converts free radicals into a stable form that cannot further react with nearby molecules and this reaction is called “the prevention of free radical formation”. On the other hand, the secondary antioxidant such as vitamin A, C, E, carotenoid and flavonoid are only derived from the natural sources. They act as an inhibiting molecule responsible for scavenging reactive oxygen species (ROS) occurred in the cell. The secondary antioxidant often function together with primary antioxidant to promote its scavenging activity.[96] (**Figure 2.15**)

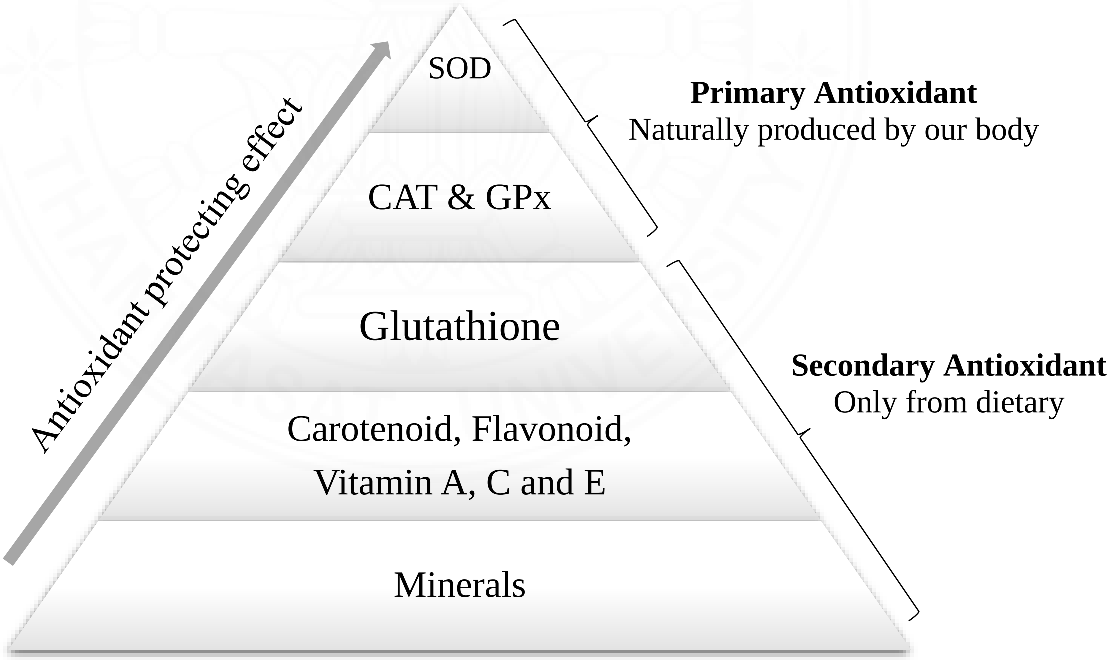


Figure 2.15 Classification of antioxidant

2.3.4 Antioxidants defense mechanism

The antioxidant protection mechanisms against oxidative damage can occur via the following mechanisms.

2.3.4.1 The scavenging of free radical

Antioxidant molecule serves as proton or electron donor to free radicals to become a more stable molecule. When an antioxidant donate proton or an electron, a new free radical is formed. In addition, this new free radical might react with another antioxidant molecule to establish a stable product. In similar way, antioxidant molecule can donate an electron or proton resulting in stable product. These cascade reactions were also called as the interruption of radical chain reactions.[97, 98] The scavenging of free radical was demonstrated in **Figure 2.16**.

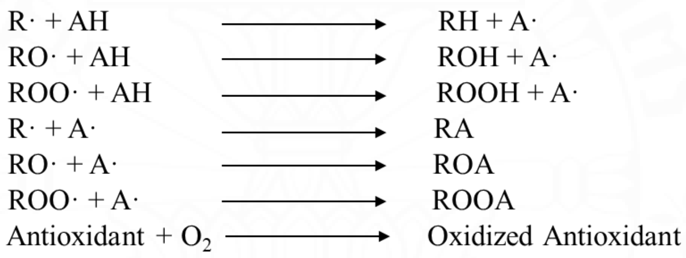


Figure 2.16 The scavenging of free radical

2.3.4.2 Metal chelating

Metals are an important part of several functional and structural components in living organism. The metal such as iron and copper acts as the intermediate in the formation of free radicals causing cell damage.[99] The increasing level of oxidation reaction in body resulted from biological metal cation such as Fe^{2+}/Fe^{3+} and Cu^{2+}.[100] These metals accelerate the formation of various free radicals such as peroxyl radical, hydroxyl radical and alkyl radical, as well as singlet oxygen.[101] Therefore, having antioxidant substances bind to these heavy metals could help to slow down the formation of free radicals in the body. The antioxidant substances that work through this mechanism include vitamin C, citric acid, flavonoids and etc.[102-105]

2.3.4.3 Quenching of singlet oxygen

Singlet oxygen($^1O_2$) is a major member of reactive oxygen species (ROS). It is unstable and highly reactive causing oxidative stress and eventually diseases in human.[106] Carotenoids are known to be efficient quenchers of singlet molecular oxygen.[107] It worked by inhibiting the function of singlet oxygen by converting singlet oxygen molecule ($^1O_2$) to triplet oxygen ($^3O_2$) and released the energy in term of heat.[108]

2.3.4.4 Enzyme inhibitor

The inhibitor acted by inhibiting the enzymes involved in the formation of free radical. For example, lipoxygenase (LOX), a member of nonheme iron-containing enzymes is a key enzyme that accelerate reaction of the deoxygenation of polyunsaturated fatty acids (PUFAs) resulting in the production of hydroperoxyl derivatives involved in cellular damaged.[109] Several studies demonstrated that phenolic compound derived from food sources like red wine and green coffee could inhibit the activity of lipoxygenase by binding to an iron, a cofactor, leading to enzyme inactivation.[108, 110]

2.4 Palm

Palm (*Elaeis guineensis Jacq*) is a member of palm tree family. It has been originated from the Gulf of Guinea, the tropical rain forest region of west and southwest Africa. The oil productions obtained from two different parts of palm which are mesocarp and endocarp are palm oil and palm kernel oil, respectively.[111]

The scientific classification of palm

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Liliopsida
Subclass	Arecidae
Order	Arecales
Family	Arecaceae
Genus	*Elaeis* Jacq.
Species	*Elaeis guineensis* Jacq.

The composition of palm oil fruit was shown in **Figure 2.17**. Palm seed (*Elaeis guineensis* Jacq) consists of 3 parts including exocarp, mesocarp and shell. The color of exocarp, an outer layer, varies depending on the species. However, the species found in Thailand has a red outer layer. The next layer is mesocarp, a layer that contains oil and fiber. The oil content in this layer is around 45-56%. Next to the seed layer is shell. The innermost layer is the endosperm of the palm which is called the kernel. The innermost kernel also contains large amount of oil content which is around 50%.[112]

In the palm oil production process, there are by-products or leftover materials consisting of palm oil sludge, palm press fiber, palm kernel, palm nut shells and bunch trash as shown in **Figure 2.18**.

2.4.1 Palm oil sludge

Palm oil sludge is brown semiliquid mixture consisting of 4-5% solids (mainly organic), 0.5-1% residual oil, and approximately 95% water.[113] It is generally disposed in palm oil plantations, which can cause the environmental problems.[114]

2.4.2 Palm press fiber

It is the shell part of the palm fruit that has been separated from palm bunches. It is mostly used as fuel in factories.

2.4.3 Palm kernel meal

The palm kernel meal has the lowest content (4-5%) when compared with other by-products. It is separated from the shell. When the oils were extracted, the obtained fiber residues called palm kernel cake (dry, hard and sheet) and palm kernel meal (fine powder).

2.4.4 Palm nut shell

Palm kernel shell is fibrous material containing approximately 8% of whole palm bunches and can be us as the biomass fuel.

2.4.5 Palm bunch trash

It is the major by-product of the whole palm bunch after oil extraction and used as a biomass fuel.

Figure 2.17 The composition of palm oil fruit

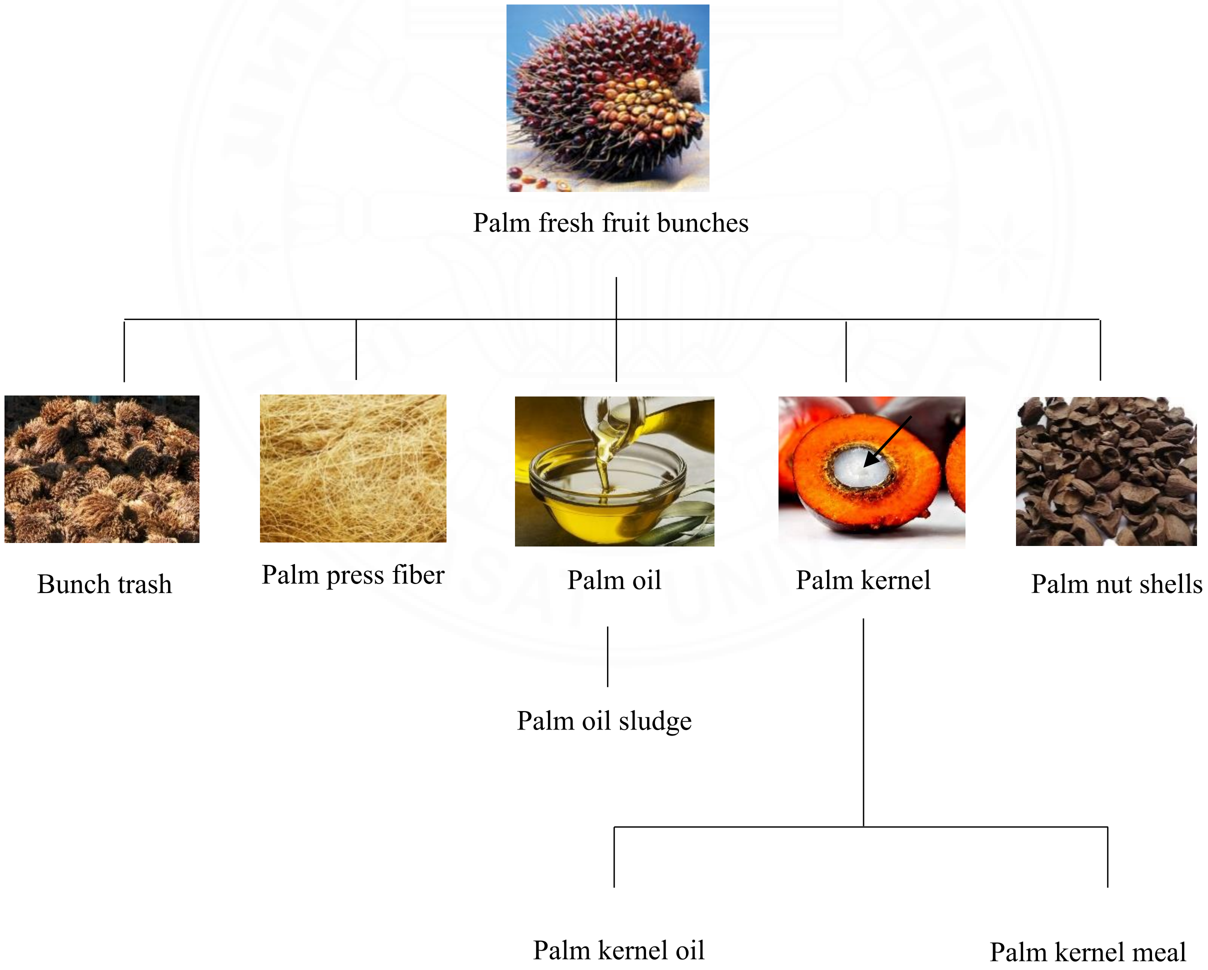


Figure 2.18 The product and by-products from palm oil extraction

Palm kernel meal (PKM) is one of by-product from palm oil milling or palm oil industry. The chemical composition of PKM was shown in **Table 2.4**. In the agriculture, various by-products such as fronds, trunks, press fiber, empty fruit bunches, kernel meal and kernel shells are generated from oil expeller extraction and used for animal feed and animal nutrition food. PKM was used as a dietary ingredient of poultry feeding instead of groundnut cake (GNC).[115] In addition, PKM is an alternative low cost energy food for lambs and other livestock.[116] Mohamed et al., 2003 reported the usage of PKM ingredients feed for feeding livestock. The recommended level of palm kernel cake fed in swine is 15-25%. However, in some area, they fed (PKC or PKM) to swine up to 40% without side effect. In addition, PKM or PKC is used as a source of energy in cow. It commonly fed together with grass and other concentrates around 30-50% because it contains high fiber.[117]

Table 2.4 The chemical composition of palm kernel meal[115]

Chemical contents	Palm kernel meal (%dry matter)
Oil content	2-8
Protein content	16-19.2
Crude fiber	16.7
Moisture	6.5
Ash	3.9
Fat	12-20
Carbohydrate	50

2.5 Protein hydrolysate

Protein hydrolysate is obtained by the breakdown of peptide bond in proteins resulting in peptides with different molecular sizes and free amino acids. The preparation of protein hydrolysate by structural alteration of their original proteins could affect the physical and chemical properties of the protein such as molecular weight, polarity and solubility.[118] The production of protein hydrolysate was generated by various methods including physical, mechanical and chemical method.

2.5.1 Physical method

The transformation of the protein structure or the process of protein degradation can be performed in various ways such as heat-based physical method. It is a traditional way to eliminate the microorganisms and reduce the toxicity[119].

2.5.2 Mechanical method

Mechanical method such as high-pressure processing (HPP) and ultrasound wave (ultrasound) resulted in the breaking of bonds within protein molecules. It was found to be effective in improving protein structure, resulting in alteration of protein properties including the reduction in toxicity, allergy as well as microbial elimination[120].

2.5.3 Chemical method

Chemical method is the most commonly used methods. It can be divided into three categories: acid, alkaline and enzymatic hydrolysis.

2.5.3.1 Acid hydrolysis

Acid hydrolysis was obtained by using inorganic acids, sulfuric acid or hydrochloric acid. The use of sulfuric acid to digest protein will result in a poor odor of protein, and when neutralized, calcium sulfate sediment is formed. In addition, the usage of hydrochloric acid to hydrolyze protein will generate sodium chloride or potassium chloride salt. In industry, hydrochloric acid is used to digest protein since the resulting salt is commonly used in food and it has low or no negative effect on the product. The process is uncomplicated, low cost and time saving. However, there was the loss of amino acid typically tryptophan and may destroy the amino acid particularly cysteine, serine and methionine.[121]

2.5.3.2 Alkaline hydrolysis

Alkali hydrolysis using sodium hydroxide, barium hydroxide and potassium hydroxide caused less of tryptophan destruction than using acid hydrolysis. However, some amino acids can undergo a racemization reaction, which is the conversion of an L-form into a D-form. Moreover, it also destroys some amino acids such as arginine, threonine and cysteine.[122, 123]

2.5.3.3 Enzymatic hydrolysis

Enzymatic hydrolysis is one of the most popular methods used because it showed high specificity, mild conditions and can control the digestion level. In this method, the degradation rate is quite high compared to acid and base hydrolysis. This is due to the specificity of an enzyme in cutting peptide bond. There are two factors involved in enzymatic hydrolysis including type of enzyme and molecular size of the protein substrate. For example, a large of protein size could provide the cleavage site of enzyme more than small protein but the large protein might be given small amount of single amino acid. Because of small proteins are short chain peptides which contribute to more specific cleavage site, so they can give single amino acid than a large protein. In addition, the enzyme-to-substrate ratio, duration, enzyme pH and temperature affected the rate of hydrolysis as well. Typically, the hydrolysis process is performed at the proper pH and temperature of individual type of enzyme, using proper digestion conditions for enzyme activity to obtain the desired product.[124]

Protein hydrolysate production using protease digestion is highly efficient method since enzyme is specific to protein substrate and pH value. Proteolytic enzymes also known as protease that catalyzed proteolysis in to short chain peptides. The protease properties are diverged depending on their property such as optimal pH and temperature, substrate specificity, active site and catalytic mechanism and stability profile.[125] They could be categorized into two types which are endoprotease and exoprotease. Endoproteases such as pepsin and trypsin, are enzyme act in interior site of protein by digesting peptide bonds within protein molecules and generating peptide chains. Carboxypeptidase and amino peptidase are example of exoprotease which they will break down peptide bond at terminal site to release single amino acid.

