OPTIMIZATION OF PROTEIN HYDROLYSATE PREPARATION FROM SPLIT GILL MUSHROOM *Schizophyllum commune*

Miss Aunchalee Wongaem

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การหาค่าเหมาะสมที่สุดสำหรับการเตรียมโปรตีนไฮโดรไลเสตจากเห็ดแครง *Schizophyllum commune*

น.ส.อัญชลี วงษ์เอี่ยม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Advisor	Associate Professor APHICHART KARNCHANATAT, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the Faculty of Science
(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Associate Professor SEHANAT PRASONGSUK, Ph.D.)

..... Thesis Advisor
(Associate Professor APHICHART KARNCHANATAT, Ph.D.)

..... Examiner
(Assistant Professor SANIT PIYAPATTANAKORN, Ph.D.)

..... Examiner
(Assistant Professor Onrapak Reamtong, Ph.D.)

อัญชลี วงษ์เอี่ยม : การหาค่าเหมาะสมที่สุดสำหรับการเตรียมโปรตีนไฮโดรไลเสตจากเห็ดแครง *Schizophyllum commune* . (OPTIMIZATION OF PROTEIN HYDROLYSATE PREPARATION FROM SPLIT GILL MUSHROOM *Schizophyllum commune*) อ.ที่ปรึกษาหลัก : รศ. ดร. อภิชาติ กาญจนทัต

การศึกษาสภาวะที่เหมาะสมในการผลิตไฮโดรไลเสตจากโปรตีนเห็ดแครงโดยใช้เอนไซม์โปรติเอสจากจุลินทรีย์ (อัลคาเลส) การศึกษาได้ดำเนินการโดยใช้การออกแบบส่วนประสมกลาง (CCD) และวิธีพื้นผิวการตอบสนอง (RSM) ถูกนำมาใช้ในการกำหนดเงื่อนไข ซึ่งมีตัวแปรที่ศึกษา 3 ตัว ได้แก่ อุณหภูมิการไฮโดรไลซิส (45, 50, 55 องศาเซลเซียส); เวลาการไฮโดรไลซิส (60, 120, 180 นาที); และอัตราส่วนของเอนไซม์ต่อสารตั้งต้น (2, 4, 6 ร้อยละโดยปริมาตรต่อมวล) ถูกเปลี่ยนแปลงในขณะที่ค่า pH คงที่ที่ pH 8.0 จากการศึกษาพบว่า ปัจจัยทั้งสามนี้มีผลต่อกิจกรรมระดับการย่อยสลาย (DH) และการกำจัดอนุมูลอิสระโดยวิธี ABTS อย่างมีนัยสำคัญ ($p < 0.05$) ซึ่งมีสภาวะที่เหมาะสมที่ได้จากการทดลองคือเวลา 161.4 นาที อุณหภูมิ 55 องศาเซลเซียส และอัตราส่วนของเอนไซม์ต่อสารตั้งต้นร้อยละ 2 โดยปริมาตรต่อมวล ให้ค่าระดับการย่อยสลายเท่ากับร้อยละ 50.882 และมีฤทธิ์การต้านอนุมูลอิสระซึ่งจะแสดงเป็นค่าความเข้มข้นของสารที่สามารถยับยั้งอนุมูลอิสระได้ร้อยละ 50 (IC_{50}) เท่ากับ 0.205 ไมโครกรัมต่อมิลลิลิตร โดยมีสัมประสิทธิ์การตัดสินใจเท่ากับ 0.997 และ 0.972 ตามลำดับ จากนั้นไฮโดรไลเสตจะถูกคัดแยกโดยน้ำหนักโมเลกุล (MW) ด้วยขนาด 10, 5, 3 และ 0.65 กิโลดาลตัน (kDa) ซึ่งพบว่า น้ำหนักโมเลกุลน้อยกว่า 0.65 kDa มีความสามารถในการกำจัดอนุมูลอิสระสูงสุด (IC_{50} = 0.026 ± 0.004 ไมโครกรัมต่อมิลลิลิตร) นำมาทำให้บริสุทธิ์โดยเทคนิคโครมาโตกราฟีของเหลวประสิทธิภาพสูง (HPLC) ได้ 5 พีค ($F_{1–5}$) โดย F_4 แสดงกิจกรรมการกำจัดอนุมูลอิสระ ABTS สูงสุด และนำไปพิสูจน์เอกลักษณ์ด้วยเทคนิคแมสสเปคโตรเมตรี พบลำดับกรดอะมิโนที่ออกฤทธิ์ต้านอนุมูลอิสระและยับยั้งการเพิ่มจำนวนเซลล์มะเร็ง 5 ลำดับ นอกจากนี้การศึกษาความสามารถการป้องกันความเสียหายของ DNA ที่เกิดจากการออกซิเดชันในพลาสมิด pBR322, pKS, และ pUC19 พบว่า MW < 0.65 kDa มีความสามารถในการป้องกันความเสียหายของ DNA และ MW < 0.65 kDa ยังแสดงฤทธิ์ต้านอนุมูลอิสระของเซลล์มะเร็งในเซลล์มะเร็งลำไส้มนุษย์ (HT-29) ซึ่งความสามารถในการยับยั้งขึ้นอยู่กับความเข้มข้นของเปปไทด์ ถึงแม้ว่าฤทธิ์ต้านอนุมูลอิสระไม่สัมพันธ์กับสัดส่วนของค่าระดับการย่อยสลาย เนื่องจากโครงสร้างและน้ำหนักโมเลกุลรวมถึงลำดับของกรดอะมิโนที่เกิดขึ้น อย่างไรก็ตามผลการวิจัยชี้ให้เห็นว่า RSM ที่มีการออกแบบส่วนประสมกลางได้พิสูจน์แล้วว่าเป็นเครื่องมือที่มีประสิทธิภาพสำหรับการเพิ่มประสิทธิภาพของพารามิเตอร์ในการหาค่าระดับการย่อยสลาย และกิจกรรมต้านอนุมูลอิสระ ผลการวิจัยเหล่านี้ชี้ให้เห็นว่าโปรตีนไฮโดรไลเสตจากเห็ดแครงเป็นแหล่งของเปปไทด์ที่มีฤทธิ์ทางชีวภาพที่ดีและมีศักยภาพในการเป็นสารต้านอนุมูลอิสระตามธรรมชาติในการผลิตอาหารและผลิตภัณฑ์เครื่องสำอาง

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Aunchalee Wongaem : OPTIMIZATION OF PROTEIN HYDROLYSATE PREPARATION FROM SPLIT GILL MUSHROOM *Schizophyllum commune* . Advisor: Assoc. Prof. APHICHART KARNCHANATAT, Ph.D.

The optimum conditions for hydrolysate production from split gill mushroom protein using the microbial protease, Alcalase were investigated. The study was conducted using central composite design and response surface methodology. Three independent factors: hydrolysis temperature (45, 50, and 55 °C); hydrolysis time (60, 120, and 180 min); and enzyme to substrate ratio (2%, 4%, and 6% w/v) were varied, while the pH was fixed at pH 8. These three factors had a significant effect on the DH and ABTS radical-scavenging activity ($p < 0.05$). The optimum conditions obtained from experiments were: enzyme to substrate ratio 2%; hydrolysis time 161.4 min; and temperature 55 °C. The coefficient of determination (R^2) of total protein and the IC_{50} value for ABTS radical-scavenging activity were 0.972 and 0.205 µg/mL, respectively, and the DH value was 50.882%. The hydrolysate was fractionated by molecular weight (MW) cut-off membranes (10, 5, 3, and 0.65 kDa). The < 0.65 kDa fraction had the highest radical scavenging ability (IC_{50} = 0.026 ± 0.004 µg/mL). The MW < 0.65 kDa fraction was separated by reversed-phase HPLC to yield four subfractions ($F_{1–5}$). The F_4 subfraction showed the highest maximum ABTS radical-scavenging activity and was selected for further analysis by quadrupole-time-of-flight-electron spin induction–mass spectrometry-based. Five antioxidant peptides were identified. In addition, the MW< 0.65 kDa fraction protected against oxidation-induced DNA damage in pBR322, pKS, and pUC19 plasmids. Furthermore, the MW < 0.65 kDa fraction, exhibited cellular antioxidant activity in a human intestinal cancer cell line (HT-29), which was dependent on the peptide concentration. These results suggested that split gill mushroom protein hydrolysate is a good source of bioactive peptides and has potential as a natural antioxidant in food production and cosmetic products.

