Doctor of Philosophy (Food Science)

DEGREE

Food Science | Food Science and Technology

FIELD | **DEPARTMENT**

TITLE: Isolation and Characterization of Antioxidative Peptides from Khao Dawk Mali (KDML) 105 Rice Bran Protein Hydrolysates

NAME: Mrs. Ladda Wattanasiritham

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Associate Professor Chockchai Theerakulkait, Ph.D.)

THESIS CO-ADVISOR

(Associate Professor Parichat Hongsprabhas, Ph.D.)

DEPARTMENT HEAD

(Assistant Professor Wannee Jirapakkul, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON .....

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

ISOLATION AND CHARACTERIZATION OF ANTIOXIDATIVE PEPTIDES FROM KHAO DAWK MALI (KDML) 105 RICE BRAN PROTEIN HYDROLYSATES

LADDA WATTANASIRITHAM

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Food Science)
Graduate School, Kasetsart University
2015

Ladda Wattanasiritham 2015: Isolation and Characterization of Antioxidative Peptides from Khao Dawk Mali (KDML) 105 Rice Bran Protein Hydrolysates. Doctor of Philosophy (Food Science), Major Field: Food Science, Department of Food Science and Technology. Thesis Advisor: Associate Professor Chockchai Theerakulkait, Ph.D. 130 pages.

Antioxidant is very important in inhibiting oxidation process in food and biological systems. Antioxidative peptide is considered as antioxidant and can be produced through protein hydrolysis. This study was to prepare rice bran protein hydrolysates with antioxidant activity by enzymatic hydrolysis and the antioxidative peptides in the hydrolysate were isolated and identified.

Rice bran protein extracted from defatted rice bran using alkali extraction and isoelectric precipitation (AE-RBP) was prepared. AE-RBP was hydrolyzed with Alcalase 2.4L or papain and antioxidant activity of the hydrolysates were determined by 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and Ferric Reducing Ability Power (FRAP) assay at hydrolysis time of 0, 30, 60, 90 and 120 min. The DPPH free radicals scavenging activity and FRAP value of the alcalase AE-RBP hydrolysates (AE-RBPHs) were 32.1 - 35.5 % and 951 - 1,018 µmol $FeSO_4$/ml of hydrolysate, respectively and were not significantly different ($p > 0.05$) at different hydrolysis times. AE-RBP was freeze-dried (FD-AE-RBP) and it was found that freeze-drying did not effect on antioxidant activity of AE-RBP. DPPH radical scavenging activity and FRAP of the FD-AE-RBP was 41.9% and 92.6 µmol $FeSO_4$ /g protein, respectively. FD-AE-RBP was also combined with butyrate hydroxylanisol (BHA) or α-tocopherol and their antioxidant activities were evaluated.

Albumin, globulin, glutelin and prolamin were extracted from defatted KDML 105 rice bran based on the difference in their solubility. These protein fractions (native form and that denatured by dithiothreitol) were hydrolyzed with papain and trypsin at 37 °C for 3 h. The antioxidant activity of them and their hydrolysates were evaluated by Oxygen Radical Absorbance Capacity (ORAC). Among these protein fractions and their hydrolysates, trypsin-hydrolyzed denatured albumin hydrolysate exhibited the highest antioxidant activity and its ORAC value was 4.07µmol Trolox eq./mg protein. The trypsin-hydrolyzed denatured albumin hydrolysate was separated by RP-HPLC. The peptide fractions that showed high antioxidant activity were identified by UPLC MS/MS. The main MW of peptides were in the range of 800-1,500 Da and consisted of 6 to 20 amino acid residues. The peptide fractions that observed to be the highest antioxidant activity demonstrated typical characteristics of well-known antioxidative peptides with hydrophobic and aromatic amino acid residues. The amino acid sequence of copper ion (Cu^{2+}) -chelating peptides were also demonstrated. The peptides with molecular weight of approximately 800-1,500 Da had high ability to donate an electron to free radical.

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENT

I am extremely grateful to my thesis advisor, Associate Professor Dr. Chockchai Theerakulkait, who has supported me throughout my thesis with his patience, kindness, encouragement, advice, knowledge and financial supporting.

I would like to express my appreciation to my co-advisor, Associate Professor Dr. Parichat Hongsprabhas for her valuable advice and constructive comments throughout the work. I also would sincerely like to thank my committee members, Dr. Pintip Rumpagaporn and Dr. Chitsuda Chaisukdanukul for their valuable comments and suggestion. I am extremely grateful to Associate Professor Dr. Jan Fred Stevens for providing me valuable educational opportunities, his kindness to help and give me access to his laboratory facilities at Oregon State University (OSU) during my research work. Moreover, I would like to thanks the member of Biomolecular Mass Spectrometry Core Center at OSU to analyze for sequencing of peptides. Sincere thanks to Wickramasekara Samanthi for her assistance in data analysis of sequencing of peptides. I am heartfelt thank to my co-workers in the Institute of Food Research and Product Development, Kasetsart University and my graduate friends for their assistance.

I am gratefully acknowledged the funding grant by National Research Council of Thailand, Kasetsart University Research and Development Institute and Thailand Toray Science Foundation for the financial support of this research. Special thanks are extended to the Patum Rice Mill and Granary Co., Ltd, for providing the rice bran.

Finally, I am especially appreciated my family for their understanding, continuing encouragements and endurance. I am appreciated my sisters and brother for their encouragement and taking care my mother. I also deeply thank Ms. Arthorn Yamhongtong who take care of my family during my study.

Ladda Wattanasiritham

April 2015

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

µg	=	Microgram
µl	=	Microliter
µM	=	Micromolar
µmol	=	Micromole
$^1O_2$	=	Singlet oxygen
$A^{\bullet}$	=	Antioxidant radicals
AAPH	=	2,2'-Azobis (2-methylpropionamidine) dihydrochloride
AE-RBP	=	Alkali extracted rice bran protein
AUC	=	Area under the curve
BHA	=	Butylated hydroxyanisole
BSA	=	Bovine serum albumin fraction V
CID	=	Collision induced dissociation
Cu^{2+}	=	Copper (II) ion
$CuSO_4$	=	Copper Sulphate
DH	=	Degree of hydrolysis
DPPH	=	2,2-Diphenyl-1-picrylhydrazyl
DTT	=	DL-Dithiothreitol:
EDTA	=	Ethylenediaminetetraactic acid Dipotassium salt
Eq.	=	Equation
ESI	=	Electrospray ionization
ESR	=	Electron spin resonance
FD-AE-RBP	=	freeze-dried Alkali extracted rice bran protein
Fe^{2+}	=	Ferric(II) ion
$FeCl_3$	=	Ferric chloride
FRAP	=	Feric Reducing Ability Power
FT-ICR	=	Fourier transform ion cyclotron resonance
g	=	Gram

LIST OF ABBREVIATIONS (Continued)

GA	=	Gallic acid equivalent
GRAS	=	Generally Recognized As Safe
H_2O_2	=	Hydrogen peroxide
HAT	=	Hydrogen atom transfer
HNO_2	=	Nitrous acid
$HO^{\bullet}$	=	Hydroxyl radical
HOCl	=	Hypochlorite
$HOO^{\bullet}$	=	Hydroperoxyl radical
HPLC	=	High-performance thin layer chromatography
h	=	Hour
IC_{50}	=	The half maximal inhibitory concentration
KDML	=	KHAO DAWK MALI
kg	=	Kilogram
$L^{\bullet}$	=	Lipid radical
LAPS	=	Linoleic acid peroxcidation system
$LO^{\bullet}$	=	Lipid alkoxyl radical
$LOO^{\bullet}$	=	Lipid peroxyl radical
LOOH	=	Lipid hydroperoxide
LTQ-FT-ICR	=	Linear ion trap-fourier transform ion cyclotron resonance
M	=	Molar
m/z	=	Mass-to-charge ratio
mg	=	Milligram
min	=	Minute
ml	=	Milliliter

LIST OF ABBREVIATIONS (Continued)

mM	=	Millimolar
MS	=	Mass spectrometry
MWCO	=	Molecular weight cut off
NaCl	=	Sodium chloride
NaOH	=	Sodium hydroxide
nM	=	Nanomolar
nm	=	Nanometer
$O_2^{\bullet-}$	=	Superoxide radical
°C	=	Degree celcious
ORAC	=	Oxygen radical absorbance capacity
PG	=	Propyl gallate
RBPF	=	Rice bran protein fraction
RNS	=	Reactive nitrogen species
$ROO^{\bullet}$	=	Peroxyl radical
ROS	=	Reactive oxygen species
RP-HPLC	=	Reversed-phase high-performance liquid chromatography
SET	=	Single electron transfer
SOD	=	Superoxide dismutase
TBHQ	=	*Tert*-butyl hydroquinone
TCA	=	Trichloroacetic acid
TE	=	Trolox equivalent
TFA	=	Trifluoroacetic acid
TPTZ	=	Tripyridyltriazine
v/v	=	Volume/volume
w/v	=	Weight/volume

LIST OF ABBREVIATIONS (Continued)

Name	Symbol	
	3-Letter	1-Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

ISOLATION AND CHARACTERIZATION OF ANTIOXIDATIVE PEPTIDES FROM KHAO DAWK MALI (KDML) 105 RICE BRAN PROTEIN HYDROLYSATES

INTRODUCTION

Oxidation reactions play a significant role in food industry because they cause loss of color, nutritional value and functionality as well as undesirable off-flavors and toxic compounds. Accumulation of the toxic products may be dangerous to the health of consumers (Nawar, 1996; Paek, *et al.*, 2001). An oxidation can generate free radicals that react with lipids and proteins to bring about food deterioration. In addition, free radicals are known to influence various diseases such as cancer, multiple sclerosis, diabetes mellitus, neurodegenerative, cardiovascular disease and inflammatory diseases (Halliwell & Gutteridge, 1990; Butterfield *et al*., 2002; Pryor, 1982). Therefore, it is important to inhibit oxidation reactions and formation of free radicals in food products and the living body to prevent foods from deterioration and protect against numerous diseases. An antioxidant is defined as any substances that significantly delays or inhibits oxidation of a substance when it is present at low concentrations compared to that of an oxidizable substrate (Halliwell, 1995; Halliwell and Gutteridge, 1989). Antioxidants are used to preserve food products by retarding deterioration and discoloration as a result of oxidation (Decker *et al.*, 2005). Currently, α-tocopherols as natural ones are practically used and some synthetic antioxidants such as propyl gallate (PG), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used to act against free radicals in food. Although the synthetic antioxidants are effective and inexpensive compared to natural ones, their applications are restricted since these synthetic antioxidants are suspected to be carcinogenic (Madhavi *et al.*, 1996). Therefore, there has been a great interest in finding new antioxidants from natural sources to replace synthetic antioxidants for using in food. Different extracts from plants (Martinez-Villaluenga *et al.*, 2009; Watchararuji *et al.*, 2008; Yang and Zhai, 2010) and animals (Je *et al*.,

2007; Bougatef, *et al*., 2010) have already been reported to exhibit antioxidative properties in different capacities to inhibit lipid peroxidation. α-Tocopherols, carotenoids and phenolic compounds were extracted from plants and well-known popular natural antioxidants.

Recently, hydrolyzed proteins from various animal and plant sources have been found to possess antioxidant activity such as fish (Bougatef *et al.*, 2010; Cheung *et al.*, 2012; Nazeer and Kulandai, 2012), soybean (Park *et al.*, 2008; Zhang *et al.*, 2010), cereal and legume (Zilic *et al.*, 2012), rice (Adebiyi *et al.*, 2009; Zhang *et al.*, 2010), oat (Jodayree *et al.*, 2012; Tsopmo *et al.*, 2010), egg (Tanzadehpanah *et al.*, 2012; You *et al.*, 2010), barley (Xia *et al.*, 2012) and potato (Cheng *et al.*, 2010). Rice bran is one of the most abundant by-product from rice milling industry. It contains a substantial amount of protein ranging from 12 to 20%. Rice bran protein has high nutrition value and is also hypoallergenic (Helm and Burks, 1996). Thailand produces rice bran over 2.0 million ton / year, about 40% is used to produce edible rice bran oil. The rest of this is used as feed for animals and this part of rice bran contains_0.143-0.189 million ton of protein. Kokkeaw and Thawornchinsombut (2007) prepared rice bran protein hydrolysates using commercial proteolytic enzymes, Protex 6L. It's maximal radical scavenging activity was only 27.08%. Chunput *et al*. (2009) reported that KDML 105 rice bran proteins fraction, digested with pepsin and followed by trypsin revealed high antioxidant activity. Protein from the Japanese rice bran was fractionated and hydrolyzed with protease M, N, S, and P, the peptides that composed of 6-30 amino acid residues show high antioxidant activity (Adebiyi *et al.*, 2009). There are only a little information on preparation and characteristics of antioxidative peptides from Thai rice bran protein hydrolysates. Rice bran can be converted to value-added products as protein hydrolysates that could be potentially used as natural antioxidant in food. The aim of this study was to fractionate protein from KDML 105 rice bran, prepare enzymatically rice bran hydrolysates, isolate antioxidative peptides and characterize these peptides.

OBJECTIVES

1. To prepare protein hydrolysates from KDML 105 rice bran with high antioxidant activity by enzymatic hydrolysis.

2. To separate the generated antioxidative peptides from rice bran protein hydrolysates and determine their antioxidant activity compared with synthetic antioxidant BHA or Trolox.

3. To identify antioxidative peptides that separated from rice bran protein hydrolysates.

4. To determine antioxidant activity of antioxidative peptides in different oxidation systems and evaluate their possible antioxidative mechanism.

LITERATURE REVIEW

1. Free Radical

A free radical is a molecule that contains an unpaired electron in its outer orbit and it can exist independently (Clarkson and Thompson, 2000). They are highly unstable molecules. Reactive oxygen species (ROS) are either oxygen radicals that contain at least one unpaired electron or reactive non-radical derivatives of oxygen, capable of oxidizing biomolecules (Gulcin, 2012). In addition to ROS, there are reactive nitrogen species (RNS) that are able to oxidize the biomolecules as well. ROS, RNS and non-free-radical species molecules were shown in Table 1.

Table 1 Reactive oxygen species (ROS), reactive nitrogen species (RNS) and non-free-radical species.

Free radical species		Non-free-radical species	
Superoxide radical	$O_2^{\bullet -}$	Hydrogen peroxide	H_2O_2
Hydroxyl radical	$HO^{\bullet}$	Singlet oxygen	$^1O_2$
Hydroperoxyl radical	$HOO^{\bullet}$	Ozone	O_3
Lipid radical	$L^{\bullet}$	Lipid hydroperoxide	LOOH
Lipid peroxyl radical	$LOO^{\bullet}$	Hypochlorite	HOCl
Peroxyl radical	$ROO^{\bullet}$	Peroxynitrite	ONOO-
Lipid alkoxyl radical	$LO^{\bullet}$	Dinitrogen trioxide	N_2O_3
Nitrogen dioxide	$NO^{\bullet}_2$	Nitrous acid	HNO_2
Nitric oxide	$NO^{\bullet}$	Nitryl chloride	NO_2Cl
Thiyl radical	$RS^{\bullet}$	Nitroxyl anion	NO^-
		Peroxynitrous acid	ONOOH

Source: Modified from Gulcin (2012)

ROS has been reported to be the most important class of radical species, occurred in foods and generated in living systems. ROS are mainly responsible for initiation of oxidation reaction in foods by reacting with lipids, proteins, sugars, and vitamins that produced undesirable volatile compounds and carcinogens, destroyed essential fatty acids, amino acids and vitamins. ROS play a very important role in human health (Choe and Min, 2006). The complete reduction of oxygen is summarized in the following equations (Eq.):

$O_2^{\bullet-} + e^- \longrightarrow O_2^{\bullet-}$ superoxide radical (1)

$O_2^{\bullet-} + H_2O \longrightarrow HO_2^{\bullet} + OH^-$ hydroperoxyl radical (2)

$HO_2^{\bullet} + e^- + H \longrightarrow H_2O_2$ hydrogen peroxide (3)

$H_2O_2 + e^- \longrightarrow OH^{\bullet} + OH^-$ hydroxyl radical (4)

Each of these oxygen-derived intermediates is considered highly reactive, this might be due to its unstable electron configurations. The superoxide radical ($O_2^{\bullet-}$) is the most well-known oxygen-derived free radical (Yu, 1994) and unlike the other oxygen-derived intermediates, can lead to the formation of additional reactive species. In particular, the protonation of $O_2^{\bullet-}$ results in the formation of hydroperoxyl radical ($HO^{\bullet}$), a much stronger radical than $O_2^{\bullet-}$. Hydroxyl radical is the most reactive ROS, followed by singlet oxygen. The generation of hydroxyl radicals from hydroperoxide provides the development of oxidative stress: DNA damage; carboxylation of proteins and lipid peroxidation including lipids of mitochondrial membranes. By these pathways, oxidative damage leads to cellular death (García-Fernández *et al*., 2008). Radicals may occur naturally as relatively stable chemical compounds. The most common example is molecular dioxygen (O_2) whose stable ground state has two unpaired electrons.

2. Lipid oxidation

Lipid oxidation refers to the oxidative degradation of lipids. In food system, it occurs autocatalytically during processing, storage and cooking through free-radical

intermediates and is generally initiated by trace metals and peroxides presented as ubiquitous impurities in food. This direct reaction of a lipid molecule with the singlet oxygen ($^1O_2$), termed autoxidation, is a free-radical chain reaction. The free radical chain reaction of unsaturated lipid/fatty acids with free radical can be described in three distinct steps including initiation, propagation and termination (Pokorny *et al.*, 2001).

1. Initiation

The autoxidation of lipid is thought to be initiated with the formation of free radicals. The initiation process can also start, via reaction of Eq. 5 by a loss of hydrogen radical ($H^{\bullet}$) in the presence of heat, light exposure or pro-oxidant metals. The resulting lipid (LH) react with oxygen to form lipid peroxyl radicals ($LOO^{\bullet}$) in the propagation reaction which is very fast in the presence of air. The formation of free radical ($LO^{\bullet}$, $LOO^{\bullet}$) in the initiation stage as Eq. 6, 7and 8 can occur by thermal, transition metal (M) catalysis or photodecomposition of hydroperoxides (LOOH).

$$LH \xrightarrow[\text{or Metals}]{\text{Heat or Light}} L^{\bullet} + H^{\bullet} \qquad (5)$$

(unsaturated fatty acid) (Free radical)

$$LOOH + M^{3+} \longrightarrow LOO^{\bullet} + H^{\bullet} + M^{2+} \qquad (6)$$

$$LOOH + M^{2+} \longrightarrow LO^{\bullet} + OH^{\bullet} + M^{3+} \qquad (7)$$

$$2LOOH \longrightarrow LO^{\bullet} + LOO^{\bullet} + H_2O \qquad (8)$$

2. Propagation

In propagation reactions, free radicals are converted into other radicals. The propagation of free-radical oxidation processes occurs in the case of lipids by chain reactions that consume oxygen and has affected new free-radical species (lipid peroxyl

radicals, $LOO^{\bullet}$) or by the formation of lipid hydroperoxides (LOOH) as in Eq. 9 or 10, respectively.

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \quad (9)$$

$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet} \quad (10)$$

At atmospheric oxygen pressure, the reaction of Eq. 9 is very rapid $>10^6$ – fold greater than the reaction of Eq. 10 which means that the concentration of $L^{\bullet}$ are very low compared to the concentrations of $LOO^{\bullet}$. The products ($L^{\bullet}$ and $LOO^{\bullet}$) can further propagate free-radical reactions. $LOO^{\bullet}$ initiate a chain reaction with other lipid molecules, resulting in the formation of LOOH and free radicals. This reaction, when repeated many times, produces an accumulation of hydroperoxides. The propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available.

3. Termination

The termination step involves the formation of non-radical, stable products by the interaction of $L^{\bullet}$, $LO^{\bullet}$ and $LOO^{\bullet}$ through reactions (11-13). The termination reaction leads to interruption of the repeating sequence of propagating steps of the chain reaction.

$$L^{\bullet} + L^{\bullet} \longrightarrow L\text{-}L \quad (11)$$

$$L^{\bullet} + LOO^{\bullet} \longrightarrow LOOL \quad (12)$$

$$LO^{\bullet} + LO^{\bullet} \longrightarrow LOOL \quad (13)$$

Food processing operations, such as grinding, mixing, and homogenizing operations, increase oxidation reaction by introducing oxygen, removal of natural antioxidant, destruction of endogenous antioxidant and increasing pro-oxidative factors such as light exposure that generates singlet oxygen, and thermal treatments which release protein-bound transition metal (Qian *et al.*, 2008). Oxidation of lipids in food is a big problem in food industry because it leads to the formation of various

products and development of various unpleasant odors and flavors, generally called rancid in oils and lipid-containing foods which deteriorate the quality of foods and shorten the shelf life. The mechanism of lipid oxidation is shown in Figure 1.

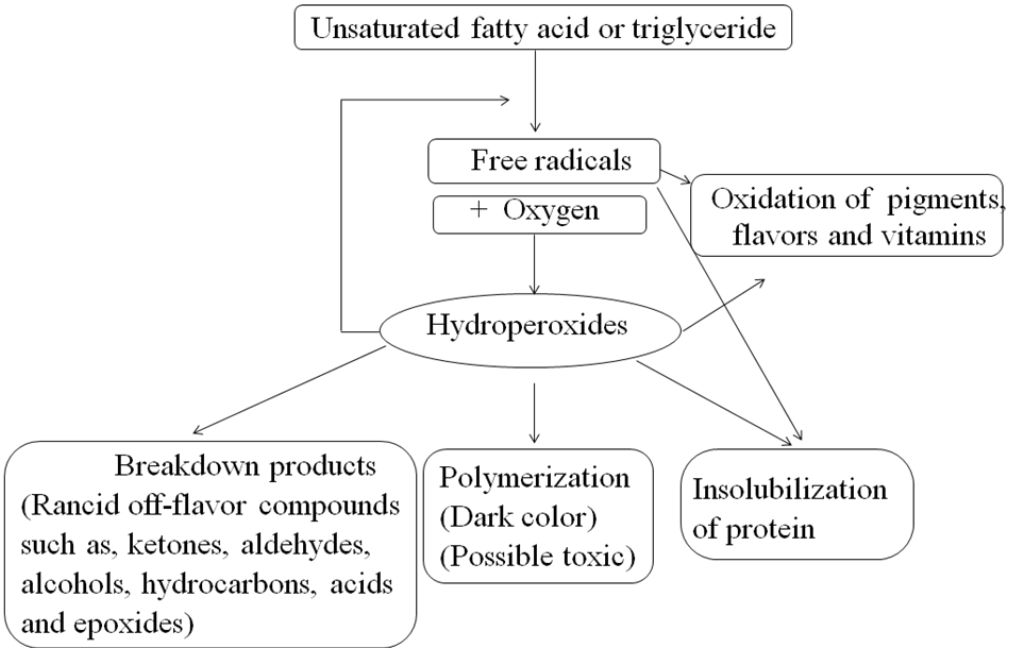


Figure 1 Mechanism of lipid oxidation

Source: Modified from Madhavi *et al.*(1996)

In biological systems, lipid oxidation can take place mainly in biomembranes, which contain highly unsaturated fatty acids. Lipid oxidation is a very complicated chemical and biochemical reaction processes involving free radicals, oxygen, metal ions and a number of other factors.

3. Antioxidant

An antioxidant is defined as any substance that significantly delays or inhibits oxidation of a substance when it is present at low concentration compared to that of an oxidizable substrate (Halliwell, 1995; Halliwell and Gutteridge, 1989). For another meaning, it is a chemical compound that inhibits oxidative damage by either donating

or receiving an electron with a free radical or donating hydrogen radical (Madhavi *et al.*, 1996). Antioxidants are divided into two types: primary or chain-breaking and secondary antioxidants. Primary antioxidants are compounds that terminate the free-radical chain reaction by donating hydrogen or electrons to free radicals before they react with further unsaturated lipid molecules and convert them to more stable products. Secondary antioxidants are compounds that delay the rate of the chain initiation step by such processes as binding metal ions, scavenging singlet oxygen, decomposing hydroperoxides to nonradical products, absorbing UV radiation, and deactivating singlet oxygen (Gordon, 1990).

Oxidation or free radical reactions in the human are reported to be the causes of cancer, coronary heart disease, Alzheimer's diseases and inflammatory diseases (Butterfield *et al.*, 2002; Qian *et al.*, 2008). Under normal conditions, ROS and free radicals are effectively eliminated by the human body's antioxidative defense systems including an enzymatic and non-enzymatic system. Virtually every cell produces antioxidant enzymes called superoxide dismutase (SOD), catalase and glutathione peroxidase. Free radicals are reduced into water with the cooperation of the three main antioxidant enzymes as shown in Figure 2.

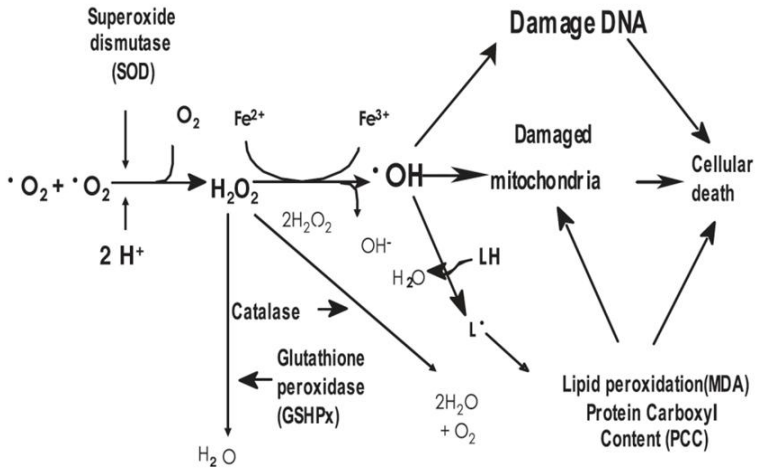


Figure 2 Antioxidative enzyme system and mechanisms of oxidative cellular damage

Source: García-Fernández *et al*. (2008)

The non-enzymatic system includes dietary antioxidant; antioxidant is defined as "a substance in food that significantly decreases the adverse effects of reactive species, such as ROS and RNS, on normal physiological function in humans" (Young *et al*., 1998). The well known dietary antioxidants are beta-carotene, vitamins A, C and E, minerals and proteins. Beta-carotene and vitamins E and C have been well studied, possess strong antioxidant activities and are well absorbed, with a relatively high bioavailability (http://www.sebiology.org/publications/Bulletin/January2008/antioxidants.html).

4. Mechanism of Antioxidants

Antioxidants can inhibit or retard oxidation in two ways: either by scavenging free radicals, in which case the compound is described as a primary antioxidant, or by a mechanism that does not involve direct scavenging of free radicals. Primary antioxidants include phenolic compounds such as vitamin E (α-tocopherol). Secondary antioxidants operate by variety of mechanisms including binding of metal ions, scavenging oxygen, converting hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen. Normally, secondary antioxidants only show antioxidant activity when a second minor component is present. This can be seen in the case of sequestering agents such as citric acid which are effective only in the presence of metal ions, and reducing agents such as ascorbic acid which are effective in the presence of tocopherols or other primary antioxidants (Pokorny *et al.*, 2001). The ability of peptides to interact with free radicals has been documented in many systems.

4.1 Free radicals scavenging

Antioxidants scavenge free radicals in foods by donating hydrogen or electron to $L^{\bullet}$ in initiation step (Eq. 14) or $LO^{\bullet}$ and $LOO^{\bullet}$ in propagation step (Eq. 15 and 16) and convert them to more stable products.

$$L^{\bullet} + AH \longrightarrow LH + A^{\bullet} \quad (14)$$

$$LO^{\bullet} + AH \longrightarrow LOH + A^{\bullet} \quad (15)$$

$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet} \quad (16)$$

The antioxidant radicals ($A^{\bullet}$) has low standard reduction potential, less than 500 mV which is lower than hydroxyl, alkyl, alkoxyl, alkyl peroxyl, and superoxide anion radicals whose reduction potentials are approximately 2300, 600, 1600, 1000 and 940 mV respectively. Free radicals, which have very high standard reduction potentials, are strong oxidizing agents and powerful electrophilic radicals (Choe and Min, 2006). The antioxidant free radical may further obstruct the chain-propagation reactions by forming peroxy antioxidant compounds (Eq. 17-19).

$$L^{\bullet} + A^{\bullet} \longrightarrow LA \quad (17)$$

$$LO^{\bullet} + A^{\bullet} \longrightarrow LOA \quad (18)$$

$$LOO^{\bullet} + A^{\bullet} \longrightarrow LOOA \quad (19)$$

4.2 Metal chelating

Transition metal ions such as copper and iron that are found in food ingredients can catalyse reduction of lipid hydroperoxides (LOOH) to reactive radical species in the initiation step as previously mentioned (Eq. 2 and 3) (Jadhav *et al.*, 1996). Copper is mainly present in food at lower concentration than iron but it is a more effective catalyst than iron in decomposition of hydroperoxide (Halliwell and Gutteridge, 1990). Metals catalyse food radical formation by abstracting hydrogen. They also produce hydroxyl radicals by catalysing the decomposition of hydrogen peroxide or hydroperoxides (Graf and Eaton, 1990). Antioxidants such as proteins delay oxidation by chelating metal or preventing metal redox cycling by changing the physical location of transition metals (e.g. partitioning metals away from oxidatively labile lipids or hydroperoxides), forming insoluble metal complexes, or providing steric hindrance between metals and food components or their oxidationintermediates (Diaz *et al.*, 2003; Graf and Eaton, 1990). EDTA and citric

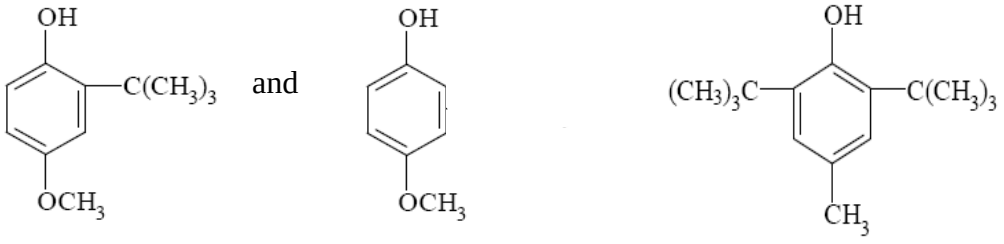
acid are the most common compounds to chelate metal in foods, so called metal chelators.

4.3 Reducing of lipid hydroperoxides

Antioxidant can reduce lipid hydroperoxides (LOOH) to relatively nonreactive lipid hydroperoxides by non-radical reactions, which in turn interferes with lipid oxidation propagation (Elias *et al.*, 2008). This activity was assessed by the ability to protect linoleic acid against oxidation.

5. Synthetic antioxidants

Many synthetic antioxidants are used as food additives to prevent lipid peroxidation and extend the shelf life of a wide variety of food products. Most of the synthetic antioxidants are phenolic type. The differences in antioxidant activities are related to their chemical structures. The commercially available and currently used synthetic antioxidants to preserve food are BHA, PG and *tert*-butyl hydroquinone (TBHQ) as shown in Figure 3. They are used extensively as antioxidants in the food industry and also in fats and oils, fat-containing foods, confectioneries, essential oils and food-coating materials. BHA is a mixture of two isomers, 2-*tert*-butyl-4-hydroxyanisole (2-BHA) and 3-*tert*-butyl-4-hydroxyanisole (3-BHA). BHT is also widely used by combination with other antioxidants such as BHA, PG and citric acid for stabilization of the oils and high-fat foods (Madhavi *et al*., 1996). The four major synthetic antioxidants could be used in maximum level of 200 mg/1 kg of the fat or oil or food products (JECFA, 2013). The toxicological effects of BHA and BHT have been reported to have a promoting effect on urinary bladder, thyroid, and lung carcinogenesis. TBHQ has also been reported to have a weak promoting effect on urinary bladder carcinogenesis (Wichi, 1988).



2-BHA 3-BHA

Butylated hydroxyanisole (BHA) *p*-hydroxyltoluene (BHT)

Propyl gallate (PG) *tert*-butyl hydroquinone (TBHQ)

Figure 3 Structures of synthetic antioxidants

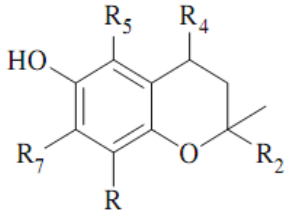
Source: Modified from Pokorny *et al*. (2001)

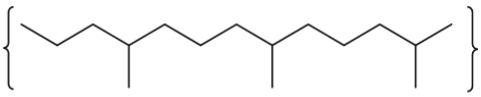
6. Natural antioxidant

The replacement of synthetic antioxidants by natural ones may have benefits due to health implications and functionality such as solubility in both oil and water in food systems. Therefore, there is a growing interest to identify antioxidative properties in many natural sources including vitamin, pro-vitamins, flavonoids in fruits and vegetables and some dietary protein compounds. Natural antioxidants are found in plants, animals, microorganisms and fungi (Pokorny *et al.*, 2001). Numerous researchers reported the isolation and identification of natural antioxidants including ascorbic acid, tocopherols, carotenoids and flavonoids. Tocopherols (vitamin E) are the best known and most widely used antioxidants. They can be classified as tocopherols and tocotrienols, and within each of these two classes, there are four

isomers (∝, ß, γ, and δ) (Figure 4). The most important antioxidant in this group is ∝-tocopherol. It reacts as an antioxidant by donating the hydrogen of the hydroxyl group to the lipid peroxyl radical. The radical formed from ∝-tocopherol is stabilized through delocalization of the single electron over the aromatic ring structure (Pokorny *et al.*, 2001). In general, food sources with the highest concentrations of vitamin E are vegetable oils, followed by nuts and whole grains (Gulcin, 2012). They have also been synthesized on a commercial scale (Madhavi *et al.*, 1996).