An example of a plant-based protease is papain and bromelain. An example of protease from animal is pepsin. Nowadays, protease enzyme from microorganisms such as alcalase, flavourzyme and neutrase enzyme were used in the industry. Alcalase was extracted from *Bacillus subtilis* and can be classified as endoprotease with optimum temperature during 55-60 °C and has an optimal pH in the range of 8-8.5.[126] Flavourzyme was produced from *Aspergillus sp*. It is classified as both endo-peptidase, an enzyme that breaks down the internal peptide bonds of polypeptide and exo-peptidase to breakdown peptide bonds from the ends of the polypeptide or protein chain. The optimum temperature is 50-60 °C and the optimum pH is in the range of 6-7.[127, 128] The neutrase enzyme is produced from *Bacillus amyloliquefaciens*. It is an endoprotease with the optimum temperature at 45-55 °C and the optimum pH is in the range of 5.5-7. Additionally, there are other protease, such as trypsin, protamex, and pronase, etc. Although the protein hydrolysis using protease enzymes is highly efficient but it is high cost. In addition, during protein hydrolysis process, degree of hydrolysis (DH) should be considered. Degree of hydrolysis (DH) is defined as the percentage of cleaved peptide bonds in a protein hydrolysate.[129]

2.5.4 Biological activity of protein hydrolysate

2.5.4.1 Antioxidant activity

Protein hydrolysate is a source of bioactive substances, especially bioactive peptides. The role of protein and peptide is related to antioxidant. For example, the ability of protein and peptide to reduce oxidative rancidity, an important issue in the food industry. There were several pathways in which protein or peptide could act as antioxidative substance such as the inhibition of the singlet oxygen causing lipid peroxidation and DNA damage through constitutive antioxidant defense systems such as free radical scavenging. In addition, chelating agents can also stabilize prooxidative transition metals which could promote antioxidant capacity. In amino acid molecules, it consists of R group such as hydrophobic and hydrophilic. As a result, the properties of amino acids such as water solubility, acidity, base, polarity and non-polarity are different. Hence, the antioxidant activity of protein hydrolysate depends on their peptides and amino acid compositions. Basic amino acids containing-basic side chains (histidine, arginine and lysine), sulfur-containing amino acid (cysteine and methionine) or amino acid with aromatic side chain (tryptophan, tyrosine, and phenylalanine) were reported to have a strong antioxidant activity.[130] The chelation of prooxidative transition metals was reported involving the -NH group of the imidazole ring which also inhibit free radicals reaction by proton donating capability.[131]

There were several studies reported the antioxidant activity of protein hydrolysate. The hydrolyzed protein of *Moringa oleifera* seed protein provided better antioxidant activity than the unhydrolyzed proteins.[132] Chickpea protein hydrolysate (CPH) obtained by enzymatic hydrolysis showed the ability to fight against free radicals. The amino acid composition and hydrophobicity of CPH was found to be related with its antioxidant property[133]. The protein hydrolysate of *Hordeum vulgare* L., known as barley, exhibited antioxidant activity. The protein hydrolysate of *Hordeum vulgare* L. hydrolyzed with flavourzyme and alcalase had higher free radical scavenging activity, Fe^{2+} -chelating ability and superoxide radical scavenging capacity than pepsin enzyme. Bamdad et al., 2011 reported that the molecular weight of peptide in protein hydrolysate was also related to the antioxidant activity in which medium and large sized barley hydrolysate fraction was most contributed to antioxidant activity. It because of the specific structure of the hordein which is a storage protein of barley. It contains high amount of hydrophobic group so they can act as hydrogen donor.[134] Palm kernel meal hydrolyzed with different proteases (alcalase, chymotrypsin, papain, pepsin, trypsin, flavourzyme, and bromelain) showed high antioxidant activity.[135] Protein hydrolysate is a natural bioactive substance that is suitable as an ingredient in food and cosmetics, especially its anti-oxidation properties which are of particular interest due to its ability to conduct to replace synthetic antioxidants such as BHA, BHT, and TBHQ.

2.5.4.2 Antimicrobial activity

In recent year, a crucial problem caused by living microorganism especially bacteria have gained much attention in many fields such as dietary, cosmetics and pharmaceutical industries. The protein hydrolysate isolated from various natural sources exhibited antimicrobial activity. Lucie et al., 2015 showed the first report on the antibacterial peptide derived from brown algae protein hydrolysate. The hydrolysate with molecular size >10 kDa was able to decrease the growth rate of *Staphylococcus aureus*.[136] Puji et al., 2019 reported that Etawa goat milk hydrolysate hydrolyzed by bromelain enzyme displayed antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*.[137] Bioactive peptide derived from chia seed with antibacterial property was reported by Aguilar et al., 2020. The research demonstrated that chia seed protein hydrolysate with molecular weight < 3 kDa could inhibit the growth of *Escherichia coli, Salmonella enterica,* and *Listeria monocytogenes.* This could be help to decrease or prevent the food-borne diseases and food spoilage.[138] Rameshkumar et al., 2009 showed that the peptide derived from the haemolymph of *Thalamita crenata* crabs possessed an antimicrobial activity.[139] In addition, The antimicrobial activity of protein hydrolysate applied as cosmeceuticals was also studied. The collagen hydrolysates from skin of marine fish (*Chanos chanos*) hydrolyzed using *Bacillus* proteases. possessed an antioxidant activity and antifungal activity against *C. albicans*.[140]

2.5.4.3 Immunomodulatory activity

Several diseases for example, rheumatoid arthritis, anti-inflammatory, diabetes, asthma and allergic disease are related to immunity system, so many researchers are interested in studying and developing these immunomodulatory products.[141, 142] In addition, immunomodulatory products are also received attention in industrial field. The different length of hydrolysate is responsible for different target. There were 3 levels of protein hydrolysis which are extensive, partial and mild hydrolysis. Extensive hydrolysis and partial hydrolysis can prevent symptoms in allergic individuals and prevent allergic sensitization, respectively. In part of mild hydrolysis, it is help to improve digestion. Parker et al., 1984 reported the protein hydrolysate from casein can activate an antibodies generation in mouse, and can increase phagocytosis ability of mouse macrophages against sheep erythrocytes. *In vivo* study reported that the injection of casein hydrolysate to mature mice can prevent the infection of gram-negative bacteria (*Klebsiella pneumoniae*).[143] Moreover, Van et al., 2011 reported that a decrease in acute allergic skin symptoms in mouse model was investigated after exposure with partial whey hydrolysate.[144] Protein hydrolysate can contribute immunomodulatory effects in the cell by several mechanisms. Protein hydrolysate can directly stimulate receptor signaling or can be brought into the cell via a protein transporter and interfere the inflammatory signaling pathways. Besides this, the endocytosis and inhibit inflammatory signaling pathways can support protein uptake into the cell. Toll-like receptors (TLRs) play critical roles in the innate immune receptor signaling. Iskandar et al., 2012 reported that whey protein hydrolysates could decrease IL-8 secretion and involved in Toll-like receptors (TLRs) pathway which could decrease the binding of lipopolysaccharides to Toll-like receptor. This might be help to increase extracellular antioxidant ability.[145]

2.5.4.4 Cytoprotective activity

The numerous studied reported that protein hydrolysate could protect the cells. Zhang et al., 2019 showed the cytoprotective effect of the edible sac fungi (*Morchella esculenta*) protein hydrolysate. It could protect Caco-2 cell against H_2O_2 which can cause oxidative stress, cell damage, cell injury and suppressed cell apoptosis by increasing cell viability, enhancing the inhibition level of intracellular ROS and malondialdehyde (MDA) generationtion.[146] Cai et al., 2019 also demonstrated pentapeptides derived from protein hydrolysate of swim bladder from marine fish (*Miichthys miiuy*) could improve the survival rate of cells from oxidative damage. The researcher explained that the action of protein hydrolysate depends on the composition, length and molecular size of peptide and amino acid. The hydrolyzed protein containing short sequences with 2–20 amino acid residues exhibited high activity since they were digested into small peptide chain which could more easily react with other radicals to protecting cell from oxidative injury.[147] [148] In addition, there were reports on the cytoprotective effect of protein hydrolyzed from plant sources. Liu et al., 2017 indicated that the antioxidant peptides fraction from hazelnut protein hydrolysate could affect cell viability by protecting cell against Ang II via inhibiting oxidative system and DNA damage.[149] Mung bean protein hydrolysate had no cytotoxicity against mouse liver cell line, Nctc-1469 cell. They could protect Nctc-1469 cell from hydrogen peroxide-induced oxidative damage through decreasing the content of lactate dehydrogenase (LDH) and reduce the production of MDA.[150] Moreover, peptide EDIVCW, MEPVW, and YWDAW from *Lophius litulon* protein hydrolysate demonstrated significant protection of HepG2 cells from hydrogen peroxide-induced cell damage by decreasing MDA and ROS level including stimulating intracellular antioxidant enzymes of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in the cells.[151]

2.6 Palm kernel meal hydrolysate

In recent year, protein hydrolysate derived from palm kernel meal much gain attention to research. Because of it contains 16-18 % of protein. In the agriculture industry, palm kernel meal is a by-product of palm oil extraction result in a numerous of waste but they could exert the beneficial in terms of use as animal feeding. Moreover, protein hydrolysate which modified from parent protein could exhibit functional activity more than their native form. **Table 2.5** showed several studies reported their multifunctional bioactivity of palm kernel meal protein hydrolysate.

2.6.1 Palm kernel cake hydrolysate with antioxidant activity and toxicity

There were several studies reported the antioxidant activity of palm kernel cake. Zarei et al., 2014 demonstrated the peptide sequences of palm kernel cake protein hydrolysate which showed high radical scavenging activity of 71% as determined by by DPPH assay.[152] In addition, The palm kernel cake protein hydrolysate hydrolyzed by papain enzyme revealed a strong antioxidant activity (65 ± 0.25%) when compared to other hydrolysates such as trypsin, pepsin, alcalase, chymotrypsin, bromelain, and flavourzyme.[135] A previous study also evaluated the antioxidant activity and toxicity of palm kernel cake protein hydrolysate (PKCPH) hydrolyzed by alcalase enzyme. Results revealed that PKCPH fraction 1 contained a moderate amount of hydrophobic (23.60%) and amino acids with antioxidant activity (26.10%) such as Asp, Glu, His, Lys, Met and Tyr. As a result, it has the highest antioxidant activity. Furthermore, the highest dosages of PKCPH (2 mg/mL) and PKCPH1 (1 mg/mL) showed high cell survival (87–92%) and slight cytotoxic activity. Therefore, palm kernel cake had a potential as a valuable source of natural antioxidants.

Table 2.5 The bioactivity of palm kernel meal protein hydrolysate.

Source of protein	**Hydrolyzed enzymes**	**Bioactivity results**	**Reference**
Palm kernel cake	Alcalase, chymotrypsin, papain, pepsin, trypsin, flavourzyme, and bromelain	DPPH•radical scavenging activity and OPA assay assay, were used to evaluate antioxidant activity and degree of hydrolysate respectively.	Zarei et al., 2012
Palm kernel cake	Papain, pepsin, trypsin, flavourzyme, bromelain and chymotrypsin	The antioxidant peptides of PKC that isolated from papain Enzyme showed DPPH• radical scavenging activity and metal chelating activity.	Zarei et al., 2014
Palm kernel expeller glutelin-2 (PKEG)	Alcalase, pepsin, trypsin and flavourzyme	Bioactive peptides isolated from PKEG exhibited potent non-competitive ACE inhibition, non-toxic to EA.hy 926 cells and they could exert antihypertensive capability via down-regulate the intracellular ET-1 content	Yajun et al., 2017
Palm kernel cake	Trypsin	The PKC protein hydrolysates exhibited potent antiradical property through using DPPH radical scavenging capacity and ABTS•+ radical scavenging capacity assay.	Ng et al., 2013

Table 2.5 The bioactivity of palm kernel meal protein hydrolysate. (Cont.)

Source of protein	**Hydrolyzed enzymes or fermentative microorganism**	**Bioactivity results**	**Reference**
Palm kernel cake	*Lactobacillus casei* ATCC334	Low molecular weight peptides generated from PKC showed antifungal activity in bread which could reduce the amount of conidia in bread result in slow down growth rate of fungi.	Mohamad et al.,2020
Palm kernel cake	Novozyme, gammanase, mannaway, ultraflo, pectinex, celluclast and mannosidase	The carbohydrates in PKC were demonstrated a potentially utilized for liquid fuel production and they could be promoted protein content in animal feed due to after extracting sugars for fermentation, the residues contained higher protein content.	Cerveró et al., 2020
Palm kernel cake	Alcalase and tryptic	Palm kernel cake hydrolyzed by two enzymatic, both of them showed antibacterial activity which they could disrupt membrane integrity of *B. cereus*.	Tan et al., 2013

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Protein isolation from palm kernel meal

The palm kernel meal protein (PKMP) wase isolated using a modified method of Zarei et al., 2012.[153] Fifty grams of PKM was grinded and defatted with hexane. The defatted PKM was dried overnight or until hexane was completely evaporated. The protein was isolated from defatted PKM by dissolving PKM in 0.03 N Sodium hydroxide (NaOH) solution at ratio of 1:10 (w/v) and extracted by stirring at room temperature for 1 h. During stirring, the pH value of the sample solution was continuously adjusted to 11. After centrifugation at 10,000 × g, 4 °C for 20 min, the pH of the supernatant was adjusted to 3.5 which is the isoelectric point of PKM protein. Then, the precipitate was obtained by centrifugation at 8,820 × g, 4 °C for 10 min. The precipitate was dissolved with deionized water, the pH was adjusted to 7 and protease inhibitor was added immediately to avoid protein degradation. The protein was stored at −20 °C until used.

3.2 Preparation of PKM protein hydrolysate

Palm kernel meal protein hydrolysates (PKMPH) was prepared according to the method described by Zarei et al., 2012 with minor modification.[153] The pH of PKM protein was adjusted to 7.5 and heat to 55 °C for 2 minutes. After that, the mixture was mixed with 2% alcalase enzyme at ratio 1: 50 (w/v) and incubated at 55 °C for 15, 30, 60 and 120 minutes. The reactions were stopped by heating the mixture at 100 °C for 10 min. After centrifugation (4,000 rpm, 4 °C, 20 min), the supernatant was collected and stored at −20 °C until use.

3.3 Degree of hydrolysis determination

Degree of hydrolysis (%DH) is defined as a ratio of cleaved peptide bond of protein hydrolysate, was calculated by using o-phthaldialehyde (OPA). DH was prepared according to the method described by Nielsen et al., 2001 with slightly modification. Firstly, sample was mixed with 215 µl of OPA reagent. The OPA reagent composed of 25 mL of 100 mM sodium tetraborate, 2.5 mL of 20%(w/v) SDS solution, 1.0 mL of 40 mg/mL of OPA dissolved in methanol and 100 µL of beta-mercaptothion. After that, the mixture was mixed for 5 second and incubated for 2 minutes at room temperature. The absorbance was measured at 340 nm and the obtained absorbance was calculated with following equation.

Equation:

$$\%\mathbf{DH} = \frac{\mathrm{OD}_{\mathrm{sample}} - \mathrm{OD}_{\mathrm{control}} - \mathrm{OD}_{\mathrm{standard}}}{\mathrm{OD}_{\mathrm{sample}}} \times 100$$

3.4 Protein and peptide analysis by SDS PAGE

Electrophoresis was prepared according to method described by Yajun et al.,2015.[154] SDS-PAGE was performed on 5 % acrylamide gel and 12% acrylamide separating gel using gel electrophoresis. The sample was directly dissolved with deionized water and 4X sample buffer. After that, sample was incubated at 95 °C for 5 minutes. The electrophoresis was run at 50 V in stacking gel and 120 V in separating gel until the tracking dye reached the bottom of gel. After that, the gels were fixed with fixative solution (40% methanol + 10% acetic acid and 50% deionized water) and washed three times with deionized water before staining with Coomassie Brilliant Blue R-250 for overnight. After that, gels were destained in a deionized water. Molecular weight of the protein bands was estimated using protein marker.