Field of Study:	Biotechnology	Student's Signature .....
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ABBREVIATIONS

A	Absorbance
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CAA	Cellular antioxidant activity
CCD	Central Composite Design
et al.	and others
etc.	et cetera
FRs	Free radicals
H_2O_2	Hydroxyl peroxide
HPLC	High-performance liquid chromatography
IC_{50}	The half maximal inhibitory concentration
kDa	Kilodalton
M	Molar
min	minute
mL	Milliliter
mM	Millimolar
MS/MS	Tandem mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut off
NaCI	Sodium chloride
nm	Nanometer
O^{2-}	Superoxide anion
OH	Hydroxyl radical
OS	Oxidative stress
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase high-performance liquid chromatography
rpm	Revolutions per minute
RSM	Response surface methodology
SDS	Sodium dodecyl sulfate
sec	Second
UF	Ultrafiltration
v/w	Volume by weight
^{o} C	Degree Celsius
α	Alpha
β	Beta
µg	Microgram
/	Per
%	Percentage
:	Ratio

CHAPTER 1
INTRODUCTION

Free radicals are highly reactive chemicals that can harm cells. Free radicals are constantly generated in humans through normal physiological reactions involved in diverse functions, including respiration, exercise, digestion, and defense against infections (Dhaval et al., 2016). However, when produced in excess, because free radical molecules do not have a complete electron shell, they are more likely to react chemically with nearby molecules, which can cause damage to the adjoining cells. The main problem caused by free radicals is cell damage as a result of the interaction between reactive species and cellular structures, including lipids and membranes, proteins, sugars, and DNA. Reactive species are involved in carcinogenesis through direct damage to DNA, cell growth and proliferation, anti-apoptosis, aggressiveness, and metastasis. Previous studies have found a higher level of reactive species in cancer patients, compared with controls, and these levels were correlated with poor clinical outcomes (Kumari et al., 2018; Liou and Storz, 2010).

Antioxidants play an important role in the detoxification of reactive species (Lee et al., 2004). Therefore, natural antioxidants from food protein-derived bioactive peptides have been the focus of numerous studies. Enzymatic hydrolysis exposes antioxidant amino acids in proteins resulting in peptides with high antioxidant activity. The antioxidant activity of bioactive peptides can be attributed to their ability for metal-ion chelation, radical scavenging, and inhibition of lipid peroxidation. Antioxidant peptides can also quench free radicals and up regulate the expression of proteins and enzymes involved in reducing oxidative stress in the human body. It has been reported that the antioxidant activity of peptides from protein hydrolysates depends on the peptide size and the amino acid sequence of the peptide, which are influenced by the source of the protein, the type of protease used, and the hydrolysis conditions (Elias et al., 2008). Response surface methodology (RSM) is an efficient and widely used tool for optimization of enzymatic hydrolysis conditions (i.e. enzyme to substrate ratio, hydrolysis time, hydrolysis temperature, and pH) and other process conditions to achieve a desired response (Qu et al., 2010; Shafisolani et al., 2014).

Many studies have focused on developing compounds with biological and pharmaceutical activity from plants, but there is a lack of similar investigations in mushrooms. Hence, studies should be conducted to explore the potential use of

mushrooms and their metabolites for the treatment of a variety of human ailments. Since ancient times, mushrooms have been widely appreciated worldwide. Mushrooms have been shown to have profound health-promoting benefits and studies have confirmed their medical use and many of the bioactive molecules present in mushrooms have been identified (Wasser & Weis, 1999a). Studies have shown that mushrooms have a low fat and high protein content, and a high content of several vitamins (B, C, D, and K), minerals (potassium, phosphorus), and trace elements (selenium). Mushrooms contain a wide variety of bioactive compounds, including terpenoids, steroids, phenols, nucleotides and their derivatives, glycoproteins, and polysaccharides (Borchers et al., 1999). The many and diverse species of mushrooms provide a rich source of bioactive molecules, which have recognized potential in drug discovery and development, including species such as *Lentinula edodes*, *Ganoderma lucidum*, *Schizophyllum commune*, *Trametes versicolor*, *Inonotus obliquus*, and *Flammulina velutipes* (Wasser & Weis, 1999b).

S. commune, or split gill mushroom, is a common species of *Schizophyllum* found worldwide that has long been known as an edible mushroom (Ohm et al., 2010). It is the most common mushroom or shelf fungus belonging to the order *Agaricales*, and is found on, or in the environment of, dead and decaying trees, especially on rotten wood (Sigler & Abbott, 1997). *S. commune* has been reported to have medicinal properties, including antitumor, anticancer, and immunomodulating activities (Lemieszek & Rzeski, 2012; Patel & Goyal, 2012). However, published studies on these mushrooms are quite limited. Hence, the biological activity of split gill mushroom metabolites needs to be explored for the potential use of the split gill mushroom as a source of drugs or functional foods for the treatment of a variety of human ailments.

The objective of this study was to determine the optimal enzymatic parameters for the hydrolysis of split gill mushrooms to obtain the maximum degree of hydrolysis (DH) using RSM, and to evaluate the antioxidant activity *in vitro*. The crude protein hydrolysate obtained was further partially purified by consecutive ultrafiltration and RP-HPLC. The peptide sequences were analyzed by LC-MS/MS. The ability of a peptide fraction to protect against oxidative damage was investigated using a human intestinal cell line (HT-29) as a model.

CHAPTER 2
LITERATURE REVIEW

2.1 Free radicals and the occurrence of apoptosis

A researcher found cancer patients have a higher increased oxidative stress (OS) than normal people. Moreover, previous studies suggested have been correlated between antioxidants and cancer. Thus, we can be explained the relationship between free radicals and cancer as the following:

Important role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) involved in and cell proliferation and tissue repair regeneration leading to the creation and release of free radicals which results in damage or modify the structure and function of biological molecules such as lipids, proteins, and DNA (Manda et al., 2009). When cell and tissue damage resulted in the stimulation of transcription factors pass signal transduction pathways such as mitogen-activated protein kinase (MAPK) and protein kinase C (PKC). Cause free radicals are in constantly and caused an imbalance with antioxidants, result in caused oxidative stress which can lead to the destruction of the structure of the genetic material to cause cell death (apoptosis) or stimulation an increasing number of cells progressing through to cancer (Mut-Salud et al., 2016; Sies, 1997). The evidence manifested as that free radicals are important for cancer by Zimmerman and Cerutti (1984), studied in fibroblast of rats with an exposed to oxygen free radicals found that fibroblast cells are the cancer cells which the effects of free radicals on cells may depend on many factors such as the type or the level of free radicals, duration of exposure the amount of antioxidation within the cell and function of cellular repair system (Valko et al., 2007). Several scientific reports suggest that chronic oxidative stress (COS) conditions are strongly associated with carcinogenesis (Hwang & Bowen, 2007). Generation of ROS and OS conditions results in modification of DNA bases which leads to abnormality (mutations, translocations, gene inactivation) at the genomic level (Toyokuni, 2006). Besides, they have been similar studies on reported, as the following in **Table 2.1.**

Table 2.1 The effects of free radicals cause cancer. The main mechanisms are as follows:

Evaluate Activity	Result	References
Direct damage to DNA	➢ Oxidation yield can stimulate of proto-oncogene and tumor-suppressor gene. ➢ In addition, has been reported that exposure of radiations or free radical for a long time affect the living system in reproductive failure of cells and could result in cellular death, leading to damage to DNA which resulted in mutations.	(Evans et al., 2004; Gutteridge & Halliwell, 1992) (Desouky et al., 2015; Scott, 2008; Waldren, 2004)
Cell proliferation	The effect of free radical on p53 protein as following: ➢ Stimulation of transcription, Expression of genes encoding the enzymes, to counteract the free radical such as manganese-containing superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) etc., resulted in senescence and apoptosis. ➢ Inhibit the activity of p53 protein and cause cancer.	(Bensaad & Vousden, 2005) (Cobbs et al., 2001)
Apoptosis	➢ A study of melanoma skin cancer cell line showed that levels of superoxide anion decreased by expression of the copper- and zinc-containing superoxide dismutase (CuZnSOD) enzymes stimulated the process of programmed cell death, or apoptosis.	(Pervaiz & Clément, 2004)
Metastasis	➢ A study the level of 8 OHdG, 4 - hydroxynonenal (4-HNE) and the substance generated from lipid peroxidation in prostate cancer cells found higher level in the spread range than the initial stage. ➢ The study of breast cancer cell showed that higher of lysyl oxidase (LOX) is an elastin crosslinking enzyme that produces H_2O_2 as a by-product in the spread than the initial stage.	(Oberley, 2002) (Payne et al., 2005)