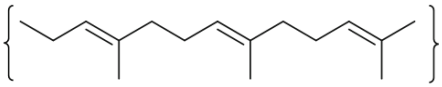
Tocopherols



$R_2 =$ 

	R_4	R_5	R_6	R_7
∝-Tocopherol	H	CH_3	CH_3	CH_3
ß-Tocopherol	H	CH_3	H	CH_3
γ-Tocopherol	H	H	CH_3	CH_3
δ-Tocopherol	H	H	H	CH_3

Tocotrienol**s**

$R_2 =$ 

	R_4	R_5	R_6	R_7
∝-Tocotrienol	H	CH_3	CH_3	CH_3
ß-Tocotrienol	H	CH_3	H	CH_3
γ- Tocotrienol	H	H	CH_3	CH_3
δ-Tocotrienol	H	H	H	CH_3

Figure 4 Chemical structures of tocopherols and tocotrienols

Source: Modified from Gulcin (2012)

Flavonoids are a group of naturally occurring plant phenolics. They are found in almost all parts of the plant. The major subgroups are flavonols, flavones, isoflavones, catechins, proanthocyanidins, and anthocyanins. It was reported that flavonoids may perform as antioxidants by scavenging radicals such as superoxide anion radicals (Hu, *et al.*, 1995; Robak and Gryglewski, 1988), lipid peroxyl and hydroxyl radicals, singlet oxygen quenching (Takahama, 1984), metal ions chelating (Ramanathan and Das, 1993) and lipoxygenases inhibition (Voss *et al.*, 1992).

Carotenoids, a class of natural fat-soluble compounds, are natural colorants and also have antioxidant properties. They act as antioxidant by scavenging singlet molecular oxygen (Mascio *et al.*, 1989) and peroxyl radicals (Stahl and Sies, 2003). Carotenoids such as ß-carotene, zeaxanthin, astaxanthin, lycopene, and lutein are found in fruits and vegetables.

7. Amino acids, peptides and proteins as antioxidants

7.1 Amino acids

Amino acids generally can be divided into hydrophobic and hydrophilic residues. The hydrophobic residues include amino acid with aliphatic side chains, such as alanine, valine, isoleucine, leucine, and methionine, and aromatic side chains, such as phenylalanine, tyrosine, and tryptophan. The hydrophilic residues include (1) amino acids with neutral, polar side chains, such as serine, threonine, asparagine, and glutamine; (2) those with acidic side chains, such as aspartic acid and glutamic acid; and (3) those with basic side chains such as histidine, lysine, and arginine (Grant, 2002). Several amino acids, such as Tyr, Met, His, Gly, Lys, Pro, and Trp are generally accepted as antioxidants (Marcuse, 1960, 1962; Reische *et al.*, 2002). Most of the amino acids have antioxidant properties depending on the pH of the media and their concentration. Glycine has been listed as a Generally Recognized As Safe (GRAS) substance for addition to fats and oils at concentrations up to 0.01% (Madhavi *et al.*, 1996). Glycine, methionine, histidine, tryptophan, proline, lysine showed their antioxidant activities in the oxidation of lipid-containing foods (Reische

et al., 2002). All 20 biologically-derived amino acids are potentially oxidizable, the most reactive amino acids tend to be those containing either nucleophilic sulfur-containing side chains (cysteine and methionine) or aromatic side chains (tryptophan, tyrosine, and phenylalanine) from which hydrogen is easily abstracted. Histidine's imidazole-containing side chain is also oxidative labile. The oxidation chemistry of free amino acids is believed to be very similar to the products detected on amino acids in peptides or proteins (Davies and Dean, 1997).

7.2 Peptides and protein hydrolysates

Antioxidative peptides occurred naturally as such in food. For instance, glutathione (γ-Glutamyl-cysteinylglycine), carnosine (ß-alanylhistidine), anserine (ß-alanyl-L-1-methylhistidine) and ophidine (ß-alanyl-L-3-methylhistidine) are antioxidative peptides naturally present in muscle tissues (Babizhayev *et al.*, 1994; Chan and Decker, 1994b). Carnosine can act as a free radical scavenger as well as a metal ion chelator (Kang *et al.*, 2002). Numerous antioxidative peptides derived from food proteins has been reported as shown in Table 2. It demonstrated the antioxidant capacity of protein hydrolysates from several sources as well as characteristic of peptides and hydrolysates in decade.

8. Relationship between peptides structure and antioxidant activities

Peptides have substantially higher activity than intact proteins. While hydrolyzed proteins have good antioxidant, it is still not well-understood how the composition of peptides influences their ability to inhibit lipid oxidation. Understanding the relationships between peptide composition and antioxidant activity could lead to the development of new classes of extremely effective, multifunctional, GRAS antioxidants. It could be used in many food applications, including the development of functional foods fortified with unsaturated fatty acids and food antioxidant additives. The antioxidant activity of peptides is affected by their chemical structures. Amino acids have also been accepted to exhibit antioxidant

Table 2 Antioxidative capacity of peptides and protein hydrolysates

Source of protein hydrolysates	Peptides characteristic	Preparation	Antioxidant activity assays	Antioxidant activity	References
Bovine ∝-lactalbumin and β-lactoglobulin	WYSLAMAASDI	**Corolase PP**, pepsin, trypsin, chymotrypsin, thrermolysin	ORAC assay	2.621 μM TE/μmol	Herna´ndez-Ledesma *et al.* (2005)
Alaska Pollack (Theragra *frame*) protein	LPHSGY	Mackerel intestine crude enzyme	LAPS, $OH^{\bullet}$ scavenging ability (ESR^{e})	35% $OH^{\bullet}$ scavenging at 53.6 μM	Je *et al.* (2005)
Jumbo squid (*Dosidicus gigas*) skin gellatin	FDSGPAGVL NGPLQAGQPGER	**Trypsin**, ∝-chymotrypsin, pepsin	$OH^{\bullet}$ and C-centered radical scavenging capacity (ESR), metal-chelating activity	IC_{50} (μM) $OH^{\bullet}$: 90.90 C-centered radical scavenging: 130.68 $OH^{\bullet}$: 100.72 C-centered radical scavenging: =141.01	Mendis *et al.* (2005b)
Giant squid (*Dosidicus gigas*) muscle	NADFGLNGLEGL-A	**Trypsin,** ∝-chymotrypsin, pepsin	$OH^{\bullet}$, C-centered radical and $O_2^{\bullet-}$ scavenging capacity (ESR), LAPS	IC_{50} (μM) $OH^{\bullet}$: 497.32 C-centered radical scavenging: 396.04 $O_2^{\bullet-}$: 669.34	Rajapakse *et al.* (2005a)

Table 2 (Continued)

Source of protein hydrolysates	Peptides characteristic	Preparation	Antioxidant activity assays	Antioxidant activity	References
Giant squid (*Dosidicus gigas*) muscle	NGLEGLK	**Trypsin,** ∝-chymotrypsin, pepsin	$OH^{\bullet}$, C-centered radical and $O_2^{\bullet-}$ scavenging capacity (ESR), LAPS	IC_{50} (μM) $OH^{\bullet}$: 428.54 C-centered radical scavenging: 396.04 $O_2^{\bullet-}$: 573.83	Rajapakse *et al.* (2005a)
Conger eel (*Conger myriaster*) muscle	LGLNGDDVN	Trypsin	LAPS, $OH^{\bullet}$ and C-centered radical scavenging activity (ESR)	IC_{50} (μM) $OH^{\bullet}$: 74.1 C-centered radical scavenging: 78.5	Ranathunga *et al.* (2006)
Tuna back bone	VKAGFAWTANQQLS	**Pepsin**, Alcalase, ∝-chymotrypsin, Neutrase, papain, trypsin	$OH^{\bullet}$, DPPH and $O_2^{\bullet-}$ radical scavenging capacity (ESR), LAPS	IC_{50} (mg/ml) $OH^{\bullet}$: 0.032 DPPH: 0.032 $O_2^{\bullet-}$: 0.70	Je *et al.* (2007)
Hoki (*Johnius belengerii*) frame protein	ESTVPERTHPACPD-FN	**Pepsin**, Alcalase, ∝-chymotrypsin, neutrase, papain, trypsin	$OH^{\bullet}$, DPPH, $O_2^{\bullet-}$ and $ROO^{\bullet}$ scavenging capacity (ESR), LAPS and protection against *t*-butylhydroperoxide-induced cytotoxicity on human embryonic lung fibroblasts	IC_{50} (μM) $OH^{\bullet}$: 17.77 DPPH: 41.37 $O_2^{\bullet-}$: 172.10 $ROO^{\bullet}$: 18.99	Kim *et al.* (2007)

Table 2 (Continued)

Source of protein hydrolysates	Peptides characteristic	Preparation	Antioxidant activity assays	Antioxidant activity	References
Human milk	WSVPQPK QVVPYPQ	Both of pepsin and pancreatin	ABTS[g] radical scavenging capacity	TEAC value (µmol/ µmol) 1.297 0.931	Herna´ndez-Ledesma *et al.* (2007)
Bullfrog skin	LEELEEELEGCE	Pepsin, **Alcalase**, ∝-chymotrypsin, neutrase, papain, trypsin	$OH^{\bullet}$, DPPH, $O_2^{\bullet-}$ and $ROO^{\bullet}$ radical scavenging activity	IC_{50} (µM) $OH^{\bullet}$: 12.8 DPPH: 16.1 $O_2^{\bullet-}$: 34.0 $ROO^{\bullet}$: 32.6	Qian *et al*. 2008
Algae (*Chlorella vulagaris*) protein waste	VECYGPNRPQF	**Pepsin**	TEAC assay, ORAC assay, $OH^{\bullet}$, $DPPH^{\bullet}$, and $O_2^{\bullet-}$ scavenging capacity, protection against oxidation-induce DNA and cell damage	IC_{50} (µM) $OH^{\bullet}$: 8.3 DPPH: 7.82 $O_2^{\bullet-}$: 22.02	Sheih *et al*. (2009)
Venison	MQIFVKTLTG	**Papain**, pepsin, trypsin, α-chymotrypsin	$OH^{\bullet}$, DPPH, $O_2^{\bullet-}$ and $ROO^{\bullet}$ scavenging activity (ESR)	IC_{50} (µM) $OH^{\bullet}$: 4.47 DPPH: 7.82 $O_2^{\bullet-}$: 22.02 $ROO^{\bullet}$: 8.62	Kim *et al.* (2009)

Table 2 (Continued)

Source of protein hydrolysates	Peptide characteristic	Preparation	Antioxidant activity assays	Antioxidant activity	References
Venison	DLSDGEQGVL	**Papain**, pepsin, trypsin, α-chymotrypsin	$OH^{\bullet}$, DPPH, $O_2^{\bullet-}$ and $ROO^{\bullet}$ scavenging activity (ESR)	$OH^{\bullet}$: 6.12 DPPH: 9.54 $O_2^{\bullet-}$: 53.24 $ROO^{\bullet}$: 14.54	Kim *et al.* (2009)
Rice bran	YLAGMN	Proteases M, N, P, S and pepsin	ABTS radical scavenging activity	TEAC (mmol) 0.192 at 0.0005%)	Adebiyi *et al.*(2009)
Human milk	YGYTGA ISELGW	Pepsin and pancreatin	ORAC assay, LAPS	ORAC assay 5169 µM TE/mmol) 4479 µM TE/mmol)	Tsopmo *et al.* (2011)
Sea cucumber (*Stichopus Japonicus)*	GPEPTGPTGAPQ WLR	Pepsin, trypsin and papain	$OH^{\bullet}$ and $O_2^{\bullet-}$ radical scavenging activity	IC_{50} (µM) $OH^{\bullet}$: 138.9 $O_2^{\bullet-}$: 353.9	Zhou *et al.* (2012)

[a] LAPS: Linoleic acid peroxidation system [b] AAPH: 2,2-azobis(2-amidinopropane) [c] DPPH: 2,2-Diphenyl-1-picrylhydrazyl

[d] ORAC: Oxygen radical absorbance capacity [e] ESR: Electron spin resonance [f]TEAC: Trolox equivalent antioxidative substances

[g]ABTS: 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

The enzyme indicated in **bold** font is the most effective of the enzymes reported to produce antioxidative peptides

Source: Modified from Samaranayaka and Li-Chan (2011)

activity, which is greater when they are incorporated in dipeptides (Yamaguchi *et al.*, 1975). Chen *et al*. (1995) isolated six antioxidant peptides from the proteolytic digest of a soybean protein. The peptides were composed of 5-16 amino acid residues, including hydrophobic amino acids, Val or Leu, at the N-terminal positions, and Pro, His, or Tyr in the sequences. It was revealed that the antioxidant activity of a peptide was more dependent on His-His segment in the Leu-Leu-Pro-His-His domain and its activity was decreased by removing a His residue from the C-terminus. According to the result from the same study, Pro-His-His (PHH) was found to be the most active among the peptides tested. Further study with 22 synthetic peptides containing His residues demonstrated that His-containing peptides can act as a metal-ion chelator, an active-oxygen quencher and a hydroxyl radical scavenger (Chen *et al.*, 1998). Saito *et al.* (2003) studied on antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry and indicated that the antioxidant activities of peptides were closely related to their sequences, their amino acid constituents, peptides size and hydrophobicity. Amino acids with aromatic residues can donate protons to electron deficient radicals and this improves the radical-scavenging properties of the amino acid residues. It is proposed that the antioxidant activity of His-containing peptides is in relation with the hydrogen-donating, lipid peroxyl radical trapping and/or the metal ion-chelating ability of the imidazole group (Chan and Decker, 1994a; Rajapakse *et al.*, 2005b). Further information regarding the effect of amino acid compositions and their correct positioning in peptide sequences are shown in Table 3. The antioxidative peptides often include hydrophobic amino acid residues such as Val or Leu at the N-terminus of the peptides, and Pro, His, Tyr, Trp, Met, and Cys in their sequences (Chen *et al.*, 1995; Elias *et al*., 2008; Uchida and Kawakishi, 1992).

9. Antioxidant activity measurements

There are numerous methods for measuring antioxidant activity. In vitro assays based on chemical reactions for measuring antioxidant capacity are divided into two groups: methods based on hydrogen atom transfer (HAT) and methods based on single electron transfer (SET). HAT-based methods measure the classical ability

Table 3 Amino acid compositions and their positioning in relation with antioxidant activity of peptides.

Amino acids	Mechanism of action	Examples
Aromatic amino acids	Converting radicals to stable molecules by donating electron, while keeping their own stability by resonance structure Improving the radical-scavenging properties of the amino acids residues (Rajapakse *et al.*, 2005b)	His at N-termini as an effective metal ion chelator His at C-termini as an effective scavenger against various radicals (Chen *et al.*, 1998) Tripeptides with Trp or Tyr at C-termini as strong radical scavengers but weak peroxynitrile scavengers (Saito *et al.*, 2003)
Hydrophobic amino acids	Enhancing the solubility of peptide in lipid which facilitates accessibility to hydrophobic radical species and to hydrophobic PUFAs (Chen *et al.*, 1998; Qian *et al.*, 2008; Suetsuna and Chen, 2002) Gly as hydrogen donor (Qian *et al.*, 2008)	Val or Leu, at the N-termini and Pro, His, or Tyr in the sequences (Chen *et al.*, 1995) High reactivity of aliphatic groups in Ala, Val, Leu to hydrophobic PUFAs (Qian *et al.*, 2008) Ala or Leu at the terminus, Gln and a Pro residue in the sequences of peptide from gluten (Suetsuna and Chen, 2002)
Acidic and basic amino acids	Carboxyl and amino groups in the side chains as chelator of metal ions (Suetsuna *et al.*, 2000), as hydrogen donor (Qian *et al.*, 2008)	Asp (acidic amino acid) and His (basic amino acid) residues in peptide purified from fermented mussel sauce (Rajapakse *et al.*, 2005b)
Cysteine	SH group as radical scavenger (Patterson and Rhoades, 1988), protecting tissue from oxidative stress, improving the glutathione activity (Selvam and Devaraj, 1996)	Tripeptides with Cys as strong scavengers against peroxynitrite radicals (Saito *et al.*, 2003) In curry leave protein SH group together with other functional groups involved in its antioxidant activity (Ningappa and Srinivas, 2008)

Source: Modified from Sarmadi and Ismail (2010)

of an antioxidant to quench free radicals by hydrogen donation:

$X^{\bullet} + AH \longrightarrow XH + A$

$X^{\bullet}$: Free radicals

HAT reactions are depended on solvent and pH and usually quite rapid, typically completed in seconds to minutes. These reactions include the following methods (Huang, D. *et al.*, 2005):

1. Oxygen radical absorbance capacity (ORAC)
2. Total radical-trapping antioxidant parameter (TRAP)
3. Inhibition of induced LDL oxidation
4. Total oxyradical scavenging capacity assay (TOSCA)
5. Crocin-bleaching assays
6. Chemiluminescent assay

SET-based methods measure the ability of an antioxidant to transfer one electron to reduce any compounds, including metal ions, carbonyls and radicals and depend on pH (Wright *et al.*, 2001). They include the following assay:

1. Total phenolics assay by Folin-Ciocalteu reagent assay
2. Trolox equivalence antioxidant capacity (TEAC) assay
3. Ferric ion reducing antioxidant power (FRAP) assay
4. Total antioxidant potential assay, using a Cu^{2+}-complex as an oxidant
5. 2,2-Diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging
6. 2,2-Azinobis 3-ethylbenzthiazoline-6-sulphonic acid radical ($ABTS^{\bullet+}$) scavenging assay
7. N,N-dimethyl-p-phenylenediamine radical ($DMPD^{\bullet+}$) scavenging assay

In addition to HAT- and SET-based methods assays, there are other methods to measure antioxidant activity. These assays include superoxide and hydroxyl radical, hydrogen peroxide, single oxygen and peroxynitrite scavenging assays and metal chelating assays. Although there are many methods to evaluate antioxidant

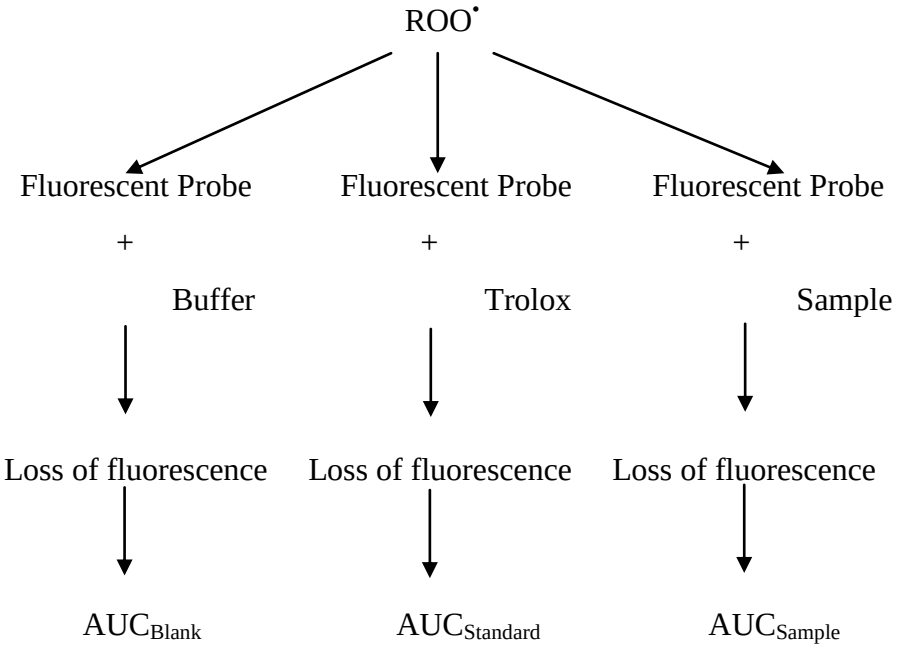
capacity of substances, no single assay will actually reflect all of the radical sources or all antioxidants in a complex system. It is suggested that measuring antioxidant capacity of a food or a food constituent of interest should be determined more than one assay due to the differences in the mechanism of antioxidative action measured and/or reaction conditions of each assay. The test sample may show different results for antioxidative capacity depending on the assay system used. In addition, solubility of antioxidants in the reaction solution also plays an important role in their antioxidative capacity (Samaranayaka and Li-Chan, 2011).

9.1 ORAC

The ORAC assay using fluorescein (FL) as fluorescent probe was developed and validated by Ou *et al*. (2001) and modified by Davalos *et al*. (2004). ORAC assay provides a direct measure of the hydrophilic and lipophilic chain-breaking antioxidant capacity against peroxyl radicals induced by 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) by altering the radical source and solvent (Huang *et al.*, 2002; Prior *et al.*, 2005). The principle of ORAC assay is demonstrated in Figure 5. The loss of fluorescence of FL is an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant is measured by evaluation of the area under the curve (AUC) of the sample as compared with the blank in which no antioxidant is present. A typical ORAC assay kinetic curve is shown in Figure 6. The antioxidant activity is evaluated by comparing the ability of Trolox (the water-soluble vitamin E analogue) to delay oxidation of peroxyl radicals. A series of Trolox solution with varying concentrations is used as antioxidant standard to obtain a standard curve (Trolox concentration versus AUC) and ORAC values are reported as Trolox equivalents.

9.2 FRAP assay

FRAP assay is a typical ET-based method that determines the total antioxidant activity of test samples. It was originally used to measure reducing power



Antioxidant capacity = AUC_{Sample} - AUC_{Blank}

Figure 5 Principle of the ORAC assay

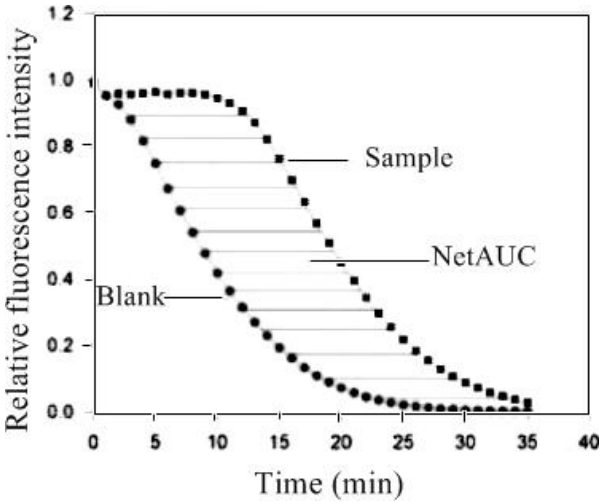
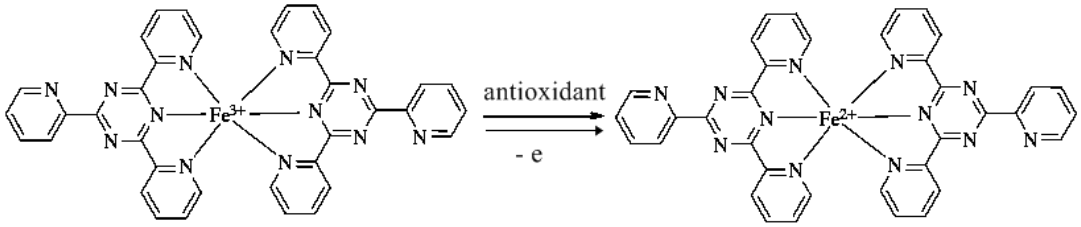


Figure 6 Kinetic curve of ORAC assay

Source: Modified from Prior *et al*. (2005)

but has been extended for assessing antioxidant activity in other biological fluids,

foods, and plant extracts. It was evaluated by the measurements of Fe^{+2}/TPTZ-complex at low pH by colorimetric method with a spectrophotometer. A ferric-tripyridyltriazine (Fe^{+3}-TPTZ) complex is reduced to ferrous (Fe^{+2}) form, resulting in an intense blue color with an absorption maximum at 593 nm whose reaction is shown in Figure 7.



Fe^{3+}-TPTZ Fe^{2+}-TPTZ

Figure 7 $[Fe^{3+}\text{-}(TPTZ)2]^{3+}\text{-}[Fe^{2+}\text{-}(TPTZ)2]^{2+}$ Reduction reaction for FRAP assay

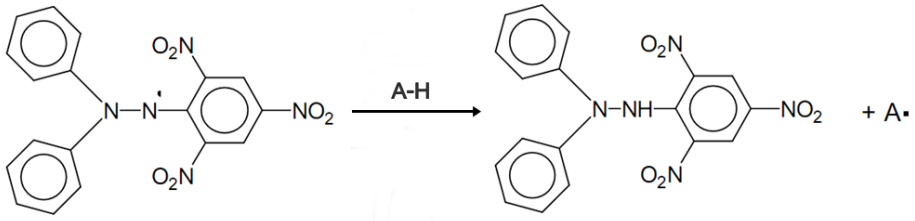
Source: Modified from Gulcin (2012)

Reducing activity value is obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those of samples containing ferrous ions in known concentrations and the results are expressed as µmole Fe^{2+} equivalents or relative to an antioxidant standard (Benzie and Strain 1996; Shahidi *et al*., 2007).

9.3 DPPH radical scavenging activity

The $DPPH^{\bullet}$ method is a rapid, simple and inexpensive method to measure antioxidant capacity of compounds. It is commonly used to screen for antioxidant activity because of its simplicity (Huang *et al.*, 2005). $DPPH^{\bullet}$ scavenging activity is widely used to evaluate the ability of peptides derived from food protein to act as free radical scavengers or hydrogen donors as indicated in Table 2. A simple method that has been developed to determine the antioxidant activity in natural compounds utilizes the stable DPPH radical. The structure of DPPH and its reduction by an antioxidant

(A-H) is shown in Figure 8. The DPPH$^{\bullet}$ is a stable free radical that gives a strong absorption maximum at 517 nm and is dark purple in color. The color turns from dark



DPPH$^{\bullet}$ DPPH-H

Figure 8 DPPH$^{\bullet}$ chemical structure and its reaction with a scavenger, indicated by A-H.

Source: Modified from Scalzo (2008)

purple to yellow when DPPH radical abstracted hydrogen from antioxidant to form the reduced DPPH-H. The concentration of a sample with a decrease in the initial DPPH concentration by 50% is defined as IC_{50}. The IC_{50} value is used to compare the capacities of different antioxidants. As a result of these differences in the reaction conditions, these values for even the standard antioxidants like ascorbic acid and BHT vary greatly. Therefore, it is not possible to compare the IC_{50} values of the same antioxidants performed from different laboratories (Sharma and Bhat, 2009).

9.4 Metal ion chelating activity

Transition metals such as Fe^{2+} and Cu^{2+} can catalyze the generation of ROS. Especially, Fe^{2+}, generates OH$^{\bullet}$ which accelerates the lipid peroxidation chain reaction (Stohs and Bagchi, 1995). A spectrophotometer is used to measure the chelating activity and EDTA is used as a positive control.

10. Antioxidative peptide and protein hydrolysates of food protein by enzymatic hydrolysis.

Enzymatic hydrolysis has been the most common way for producing antioxidative peptides from food proteins. Various studies have been conducted to investigate antioxidant properties of hydrolysates from plant or animal sources such as soybean (Chen *et al*., 1998; Li *et al*., 2006; Park *et al.*, 2008; Zhang *et al.*, 2010), wheat (Suetsuna and Chen, 2002; Zilic *et al.*, 2012), rice (Chunput *et al*., 2009; Adebiyi *et al.*, 2009; Zhang *et al.*, 2010), oat (Jodayree *et al.*, 2012; Tsopmo *et al.*, 2010), barley (Xia *et al.*, 2012), alfalfa leaf (Xie *et al.*, 2008), potato (Cheng *et al.*, 2010), fish (Wu *et al*., 2003; Bougatef *et al.*, 2010; Cheung *et al.*, 2012; Nazeer and Kulandai, 2012) and egg (Tanzadehpanah *et al.*, 2012; You *et al.*, 2010). Industrial food-grade proteinases such as Alcalase®, Flavourzyme® and Protamex® derived from microorganisms, as well as enzymes from plant (e.g. papain) and animal sources (e.g. chymotrypsin, pepsin and trypsin have been widely used to produce antioxidative protein hydrolysates and peptides (Samaranayaka and Li-Chan, 2011). In different studies, it has been found that selection of proteases for hydrolysis and preparation condition such as temperature and pH for the optimal activity of enzyme, as well as the control of hydrolysis time plays critical roles in generating of antioxidative peptides. Protease specificity and source of proteins affect size, amount, peptides and their amino acid sequences which in turn influence the antioxidant activity of the hydrolysates. For example, Pena-Ramos and Xiong (2002) used three purified (pepsin, papain and chymotrypsin) and three crude (Alcalase®, Protamex™ and Flavourzyme™) proteases to produce hydrolysates from native and heated soy protein isolates. They found that the heated soy protein isolates was hydrolyzed with chymotrypsin and flavourzyme in 0.5 h and had the greatest antioxidant activity which was determined by inhibiting lipid oxidation. Zhang *et al*. (2009) used five different enzymes (alcalase, chymotrypsin, neutrase, papain and flavorase) to hydrolyse rice endosperm protein and also determined their antioxidant activity. The rice endosperm proteins were hydrolyzed for 4 h based on their optimum hydrolysis conditions. The results showed that the protein hydrolysates prepared from the enzyme neutrase revealed maximum antioxidant activity with its sequence of Lys-His-Asn-Arg-Gly-Asp-Glu-Phe and the molecular mass of 1002.5217 Da. Recently, Xia *et al*. (2012) reported that alcalase-derived hydrolysates from enzymatic digestion

of barley glutelin had significantly higher antioxidant capacity than those being treated by flavourzyme. The amino acid sequences of the peptides with high antioxidant activity were Gln-Lys-Pro-Phe-Pro-Gln-Gln-Pro-Pro-Phe, Pro-Gln-Ile-Pro-Glu-Gln-Phe, Leu-Arg-Thr-Leu-Pro-Met and Ser-Val-Asn-Val-Pro-Leu. The antioxidant properties of the hydrolysates, largely depended on protease specificity, degree of hydrolysis (DH) and nature of released peptides (e.g. molecular weight and amino acid composition). They have been attributed to cooperative or combined effects of a number of properties, including their ability to scavenge free radicals, to act as chelating agents of metal ions or act as hydrogen donor (Tang *et al.*, 2009). However, many peptides released by enzymatic hydrolysis commonly have a bitter off-taste which make them be undesirable as food ingredient. Humiski and Aluko (2007) investigated the effects of different proteolytic treatments on physiochemical and bitterness properties of peanut protein hydrolysates. The results showed that the alcalase hydrolysates was the most bitter one while papain and α-chymotrpsin hydrolysates were the least.

11. Proteolytic enzymes

11.1 Alcalase

Alcalase is proteolytic enzyme produced by submerged fermentation of a selected strain of *Bacillus licheniformis*. The main enzyme component, Subtilisin A (= Subtilisin Carlsberg) is an endoprotease which cleaves peptide bonds with broad specificity. It hydrolyses peptide bonds in oxidized ß-chain of insulin 3 as shown in Figure 9. It has a size of approximately 27.30 kDa. The optimal conditions for alcalase activity are temperatures between 55 °C and 70 °C, depending on the type of substrate and pH values between 6.5-8.5. It can be inactivated in 30 min at 50 °C at pH 4 in 10 min at 85 °C or higher at pH 8. However, the inactivation is depended on various condition such as substrate concentration, pH, etc. (Novozymes, 2001). It has been used in several previous studies to produce protein hydrolysates from soy (Seo *et al.*, 2008) fish (Klompong *et al.*, 2008) and barley (Bamdad and Chen, 2013;

Xia *et al*., 2012)

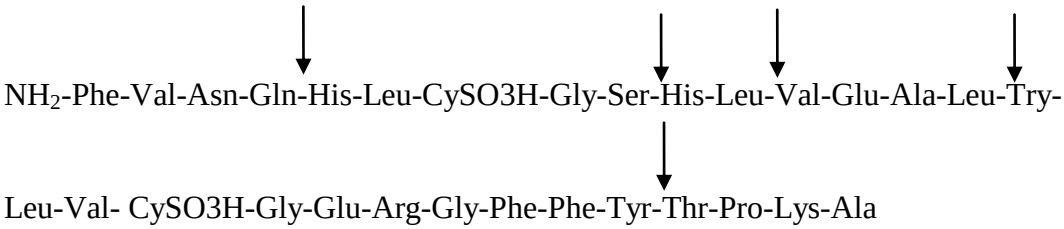


Figure 9 Bonds attacked in oxidized ß-chain of insulin 3 by alcalase

Source: Johansen *et al.* (1968)

11.2 Papain

Papain is a cysteine hydrolase that is stable and active under a wide range of conditions. It is very stable even at high temperatures (Cohen *et al.*, 1986). Papain is a single-chained polypeptide with three disulfide bridges and a sulfhydryl group necessary for the activity of the enzyme. The optimal pH for activity is 6.0-7.0, the temperature optimum is 65 °C and pI for it: 8.75 and 9.55. It has fairly broad specificity showing endopeptidase, amidase, and esterase activities. Papain digests most protein substrates more extensively than the pancreatic proteases. Papain cleaves the peptide bond on the carboxyl side of lysine, arginine or the amino acid next to phenylalanine (International Union of Biochemistry, 1984; Lauwers and Scharpe, 1997). It is soluble in water at 10 mg/ml. Immediately prior to use, the enzyme is typically diluted in buffer containing ~5 mM L-cysteine. Activation/stabilizing agents include EDTA, cysteine, and dimercaptopropanol (Arnon, 1970). Although papain solutions have good temperature stability, the solution stability is pH dependent. Papain solutions are unstable under acidic conditions, i.e., at pH values below 2.8, there is a significant loss in activity. For the active enzyme in solution, the loss in activity is about 1-2% per day, probably as a result of autolysis and/or oxidation (Mitchel *et al.*, 1970; Schomburg and Schomburg, 2002). It was used to prepare protein hydrolysates from plant and animal proteins for

improving antioxidative properties (Saiga *et al.*, 2003; Wang *et al.*, 2007; You *et al.*, 2010).

11.3 Trypsin

Trypsin is a pancreatic serine protease with substrate specificity based upon positively charged lysine and arginine side chains (Brown and Wold, 1973) which cleaves proteins on the C-terminal sides of lysine and arginine residues except when proline is at the carboxylic side of lysine or arginine. It is produced in the pancreas as the inactive proenzyme trypsinogen. If an acidic residue is on either side of the cleavage site, the rate of hydrolysis is slower. Trypsin has an optimal operating pH of about 7.5-8.5 and optimal operating temperature of about 37 °C. It is used for generating peptides from several protein sources to produce bioactive peptides (Mendis *et al.*, 2005a; Qin *et al.*, 2011; Rajapakse *et al.*, 2005a; Ranathunga *et al.*, 2006; Rival *et al.*, 2001b).