3.5 Determination of free radical scavenging activity

3.5.1 DPPH radical scavenging activity

DPPH assay was used to study the antioxidant activity of protein hydrolysate.[155] 2,2-Diphenyl-1-picrylhydrazyl, a stable radical substance that used to test the capacity of organic compound to serve as unpaired. When DPPH reacts with a sample that act as proton-donating substance, the radical will be scavenged. The antioxidant reaction was investigated by colorimetric change of mixture solution from purple to yellow. The DPPH assay was performed in accordance with Yazdi et al., 2013 with slightly modification. One hundred µl of sample (at concentration of 500-0.97 µg/mL) was mixed with 100 µl of freshly prepared DPPH reagent and incubated at room temperature for 30 minutes. After incubation, the absorbance at 517 nm of mixture was measured using microplate reader, and the obtained absorbance was calculated as % decolorization with following equation. IC_{50} or half maximal inhibitory concentration was determined by nonlinear regression (curve fit) using GraphPad Prism 7.00 program. L-Glutathione (at concentration of 500-0.97 µg/mL) and water were used as positive and negative control, respectively.

$$\%\mathbf{Decolorization} = 1 - \left(\frac{\mathrm{A_{sample}}}{\mathrm{A_{control}}}\right) \times 100$$

3.5.2 ABTS radical scavenging activity

2,2'-azino-bis (3- ethlybenzthiazoline-6-sulphonic acid) or ABTS in combination with potassium persulfate is converted to cation radical ($ABTS^+$). The antioxidant will serve as free radical cation donation to $ABTS^+$ and convert to ABTS, which is more stable. The scavenging reaction was observed by the measurement of absorbance at 734 nm. Fifty µl of sample (at concentration of 500-0.97 µg/mL) was mixed with 200 µl of freshly prepared ABTS solution and the absorbance of the mixture was immediately measured at 734 nm using microplate reader, and the obtained absorbance was calculated as % ABTS with following equation. IC_{50} or half maximal inhibitory concentration was determined by nonlinear regression (curve fit) using

GraphPad Prism 7.00 program. L-Glutathione (at concentration of 500-0.97 μg/mL) and water was used as positive and negative control, respectively.

$$\%\mathbf{ABTS} = \left(\frac{\mathrm{A_{control}} - \mathrm{A_{sample}}}{\mathrm{A_{control}}}\right) \times 100$$

3.5 Cytotoxicity determination

MTT assay is a technique for measuring cellular metabolic activity, an indication of whether the cell is capable of proliferating cells. When MTT was reduced by mitochondrial reductase enzyme in mitochondria of living cells, the MTT reagent (yellow dye) was converted to an insoluble purple formazan and the absorbance was measured at 570 nm. Cell viability was investigated by the increasing of purple color of mixture solution. [156] The cytotoxic capacity was observed using MTT assay according with Frederico et al., 2009. L-glutathione is an antioxidant compound produced by human body. It is a protein protecting cells and tissues from being destroyed by free radicals that accumulate in various parts of the body. Thus L-glutathione (500-0.97 μg/mL) was used as a positive control. L929 mouse fibroblast cells (10^3 cell/well) were seeded in Gibco Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and 1% of penicillin-streptomycin (pen-strep) in 96 well culture plate. After that, plate was incubated with 5% CO_2, 37 °C for 24 h until total adhesion to plate surface. The medium was replaced with fresh supplemented medium containing different concentration of palm kernel meal hydrolysate (500-0.97 μg/mL) and 0.4 mg/mL stock solution of MTT reagent in PBS was added (100 μl). Then, cells were incubated at 37°C 5% CO_2 for 3 h. After incubation, MTT solution was discarded and 100 μl of 100% DMSO was added. The extent of MTT reduction to formazan within cells were measured by absorbance at 570 nm using a microplate reader.

$$\%\mathbf{Cell\ viability} = \left(\frac{\mathrm{Absorbance_{sample}}}{\mathrm{Absorbance_{control}}}\right) \times 100$$

3.6 Cytoprotective effect determination

The colorimetric MTT assay was used to determined cytoprotective effect according with Benoit et al., 2017.[157] L-glutathione (500-0.97 μg/mL) was used as a positive control. L929 mouse fibroblast cells were seeded in Gibco Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and 1% of penicillin-streptomycin (pen-strep) at density of 10^3 cells/well culture plates. After that, plate was incubated with 5% CO_2, 37 °C for 24 h until total adhesion to plate surface. After 24 h, cells were treated different concentration of palm kernel meal hydrolysate (500-0.97 μg/mL) and the plate was incubated at 37°C 5% CO_2 for 3 h. After that sample was removed and replaced with 100 μl of 1,000 μM H_2O_2.Then, cells were incubated at 37°C 5% CO_2 for 3 h. After that, 1,000 μM**.** H_2O_2 was discarded and replaced with 0.4 mg/mL stock solution of MTT reagent in PBS (100 μl) and incubated again for 3 h at 37°C, 5% CO_2. After this time, MTT solution was discarded and 100 μl of 100% DMSO was added. The extent of MTT reduction to formazan within cells were measured by absorbance at 570 nm using a microplate reader. Cell viability percentage was determined by MTT reduction assay and was measured by following equation:

$$\%\mathbf{Cell\ viability} = \left(\frac{\mathrm{Absorbance}_{\mathrm{sample}}}{\mathrm{Absorbance}_{\mathrm{control}}}\right) \times 100$$

3.7 Purification of antioxidant peptide

In order to purify the antioxidant peptide in the PKMPH, Fast protein liquid chromatography (FPLC) was performed. Separation of protein and peptide in mixture rely on different affinity for two materials, a mobile phase (buffer + protein) and a stationary phase (resin beads), in column. There were various kinds of column used for protein purification including ion exchange, gel filtration, reversed phase (hydrophobic interaction) and affinity columns.[158] The antioxidant peptide obtained from enzymatic hydrolysate was purified by using FPLC on a DEAE Sepharose Fast Flow anion exchange column. The sample was loaded onto DEAE Sepharose Fast Flow anion exchange column and equilibrated with 2.5 mM Tris-HCl buffer (PH 8.0). After that, the peptide was eluted using linear gradient of NaCl (0-1 M) in a same buffer (2.5 mM Tris-HCl buffer (PH 8.0) at flow rate of 0.5 mL/min. The fraction was collected and stored at -20 °C until use. The protein concentration and antioxidant activity of each fraction was measured using Bradford assay and DPPH and ABTS radical scavenging activity, respectively.

3.8 Mass spectrophotometry (LC/MS MS)

Mass spectrometry (MS) has the ability to separate organic molecules according to their molecular mass and provide to their detection and quantitation with highly sensitivity and selectivity.[159] The active fraction with antioxidant capability after FPLC purification was digested with trypsin enzyme and subjected to mass spectrophotometry (LC-MS/MS) using Thermo Scientific^{TM} UltiMate^{TM} 3000 RSLC nano system. The antioxidant peptide was separately infused into the electrospray source following dissolve in solvent A (2% acetonitrile,0.1% formic acid) and solvent B (80% acetonitrile,0.1% formic acid) with flowrate of 0.3 µl/min. Spectra were analyzed in positive ion reflector mode with mass/charge (m/z) rang of 350-1500 Da in MS mode and 350-1000 Da in MS/MS mode. Peptide sequencing was performed by processing the MS/MS spectra using SCIEX TripleTOF® 6600+. All data was performed using PEAK studio database to obtain peptide sequencing.

CHAPTER 4

RESULTS

4.1 Protein extraction

In the utilization of palm kernel meal protein (PKMP), it is necessary to extract or separate proteins from palm kernel meal. The most commonly used protein extraction process is alkaline extraction method. After extraction, enzymatic digestion is performed which will hydrolyze the polypeptide chains into free amino acids or short chain peptides in order to improve nutrition or some properties such as radical-scavenging capacity. In this study, PKMP was extracted by using 0.03 N NaOH and protein precipitation was employed by using 6 M HCl. The results showed that %yield of total palm kernel meal protein extraction was 0.58 ± 0.012% and the protein concentration was 2.15 ± 0.047 mg/mL.

4.2 Protein hydrolysis

Protein hydrolysates are protein products obtained by hydrolysis of proteins by proteolytic enzymes, acids or bases in which the protein molecules are cleaved into smaller peptides or free amino acids.[160] In this study, PKMP was hydrolyzed with alcalase enzyme at various time (0, 15, 30, 60 and 120 minutes) to obtain the palm kernel meal protein hydrolysate (PKMPH). **Table 4.1** showed that the protein concentration of PKMPH was decreased when the hydrolysis time was longer revealing more hydrolysis. The results indicated that the hydrolysis time significantly affects the protein concentration of PKMPH.

Table 4.1 Protein concentration of a palm kernel meal protein hydrolysate (PKMPH)

Time of hydrolysis (min)	Protein concentration (mg/mL)
0	2.15 ± 0.108
15	1.80 ± 0.101
30	1.77 ± 0.097
60	1.77 ± 0.073
120	1.75 ± 0.080

4.3 SDS-PAGE analysis of PKMP and PKMPH

Gel electrophoresis is a technique used to separate proteins, nucleic acids or macromolecules based on the principle of motion on a gel under an electric field. Protein separation by polyacrylamide gel electrophoresis can be used to determine the molecular weight.[161] In this study, PKMP and their hydrolysates were seperated on 12% (w/v) polyacrylamide gel and stained with Comassie Brillian Blue. All samples including PKMP and PKMPH showed smear band of protein as shown in **Figure 4.1**.

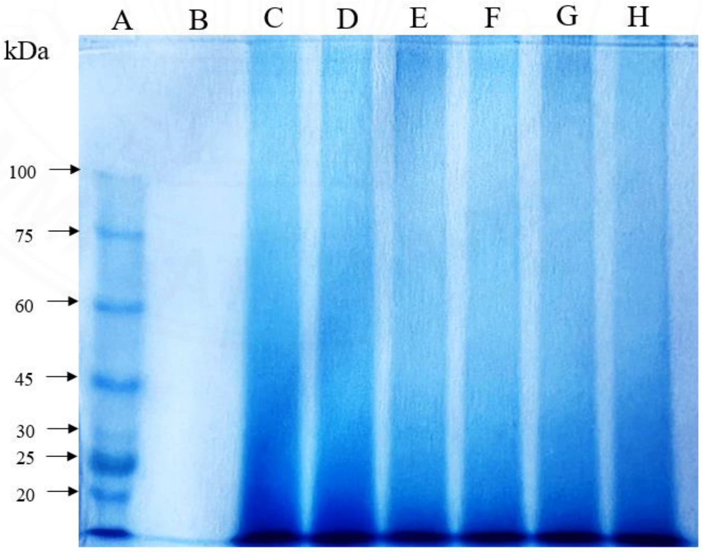


Figure 4.1 SDS-PAGE patterns and molecular weight distribution profile of palm kernel meal protein and their hydrolysates. Lane A: protein marker, Lane B: 4x sample buffer (negative control), Lane C: PKMP, Lane D-H: PKMPH after 0, 15, 30, 60 and 120 minutes hydrolysis, respectively.

4.4 Degree of hydrolysis of PKMPH

Degree of hydrolysis (DH) indicates the efficiency of enzymatic protein digestion. The peptide bonds of proteins in palm kernel meal are hydrolyzed into short chain peptides and amino acid residues. As shown in **Figure 4.2**, the degree of hydrolysis of PKMP with alcalase enzyme at various time (15, 30, 60, and 120 minutes) was not different. The degree of hydrolysis at 0, 15, 30, 60, and 120 minutes was 0%, 89.48±0.67%, 89.47±0.72%, 89.08±0.71 % and 89.31±0.90 %, respectively. The results showed that rapid enzymatic hydrolysis resulted in a significant change in the degree of hydrolysis.

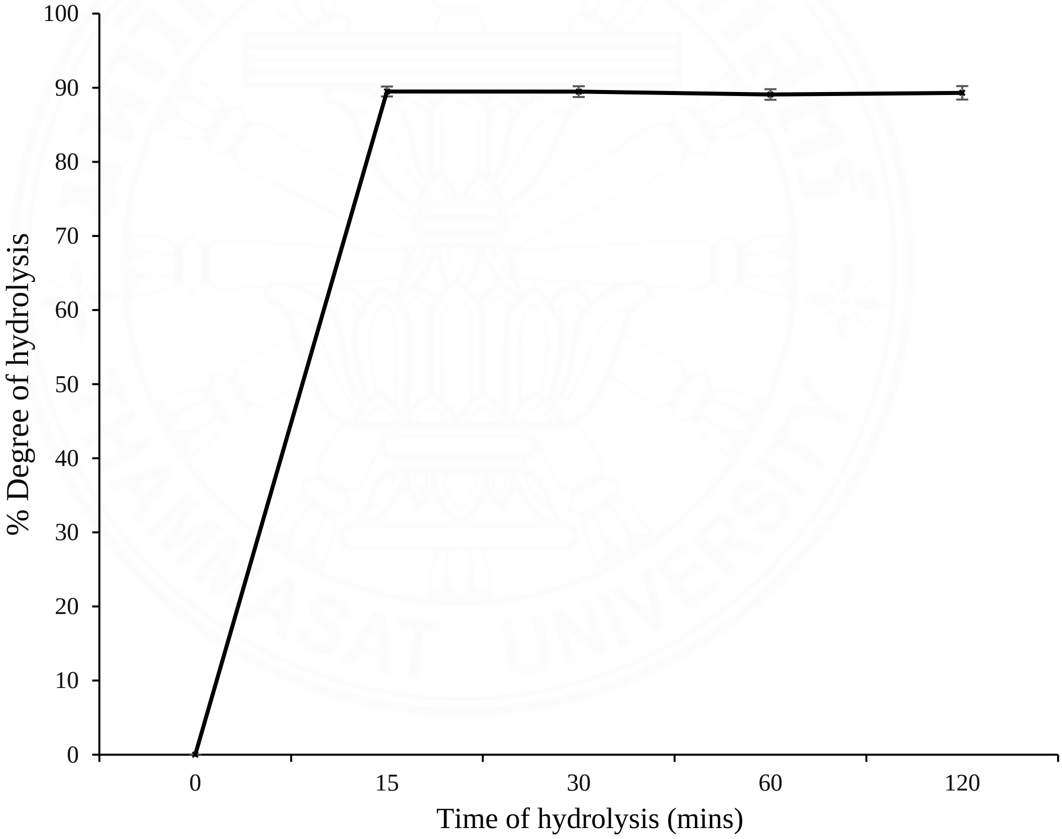


Figure 4.2 Degree of hydrolysis of palm kernel meal protein after 0, 15, 30, 60, and 120 minutes hydrolysis with alcalase at 55 °C

4.5 *In vitro* antioxidant activity of PKMP and PKMPH

Several studies have reported that various plant protein hydrolysate extracts showed biological activity including antimicrobial, anti-inflammatory, anti-hypertensive, and antioxidant activity. The antioxidant capacity of palm kernel meal protein and their hydrolysate was evaluated using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS•+) antiradical scavenging activity.

4.5.1 DPPH radical scavenging activity

DPPH assay is one of commonly used methods to determine the antioxidant activity by using a free DPPH radical (2,2-diphenyl-1-picrylhydrazyl). When the DPPH radical interacts with methanol-soluble antioxidants, it will fade from purple to pale yellow or colorless, which is reduced by the antioxidant compound.[162, 163] The anti-oxidative activity by DPPH assay was reported as IC_{50}. The IC_{50} value is a commonly used criterion for evaluating the antiradical scavenging properties of samples. It is measured as the concentration of antioxidants required to reduce the initial DPPH concentration by 50%. Accordingly, the lower IC_{50} value indicated the higher antioxidant properties. In addition, L-glutathione was used as a control in this experiment. As shown in **Figure 4.3**, PKMP and PKMPH at various time point (0, 15, 30, 60, and 120 minutes) exhibited IC_{50} value ranging from 5.73±0.23 to 7.43±0.22 μg/mL which was lower than that of L-glutathione (IC_{50} value of 28.25±0.97 μg/mL) indicating higher DPPH radical scavenging activity ($p<0.05$). By comparing IC_{50} values of PKMPH after hydrolysis, it was found that PKMPH after 60 minutes hydrolysis showed the highest antioxidant activity as shown by its lowest IC_{50} value (5.73±0.23 μg/mL) when compared to control group ($p<0.05$). It was observed that whenever the concentration of PKMPH was increased, the antioxidant capacity was increased as well.

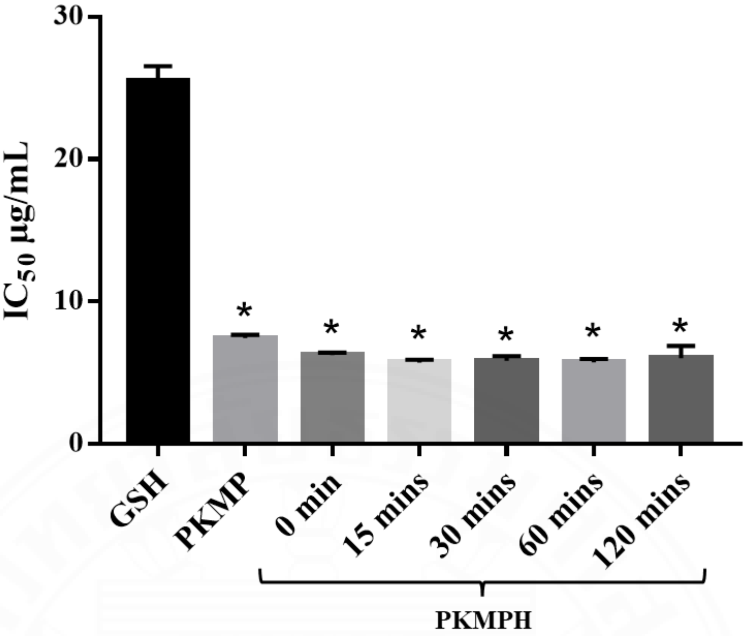


Figure 4.3 IC_{50} value of palm kernel meal protein and their hydrolysates at various time determined by DPPH free radical scavenging activity. L-glutathione (GSH) was used as a positive control. The values are presented as the mean ± stand deviation (SD). Asterisks (*) indicate statistically significant differences when compared to the positive control group at $p<0.05$ using one way ANOVA and multiple comparison tests.