Source: Srinagarind Medical Journal 2014; 29 (2)

2.2 DNA damage

There is evidence for mutagenic DNA damage induced by oxidative stresses (Daviet et al., 2007). An important role of damage to DNA could cause by endogenous and exogenous sources which may result in response to higher amount of oxygen species (ROS) and free radicals (FRs) cause damage or cellular injury because of uncontrolled tumor growth and may lead to mutation, cancer, and cellular or organismic death by a variety of mechanisms (Kryston et al., 2011; Mantovani et al., 2008). In endogenous oxidation have reaction conditions of cause free radical-induced DNA damage in living organisms by the most caused by hydroxyl radicals reacts with the heterocyclic DNA bases (purine and pyrimidine) and the sugar moiety near or at diffusion-controlled rates (Balasubramanian et al., 1998; Cadet et al., 1999). Besides, has been reported that exposure to ionizing radiations (UV, X-rays, γ-rays, alpha particles) causes DNA damage (Desouky et al., 2015). However, the extent of DNA damage depends on the nature of the bases and the flexibility of DNA. Therefore, Fenton reaction is used to detect DNA damage in organisms. The Fenton reaction is oxidation reaction occurs rapidly of an organic compound in H_2O_2 solution with an iron of ferrous which causes hydroxyl radicals (H_2O_2) as following (Walling, 1975). As shown below:

O_2^- reduce Fe^{2+} $\quad O_2^{\bullet -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$ (Ferrous)

Fenton $\quad H_2O_2 + Fe^{2+} \longrightarrow OH^{\bullet} + {}^{\cdot}OH + Fe^{3+}$ (Ferric)

Haber - Weiss $\quad O_2^{\bullet -} + H_2O_2 \longrightarrow OH^{\bullet} + O_2 + {}^{\cdot}OH$

2.3 Cell death

The change of the cell damage that may be able to return to normal or may be caused cell death. Depending on the severity of the harm that has been and is an adaptation of the cell. This form of the death of the cell are two major forms of necrosis and apoptosis, both are different.

2.3.1 Necrosis

Necrosis is a process in which our body cells face unnatural death or non-programmed cell death. This could occur due to a lack of oxygen to a certain part or tissue, which results in the cells dying by hypoxia, or it could be caused by an infection or harmful toxin that kills our body's cells before their programmed death. Any form of mechanical injury could also lead to necrosis. Necrosis can even occur with exposure to extreme external or environmental factors, such as heat, cold, electricity, etc. It causes a great deal of damage and can be fatal if left unchecked. Based on the cause of cell death, the location of the affected body part, and the duration.

2.3.2 Apoptosis

Apoptosis is an important part of our natural functioning and is used to maintain a healthy number of cells. This cannot be done by controlling cell division alone. Apoptosis is also used when a body structure becomes redundant. Apoptosis plays a role in the development of certain body parts when the cells between individual digits die by apoptosis. Therefore, apoptosis plays an important role in normal development, eliminate unwanted cells.

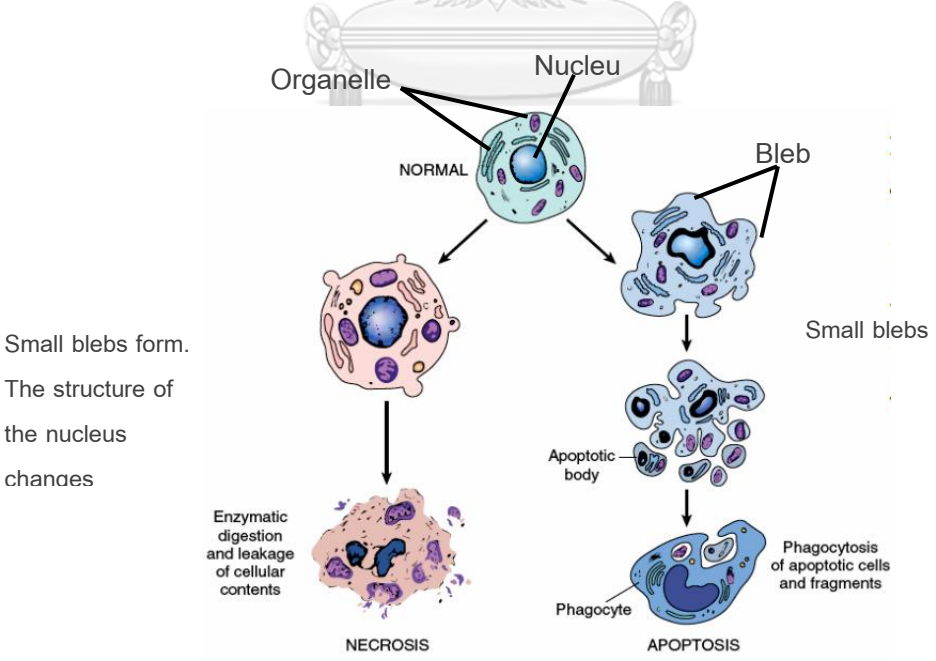

Figure 2.1 Structural changes of cells undergoing necrosis and apoptosis. (Nash et al., 1999)

2.4 Free radical and Reactive oxygen species (ROS)

Free radicals are atoms or molecules with one or more unpaired electrons. Radicals can have a positive, negative or neutral charge, such as superoxide anion (O_2^-) and hydroxyl radical (OH) will occur when the bond between atoms break out. The presence of unpaired electrons makes these species unstable and reacts quickly, which free radicals being very reactive to the interaction with other molecules because they seek another electron to achieve a more stable, less reactive configuration. The consequences are adjacent or molecular lose electrons its electron it becomes a free radical itself, beginning a chain reaction (Cornelli, 2009). They are formed as necessary intermediates in a variety of normal biochemical reactions, but when excess or uncontrolled free radical production is detrimental to cells either by direct damage to macromolecules or liberation of toxic byproducts. Therefore, what should be used to tell the toxic level should be the ability to oxidize. Biomolecules in the body than substance the ability to oxidize. In the biology system, the free radicals are often derived from oxygen, nitrogen and sulfur molecules. These free radicals are parts of groups of molecules called reactive oxygen species (ROS), reactive chlorine species (RCS) and reactive nitrogen species (RNS) by molecules that can, cause oxidation reaction could be found in the form of lipid radical or genetic radical RS that no water is to be in the form of a free radical always. A compound of some molecules in the form non-radical but sensitive to reaction oxidation such as H_2O_2 is classified as reactive species (RS). As shown the **Table 2.2.**

Table 2.2 Some of the main RS divided according to the nature of the substance, free radical or nonradical, and grouped by the element that determines oxidation.

Reactive oxygen species (ROS)			
Free radicals	**Formula**	**Non-radicals**	**Formula**
Oxygen	$O_2^{\bullet}$ [a]	Singlet oxygen	$^1O_2^*$
Superoxide	$O_2^{\bullet-}$ [a]	Hydrogen peroxide	H_2O_2
Hydroxyl	$OH^{\bullet}$ [a]	Ozone	O_3
Hydroperoxil	$HO_2^{\bullet}$ [a]	Hypochlorus acid	HOCl
Peroxyl	$RO_2^{\bullet}$	Hypobromous acid	HOBr
Alcoxyl	$RO^{\bullet}$	Organic peroxides	ROOH
Carbonate	$CO_3^{\bullet-}$		
Carbon dioxide	$CO_2^{\bullet-}$		
Reactive chlorine species (RCS)			
Atomic chlorine	$Cl^{\bullet}$	Hypochloric acid	HOCl
		Nitryl chloride	NO_2Cl
		Clorine gas	Cl_2
Reactive nitrogen species (RNS)			
Nitric oxide	$NO^{\bullet}$	Nitrous acid	HNO_2
Nitrogen dioxide	$NO_2^{\bullet}$	Peroxynitrite	$ONOO^-$
		Peroxynitrous acid	ONOOH
		Alchyl peroxynitrite	ROONO
		Nitryl chloride	NO_2Cl

Source: Nutraceuticals: A Guide for Healthcare Professionals (Lockwood, 2007)

[a] Intermediate step of the transformation (quenching) of O_2 into H_2O.