12. Rice bran

Rice (*Oryza sativa*) is one of the most important staple food for a large part of the world's population, mostly in Asian countries (Sharif *et al.*, 2013). In 2012 in Thailand, produced milled-rice is about 22.8 million tons (FAO Report, 2013). Rough rice grain is consisted of a white starchy rice kernel tightly covered by a coating of bran, enclosed in a tough siliceous hull (Lakkakula *et al.*, 2004) as shown in Figure 10. Rice bran is a by-product of rice milling process and produced annually around 40-50 million in the global while Thailand shares ultimately 2.0 million tons. Around 40% is used to produce edible rice oil and the rest of this is used as feed for animals or discarded. Rice bran is consisted of pericarp, aleurone, sub-aleurone, seed coat, nucellus along with the germ and a small portion of endosperm (Hargrove, 1994; Hu *et al.*, 1996; Salunkhe *et al.*, 1992). The composition of rice bran is 15-22% lipids, 34.1-52.3% carbohydrates, 7-11.4% fiber, 6.6-9.9% ash, 8-12% moisture, and 10-16% highly nutritional protein (Juliano, 1985; Saunders, 1990).

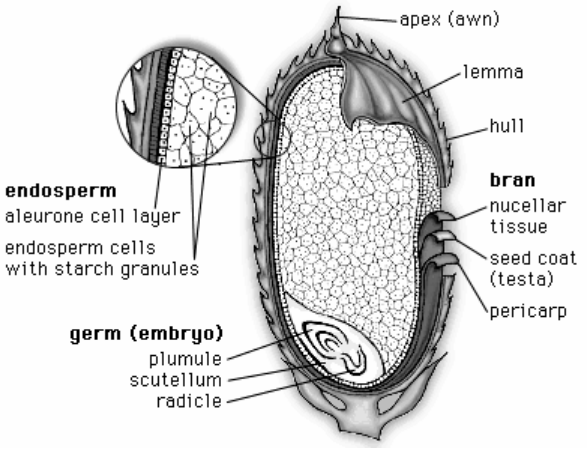


Figure 10 Structure of a rough rice grain

Source: Modified from Britannica and Edwards (1996)

Rice bran proteins have high nutritional value (Kennedy *et al.*, 1996) and are hypoallergenic (Tsuji *et al.*, 2001). These proteins are rich in lysine content, hence can be used as an ingredients in food recipes (Wang *et al.*, 1999). Recently, enzymatic rice bran protein hydrolysate revealed antioxidant activity and improved functional properties (Adebiyi *et al.*, 2009; Chanput *et al.*, 2009; Zhou *et al.*, 2013)

13. Rice bran protein

Rice bran is a good source of protein which contains a substantial amount of protein ranging from 12 to 20%. Based on solubility, it could be classified into albumin, globulin, prolamine and glutelin which are solubilized in distilled water, sodium chloride, ethanol and sodium hydroxide, respectively. These proteins are mostly storage proteins and have high nutrition value (Hamada, 1997; Juliano, 1985). The composition of the protein in rice bran may be varied depending on differences in extraction methods and varieties (Table 4).

Table 4 Varietal difference in solubility fractions of rice bran proteins (%)

Cultivar	Albumin	Globulin	Prolamine	Glutelin
Spanish	26.5	13.6	1.8	24.0
Bengal	34.7	17.4	6.7	11.9
Cypress	33.0	13.7	7.8	9.7
Della	32.2	13.5	5.7	10.4
Mars	39.5	17.0	5.3	12.1
Maybelle	33.4	12.8	5.9	14.7
Toro-2	30.2	14.3	5.3	6.6
Hitimebore (Japanese rice bran)				
By different extraction methods:				
Ju *et al*. (2001)	39	27	1	33
Hamada and Jamel (1999)	24	29	3	41
Adebiyi *et al*. (2009)	24-39	27-30	1-3	33-42

Source: Betschart *et al.* (1977), Hamada (1997), Abayomi *et al*. (2009)

Protein from defatted rice bran was fractionated and showed strong SOD-like activity (Tatsro *et al.*, 2002). Kokkeaw and Thawornchinsombut (2007) prepared rice bran protein hydrolysates using Protex 6L. whose maximal radical scavenging activity was only 27.08 %. Recently, albumin, globulin, glutelin and prolamin from Hitimebore (Japanese rice bran) were hydrolyzed with proteases M, N, P, S and pepsin. The globulin hydrolysate from pepsin hydrolysis for 2 h showed high antioxidant activity and nineteen antioxidative peptides were isolated. These peptides were composed of 6–30 amino acid residues with molecular masses ranging from 670 - 3,611 Da. Among them Tyr-Leu-Ala-Gly-Met-Asn had the highest antioxidant activity (Adebiyi *et al.*,2009). Chanput *et al*. (2009) fractionated albumin, globulin, prolamine and glutelin from KDML 105 rice bran and prepared the protein hydrolysates by

enzymatic hydrolysis of these protein fractions using trypsin and chymotrypsin. It was found that the protein hydrolysate contained higher antioxidant activity than original proteins. The antioxidant properties of the hydrolysates, largely depend on protease specificity, degree of hydrolysis (DH) and nature of released peptides (e.g., molecular weight and amino acid composition). They have been attributed to cooperative or combined effects of a number of properties, including their ability to scavenge free radicals, to act as chelating agents of metal ions, or act as hydrogen donor (Tang, *et al.*, 2009).

14. Isolation of antioxidative peptides by reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase. Figure 11 shows the binding of a peptide to a reversed-phase surface.

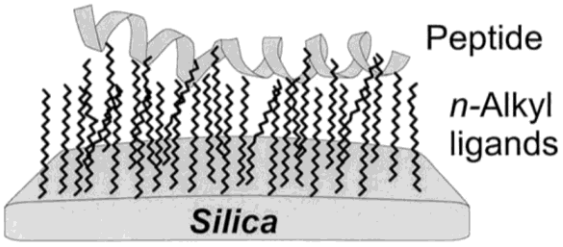


Figure 11 Schematic representation of the binding of a peptide to an RP-HPLC silica-based sorbent.

Source: Modified from Aguilar (2004)

The peptide interacts with the immobilized hydrophobic ligands through the hydrophobic chromatographic contact region (Aguilar, 2004). RP-HPLC is a very powerful technique for the analysis of peptides and proteins as a result of four factors.

First, the excellent resolution that can be achieved under a wide range of chromatographic conditions for very closely related molecules as well as structurally quite distinct molecules. Second, the experimental ease of which chromatographic selectivity can be manipulated through changes in mobile phase characteristics. Third, the excellent reproducibility of repetitive separations carried out over a long period of time. And finally, it is generally high recovery and high productivity in term of cost parameters (Aguilar, 2004). The RP-HPLC experimental system for the analysis of peptides and proteins usually consists of an n-alkyl silica-based sorbent like C18-based sorbent from which the solutes are eluted with gradients of increasing concentrations of organic solvent such as acetonitrile containing an ionic modifier such as trifluoroacetic acid (TFA) (Aguilar and Hearn, 1996). TFA is normally used to be added to a mobile phase at concentration of about 0.1%(w/v). Separations can be easily manipulated by changing the gradient slope, the operating temperature, the ionic modifier or the organic solvent composition. Numerous antioxidative peptides from enzymatic hydrolysis of animal and plant proteins used RP-HPLC to separate and purify before the peptides were characterized. Example of these enzymatic hydrolysates are the antioxidative peptides from squid (Mendis *et al.*, 2005), fish (Bougatef *et al.*, 2010; Kim *et al.*, 2007), bullfrog skin (Qian *et al.*, 2008) and rice bran (Adebiyi *et al.*, 2009).

15. Identification of antioxidative peptides by MS/MS

Mass spectrometry (MS) is a powerful method for the characterization of biological molecules by measuring their mass-to-charge ratio (m/z). Mass spectrometric measurements are carried out on ions in the gas phase. All mass spectrometers consist of three basic components: an ionization source which convert the aqueous phase ions to multiple protonated ions in gas phase, a mass analyzer that separate the ions according to their m/z and a detector to record the number of ions at each m/z. The ‘soft’ ionization techniques, electrospray ionization (ESI) (Fenn *et al.*, 1989) and matrix-assisted laser desorption ionization (MALDI) (Tanaka *et al.*, 1988) have provided for analysis of peptides, proteins, and most other biomolecules and are

now widespread ionization sources for biomolecular mass spectrometry. The most common mass analyzers used for biomolecular analysis contain the quadrupole (Q), ion trap (IT), time-of-flight (TOF), Fourier transform ion cyclotron resonance (FT-ICR), and the more recently developed orbitrap (OT). Tandem mass spectrometry, MS/MS employs two stages of mass spectrometric analysis. This type of analysis is performed using hybrid mass spectrometers that contain two mass analyzers such as; the quadrupole-time-of-flight (Q-TOF), time-of-flight/time-of-flight (TOF/TOF), and linear ion trap-fourier transform ion cyclotron resonance (LTQ-FT-ICR). The peptide ions have to be evaporated in the ion source, then the first MS records the mass to charge ratio (m/z) value and allows the selected peptide ions to pass to the second MS where the ions undergo fragmentation to produce daughter ions for sequence information. The Thermo Scientific LTQ-FT-ICR Ultra hybrid mass spectrometer is a linear ion trap-Fourier transform ion cyclotron resonance instrument (Figure 12) operated primarily with an ESI source used to identify peptides.

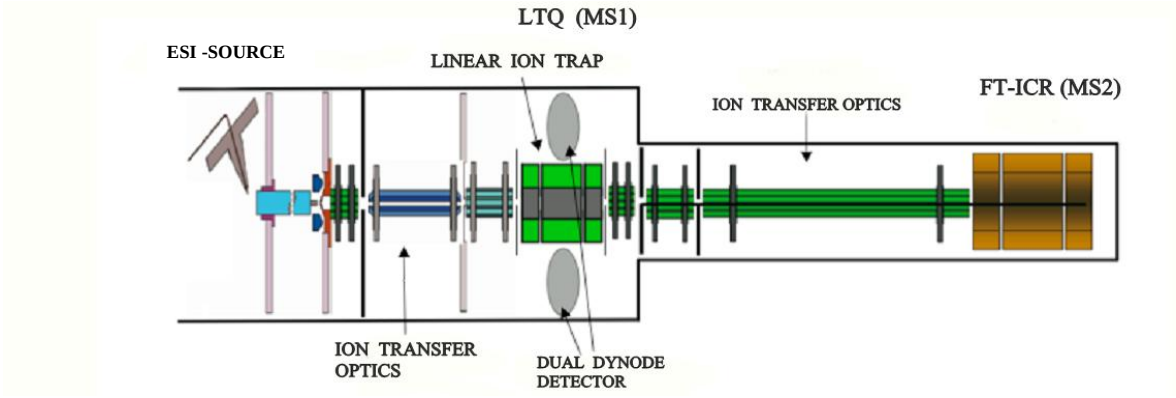


Figure 12 A schematic of the hybrid instruments

Source: Modified from Thermo Fisher Scientific (Waltham, MA).

In general, peptides can be identified by fragmenting them in a mass spectrometer. A nomenclature of peptide fragments that arise from a tandem mass spectrum was proposed by Roepstorff and Fohlman (1984) and modified by Biemann (1990) as shown in Figure. 13.

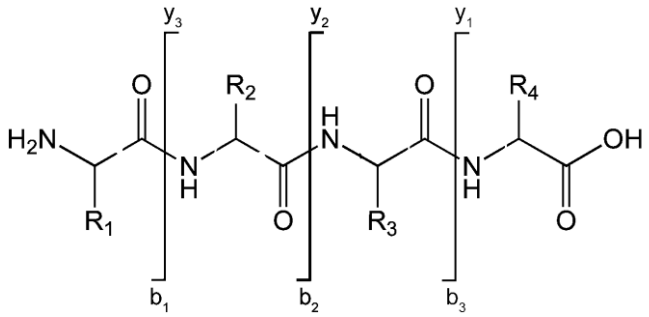


Figure 13 The nomenclature of peptide fragmentation

Source: Modified from Roepstorff and Fohlman (1984), Biemann (1990)

The peptide fragment ions are indicated by b if the charge is retained on the N-terminus and by y if the charge is maintained on the C-terminus. According to this nomenclature, at low energy collision induced dissociation (CID) processed to generate b_1 and y_3 fragment ions as a result of cleavage of peptide bonds.

MATERIALS AND METHODS

Materials

1. Raw materials

Full-fat rice bran (KDML 105) was obtained from Patum Rice Mill and Granary Plubic Co. Ltd. and packed in aluminum foil bag and kept at -20 °C until use.

2. Enzymes

2.1 Alcalase 2.4L activity; 2.4 AU/g (Novo Co., Bagsvaerd, Denmark)

2.2 Papain 3.11 U/mg. Isolated from Papaya (*Carica papaya*) Latex (Fluka BioChemika, Buchs, Switzerland)

2.3 Trypsin 15,000 U/mg (Gold mass spectrometry grade; Promega Corporation., Madison, WI, USA.).

3. Chemicals

3.1 2,2'-Azobis (2-methylpropionamidine) dihydrochloride: AAPH (Analytical reagent; Sigma Co. St. Louis, MO, USA.)

3.2 2,2-diphenyl-2-picrylhydrazyl: DPPH (Analytical reagent; Sigma Co. St. Louis, MO, USA.)

3.3 2-Chloroacetamide (Synthesis reagent; Merck, Darmstadt, Germany)

3.4 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid: Trolox (Analytical reagent; Sigma Co. St. Louis, MO, USA.)

3.5 3-tert-butyl-4-hydroxyanisol: BHA (Analytical reagent; Fluka BioChemika, Buchs, Switzerland)

3.6 Acetic acid (Analytical reagent; Merck, Darmstadt, Germany)

3.7 Acetronitrile (HPLC grade; Lab Scan, Dublin, Ireland)

3.8 Ammonium bicarbonate (Analytical reagent; Sigma Co. St. Louis, MO,

USA.)

3.9 Ammonium sulphate (Analytical reagent; Ajex Finechemical Pty. Ltd., NSW, Australia)

3.10 Bio-Rad Protein Assay Dye Reagent Concentrate (Analytical reagent; Bio-Rad Laboratories Ltd., Hercules, CA, USA.)

3.11 Bovine serum albumin fraction V: BSA (Analytical reagent; Sigma Co. St. Louis, MO, USA.)

3.12 DL-Dithiothreitol: DTT (Analytical reagent; Sigma Co. St. Louis, MO, USA.)

3.13 Ethanol (Analytical reagent; Merck, Darmstadt, Germany)

3.14 Ethylenediaminetetraactic acid Dipotassium salt: EDTA (Analytical reagent; Fluka, Buchs, Switzerland)

3.15 Ferric chloride (Analytical reagent; Sigma Co. St. Louis, MO, USA.)

3.16 Ferrous sulfate (Analytical reagent; Ajex Finechemical Pty. Ltd., NSW, Australia)

3.17 Ferrozine (Analytical reagent; Sigma Co. St. Louis, MO, USA.)

3.18 Fluorescein Sodium salt (Analytical reagent; Sigma Co. St. Louis, MO, USA.)

3.19 Folin & Ciocalteau phenol reagent (Analytical reagent; Fluka, Buchs, Switzerland)

3.20 Gallic acid (Analytical reagent; Sigma Co. St. Louis, MO, USA.)

3.21 Hexane (commercial grade)

3.22 Hydrochloric acid (Analytical reagent; Merck, Darmstadt, Germany)

3.23 L-Cysteine (Analytical reagent; Fluka, Buchs, Switzerland)

3.24 Methanol (Analytical reagent; Merck, Darmstadt, Germany)

3.25 Potassium dihydrogen orthophosphate (Analytical reagent; Ajex Finechemical Pty. Ltd., NSW, Australia)

3.26 Potassium phosphate Dibasic anhydrous (Analytical reagent; Ajex Finechemical Pty. Ltd., NSW, Australia)

3.27 ProteaseMAX™ surfactant (Promega corporation, Madison, USA)

3.28 Pyrocatechol violet (Analytical reagent; Tokyo Chemical Indrstry Co. Ltd., Tokyo, Japan)

3.29 Sodium acetate trihydrate (Analytical reagent; Ajex Finechemical Pty. Ltd., NSW, Australia)

3.30 Sodium carbonate anhydrous (Analytical reagent; Ajex Finechemical Pty. Ltd., NSW, Australia)

3.31 Sodium chloride: NaCl (Analytical reagent; Merck, Darmstadt, Germany)

3.32 Sodium hydroxide (Analytical reagent; Ajex Finechemical Pty. Ltd., NSW, Australia)

3.33 Sulphuric acid (Analytical reagent; Merck, Darmstadt, Germany)

3.34 Trifluoroacetic acid (Spectroscopy Uvaso reagent; Merck, Darmstadt, Germany)

3.35 Tripyridyltriazine: TPTZ (Fluka BioChemika, Buchs, Switzerland)

3.36 Tris(hydroxymethy)aminomethan (Analytical reagent; Bio-Rad Laboratories Ltd., Hercules, CA, USA.)

3.37 Formic acid (Analytical reagent; Merck, Darmstadt, Germany)

4. Equipments

4.1 Auto pipette 0.5-10 µl, 10-100 µl 8-multichannel 10-200 µl (Axygen Biosciences, CA, USA.)

4.2 Centrifugal evaporation (CVE-200D Eyela, Tokyo, Japan)

4.3 Deep freezer -40 °C (HLLF-370, Jouan Nordic, Allerrod, Denmark)

4.4 Dialysis membrane tube (Cellu-Sep T2 Nominal MWCO: 6,000 - 8,000, Membrane Filtration Products, Inc.,Texas, USA.)

4.5 Five digit-balance (Sartorius, Goettingen, Germany)

4.6 Freeze dryer (LyoLab 3000, Jouan Nordic, Allerrod, Denmark)

4.7 High Performance Liquid Chromatography: HPLC (series 1100, Agilent Technologies, California, USA.)

4.8 Incubator (FD53, BINDER Gmbh, Tuttlingen Germany)

4.9 Microplate reader (InfinteM200; TECAN, Salzburg, Austria)

4.10 Microplate reader (sunrise TECAN; Salzburg, Austria)

4.11 Milti Position Magnetic Stirrer (RO-10; IKA, Staufen, Germany)

4.12 pH meter (Do-cu pH meter; Sartorius, Goettingen, Germany)

4.13 Refrigerator SJ-D50H (Sharp Thai Co., Ltd., Bangkok, Thailand)

4.14 Refrigerated centrifuge (Sorvall RC-5C Plus; Dopont, Delaware, USA.)

4.15 Two digit-balance (Sartorius, Goettingen, Germany)

4.16 Ultra filtration concentrators (Vivaspin 20 MWCO: 5,000, Vivaspin 2 MWCO: 3,000; GE Healthcare Bio-Sciences AB., Uppsala, Sweden)

4.17 Vortex (Pro VSM-3; Oxford, CT, USA.)

4.18 EVEREST ™C18, 250 x 10 mm, 5 µm (Grace Davison Discovery Sciences, IL, USA.)

4.19 LTQ-FT Ultra mass spectrometer (Finnigan™ LTQ FT MS™; Thermo Electron Corporation, CA, USA.)

4.20 nanoAcquity UPLC (Waters Corporation, MA, USA.)

4.21 Zorbax 300SB-C18, 250 x 0.3 mm, 5 µm (Agilent Technologies, CA, USA.)

Methods

1. Preparation of rice bran protein and their hydrolysates

Preparation of alkali extracted rice bran protein (AE-RBP)

Full-fat rice bran (KDML 105) was defatted by extracting twice with three volumes of hexane. The defatted rice bran was air-dried overnight under fume hood, then ground and sieved through an 0.5 mm screen (Wang *et al*., 1999).

AE-RBP was prepared using alkali extraction method of followed by isoelectric point precipitation (Adebiyi *et al*., 2009; Gnanasambandam and Hettiarachchy, 1995) with some modification. The defatted rice bran (200 g) was

stirred in water (rice bran : water, 1:4) and control pH at 9.5 with 1 N NaOH for 45 min. The slurry was centrifuged at 10,000 × g, 25 °C for 30 min. The sediment was discarded and the supernatant was collected. The pH of the supernatant was adjusted to 4.5 and left to stand for 1 h at 4 °C. The precipitate was washed twice with distilled water by centrifuging at 10,000 × g, 25 °C for 30 min. The sediment was alkali extracted rice bran protein (AE-RBP). AE-RBP was dispersed in distilled water, and the pH was adjusted to 7.0 and lyophilized. The freeze-dried AE-RBP (FD-AE-RBP) was stored at -20 °C until use (Gnanasambandam and Hettiarachchy, 1995). Crude protein and moisture content in defatted rice bran, AE-RBP and FD-AE-RBP were determined by the Kjeldahl method (AOAC, 1995). Total phenolic compounds in FD-AE-RB were determined according to the method of Tangkanakul *et al*. (2005) with slightly modifications. The modified method of Gnanasambandam and Hettiaracchchy (1995) was used to measure the nitrogen solubility of FD-AE-RBP.

Preparation of rice bran protein hydrolysates from AE-RBP

AE-RBP was suspended in 50 mM Tris-HCl buffer, pH 8.0 (50 ml/g rice bran protein, 2% protein) and hydrolyzed with alcalase (enzyme:substrate; 20 µl:g rice bran protein) or papain (enzyme:substrate; 1:100 w/w) at 50 and 37 °C in a water bath with constant agitation, respectively. The degree of hydrolysis (DH) was determined at hydrolysis times of 30, 60 and 90 min. The enzymatic hydrolysis was terminated by heating in a boiling water bath for 5 min. The protein hydrolysates were centrifuged at 12,000 × g, 15 °C for 20 min. The supernatants were stored at -20 °C until use.

2. Effect of using FD-AE-RBP combined with commercial antioxidant

The commercial antioxidants namely BHA and α-tocopherol were normally used to prevent oxidation in food. To replace or decrease these commercial antioxidants by using rice bran protein, an effect of RBP on them need to be studied. FD-AE-RBP combined with BHA or α-tocopherol and their antioxidant activities

were determined. DPPH radical scavenging activity of FD-AE-RBP, BHA, α-tocopherol, the mixture of FD-AE-RBP and BHA and the mixture of FD-AE-RBP and α-tocopherol was determined according to the methods as described below by preparing an amount of protein for each sample in the range of 20-60% DPPH radical scavenging activity. of These samples were also used to determine antioxidant activity by FRAP.

3. Preparation of rice bran protein fraction (RBPF) and their enzymatic hydrolysates

Free phenolic compounds were extracted from defatted rice bran following the method of Adom and Liu (2002) with a few modifications. The defatted rice bran was shaking with 80% ethanol (defatted rice bran: 80% ethanol 1:5) for 30 min. After centrifugation at 3,600 × g for 15 min, the supernatant was discarded and the extraction was repeated one more time. The residue was air-dried overnight under the fume hood and was ground.

RBPF was fractionated according to the method of Chanput *et al*. (2009) with slight modification. The defatted rice bran which free phenolic compounds have been removed (10.0 g) was first fractionated by extracting with distilled water (rice bran: water 1:6) by stirring on a magnetic stirrer for 60 min, filtered pass nylon cloth 100 mesh and centrifuging at 10,000 × g, 20 °C for 30 min to obtain the albumin fraction. The rice bran residue was repeatedly extracted with 40 ml of distilled water and the supernatant was pooled for albumin fraction. This fraction was saturated to 70% ammonium sulfate at final concentration, the precipitate was dispersed in distilled water then it was desalted in water using dialysis tube (MWCO: 6,000 - 8,000) for 15 h at 25 °C. The rice bran residue after extraction of albumin was extracted with 60 ml of 2% NaCl to recover the globulin fraction (supernatant). The residue after extraction of globulin was further extracted with 0.1 N NaOH to yield the glutelin fraction (supernatant), then the rice bran residue was extracted with 70% ethanol to obtain prolamin fraction. Each extraction step was performed the same method as

albumin extraction. The supernatant of globulin and glutelin fraction were desalted in 50 mM ammonium bicarbonate buffer pH 7.8 using dialysis tube (MWCO: 6,000 - 8,000) for 15 h at 25 °C.

Each fraction was concentrated and desalted using centrifugal ultra filtration MWCO 5,000 Da. The concentrated protein fractions were freeze-dried and stored at -20 °C until use. Crude protein was determined by Bradford's method (Bradford, 1976) and the Kjeldahl method AOAC (1990).

Preparation of rice bran protein hydrolysates from RBPFs

RBPFs were hydrolyzed with trypsin under the conditions recommended by the manufacturer of Promega Corporation (Technical Bulletin No.373). One hundred µg of RBPF were dispersed in 91.5 µl of 50 mM ammonium bicarbonate buffer pH 7.8, then 2 µl of 1% ProteaseMAX™ surfactant in 50 mM ammonium bicarbonate buffer pH 7.8 and 1 µl of 0.5 M DTT were added. The solution were incubated at 56 °C for 20 min, then 2.7 µl of 0.55 M chloroacetamide was added and incubated in the dark at room temperature for 15 min. After that 1 µl of 1% ProteaseMax™ surfactant and 1.8 µl of 1 µg/µl trypsin in 50 mM acetic acid (enzyme : protein 1.8:100 w/w) were added. The hydrolysis was carried out at 37 °C for 3 h. The enzyme was rapidly inactivated by adding TFA to a final concentration of 0.5%, mixed and incubated at room temperature for 5 min, then centrifuged at 12,000 × g 20 °C 10 min. The supernatant was freeze-dried and kept at -20 °C for further studies. Trypsin hydrolysis for native protein was done without DTT and chloroacetamide, briefly, 100 µg of RBPF were dispersed in 98.2 µl of 50 mM ammonium bicarbonate buffer pH 7.8 and add 1.8 µl of 1 µg/µl trypsin. The hydrolysis was the same trypsin digestion process as described above.

RBPFs were hydrolyzed with papain by using 20 mM phosphate buffer pH 7.0 containing 2 mM EDTA and 5 mM cysteine instead of 50 mM ammonium

bicarbonate buffer pH 7.8 and enzyme:protein was 5:100 w/w. The denatured and the native protein were hydrolyzed as same as trypsin hydrolysis process.

4. Determination of antioxidant activities

4.1. DPPH radical scavenging assay

DPPH radical-scavenging activity was determined as described by Wu *et al*. (2003) with a slight modification. To dilute sample (0.1 ml), 1.5 ml of 0.10 mM DPPH in ethanol were added. The mixture was then mixed vigorously and allowed to stand in dark at room temperature for 60 min. The samples were diluted to result of 50-80% DPPH radical scavenging activity range. The absorbance of the resulting solution was read at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that distilled water was used instead of a sample. BHA (5-15 µg) was used for the calibration curve. The scavenging activity was calculated as follows:

$$\text{Radical-scavenging activity (\%)} = \frac{(B - A) \times 100}{B}$$

Where A is absorbance of the sample at 517 nm and B is absorbance of the blank at 517 nm.

4.2 Ferric Reducing Ability Power (FRAP)

The reducing ability of samples was measured using a modified method described by Benzie and Strain (1996). AE-RBP, AE-RBP's hydrolysates and FD-AE-RBP were measured by using test tube assay and microplate assay were used for RBPFs and peptide fractions which were separated from trypsin-hydrolyzed denatured albumin hydrolysates by using RP-HPLC.

Test tube assay

The FRAP reagent consisted of 3.0 ml of 10 mM tripyridyltriazine (TPTZ) in 40 mMHCl, 3.0 ml of 20 mM $FeCl_3 \times 6H_2O$ in distilled water and 30 ml of 300 mM sodium acetate buffer pH 3.6. The solution must be freshly prepared and was also used for blank. One milliliter and 800 µl FRAP reagent was added to test tube, warmed to 37 °C; and 50 µl of sample, along with 150 µl of H_2O, was then added. Absorbance at 593 nm of samples as well as blank solutions was measured at 6 min. The absorbance at 6 min was compared with standard aqueous Fe^{2+} ($FeSO_4 \times 7H_2O$) and expressed the amount of Fe^{2+} from the reduction of Fe^{3+}.

Microplate assay

An aliquot (40 µl) of each sample (with appropriate dilution, if necessary) or standard Trolox was added to the well of a 96-well flat bottom microplate in duplicates followed by addition of 300 µl of pre-warm FRAP reagent (preparation as above). Absorbance (593 nm) was taken after 15 min incubation at 37 °C in a microplate reader (TECAN model Infinite M200). Antioxidant activity was calculated against a standard solution of Trolox and expressed as µmol Trolox equivalents or ferrous sulfate per g protein (µmol of TE/g protein) of sample.

4.3 Oxygen radical absorbance capacity (ORAC)

ORAC was measured according to the method of Huang *et al*. (2002) with some modifications using microplate reader (TECAN model Infinite M200). The method measures the scavenging activity against peroxyl radical generated by decomposition of AAPH at 37 °C and fluorescein was used as the substrate. All reagents and samples were prepared in 75 mM of potassium phosphate buffer (PBS), pH 7.4. Fluorescence condition were as follow: excitation at 485 nm and emission at 535 nm. Trolox was used for defining the standard curve. The net area under the curve (AUC) was obtained by subtracting the area under the curve of the blank from that of

the sample. The antioxidant activity were expressed as TE using the standard curve in µmol of TE/g protein.

4.4 Copper chelating activity

Cu^{2+}-chelating activity as measured according to Carrasco-Castilla *et al*. (2012) with a slightly modification. Sodium acetate buffer, pH 6 (280 µl, 50 mM), 6 µl of 4 mM pyrocatechol violet prepared in the same buffer, and 10 µl of 1 µg/µl $CuSO_4 \times 5H_2O$ were added to hydrolysates or peptide fractions and ethylenediaminetertraacetic acid (EDTA) was used as a positive control. Distilled water was used as a blank instead of a sample. Absorbance at 632 nm was measured using a microplate reader (InfinteM200, TECAN, Salzburg, Austria). Copper chelating activity was calculated as described follow:

$$\% \text{ Chelating activity } = \frac{(A_{control} - A_{sample}) \times 100}{A_{control}}$$

Where $A_{control}$ is absorbance of the blank at 632 nm and A_{sample} is absorbance of the sample at 632 nm.

4.5 Total free phenolic compounds

Total phenolic content was estimated by analysis of the content of free phenolic compounds potentially oxidizable groups by means of the Folin Ciocalteu method in a 96-well microplate as described by Tangkanakul *et al*. (2005) with a few modifications. Aliquot (20 µl) of sample was added with 100 µl of 0.2 N Folin Ciocalteu reagent and stood at room temperature for 8 min. Then 80 µl of 7.5% sodium bicarbonate and 50 µl of water were added to each well. Absorbance (765 nm) was taken after 30 min incubation at 40 °C in a microplate reader (TECAN model Infinite M200). Measurements were carried out in triplicate. Data were expressed as milligrams of gallic acid equivalent (GA)/100 g of product. A calibration curve of

gallic acid (25-150 mg/ml) was constructed and employed for free phenolic compounds determination.

5. Degree of hydrolysis

The degree of hydrolysis (DH) was determined by the ratio of the percentage of 10 % trichloroacetic acid (TCA)-soluble nitrogen to total nitrogen in the sample (Qi *et al.,* 1997). An aliquot of hydrolyzed AE-RBP was mixed with 20% TCA to create 10 % TCA and then centrifuged at 9,800 x g, 25 °C for 15 min. The soluble nitrogen in the supernatant was determined by the Kjeldahl method (AOAC, 1990). The percent DH was calculated as follows:

$$\text{DH (\%)} = \frac{\text{Soluble nitrogen in 10 \% TCA solution (mg)}}{\text{Total nitrogen in AE-RBP (mg)}} \times 100$$

6. Isolation of antioxidative peptides

The HPLC system (Agilent1100 series) equipped with vacuum degassing, quaternary pump, diode array detector, auto sampler computer with chemstation software and fraction collector (Bio-Rad Model 2110). The lyophilized albumin RBPH which showed the highest antioxidant activity (1 mg protein/ml) was dissolved in 0.1% TFA in water, centrifuge at 12,000 × g, 20 °C for 15 min and the supernatant was performed on a Vydac C18 column EVEREST column (10 × 250 mm). The mobile phase was 0.085% TFA in acetonitrile as eluent A and 0.1% TFA in water v/v) as eluent B. The flow rate was 1.0 ml/min and gradient applied was 100% (v/v) B over 5 min, then 0–80% (v/v) A over 45 min, 80-100% (v/v) A over 10 min. Online UV absorbance were detected at 215 and 280 nm. The elution fractions were collected at a volume of 2.0 ml and concentrated using centrifugal evaporation at 5,000 rpm 40 °C under vacuum then freeze-dried and kept at -20 °C for further use. Each fraction from the RP-HPLC was suspended in suitable solution to evaluate antioxidative capacity and its amino acid sequence was identified.

7. Identification of peptides sequence by LC–MS/MS using LTQ-FT Ultra

The freeze-dried sample was dissolved in 0.1% formic acid. After cleaning up using C18 ZipTip pipette tips, 2 µl of each sample was analyzed on a LTQ-FTICR (Fourier transform ion cyclotron resonance) with an IonMax electrospray ion source (Thermo) to perform data dependent MS/MS analysis. The mass spectrometer was coupled to a nanoAcquity Ultra performance LC system (Waters) equipped with a Michrom Peptide CapTrap column and a C18 column (Zorbax 300SB-C18, 250 × 0.3 mm, 5 µm). A binary gradient system was used consisting of solvent A, 0.1% aqueous formic acid and solvent B, acetonitrile containing 0.1% formic acid. Peptides were trapped and washed with 1% solvent B for 3 min. Peptide separation was achieved using a linear gradient from 3% B to 30% B at a flow rate of 4 µl /min over 35 min. The mass spectrometer was operated in a data-dependent mode. A full FT-MS scan (m/z 350-2000) was alternated with collision-induced dissociation (CID) MS/MS scans of the five most abundant doubly or triply charged precursor ions. The survey scan was acquired in the ICR cell, while the CID experiments were performed in the linear ion trap where precursor ions were isolated and subjected to CID in parallel with the completion of the full FT-MS scan. CID was performed with helium gas at a normalized collision energy 35% and activation time of 30 ms. Automated gain control (AGC) was used to accumulate sufficient precursor ions (target value, 5×10^4/micro scan; maximum fill time 0.2 s). Dynamic exclusion was used with a repeat count of 1 and exclusion duration of 60 s. Data acquisition was controlled by Xcalibur (version 2.0.5) software (Thermo).