4.5.2 ABTS radical scavenging activity

Besides DPPH radical scavenging activity method, ABTS radical scavenging assay was also applied to measure the antioxidation capability of compounds. The sample was tested by mixing the sample solution with ABTS•+ solution and measuring the absorbance at 734 nm. The antioxidant activity of the samples was determined by calculating the fading color of the ABTS•+ radical inhibition.[164] The antioxidant activity of PKMP and PKMPH at 0, 15, 30, 60, and 120 minutes was compared with that of L-glutathione standard solution in terms of IC_{50} value. Palm kernel meal protein and their protein hydrolysates expressed antioxidant activity against ABTS radicals. The results demonstrated that PKMP and its hydrolysate with alcalase enzyme for 0, 15, 30, 60, and 120 minutes showed IC_{50} value ranging from 7.84±0.89 to 14.96±0.58 µg/mL which was lower than that of L-glutathione (IC_{50} value of 14.03±0.58 µg/mL) with $p<0.05$ (**Figure 4.4**). Moreover, The PKMPH after 60 minutes hydrolysis had the highest antioxidant efficiency when compared to their companies. The other hydrolysis time also showed various DPPH radical-scavenging activity in the order of 120 mins, 30 mins, and 15 mins (IC_{50} value of 7.93±0.87, 10.58±0.06, and 12.31±0.53 µg/mL, respectively).

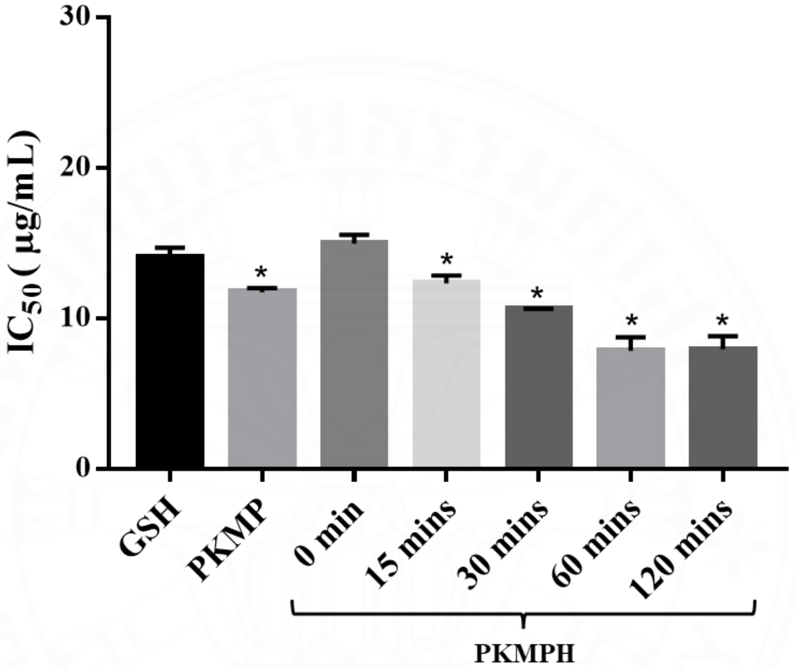


Figure 4.4 IC_{50} value of palm kernel meal protein and their hydrolysates at various time determined by ABTS free radical scavenging activity. L-glutathione (GSH) was used as a positive control. The values are presented as the mean ± stand deviation (SD). Asterisks (*) indicate statistically significant differences when compared to the positive control group at $p<0.05$ using one way ANOVA and multiple comparison tests.

4.5.3 The correlation between *in vitro* antioxidant activity of PKMPH and degree of hydrolysis

In this experiment, the radical scavenging activity (%) and degree of hydrolysis of palm kernel meal protein were compared (%). The comparative results revealed that palm kernel meal protein hydrolysate had the highest antioxidant properties at PKMPH degradation levels at 60 minutes, followed by a slight decrease in antioxidant properties at 120 minutes. The results showed that when the degree of hydrolysis of palm kernel meal protein hydrolysate was increased, the antioxidant efficiency of the sample was increased (**Figure 4.5** and **4.6**).

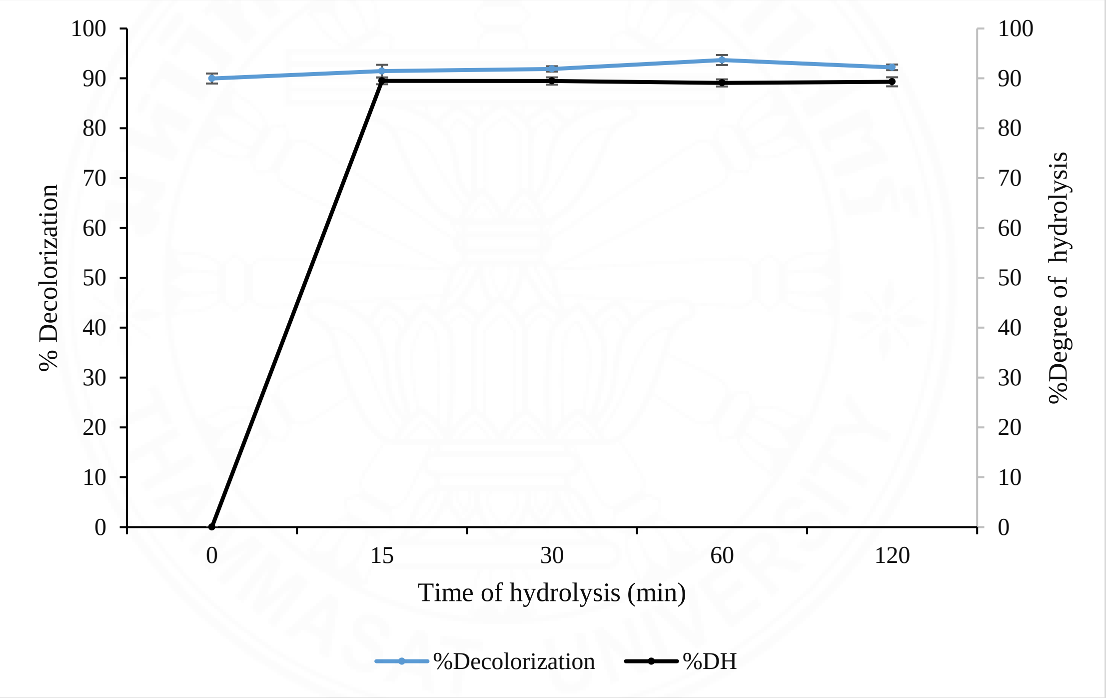


Figure 4.5 The antioxidant activity of palm kernel meal protein hydrolysate on diphenyl-1picrylhydrazyl radical (%decolorization) compared with degree of hydrolysis (%). All data are presented as the mean ± SD (n = 3).

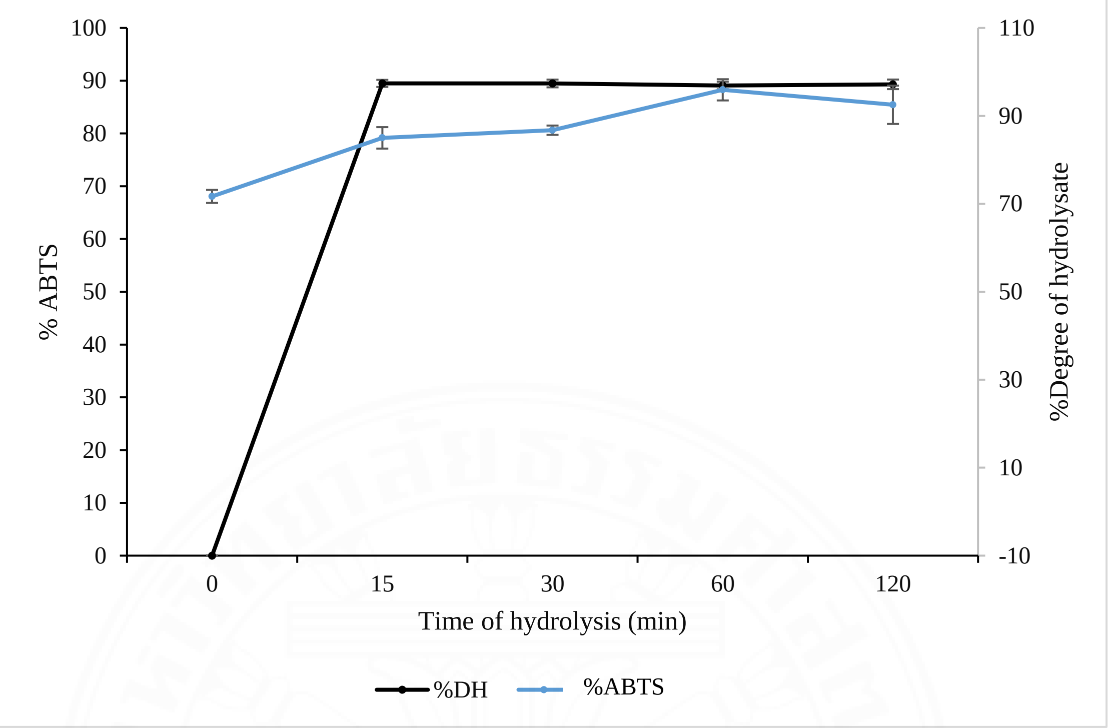


Figure 4.6 The antioxidant activity of palm kernel meal protein hydrolysate on 2,2-azino-bis (3-ethylbenzothia zoline-6-sul-fonic acid) diammonium salt radical (%ABTS) compared with degree of hydrolysis (%). All data are presented as the mean ± SD (n = 3).

4.6 Cytotoxicity determination of PKMP and PKMPH

The MTT assay is an enzyme-based method used to determine the number of viable cells, cell proliferation, and also toxicity of the cells.[165] The cytotoxicity of protein and protein hydrolysate from palm kernel meal against the L929 cells was performed by MTT assay. In this experiment, L929 mouse fibroblast cells were treated with PKMP and PKMPH at a concentration of 500-0.97 µg/mL for 24 hours. L-glutathione is an antioxidant compound produced by human body. It is a protein protecting cells and tissues from being destroyed by free radicals that accumulate in various parts of the body.[166] Thus, L-glutathione was used as a positive control. The cell survival rate was presented as % cell viability. The results in **Figure 4.7** demonstrated the cell survival of L929 cells after exposure to PKMP and PKMPH at various concentrations. It was found that PKMP and PKMPH at concentration more than or equal to 250 µg/mL resulted in reduction of cell survival (%) compared to that of control group. In addition, % cell viability at the concentration of 500 µg/mL of PKMP and PKMPH were 74.42 ± 2.08 and 48.75 ± 2.06 µg/mL, respectively. Moreover, when compared at the same concentration, PKMPH (500 µg/mL) resulted in a significant reduction of cell viability (%) compared to GSH (at concentration of 500 µg/mL). Results indicated that PKMP and PKMPH at high concentration showed toxicity to the cell. The cell viability when incubated with PKMP and PKMPH at concentration less than or equal to 125 µg/mL was high (up to 91%). It can be suggested that PKMP and PKMPH at the concentrations less than or equal to 125 µg/mL had no toxicity to L929 cells.

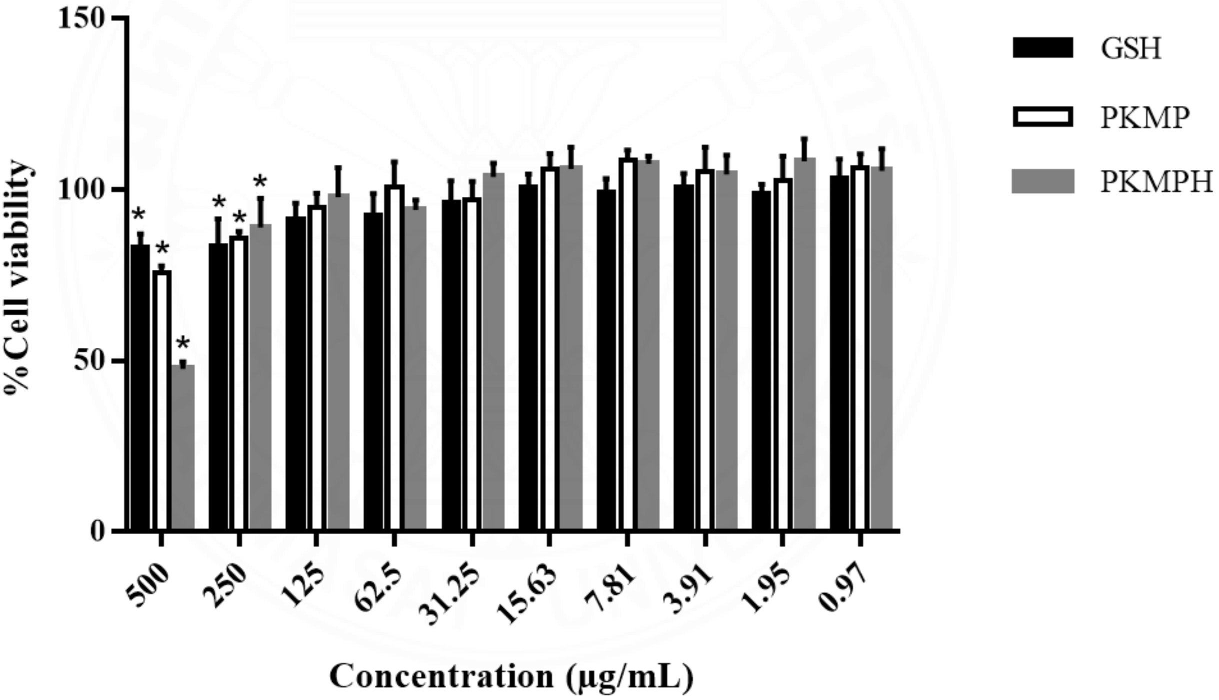


Figure 4.7 Cytotoxicity of PKMP and PKMPH on L929 mouse fibroblast cells viability. L-glutathione (GSH) was used as a positive control. The % cell viability are presented as the mean ± SD. Asterisks (*) indicate statistically significant differences when compared to the negative control group at p<0.05 using one way ANOVA and multiple comparison tests.

4.7 Cytoprotective effect of PKMPH on oxidative damaged L929 mouse fibroblast cells.

Hydrogen peroxide (H_2O_2) was first identified as a reactive oxygen species, capable of contributing damage to a wide range of the cellular target. Thus, H_2O_2 was used to establish an oxidative stress-induced cell damage model.[167] The toxicity of H_2O_2 was performed by incubating L929 mouse fibroblast cell line with various concentration of H_2O_2 (0 - 1000 µg/mL) for 3 h. Thereafter, the cell survival rate was examined with MTT assay and the obtained data was calculated as % cell viability. Results of cell survival rate after exposure to H_2O_2 were demonstrated in **Figure 4.8**. The cytotoxicity of H_2O_2 was found to be dose-dependent manner ($p<0.05$). To observe the cytoprotective effect of PKMPH, 1000 µg/mL H_2O_2 treated for 3 h was selected.

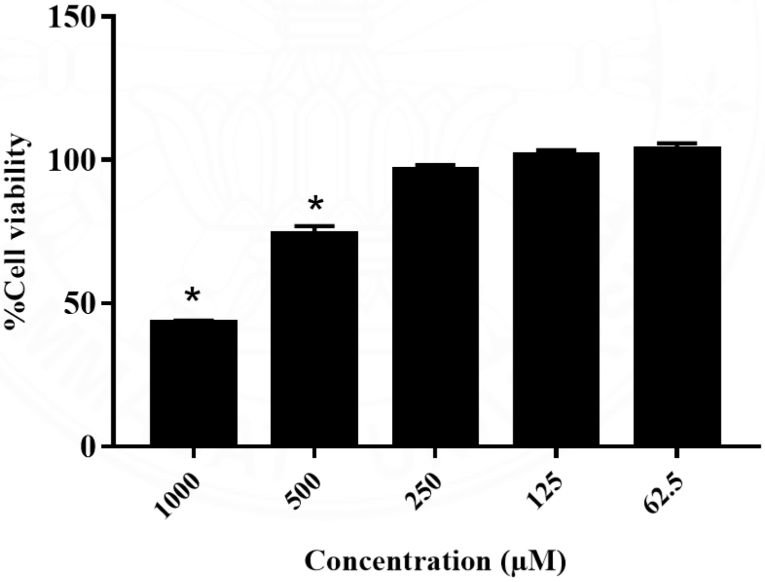


Figure 4.8 %Cell viability of L929 mouse fibroblast cells after treated with various concentration of H_2O_2 for 3 hours. The %cell viability are presented as the mean ± SD. Asterisks (*) indicate statistically significant differences when compared to the negative control group at $p<0.05$ using one way ANOVA and multiple comparison tests.