2.4.1 Sources of free radical

Free radicals are generated through normal reactions within the body in organisms use oxygen. The free radicals can be produced from either endogenous or exogenous sources as follows:

2.4.1.1 Endogenous sources

The endogenous sources of free radical were found from different cellular organs such as mitochondria, peroxisomes, endoplasmic reticulum, and phagocytic cells, etc. where the oxygen consumption is high. The examples of reactions that cause free radicals include

1. Autoxidation is a complex oxidation mechanism that proceeds through a free radical chain process. It is a common degradation mechanism for unsaturated fats such as lipid oxidation. In general, the free radical chain process consists of three steps (Nawar, 1996). As follows:

1.1 Initiation processes; the attacked molecule loses its hydrogen and becomes free radicals, which frequently through the thermal or light cleavage by use catalyzed are metal ions.

$$RH + \text{initiator} \xrightarrow[\text{Light}]{T} R^{\bullet} + H^{\bullet} \qquad (2.1)$$

1.2 Propagation processes; Propagation processes; free radicals react with molecular oxygen to form peroxy radical (**Eq. 2.2**) and then the can also reaction with lipid acid can produce hydroperoxide molecules and free radical (**Eq. 2.3**), which if have thermal or light is catalyzed can produce many free radical molecules and the chain can be quite long.

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet} \qquad (2.2)$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet} \qquad (2.3)$$

1.3 Termination processes**;** the chain reaction is broken when two free radicals reactions are extremely efficient and lead to the disappearance of free radicals to form nonradical products.

$$R^{\bullet} + R^{\bullet} \longrightarrow RR \qquad (2.4)$$

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2 \qquad (2.5)$$

Where RH is an unsaturated fatty acid; $R^{\bullet}$ is a free radical formed by removing labile hydrogen from a carbon atom adjacent to a double bond; ROOH is a

hydroperoxide, one of the major initial oxidation products that decompose to form compounds.

2.4.1.2 Exogenous sources

ROS generated from exogenous sources such as pollution, alcohol, diet, ionizing radiation (ultraviolet light, x-ray, grammar-rays), tobacco smoke (NO, NO_2, $ONOO^-$, SO_2, CCI_4), transition metals (Cd, Hg, Pb, As), Heavy metals (Fe, Cu, Co, Cr), industrial solvents, pesticides, high temperature, Cooking (smoked meat, used oil, fat), certain drugs (halothane, paracetamol), chemicals that react to form peroxides (ozone and singlet oxygen), etc.

2.4.2 Classified of free radical

Free radicals can be classified into 3 types as follows:

2.4.2.1 Oxygen-centered radicals are created from mitochondria by through electron transport chain (ETC) and cardiovascular system are unusual which is the free radicals in the form of superoxide anion, hydrogen peroxide (H_2O_2), hydroxyl radical and singlet oxygen, etc.

2.4.2.2 Carbon-centered radicals such as peroxyl and alkoxyl radicals

2.4.2.3 Nitrogen-centered free radicals such as a nitric oxide (NO) and a by-product of nitric oxide [nitrate (NO_3^-), nitrite (NO_2^-), peroxynitrite ($ONOO^-$), 3 nitrotyrosine and nitrogen dioxide] (Mut-Salud et al., 2016).

2.5 Antioxidants

Antioxidants are substances that can prevent or slow damage to cells caused by free radicals, unstable molecules that the body produces as a reaction to environmental and other pressures (Halliwell, 2009). Antioxidants have an important role as antagonists of autoxidation processes, which can be found from natural such as phenolic compounds, nitrogen compounds, and carotenoid compounds (Velioglu et al., 1998) and synthetic. The reaction mechanisms of antioxidants such as preventive, scavenge, chelate for protection from free radical (Sies, 1997).

2.5.1 Sources of Antioxidants

In general, in food systems and biological systems are usually subdivided into synthetic and natural antioxidants. Commonly the molecular structures of the aromatic ring have one or more hydroxy groups to provide labile hydrogen.

2.5.1.1 Synthetic antioxidants

Some of the best-known synthetic antioxidants such as propyl gallate, 2-butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and Ethylenediaminetetraacetic acid (EDTA). However, in food systems, the use of synthetic antioxidants has been put under strict regulation due to their potential health risks associated with long-term use (Pokorny et al., 2001; Yang et al., 2010).

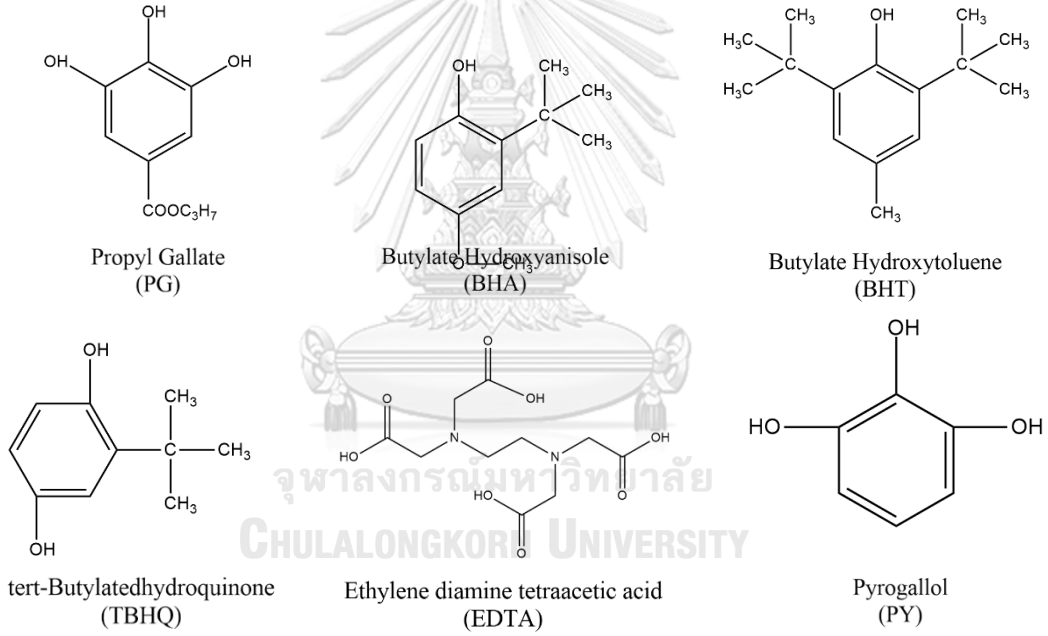

Figure 2.2 Chemical structures of the synthetic

2.5.1.2 Natural antioxidants

Natural antioxidants are found in numerous fruits and vegetables such as Vitamin E, Vitamin C, carrots (beta-carotene), tomatoes (lycopene), walnuts (tocopherols), broccoli (glutathione) and apple (quercetin), which can be divided into three groups: vitamins, carotenoids, and phenolic compounds. Commonly the molecular structures of the aromatic ring have one or more hydroxy groups to provide labile hydrogen.

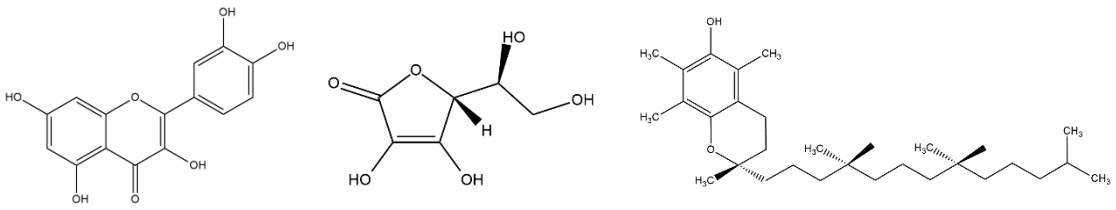

Figure 2.3 Chemical structures of the natural antioxidants

2.5.2 Classification of antioxidants

Antioxidants exist both in enzymatic and non-enzymatic forms in the intracellular and extracellular environment. Antioxidants can be categorized in multiple ways. Based on their activity, they can be categorized as enzymatic and non-enzymatic antioxidants (Shahidi & Zhong, 2010).

2.5.2.1 Enzymatic

Enzymatic antioxidants work by breaking down and removing free radicals. The antioxidant enzymes convert dangerous oxidative products to hydrogen peroxide (H_2O_2) and then to water, in a multi-step process in the presence of cofactors such as copper, zinc, manganese, and iron. Certain compounds act as in vivo antioxidants by raising the levels of endogenous antioxidant defenses. Expression of genes encoding the enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) increases the level of endogenous antioxidants (Thomas, 1997).