8. Database search

Thermo RAW data files were processed with Proteome Discoverer version 1.2 using default parameters. A Mascot (version 2.3.0) search against whole SwissProt 2010 database or the Oryza sativa Uniprot database (unknow version, 60544 entries) was performed using Proteome Discoverer with the following parameters: the digestion enzyme was set to Trypsin/P and one missed cleavage site was allowed. The

precursor ion mass tolerance was set to 5 ppm, while fragment ion tolerance of 0.8 Da was used. Dynamic modifications included carbamidomethyl (+57.0214 Da) for Cys and oxidation (+15.9994 Da) for Met. Results of protein IDs were summarized by Scaffold 3 (Proteome Software, OR).

9. Statistical Analysis

Three sample replications (three replicate preparations and hydrolysis of each sample) were performed. Data were analyzed by ANOVA and least significant difference (LSD) procedures to separate means, and differences were reported as significant at $p \leq 0.05$, using standard statistical software package.

RESULTS AND DISCUSSION

1. Preparation of rice bran protein and their hydrolysates

Preparation of alkali extracted rice bran protein (AE-RBP)

Protein content in defatted rice bran KDML 105 that determined by Kjedahl method was 14.66% (w/w). The protein content of the AE-RBP was 16-17% (w/w) on a wet basis. The yield of protein in AE-RBP was 23.21 g/100 g of total protein in rice bran. RBP was classified base on its solubility properties in water (albumin), salt (globulin), alcohol (prolamine) and alkaline (glutelin). The protein in AE-RBP might be mainly of glutelin because it is mainly alkali-soluble protein in rice bran (Hamada, 1997; Adebiyi *et al*., 2007; 2009).

Enzymatic hydrolysis of AE-RBP

The hydrolysis of rice bran protein with Alcalase 2.4L proceeded at a rapid rate during the initial 30 min and then slowed down afterward (Figure 14). DH values

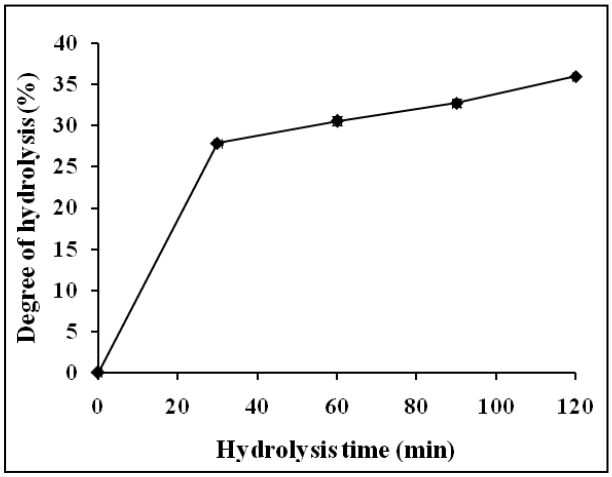


Figure 14 Enzymatic hydrolysis of rice bran protein with Alcalase 2.4L
Data represents the average ± SD of two independent experiments.

varied from 27.8% to 35.9% during 30-120 min of hydrolysis time. This was typical for hydrolysis curves reported by Zheng *et al*. (2006). They studied the hydrolysis of corn gluten with Alcalase 2.4L where the DH value was 24.0-31.2% during the same incubation time. Abayomi *et al*. (2008) also reported that rice bran protein hydrolyzed with protease showed a high rate of hydrolysis for the first hour and DH slightly increased thereafter.

2. Antioxidant activities of AE-RBP and its hydrolysates (AE-RBPHs)

Because the results of antioxidant activity depend on many factors in a given evaluation, the antioxidant activity of AE-RBP and AE-RBPHs can be better characterized with different assays based on different mechanisms. DPPH radical scavenging activity and the other method based on the reducing power were used. The DPPH radical scavenging activity of AE-RBP and alcalase-hydrolyzed AE-RBP are shown in Figure 15.

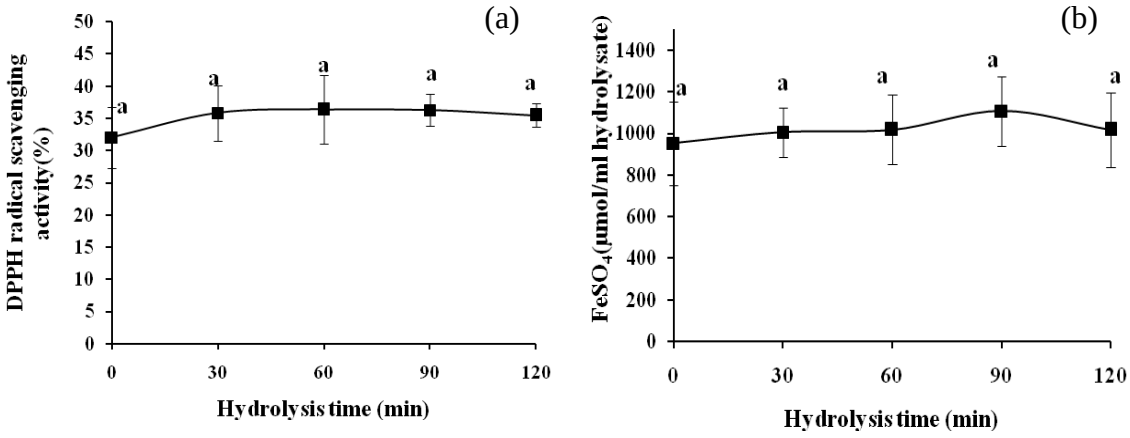


Figure 15 Antioxidant activity on DPPH radical scavenging activity (a) and FRAP assay (b) of alcalase-hydrolyzed AE-RBP with different time of hydrolysis.

Bars marked by the same letter are not significantly different ($p > 0.05$).

The DPPH radical scavenging activity of AE-RBP and alcalase-hydrolyzed

AE-RBP during hydrolysis times of 30 to 120 min was 32.1±4.75 to 35.5±1.83%. FRAP value of AE-RBP and its hydrolysates showed similar trend as DPPH radical scavenging activity. They were 951 ± 201 to 1018 ± 178 µM of Fe^{+2} /ml hydrolysate. The DPPH radical scavenging activity and FRAP value of the alcalase-hydrolyzed AE-RBP were not significantly different from those of AE-RBP. Several studies have displayed that aggregation of hydrolysate is the consequence of hydrolysis by glutamyl endoprotease enzymes (Creusot and Gruppen, 2007). Alcalase performs both subtilisin and glutamyl endoprotease activity, so the peptides in alcalase-hydrolyzed AE-RBP possibly co-aggregated during hydrolysis times of 30 to 120 min. At hydrolysis time of 30 to 60 min, the DPPH radical scavenging activity and FRAP value of papain-hydrolyzed AE-RBP were 49 to 56% and 3,993 to 4,257 µM of Fe^{+2}/ml hydrolysate, respectively (Figure 16).

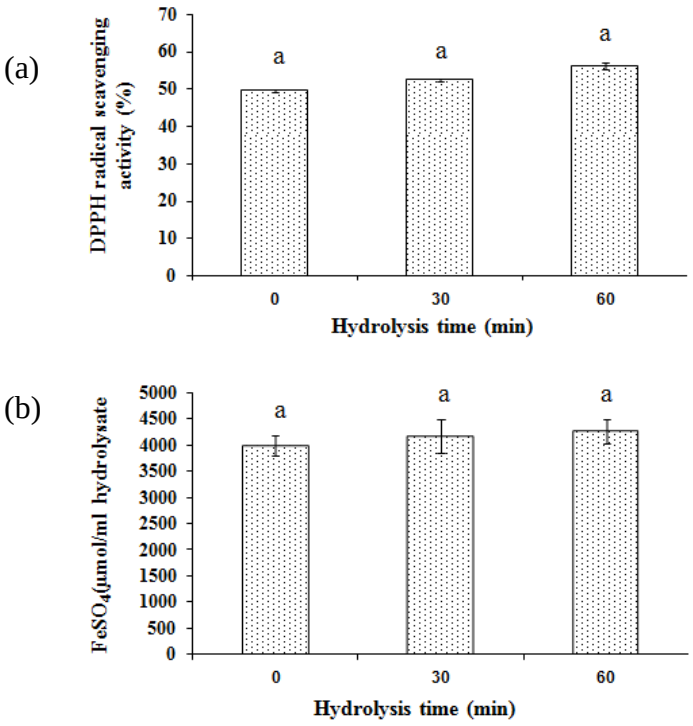


Figure 16 Antioxidant activity on DPPH radical scavenging activity (a) and FRAP assay (b) of papain-hydrolyzed AE-RBP with different time of hydrolysis. Bars marked by the same letter are not significantly different ($p > 0.05$).

The DPPH radical scavenging activity and FRAP value of both papain-hydrolyzed and alcalase-hydrolyzed AE-RBP were non-significantly different from AE-RBP. One reason could be because AE-RBP that contained main glutelin was high molecular weight and difficult to be hydrolyzed (Fabian and Ju, 2011). It can be hydrolyzed into small peptides for strong condition. Therefore, the antioxidant activity of AE-RBP was compared to BHA. As shown in Figure 17, BHA, a synthetic antioxidant displayed 50% DPPH radical scavenging activity at 11.90 µg (Figure 17 a) while AE-RBP exhibited 50% at 1.45 mg protein. (Figure 17b). It suggested that AE-RBP may be used to replace BHA in food products. However, the antioxidant AE-

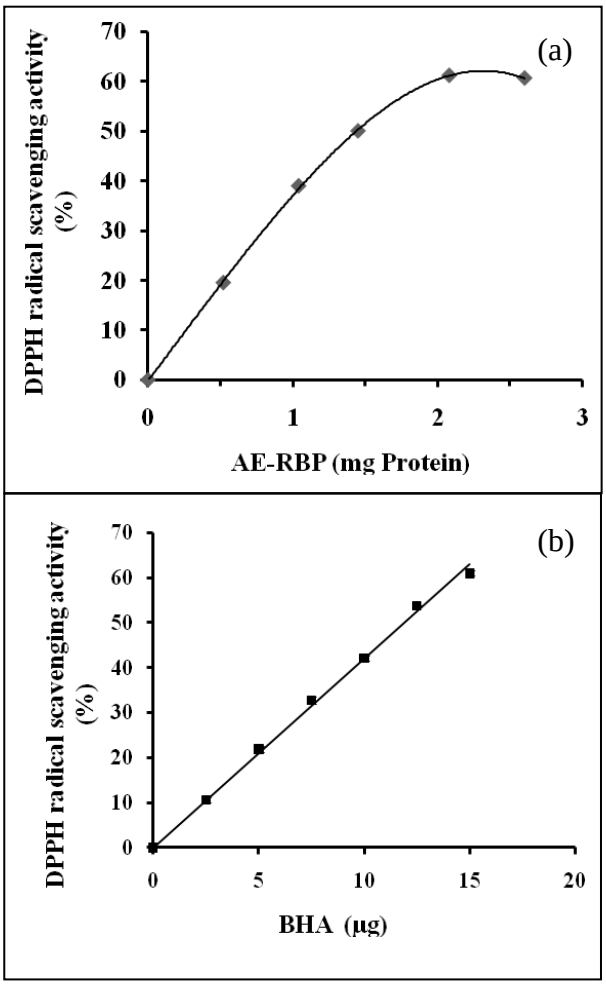


Figure 17 DPPH radical scavenging activity of AE-RBP (a) and BHA (b)
Data represents the average ± SD of two independent experiments

RBP in liquid form might not be stable, therefore, solid form of AE-RBP should be prepared and further studied. The freeze-dried AE-RBP (FD-AE-RBP) was prepared. The moisture content, protein and total phenolic content were 4.09%, 53.65% and 18.78 µg/g FD-AE-RBP, respectively. The nitrogen solubility of FD-AE-RBP was 23.59 % (dry basis).

Antioxidant activities of AE-RBP and FD-AE-RBP

The effect of freeze-drying on antioxidant activity of AE-RBP was investigated. It was found that DPPH radical scavenging activity of AE-RBP and FD-AE-RBP was not significantly different with the values of 39.10 ± 4.97 and 41.91 ± 3.44%, respectively (Table 5). FRAP values of AE-RBP and FD-AE-RBP were also not significantly different, they were 94.8 and 92.6 µmol $FeSO_4$/g protein, respectively. It indicated that freeze-drying has no significantly effect on antioxidant activity of RBP. Moreover, FD-AE-RBP combined with commercial antioxidant including BHA and α-tocopherol were further investigated.

Table 5 Antioxidant activities of AE-RBP and FD-AE-RBP

Sample	DPPH scavenging activity (%)	FRAP (µmol $FeSO_4$ /g protein)
AE-RBP	39.10 ± 4.97[a]	94.8 ± 15.6[a]
FD-AE-RBPE	41.91 ± 3.44[a]	92.6 ± 6.7[a]

Values are mean ± SD (n = 3). Means in the same column with different letters are significantly different ($p \leq 0.05$)

3. Using FD-AE-RBP combined with commercial antioxidants

DPPH radical scavenging activity and FRAP value of FD-AE-RBP, BHA and α-tocopherol at different amount are shown in Figure 18 and 19, respectively. The

relationship of DPPH radical scavenging activity and BHA or α-tocopherol content are linear at 0-15 µg while the relation of DPPH radical scavenging activity and protein content of FD-AE-RBP is linear to 1500 µg (Figure 18).

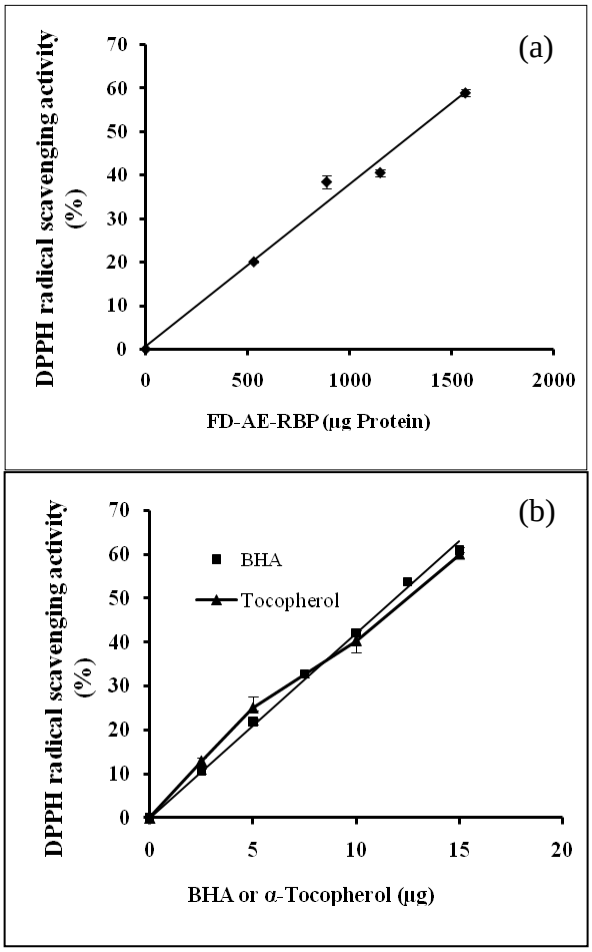


Figure 18 Effect of the amount of FD-AE-RBP (a), BHA and α-tocopherol (b) on DPPH radical scavenging activity.
Data represents the average ± SD of two independent experiments

The relationship of FRAP value and BHA or α-tocopherol content are linear at 0-4 µg while the relationship of FRAP value and protein content of FD-AE-RBP is linear at 0-636 µg (Figure 19). When protein content was higher than 636 µg, FRAP only slightly increased and its relation was no longer linear. The results indicated that

protein content should be in the range of 0-636 µg for the FRAP assay. Therefore, the amount of

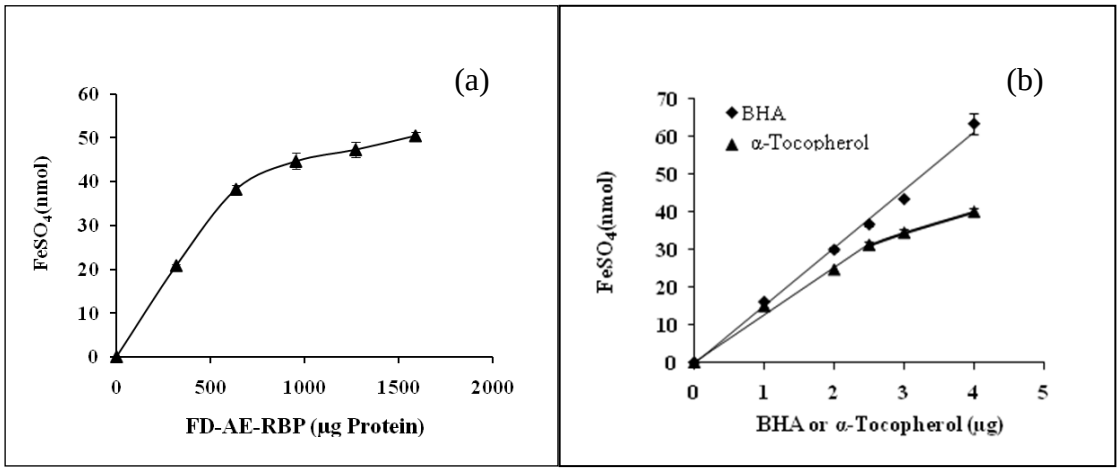


Figure 19 Effect of the amount of FD-AE-RBP (a), BHA and α-tocopherol (b) on FRAP value.
Data represents the average ± SD of two independent experiments

protein must be in the range of 0-636 µg when the effect of FD-AE-RBP combined with BHA or α-tocopherol is studied.

DPPH scavenging activity of FD-AE-RBP, BHA, α-tocopherol and mixture of FD-AE-RBP and BHA or α-tocopherol are shown in Table 6. DPPH radical scavenging activity of 530 µg protein of FD-AE-RBP was not significantly different from the 5 µg of BHA or α-tocopherol ($p > 0.05$). The antioxidant activity of 530 µg protein of FD-AE-RBP combined with 5 µg of BHA was closed to the sum of antioxidant activity of FD-AE-RBP and 5 µg of BHA. Although, antioxidant activity of 530 µg protein of FD-AE-RBP combined with 5 µg of α-tocopherol was less than the sum of FD-AE-RBP and α-tocopherol, it was not significantly different from mixture of FD-AE-RBP and 5 µg of BHA ($p > 0.05$). The results suggested that FD-AE-RBP can replace BHA or α-tocopherol by using of 106 µg protein of FD-AE-RBP to replace 1 µg BHA or α-tocopherol or used FD-AE-RBP combined with BHA or α-tocopherol to inhibit lipid peroxidation. FRAP value of FD-AE-RBP, BHA, α-

tocopherol and a mixture of FD-AE-RBP and BHA or α-tocopherol are shown in Table 7.

Table 6 DPPH scavenging activity of individual FD-AE-RBP, BHA, α-tocopherol and their combination

Sample	DPPH scavenging activity (%)
FD-AE-RBP (530 µg protein)	20.12 ± 0.28^{a}
BHA (5 µg)	22.16 ± 0.61^{a}
α-tocopherol (5 µg)	22.12 ± 5.11^{a}
FD-AE-RBP(530 µg protein) + BHA(5 µg)	41.79 ± 1.46^{b}
FD-AE-RBP (530 µg protein) + α-tocopherol (5 µg)	36.34 ± 1.10^{b}

Values are mean ± SD (n = 3). Means with superscripts are significantly different ($p \leq 0.05$)

Table 7 FRAP value of FD-AE-RBP, BHA, α-tocopherol and their combination

Sample	FRAP (nmol $FeSO_4$)
FD-AE-RBP (640µg protein)	38.30 ± 0.98^{a}
BHA (2 µg)	30.01 ± 0.61^{b}
FD-AE-RBP(640 µg protein) + BHA(2 µg)	62.07 ± 1.46^{c}
FD-AE-RBP (950 µg protein)	46.47 ± 2.42^{d}
α-tocopherol (2.5 µg)	24.67 ± 2.42^{e}
FD-AE-RBP (950 µg protein) + α-tocopherol (2.5 µg)	71.35 ± 3.85^{f}

Values are mean ± SD (n = 3). Means with different letters are significantly different ($p \leq 0.05$)

The concentration of FD-AE-RBP, BHA, α-tocopherol and combination of them were different in each sample due to the limitation for each sample assay

(Figure 19). When the concentration of protein increased more than 640 µg, the relation of FRAP value and protein concentration was no longer linear. FRAP value of mixture of FD-AE-RBP (640 µg protein) and BHA (2 µg) was lower than the sum of FD-AE-RBP and BHA, while 950 µg protein of FD-AE-RBP combined with α-tocopherol (2.5 µg) was closed to the sum of them (Table 7). This revealed that FD-AE-RBP effected on FRAP value of BHA when it was combined with BHA. FD-AE-RBP had no effect on FRAP value of α-tocopherol when they were combined. These results suggested that FD-AE-RBP should not be used to combine with BHA but it should be considered to replace BHA in food products. The amount of FD-AE-RBP to be used as a replacement of BHA or α-tocopherol should be determined equivalent to BHA or α-tocopherol and depended on the type of antioxidant activity assay.

RBP contained albumin, globulin, prolamine and glutelin that they are soluble in water, salt solution, alcohol solution and alkali solution, respectively. From the preliminary study, AE-RBP was extracted from rice bran by 1 N NaOH at pH 9.5, so the protein in AE-RBP shoud be mainly glutelin. Yield of protein from rice bran was little that it was only 23.21% of total protein in rice bran. Therefore, the further study, RBP was fractionated base on solubility properties of protein in order to increase protein's yield. In addition, AE-RBP hydrolysates obtained from alcalase and papain hydrolysis were not remarkable antioxidant activity. It's possible that enzymatic condition, protein source and kind of enzyme were not reasonable, for this reason, RBP was denatured before hydrolysis. Papain and trypsin would be used to study due to optimum temperature was the same as 37 °C.

3. Preparation of rice bran protein fractions (RBPF)

RBPF was extracted from defatted rice bran that removed phenolic compounds by 80% methanol with difference solvent solubility. RBP was classified base on its solubility properties in water (albumin), salt (globulin), alcohol (prolamin) and alkaline (glutelin). Protein contents of RBPFs are shown in Table 8. Total protein of RBPFs was 7.76 % w/w of rice bran. The protein content in rice bran was 14.66%

(w/w) as reported above. Thus, yield of protein was 52.93% of total protein. The low yields obtained in this experiment because it may be lose during extraction processes.

Table 8 Protein contents of RBPFs.

Rice bran protein fractions	Protein (% w/w of rice bran)	Protein (g/100 g total protein in rice bran)[1]
Albumin	0.98 ± 0.01^{a}	12.53
Globulin	1.00 ± 0.03^{a}	13.85
Prolamin	0.23 ± 0.00^{b}	2.89
Glutelin	5.55 ± 0.03^{c}	70.75
Total	7.76	

Values are mean $\pm$ SD (n = 3), Mean in the same column with different superscripts are significantly different ($p \leq 0.05$).

[1]Protein content equivalents to BSA

In addition, the poor solubility of rice bran protein that cause of its strong aggregation and extensive disulfide bond cross-linking (Hamada, 1997) had effect on the yield. Rice bran proteins are contained protein bodies inside plant cell walls that was disrupted before they can be totally solubilized and extracted (Fabian and Ju, 2011). Therefore, before RBP extraction, cell disruption is also need to use the efficient method for increasing the protein yield. RBP was fractionated to albumin, globulin, prolamin and glutelin and calculated as 12.5, 13.9 2.9 and 70.8% of the total rice bran protein respectively. Hamada (1997) reported the average rice bran protein composition of 6 varieties of rice bran as albumin, globulin, prolamin and glutelin to be 34%, 15%, 6% and 11%, respectively. Adebiyi *et al*. (2009) studied the optimization of RBP extraction method using a Japanese rice variety and reported the contents of albumin, globulin, prolamin and glutelin to be 24-39, 27-30, 1-3% and 33-42%, respectively. The percent protein ratio of RBPFs are different from those

reports that fractionated using the same solvent. This could be due to the variety of rice bran, extraction and protein determination methods

4. Antioxidant activity of RBPFs and their hydrolysates

RBPFs included albumin, globulin, glutelin and prolamin that are soluble in different solvents (water, NaCl solution, NaOH solution and 70% ethanol). Each fraction was extracted at room temperature, so it's possible that RBP were native form. RBP were poor soluble because of its strong aggregation and extensive disulfide bond cross-linking (Hamada, 1997). Thus, RBPFs were denatured by treatment with surfactant, reducing agent at 56 °C before enzymatic hydrolysis. ProteaseMAX™ Surfactant was used to solubilizes proteins and enhances protein digestion by providing a denaturing environment prior to protease addition. DTT was reducing agent that break the hydrogen bonds and the disulfide bond cross-linking. Then, chloroacetamide was used to alkylate sulhydryl group for preventing disulfide cross-linking. The RBPFs were hydrolyzed by using trypsin and papain for 3 h except none for prolamin because of its low yield. Albumin fraction was precipitated with saturated 70% of ammonium sulfate then concentrated using centrifugal ultrafiltration MWCO 5,000 Da. Globulin and glutelin fraction were concentrated by centrifugal ultrafiltration MWCO 5,000 Da. The free phenolic compounds in RBPFs were decreased after treated by these methods (data not shown). The antioxidant activity of hydrolyzed native and denatured RBPFs were determined by ORAC assay as shown in Figure 20. In considering ORAC value of RBPFs and their hydrolysates which hydrolyzed with trypsin, ORAC value of hydrolysates obtained from denatured RBPFs (1.096-4.067 µmol of TE/mg protein) were significantly higher than that obtained from native RBPFs (0.450-2.301 µmol of TE/mg protein) and non-hydrolyzed denatured RBPFs ($p \leq 0.05$). This might be due to the denaturation of RBPFs, trypsin can cleave all specific peptide bonds in RBPFs. Therefore, the low molecular weight peptides were liberated from denatured RBPFs more than native RBPFs. It had been reported that low molecular weight peptides were more potent as antioxidant activity (Kim *et al.*, 2007; Kitts and Weiler, 2003; Qian *et al.*, 2008).

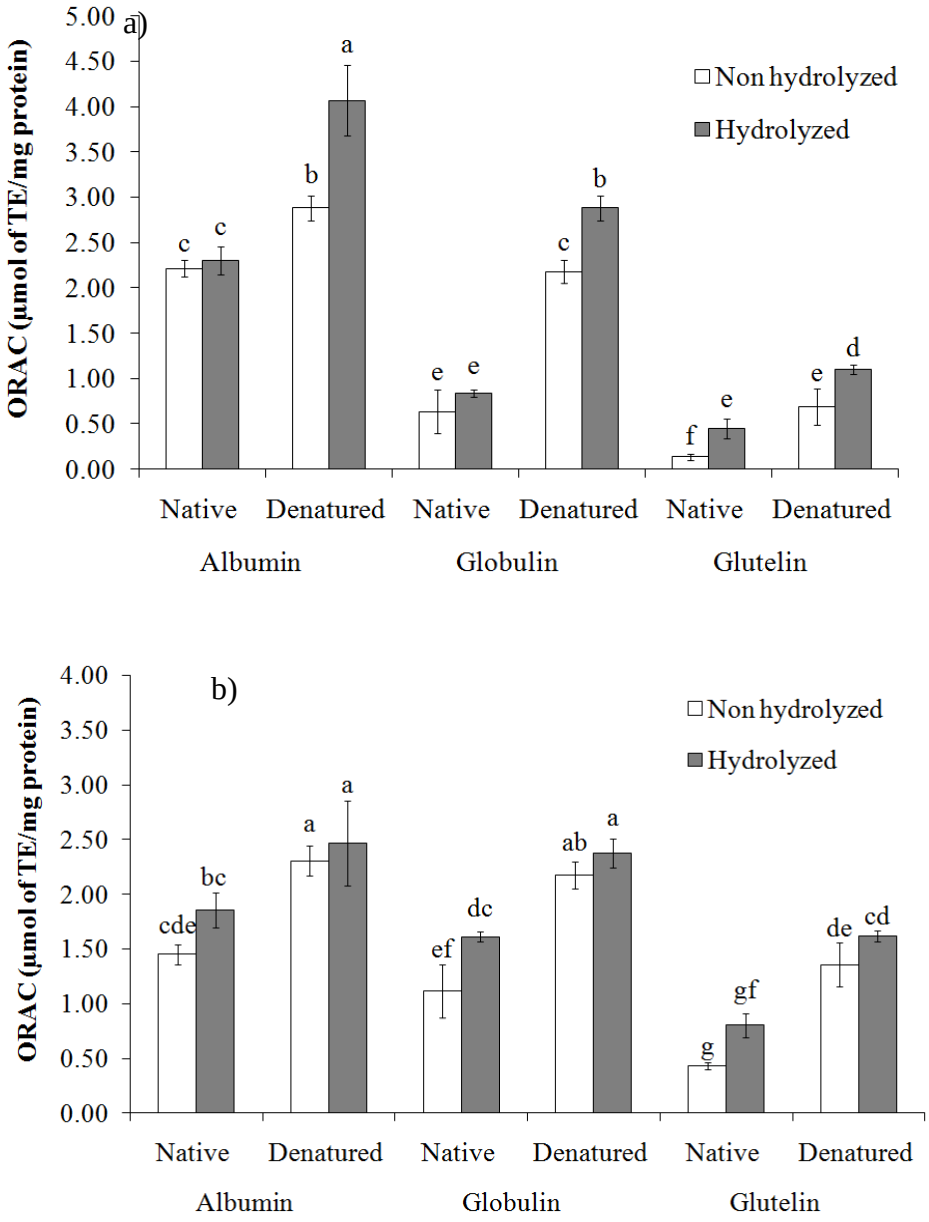


Figure 20 Antioxidant activity of native and denatured rice bran protein that hydrolyzed with trypsin (a) and papain (b) measured by ORAC assay. Bars marked by the different letters are significantly different ($p \leq 0.05$).

When RBPFs were hydrolyzed with papain, the antioxidant activity of hydrolyzed RBPFs were 0.803-2.467 µmol of TE/mg protein and non- significantly different from non-hydrolyzed RBPFs (0.433 - 2.307 µmol of TE/mg protein) ($p > 0.05$) except for the hydrolyzed native globulin. The ORAC values of the papain

hydrolyzed and non-hydrolyzed denatured albumin and globulin were higher than the others. However, the overall results demonstrated that the ORAC values of trypsin hydrolyzed denatured albumin was the highest (4.067 µmol of TE/mg protein). Pena-Ramos and Xiong (2001) also found that whey protein hydrolyzed with trypsin for 6 h showed higher antioxidant activity than that hydrolyzed with papain. Trypsin hydrolyzed peptides liberated from sea cucumber (*Stichopus japonicas*) protein exhibited higher antioxidant activity than papain hydrolyzed peptides (Zhou *et al.*, 2012). It were also observed that antioxidant activity of Ostrich (*Struthio camelus*) egg white proteins hydrolyzed with trypsin was higher than that hydrolyzed with papain. These findings indicate that trypsin hydrolysis produces peptides with higher antioxidant activity than papain hydrolysis. Trypsin is endopeptidase that cleaves peptide bonds at the C-terminal side of lysine and arginine, resulting in the numbers of peptides chains containing independent lysine and arginine at the C-terminal. It has been reported that antioxidant peptide such as Phe-Lys, Asn-Gly-Leu-Glu-Gly-Leu-Lys Phe-Arg, Leu-Asp-Arg, Ile-Arg from royal jelly protein hydrolysates containing lysine or arginine at the C-terminal had high antioxidant activity (Guo *et al.*, 2009). Therefore, the results indicate that the antioxidant activity of the hydrolysates depend on the enzyme specificity and the amino acid sequences of derived peptides.

5. Isolation of antioxidative peptides

The trypsin-hydrolyzed denatured albumin which showed the highest antioxidant activity was isolated by preparative HPLC. Ninety µg of protein were loaded on RP-HPLC column and each fraction (2.0 ml) was collected separately. The HPLC profile of trypsin-hydrolyzed denatured albumin hydrolysate is shown in Figure 21. It was fractionated according to hydrophobicity binding of peptides with stationary phase. The low molecular weight (MW) peptides and low hydrophobicity were eluted at the first and high MW peptides with high hydrophobicity were eluted thereafter. Thirty fractions were collected and their antioxidant activity were evaluated by ORAC assay as shown in Figure 22. ORAC assay directly measures the antioxidant activity of chain-breaking antioxidant against $ROO^{\bullet}$. The fractions no.(F)

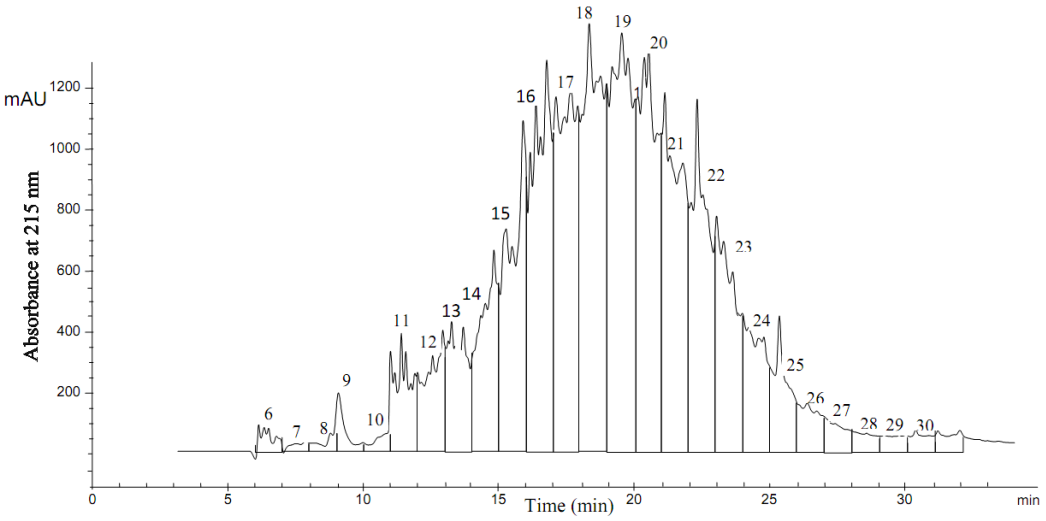


Figure 21 HPLC profile of albumin RBPH. Separation was carried out on a Vydac C18 Everest column (4.6 mm i.d. x 250 mm) using a linear gradient of 0.1% TFA in water and 0.085% TFA in acetonitrile; flow rate 2.0 ml/min; detection absorbance at 215 nm.