This study examined the ability of PKMPH to protect cells from hydrogen peroxide (H_2O_2). The L929 mouse fibroblast cell lines were treated with PKMPH at various concentrations, incubated for 24 hour and followed by 1,000 µM H_2O_2 for 3 hours. **Figure 4.9** demonstrated the ability of PKMPH to protect cells from H_2O_2-induced oxidative stress. PKMPH at concentration of 500-0.97 µg/mL can promote cell survival with an increase in % cell viability when compared to that of H_2O_2 treated group ($p<0.05$). PKMPH at concentration of 1.95-0.97 µg/mL showed a slightly decrease in % cell viability when compared to PKMPH at concentration of 31.25-3.91 µg/mL. At very high concentration of PKMPH (250-500 µg/mL), a relatively small percentage of cell survival was observed. It was found that cell survival (%) were 65.12±5.45 and 56.16±4.5, respectively. Accordingly, PKMPH at concentration of 500-0.97 µg/mL is capable in protecting cells from oxidative damage by H_2O_2.

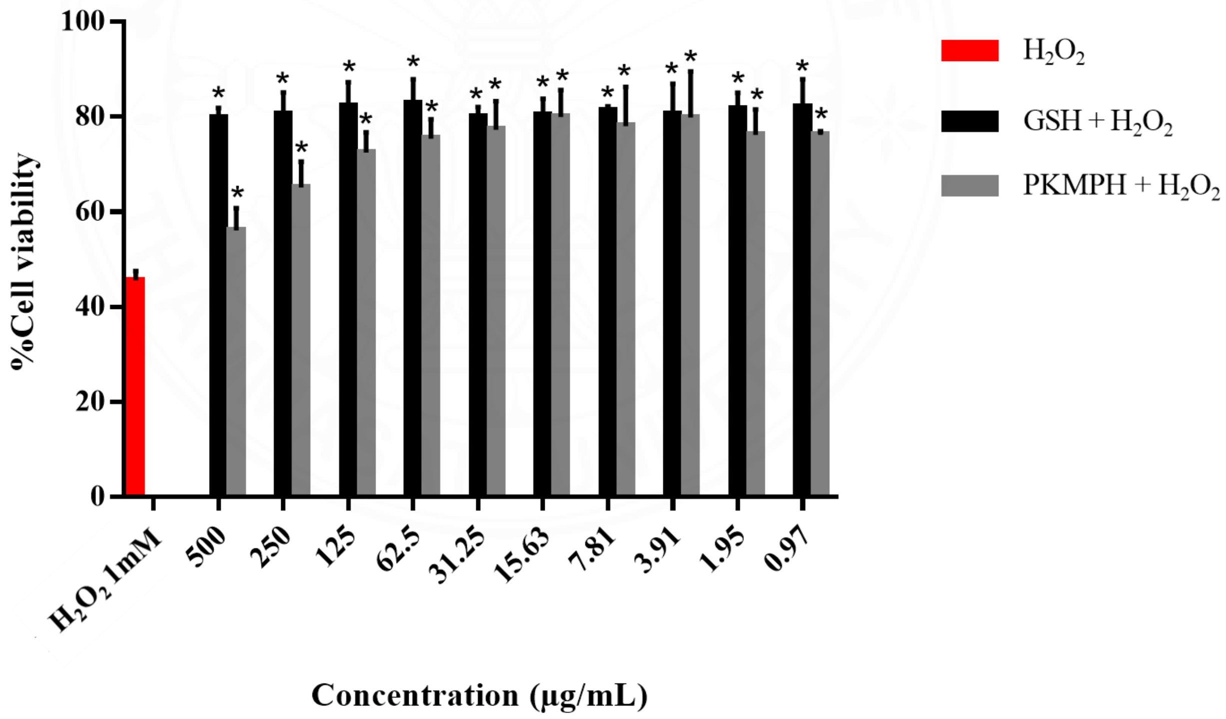


Figure 4.9 % Cell viability of L929 mouse fibroblast cells after treated with PKMPH for 24 h, followed by a 1,000 µM H_2O_2 for 3 h. L-glutathione (GSH) was used as a positive control. The % cell viability are presented as the mean ± SD. Asterisks (*) indicate statistically significant differences when compared to H_2O_2-treated group at $p<0.05$ using one way ANOVA and multiple comparison tests.

4.8 Purification of antioxidant protein

4.8.1 Ion exchange chromatography

Ion exchange chromatography is a technique used to separate proteins based on the property of each protein that has a different charge. In the purification of PKMPH, HiTrap DEAE Sepharose FF column and 25 mM Tris HCl buffer pH 8.0 were used. As shown in **Figure 4.10**, two major protein peaks (P1 and P2) demonstrated in the blue line were eluted from the column with NaCl. All fractions were tested for antioxidant activity again before further identification of peptide sequences by LC MS/MS mass spectrometry.

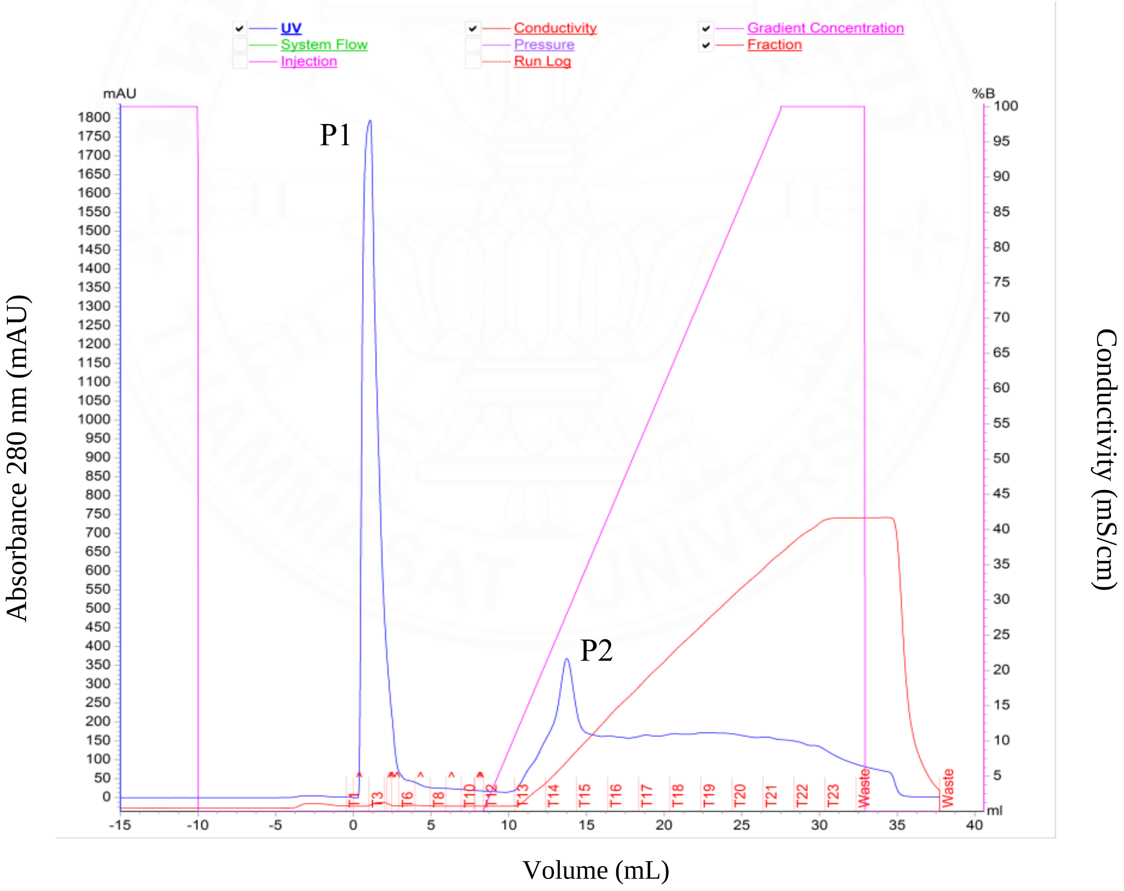


Figure 4.10 Fast protein liquid chromatography (FPLC) chromatogram of PKMPH after loading in HiTrap DEAE Sepharose FF column and elution with NaCl at flow rate of 1 mL/min.

4.8.2 Antioxidant activity of PKMPH after purification

The antioxidant activity of PKMPH fraction number 1 to 23 determined by DPPH and ABTS radical scavenging assay was shown in **Figures 4.11** and **4.12**. PKMPH fraction numbers 13, 14, and 15 tend to have relatively high antioxidant activity which was consistent with peak P2. When comparing the antioxidant activity by two antioxidant radical scavenging assays in order to select only one candidate fraction to continue identifying sequences. It was found that fraction number 15 is the most promising fraction-containing high antioxidant activity. Therefore, PKMPH fraction number 15 was selected for further identifying sequences.

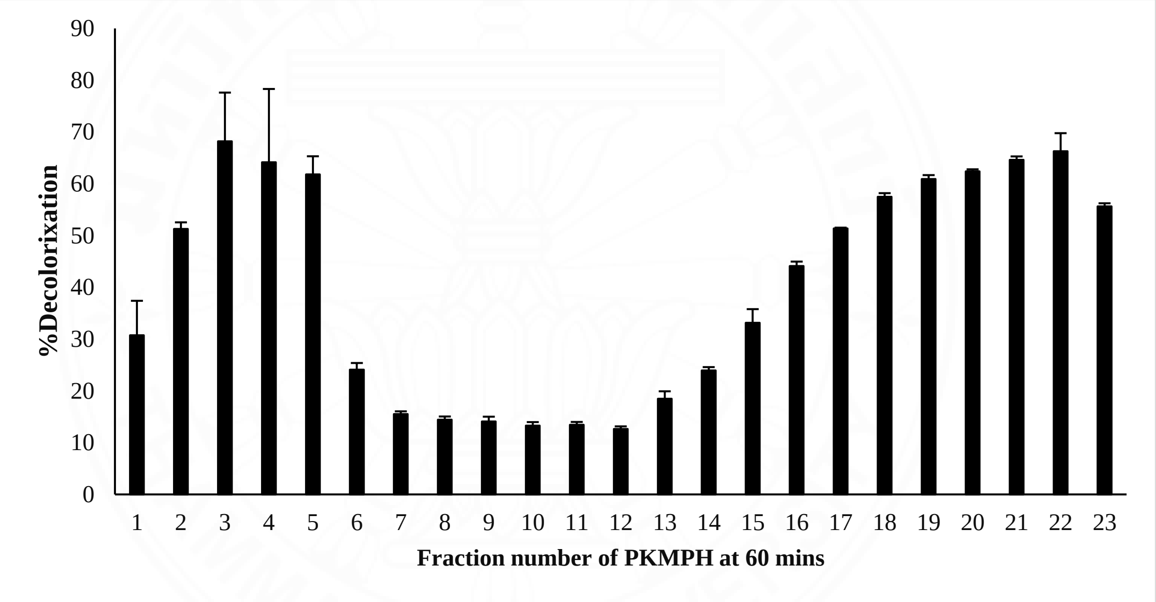


Figure 4.11 The antioxidant activity of palm kernel meal protein hydrolysate on diphenyl-1picrylhydrazyl radical (% decolorization). All data are presented as the mean ± SD (n = 3).

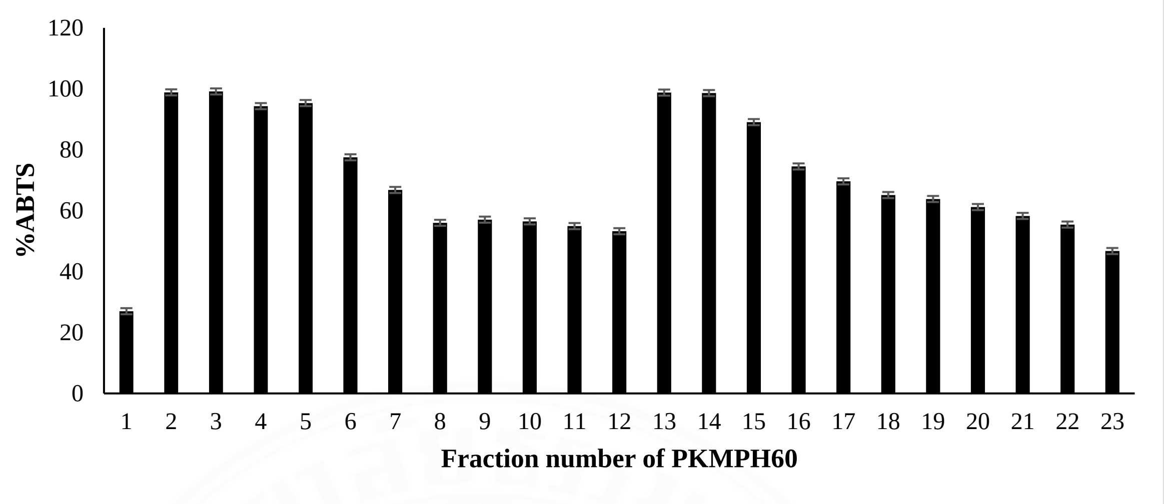


Figure 4.12 The antioxidant activity of palm kernel meal protein hydrolysate on 2,2-azino-bis (3-ethylbenzothia zoline-6-sul-fonic acid) diammonium salt radical (%ABTS). All data are presented as the mean ± SD (n = 3).

4.9 Identification of peptide sequences by LC MS/MS tandem mass spectrometry

The trypsin-digested peptide samples were injected into the LC-MS/MS machine for amino acid sequence separation and analysis. The obtained data will be analyzed by bioinformatics technical tools. The tandem mass spectra were analyzed with a database search engine such as PEAKs to determine the amino acid sequence of the peptide. **Table 4.2** presented nine sequences and molecular weights of peptide from fraction number 15 obtained from purification of PKMPH with HiTrap DEAE Sepharose FF column. All peptide sequences were released from the group of proteins such as globulin, vicilin-like antimicrobial peptides, cocosin, and carboxypeptidase. In addition, they are made up of 9 to 10 free amino acid residues. Furthermore, the molecular weight ranges from 1084 to 1291 Da.

Table 4.2 Peptide sequences of palm kernel meal hydrolysate fraction number 15 by LC MS/MS mass spectrometry

Peptide sequences	Peptide -10lgP	Length	Molecular weight (Da)	Protein family
VDEVLNAPREE	51.63	11	1270	7S globulin, vicilin-like antimicrobial peptides
FFDEESFLH	51.01	9	1169	7S globulin, vicilin-like antimicrobial peptides 2-2
AGITDYFDED	35.79	10	1144	cocosin 1-like
EADRTDYPE	33.38	9	1094	63 kDa globulin-like protein
ISDETIDAIH	32.61	10	1112	serine carboxypeptidase II-3, Carboxypeptidase,
LRPPSEEEE	32.41	9	1084	cocosin 1, cocosin 1 isoform X1 and X2
VLRPPSEEEE	30.14	10	1183	cocosin 1, cocosin 1 isoform X1 and X2
READSDDYPE	27.29	10	1195	7S globulin, vicilin-like antimicrobial peptides
SFDQPAREVDE	25.23	11	1291	63 kDa globulin-like protein

CHAPTER 5
DISCUSSION

Free radicals are substances that have lone pair electrons within an atom or molecule. If the body contains a high number of free radicals, it may cause several serious problems. In particular, reactive oxygen species (ROS) is one of the free radical derivatives that damage cells and lead to human dysfunction. Since it is a waste product caused by metabolism in the body and also unstable and reacts rapidly with biomolecules such as proteins, lipid, DNA and RNA, leading to tissue damage. Several diseases are caused by free radicals, such as aging and cancer, ischemic heart disease, Alzheimer's disease, allergic disease, high blood pressure eye disorder and nervous system.[10, 33, 168, 169] Therefore, the elimination of ROS and free radicals is the most significant mechanism of living organisms. It must be eliminated to maintain this balance in order to inhibit or prevent the occurrence of diseases as mentioned above. Interestingly, the bioactive peptide in plants have been found to affect biological activity within living cells, such as the transport of nutrients and minerals, cell communication, immunity against invaders such as fungi, viruses, and bacteria. In addition, it also helps to protect cells from free radical damage in the body.[15, 170-172]

Plant protein hydrolysate plays a crucial role in various applications. The bioactive compounds of plant protein hydrolysate also play an important role in human health. They could regulate the functions of various systems within the body and help to reduce the risk of developing certain diseases, including the cardiovascular system, diabetes, obesity, hypertension, low-grade systemic inflammation, and Alzheimer's disease.[173] There were report that egg white protein hydrolysate-derived peptides could inhibit α-glucosidase activity in the intestinal that involves in the reduction of blood glucose concentration.[174] Soy protein hydrolysate derived-peptides can also control body weight by modulating glucose metabolism modulate glucose homeostasis in type 2 diabetic.[175]