1. Superoxide dismutase (SODs) is an enzyme that alternately catalyzes the dismutation of the superoxide (O_2^-) radical into either ordinary molecular hydrogen peroxide (H_2O_2), which a common enzyme found in all living cells.

$$2O_2^{\bullet-} + 2H \longrightarrow H_2O_2 + O_2 \qquad (2.6)$$

2. Catalase (CAT), an enzyme that catalyzes the decomposition of converts two hydrogen peroxide molecules to oxygen and water. It has manganese (Mn^{+4})/iron (Fe^{+3}) cofactor, which a common enzyme found in a eukaryotic cell such as bacteria, plants, and animals.

$$2H_2O_2 \longrightarrow O_2 + H_2O \qquad (2.7)$$

3. Glutathione peroxidases (GTPx), an enzyme that catalyzes the reduction of hydrogen peroxide. (H_2O_2), which can convert of H_2O_2 molecules to water, can be written as follows:

$$2\ GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O \quad (2.8)$$

$$2\ GSH + ROOH \longrightarrow GSSG + ROH + 2\ H_2O \quad (2.9)$$

2.5.2.2 Non Enzymatic

Non-enzymatic antioxidants work by interrupting free radical chain reactions. Few examples of

1. Metabolic antioxidants (endogenous) glutathione, L-arginine, Coenzyme Q, melatonin, uric acid, transferring.

2. Nutrient antioxidants (exogenous) vitamin E, vitamin C, Beta-carotenoids, trace elements (selenium, Zinc, Manganese), flavonoids, omega 3 and omega 6 fatty acids.

2.5.3 The mechanism of antioxidant activity

2.5.3.1 Free radical scavenging to show free radical scavenging activity that can donate an electron or hydrogen radical to become a stable molecule. The active substance that exhibited this mechanism for example butylated hydroxyl anisole (BHA), vitamin E, etc. Can be written as follows (Vertuani et al., 2004):

$$R^{\bullet} + AH \longrightarrow RH + A^{\bullet} \quad (2.10)$$

$$RO^{\bullet} + AH \longrightarrow ROH + A \quad (2.11)$$

$$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet} \quad (2.12)$$

$$R^{\bullet} + A^{\bullet} \longrightarrow RA \quad (2.13)$$

$$RO^{\bullet} + A^{\bullet} \longrightarrow ROA \quad (2.14)$$

$$ROO^{\bullet} + A^{\bullet} \longrightarrow ROOA \quad (2.15)$$

$$Antioxidant + O_2 \longrightarrow Oxidized\ Antioxidant \quad (2.16)$$

2.5.3.2 Singlet oxygen quenching ($^1O_2^{\bullet}$) have an ability inhibit the reaction of singlet oxygen by to convert singlet oxygen ($^1O_2^{\bullet}$) to triplet oxygen ($^3O_2$) and release the energy received in the form of heat. The active substance that exhibited this mechanism for example carotenoids. Can be written as follows:

$$^1O_2^{\bullet} + {}^1Car \longrightarrow 3O_2 + {}^3Car^{\bullet} \quad (2.17)$$

$$^3Car^{\bullet} \longrightarrow {}^1Car + thermal\ energy \quad (2.18)$$

2.5.3.3 Metal chelating such as Fe^{2+}/Fe^{3+} and Cu^{2+} have the effects of accelerating the oxidation reaction in the body which this reaction by stimulate to generate free radical such as peroxyl radical, hydroxyl radical and alkyl radical, including singlet oxygen so can be attached to iron ions. As a result, decreased to generate free radical in the reaction of the body. The active substance that exhibited this mechanism for example flavonoids, phosphoric acid, and ascorbic acid, etc.

2.5.3.4 Enzyme inhibition; some phenolic compound such as flavonoids phenolic acid and gallates can be inhibited the reaction of lipoxygenase by can be attached to iron ions which are cofactor to cause enzyme cannot active.

Table 2.3 Antioxidant effects of bioactive compounds.

Antioxidant compound	**Perceived health benefit**
➢ β -Carotene, lutein	• Antimutagenic • Protective against breast cancer
➢ Bromophenol	• α-Glucosidase inhibition
➢ Carraggeenan, oligosaccharide	• Anti-tumor
➢ Fucoidan	• Anti-HIV • Ameliorales hyperoxaluria • Anticancer • Protection against neurodegenerative disorder
➢ Fucophlorethols	• Chemo preventive effects
➢ Fucoxanthin	• Antiangiogenic • Protective effects against retinol deficiency
➢ Galactan sulfate	• Anti-viral
➢ Phlorotannins	• Anti-inflammatory • Bactericide • Inhibits H_2O_2 mediated DNA damage • Hypertension • Photochemopreventive effects
➢ Phycoerythrin	• Amelioration of diabetic complications
➢ Polyphenols	• Vascular chemoprotection • Antiproliferation • Antimicrobial • α-Glucosidase inhibition
➢ Porphyran, shinorine	• Delays aging process

Source: Acad. J. of Kalasin Rajabhat University Vol. 1 No. 1 May-August 2011 (Jirum & Srihanam, 2011).

2.5.4 Methods of Antioxidant Capacity Assessment

Various *in vitro* or in vivo methods are available for the evaluation of the antioxidant activity of different any compound that can be carried out. Which to be popular (for example, DPPH, ABTS, etc.) due to their simplicity, provides a short experimental time, sensitive and easy, and the employment of the inexpensive for measuring (Mishra et al., 2012).

2.5.4.1 ABTS assay

ABTS (2, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) is a stable (in powder form) free radical which was then reacted with potassium persulfate to generate a radical cation ($ABTS^{\bullet+}$), that is green in color and can be measured by absorbance at 734 nm (van den Berg et al., 1999). The ABTS assay are these antioxidants suppress this reaction by electron donation radical scavenging and inhibit the formation of the colored ABTS radical which is the decolorization of preformed ABTS can be written as;

$$ABTS^{\bullet+} + AH \text{ (antioxidants)} \longrightarrow ABTS \text{ (decolorization)} + A^{\bullet} \qquad (2.19)$$

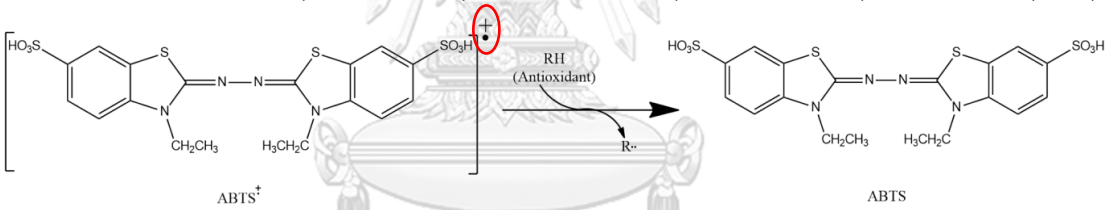

Figure 2.4 Reaction between antioxidant and ABTS radical

2.5.4.2 Cellular antioxidant activity (CAA) assay

The CAA assay is considered to be more biologically relevant method than the popular chemistry antioxidant activity assays (DPPH, ABTS, etc.) because it simulates cellular processes, takes into consideration physiological conditions, such as pH, temperature, bioavailability, including metabolism, and distribution to predict the behavior of the tested antioxidant compounds within cells (Wolfe & Liu, 2007). The method measures the ability the capacity of antioxidants to prevent oxidation of DCFH and reduce the formation of fluorescent dichlorofluorescein (DCF) by addition 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH). The main principles of the CAA assay are as follows: first, the fluorescent probe (DCF) or dye DCFH-DA which is nonionic and non-polar reacts

with the AAPH which generated peroxyl radicals (ROO•) and attack the cell membrane to produce more radicals. Then, DCFH is rapidly oxidized by free radicals, thus the addition of AAPH, a peroxyl radical initiator, leads to the oxidation of DCFH to the fluorescent DCF proportional to the level of oxidation, which is measured spectrophotometrically. The reduced in cellular fluorescence when compared to the control cells, which takes into account relative areas under the curves of sample wells and control wells, indicates a sample has the antioxidant capacity within the cell and the results were expressed as micromoles of quercetin per 100 g of dry weight (QE/100 g DW). The cellular antioxidant activity (CAA) was conducted according to Wolfe and Liu with minor modifications, which is a more accurate measure to analyze the antiproliferative activity against human cancer cells and quercetin was used as a standard to calculate the cellular antioxidant activity value.

2.6 Characteristics of *Schizophyllum commune*

Schizophyllum commune or split gill mushroom has long been known as an edible mushroom commune found in in the worldwide distribution which a single common species of *Schizophyllum* (Alexopoulos et al., 1996). It is the most common mushroom or shelf fungus belonging to the order *Agaricales*, being found on in the environment on dead and decaying basidiomycete, especially the rotten wood to be used as a substrate (Sigler & Abbott, 1997). However, that has been reported for its significant medical importance such as antitumor, anticancer and immunomodulating activities (Kidd, 2000; Smith et al., 2002).