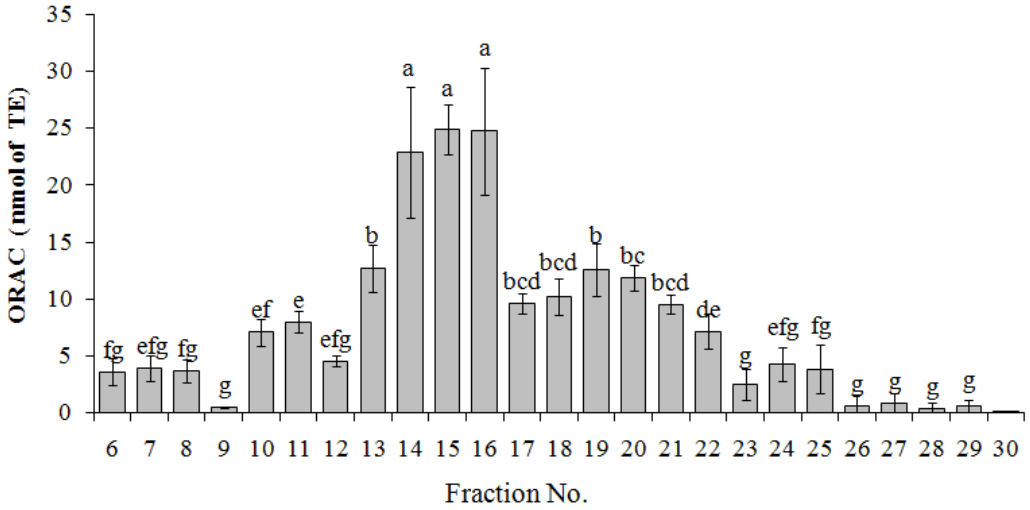


Figure 22 Comparison of the antioxidant activity of peptide fractions were isolated by RP-HPLC. Each fraction (2.0 ml) was freeze-dried and reconstituted in 0.100 ml of distilled water.

Bars marked by different letters are significantly different ($p \leq 0.05$).

13-F24 that the antioxidant activity with the values of 2.52 ± 0.78 to 24.90 ± 1.37 nmol TE/% Peak area (Figure 22) were subjected to UPLC MS/MS for amino acid sequencing of peptides. There are 30, 27, 31, 30, 28, 37, 39, 68, 76, 67, 45 and 81 peptides in the F13-F24, respectively and amino acid sequence of the peptides are shown in Appendix Table D1. The probability of certainty was more than 97%. The almost peptides have R and K at C-terminal and main amino acids composition in peptides are G, A, E and D. The molecular weight (MW) of the identified peptides in F13-F24 were in the range of 701 to 4,191 Da that consisting of 6 to 33 amino acid residues. The MW distribution of peptides are shown in Table 9.

Table 9 Molecular weight distribution of peptide fractions separated on RP-HPLC from trypsin-hydrolyzed denatured albumin. (Calculated from MS/MS data in the Appendix Table D1)

Fraction no.(F)	Molecular weight distribution (%)			
	700-800	800-1000	1000-1500	> 1500
13	22.2	25.9	37.0	14.8
14	9.1	36.4	31.8	22.7
15	9.5	23.8	42.9	23.8
16	0.0	8.7	56.5	34.8
17	0.0	0.0	35.0	65.0
18	0.0	0.0	62.1	37.9
19	0.0	12.1	45.5	42.4
20	1.9	7.5	47.2	43.4
21	0.0	9.6	44.2	46.2
22	0.0	3.7	37.0	59.3
23	0.0	5.6	33.3	61.1
24	0.0	1.6	37.5	60.9

The main MW of peptides in the fractions no. 13-21 were ranging around 800-1,500 Da except for F17. The main MW distribution (> 50%) in the F22-F24 were over 1,500 Da. The peptides in F13-F21consisting of 6-20 amino acid residues and MW of peptides in the range of 800-1,500 Da displayed high antioxidant activity.

The result indicated that peptides with low MW revealed higher antioxidant activity (ORAC) than high MW peptides (Figure 22 and Table 9). It was also reported that low molecular weight peptides were more potent as antioxidant and generally consisted of 2 to 20 amino acids (Kitts and Weiler, 2003; Qian *et al*., 2008). Many researchers have revealed that the antioxidant activity of peptides was highly dependent on their sequence and the amino acid composition. Mendis *et al*. (2005) purified the peptides from jumbo squid skin gelatin and reported that the peptides contained Pro, Gly, Ala, Val and Lue in the peptides sequence had strong antioxidant activity. They suggested that hydrophobic amino acids presented in peptides contributed antioxidant activity. Ranathunga *et al*. (2006) purified and characterized the antioxidative peptide from Conger eel (*Conger myriaster*). They reported that these peptides consisted of hydrophobic residues such as, Leu, Gly and Val that represented about 55% of the sequence. In addition to the molecular size, hydrophobicity of peptides or the proportion of hydrophobic amino acids presented in peptides, is widely reported to correlate with antioxidant activity of the peptides (Byun *et al*., 2009; Park *et al*., 2010; Tang *et al*., 2010). According to the F14-F16, they were observed to be the highest antioxidant activity by chain-breaking reaction against $ROO^{\bullet}$ (ORAC). The peptides in the F14 have hydrophobic amino acids over 50% in sequence were identified as VAGAEDAAK (830 Da), AGDMAGQTK (877 Da), GGPAAAMESAASR (1,190 Da), NTIAGGGGGGHGGNNGGER (1,638 Da) and the spectra are shown in Figure 23. Three peptides in fraction F15 that also consisted of a high percentage of hydrophobic amino acids had the sequences EAAANVGASAR, NAADKDAAEVR and AKDAADMAQGTAR and the spectra are shown in Figure 24. Likewise, the peptides in F16 had a high proportion of hydrophobic amino acid: EGQTVVPGGTGGK, IPGPGSGGAGAGAAAGEGK and DKIPGPGSGGAGAGA-AAGEGK and the spectra are shown in Figure 25. The amino acid composition of the peptides in the F14-F16 was rich in Gly (G) and Ala (A). Hence, these findings suggest that the presence of these hydrophobic amino acids in the sequence contribute to the antioxidant activity of the denatured albumin peptides.

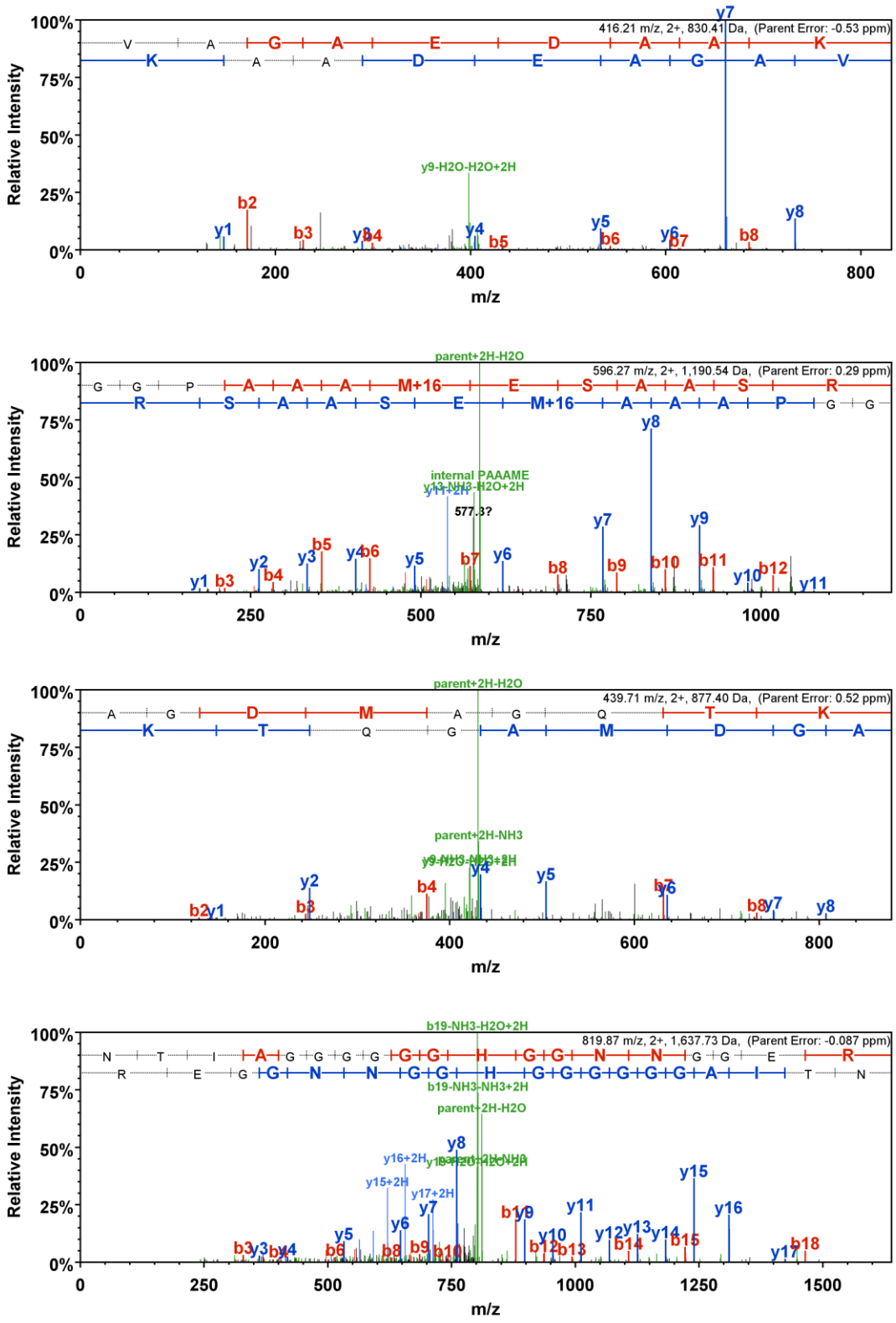


Figure 23 Tandem mass spectra of peptides (F14 in Figure 22) from trypsin-hydrolyzed denatured albumin.

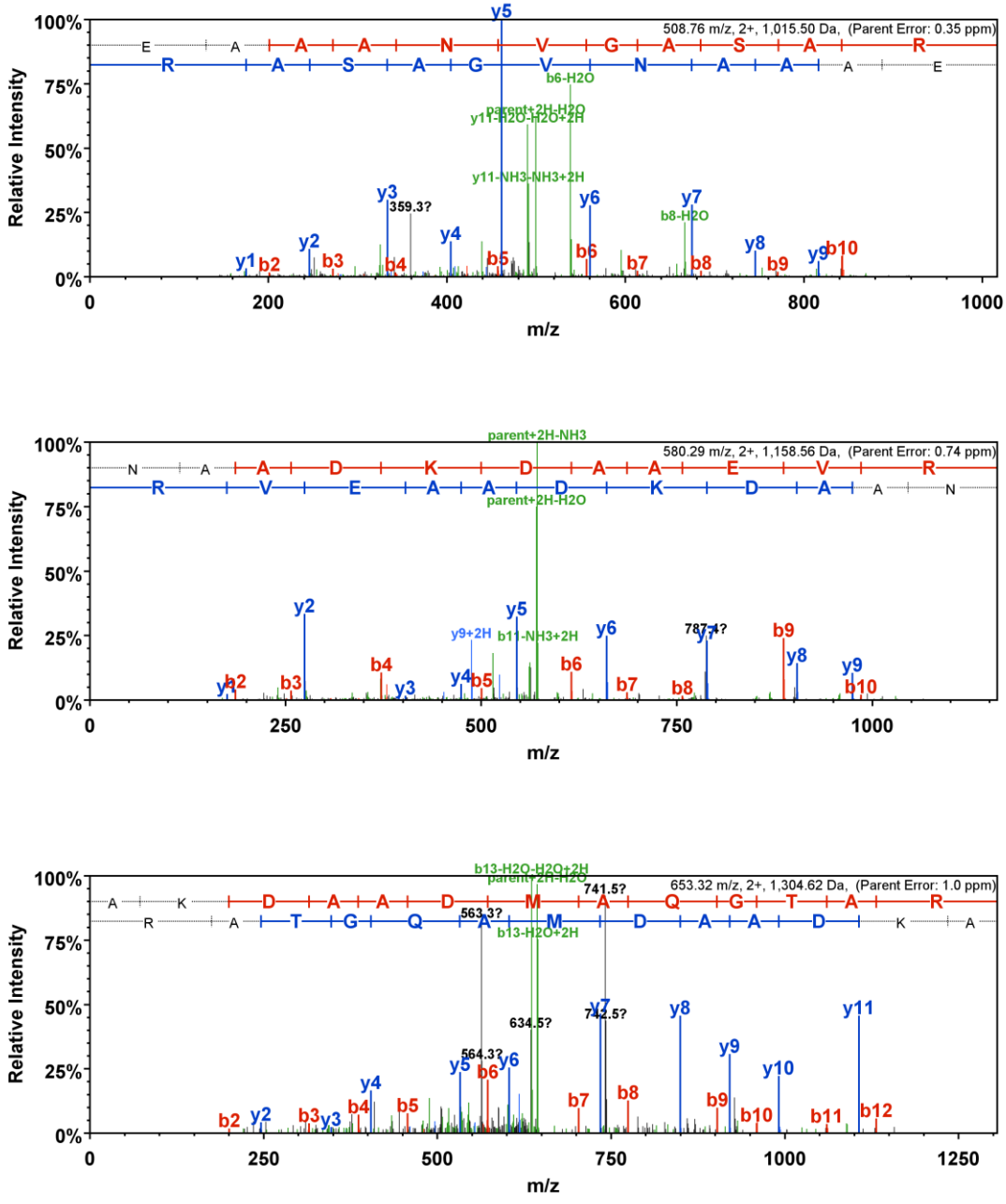


Figure 24 Tandem mass spectra of peptides (F15 in Figure 22) from trypsin-hydrolyzed denatured albumin.

By contrast, Hernandez-Ledesma *et al*. (2005) reported that Trp showed the highest antioxidant activity followed by Tyr, Met, Cys, His and Phe. The other amino acids including Arg, Asn, Gln, Asp, Pro, Ala, Val, Lys, Ile, Thr, Leu, Glu, and Gly did not exhibit antioxidant activity as determined by the ORAC assay.

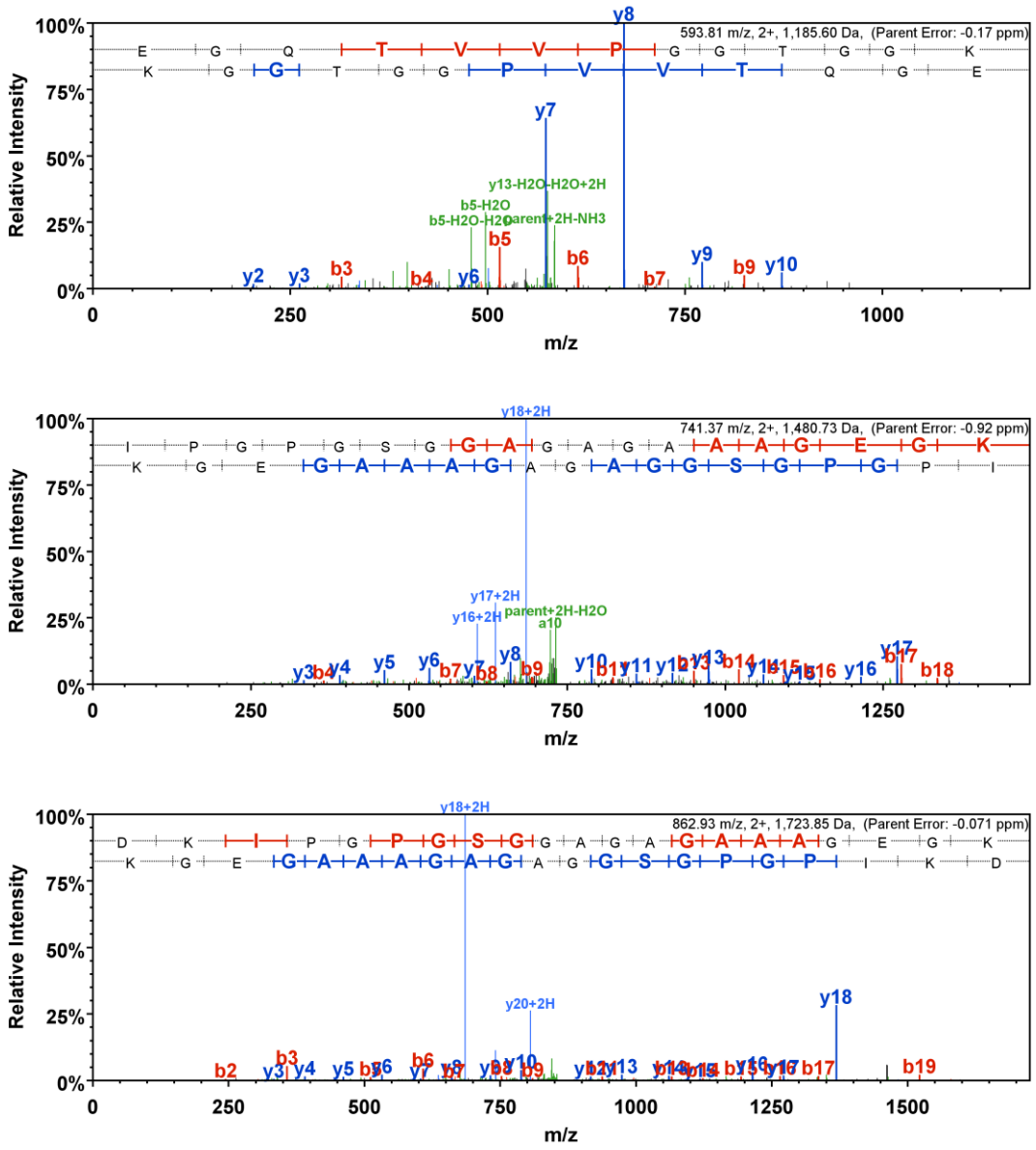


Figure 25 Tandem mass spectra of peptides (F16 in Figure 22) from trypsin-hydrolyzed denatured albumin.

Trp (W) contains an indole group that can stabilize radicals through resonance or delocalization of free radical (Elias *et al*., 2005). Two peptides containing W in the sequence have been found in F16 (CQHHHDQWK and WEAGQDQR). The spectra are shown in Figure 26. The phenolic hydroxyl group of Tyr (Y) has the capacity to donate hydrogen atom to free radicals (Zhang, 2005). F14, F15 and F16 contained 3,

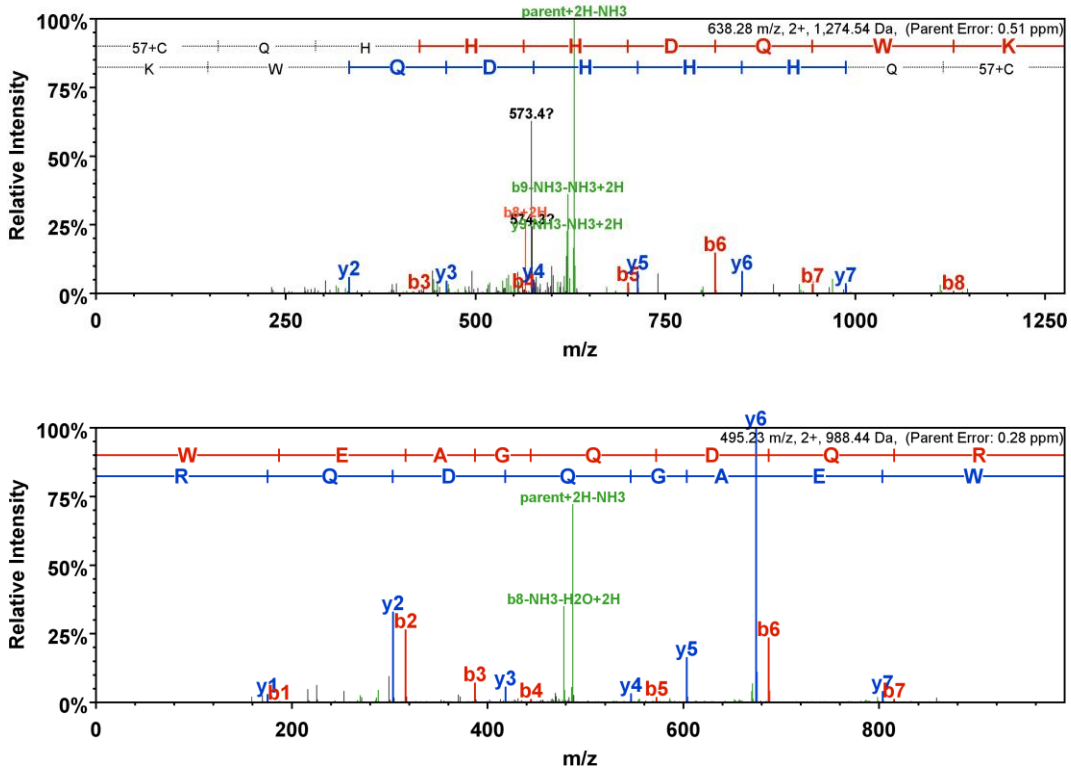


Figure 26 Tandem mass spectra of peptides (F16 in Figure 22) from trypsin-hydrolyzed denatured albumin.

2 and 6 peptides with Y in the sequence (Appendix Table D1). Several reports have also found Tyr was the common amino acid in the sequence and at both termini of the antioxdative peptides from sweet potato protein (Zhang *et al*., 2014), potato protein (Cheng *et al*., 2010), rice bran protein (Adebiyi *et al*., 2009) and royal jell protein (Guo *et al*., 2009).

Met (M) is an efficient scavenger of reactive oxygen species by reacting with oxidant to form methionine sulfoxide (Levine *et al*., 1996). The peptides with M in the sequence were found in F14, F15 and F16 that contained 6, 4 and 7 peptides (Appendix Table D1), respectively.

Cys (C) is a reducing SH group-containing amino acid that acts direct with radicals (Elias *et al*., 2005; Qian *et al*., 2008). Cys-containing peptides were found in

three peptides of F15 and F16 and in two peptides of F14. Two and three Cys residues were present in the sequence of peptides from both fractions F15 and F16 (Appendix Table D1).

The imidazole group of His (H), a hydrogen donating moiety, has the ability to scavenge reactive oxygen species. His-containing peptides can also act as a metal-ion chelator, thereby preventing redox-active metal ions to catalyze radical-mediated reactions (Chen *et al*., 1998). Three His-containing peptides were found in F14 and F15 and six in fraction F16. Moreover, the presence of amino acids with antioxidant activity in their sequence play an importance role in the antioxidant activity of the peptides. The hydrophobic properties of amino acids at the C- and N-terminal contributed antioxidant activity. It was reported that peptides containing Val, Leu , Ile, Ala, Phy, and Lys residues at the N-terminal from soybean protein, royal jelly and Ostrich (*Struthio camelus*) egg white protein exhibited high antioxidant activity (Chen *et al*., 2009; Tanzadehpanah *et al*., 2012). Li and Li (2013) recently characterized the structure–antioxidant activity relationship of peptides in free radical systems and found that the bulky hydrophobic amino acid with low electronic or steric/hydrogen bonding properties such as W, Y, F, M, L and I at the third position of amino acid next to C-terminal contributed antioxidant activity that measured using an ORAC assay. The peptides sequence KEEIER, DKTAETAEGAMDR, APGGEAYTR and FIECCSCGEYLKK showed these features. The MS/MS spectra of them are shown in Figure 27. Several peptides from fractions F14, F15 and F16 comprised more than one of these features (structural features, proportion of hydrophobic amino acids and the proper of amino acid in the sequence) for antioxidant activity. Many peptides from those fractions contained two features for antioxidant activity such as VAGAEDAAK, AAVQGQVEK, GGHELSK, KEQMGEEGYR, VAAGTATDYAR, FIECCSCGEY-LKK, CQHHHDQWK and APGGEAYTR. The peptide FIECCSCGEYLKK draws interest because it contains amino acids with antioxidant activity in its sequence including F, three C and Y as well as a hydrophobic amino acid residue (F) at the N-terminal and a bulky hydrophobic amino acid (L) at the third position of amino acid adjacent to the C-terminus.

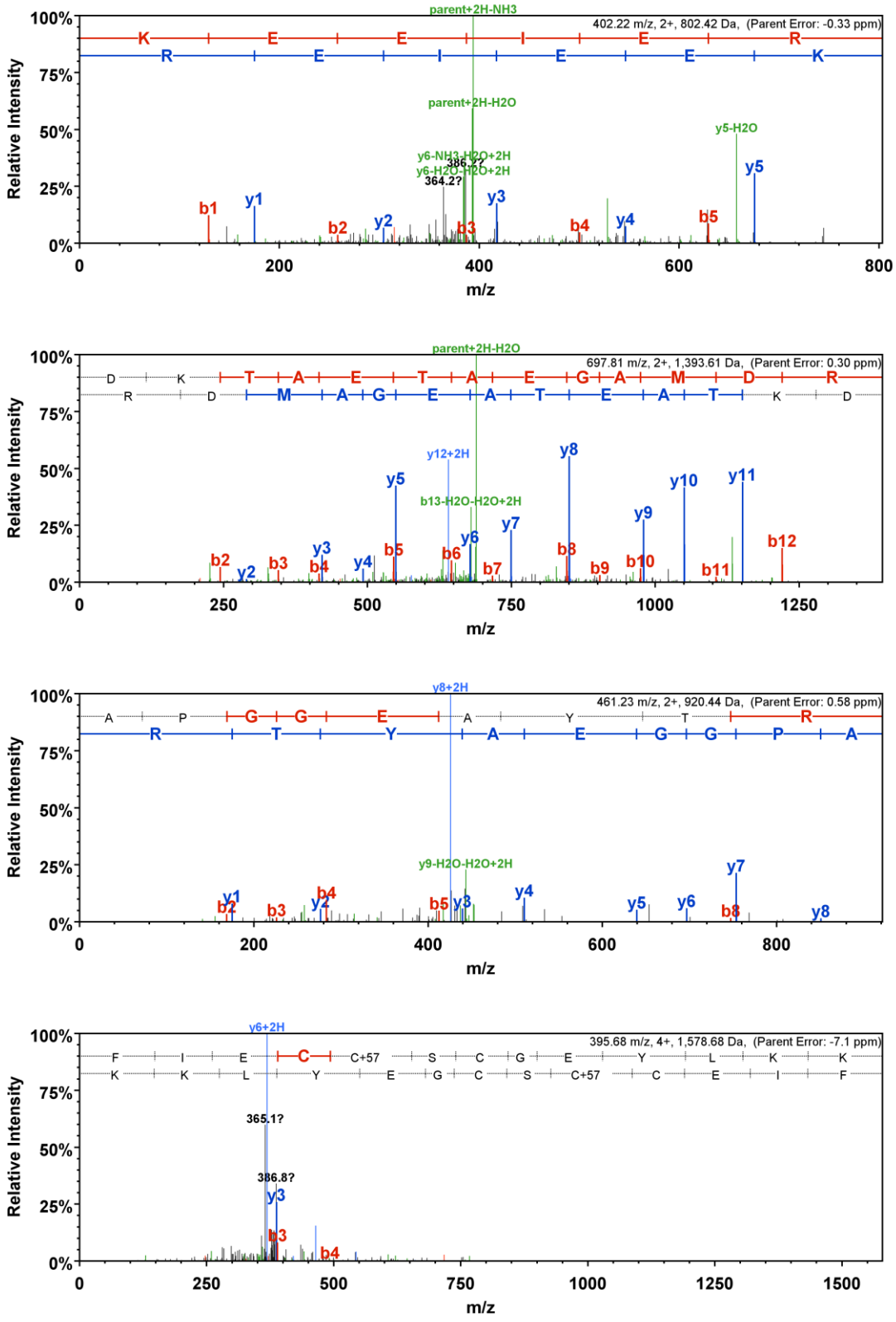


Figure 27 Tandem mass spectra of peptides (F15 and F16 in Figure 22) from trypsin- hydrolyzed denatured albumin**.**

Copper chelating activity

Chelation of metal ions have results in antioxidative effect. Since the transition metal ions (iron and copper) catalyse the production of reactive oxygen species, including hydroxyl radical ($HO^{\bullet}$) and superoxide radical ($O_2^{\bullet-}$) from oxidation of unsaturated lipids. Therefore, the chelation of metal ions contributes to antioxidation. The activity of each peptide fraction in chelating transition metal ion was tested by Cu^{2+}-chelation of 0.270 µg protein. EDTA was used as a positive control to compare the activity with each fraction. All fractions displayed chelating activity ranging from 12.01 ± 0.52 to 27.9 ± 4.16% as shown in Figure 28.

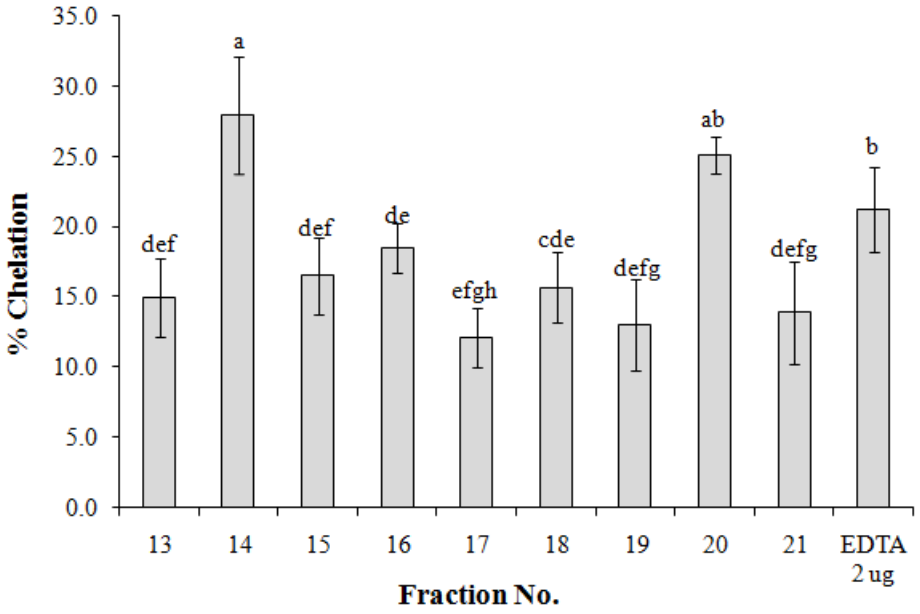


Figure 28 Cu^{2+}-chelating activity of peptide fractions of trypsin-hydrolyzed denatured albumin hydrolysates separated by RP-HPLC.
F13-F21: 0.270 µg protein/well; EDTA: 2 µg /well.
Bars marked by the different letters are significantly different ($p \leq 0.05$)

Considering the chelating activity of these fractions compared with 2 µg of EDTA, the peptide fractions can be used to replace EDTA by increasing their protein content in order to increase % of chelation. Saiga *et al*. (2003) investigated the

antioxidant activity of porcine myofibrillar protein hydrolysates and showed that the sequence of peptides with high chelating activity were DSGVT, IEAEGE, DAQEKLE, EELDNALN and VPSIDDQEELM. They proposed that the acidic amino acids in the peptides such as D, E played an important role in the chelating activity (Saiga *et al*., 2003). The chelating activity of the peptides in the F14 and F20 were the highest. The high content of peptides in the F14 that carried acidic amino acid more than 30% of total residues might have an effect on high chelating activity. The amino acid sequence of peptides in F14 which carried high amino acid were EVESGDAAR, RGEGSSEEEDEGRER, RREEEQGEEEHGER (Appendix Table D1). The MS/MS spectra of them are shown in Figure 29. Cu^{2+}-chelating activity of the peptide in the F20 might be due to the peptides with highly containing acidic amino acid 30-50% of total residues. Three peptides in F20 that contained high acidic amino acid had the sequence IEDAIEDAIK and DTEFFK (Appendix Table D1). The MS/MS spectra of them are shown in Figure 30. Copper is a prooxidative transition metal in lipid system and usually found in food ingredients. It is more effective catalyst in the generation of ROS such as $OH^{\bullet}$ and $O_2^{\bullet-}$ than iron (Halliwell and Gutteridge, 1990). The chelating peptides remove the copper ion from food and when copper ion is chelated, it may lose its prooxidant activity. Food mineral supplementation is applied to increase absorption of essential minerals such as iron or calcium (Fairwheater-Tait and Teucher, 2002). However, mineral fortification frequently generates undesired secondary effects, such as food lipid peroxidation or flavor and appearance deterioration. Thus, trypsin-hydrolyzed denatured albumin hydrolysate may be useful not only in the food to prevent oxidative activity of pro-oxidant metals but also useful for the chelating of other minerals, in human nutrition, such as calcium or zinc to increase mineral bioavailability while they maintain food quality and appearance.

FRAP assay

FRAP assay is used to measure the ability of natural antioxidants to donate an electron to free radicals. Free radical accepted electron and turned into stable form and therefore the free radical chain reactions are inhibited (Xia *et al*., 2012).