In this study, palm kernel meal protein (PKMP) was extracted by alkali extraction method and the protein concentration was measured by Coomassie dye-binding (Bradford assay). The hydrolysate process is the digestion of proteins to be smaller proteins or peptides. In this study, alcalase enzyme was used to digest the peptide chain by random digestion to obtain the palm kernel meal protein hydrolysate (PKMPH). The results revealed that when time of hydrolysis was increased, the concentration of PKMPH was also decreased. The decreasing of PKMPH concentration indicated the short chain peptides produced by enzymatic digestion and they were normally too small to be determined by Bradford assay. The Bradford assay's general principle is the reaction of Coomassie brillant blue G-250 with proteins will produce blue color. It was used to measure the absorbance and eventually calculated as the protein concentration.[176] The protein concentration of PKMPH indicated that the PKMP was digested to small peptides that Coomassie brillant blue G-250 is unable to bind. Furthermore, when the time of hydrolysis was increased, % degree of hydrolysis was also reached 89%. Whenever the hydrolysis was complete, the hydrolysis remained constant because it reached to the plateau and it was correlated to the study of Zarei et al., 2012.[135]

The molecular weight pattern of PKMP and their hydrolysate (PHKMPH) was evaluated by SDS-PAGE. Unfortunately, the results showed that protein bands were not clearly separated within the range of 20 to 100 kDa which was similar to You et al., 2013. The proteins might be denatured during the manufacturing process which using a very high temperature for oil extraction. Moreover, the solvent used to extract protein might affect the proteins. According to You et al., 2013 , they found that the degradation of silk fibroin was involved with different ionic liquid solvents.[177]

An antioxidant compound is a substance that can inhibit or slow down the oxidation reaction, which is the cause of free radicals such as lipid oxidation reaction.[178] These antioxidant active compounds may naturally be found in various sources, such as phytochemicals in plants, animals, microorganisms, and dietary.[11] Numerous studied showed that the bioactive peptide or protein hydrolysate from several sources such as rice, mung bean, soy bean, chick pea, cheese whey, cotton seed, rice band, and chicken breast exhibited antioxidant activity.[179, 180-182] In this research, protein hydrolysate was obtained from enzymatic hydrolysis of palm kernel meal (*Elaeis guineensis*). It was hydrolyzed with 2% (w/w) alcalase enzyme. The antioxidant activity based on DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) methods was determined and compared to that of L-glutathione standard. The results showed that the DPPH and ABTS free radical scavenging of palm kernel meal protein hydrolysate (PKMPH) has high antioxidant properties with IC_{50} of 5.73 ± 0.23 and 7.84 ± 0.89 µg/mL, respectively. Zielińska et al., 2017 found that the highest antioxidation activity against DPPH• (2,2-diphenyl-1-picrylhydrazyl radical) and ABTS•+(2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) from baked cricket *Gryllodes sigillatus* hydrolysate revealed IC_{50} value of 10.9 and 5.3 µg/mL, respectively.[183] Fish muscle-papain hydrolyze protein presented the highest antiradical scavenging activity with IC_{50} value of 2.08-3.18 mg/mL and 0.12-0.60 mg/mL on DPPH and ABTS methods, respectively.[184] Egg white protein hydrolyzed by neutrase enzyme exhibited high ABTS radical scavenging activity with IC_{50} value of 3.6 mg/mL. Moreover, there were several reports on the antioxidant activity of plant hydrolyzed-protein. Chen et al., 2021 reported rice derived protein hydrolysates exhibited dose-dependent antioxidation activity determined by DPPH and ABTS assay with IC_{50} value of 42.58 ± 2.1 mg/g and 2.11 ± 0.88 mg/g, respectively. The DPPH and ABTS radical-scavenging activity of *Cardamine violifolia* protein hydrolysate demonstrated the antiradical activity with IC_{50} of 0.58 mg/mL and 0.193 mg/mL, respectively.[185] Three kernel cake peptide sequences, AWFS, WAF, and LPWRPATNVF exhibited high antioxidation activity of 71%, 56%, and 50%, respectively. The highest IC_{50} value of peptide measured by DPPH radical scavenging assay was obtained from peptide sequence GIFE, GVQEGAGHYALL, and GGIF at 0.02 µM, 0.09 µM and 0.35 µM, respectively,[152] In addition, Zarei et al., 2012 revealed that palm kernel cake produced by papain enzyme had the highest DPPH• radical scavenging activity (73.5 ± 0.25%).[135] Furthermore, previous research revealed that palm kernel cake protein hydrolysate fraction 1 had the highest antioxidant activity, with moderate amount of hydrophobic (23.60%) and containing Asp, Glu, His, Lys, Met and Tyr, which showed antioxidant activity. Our study showed different results to those previous studies and it might be because of the difference in extraction process or material source.

The antioxidant capacity of proteins depends on the amino acids presenting in the protein. The amino acids with high antioxidant capacity mostly have sulfur-containing side chains (nucleophilic sulfur-containing side chains), such as cysteine and methionine or contain hydrogen atoms that can be easily removed. In addition, the imidazole groups of histidine also exhibit antioxidant properties.[186, 187, 188] In this study, the obtained amino acid sequences of PKMPH fractions number15 were identified as VDEVLNAPREE (P1), FFDEESFLH (P2), AGITDYFDED (P3), EADRTDYPE (P4), ISDETIDAIH (P5), LRPPSEEEE (P6), VLRPPSEEEE (P7), READSDDYPE (P8), and SFDQPAREVDE (P9). All peptide sequences contain hydrophobic amino acids including Ala(A), Val(V), Ile(I), and Leu and negatively charged amino acids such as Glu(E) and Asp(D). The increasing of hydrophobicity will result in the solubility in lipid and involve with antioxidative activity.[189] Our results were in agreement with previous study by Zarei et al., 2014. They reported that palm kernel cake protein antioxidant peptides WAF, YLLLK, WAFS and AWFS exhibited the highest percentage of hydrophobic amino acid residues with 100%, 80%, 75% and 75%, respectively.[152] Other study also reported that chickpea protein hydrolysate was composed of antioxidative peptide and they are rich in Leu and Ala in their sequences.[133] In this study, most of antioxidant peptides from PKMPH composed of negatively charged and hydrophobic amino acids. These amino acid groups have the ability to donate proton or electron to free radical molecules due to the presence of excess electrons, exhibit free radical quenching activity. Hence, the molecule returned to its stable state, with no more free radicals. This result was consistent with the reports of Liu et al., 2004. They reported that aspartic acid and glutamic acid monohydroxamates played important roles in the antioxidative activity, which protected against peroxynitrite-mediated dihydrorhodamine 123 oxidations and hydroxyl radical-mediated DNA damage.[190]

Several studies suggested that high degrees of hydrolysis have a positive correlation with the DPPH and ABTS antioxidation capacity. In this experiment, the antioxidant activity of PKMPH revealed the highest antioxidant activity on DPPH and ABTS scavenging assay when the time of hydrolysis reached 60 minutes with approximately 89% digestibility. The antioxidant activity measured by β-carotene bleaching, reducing power, and 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging activity assay of chickpea protein isolates hydrolyzed by alcalase enzyme for 1 h followed by flavourzyme enzymatic hydrolysis for 2 hours was higher than that of protein before digestion and it showed around 70% digestibility.[191] The enzymatic hydrolysis such as alcalase, pepsin and chymotrypsin were increased in degree of hydrolysis with high antioxidant activity at the first step. After 10 hours, they found that the antiradical scavenging activity was significantly decreased.[153] The antioxidant capacity of proteins is related to the presence of a tertiary structure of the protein. Therefore, the enzymatic degradation of proteins is a change in the tertiary structure resulting in the release of the amino acid side chains with antioxidant properties.[192]

One of the factors that contribute to the development of various diseases in the human body is the accumulation of ROS within the body. It was discovered that free radicals were produced and accumulated significantly more in the tissues of the elderly. Cells and tissues are harmed as a result of this occurrence.[193] In order to assess the potential protective effect of PKMPH, the antioxidant activity and cytoprotective effect of PKMPH was determined in L929 mouse fibroblast cells. The PKMPH could significantly protect L929 cells from H_2O_2-induced oxidative damage, particularly at concentration ranging from 500-0.97 μg/mL μg/mL with a relatively high survival rate of 56-76% when compare to control group ($p<0.05$). It could be concluded that, despite being exposed to high concentrations of antioxidant-rich samples, L929 cells' metabolic efficiency did not deteriorate. Because the accumulation of oxidative substances such as ROS has been linked to cell death or cell damage and they are directly related to mitochondrial dehydrogenase activity. When this enzyme is disrupted by these harmful free radicals, oxidative stress occurs. The cells will be harmed until they die as a result of it. It can be used to calculate cell survival as a measure of cell protection.[194] At the concentration of 500 μg/mL, PKMPH showed low percentage of cell survival when compare to control group ($p<0.05$). This was due to the toxicity of PKMPH at 500 μg/mL on L929 cells. There were several studies reported the cytoprotective capability of protein hydrolysate from various sources. Hazelnut protein hydrolysate can inhibit ROS accumulation in HUVECs cells, implying that they have a strong protective effect on cells.[149] Zhang et al., 2019 also demonstrated that soybean protein hydrolysate hydrolyzed by alcalase enzyme had a strong anti-oxidative capacity in human intestinal Caco-2 cells.[195] Our results, however, revealed that at low concentration (0.97-1.95 μg/mL) of PKMPH, there was no effect on cell viability when compare to control group. These findings were inconsistent with the findings of Hu et al., 2020, which discovered that *Lophius litulon* protein hydrolysate had a lower survival rate at lower sample concentrations.[151]

CHAPTER 6
CONCLUSION

Palm kernel meal protein hydrolysate is produced by degrading palm kernel meal proteins into amino acids and peptides, with biological activity such as antioxidant activity. The preparation of protein hydrolysate from palm kernel meal to obtain amino acids with these properties is dependent on several factors such as the source of protein precursors for protein hydrolysate preparation, as well as the type and activity of the enzymes used for the digestion, and the degree of hydrolysate, etc. Based on these factors, there is a desire to investigate the preparation of protein hydrolysate from palm kernel meal in order to add value to locally available raw materials. The aim of this study was to prepare the palm kernel meal protein hydrolysate (PKMPH) and evaluate its antioxidant and cytoprotective activity. The protein was extracted from palm kernel meal and protein hydrolysate was generated by alcalase enzyme. The results showed that protein hydrolysate with a hydrolysis time of 60 minutes showed the highest antioxidant activity, with IC_{50} values of 5.73±0.23 and 7.84±0.89 µg/mL as determined by DPPH and ABTS assay, respectively. The PKMP and PKMPH at low concentration (less than or equal to 125 µg/mL) were not toxic to the L929 cells, while at high concentration (500 µg/mL), they showed moderate toxicity to L929 cells. At concentration ranging from 500-0.97 µg/mL, PKMPH could protect L929 cells from H_2O_2-induced damage. However, low survival rate of L929 was observed at high concentration of PKMPM (250-500 µg/mL). The antioxidant peptides such as VDEVLNAPREE, FFDEESFLH, AGITDYFDED, EADRTDYPE, ISDETIDAIH, LRPPSEEEE, VLRPPPSEEEE, READSDDYPE, and SFDQPAREVDE from PKMPH were identified. This study revealed the significance and antioxidant activity of palm kernel meal protein and palm kernel meal protein hydrolysate. They might be further studied and developed as a natural antioxidant source or animal feed supplement. Furthermore, it might be observed in the future for other bioactivities such as antibacterial testing for use in animal products.

REFERENCES

1. Ray PD, Huang B-W, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal. 2012;24(5):981-90.
2. Sugamura K, Keaney JF. Reactive oxygen species in cardiovascular disease. Free Radical Biology and Medicine. 2011;51(5):978-92.
3. Wu D, Cederbaum AI. Alcohol, oxidative stress, and free radical damage. Alcohol Res Health. 2003;27(4):277-84.
4. Chatterjee S. Chapter Two - Oxidative Stress, Inflammation, and Disease. In: Dziubla T, Butterfield DA, editors. Oxidative Stress and Biomaterials: Academic Press; 2016. p. 35-58.
5. Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. Journal of Botany. 2012;2012:217037.
6. Jenner P. Oxidative stress in Parkinson's disease. Annals of Neurology. 2003;53(S3):S26-S38.
7. Madamanchi Nageswara R, Vendrov A, Runge Marschall S. Oxidative Stress and Vascular Disease. Arteriosclerosis, Thrombosis, and Vascular Biology. 2005;25(1):29-38.
8. Golden TR, Hinerfeld DA, Melov S. Oxidative stress and aging: beyond correlation. Aging Cell. 2002;1(2):117-23.
9. Nunomura A, Castellani RJ, Zhu X, Moreira PI, Perry G, Smith MA. Involvement of Oxidative Stress in Alzheimer Disease. Journal of Neuropathology & Experimental Neurology. 2006;65(7):631-41.
10. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev. 2010;4(8):118-26.
11. Lourenço SC, Moldão-Martins M, Alves VD. Antioxidants of Natural Plant Origins: From Sources to Food Industry Applications. Molecules. 2019;24(22):4132-1.

12. Lourenço SC, Moldão-Martins M, Alves VD. Antioxidants of Natural Plant Origins: From Sources to Food Industry Applications. Molecules. 2019;24(22):4132-2.
13. Xie Z, Huang J, Xu X, Jin Z. Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. Food Chemistry. 2008;111(2):370-6.
14. Park SY, Lee J-S, Baek H-H, Lee HG. PURIFICATION AND CHARACTERIZATION OF ANTIOXIDANT PEPTIDES FROM SOY PROTEIN HYDROLYSATE. Journal of Food Biochemistry. 2010;34(s1):120-32.
15. Habinshuti I, Chen X, Yu J, Mukeshimana O, Duhoranimana E, Karangwa E, et al. Antimicrobial, antioxidant and sensory properties of Maillard reaction products (MRPs) derived from sunflower, soybean and corn meal hydrolysates. LWT. 2019;101:694-702.
16. Malomo SA, Onuh JO, Girgih AT, Aluko RE. Structural and Antihypertensive Properties of Enzymatic Hemp Seed Protein Hydrolysates. Nutrients. 2015;7(9).
17. Harnedy PA, FitzGerald RJ. In vitro assessment of the cardioprotective, anti-diabetic and antioxidant potential of Palmaria palmata protein hydrolysates. Journal of Applied Phycology. 2013;25(6):1793-803.
18. Marcone S, Haughton K, Simpson PJ, Belton O, Fitzgerald DJ. Milk-derived bioactive peptides inhibit human endothelial-monocyte interactions via PPAR-γ dependent regulation of NF-κB. Journal of Inflammation. 2015;12(1):1.
19. Tonolo F, Sandre M, Ferro S, Folda A, Scalcon V, Scutari G, et al. Milk-derived bioactive peptides protect against oxidative stress in a Caco-2 cell model. Food & Function. 2018;9(2):1245-53.
20. Betteridge DJ. What is oxidative stress? Metabolism. 2000;49(2 Suppl 1):3-8.
21. Rahman T, Hosen I, Islam M, Shekhar H. Oxidative stress and human health. Advances in Bioscience and Biotechnology. 2012;03:997-1019.
22. Senoner T, Dichtl W. Oxidative Stress in Cardiovascular Diseases: Still a Therapeutic Target? Nutrients. 2019;11(9):2090.

23. Cichoż-Lach H, Michalak A. Oxidative stress as a crucial factor in liver diseases. World J Gastroenterol. 2014;20(25):8082-91.

24. Liguori I, Russo G, Curcio F, Bulli G, Aran L, Della-Morte D, et al. Oxidative stress, aging, and diseases. Clin Interv Aging. 2018;13:757-72.

25. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010;107(9):1058-70.

26. Saha SK, Lee SB, Won J, Choi HY, Kim K, Yang G-M, et al. Correlation between Oxidative Stress, Nutrition, and Cancer Initiation. International journal of molecular sciences. 2017;18(7):1544.

27. Gella A, Durany N. Oxidative stress in Alzheimer disease. Cell Adh Migr. 2009;3(1):88-93.

28. Girotti AW. Mechanisms of lipid peroxidation. Journal of Free Radicals in Biology & Medicine. 1985;1(2):87-95.

29. Balasubramanian B, Pogozelski WK, Tullius TD. DNA strand breaking by the hydroxyl radical is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone. Proceedings of the National Academy of Sciences. 1998;95(17):9738-43.

30. Mukherji S, Singh SP. Reaction mechanism in organic chemistry: Macmillan; 1984.

31. Nafiu MO, Salawu MO, Kazeem MI. 21 - Antioxidant Activity of African Medicinal Plants. In: Kuete V, editor. Medicinal Plant Research in Africa. Oxford: Elsevier; 2013. p. 787-803.