The taxonomic hierarchy of *Schizophyllum commune.*

Kingdom: Fungi

Division: Basidiomycotina

Class: Agaricomycetes

Order: Agaricales

Family: Schizophyllaceae

Genus: *Schizophyllum*

Species: *S. commune*

Table 2.4 Biological activity from edible mushrooms [Sánchez, C. (2017)]

Mushroom	Bioactive Compounds	Bioactivity	References
Agaricus subrufescens.	Protein & polysaccharides fractions	Immunomodulatory	(Jeurink et al., 2008)
Boletus spp.	2,4,6-trimethylacetophenone imine, glutamyl tryptophan, azatadine, lithocholic acid glycine conjugate	Antioxidant	(Yuswan et al., 2015)
Calvatia gigantean	Calvacin	Antitumor	(Rathee et al., 2012)
Clitocybe maxima	Laccase	Antitumor	(Zhang et al., 2010)
Flammulina velutipes	Peptidoglycan	Immunomodulatory	(Yin et al., 2010)
Ganoderma lucidum	Ling zhi-8 (protein) Ganodermin (protein)/ Triterpenoid Se-containing protein	Immunomodulatory Antifungal Antitumor	(Dudhgaonkar et al., 2009; Kino et al., 1989) (Akihisa et al., 2005; Iwatsuki et al., 2003) (Du et al., 2007)
Grifola Frondosa	Low-molecular weight protein fraction	Antitumor	(Kodama et al., 2002)
Hericium erinaceus	Lectin (glycoprotein)	Antitumor, Anti-virus	(Friedman, 2015; Lee et al., 2009)
Inonotus obliquus	β-D-glucans	Antioxidant, cancer	(Rathee et al., 2012)
Lentinula edodes	Lentin (protein)	Antifungal	(Ngai & Ng, 2003)
Lignosus rhinoceros	Polysaccharides-protein	Anticancer	(Gupta et al., 2015)
Pleurotus citrinopileatus	Glycoprotein (PCP-3A)	Antitumor, Immune	(Chen et al., 2010)
Pleurotus ostreatus	Proteoglycan	Antioxidant, Antitumor	(Tong et al., 2009)
Russula lepida	Lectin (glycoprotein)	Antitumor	(Zhang et al., 2010)
Tricholoma giganteum	Trichogin (protein)	Antifungal	(Guo et al., 2005)
Xylaria hypoxylon	Lectin (glycoprotein)	Antimitogenic, antitumor	(Liu et al., 2006)

2.7 Protein Hydrolases

Proteins are very large molecules containing many amino acid residues linked together in a very specific order then 50 amino acids are known as peptides. Hydrolysis to hydrolysate proteins is molecules essential to the structure and function of all living organisms. They are made up of chains of amino acids linked by peptide bonds and folded in a variety of complex structures. Proteins must be broken down into amino acids and peptides by hydrolysis, hydrolysis process using acid-catalyzed hydrolysis and enzyme-catalyzed hydrolysis. The difference between acid and enzymatic hydrolysis is given in **Table 2.5**.

Li et al. (2008) was studied the antioxidant activity and free radicals scavenging properties of Chickpea protein hydrolysate (CPH) by gel filtration on Sephadex G-25. Analyses showed that amino acid composition and molecular weight distribution have relationship with the antioxidant activity.

Cheng et al. (2010) studied potato protein hydrolysate (PPH) by using Alcalase and purified by RP-HPLC. They found that the peptide with the antioxidant activity are Ser-Ser-Glu-Phe-Thr-Tyr and Ile-Tyr-Leu-Gly-Gln in P50 to be the dominant peptides that matched the sequences in metallocarboxypeptidase inhibitor and lipoxygenase 1, respectively.

Umayaparvathi et al. (2014) was studied the antioxidant and anticancer activities of bioactive peptide isolated from oyster (*Saccostrea cucullata*) protein hydrolysate. They found that the peptide with the antioxidant activity were Leu-Ala-Asn-Ala-Lys (MW = 515.29 Da), Pro-Ser-Leu-Val-Gly-Arg-Pro-Pro-Val-Gly-Lys-Leu-Thr-Leu (MW = 1432.89 Da) and Val-Lys-Val-Leu-Leu-Glu-His-Pro-Val-Leu (MW = 1145.75 Da), respectively. Moreover, oyster peptide (Leu-Ala-Asn-Ala-Lys) had anticancer activity against human colon carcinoma (HT-29) cell lines.

Chi et al. (2015) was studied two peptides isolated from the protein hydrolysate of blood clam (*Tegillarca granosa*) muscle were identified as Trp-Pro-Pro (BCP-A) and Gln-Pro (BCP-B), with molecular weights of 398.44 Da and 243.23 Da, respectively. They found that Trp-Pro-Pro showed the highest radical scavenging activity and showed strong anticancer activities on PC-3, DU-145, H-1299 and HeLa cell lines.

Pan et al. (2016) was studied the antioxidant hydrolysate from skate cartilage was obtained by Trypsin and Alcalase using ultrafiltration and chromatographic methods. They found that the peptide with the antioxidant activity were Phe-Ile-Met-Gly-Pro-Tyr, Gly-Pro-Ala-Gly-Asp-Tyr and Ile-Val-Ala-Gly-Pro-Gln with molecular weights of 726.90, 578.58 and 583.69 Da, respectively.

Table 2.5 Differences between acid and enzymatic hydrolysis.

Comparing variable		Hydrolysis	
		Chemical	**Enzyme**
Hydrolysis	**Reaction**	A fairly harsh process	Do not strong reaction
	Target to cleavage site	Non-specific but random	Specific peptide bonds.
	Temperature	Carried out at high	Do not require high temperature
	Time of Hydrolysis	Occurs is shorter time	Takes longer duration
	Yields	Low to middle	High
	Cost of catalyst	Low	High

2.7.1 Enzymatic Hydrolysis

Enzymatic hydrolysis is a process in which enzymes facilitate the cleavage of bonds in molecules with the addition of the elements of water. It plays an important role in the digestion of food. Proteolytic enzymes hydrolyze proteins more gently than acids, do not require high temperature and usually target specific peptide bonds and can control the quality of the product because proteases digest protein at the polypeptide bond. Hydrolyzed proteins have a higher solubility and changed character due to their group being more carboxylic.

2.7.2 Proteases Enzyme

Proteases (EC 3.4.21.62) are enzymes which essential in the processes of building up or breaking down of cellular components. Proteases have a physiological role in all living organisms and therefore, they are present in a wide range of sources such as plant, animal, and microbial. Due to, an enzyme can catalyze the reaction of hydrolysis of various bonds with the participation of a water molecule and breaking down proteins or polypeptides into short peptides or free amino acids. Thus, proteases are widely used in the food and pharmaceutical industry.

Alcalase® is an endo-protease of the serine type and acts as an esterase, enabling it to catalyze stereoselective hydrolysis of some esters. It has very broad substrate specificity. In other words, it can hydrolyse most peptide bonds within a protein molecule. Peptides and amino acids are formed which are either dissolved or dispersed in the washing water. Alcalase® is protease that works well in the alkaline, high-temperature conditions. It was produced by submerged fermentation of a selected strain of *Bacillus licheniformis*.

Table 2.6 Comparing different of commercial enzymes to estimate *in vitro* proteolysis

Product name	EC number	Source	Class	Type	Preferential specificity	Form	Optimum Condition	
							Temp (°C)	pH
Plant Enzymes								
▪ Papain	3.4.22.2	Pawpaw latex	Endo	cysteine protease	Broad specificity, mainly P2: hydrophobic amino	Dry powder	65 - 80	5 - 7
▪ Bromelain	3.4.22.32	Pineapple latex	Endo	cysteine protease	acid	Dry powder	40 - 65	5 - 8
Animal Enzymes								
▪ Trypsin	3.4.21.4	Pancreas	Endo	Serine protease	Lys, Arg	Granulate	30 - 55	7.8–8.0
▪ Chymotrypsin	3.4.21.1	Pancreas	Endo	Serine protease	Phe, Tyr, Trp, Val, Leu	powder	40 - 50	7.8 – 8.0
▪ Pepsin	3.4.23.1	Pancreas	Endo	Aspartic protease	Phe, Tyr, Trp, Met	powder	37	1 - 4
Microbial & Fungal Enzymes								
▪ Alcalase®	3.4.21.62	*Bacillus licheniformis*	Endo	Serine endo-peptidase (mainly subtilisin A)	P1: alanine	Liquid	30–65	7–9
▪ Neutrase®	3.4.24.28	*Bacillus amyloliquefaciens*	Endo	Metalloprotease	except proline, arginine, lysine	Liquid	40–50	5.5 - 7

Note: ®Commercial preparations

Source: Adler-Nissen, 1993 Novozymes

2.7.3 Degree of protein hydrolysis (DH)

The degree of hydrolysis (DH) is defined as the proportion of cleaved peptide bonds in a protein hydrolysate. Several methods exist for determining DH; the most commonly used of these include the o-phthaldialdehyde (OPA), which methods are based on the measurement of amino groups generated from hydrolysis.