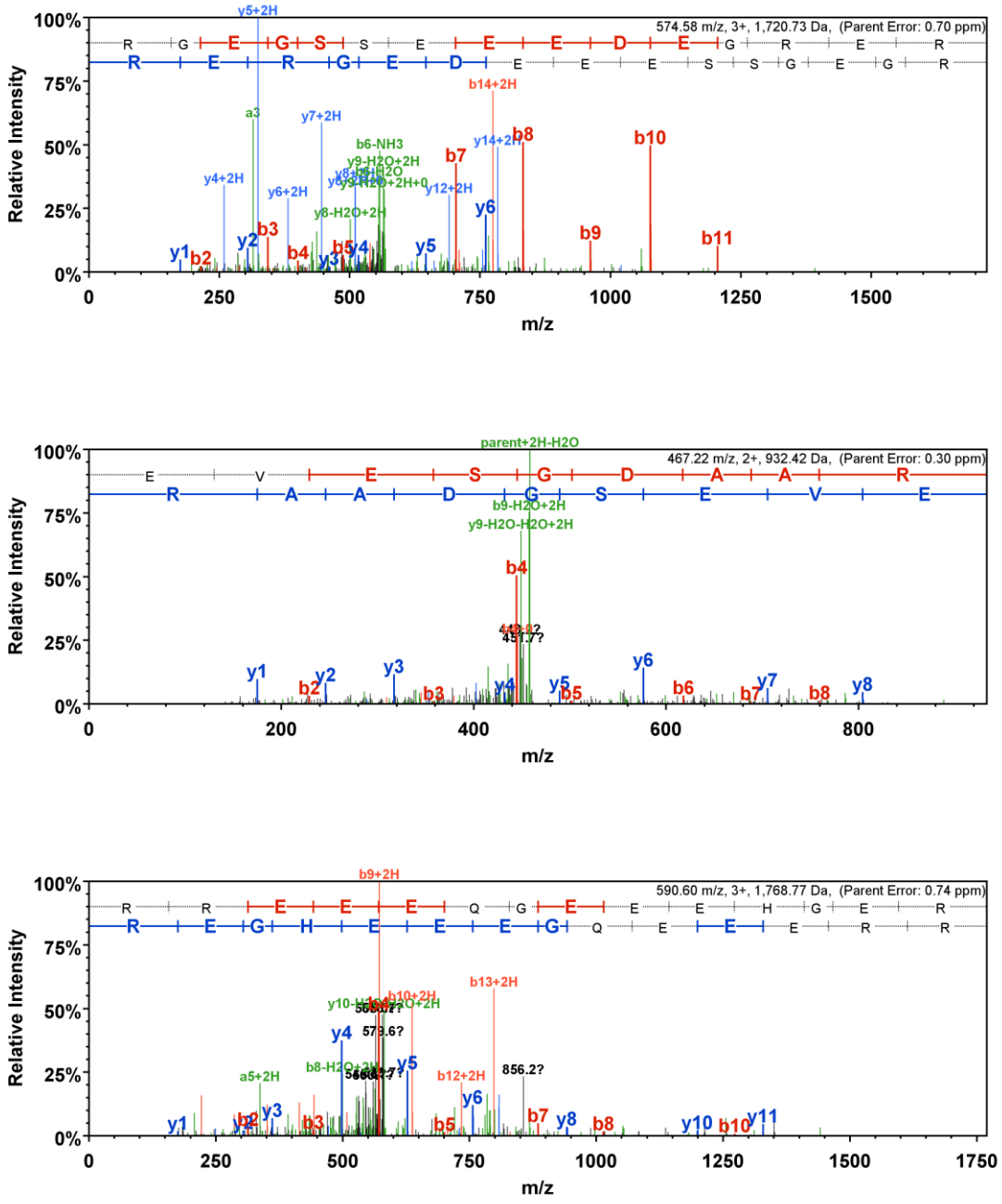


Figure 29 Tandem mass spectra of peptides (F14 in Figure 22) from trypsin-hydrolyzed denatured albumin.

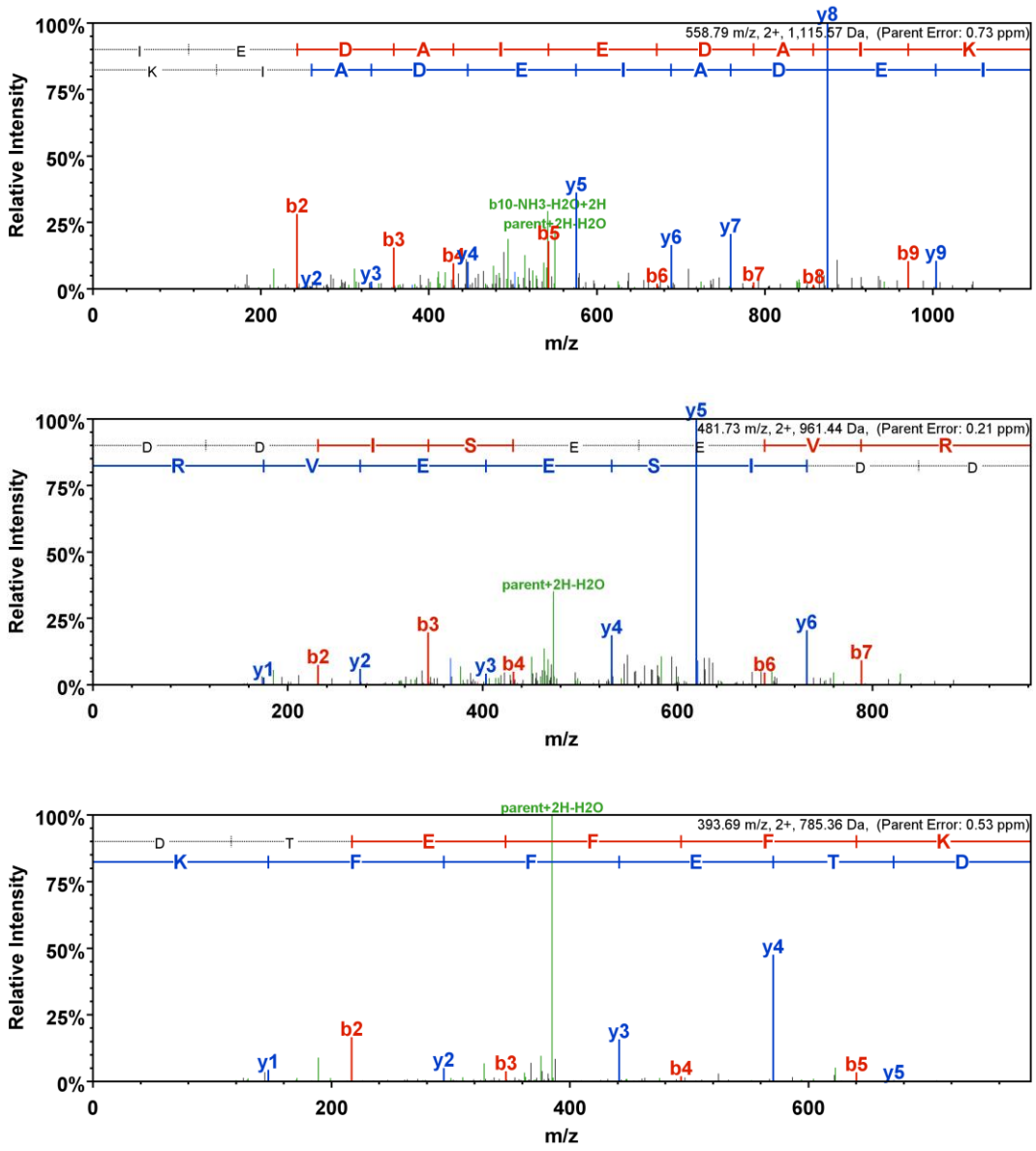


Figure 30 Tandem mass spectra of peptides (F20 in Figure 22) from trypsin-hydrolyzed denatured albumin

In FRAP assay, the presence of antioxidants in the samples resulted in reducing the Fe^{3+} to the Fe^{2+} form in acidic condition (pH 3.6). FRAP values of the peptide fractions which separated from albumin RBPH by RP-HPLC are shown in Figure 31.

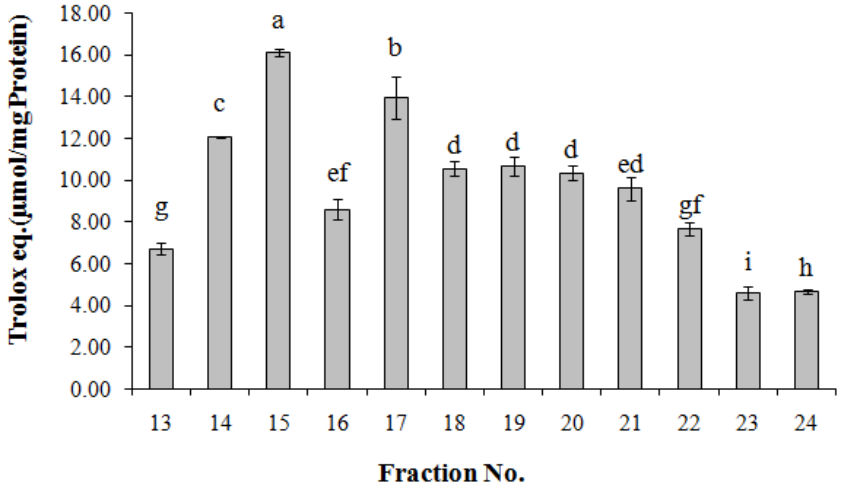


Figure 31 FRAP value of peptide fractions of trypsin-hydrolyzed denatured albumin hydrolysates separated by RP-HPLC.
Bars marked by the same letter are not significantly different ($p \leq 0.05$).

The FRAP values of these peptide fractions were remarkably high in F14, F15 and F17. It showed decreases in F18-F24. Several researchers have reported that the antioxidant activity of hydrolysates depended on their molecular weight (MW) distribution (Moure *et al*., 2006; Pena-Ramos *et al*., 2004; Wang *et al*., 2007). The most molecular weight distribution of peptides in the F14 and F15 were in the range 800-1,000 and 1,000-1,500 Da (Table 9), respectively and they possessed higher FRAP values than other fractions. The peptides in F14 had the sequence of amino acid as GGHELSKT (726 Da) that contained low percentage of hydrophobic amino acid/total residues (28.57%) in the peptides. The MW of peptides in F15 were 700-800 Da, and the sequence of amino acids were RGDLER (744 Da) and ARPSPPE (752 Da). The percentages of hydrophobic amino acid / total residues of the both of them were 33.33 and 57.14%, respectively. Thus, they might not affect on FRAP value in F14 and F15.

However, the percentage of hydrophobic amino acid/total residues in peptides were lower than peptides with MW 800-1,500 Da that were mainly in range 40-60%

(Appendix Table 1). These results were in agreement with Wu *et al*., (2003) that found the antioxidant activity of peptides from protein hydrolysate of mackerel with molecular weight of approximately 1,400 Da to be stronger than those of the 900 and 200 Da. peptides. Li *et al*. (2008) also found that the antioxidant activity of corn gluten meal hydrolysates with molecular weight of 500-1,500 Da was stronger than that of peptides above 1,500 Da and peptides below 500 Da. The F18-F21 contained peptides with MW distribution ranging from 800-1,500 Da. more than F F22-F24 and peptides with MW of above 1,500 Da less than F22-F24. The FRAP values of peptides F18-F21 are higher than F22-F24. It is possible that peptides from trypsin-hydrolyzed denatured albumin hydrolysate with MW in the range 800-1,500 Da exhibit higher antioxidant activity than peptides with MW over 1,500 Da. These results suggested that the antioxidant activity of peptides from trypsin-hydrolyzed denatured albumin with molecular weight of approximately 800-1,500 Da was stronger than that of the peptides above 1,500 Da and below 800 Da.

Albumin RBPHs was separated by RP-HPLC to obtain 30 peptide fractions. Each peptide fractions acts as antioxidant in different mechanisms. Three mechanisms investigated in this study are proposed, i) radical scavenging activity against $ROO^{\bullet}$ (ORAC assay), ii) Cu^{2+}-chelating activity and iii) the ability to donate electron to free radicals (FRAP assay).

CONCLUSION AND RECOMMENDATIONS

Conclusion

1. RBP extracted from defatted rice bran with alkaline solution and precipitated at pH 4.5 (AE-RBP) was prepared. It was hydrolyzed with alcalase and papain and antioxidant activity of AE-RBP and its hydrolysates were evaluated by DPPH radical scavenging activity and FRAP assay. Antioxidant activities of AE-RBP and their hydrolysates were not significantly different. Then AE-RBP was freeze dried (FD-AE-RBP) and antioxidant activity of them were investigated. DPPH radical scavenging activity and FRAP assay of AE-RBP were at similar extent to those of FD-AE-RBP. The FD-AE-RBP had no effect on antioxidant activity of BHA or α-tocopherol when it was combined with them. It suggested that FD-AE-RBP can be used to replace BHA, α-tocopherol or combine with α-tocopherol.

2. RBP was fractionated on the basis of differences in its solubility properties. They included albumin, globulin, glutelin and prolamin fractions. Denatured RBPFs, that were denatured by reducing agent (DTT), alkylation by chloroacetamide and native RBPFs were hydrolyzed with trypsin and papain under optimum conditions. Denatured albumin hydrolyzed with trypsin generated peptides with the strongest antioxidant activities and its ORAC value was 4.067 μmol TE /μg protein.

3. The peptides that obtained from the trypsin-hydrolyzed denatured albumin that separated by RP-HPLC were identified by MS/MS. The main MW of peptides were in the range of 800-1,500 Da and consisted of 6 to 20 amino acid residues. The peptide fractions with the highest antioxidant activity demonstrated typical characteristics of well-known antioxidative peptides with hydrophobic and aromatic amino acid residues.

4. Antioxidant activity of antioxidative peptides in each peptide fraction from trypsin-hydrolyzed denatured albumin hydrolysate were depended on amino acid

composition, amino acid sequence, hydrophobicity and MW of peptides. The peptide fractions that observed to be the highest antioxidant activity by chain-breaking reaction against $ROO^{\bullet}$ (ORAC assay) have high hydrophobic amino acid in the sequence and rich in Gly and Ala. The trypsin-hydrolyzed denatured albumin hydrolysate contained Cu^{2+}-chelating peptides whose Cu^{2+}-chelating activity depended on percentage of Asp and Glu in the peptides. Peptides from trypsin-hydrolyzed denatured albumin with molecular weight of approximately 800-1,500 Da have high ability to donate an electron to free radicals.

Recommendations

1. The antioxidative activity of peptides from trypsin-hydrolyzed denatured albumin hydrolysate in other *in vitro* chemical assays such as inhibition of lipid peroxidation, $OH^{\bullet}$ radical scavenging activity should be performed. Moreover, further detailed study on the antioxidative of the peptides activity *in vivo* are also needed.

2. Antioxidative peptides from trypsin-hydrolyzed denatured albumin hydrolysates can be possibly incorporated into food matrices as a natural food antioxidant. However, the feasibility study for commercial preparation of antioxidative peptides such as safety and cost should be further investigated. In addition, crude peptides may be used in food products because they contain various antioxidative peptides.

.

LITERATURE CITED

Addis, P.B. 1986. Occurrence of lipid oxidation products in foods. **Food Chemistry Toxic.** 24: 1021-1030.

Adebiyi, A.P., A.O. Adebiyi, T. Ogawa and K. Muramoto. 2007. Preparation and characterization of high-quality rice bran proteins. **J. Sci. Food Agric.**. 87: 1219-1227.

Adebiyi, A.P., A.O. Adebiyi, Y. Hasegawa, T. Ogawa and K. Muramoto. 2009. Isolation and characterization of protein fractions from deoiled rice bran. **Eur. Food Res Technol**. 228: 391-401.

Adom, K.K. and R.H. Liu. 2002. Antioxidant activity of grains. **J. Agric. Food Chem.** 50: 6182-6187.

Aguilar, M.-I. and M.T.W. Hearn. 1996. [1] high-resolution reversed-phase high-performance liquid chromatography of peptides and proteins, pp. 3-26. *In* B.L. Karger and W.S. Hancock. eds. **Methods in Enzymology.** Academic Press

Aguilar, M.I. 2004. Reversed-phase high-performance liquid chromatography, pp. *In* M.-I. Aguilar. eds. **HPLC of peptides and proteins methods and protocols**. Humana Press Inc., Totowa.

AOAC. 1995. **Official Methods of Analysis**. 15th. Association of Official Analytical Chemist, Washington, DC.

Arnon, R. 1970. [14] papain, pp. 226-244. *In* L.L. Gertrude E. Perlmann. eds. **Methods in Enzymology**. Academic Press.

Babizhayev, M.A., M.C. Seguin, J. Gueyne, R.P. Evstigneeva, E.A. Ageyeva and G.A. Zheltukhina. 1994. L-carnosine (beta-alanyl-l-histidine) and carcinine (beta-alanylhistamine) act as natural antioxidants with hydroxyl-radical-scavenging and lipid-peroxidase activities. **Biochem. J**. 304: 509-516.

Bamdad, F. and L. Chen. 2013. Antioxidant capacities of fractionated barley hordein hydrolysates in relation to peptide structures. **Mol. Nutr. Food Res**. 1-11.

Benzie, I.F.F. and J.J. Strain. 1996. The ferric reducing ability of plasma (frap) as a measure of "antioxidant power": The frap assay. **Anal. Biochem**. 239: 70-76.

Betschart, A.A., R.Y. Fong and R.M. Saunders. 1977. Rice by-products: Comparative extraction and precipitation of nitrogen from u.S. And spanist bran and germ. **Journal of Food Science**. 42: 1088-1093.

Biemann, K. 1990. Sequencing of peptides by tandem mass spectrometry and high-energy collision-induced dissociation. **Methods in Enzymology.** 93: 455-479.

Biochemistry, I.U.O. 1984. **Nomenclature committee: Enzyme nomenclature**. Academic Press, Inc, London.

Boonmee, A., C. Srisomsap, A. Karnchanatat and P. Sangvanich. 2011. An antioxidant protein in *curcuma comosa* roxb. Rhizomes. **Food Chemistry**. 124: 476-480.

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

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem.** 72: 248-254.

Britannica, E. and G.M. Edwards. 1996. **Britannica book of the year: 1996**. Encyclopaedia Britannica.

Brown, W.E. and F. Wold. 1973. Alkyl isocyanates as active-site-specific reagents for serine protease. Identification of the active-site serine as the site of reaction. **Biochemistry**. 12: 835-840.

Butterfield, D.A., A. Castenga, C.B. Pocernich, J. Drake, G. Seapagnini and V. Calabrese. 2002. Nutritional approaches to combat oxidative stress in alzheimer's disease. **J. Nutr. Biochem**. 13: 444-446.

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

Carrasco-Castilla, J., A.J. Hernández-Álvarez, C. Jiménez-Martínez, C. Jacinto-Hernández, M. Alaiz, J. Girón-Calle, J. Vioque and G. Dávila-Ortiz. 2012. Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. **Food Chemistry**. 135: 1789-1795.

Chan, K.M. and E.A. Decker. 1994b. Endogenous muscle antioxidants. **Critical Reviews in Food Science and Nutrition**. 34: 403-426.

Chanput, W., C. Theerakulkait and S. Nakai. 2009. Antioxidative properties of partially purified barley hordein, rice bran protein fractions and their hydrolysates. **Journal of Cereal Science**. 49: 422-428.

Chen, H.M., K. Muramoto and F. Yamauchi. 1995. Structural analysis of antioxidative peptides from soybean -conglycinin. **J. Agric. Food Chem**. 43: 574-578.

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

Cheng, Y., Y.L. Xiong and J. Chen. 2010. Fractionation, separation, and identification of antioxidative peptides in potato protein hydrolysate that enhance oxidative stability of soybean oil emulsions. **Journal of Food Science**. 75: C760-C765.

Cheung, I.W.Y., L.K.Y. Cheung, N.Y. Tan and E.C.Y. Li-Chan. 2012. The role of molecular size in antioxidant activity of peptide fractions from pacific hake *(merluccius productus)* hydrolysates. **Food Chemistry**. 134: 1297-1306.

Choe, E. and D.B. Min. 2006. Chemistry and reactions of reactive oxygen species in foods. **Critical Reviews in Food Science and Nutrition**. 46: 1-22.

Clarkson, P.M. and H.S. Thompson. 2000. Antioxidants: What role do they play in physical activity and health? **Am J. Clin. Nutr.** 72: 637S-646S.

Cohen, L., V. Coghlan and L. Dihel, 48, 219, 1986. 1986. Cloning and sequencing of papain-encoding cdna. **Gene**. 48: 219-227.

Creusot, N. and H. Gruppen. 2007. Enzyme-induced aggregation and gelation of proteins. **Biotechnology Advances**. 25: 597-601.

Davalos, A., M. Miguel, B. Bartolome and R. Lopez-Fandino. 2004. Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. **Journal of Food Protein**. 67: 1939-1944.

Davies, M.J. and R.T. Dean. 1997. **Radical-mediated protein oxidation : From chemistry to medicine**. Oxford University Press, New York.

Decker, E.A., K. Warner, M.P. Richards and F. Shahidi. 2005. Measuring antioxidative effectiveness in food. **J. Agric. Food. Chem.** 53: 303-4310.

Diaz, M., C.M. Dunn, D.J. McClements and E.A. Decker. 2003. Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. **J. Agric. Food Chemistry**. 51: 2365-2370.

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

Fabian, C. and Y.-H. Ju. 2011. A review on rice bran protein: Its properties and extraction methods. **Critical Reviews in Food Science and Nutrition**. 51: 816-827.

FAO Report. 2013. **Food outlook biannual report on global food markets** http://www.fao.org/docrep/018/al999e/al999e.pdf.

Fenn, J., M. Mann, C. Meng, S. Wong and C. Whitehouse. 1989. Electrospray ionization for mass spectrometry of large biomolecules. **Science**. 246: 64-71.

Gnanasambandam, R. and N.S. Hettiarachchy. 1995. Protein concentrates from unstabilized rice bran: Preparation and properties. **Journal of Food Science**. 60: 1066-1074.

Graf, E. and J.W. Eaton. 1990. Antioxidant functions of phytic acid. **Free Radical Biol Med**. 8: 61-69.

Grant, G.A. 2002. **Synthetic peptides : A user's guide:** Oxford University Press, Inc., New York.

Gulcin, I. 2012. Antioxidant activity of food constituents: An overview. **Arch Toxicol** 86: 345-391.

Guo, H., Y. Kouzuma and M. Yonekura. 2009. Structures and properties of antioxidative peptides derived from royal jelly protein. **Food Chemistry**. 113: 238-245.

García-Fernández, M., G. Delgado, J. E. Puche, S. González-Barón and C. I. Castilla. 2008. Low doses of insulin-like growth factor I improve insulin resistance, lipid metabolism, and oxidative damage in aging rats. **Endocrinology**. 149: 2433-2442

Halliwell, B. 1995. Antioxidant characterization; methodology and mechanism. **Biochem Pharmacol**. 49: 1341-1348.

Halliwell, B. and J.M.C. Gutteridge. 1989. **Free radicals in biology and medicine**. Clarendon Press, Oxford.

Halliwell, B. and J.M. Gutteridge. 1990. Role of free radicals and catalytic metal ions in human disease: An overview. **Methods. Enzymol**. 186: 1-85.

Hamada, J.S. 1997. Characterization of protein fractions of rice bran to devise effective methods of protein solubilization. **Cereal Chem**. 74: 662-668.

Hamada, J.S. 1999. Use of proteases to enhance solubilization of rice bran proteins1. **Journal of Food Biochemistry**. 23: 307-321.

Hargrove, K.L. 1994. Processing and utilization of rice bran in the united states, pp. *In* W.E. Marshall and J.I. Wadsworth. eds. **Rice science and technology**. Marcel Dekker Inc, New York.

Helm, R.M. and A.W. Burks. 1996. Hypoallerginicity of rice protein. **Cereal Foods World**. 41: 839-842.

Herna´ndez-Ledesma, B., A. Davalos, B. Bartolome and L. Amigo. 2005. Preparation of antioxidant enzymatic hydrolysates from a-lactalbumin and b-lactoglobulin. Identification of active peptides by hplc-ms/ms. **J. Agric. Food Chem.** 53: 588-593.

Hu, J.P., M. Calomme, A. Lasure, T. De Bruyne, L. Peters, A. Vlietinck and D.A. Van den Berghe. 1995. Structure-activity relationship of flavonoids with superoxide scavenging activity. **Biol Trace Element Res**. 47: 327-331.

Hu, W., J.H. Wells, T.S. Shin and J.S. Godber. 1996. Comparison of isopropanol and hexane for extraction of vitamin e and oryzanols from stabilized rice bran. **J. Am. Oil Chem. Soc**. 73: 1653-1656.

Huang, D., B. Ou and R.L. Prior. 2005. The chemistry behind antioxidant capacity assays. **J. Agric. Food Chem..** 53: 1841-1856.

Huang, D., B. Ou, M. Hampsch-woodill, J.A. Flanagan and R.l. Prior. 2002. High-throughput assay of oxygen radical absorbance capacity (orac) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. **J. Agric. Food Chem.** 50: 4437-4444.

Humiski, L.M. and R.E. Aluko. 2007. Physicochemical and bitterness properties of enzymatic pea protein hydrolysates. **Journal of Food Science**. 72: S605-S611.

Jadhav, S.J., S.S. Nimbalkar, A.D. Kulkami and D.L. Madhavi. 1996. Lipid oxidation in biological and food systems, pp. 5-64. *In* D.L. Madhavi, S.S. Deshpande and D.K. Salunkhe. eds. **Food antioxidants**. Marcel Dekker Inc., New York.

Jao, C.L. and W.C. Ko. 2002. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging by protein hydrolyzates from tuna cooking juice. **Fisheries Science**. 430-435.

JECFA, 2013. **Codex general standard for food additives**. CODEX STAN 192-1995

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

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

Jodayree, S., J.C. Smith and A. Tsopmo. 2012. Use of carbohydrase to enhance protein extraction efficiency and antioxidative properties of oat bran protein hydrolysates. **Food Research International**. 46: 69-75.

Johansen, J.T., M. Ottesen, I. Svendsen and G. Wybrandt. 1968. The degradation of the b-chain of oxidized insulin by two subtilisins and their succinylated and n-carbamylated derivatives. **C.R. Trav. Lab. Carlsberg**. 36: 365-384.

Juliano, B.O. 1985. Rice bran, pp. 647-680. *In* B.O. Juliano. eds. **Rice chemistry and technology**. American Association of Cereal Chemists, St. Paul, MN.

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

Kang, J.H., K.S. Kim, S.Y. Choi, H.Y. Kwon, M.H. Won and T.C. Kang. 2002. Carnosine and related dipeptides protect human ceruloplasmin against peroxyl radical-mediated modification. **Molecules and Cells**. 13: 498-502.

Kennedy, L.L., H.Y. Chung and M. Hegsted. 1996. New food products developed from rice bran fractions, pp. 56. *In* eds. **IFT annual meeting: Book of abst**. Institute of Food Technologists, USA.

Kim, S.Y., J.Y. Je and S.K. Kim. 2007. Purification and characterization of antioxidant peptide from hoki (j*ohnius belengerii*) frame protein by gastrointestinal digestion. **Journal of Nutritional Biochemistry**. 18: 31-38.

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

Kitts, D.D. and K. Weiler. 2003. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. **Current Pharmaceutical Design**. 9: 1309-1323.

Klompong, V., S. Benjakul, D. Kantachote, K.D. Hayes and F. Shahidi. 2008 Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from alcalase and flavourzyme. **International Journal of Food Science and Technology**. 43: 1019-1026.

Kokkeaw, H. and S. Thawornchinsombut. 2007. Process optimization of jasmine rice bran protein hydrolysates and its radical scavenging property. **Agric Sci. J.** 38: 177-180.

Lakkakula, N.R., M. Lima and T. Walker. 2004. Rice bran stabilization and rice bran oil extraction using ohmic heating. **Bioresour. Technol**. 92: 157-161.

Lauwers, A. and S. Scharpe. 1997. **Pharmaceutical enzymes, drugs and pharmaceutical sciences**. Marcel Dekker, Inc, New York-Basel-Hong Kong.

Li, X., H.-m. Li, Y.-b. Huang, H. Wang, G.-j. Nie and X.-z. Zang. 2006. A study on anti-oxidative activity of soybean peptides with linoleic acid peroxidation systems. **Chemistry Research Chinese University.** 22: 205-208.

Li, X.X., L.J. Han and L.J. Chen. 2008. *In vitro* antioxidant activity of protein hydrolysates prepared from corn gluten meal. **J Sci Food Agric**. 88: 160-166.

Madhavi, D.L., S.S. Deshpande and D.K. Salunkhe. 1996. **Food antioxidants: Technological,toxicological and health perspectives**. Marcel Dekker, Inc., New York.

Marcuse, R. 1960. Antioxidative effect of amin acids. **Nature**. 186: 886-887.

Marcuse, R. 1962. The effect of some amino acids on the oxidation of linoleic acid and its methyl ester. **J. Am.Oil Chem. Soc.** 39: 97-103.

Martinez-Villaluenga, C., H. Zielinski, J. Frias, M.K. Piskula, H. Kozlowska and C.N. Vidal-Valverde. 2009. Antioxidant capacity and polyphenolic content of high-protein lupin products. **Food Chemistry**. 112: 84-88.

Mascio, P., S. Kaiser and H. Sies. 1989. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. **Arch Biochem Biophy**. 274: 532-538.

Mendis, E., N. Rajapakse and S.-K. Kim. 2005a. Antioxidant properties of radical scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. **J. Agric. Food Chem.** 53: 581-587.

Mendis, E., N. Rajapaksea, H.-G. Byunb and S.-K. Kima. 2005b. Investigation of jumbo squid (*dosidicus gigas*) skin gelatin peptides for their in vitro antioxidant effects. **Life Sciences**. 77: 2166-2178.

Mitchel, R.E.J., I.M. Chaiken and E.L. Smith. 1970. The complete amino acid sequence of papain: Additions and corrections. **Journal of Biological Chemistry**. 245: 3485-3492.

Moure, A., H. Dominguez and J.C. Parajo. 2006. Antioxidant properties of ultra filtration-recovered soy protein fractions from industrial effluents and their hydrolysates. **Process Biochemistry**. 41: 447-456.

Nawar, W. W. 1996. Lipids. In O.R. Fennema (Ed.), Food Chemistry, (pp. 225-319). New York: Dekker.

Nazeer, R.A. and K.A. Kulandai. 2012. Evaluation of antioxidant activity of muscle and skin protein hydrolysates from giant kingfish, *caranx ignobilis* (forsskål, 1775). **International Journal of Food Science and Technology**. 47: 274-281.

Ningappa, M. and L. Srinivas. 2008. Purification and characterization of ~35 kda antioxidant protein from curry leaves (*murraya koenigii l.*). **Toxicol In Vitro**. 22: 699-709.

Ou, B., M. Hampsch-Woodill and R.L. Prior. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. **J. Agric. Food Chem.** 49: 4619-4626.

Paek, P.J., W.K. Jung, K.S. Nam, F. Shahioli and S.K. Kim. 2001. Purification and characterization of antioxidative peptides from lecithin-free egg york protein. **J. Am.Oil Chem. Soc.** 78: 651-656.

Park, E.Y., M. Morimae, Y. Matsumura, Y. Nakamura and K. Sato. 2008. Antioxidant activity of some protein hydrolysates and their fractions with different isoelectric points **J. Agric. Food Chem.** 56: 9246-9251.

Park, S.Y., J.S. Lee, H.H. Baek and H.G. Lee. 2010. Purification and characterization of antioxident peptides from soy protein hydrolysate. **J. Food Biochem**. 34: 120-132.

Patterson, C.E. and R.A. Rhoades. 1988. Protective role of sulfhydryl reagents in oxidant lung injury. **Exp. Lung Res.** 14: 1005-10109.

Paek, P. J., W. K. Jung, K. S. Nam, F. Shahioli and S. K Kim. 2001. Purification and characterization of antioxidant peptides from lecithin-free egg york protein. **Journal of American Oil Chemistry Society**, 78: 651-656.

Pena-Ramos, E.A. and Y.L. Xiong. 2001. Antioxidative activity of whey protein hydrolysates in a liposomal system. **J. Dairy Sci.** 84: 2577-2583.

Pena-Ramos, E.A., Y.L. Xiong and G.E. Arteaga. 2004. Fractionation and characterization for antioxidative activity of hydrolyzed whey protein. **J. Sci. Food Agric.** 84: 1908-1918.

Peña-Ramos, E.A. and Y.L. Xiong. 2002. Antioxidant activity of soy protein hydrolysates in a liposomal system. **Journal of Food Science**. 67: 2952-2956.

Pokorny, J., N. Yanishlieva and M. Gordon. 2001. **Antioxidants in food practical applications**. Woodhead Publishing Limited, Cambridge.

Prior, R.L., X. Wu and K. Schaich. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. **J. Agric. Food Chem.** 53: 4290-4302.

Pryor, W.A. 1982. Free radical biology: Xenobioties cancer and aging. **Ann. N Y Acad. Sci.** 393: 1-22.

Qi, M., N.S. Hettiarachchy and U. Kalapathy. 1997. Solubility and emulsifying properties of soy protein isolates modified by pancreatin. **Journal of Food Science**. 62: 1110-1115.

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

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

Rajapakse, N., E. Mendis, W.K. Jung, J.Y. Je and S.K. Kim. 2005b. Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. **Food Res Int**. 38: 175-182.

Ramanathan, L. and N.P. Das. 1993. Effect of natural copper chelating components on the pro-oxidant activity of ascorbic acid in steam-cooked ground fish. **Int. J. Food Sci. Technol**. 28: 279-288.

Ranathunga, S., N. Rajapakse and S.K. Kim. 2006. Purification and characterization of antioxidative peptide derived from muscle of conger eel (*conger myriaster*). **Eur. Food Res. Technol.** 222: 310-315.

Reische, D.W., D.A. Lillard and R.R. Eitenmiller. 2002. Antioxidants, pp. 489-516. *In* C.C. Akoh and D.B. Min. eds. **Food lipids**. Marcel Dekker Inc., New York.

Rival, S.G., C.G. Boeriu and H.J. Wichers. 2001a. Caseins and casein hydrolysates. Ii. Antioxidative properties and relevance to lipoxygenase inhibition. **J. Agric. Food Chemistry**. 49: 295–302.

Rival, S.G., C.G. Boeriu, S. Fornaroli and H.J. Wichers. 2001b. Caseins and casein hydrolysates. I. Lipoxygenase inhibitory properties. **J. Agric. Food Chem.** 49: 287–294.

Robak, J. and R.J. Gryglewski. 1988. Flavonoids are scavengers of superoxide anions. **Biochem Pharmacol**. 37: 837-841.

Roepstorff, P. and J. Fohlman. 1984. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. **Biomed. Mass Spectrom**. 11: 601.

Saiga, A., S. Tanabe and T. Nishimura. 2003. Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. **J. Agric. Food Chemistry**. 51: 3661-3667.

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

Salunkhe, D.K., J.K. Chavan, R.N. Adsule and S.S. Kadam. 1992. Rice, pp. 424-448. *In* eds. **World Oilseeds: Chemistry, Technology and Utilization**. Van Nostrand Reinhold, New York.

Samaranayaka, A.G.P. and E.C.Y. Li-Chan. 2011. Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. **Journal of Functional Foods**. 3: 229-254.

Sarmadi, B.H. and A. Ismail. 2010. Antioxidative peptides from food proteins: A review. **Peptides**. 31: 1949-1956.

Saunders, R.M. 1990. The properties of rice bran as a food stuff. **Cereal Foods World**. 35: 632-662.