32. Cornelli U. Antioxidant use in nutraceuticals. Clin Dermatol. 2009;27(2):175-94.

33. Cheeseman KH, Slater TF. An introduction to free radical biochemistry. British medical bulletin. 1993;49(3):481-93.

34. Zhang J, Wang X, Vikash V, Ye Q, Wu D, Liu Y, et al. ROS and ROS-Mediated Cellular Signaling. Oxidative medicine and cellular longevity. 2016;1245-53.

35. Phaniendra A, Jestadi DB, Periyasamy L. Free radicals: properties, sources, targets, and their implication in various diseases. Indian J Clin Biochem. 2015;30(1):11-26.

36. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. Int J Biomed Sci. 2008;4(2):89-96.
37. Reisz JA, Bansal N, Qian J, Zhao W, Furdui CM. Effects of ionizing radiation on biological molecules--mechanisms of damage and emerging methods of detection. Antioxid Redox Signal. 2014;21(2):260-92.
38. van Leeuwen I, Kelpin F, Kooijman S. A mathematical model that accounts for the effects of caloric restriction on body weight and longevity. Biogerontology. 2002;3:373-81.
39. Hancock JT, Desikan R, Neill SJ. Role of reactive oxygen species in cell signalling pathways. Biochem Soc Trans. 2001;29(Pt 2):345-50.
40. Alhasawi A, Legendre F, Jagadeesan S, Appanna V, Appanna V. Chapter 10 - Biochemical Strategies to Counter Nitrosative Stress: Nanofactories for Value-Added Products. In: Das S, Dash HR, editors. Microbial Diversity in the Genomic Era: Academic Press; 2019. p. 153-69.
41. Nitrosative Stress. Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine. Berlin, Heidelberg: Springer Berlin Heidelberg; 2006.
42. Kaludercic N, Giorgio V. The Dual Function of Reactive Oxygen/Nitrogen Species in Bioenergetics and Cell Death: The Role of ATP Synthase. Oxidative Medicine and Cellular Longevity. 2016;2016:3869610.
43. Alfadda AA, Sallam RM. Reactive Oxygen Species in Health and Disease. Journal of Biomedicine and Biotechnology. 2012;2012:936486.
44. Liu Z, Ren Z, Zhang J, Chuang C-C, Kandaswamy E, Zhou T, et al. Role of ROS and Nutritional Antioxidants in Human Diseases. Frontiers in Physiology. 2018;(477):9.
45. Spooner R, Yilmaz O. The role of reactive-oxygen-species in microbial persistence and inflammation. International journal of molecular sciences. 2011;12(1):334-52.
46. Halliwell B. Antioxidant characterization. Methodology and mechanism. Biochem Pharmacol. 1995;49(10):1341-8.
47. Boots AW, Haenen GRMM, Bast A. Health effects of quercetin: From antioxidant to nutraceutical. European Journal of Pharmacology. 2008;585(2):325-37.

48. Huang D, Ou B, Prior RL. The Chemistry behind Antioxidant Capacity Assays. Journal of Agricultural and Food Chemistry. 2005;53(6):1841-56.
49. Silverstein TP. Oxidation and Reduction: Too Many Definitions? Journal of Chemical Education. 2011;88(3):279-81.
50. Abushita AA, Hebshi EA, Daood HG, Biacs PA. Determination of antioxidant vitamins in tomatoes. Food Chemistry. 1997;60(2):207-12.
51. Ahmad P, Jaleel CA, Salem MA, Nabi G, Sharma S. Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. Critical Reviews in Biotechnology. 2010;30(3):161-75.
52. Nimse SB, Pal D. Free radicals, natural antioxidants, and their reaction mechanisms. RSC Advances. 2015;5(35):27986-8006.
53. Singh S. Nanomaterials Exhibiting Enzyme-Like Properties (Nanozymes): Current Advances and Future Perspectives. Frontiers in Chemistry. 2019;7(46).
54. Tang J, Yin HY, Zhang JL. Chapter One - Luminescent Zinc Complexes as Bioprobes for Imaging Molecular Events in Live Cells. In: Lo KK-W, editor. Inorganic and Organometallic Transition Metal Complexes with Biological Molecules and Living Cells: Academic Press; 2017. p. 1-53.
55. Fridovich I. Superoxide radical and superoxide dismutases. Annu Rev Biochem. 1995;64:97-112.
56. Noor R, Mittal S, Iqbal J. Superoxide dismutase--applications and relevance to human diseases. Med Sci Monit. 2002;8(9):Ra210-5.
57. Van Camp W, Bowler C, Villarroel R, Tsang EW, Van Montagu M, Inzé D. Characterization of iron superoxide dismutase cDNAs from plants obtained by genetic complementation in Escherichia coli. Proceedings of the National Academy of Sciences. 1990;87(24):9903.
58. Li C, Zhou H-M. The Role of Manganese Superoxide Dismutase in Inflammation Defense. Enzyme Research. 2011;2011:387176.
59. Wu Q-S, Ying-Ning Z, Abd-Allah E. Mycorrhizal Association and ROS in Plants. 2014. p. 453-75.

60. Youn HD, Kim EJ, Roe JH, Hah YC, Kang SO. A novel nickel-containing superoxide dismutase from Streptomyces spp. Biochem J. 1996;318 (Pt 3)(Pt 3):889-96.
61. Priya B, Premanandh J, Dhanalakshmi RT, Seethalakshmi T, Uma L, Prabaharan D, et al. Comparative analysis of cyanobacterial superoxide dismutases to discriminate canonical forms. BMC Genomics. 2007;8(1):435.
62. Ryan KC, Guce AI, Johnson OE, Brunold TC, Cabelli DE, Garman SC, et al. Nickel superoxide dismutase: structural and functional roles of His1 and its H-bonding network. Biochemistry. 2015;54(4):1016-27.
63. Nandi A, Yan L-J, Jana CK, Das N. Role of Catalase in Oxidative Stress- and Age-Associated Degenerative Diseases. Oxidative Medicine and Cellular Longevity. 2019;2019:9613090.
64. Lück H. Catalase. In: Bergmeyer H-U, editor. Methods of Enzymatic Analysis: Academic Press; 1965. p. 885-94.
65. Aebi H. Catalase. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis (Second Edition): Academic Press; 1974. p. 673-84.
66. Michaelsen JT, Dehnert S, Giustarini D, Beckmann B, Tsikas D. HPLC analysis of human erythrocytic glutathione forms using OPA and N-acetyl-cysteine ethyl ester: Evidence for nitrite-induced GSH oxidation to GSSG. Journal of Chromatography B. 2009;877(28):3405-17.
67. Brown KM, Arthur JR. Selenium, selenoproteins and human health: a review. Public Health Nutrition. 2001;4(2b):593-9.
68. Somani SM, Husain K. Part IX • Chapter 26 - Influence of exercise-induced oxidative stress on the central nervous system. In: Sen CK, Packer L, Hänninen OOP, editors. Handbook of Oxidants and Antioxidants in Exercise. Amsterdam: Elsevier Science B.V.; 2000. p. 713-51.
69. Weydert CJ, Cullen JJ. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat Protoc. 2010;5(1):51-66.
70. Palb SBNaD. Free radicals, natural antioxidants, and their reaction mechanisms. The royal society of chemistry. 12th March 2015;5:27986-8006.

71. Mirończuk-Chodakowska I, Witkowska AM, Zujko ME. Endogenous non-enzymatic antioxidants in the human body. Advances in Medical Sciences. 2018;63(1):68-78.
72. Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, et al. Vitamin C as an antioxidant: evaluation of its role in disease prevention. J Am Coll Nutr. 2003;22(1):18-35.
73. Telang PS. Vitamin C in dermatology. Indian Dermatol Online J. 2013;4(2):143-6.
74. Duarte TL, Lunec J. Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. Free Radic Res. 2005;39(7):671-86.
75. May JM. Ascorbate function and metabolism in the human erythrocyte. Front Biosci. 1998;3:d1-10.
76. Timoshnikov VA, Kobzeva TV, Polyakov NE, Kontoghiorghes GJ. Redox Interactions of Vitamin C and Iron: Inhibition of the Pro-Oxidant Activity by Deferiprone. International Journal of Molecular Sciences. 2022 May 31;21(11):3967.
77. Packer JE, Slater TF, Willson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature. 1979;278(5706):737-8.
78. Ahsan H, Ahad A, Siddiqui WA. A review of characterization of tocotrienols from plant oils and foods. J Chem Biol. 2015;8(2):45-59.
79. Brigelius-Flohé R, Traber MG. Vitamin E: function and metabolism. Faseb j. 1999;13(10):1145-55.
80. El-Demerdash FM. Antioxidant effect of vitamin E and selenium on lipid peroxidation, enzyme activities and biochemical parameters in rats exposed to aluminium. J Trace Elem Med Biol. 2004;18(1):113-21.
81. Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radic Biol Med. 2007;43(1):4-15.
82. Pullar JM, Carr AC, Vissers MC. The roles of vitamin C in skin health. Nutrients. 2017 Aug 12;9(8):866.

83. Wang X, Quinn PJ. Vitamin E and its function in membranes. Prog Lipid Res. 1999;38(4):309-36.

84. Pak RW, Lanteri VJ, Scheuch JR, Sawczuk IS. Review of vitamin E and selenium in the prevention of prostate cancer: implications of the selenium and vitamin E chemoprevention trial. Integr Cancer Ther. 2002;1(4):338-44.

85. Traber MG, Stevens JF. Vitamins C and E: beneficial effects from a mechanistic perspective. Free Radic Biol Med. 2011;51(5):1000-13.

86. Ryan MJ, Dudash HJ, Docherty M, Geronilla KB, Baker BA, Haff GG, et al. Vitamin E and C supplementation reduces oxidative stress, improves antioxidant enzymes and positive muscle work in chronically loaded muscles of aged rats. Exp Gerontol. 2010;45(11):882-95.

87. Nieves JW. Chapter 74 - Alternative Therapy through Nutrients and Nutraceuticals. In: Marcus R, Feldman D, Dempster DW, Luckey M, Cauley JA, editors. Osteoporosis (Fourth Edition). San Diego: Academic Press; 2013. p. 1739-49.

88. Stahl W, Sies H. Antioxidant activity of carotenoids. Molecular Aspects of Medicine. 2003;24(6):345-51.

89. Vertuani S, Angusti A, Manfredini S. The Antioxidants and Pro-Antioxidants Network: An Overview. Current pharmaceutical design. 2004;10:1677-94.

90. Shahidi F. Antioxidants in food and food antioxidants. Nahrung. 2000;44(3):158-63.

91. Shadyro OI, Sosnovskaya AA, Edimecheva IP. Flaxseed oil stabilization using natural and synthetic antioxidants. European Journal of Lipid Science and Technology. 2017;119(10):1700079.

92. Peng C, Wang X, Chen J, Jiao R, Wang L, Li YM, et al. Biology of ageing and role of dietary antioxidants. Biomed Res Int. 2014;2014:831841.

93. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr. 2004;79(5):727-47.

94. Li AN, Li S, Zhang YJ, Xu XR, Chen YM, Li HB. Resources and biological activities of natural polyphenols. Nutrients. 2014;6(12):6020-47.

95. Balmus IM, Ciobica A, Trifan A, Stanciu C. The implications of oxidative stress and antioxidant therapies in Inflammatory Bowel Disease: Clinical aspects and animal models. Saudi J Gastroenterol. 2016;22(1):3-17.
96. Shalaby E, Shanab S. Antioxidant compounds, assays of determination and mode of action. Afr J Pharm Pharmacol. 2013;7.
97. Huang D, Ou B, Prior R. The Chemistry behind Antioxidant Capacity Assays. Journal of agricultural and food chemistry. 2005;53:1841-56.
98. Romera-Castillo C, Jaffé R. Free radical scavenging (antioxidant activity) of natural dissolved organic matter. Marine Chemistry. 2015;177:668-76.
99. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact. 2006;160(1):1-40.
100. Flora SJS, Pachauri V. Chelation in metal intoxication. Int J Environ Res Public Health. 2010;7(7):2745-88.
101. Buettner GR, Jurkiewicz BA. Catalytic Metals, Ascorbate and Free Radicals: Combinations to Avoid. Radiation Research. 1996;145(5):532-41.
102. Mira L, Fernandez MT, Santos M, Rocha R, Florêncio MH, Jennings KR. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. Free Radic Res. 2002;36(11):1199-208.
103. Sahiti H, Bislimi K, Rexhepi A, Dalo E. Metal Accumulation and Effect of Vitamin C and E in Accumulated Heavy Metals in Different Tissues in Common Carp (<i>Cyprinus carpio</i>) Treated with Heavy Metals. Pol J Environ Stud. 2020;29(1):799-805.
104. Xu D, Wan J, Xu D, Luo T, Zhong Y, Yang X, et al. Chelation of metal ions with citric acid in the ammoniation process of wet-process phosphoric acid. The Canadian Journal of Chemical Engineering. 2020;98(3):665-75.
105. Peters RW. Chelant extraction of heavy metals from contaminated soils. J Hazard Mater. 1999;66(1-2):151-210.
106. Dogra V, Kim C. Singlet oxygen metabolism: from genesis to signaling. Frontiers in plant science. 2020:1640.

107. Terao J, Minami Y, Bando N. Singlet molecular oxygen-quenching activity of carotenoids: relevance to protection of the skin from photoaging. J Clin Biochem Nutr. 2011;48(1):57-62.
108. Min DB, Boff JM. Chemistry and Reaction of Singlet Oxygen in Foods. Comprehensive Reviews in Food Science and Food Safety. 2002;1:58-72.
109. Miranda-Bautista J, Bañares R, Vaquero J. Chapter 1 - The Gastrointestinal System: Anatomy and Sources of Oxidative Stress. In: Gracia-Sancho J, Salvadó J, editors. Gastrointestinal Tissue: Academic Press; 2017. p. 3-20.
110. Gawlik-Dziki U, Świeca M, Dziki D, Kowalska I, Pecio Ł, Durak A, et al. Lipoxygenase inhibitors and antioxidants from green coffee—mechanism of action in the light of potential bioaccessibility. Food Research International. 2014;61:48-55.
111. Naher L, Yusuf U, Tan S, Ismail A. Ecological status of Ganoderma and basal stem rot disease of oil palms (Elaeis guineensisJacq). Australian Journal of Crop Science. 2013;7:1723-7.
112. Singh RP, Ibrahim MH, Esa N, Iliyana MS. Composting of waste from palm oil mill: a sustainable waste management practice. Reviews in Environmental Science and Bio/Technology. 2010;9(4):331-44.
113. Onyia CO, Uyu AM, Akunna JC, Norulaini NA, Omar AK. Increasing the fertilizer value of palm oil mill sludge: bioaugmentation in nitrification. Water Sci Technol. 2001;44(10):157-62.
114. Thangalazhy-Gopakumar S, Al-Nadheri WMA, Jegarajan D, Sahu JN, Mubarak NM, Nizamuddin S. Utilization of palm oil sludge through pyrolysis for bio-oil and bio-char production. Bioresource Technology. 2015;178:65-9.
115. Onwudike OC. Palm kernel meal as a feed for poultry. 1. Composition of palm kernel meal and availability of its amino acids to chicks. Animal Feed Science and Technology. 1986;16(3):179-86.
116. Ribeiro RDX, Oliveira RL, Macome FM, Bagaldo AR, Silva MCA, Ribeiro CVDM, et al. Meat Quality of Lambs Fed on Palm Kernel Meal, a By-product of Biodiesel Production. Asian-Australas J Anim Sci. 2011;24(10):1399-406.

117. Mohamed WZ, Alimon A. Use of Palm Kernel Cake and Oil Palm By-Products in Compound Feed Use of Palm Kernel Cake and Oil Palm By-Products in Compound Feed. Palm Oil Developments. 2003;40.
118. Manninen AH. Protein hydrolysates in sports and exercise: a brief review. J Sports Sci Med. 2004;3(2):60-3.
119. Vanga SK, Raghavan V. Processing effects on tree nut allergens: A review. Crit Rev Food Sci Nutr. 2017;57(17):3794-806.
120. Rivalain N, Roquain J, Demazeau G. Development of high hydrostatic pressure in biosciences: pressure effect on biological structures and potential applications in biotechnologies. Biotechnol Adv. 2010;28(6):659-72.
121. Hill LR, Silvestri LG, Ihm P, Farchi G, Lanciani P. AUTOMATIC CLASSIFICATION OF STAPHYLOCOCCI BY PRINCIPAL-COMPONENT ANALYSIS AND A GRADIENT METHOD. J Bacteriol. 1965;89(5):1393-401.
122. Udenigwe CC, Aluko RE. Food protein-derived bioactive peptides: production, processing, and potential health benefits. J Food Sci. 2012;77(1):R11-24.
123. Wang Z, Keshwani DR, Redding AP, Cheng JJ. Sodium hydroxide pretreatment and enzymatic hydrolysis of coastal Bermuda grass. Bioresource Technology. 2010;101(10):3583-5.
124. Vanga SK, Singh A, Raghavan V. Review of conventional and novel food processing methods on food allergens. Crit Rev Food Sci Nutr. 2017;57(10):2077-94.
125. Zhu D, Wu Q, Hua L. 3.01 - Industrial Enzymes☆. In: Moo-Young M, editor. Comprehensive Biotechnology (Third Edition). Oxford: Pergamon; 2019. p. 1-13.
126. Normah I, Jamilah B, Saari N, Yaakob BCM. OPTIMIZATION OF HYDROLYSIS CONDITIONS FOR THE PRODUCTION OF THREADFIN BREAM (NEMIPTERUS JAPONICUS) HYDROLYSATE BY ALCALASE. Journal of Muscle Foods. 2005;16(2):87-102.
127. Thiansilakul Y, Benjakul S, Shahidi F. ANTIOXIDATIVE ACTIVITY OF PROTEIN HYDROLYSATE FROM ROUND SCAD MUSCLE USING

ALCALASE AND FLAVOURZYME. Journal of Food Biochemistry. 2007;31(2):266-87.