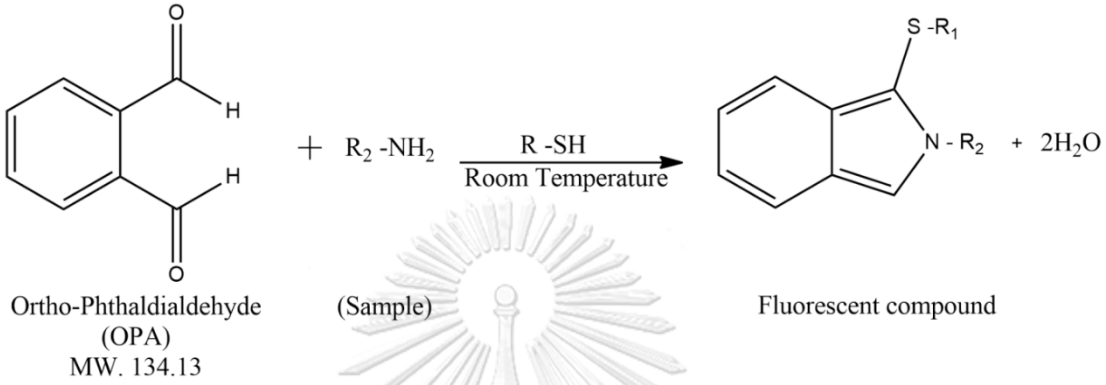

Figure 2.5 Schematic of the reaction between OPA reacts with SH-compound (dithiothreitol, DTT) and the absorbance was read at 340 nm.

DH is defined as the percentage of cleaved peptide bonds:

$$DH = h/h_{tot} \times 100\% \quad (2.20)$$

Where h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds. h_{tot} is dependent on the amino-acid composition of the raw material.

The degree of hydrolysis was calculated according to Adler-Nissen (1993) as shown in **Eq. 2.21.**

$$DH\ (\%) = [((B \times N_b)/MP) \times (1/\alpha) \times (1/h_{tot})] \times 100 \quad (2.21)$$

Where: B is the base consumption (mL), N_b is the normality of the base, α is the average degree of dissociation of the α -NH_2 groups, MP is the mass of protein (g) and h_{tot} is the total number of peptide bonds in the protein substrate (8.0 meqv/g food protein).

2.8 Response Surface Methodology (RSM)

Response surface methodology (RSM) is a collection of mathematical techniques and statistical tools suitable for developing, improving, and optimizing products and processes (Myers et al., 2016). This technique studies the relationship between an independent variable and multiple response variables and the complexity of the interactions between the variables will be analyzed until a valid model is

obtained (Bezerra et al., 2008; Montgomery, 2017). The most extensive applications of RSM and the resultant model contains all of the information on the effect of the experimental conditions, can help the user to perform the optimization of the parameters with a minimize the consumption of time, reagents consumption and number of experiments, and the appropriate optimum conditions are determined (Lundstedt et al., 1998). Besides, the model can be used to predict future observations within the model ranges.

In general, if each independent variable does not have an interaction effect, the regression equation is called the first-order model is

$$\hat{y} = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \varepsilon \qquad (2.22)$$

But, if interaction effect between each independent variable exists, the regression equation is called the second-order model is

$$\hat{y} = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1, j<1}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad (2.23)$$

Often the curvature in the true response surface is strong enough that the polynomial first-order or second order (even with the interaction term included) is inadequate. Response surface design to gain the optimal value, there are several methods such as steepest ascent and second-order models work well in solving real response surface problems there is considerable practical experience indicating that shown the **Figure 2.6.**

(a) (b)

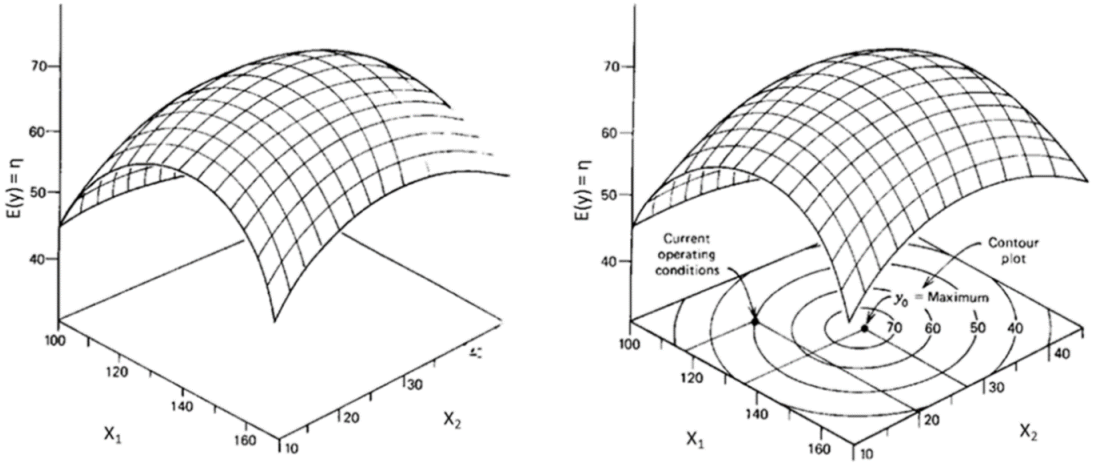

Figure 2.6 (a) A three-dimensional response surfaces. (b) A contour plot of a response surfaces.

2.8.1 Central Composite Designs

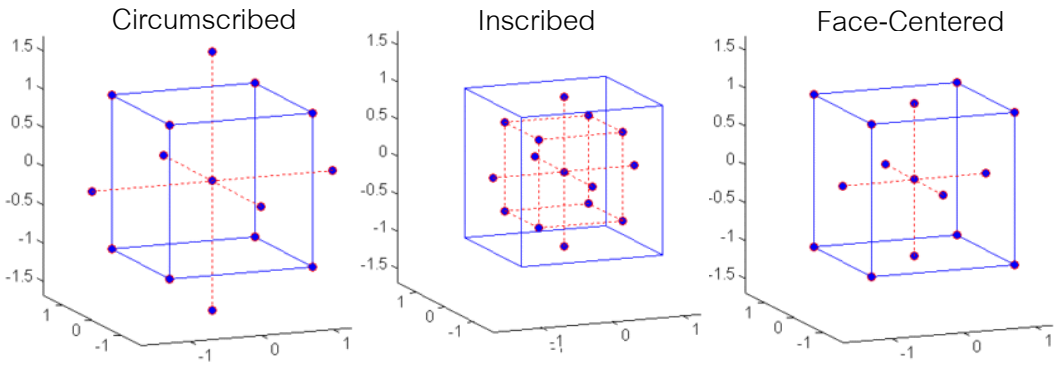

Figure 2.7 Circumscribed, inscribed and faced-centered designs.

Central Composite Designs, also known as Box-Wilson Designs, are a five-level fractional factorial design that is appropriate for calibrating the quadratic response model described in Response Surface Models. In experiments, there are three types of CCDs, namely, circumscribed, inscribed and face-centered. The geometry of CCDs is shown in **Figure 2.7**. The five-level coded values of each factor are represented by, where corresponds to the physical lower and upper limit of the explored factor space. It is obvious that it establishes new "extreme" physical lower and upper limits for all factors. The value of varies depending on design property and the number of factors in the study. For the circumscribed CCDs, considered to be the original form of Central Composite Designs, the value of is greater than one. The following is the number of all experiments (n) can be indicated from following equation

$$N = 2^k+2k + m \qquad (2.27)$$

Where k is the number of independent variables and m is the number of experiments at center points. The number of factorial runs is 2^k and the number of axials.