Scalzo, R.L. 2008. Organic acids influence on dpph• scavenging by ascorbic acid **Food Chemistry**. 107: 40-43.

Schomburg, D. and I. Schomburg. 2002. **Springer handbook of enzymes**. 2nd. Springer-Verlag Berlin Heidelberg, Berlin.

Selvam, R. and S. Devaraj. 1996. Oxalate binding to rat kidney mitochondria: Induction by oxidized glutathione. **Indian. J. Biochem. Biophys**. 33: 62-65.

Seo, W.H., H.G. Lee and H.H. Baek. 2008. Evaluation of bitterness in enzymatic hydrolysates of soy protein isolate by taste dilution analysis. **Journal of Food Science**. 73: S41-S46.

Shahidi, F. 1997. Natural antioxidants: An overview., pp. 1-11. *In* F. Shahidi. eds. **Natural antioxidants: Chemistry, health effects and applications**. The American Oil Chemists Society, Washington, DC.

Shahidi, F. and Y. Zhong. 2007. Measurement of antioxidant activity in food and biological systems, pp. 36-66. *In* eds. **ACS. symposium series; 956**. American Chemical Society, Washington, DC.

Sharif, M.K., M.S. Butt, F.M. Anjum and S.H. Khan. 2013. Rice bran: A novel functional ingredient. **Critical Reviews in Food Science and Nutrition**. 8-47.

Sharma, O.P. and T.K. Bhat. 2009. Dpph antioxidant assay revisited. **Food Chemistry**. 113: 1202-1205.

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

Spellman, D., P. Kenny, G. O'Cuinn and R.J. Fitzgerald. 2005. Aggregation properties of whey protein hydrolysates generated with *bacillus licheniformis* proteinase activities. **J. Agric. Food Chem.** 53: 1258-1265.

Stahl, W. and H. Sies. 2003. Antioxidant activity of carotenoids. **Mol Asp Med**. 24: 345-351.

Stohs, S.J. and D. Bagchi. 1995. Oxidative mechanisms in the toxicity of metal ions. **Free Radical Biol Med**. 18: 321-336.

Suetsuna, K. and J.R. Chen. 2002. Isolation and characterization of peptides with antioxidant activity derived from wheat gluten. **Food Sci.Technol Res.** 8: 227-230.

Suetsuna, K., H. Ukeda and H. Ochi. 2000. Isolation and characterization of free radical scavenging activities peptides derived from casein. **J. Nutr. Biochem**. 128-131.

Takahama, U. 1984. Hydrogen peroxide dependent oxidation of quercetin by intact spinach chloroplast. **Plant Physiol**. 74: 852-857.

Tanaka, K., H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida and T. Matsuo. 1988. Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. **Rapid Communications in Mass Spectrometry**. 2: 151-153.

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

Tang, X., Z. He, Y. Dait, Y.L. Xiong, M. Xie and J. Chen. 2010. Peptide fractionation and free radical scavenging activity of zein. **J. Agric. Food Chemistry**. 58: 587-593.

Tangkanakul, P., G. Trakoontivakorn and C. Jariyavattanaviji. 2005. Extracts of thai indigenous vegetables as rancid inhibitor in a model system. **Kasetsart Journal: Natural Science**. 39: 274-283.

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

Tatsro, K., N. Yoshiyaki, M. Ai, O. Tateaki and I. Taeko. 2002. Radical scavenging properties of protein fraction from rice bran. **New Tech. and Med**. 3: 492-496.

Torres-Fuentes, C., M. Alaiz and J. Vioque. 2011. Affinity purification and characterisation of chelating peptides from chickpea protein hydrolysates. **Food Chemistry**. 129: 485-490.

Tsopmo, A., A. Cooper and S. Jodayree. 2010. Enzymatic hydrolysis of oat flour protein isolates to enhance antioxidative properties. **Adv. J. Food Sci. Technol.** 2: 206-212.

Tsopmo, A., A. Romanowski, L. Banda, J.C. Lavoie, H. Jenssen and J.K. Friel. 2011. Novel anti-oxidative peptides from enzymatic digestion of human milk. **Food Chemistry**. 126: 1138-1143.

Tsuji, H., M. Kimoto and Y. Natori. 2001. Allergens in major crops. **Nutr. Res.** 21: 925-934.

Udenigwe, C.C., Y.-L. Lu, C.-H. Han, W.-C. Hou and R.E. Aluko. 2009. Flaxseed protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. **Food Chemistry**. 116: 277-284.

Voss, C., S. Sepulveda-Boza and F.W. Zilliken. 1992. New isoflavonoids as inhibitors of porcine 5-lipoxygenase. **Biochem Pharmacol**. 44: 157-162.

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

Wang, M., N.S. Hettiarachchy, M. Qi, W. Burks and T. Siebenmorgen. 1999 Preparation and functional properties of rice bran protein isolate **J. Agric. Food Chem.** 47: 411-416.

Watchararuji, K., M. Goto, M. Sasaki and A. Shotipruk. 2008 Value-added subcritical water hydrolysate from rice bran and soybean meal **Bioresource Technology**. 99: 6207-6213.

Wichi, H.P. 1988. Enhanced tumour development by butylated hydroxyanisole (bha) from the perspective of effect on forestomach and oesophageal quamous epithelium. **Food Chem.Toxicol**. 26: 717-723.

Wright, J.S., E.R. Johnson, G.A.P.t.a.o. DiLabio, a.o.s. phenolic antioxidants: theoretical methods, a.a.t.m.f.o.a. effects and 123:1173–1183. 2001. Predicting the activity of phenolic antioxidants: Theoretical methods, analysis of substituent effects, and application to major families of antioxidants. **J. Am. Chem. Soc.** 123: 1173-1183.

Wu, H.C., H.M. Chen and C.Y. Shiau. 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*scomber austriasicus*). **Food Research International**. 36: 349-357.

Xia, Y., F. Bamdad, M. Gänzle and L. Chen. 2012. Fractionation and characterization of antioxidant peptides derived from barley glutelin by enzymatic hydrolysis. **Food Chemistry**. 134: 1509-1518.

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

Yamaguchi, N., Y.Yokoo and M. Fujimaki. 1975. Studies on antioxidative activities of amino compounds on fats and oils. Part ii. Antioxidative activities of dipeptides and their synergistic effects of tocopherol. **Nippon Shokuhin Kogyo Gakkaishi**. 425-430.

Yang, Z. and W. Zhai. 2010. Identification and antioxidant activity of anthocyanins extracted from the seed and cob of purple corn (*zea mays* l.). **Innovative Food Science and Emerging Technologies**. 11: 169-176.

You, L., M. Zhao, J.M. Regenstein and J. Ren. 2010. Purification and identification of antioxidative peptides from loach (*misgurnus anguillicaudatus*) protein hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry. **Food Research International**. 43: 1167-1173.

You, S.-J., C.C. Udenigwe, R.E. Aluko and J. Wu. 2010. Multifunctional peptides from egg white lysozyme. **Food Research International**. 43: 848-855.

Young, V.R., J.W.J. Erdman and J.C. King. 1998. Dietary reference intakes. Proposed definition and plan for review of dietary antioxidant and related compounds. Washington D.C. National Academy Press.

Yu, B.P. 1994. Cellular defenses against damage from reactive oxygen species. **Physiol. Rev.** 74: 139-162.

Zhang, J., H. Zhang, L. Wang, X. Guo, X. Wang and H. Yao. 2010. Isolation and identification of antioxidative peptides from rice endosperm protein enzymatic hydrolysate by consecutive chromatography and maldi-tof/tof ms/ms. **Food Chemistry**. 119: 226-234.

Zhang, L., J. Li and K. Zhou. 2010. Chelating and radical scavenging activities of soy protein hydrolysates prepared from microbial proteases and their effect on meat lipid peroxidation. **Bioresource Technology**. 101: 2084-2089.

Zheng, X.-q., L.-t. Li, X.-l. Liu, X.-j. Wang, J. Lin and D. Li. 2006. Production of hydrolysate with antioxidative activity by enzymatic hydrolysis of extruded corn gluten. **Apply Microbiology Biotechnology**. 73: 763-770.

Zhou, K., C. Canning and S. Sun. 2013. Effects of rice protein hydrolysates prepared by microbial proteases and ultrafiltration on free radicals and meat lipid oxidation. **LWT Food Science and Technology**. 50: 331-335.

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

Zilic, S., G. Akıllıoglu, A. Serpen, M. Barac and V. Gokmen. 2012. Effects of isolation, enzymatic hydrolysis, heating, hydratation and maillard reaction on the antioxidant capacity of cereal and legume proteins. **Food Research International.** 49: 1-6.

http://www.sebiology.org/publications/Bulletin/January_2008/antioxidants.html

APPENDICS

Appendix A

Chemical analysis

Appendix A

Chemical analysis

Protein determination (Modified from Bradford, 1976)

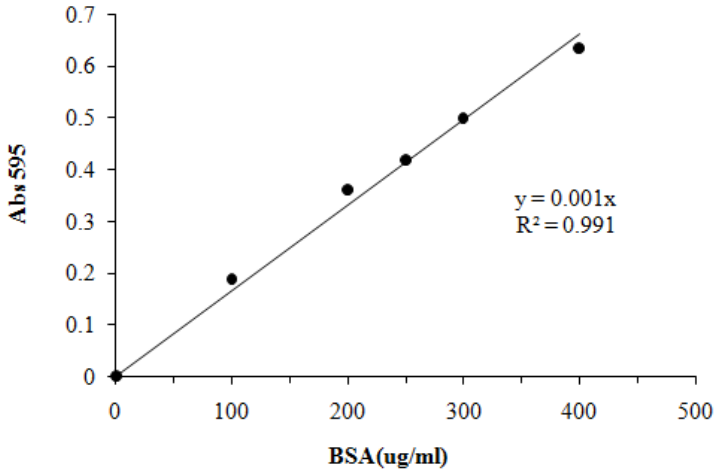
1. Chemincal reagents

- Bio Rad Protein Assay Kit (Cataloog no. 500 0001); Dilutte 1 part of Bio Rad Protein Assay Kit concentrat with 4 parts of distilled water then filter through Watman No 4 filter before using..

- BSA FractionV (standard protein.); Dissoved BSA in distilled water in rang of 50 500 µg/ml.

2. Procedure

Pipette 10 µl of standard protein (BSA fraction V; working rang 50 - 500 µg/ml) or unknown sample into 96-well flat bottom polystyrene microplate, then add 200 µl of dilutted Bio Rad Protein Assay Kit to each well and mix. Incubate at room temperature for 5 min and measure the absorbance at 595 nm with microplate Reader.



Appendix Figure A1 Standard curve of BSA (Microplate assay, Bradford method, 1976)

ORAC assay (Modified from Huang *et al*., 2002)

1. Chemincal reagents

- 75 mM potassium phosphate buffer pH 7.4 (PBS)

- 8.16 x 10^{-5} mM fluorescein; Stock solution : 4.19 x 10^{-3} mM fluorescein (MW 376.28) was made in 75 mM phosphate buffer (pH 7.4) and was kept at 4 °C in dark condition. The fluorescein stock solution at such condition can last several months. The 8.16 x 10^{-5} mM fresh fluorescein working solution was made daily by further diluting the stock solution in 75 mM phosphate buffer (pH 7.4).

- Trolox standard; 10 mM Trolox solution (2.5 mg in 1 ml methanol), diluted to 1 mM by adding 9.0 ml water, divided into 100 µl aliquots and store at -80 °C.

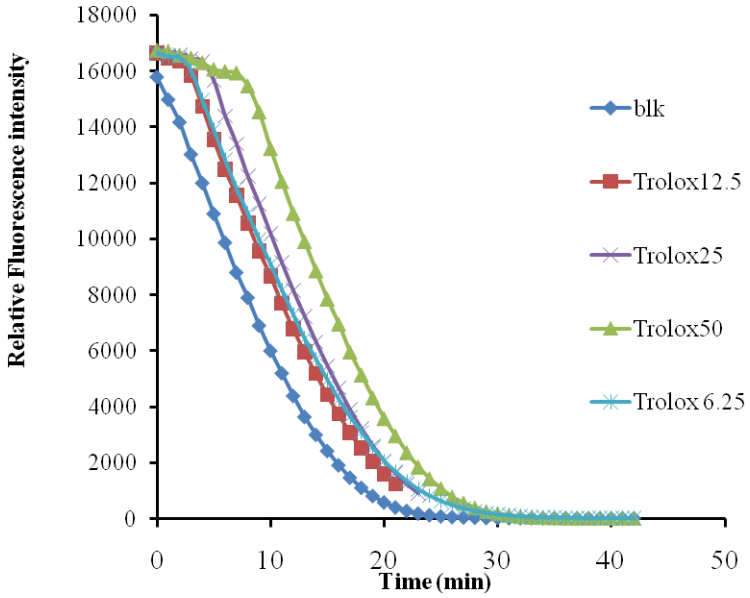
- 153 mM AAPH; 0.414 g of AAPH (MW 271.19) was dissolved in 10 ml PBS and was kept in an ice bath. The unused AAPH solution was discarded within 8 h.

2. Procedure

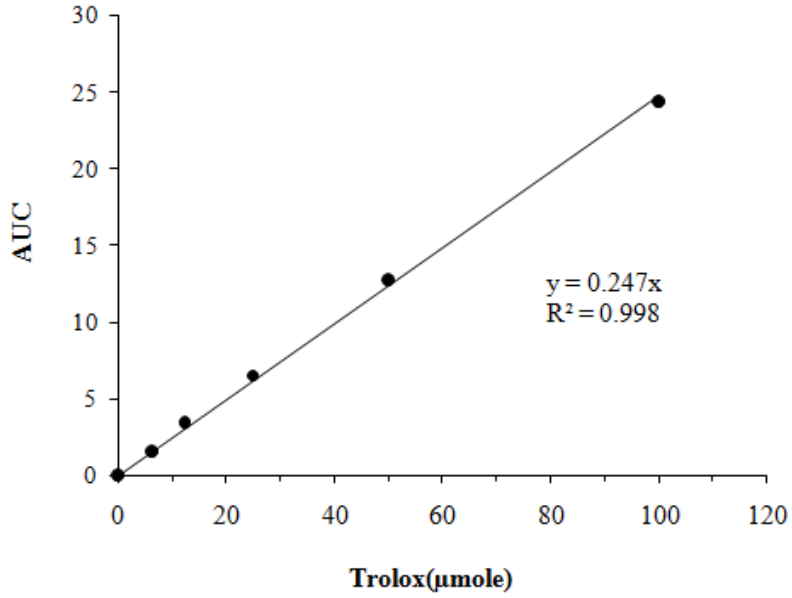
Twenty five µl of sample or standards(Trolox at final concentration of 50, 25, 12.5, and 6.25 µM), diluted in PBS pH 7.4, are added to the wells of a 96 well plate (flat bottom in triplicates, followed by addition of 150 µl/well of 8.16 x 10^{-5} mM fluorescein. Cover with the lid and warm this filled plate into 37 °C in TCAN microplate reader for for 30 s before the initial fluorescence was measured with excitation, 485 nm; emission, 535 nm, and the fluorescence was recorded at 37 °C every min until the reading remained constant. The standard curve is obtained by plotting Trolox concentration against the average net AUC of the three measurements for each concentration. Final ORAC values are calculated using the regression equation between Trolox concentration and the net AUC and are expressed as micromole Trolox equivalents per liter for liquid samples or per gram protein. The AUC is calculated as;

$$AUC = 0.5 + f_1/f_0 + ... f_i/f_0 + ... + f_{34}/f_0 + 0.5(f_{35}/f_0)$$

where f_0) initial fluorescence reading at 0 min and f_i) fluorescence reading at time *i*. The net AUC is obtained by subtracting the AUC of the blank from that of a sample.



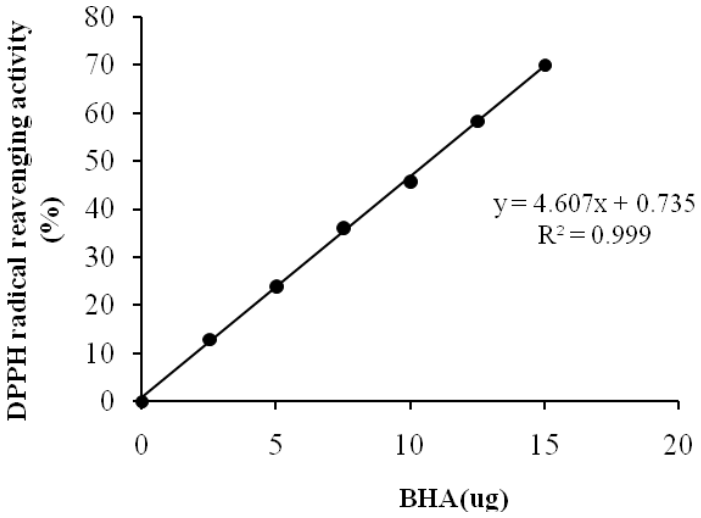
Appendix Figure A2 Kinetic curve of Trolox standard (ORAC assay)



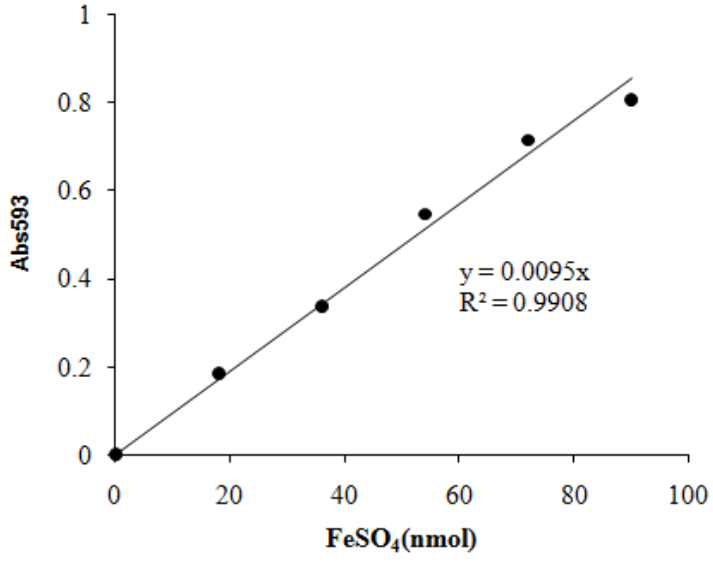
Appendix Figure A3 Standard curve of Trolox (ORAC assay)

Appendix B

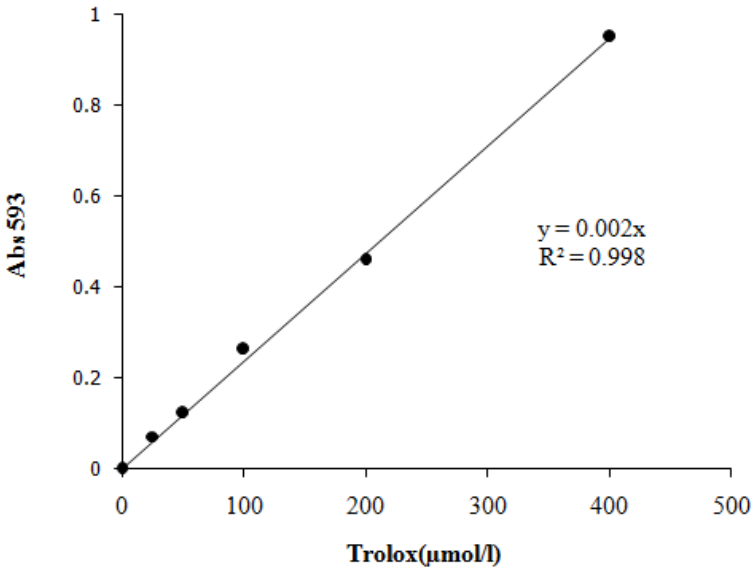
Standard curve



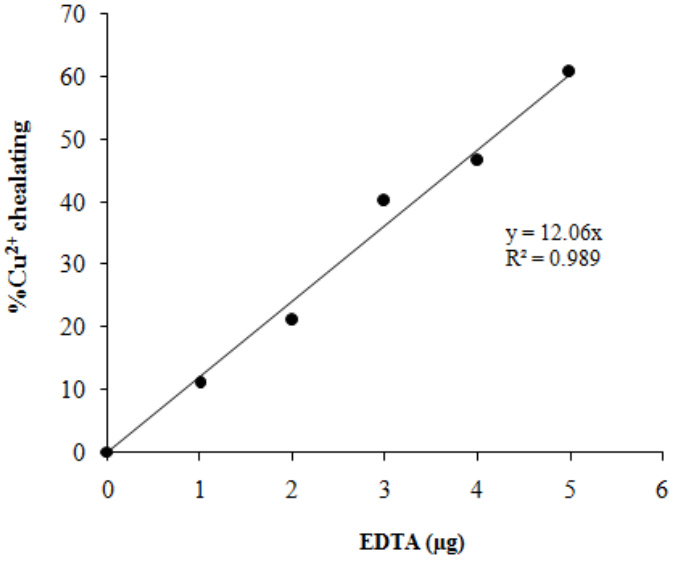
Appendix Figure B1 Standard curve of BHA (DPPH assay)



Appendix Figure B2 Standard curve of $FeSO_4$ (FRAP, test tube assay)



Appendix Figure B3 Standard curve of Trolox (FRAP, microplate assay)



Appendix Figure B4 Standard curve of EDTA (Cu^{2+}-chelating activity)

Appendix C

Statistical Data

Appendix C

Statistical Data

Appendix Table C1 Antioxidant activity of alcalase-hydrolyzed AE-RBP determined by DPPH radical scavenging activity and FRAP assay at different hydrolysis times.

Hydrolysis time(min)	DPPH radical scavenging activity (%)	$FeSO_4$(µmol/ml) hydrolysate
0	32.04 ± 4.75[a]	951.6 ± 201.3 [a]
30	35.82 ± 4.32[a]	1005.4 ± 116.8 [a]
60	36.37± 5.37 [a]	1017.0 ± 166.8 [a]
90	36.29 ± 2.53 [a]	1107.0 ± 166.8 [a]
120	35.49 ± 1.83 [a]	1018.5 ± 178.90 [a]

Mean values ± SD in the same column with same letters are not significantly different ($p > 0.05$).

Appendix Table C2 Antioxidant activity of papain-hydrolyzed AE-RBP determined by DPPH radical scavenging activity and FRAP assay at different hydrolysis times.

Hydrolysis time(min)	DPPH radical scavenging activity (%)	$FeSO_4$(µmol/ml) hydrolysate
0	49.53 ± 0.44[a]	3993.6 ± 191.0 [a]
30	52.38 ± 0.28[a]	4174.0 ± 320.9 [a]
60	56.22± 0.96 [a]	4257.0 ± 225.7 [a]

Mean values ± SD in the same column with same letters are not significantly different ($p > 0.05$).

Appendix Table C3 Antioxidant activity (ORAC assay) of native and denatured rice bran protein that hydrolyzed with trypsin.

Protein fractions	(µmol of TE /µg protein)	
	Non hydrolyzed	Hydrolyzed
Albumin		
Native	2.21 ± 0.09^{c}	2.30 ± 0.16^{c}
Denature	2.88 ± 0.14^{b}	4.07 ± 0.39^{a}
Globulin		
Native	0.63 ± 0.24^{e}	0.84 ± 0.04^{e}
Denature	2.18 ± 0.13^{c}	2.88 ± 0.14^{b}
Glutelin		
Native	0.14 ± 0.04^{f}	0.45 ± 0.11^{e}
Denatured	0.69 ± 0.20^{e}	1.10 ± 0.05^{d}

Mean values $\pm$ SD with different superscripts are significantly different ($p \leq 0.05$).

Appendix Table C4 Antioxidant activity (ORAC assay) of native and denatured rice bran protein that hydrolyzed with papain.

Protein fractions	(µmol of TE /µg protein)	
	Non hydrolyzed	Hydrolyzed
Albumin		
Native	1.45 ± 0.15^{cde}	1.85 ± 0.11^{bc}
Denature	2.31 ± 0.49^{a}	2.47 ± 0.44^{a}
Globulin		
Native	1.11 ± 0.06^{ef}	1.61 ± 0.22^{dc}
Denature	2.18 ± 0.18^{ab}	2.38 ± 0.14^{a}
Glutelin		
Native	0.43 ± 0.08^{g}	0.80 ± 0.10^{gf}
Denatured	1.36 ± 0.09^{de}	1.62 ± 0.01^{cd}

Mean values $\pm$ SD with different superscripts are significantly different ($p \leq 0.05$).

Appendix Table C5 Antioxidant activity of peptide fractions from trypsin-hydrolyzed denatured albumin that was isolated by RP-HPLC.

Fraction No.	ORAC (nmol of TE/% Area)	% Cu chealating[1]	FRAP (μmol of TE /mg protein)
6	3.61 ± 1.21^{fg}	-	-
7	3.89 ± 1.14^{efg}	-	-
8	3.69 ± 1.00^{fg}	-	-
9	0.48 ± 0.10^{g}	-	-
10	7.10 ± 1.17^{def}	-	-
11	8.00 ± 1.00^{cde}	-	-
12	4.55 ± 0.47^{efg}	-	-
13	12.66 ± 2.07^{b}	14.89 ± 2.80^{def}	6.71 ± 0.274^{g}
14	22.89 ± 5.79^{a}	27.94 ± 4.16^{a}	12.03 ± 0.045^{c}
15	24.90 ± 2.18^{a}	16.48 ± 2.68^{def}	16.10 ± 0.164^{a}
16	24.73 ± 5.59^{a}	18.47 ± 1.80^{de}	8.58 ± 0.495^{ef}
17	9.60 ± 0.90^{bcd}	12.12 ± 2.10^{efgh}	13.95 ± 1.019^{b}
18	10.19 ± 1.59^{bcd}	15.66 ± 2.49^{cde}	10.55 ± 0.321^{d}
19	12.62 ± 2.30^{b}	12.98 ± 3.28^{defg}	10.62 ± 0.448^{d}
20	11.89 ± 1.14^{bc}	25.05 ± 1.34^{ab}	10.33 ± 0.324^{d}
21	9.55 ± 0.78^{bcd}	13.85 ± 3.61^{defg}	9.58 ± 0.569^{ed}
22	7.17 ± 1.57^{def}	9.60 ± 3.01^{fgh}	7.66 ± 0.326^{gf}
23	2.52 ± 1.37^{g}	8.08 ± 0.40^{gh}	4.60 ± 0.326^{i}
24	4.30 ± 1.48^{efg}	6.97 ± 0.52^{h}	4.67 ± 0.100^{h}
25	3.85 ± 2.10^{g}	-	-
26	0.57 ± 1.05^{g}	-	-
27	0.88 ± 0.88^{g}	-	-
28	0.40 ± 0.52^{g}	-	-
29	0.57 ± 0.50^{g}	-	-
30	$0.00 \pm 0.12g$	-	-

Mean values $\pm$ SD in the same column with different superscripts are significantly different ($p \leq 0.05$). [1]F13-F24: 0.270 μg protein/well

Appendix D

MS/MS data

.Appendix D

MS/MS data

Appendix Table D1 Amino acid sequence of peptides from trypsin-hydrolyzed denatured Albumin determined by LC–MS/MS using LTQ-FTICR

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
13	GEREEEDER	1,147	100	9	1	11.1	1	11
	REEEQGEEEHGER	1,613	100	13	2	15.4	7	54
	CNDEVK	763	99	6	1	16.7	2	33
	EREEEER	975	100	7	0	0.0	5	71
	KAEEEEEGGGGK	1,219	100	12	5	41.7	5	42
	KAEEEEEGGGGKGEK	1,533	100	15	6	40.0	6	40
	LDQSHK	726	96	6	1	16.7	1	17
	HEAAEEGHGGGK	1,178	98	12	6	50.0	3	25
	AADTAQSAADR	1,075	100	11	5	45.5		0
	DAASDATGR	862	100	9	4	44.4	2	22
	DAADMAQGTAR	1,121	100	11	6	54.5	2	18
	DAAEEAK	732	96	7	3	42.9	3	43
	EAAQGVK	701	100	7	4	57.1	1	14
	CVQECK	822	96	6	1	16.7	1	17
	CVQECKDQQQQQQER	1,991	99	15	1	6.7	3	20
	EEEEEEEQQK	1,306	100	10	0	0.0	7	70
	GEGSSEEEDEGR	1,729	100	12	3	25.0	6	50
	RGEESEDEDR	1,220	100	10	1	10.0	6	60
	RGEESEDEDRR	1,377	100	11	1	9.1	6	55
	RGEGSSEEEDEGR	1,436	100	13	3	23.1	6	46
	RAEAAAGGNK	943	95	10	6	60.0	1	10
	SEPQPSSEEK	1,116	98	10	2	20.0	3	30
	EHGAPQDENR	1,152	100	10	4	40.0	3	30
	DDDMHK	759	99	6	1	16.7	3	50
	EAAEATASGASSK	1,179	100	13	6	46.2	2	15
	GAAGEATR	731	99	8	5	62.5	1	13
	GAEVHEQSK	983	97	9	3	33.3	2	22
	SAQHTASEAGR	1,114	100	11	4	36.4	1	9

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
13	AGCSNPCR	920	99	8	3	37.5	0	0
	EVESGDAAR	932	100	9	4	44.4	3	33
14	QQCMQDCR	1,124	100	8	1	12.5	1	13
	VAGAEDAAK	830	100	9	6	66.7	2	22
	LPADDKK	785	100	7	2	28.6	2	29
	EVESGDAAR	932	100	9	4	44.4	3	33
	GGPAAAMESAASR	1,191	100	13	9	69.2	1	8
	AADGAEEPSSGTADK	1,405	100	15	7	46.7	4	27
	AGDMAGQTK	877	100	9	5	55.6	1	11
	YGEGESSESETER	1,459	100	13	2	15.4	5	38
	GGHELSK	726	100	7	2	28.6	1	14
	SGSSSSSSSSEDDGMGGR	1,676	100	18	5	27.8	3	17
	AAVQGQVEK	929	100	9	5	55.6	3	33
	TKEAAEAAGER	1,132	100	11	5	45.5	3	27
	RSEPQPSSEEK	1,273	100	11	2	18.2	3	27
	RGEGSSEEEDEGRER	1,721	100	15	0	0.0	7	47
	HQQGDTAAAAAGTGR	1,411	100	15	8	53.3	1	7
	RHQQGDTAAAAAGTGR	1,567	98	16	8	50.0	1	6
	RREEEQGEEEHGER	1,769	100	14	2	14.3	7	50
	AADTAQSAADR	1,075	100	11	5	45.5	2	18
	DKAYDAK	809	97	7	1	14.3	2	29
	ASGSSSSSEEGEDDVAAQR	1,868	100	19	6	31.6	5	26
	NTIAGGGGGGHGGNNGGER	1,638	100	19	12	63.2	1	5
	KEQMGEEGYR	1,242	99	10	3	30.0	3	30
	QQHQPPPPSQPQPQQEK	1,978	100	17	6	35.3	1	6
	YRDAAEEAK	1,051	99	9	3	33.3	3	33
	MNPPTCR	890	99	7	3	42.9	0	0
	VESSEPPR	899	100	8	3	37.5	2	25
15	GTAAAAEQVR	973	100	10	6	60.0	1	10
	RGDLER	744	100	6	2	33.3	2	33
	KPLQPGSK	854	100	8	4	50.0	0	0
	GGLSTGDESGGER	1,221	100	13	6	46.2	3	23
	KEQMGEEGYR	1,242	100	10	3	30.0	3	30
	KGGLSTGDESGGER	1,349	100	14	6	42.9	3	21

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
15	SLNSAASTTADR	1,193	100	12	4	33.3	1	8
	CCHQLR	872	100	6	1	16.7	0	0
	KEQPPPPPQHGK	1,339	100	12	6	50.0	1	8
	CLMDCR	869	100	6	2	33.3	1	17
	ARPSPPE	752	100	7	4	57.1	1	14
	ECEEQCLQR	1,251	100	9	0	0.0	3	33
	KARPSPPE	880	100	8	4	50.0	1	13
	LVAQETDER	1,060	100	9	3	33.3	3	33
	EAAANVGASAR	1,016	100	11	7	63.6	1	9
	NAADKDAAEVR	1,159	100	11	6	54.5	3	27
	AKDAADMAQGTAR	1,305	100	13	7	53.8	2	15
	DAADMAQGTAR	1,105	100	11	6	54.5	2	18
	KLDPQAK	798	99	7	3	42.9	1	14
	RGEESEDEDRR	1,377	100	11	1	9.1	6	55
	KEEIER	802	99	6	1	16.7	3	50
	YKAEDEQVR	1,137	100	9	1	11.1	3	33
	RREEEQGEEEHGER	1,679	100	14	2	14.3	7	50
	YGEGESSESETER	1,459	100	13	2	15.4	5	38
	ASGSSSSSEEGEDDVAAQR	1,868	100	19	5	26.3	5	26
	GEEIGETQR	1,017	100	9	3	33.3	3	33
	LRPGDEDR	956	98	8	3	37.5	3	38
	SSSSEEGEDDVAAQR	1,566	100	15	4	26.7	5	33
	HSLGQGHPVK	1,059	98	10	5	50.0	0	0
	TGCGFDGSGNGQCQTGDCGGK	2,119	100	21	9	42.9	2	10
	QQHQPPPPSQPQPQQEK	1,978	100	17	6	35.3	1	6
16	MQCCQQLQDVSR	1,568	100	12	3	25.0	1	8
	EGQTVVPGGTGGK	1,186	100	13	8	61.5	1	8
	EQMGEEGYR	1,097	100	9	3	33.3	3	33
	KEQMGEEGYR	1,242	100	10	3	30.0	3	30
	KGGLSTGDESGGER	1,349	100	14	6	42.9	3	21
	QCAGGAVDEQVR	1,289	100	12	6	50.0	2	17
	SLNSAASTTADR	1,196	100	12	4	33.3	1	8
	KEQPPPPPQHGK	1,339	100	12	6	50.0	1	8
	TSDTIAQAGHGAGEAK	1,513	100	16	7	43.8	2	13