128. Anh TL, Hoa NT, Nguyen PD, Thanh HV, Nguyen PB, Anh LT, et al. Soybean Protein Extraction by Alcalase and Flavourzyme, Combining Thermal Pretreatment for Enteral Feeding Product. Catalysts. 2020;10(8):829.

129. Sbroggio MF, Montilha MS, FIGUEIREDO VR, Georgetti SR, Kurozawa LE. Influence of the degree of hydrolysis and type of enzyme on antioxidant activity of okara protein hydrolysates. Food Science and Technology. 2016 May 20;36:375-81.

130. Dash P, Ghosh G. Amino acid composition, antioxidant and functional properties of protein hydrolysates from Cucurbitaceae seeds. J Food Sci Technol. 2017;54(13):4162-72.

131. Liu R, Xing L, Fu Q, Zhou G-h, Zhang W-g. A Review of Antioxidant Peptides Derived from Meat Muscle and By-Products. Antioxidants. 2016;5(3):32.

132. Aderinola TA, Fagbemi TN, Enujiugha VN, Alashi AM, Aluko RE. Amino acid composition and antioxidant properties of Moringa oleifera seed protein isolate and enzymatic hydrolysates. Heliyon. 2018;4(10):e00877.

133. Li Y, Jiang B, Zhang T, Mu W, Liu J. Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). Food Chemistry. 2008;106(2):444-50.

134. Bamdad F, Wu J, Chen L. Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein. Journal of Cereal Science. 2011;54(1):20-8.

135. Zarei M, Ebrahimpour A, Abdul-Hamid A, Anwar F, Saari N. Production of Defatted Palm Kernel Cake Protein Hydrolysate as a Valuable Source of Natural Antioxidants. International Journal of Molecular Sciences. 2012;13(7):8097-111.

136. Beaulieu L, Bondu S, Doiron K, Rioux L-E, Turgeon SL. Characterization of antibacterial activity from protein hydrolysates of the macroalga

Saccharina longicruris and identification of peptides implied in bioactivity. Journal of Functional Foods. 2015;17:685-97.

137. Lestari P, Suyata. Antibacterial activity of hydrolysate protein from Etawa goat milk hydrolysed by crude extract bromelain. IOP Conference Series: Materials Science and Engineering. 2019;509:012111.

138. Aguilar-Toalá JE, Deering A, Liceaga A. New Insights into the Antimicrobial Properties of Hydrolysates and Peptide Fractions Derived from Chia Seed (Salvia hispanica L.). Probiotics and Antimicrobial Proteins. 2020;12(4):1571-81.

139. Rameshkumar G, Samuthirapandian R, Kaliyavarathan G, Ajith Kumar TT. Antimicrobial peptide from the crab, Thalamita crenata (Latreille, 1829). World Journal of Fish and Marine Sciences. 2009;1:74-9.

140. Kusumaningtyas E, Nurilmala M, Sibarani D. Antioxidant and antifungal activities of collagen hydrolysates from skin of milkfish (Chanos chanos) hydrolyzed using various bacillus proteases. IOP Conference Series: Earth and Environmental Science. 2019;278:012040.

141. Wichers H. Immunomodulation by food: promising concept for mitigating allergic disease? Analytical and Bioanalytical Chemistry. 2009;395(1):37-45.

142. Her M, Kavanaugh A. Advances in use of immunomodulatory agents--a rheumatology perspective. Nat Rev Gastroenterol Hepatol. 2015;12(6):363-8.

143. Parker F, Migliore-Samour D, Floc'h F, Zerial A, Werner GH, Jollès J, et al. Immunostimulating hexapeptide from human casein: amino acid sequence, synthesis and biological properties. Eur J Biochem. 1984;145(3):677-82.

144. van Esch BC, Schouten B, de Kivit S, Hofman GA, Knippels LM, Willemsen LE, et al. Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3+ regulatory T-cells in the mesenteric lymph nodes. Pediatr Allergy Immunol. 2011;22(8):820-6.

145. Iskandar M, Dauletbaev N, Kubow S, Mawji N, Lands L. Whey protein hydrolysates decrease IL-8 secretion in lipopolysaccharide (LPS)-stimulated

respiratory epithelial cells by affecting LPS binding to Toll-like receptor 4. The British journal of nutrition. 2013;110:1-11.

146. Zhang Q, Wu C, Sun Y, Li T, Fan G. Cytoprotective Effect of Morchella esculenta Protein Hydrolysate and Its Derivative Against H2O2-Induced Oxidative Stress. Polish Journal of Food and Nutrition Sciences. 2019;69:255-65.

147. Cai SY, Wang YM, Zhao YQ, Chi CF, Wang B. Cytoprotective Effect of Antioxidant Pentapeptides from the Protein Hydrolysate of Swim Bladders of Miiuy Croaker (Miichthys miiuy) against H(2)O(2)-Mediated Human Umbilical Vein Endothelial Cell (HUVEC) Injury. Int J Mol Sci. 2019;20(21):5425.

148. Kumar B, Hemalatha R, Tummala J. Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. Food chemistry. 2012;135:3020-38.

149. Liu C, Ren D, Li J, Fang L, Wang J, Liu J, et al. Cytoprotective effect and purification of novel antioxidant peptides from hazelnut (C. heterophylla Fisch) protein hydrolysates. Journal of Functional Foods. 2018;42:203-15.

150. Xie J, Ye H, Du M, Yu Q, Chen Y, Shen M. Mung Bean Protein Hydrolysates Protect Mouse Liver Cell Line Nctc-1469 Cell from Hydrogen Peroxide-Induced Cell Injury. Foods. 2020;9(1):14.

151. Hu X-M, Wang Y-M, Zhao Y-Q, Chi C-F, Wang B. Antioxidant Peptides from the Protein Hydrolysate of Monkfish (Lophius litulon) Muscle: Purification, Identification, and Cytoprotective Function on HepG2 Cells Damage by H2O2. Marine Drugs. 2020;18(3):153.

152. Zarei M, Ebrahimpour A, Abdul-Hamid A, Anwar F, Bakar FA, Philip R, et al. Identification and characterization of papain-generated antioxidant peptides from palm kernel cake proteins. Food Research International. 2014;62:726-34.

153. Zarei M, Ebrahimpour A, Abdul-Hamid A, Anwar F, Saari N. Production of defatted palm kernel cake protein hydrolysate as a valuable source of natural antioxidants. International journal of molecular sciences. 2012;13(7):8097-111.

154. Yajun Z, Li Y, Zhang Y, Zhang R, Zhang Qa, Zhang Y, et al. Fractionation, physicochemical properties, nutritional value, antioxidant activity and ACE inhibition of palm kernel expeller protein. RSC Adv. 2015;5:12613-23.
155. Memarpoor-Yazdi M, Mahaki H, Zare-Zardini H. Antioxidant activity of protein hydrolysates and purified peptides from Zizyphus jujuba fruits. Journal of Functional Foods. 2013;5(1):62-70.
156. Ciapetti G, Cenni E, Pratelli L, Pizzoferrato A. In vitro evaluation of cell/biomaterial interaction by MTT assay. Biomaterials. 1993;14(5):359-64.
157. Biais B, Krisa S, Cluzet S, Da Costa G, Waffo-Teguo P, Mérillon J-M, et al. Antioxidant and Cytoprotective Activities of Grapevine Stilbenes. Journal of Agricultural and Food Chemistry. 2017;65(24):4952-60.
158. Pontis HG. Chapter 3 - Protein and Carbohydrate Separation and Purification. In: Pontis HG, editor. Methods for Analysis of Carbohydrate Metabolism in Photosynthetic Organisms. Boston: Academic Press; 2017. p. 45-63.
159. Bianchi F, Riboni N, Termopoli V, Mendez L, Medina I, Ilag L, et al. MS-Based Analytical Techniques: Advances in Spray-Based Methods and EI-LC-MS Applications. Journal of Analytical Methods in Chemistry. 2018;2018:1308167.
160. Pasupuleti VK, Holmes C, Demain AL. Applications of protein hydrolysates in biotechnology. Protein hydrolysates in biotechnology: Springer; 2008. p. 1-9.
161. Dunn MJ. Gel electrophoresis of proteins: Elsevier; 2014.
162. Mishra K, Ojha H, Chaudhury NK. Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. Food Chemistry. 2012;130(4):1036-43.
163. Kaur IP, Geetha T. Screening methods for antioxidants-a review. Mini reviews in medicinal chemistry. 2006;6(3):305-12.
164. Krishnaiah D, Sarbatly R, Nithyanandam R. A review of the antioxidant potential of medicinal plant species. Food and Bioproducts Processing. 2011;89(3):217-33.

165. Meerloo JV, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. Cancer cell culture: Springer; 2011. p. 237-45.
166. Raijmakers MTM, Roelofs HMJ, Steegers EAP, Steegers-Theunissen RéPM, Mulder TPJ, Knapen MFCM, et al. Glutathione and glutathione S-transferases A1-1 and P1-1 in seminal plasma may play a role in protecting against oxidative damage to spermatozoa. Fertility and Sterility. 2003;79(1):169-72.
167. Lismont C, Revenco I, Fransen M. Peroxisomal Hydrogen Peroxide Metabolism and Signaling in Health and Disease. International journal of molecular sciences. 2019;20(15):3673.
168. Umeno A, Biju V, Yoshida Y. In vivo ROS production and use of oxidative stress-derived biomarkers to detect the onset of diseases such as Alzheimer’s disease, Parkinson’s disease, and diabetes. Free Radical Research. 2017;51(4):413-27.
169. Kawagishi H, Finkel T. Unraveling the truth about antioxidants: ROS and disease: finding the right balance. Nature medicine. 2014;20(7):711-3.
170. de Oliveira Filho JG, Rodrigues JM, Valadares ACF, de Almeida AB, Valencia-Mejia E, Fernandes KF, et al. Bioactive properties of protein hydrolysate of cottonseed byproduct: antioxidant, antimicrobial, and angiotensin-converting enzyme (ACE) inhibitory activities. Waste and Biomass Valorization. 2021;12(3):1395-404.
171. Zou Z, Wang M, Wang Z, Aluko RE, He R. Antihypertensive and antioxidant activities of enzymatic wheat bran protein hydrolysates. Journal of food biochemistry. 2020;44(1):e13090.
172. Möller NP, Scholz-Ahrens KE, Roos N, Schrezenmeir J. Bioactive peptides and proteins from foods: indication for health effects. European journal of nutrition. 2008;47(4):171-82.
173. Cam A, de Mejia EG. Role of dietary proteins and peptides in cardiovascular disease. Molecular Nutrition & Food Research. 2012;56(1):53-66.
174. Zambrowicz A, Pokora M, Setner B, Dąbrowska A, Szołtysik M, Babij K, et al. Multifunctional peptides derived from an egg yolk protein hydrolysate: isolation and characterization. Amino acids. 2015;47(2):369-80.

175. Lu J, Zeng Y, Hou W, Zhang S, Li L, Luo X, et al. The soybean peptide aglycin regulates glucose homeostasis in type 2 diabetic mice via IR/IRS1 pathway. The Journal of nutritional biochemistry. 2012;23(11):1449-57.
176. Jones CG, Daniel Hare J, Compton SJ. Measuring plant protein with the Bradford assay. Journal of Chemical Ecology. 1989;15(3):979-92.
177. You R, Zhang Y, Liu Y, Liu G, Li M. The degradation behavior of silk fibroin derived from different ionic liquid solvents. Natural Science. 2013;05:10-9.
178. Gupta D. Methods for determination of antioxidant capacity: A review. International Journal of Pharmaceutical Sciences and Research. 2015;6(2):546.
179. Athira S, Mann B, Saini P, Sharma R, Kumar R, Singh AK. Production and characterisation of whey protein hydrolysate having antioxidant activity from cheese whey. Journal of the Science of Food and Agriculture. 2015;95(14):2908-15.
180. Gao D, Cao Y, Li H. Antioxidant activity of peptide fractions derived from cottonseed protein hydrolysate. Journal of the Science of Food and Agriculture. 2010;90(11):1855-60.
181. Thamnarathip P, Jangchud K, Nitisinprasert S, Vardhanabhuti B. Identification of peptide molecular weight from rice bran protein hydrolysate with high antioxidant activity. Journal of Cereal Science. 2016;69:329-35.
182. Sun Y, Pan D, Guo Y, Li J. Purification of chicken breast protein hydrolysate and analysis of its antioxidant activity. Food and Chemical Toxicology. 2012;50(10):3397-404.
183. Zielińska E, Baraniak B, Karaś M. Antioxidant and anti-inflammatory activities of hydrolysates and peptide fractions obtained by enzymatic hydrolysis of selected heat-treated edible insects. Nutrients. 2017;9(9):970.
184. Rafieian-Kopaei M, Rezaei M, Asgharzade S, Nikoo M, Rafieia-kopai M. Antioxidant and cytotoxic properties of protein hydrolysates obtained from enzymatic hydrolysis of Klunzinger’s mullet (Liza klunzingeri) muscle. Brazilian Journal of Pharmaceutical Sciences. 2019;24:55.

185. Zhu S, Du C, Yu T, Cong X, Liu Y, Chen S, et al. Antioxidant Activity of Selenium-Enriched Peptides from the Protein Hydrolysate of Cardamine violifolia. J Food Sci. 2019;84(12):3504-11.
186. Karami Z, Akbari-Adergani B. Bioactive food derived peptides: a review on correlation between structure of bioactive peptides and their functional properties. J Food Sci Technol. 2019;56(2):535-47.
187. Xu N, Chen G, Liu H. Antioxidative Categorization of Twenty Amino Acids Based on Experimental Evaluation. Molecules. 2017;22(12):2066.
188. Olagunju AI, Omoba OS, Enujiugha VN, Alashi AM, Aluko RE. Antioxidant properties, ACE/renin inhibitory activities of pigeon pea hydrolysates and effects on systolic blood pressure of spontaneously hypertensive rats. Food Sci Nutr. 2018;6(7):1879-89.
189. Rajapakse N, Mendis E, Jung W-K, Je J-Y, Kim S-K. Purification of radical scavenging peptide from fermented mussel sauce and its antioxidant properties. Food Research International. 2005;38:175-82.
190. Liu DZ, Lin YS, Hou WC. Monohydroxamates of aspartic acid and glutamic acid exhibit antioxidant and angiotensin converting enzyme inhibitory activities. J Agric Food Chem. 2004 Apr 21;52(8):2386-90. doi: 10.1021/jf035493g. PMID: 15080651.
191. Yust M, Millán-Linares M, Alcaide JM, Millán F, Pedroche J. Hypocholesterolaemic and antioxidant activities of chickpea (Cicer arietinum L.) protein hydrolysates. Journal of the science of food and agriculture. 2012;92:1994-2001.
192. Chang C-Y, Wu K-C, Chiang S-H. Antioxidant properties and protein compositions of porcine haemoglobin hydrolysates. Food Chemistry. 2007;100(4):1537-43.
193. Fulle S, Protasi F, Tano G, Pietrangelo T, Beltramin A, Boncompagni S, et al. The contribution of reactive oxygen species to sarcopenia and muscle ageing. Exp Gerontol. 2004;39:17-24.
194. Präbst K, Engelhardt H, Ringgeler S, Hübner H. Basic colorimetric proliferation assays: MTT, WST, and resazurin. Cell viability assays: Springer; 2017. p. 1-17.

195. Zhang Q, Tong X, Li Y, Wang H, Wang Z, Qi B, et al. Purification and Characterization of Antioxidant Peptides from Alcalase-Hydrolyzed Soybean (Glycine max L.) Hydrolysate and Their Cytoprotective Effects in Human Intestinal Caco-2 Cells. Journal of Agricultural and Food Chemistry. 2019;67(20):5772-81.

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