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
16	VAAGTATDYAR	1,095	100	11	6	54.5	1	9
	FIECCSCGEYLKK	1,579	99	13	4	30.8	2	15
	CQHHHDQWK	1,275	97	9	1	11.1	1	11
	ECEEQCLQR	1,251	100	9	1	11.1	3	33
	WEAGQDQR	988	100	8	3	37.5	2	25
	DSCQDLGVSR	1,135	100	10	3	30.0	2	20
	DTSTYKPGTGSDYQ	1,519	100	14	3	21.4	2	14
	CEQDRPPYER	1,349	99	10	2	20.0	2	20
	VVHQIPADQAK	1,205	100	11	6	54.5	1	9
	DKIPGPGSGGAGAGAAAGEGK	1,724	100	21	16	76.2	2	10
	IPGPGSGGAGAGAAAGEGK	1,481	100	19	16	84.2	1	5
	DKTAETAEGAMDR	1,394	100	13	5	38.5	4	31
	TAETAEGAMDR	1,166	100	11	5	45.5	3	27
	RREEEQGEEEHGER	1,679	100	14	2	14.3	7	50
	NVAAAEEEGGQGQMHR	1,683	100	16	8	50.0	3	19
	EAIAGGSTQSMK	1,195	100	12	5	41.7	1	8
	APGGEAYTR	920	100	9	5	55.6	1	11
	HHVADVDEEEDAAR	1,592	100	14	5	35.7	6	43
	HHVADVDEEEDAARK	1,720	100	15	5	33.3	6	40
	DKAVAADQGGGGGDLR	1,486	93	16	10	62.5	3	19
	LAADKEVESGDAAR	1,431	100	14	7	50.0	4	29
17	MEDQLQSQAQGQGQGQTEAIK	2,274	100	21	8	38.1	3	14
	QCVAPGTVDEQVR	1,458	100	13	6	46.2	2	15
	RQCVAPGTVDEQVR	1,614	99	14	5	35.7	2	14
	GKDVTLSAGETAAEHAK	1,684	100	17	8	47.1	3	18
	EVASGTMPDPGSLTAGDTTR	1,978	100	20	9	45.0	3	15
	DELAPPAPTAAEHGGGK	1,617	100	17	11	64.7	3	18
	TTKDELAPPAPTAAEHGGGK	1,947	100	20	11	55.0	3	15
	RVDSEAGHTELYDDR	1,762	100	15	4	26.7	5	33
	DSCQDLGVSR	1,135	100	10	3	30.0	2	20
	MQCCQQLQDVSR	1,568	100	12	3	25.0	1	8
	QYAAQLPSMCR	1,340	100	11	5	45.5	0	0
	GPIPPSR	722	98	7	5	71.4	0	0

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
17	LVSHPIAAHEGR	1,286	100	12	7	58.3	1	8
	KGETDESAWQHGEDVR	1,843	100	16	5	31.3	5	31
	AADGCEPGQGVVPK	1,384	100	14	9	64.3	2	14
	ARDGAGQTGSYIGQTAEAAK	1,951	100	20	10	50.0	2	10
	DGAGQTGSYIGQTAEAAK	1,724	100	18	9	50.0	2	11
	IEPSNACCSVIQK	1,505	100	13	5	38.5	1	8
	GGLSTGDESGGER	1,221	100	13	6	46.2	3	23
	KEQMGEEGYR	1,226	100	10	3	30.0	3	30
	KGGLSTGDESGGER	1,349	100	14	6	42.9	3	21
	AVDSGAVPVSPPEQK	1,480	100	15	9	60.0	2	13
	RAVDSGAVPVSPPEQK	1,636	100	16	9	56.3	2	13
	DTVAGGDVDADAAMK	1,435	100	15	9	60.0	4	27
	DTVAGGDVDADAAMKEQDR	1,963	100	19	9	47.4	6	32
	QCVGHGAPGGAVDEQLR	1,750	100	17	10	58.8	2	12
	RQCVGHGAPGGAVDEQLR	1,906	100	18	10	55.6	2	11
18	EVQDSPLDACR	1,289	100	11	4	36.4	3	27
	GGLSTGDESGGER	1,221	100	13	6	46.2	3	23
	SLEAQENLAEGR	1,316	100	12	5	41.7	3	25
	AVDSGAVPVSPPEQK	1,480	100	15	9	60.0	2	13
	GAASAADEQVWQDCCR	1,823	100	16	7	43.8	3	19
	APGATVVSCVER	1,245	100	12	7	58.3	1	8
	DVALSTGGTASEYAK	1,469	100	15	7	46.7	2	13
	DVTLSAGETAAEHAK	1,499	100	15	6	40.0	3	20
	VNGDDEMLR	1,048	100	9	4	44.4	3	33
	DSCQDLGVSR	1,135	100	10	3	30.0	2	20
	KGFPVR	702	100	6	4	66.7	0	0
	DGAGQTGSYIGQTAEAAK	1,724	100	18	8	44.4	2	11
	DKTGAVLQQAGEQVK	1,571	100	15	7	46.7	2	13
	TGAVLQQAGEQVK	1,328	100	13	7	53.8	1	8
	GPIPPSR	722	98	7	5	71.4	0	0
	ATGGAGAYHPSQGAPGVDPR	1,865	100	20	13	65.0	1	5
	DTVAGGDVDADAAMK	1,435	100	15	9	60.0	4	27

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
18	EEGHTTLGETLR	1,342	100	12	4	33.3	3	25
	EEEEEEEEQQKGQEEEEEEQVGQGYETIR	3,525	100	29	5	17.2	15	52
	GQEEEEEEQVGQGYETIR	2,109	100	18	5	27.8	7	39
	GETDESAWQHGEDVR	1,714	100	15	5	33.3	5	33
	EGGEITTAPEEQIR	1,529	100	14	6	42.9	4	29
	EGEDIGTTPVVR	1,272	100	12	6	50.0	3	25
	IPSVQEVEGDGGAPR	1,510	100	15	9	60.0	3	20
	VPAVAPAAPGDTIAK	1,377	100	15	12	80.0	1	7
	DAAWETAEAAR	1,190	100	11	6	54.5	3	27
	ATAGDTHLGGEDFDNR	1,675	100	16	7	43.8	4	25
	MYQGAGADMGGAAGMDEDAPAGGSGAGPK	2,612	100	29	21	72.4	4	14
	VEIIANDQGNR	1,228	100	11	5	45.5	2	18
	ADEGYCVAVR	1,139	100	10	5	50.0	2	20
	AVVVHADPDDLGK	1,335	100	13	8	61.5	3	23
	QCVGHGAPGGAVDEQLR	1,480	100	17	10	58.8	2	12
	RQCVGHGAPGGAVDEQLR	1,906	100	18	10	55.6	2	11
	KLADLTTTK	990	99	9	3	33.3	1	11
	ELGDKVPAPVK	1,152	100	11	7	63.6	2	18
	MVEEADKFAQEDK	1,539	100	13	5	38.5	5	38
	GGPAAAMESAASR	1,191	100	13	9	69.2	1	8
19	VVYVADYCK	1,116	98	9	4	44.4	1	11
	ECVEAPGDFPR	1,276	100	11	6	54.5	3	27
	EVQDSPLDACR	1,289	100	11	4	36.4	3	27
	RPGALGLR	839	100	8	6	75.0	0	0
	SLEAQENLAEGR	1,316	100	12	5	41.7	3	25
	AYDGSDPSKPIYVSVR	1,753	100	16	7	43.8	2	13
	DDADVSGDLSGLSDK	1,493	100	15	6	40.0	5	33
	MSKDDADVSGDLSGLSDK	1,839	100	18	7	38.9	5	28
	KYEGWPAEPK	1,204	99	10	5	50.0	2	20
	VWCMEK	851	100	6	3	50.0	1	17
	GQPMDEVFPGCR	1,408	99	12	2	16.7	2	17
	NVAGSISGLNAGNAASIPSK	1,827	100	20	12	60.0	0	0
	YGQDATNVGDEGGFAPNIQENK	2,323	100	22	10	45.5	4	18

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
19	AVDSGAVPVSPPEQK	1,480	100	15	9	60.0	2	13
	GAASAADEQVWQDCCR	1,823		16	7	43.8	3	19
	PASPGHGGGLTMAPR	1,405		15	8	53.3	0	0
	TYDLNFKEENNDGSQK	1,091	100	16	3	18.8	4	25
	ATGGAGAYHPSQGAPGVDPR	1,865	100	20	13	65.0	1	5
	SKEEAMADYITK	1,385	93	12	3	33.3	3	25
	LLHAVYR	871	99	7	4	57.1	0	0
	SAEAVELVTK	1,046	100	10	5	50.0	2	20
	NAQQESAFLAGPEK	1,489	95	14	7	50.0	2	14
	GQEEEEEEQVGQGYETIRA	2,180	100	19	6	31.6	7	37
	SFHDLAEHDIR	1,339	100	11	4	36.4	3	27
	DEPGGGGGGMLGTVQESAR	1,774	100	19	11	57.9	3	16
	RDEPGGGGGGMLGTVQESAR	1,946	100	20	11	55.0	3	15
	DPAAAPAAVAPGAAGAPPAQLPR	2,036	100	23	20	87.0	1	4
	EVASGTMPDPGSLTAGDTTR	1,978	100	20	11	55.0	3	15
	ALSEAISGAVQR	1,201	100	12	7	58.3	1	8
	GYQEDVEEDKPVAMESDPEVHGAPMYDSAR	3,350	100	30	13	43.3	9	30
	IPSVQEVEGDGGAPR	1,510	100	15	9	60.0	3	20
	VPAVAPAAPGDTIAK	1,377	100	15	12	80.0	1	7
	APGGCNNACTVFR	1,423	100	13	7	53.8	0	0
	EEGHTTLGETLR	1,342	100	12	4	33.3	3	25
	TTGDVMTHSFGEGYSTR	1,845	100	17	6	35.3	2	12
	LVFGGSAAR	876	100	9	7	77.8	0	0
	CQPGMGYPMYSLPR	1,688	100	14	8	57.1	0	0
	RQCVGHGAPGGAVDEQLR	1,906	99	18	10	55.6	2	11
	AISTSNAYDDQFK	1,459	93	13	4	30.8	2	15
20	ELGAPDVGHPMSEVFR	1,756	100	16	10	62.5	3	19
	QLAAVDDSWCR	1,320	100	11	5	45.5	2	18
	IEPSNACCSVIQK	1,505	100	13	5	38.5	1	8
	DWYSTVDPGHMCTAPDQPTTK	2,406	100	21	8	38.1	3	14
	EVQDSPLDACR	1,289	100	11	4	36.4	3	27
	MQCCQQLQDVSR	1,568	100	12	3	25.0	1	8
	RPGALGLR	839	100	8	6	75.0	0	0

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
20	KEQMGEEGYR	1,226	100	10	3	30.0	3	30
	SLEAQENLAEGR	1,316	100	12	5	41.7	3	25
	GVTSNPSIFQK	1,177	100	11	5	45.5	0	0
	ANIPCLCAGVTK	1,303	100	12	7	58.3	0	0
	IEPSEACCAVWQR	1,605	99	13	6	46.2	2	15
	GQPMDEVFPGCR	1,408	100	12	3	25.0	1	8
	IEEELGAAAVYAGAK	1,491	100	15	10	66.7	3	20
	AVDSGAVPVSPPEQK	1,480	100	15	9	60.0	2	13
	ETDALTGAVQLPDSAGEF	1,829	100	18	9	50.0	4	22
	GAASAADEQVWQDCCR	1,823		16	7	43.8	3	19
	QLAAVDDGWCR	1,273	100	11	6	54.5	2	18
	AADGCEPGQGVVPK	1,364	98	14	9	64.3	2	14
	EQCEHQQDWWEK	1,702	100	12	2	16.7	4	33
	DSCQDLGVSR	1,135	100	10	3	30.0	2	20
	STEWHVGDEPLTGAR	1,336	100	15	7	46.7	3	20
	VVTGPLIGPSPSGR	1,336	100	14	10	71.4	0	0
	EYGGIQEGVNDWK	1,494	100	13	6	46.2	3	23
	ATGGAGAYHPSQGAPGVDPR	1,865	100	20	13	65.0	1	5
	DTVAGGDVDADAAMK	1,435	100	15	9	60.0	4	27
	DTVAGGDVDADAAMKEQDR	1,979	99	19	9	47.4	6	32
	SKEEAMADYITK	1,385	100	12	2	16.7	3	25
	AAEMAAQGSDVTMPGGLADQAQAAAR	2,487	100	26	17	65.4	3	12
	MEDQLQSQAQGQGQGQTEAIK	2,274	95	21	8	38.1	3	14
	CYTECFGMK	1,194	100	9	3	33.3	1	11
	GNAYAQVAIGTEDVYK	1,698	100	16	8	50.0	2	13
	QPGPLPGLNTK	1,121	100	11	7	63.6	0	0
	SAEAVELVTK	1,046	100	10	5	50.0	2	20
	VDNADFLK	920	99	8	4	50.0	2	25
	AQIHDVVLVGGSTR	1,451	100	14	7	50.0	1	7
	IEDAIEDAIK	1,116	98	10	5	50.0	4	40
	DEPGGGGGGMLGTVQESAR	1,790	100	19	12	63.2	3	16
	KGETDESAWQHGEDVR	1,843	100	16	5	31.3	5	31
	RDEPGGGGGGMLGTVQESA	1,774	100	19	12	63.2	3	16

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
20	GTSQVEGVVTLTQDDQ GPTTVNVR	2,500	100	24	10	41.7	3	13
	QIPLSGPNSVVGR	1,306	100	13	8	61.5	0	0
	RVDSEAGHTELYDDR	1,762	100	15	4	26.7	5	33
	ATGHVLWTTAR	1,212	100	11	6	54.5	0	0
	HVGAAGKPDADVQNDP MPVSDAATAGEEWK	3,062	100	30	17	56.7	6	20
	LGDLGGPVVEDPAAPR	1,562	100	16	12	75.0	3	19
	ADEGFSVTVR	1,080	100	10	5	50.0	2	20
	AVVVHADPDDLGK	1,335	100	13	8	61.5	3	23
	CDALNHMLR	1,129	100	9	4	44.4	1	11
	ESGAADAGHPMAEVFR	1,644	100	16	10	62.5	3	19
	APAALGPYSQAIK	1,286	100	13	9	69.2	0	0
	FVSESVEEQTEQVMK	1,785	100	15	5	33.3	4	27
	VDQYGNPVPPVDQYGN PVPDEPAPR	2,719	100	25	14	56.0	4	16
	EGLENLKGELK	1,229	100	11	5	45.5	3	27
	DAAWETAEAAR	1,190		11	6	54.5	3	27
	CQPGMGYPMYSLPR	1,688	95	14	8	57.1	0	0
	ELGATDVGHPMAEVFPG CR	2,058	100	19	12	63.2	3	16
	QCVGHGAPGGAVDEQLR	1,750	95	17	10	58.8	2	12
	QLAAVDDSWCR	1,320	100	11	5	45.5	2	18
	RQCVGHGAPGGAVDEQ LR	1,906	100	18	10	55.6	2	11
	DTEFFK	785	100	6	2	33.3	2	33
	TGCGFISGGSLGQCQTG DCGGTLR	2,445		24	12	50.0	1	4
	GATTIIGGGDSVAAVEK	1,545	100	17	11	64.7	2	12
	NEEMGVVGHDQATDAA AEQGVNVSDTLVPGGGR	3,296	100	33	19	57.6	6	18
21	GNAYAQVAIGTEDVYK	1,698	100	16	8	50.0	3	19
	GPTPEPLCQVMLR	1,513	100	13	8	61.5	1	8
	SAEAVELVTK	1,046	100	10	6	60.0	2	20
	SPEVVLEWPKK	1,311	100	11	5	45.5	2	18
	YTIAMLGYADEDK	1,505	100	13	6	46.2	3	23
	DLQDLIMECQK	1,392	100	11	4	36.4	3	27
	KEQMGEEGYR	1,242	100	10	3	30.0	3	30
	SLEAQENLAEGR	1,316	99	12	5	41.7	3	25
	IEPSEACCAVWQR	1,605	100	13	6	46.2	2	15

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
21	KYEGWPAEPK	1,204	99	10	5	50.0	2	20
	GQPMDEVFPGCR	1,392	97	12	6	50.0	2	17
	GYPMYPLPR	1,093	100	9	6	66.7	0	0
	NEWGWCK	978	95	7	3	42.9	1	14
	VNQIGSVTESIEAVK	1,573		15	4	26.7	2	13
	DIDGIIVR	900	100	8	5	62.5	2	25
	ETDALTGAVQLPDSAGER	1,829	100	18	9	50.0	4	22
	ESTLHLVLR	1,067	100	9	4	44.4	1	11
	TITLEVESSDTIDNVK	1,763	100	16	5	31.3	4	25
	ELGATEAGHPMAEVFPGCR	2,028	100	19	12	63.2	3	16
	AADGCEPGQGVVPK	1,386	100	14	7	50.0	2	14
	GAMAAIAEGLPGRDECDLDTR	2,217	100	21	12	57.1	5	24
	DVTLSAGETAAEHAK	1,499	100	15	6	40.0	3	20
	EKPSIGTVVAVGPGPLDDEGK	2,064	100	21	12	57.1	4	19
	EKPSIGTVVAVGPGPLDDEGKR	2,220	100	22	12	54.5	4	18
	TAGGLILTETTK	1,204	100	12	6	50.0	1	8
	DSQPVDLFDQAR	1,390	98	12	5	41.7	3	25
	TGFINGTPLEAGK	1,305	100	13	8	61.5	1	8
	INGEDLNEFVESPR	1,678	100	14	6	42.9	4	29
	ECEEQCLQR	1,251	96	9	0	0.0	3	33
	EQCEHQQDWWEK	1,702	100	12	2	16.7	4	33
	AAMEELLR	947	100	8	5	62.5	2	25
	EGLAGSYVDPQPAASAFR	1,835	100	18	10	55.6	2	11
	AWLGASQPPHVVVFK	1,635	100	15	8	53.3	0	0
	DSCQDLGVSR	1,135	100	10	3	30.0	2	20
	FTPWGGAAAPEDR	1,374	100	13	9	69.2	2	15
	KGFPVR	702	100	6	4	66.7	0	0
	VVTGPLIGPSPSGR	1,336	100	14	10	71.4	0	0
	LYALDDEVHLGNK	1,486	100	13	6	46.2	3	23
	ATGGAGAYHPSQGAPGVDPR	1,865	100	20	13	65.0	1	5
	HAPWYIEDEPR	1,412	98	11	5	45.5	3	27
	RPPIGPGSCFIQ	1,328	100	12	8	66.7	0	0
	CEQDRPPYER	1,349	100	10	2	20.0	3	30
	ELESLCNPIISK	1,402	100	12	5	41.7	2	17

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty	Total A.A (residues)	HB A.A (residues)	% HB A.A of peptides	Acidic A.A (residues)	% Acidic A.A of peptides
21	KIEDAIEDAIK	1,244	100	11	5	45.5	4	36
	DTVAGGDVDADAAMK	1,435	100	15	9	60.0	3	20
	FPDEQVVGAAVGGYR	1,564	100	15	10	66.7	2	13
	LFELTGDECR	1,239	100	10	4	40.0	3	30
	LVFGGSAAR	876	100	9	6	66.7	1	11
	GTSQVEGVVTLTQDDQG PTTVNVR	2,500		24	10	41.7	2	8
	AVVVHADPDDLGK	1,335	100	13	8	61.5	3	23
	HAGDLGNITAGADGVAN VNVSDSQIPLTGAH	2,970	100	31	18	58.1	3	10
	DAGRDWDIDYEMR	1,657	100	13	5	38.5	5	38
	LGDLGGPVVEDPAAPR	1,562	100	16	12	75.0	3	19
	ELESICNPIIAK	1,386	100	12	3	25.0	2	17
	MKELESICNPIIAK	1,645	100	14	4	28.6	2	14
	NALENYAYNMR	1,358		11	4	36.4	1	9
	IKDEEGNPAFALVNK	1,644	100	15	7	46.7	3	20
	FEALDANGDGVLSR	1,463	100	14	8	57.1	3	21
	ESGAADAGHPMAEVFR	1,644	100	16	10	62.5	3	19
	QLAAIDSSWCR	1,306	100	11	4	36.4	1	9
	LDPGQTWTINVPAGTTGC R	1,940	100	19	10	52.6	1	5
	CPDAYLFPEDNTK	1,569	100	13	5	38.5	3	23
	DAAWETAEAAR	1,190		11	6	54.5	3	27
	ITEGLGLK	829	98	8	4	50.0	1	13
	CQPGMGYPMYSLPR	1,672	100	14	8	57.1	0	0
	CSALNHMVGGIYR	1,493	100	13	6	46.2	0	0
	ELGATDVGHPMAEVFPG CR	2,042	100	19	12	63.2	3	16
	QCVGHGAPGGAVDEQLR	1,750	99	17	10	58.8	2	12
	QLAAVDDSWCR	1,320	100	11	5	45.5	2	18
	RQCVGHGAPGGAVDEQL R	1,906	100	18	10	55.6	2	11
	DVVGGAAEALPADMVA TR	1,742	100	18	13	72.2	3	17
	GVAEAVAAAAEMNEGR	1,545	100	16	11	68.8	3	19

HB A.A.; Hydrophobic amino acid: Gly (G), Ala (A), Val (V), Leu (L), Pro (P), Met (M), Phe (F), Trp (W) and Ile (I)

Acidic A.A.; Acidic amino acid: D (Asp) and E (Glu)

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty
22	LDPGQTWTINVPAGTTGGR	1,940	100
	CQPGMGYPMYSLPR	1,672	100
	CSALNHMVGGIYR	1,477	98
	ELGATDVGHPMAEVFPGCR	2,042	100
	QCVGHGAPGGAVDEQLR	1,750	100
	RQCVGHGAPGGAVDEQLR	1,906	97
	ECEEQCLQR	1,251	96
	GAASAADEQVWQDCCR	1,823	100
	ITVGDVLTDATAK	1,303	100
	WLDGNQLAEADEFEDK	1,879	100
	AAMEELLR	931	95
	NEEMGVVGHDQATDAAAEQGVNVSDTLVPGGGR	3,280	100
	VEQDDGAAVVAGVVGAAPGAVR	2,007	99
	YGDVFPVTGSLAAKPIAPR	1,958	100
	IAESEPVDLFSDAGR	1,605	100
	KIAESEPVDLFSDAGR	1,733	100
	VADSQPVDLFDQAR	1,560	100
	DVALSTGGTASEYAK	1,469	100
	DWDIDYEMR	1,242	100
	EVASGTMPDPGSLTAGDTTR	1,978	100
	NPYSHSIPICEVSYSLK	1,993	100
	AFVVHELEDDLGK	1,471	100
	EALEWLDENQTAEKEEYEEK	2,482	100
	LGIDWDEVGK	1,131	100
	TIDANVSDAEGVYSALEK	1,881	100
	DLQDLIMECQK	1,408	100
	AVVVHADPDDLGK	1,335	100
	HAGDLGNITAGADGVANVNVSDSQIPLTGAHSIIGR	3,497	100
	VACGIIGLQG	987	100
	CEAISHMLGGIYR	1,506	100
	ELGAPDVGHPMSEVFR	1,740	100
	AWLGASQPPHVVVFK	1,635	100
	DSCQDLGVSR	1,135	100

Fraction no.	Peptide sequence	MW	Probability of certainty
22	FNAATICVQSTEWHVGDEPLTGAR	2,658	100
	FTPWGGAAAPEDR	1,374	100
	PASPGHGGGLTMAPR	1,405	100
	PPHVVVFK	922	98
	VVTGPLIGPSPSGR	1,336	100
	EVQDSPLDACR	1,289	100
	SGHAIPAVGLGTWR	1,421	100
	VLTGEELFVNK	1,248	100
	LPGTPGVALHIIER	1,472	100
	KIPLYQHIANLAGNK	1,679	100
	DAAWETAEAAR	1,190	100
	SATENIYGSAASAAEAFR	1,815	100
	NVAGSISGLNAGNAASIPSK	1,827	100
	ASGSEAEKSPEVVLEWPKK	2,112	99
	GNAYAQVAIGTEDVYK	1,698	100
	GPTPEPLCQVMLR	1,497	100
	SAEAVELVTK	1,046	100
	SPEVVLEWPKK	1,311	100
	VLVDNADFLK	1,133	99
	VAYVLDGEGEAEIVCPHLSR	2,213	96
	MSHISTGGGASLELLEGK	1,786	100
	ATGGAGAYHPSQGAPGVDPR	1,865	100
	GAMAAIAEGLPGRDECDLDTR	2,217	100
	HSNSIKDEEGYPAFALVNR	2,146	99
	ILPWGDEAYAGGSANAPR	1,844	100
	IPSVQEVEGDGGAPR	1,510	100
	LGDLGGPVVEDPAAPR	1,562	100
	VPAVAPAAPGDTIAK	1,377	100
	QATVGPVTTGRPGIFNLK	1,855	96
	SKEEAMADYITK	1,385	100
	DISPPVEWYGVPDGTR	1,787	100
	KDISPPVEWYGVPDGTR	1,915	100
	GGLSTGDESGGER	1,221	100

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty
22	SLEAQENLAEGR	1,316	100
	IEPSEACCAVWQR	1,605	99
	AVDSGAVPVSPPEQK	1,480	100
	ETDALTGAVQLPDSAGER	1,829	100
	VGYVRDIDGIIVR	1,474	100
	VVETAQAIGEK	1,144	97
	GWSVPDANAEPYNSWAGR	1,976	100
	YDDGNTVFTFDR	1,449	100
23	CSALNHMVGGIYR	1,478	100
	QCVGHGAPGGAVDEQLR	1,750	100
	QLAAVDDSWCR	1,320	100
	VEKPEAELAELSFQSVGR	1,988	100
	DVALSTGGTASEYAK	1,469	100
	DVTLSAGETAAEHAK	1,499	100
	AVDSGAVPVSPPEQK	1,480	99
	DIDGIIVR	900	100
	DIDGIIVRMGSHVRA	1,638	100
	ETDALTGAVQLPDSAGER	1,829	100
	GPVEICFDYADVDAAYR	1,960	100
	WAELESGTTTIAFTPLHQR	2,157	100
	FVVAGDLGQTGWTESTLR	1,936	100
	TFAYEGTVSAAGVTGASGQLQPTTR	2,469	100
	VDQYGNPVPPVDQYGNPVPDEPAPR	2,719	100
	IAESEPVDLFSDAGR	1,605	100
	CGALDHMLSGIYR	1,508	100
	AFVVHELEDDLGK	1,471	100
	AFVVHELEDDLGKGGHELSLSTGNAGGR	2,864	100
	LGIDWDEVGK	1,131	100
	AVVVHADPDDLGK	1,335	100

Fraction no.	Peptide sequence	MW	Probability of certainty
23	HAGDLGNITAGADGVANVNVSDSQIPLTGAHSIIGR	3,497	100
	AWLGASQPPHVVVFK	1,635	100
	DSCQDLGVSR	1,135	98
	FTPWGGAAAPEDR	1,374	100
	VAQETDER	946	97
	VLPCPLLVAQETDER	1,739	100
	VLPCPLLVAQETDERR	1,895	99
	VVTGPLIGPSPSGR	1,336	100
	DISPPVEWYGVPDGTR	1,787	100
	AVDNVNSIIGPALIGK	1,580	100
	GNAYAQVAIGTEDVYK	1,698	100
	GPTPEPLCQVMLR	1,497	100
	LLHAVYR	871	99
	SPEVVLEWPK	1,183	100
	TTVIELTYNYGVTEYTK	1,194	100
	VVLVDNADFLK	1,133	100
	YDIGAGFGHFA	1,154	97
	EVDELLNAQQESAFLAGPEK	2,188	100
	ATGGAGAYHPSQGAPGVDPR	1,865	100
	AMVEVLVEEEEAPLACK	1,932	100
	AFLQPSHYDADEVFYVK	2,028	100
	LVPYNPGYQDESVLWTESR	2,252	100
	IADLAVGLATGQIK	1,369	98
	VVIGMDVAASEFYNDK	1,773	100
	IPSVQEVEGDGGAPR	1,510	100
	LGDLGGPVVEDPAAPR	1,562	100
	GLQEEFEEFAEK	1,455	100
	GFEVEVLRPGFGVPR	1,658	100
	EVEAVCNPIISAVYQR	1,847	100
	LKEVEAVCNPIISAVYQR	2,088	100
	DTVAGGDVDADAAMK	1,435	100
	DTVAGGDVDADAAMKEQDR	1,963	98

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty
24	DLQDLIMECQK	1,408	99
	MQCCQQLQDVSR	1,568	99
	SLEAQENLAEGR	1,316	100
	GLQEEFEEFAEK	1,455	100
	GNAYAQVAIGTEDVYK	1,698	100
	GPTPEPLCQVMLR	1,497	100
	IASFLDPDGWK	1,248	100
	PDGYMFELIQR	1,368	98
	SAEAVELVTK	1,046	100
	SPEVVLEWPK	1,183	100
	SPEVVLEWPKK	1,311	99
	TTVIELTYNYGVTEYTK	1,994	100
	VVLVDNADFLK	1,232	100
	VVLVDNADFLKELQ	1,602	100
	YDIGAGFGHFAIATEDVYK	2,073	100
	EVDELLNAQQESAFLAGPEK	2,187	100
	ARFEELNMDLFR	1,556	100
	MKELESLCNPIISK	1,661	100
	NALENYAYNMR	1,358	100
	GTSQVEGVVTLTQDDQGPTTVNVR	2,500	100
	LACGVVGLTPL	1,099	99
	LPGTPGVALHIIERDPAAAPAAVAPGAAGAPPAQLPR	3,490	97
	LGDLGGPVVEDPAAPR	1,562	99
	FEALDANGDGVLSR	1,463	100
	HAGDLGNITAGADGVANVNVSDSQIPLTGAHSIIGR	3,497	100
	QLAAIDSSWCR	1,306	100
	TFAYEGTVSAAGVTGASGQLQPTTR	2,469	100
	VDQYGNPVPPVDQYGNPVPDEPAPR	2,719	100
	DDLFNINAGIVK	1,318	100
	LFGVTTLDVVR	1,219	100
	VLGLGQLSDFEK	1,305	96
	SDEEGFGGVYGQNDPVFNPGTEVHPSHPE	3,097	99

Fraction no.	Peptide sequence	MW	Probability of certainty
24	SDEEGFGGVYGQNDPVFNPGTEVHPSHPEYDTSQGSEV	4,064	98
	SDEEGFGGVYGQNDPVFNPGTEVHPSHPEYDTSQGSEVK	4,192	100
	CPDAYLFPEDNTK	1,569	97
	CSFTVWPAATPVGGGVQLSPGQTWTINVPAGTSSGR	3,645	100
	DDADVSGDLSGLSDKELGVLADWETK	2,734	100
	ELGVLADWETK	1,260	100
	QLELEGVDSFKK	1,392	95
	TIDANVSDAEGVYSALEK	1,881	98
	ANIPCLCAGVTK	1,303	100
	IEPSEACCAVWQR	1,605	100
	KYEGWPAEPK	1,204	100
	QLAAIDDSFCR	1,295	98
	VNQIGSVTESIEAVK	1,573	100
	AVDSGAVPVSPPEQK	1,480	100
	DIDGIIVR	900	100
	ANIPCLCAGVTK	1,303	100
	DIDGIIVRMGSHVRA	1,638	100
	ETDALTGAVQLPDSAGER	1,829	100
	GPVEICFDYADVDAAYRR	2,116	100
	WAELESGTTTIAFTPLHQR	2,157	100
	TITLEVESSDTIDNVK	1,763	99
	AMVEVLVEEEEAPLACK	1,932	100
	DVALSTGGTASEYAK	1,469	100
	VADSQPVDLFDQAR	1,560	99
	ELQPAVADIGAAIGLPAR	1,761	100
	GSSAQADHIYSEVLNEFTK	2,095	100
	EGLAGSYVDPQPAASAFR	1,835	100
	FVVAGDLGQTGWTESTLR	1,936	100
	AWLGASQPPHVVVFK	1,635	100
	DSCQDLGVSR	1,135	100
	FNAATICVQSTEWHVGDEPLTGAR	2,658	100
	FTPWGGAAAPEDR	1,374	100

Appendix Table D1 (continued)

Fraction no.	Peptide sequence	MW	Probability of certainty
24	FNAATICVQSTEWHVGDEPLTGAR	2,658	100
	FTPWGGAAAPEDR	1,374	100
	RVVTGPLIGPSPSGR	1,492	100
	VLPCPLLVAQETDER	1,739	100
	VVTGPLIGPSPSGR	1,336	100
	MTAEIGEQVQIVGDDLLVTNPTR	2,498	100
	VNQIGSVTESIEAVK	1,573	100
	DTVAGGDVDADAAMK	1,435	100
	DTVAGGDVDADAAMKEQDR	1,963	100
	LAPFNPEYPDESVLWTESGDVGK	2,549	100
	CQPGMGYPMYSLPR	1,672	97
	CSALNHMVGGIYR	1,477	100
	ELGATDVGHPMAEVFPGCR	2,058	100
	QCVGHGAPGGAVDEQLR	1,750	98
	QLAAVDDSWCR	1,320	100
	RQCVGHGAPGGAVDEQLR	1,906	98
	GATTIIGGGDSVAAVEK	1,545	100
	MSHISTGGGASLELLEGK	1,786	99
	DVVGGAAEALPADMVATR	1,742	100
	GVAEAVAAAAEMNEGR	1,545	100
	SGPFFFISGNEANCR	1,072	100
	CGYTVWPAALPSGDGNQLDPGQSWAVYVPAGTK	3,462	99
	DGQGTTFTCPAGTDYQIVFCP	2,334	100
	TGCGFISGGSLGQCQTGDCGGTLR	2,445	98
	IGEALEAAALAAGGTPVER	1,795	96
	NEEMGVVGHDQATDAAAEQGVNVSDTLVPGGGR	3,280	100
	YGDVFPVTGSLAAKPIAPR	1,958	96

CURRICULUM VITAE

NAME : Mrs. Ladda Wattanasiritham

BIRTH DATE : June 10, 1963

BIRTH PLACE : Nonthaburi, Thailand

EDUCATION	**:YEAR**	**INSTITUTE**	**DEGREE/DIPLOMA**
	1988	Chulalongkorn University	B.Sc. (Biochemistry)
	1993	Chulalongkorn University	M.Sc. (Biochemistry)

POSITION/TITLE : Researcher

WORK PLACE : Institute of Food Research and Product Development, Kasetsart University, Bangkok, 10900, Thailand

SCHOOLARSHIP **:** National Research Council of Thailand (NRCT) 2011