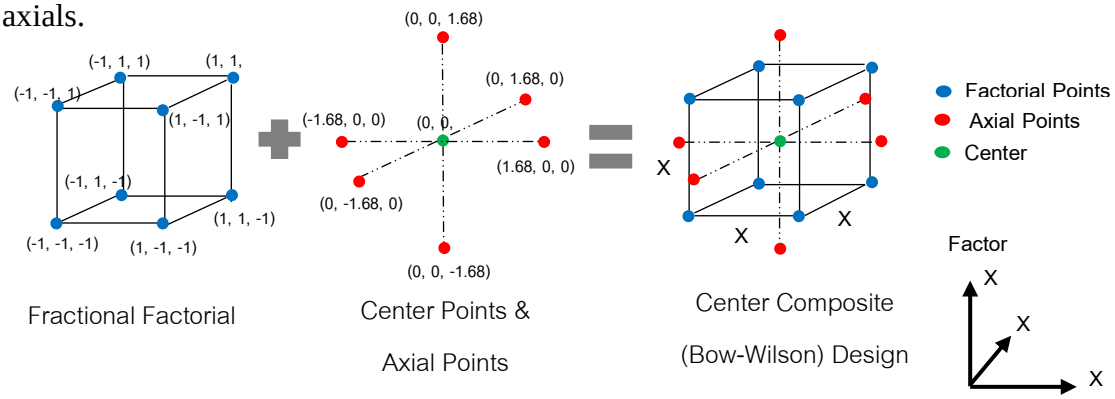

Figure 2.8 Central Composite Design for three factors
(Modified from (Salehi et al., 2012))

Each design consists of the position of the factorial and star points is determined by the number of factors and by the desired properties that allow estimation of second order effects. For a full quadratic model with *n* factors, CCDs have enough design points to estimate the $(n+2)(n+1)/2$ coefficients in a full quadratic model with n factors.

Table 2.7 Examples of parameters and responses of optimized condition

Source	Enzyme	Model	Parameter	Optimization	Reference
Catla catla	Alcalase (0.6 Anson-U/g	Factorial	Temp (35, 45, 55 °C) Time (45, 105, 165 min) E/S (0.5, 1.0, 1.5 %w/v) pH (7, 8, 9)	Temp: 50 °C Time: 135 min E/S: 1.5 %w/v) pH: 8.5	(Bhaskar & Mahendrakar, 2008)
Pumpkin oil Cake	protease from *Aspergillus niger*	Factorial	Temp (30, 40, 50 °C) Time (60, 80, 100 min) E/S (4.1, 5.3, 6.5 HUT/mg)	Temp: 40 °C E/S: 4.38 HUT/mg Time: 45 min	(Vaštag et al., 2010)
Tuna (*Euthynnus affinis*)	Alcalase	Factorial	Temp (30, 40, 60 °C) E/S (1.0, 1.5, 2.0 %w/v) Time (60, 120, 240 min) pH (7, 8, 9)	Temp: 40 °C E/S:1.5% Time: 240 min pH: 8	(Salwanee et al., 2013)
Leucaena leucocephala	Alcalase® 2.4 L (2.4 AU/g)	CCD	Temp (50, 55, 60 °C) Time (30, 60, 90 min) E/S (1, 2, 3%w/v) pH (7-9)	Temp: 55 °C Time: 90 min E/S: 2 %w/v) pH: 9	(Rafi et al., 2015)
Tilapia (Oreochromis niloticus)	Alcalase® 2.4 L	CCD	Temp (35-60 °C) E (2, 2.5, 3 %w/w) S (12.5, 15, 17.5 %w/v) pH (7, 7.5, 8)	Temp: 60 °C E: 2.5 %w/w S: 15 %w/v) pH: 7.5	(Roslan et al., 2015)
Rice bran protein	Alcalase® (2.990 U/mL)	Boxe Behnken	Microwave Power (600-100W) Time (60-120 sec) Solid/liquid (0.5-1.5 g/10mL)	microwave power: 1000 W Time: 90 s solid to liquid: 0.89 g /10 mL	(Phongthai et al., 2016)
Eel (Monopterus sp.*)*	Alcalase 2.4 L (2.4AU/g)	CCD	Temp (40, 50, 60 °C) E/S(1, 2, 3%w/v) pH (7, 8, 9)	Temp: 55.76°C E/S: 1.80% pH: 9	(Jamil et al., 2016)
Anadara granosa	Alcalase®, Protamex™ and Neutrase®	CCD	Temp (35-60 °C) Time (4-8 h) E/S (0.5-1.5 %w/v) pH (5.5-7.5)	Temp: 59.8 °C Time: 4.69 h E/S: 0.9 %w/v) pH: 5.59	(Aishah et al., 2017)

CHAPTER 3
METERIALS AND METHODS

3.1 Materials and chemicals

The split gill mushroom (*S. commune*) was collected from Baan-Hed-Krang Farm, Chana District, Songkhla, Thailand (containing 20.25% protein, 2.19% fat, 62.80% carbohydrate, 4.53% ash, and 10.23% moisture). Alcalase® from *Bacillus licheniformis* (3.018 U/mL) was purchased from Novo (Denmark). 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2, 2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 2,5-diphenyl-2H-tetrazolium bromide (MTT), L-ascorbic acid, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from Sigma (USA). Acetonitrile, formic acid, and trifluoroacetic acid (TFA) were of chromatographic grade and purchased from Thermo Fisher Scientific (Massachusetts, USA). All other chemicals used were of analytical grade.

3.2 Preparation of protein hydrolysate

3.2.1 Preparation of split gill mushroom (*Schizophyllum commune*)

The split gill mushrooms were dried at 60 °C in a hot air oven and spun by churn and then passed through a 90-micron mesh filter and kept packed in polyethylene bags and stored at 4 °C until use.

3.2.2 Preliminary study on the use protein hydrolysate preparation from split gill mushroom (*Schizophyllum commune*)

Before the optimum conditions for preparing protein hydrolysate from split gill mushroom was studied using RSM, some preliminary experiments with variations in temperature, pH, time, and E/S were performed, which the effect of different degree of hydrolysis. Each condition was selected from the activity of Alcalase. The hydrolysis was tested at pH (7 - 9); the temperature (30 - 65 ^{o}C), hydrolysis time (1-5 h), and E/S (1-3 %v/w).

3.2.3 Experimental design and optimization

The experimental design and statistical analysis were performed using Stat-Ease software (Design-Expert 11 Trial). RSM was employed to optimize the conditions for the enzymatic hydrolysis of split gill mushrooms, using Alcalase enzyme. The influence of three independent variables, A (time, h), B (temperature, °C), and C (enzyme to substrate ratio, % v/w), at five levels (–1.68, –1, 0, 1, 1.68), on the DH were investigated using central composite design (CCD) and the coded and actual values of the experimental design are shown in **Table 3.1**.

The regression model between dependent variables (Y) and independent variables (x) was according to the **Eq. (3.1)**:

$$Y_1 = \beta_0 + {}_{i=1}\Sigma\, \beta_i x_i + {}_{i=1}\Sigma\, \beta_{ii} x^2{}_i + {}_{i<j\text{-}2}\Sigma\, \beta_{ij} x_i x_j \qquad (3.1)$$

Where Y is the measured response variable, β_0, β_i, β_{ii} and β_{ij} are the constant, linear, quadratic and interaction regression coefficients of the model, respectively. x_i and x_j represent the independent variables.

Table 3.1 Experimental codes, factors, and levels of CCD.

Independent			**Code level**				
Variables	**Unit**	**Code**	**-1.68**	**-1**	**0**	**1**	**1.68**
Time	(h)	X_1	0.32	1	2	3	3.68
Temperature	(°C)	X_2	41.59	45	50	55	58.41
E/S	(%v/w)	X_3	0.64	2	4	6	7.36

X_1 = Time (h); X_2 = Temperature (°C); and X_3 = E/S (%v/w)

3.2.4 Enzymatic hydrolysis

Split gill mushrooms powder was hydrolyzed using Alcalase. For the hydrolysis, 0.64–7.36 %v/w of substrate was dissolved in 50 mL of 20 mM phosphate buffer (PB) and the pH of the suspension was adjusted to pH 8.0. The hydrolysis was conducted for 0.32–3.68 h at 41.59–58.41 °C in a shaker at 150 rotations/min; the hydrolysis conditions were investigated using RSM as shown in Table 4.1. After hydrolysis, the enzyme activity was terminated in boiling water at > 90 °C for 15 min. The mixture was immediately cooled and then centrifuged at 9880 × *g* for 30 min at

4 °C. The supernatant was collected and the slurry was cooled to room temperature and kept in a refrigerator until analysis.

3.3 Degree of hydrolysis (DH) analysis

OPA reagent was prepared according to the method described by Church et al. (1983); 7.62 g sodium tetraborate decahydrate and 0.2 g SDS were dissolved in 150 mL deionized water, after mixing, 97% *o*-phthaldehyde (OPA) in ethanol was added and mixed with 99% dithiothreitol (DTT) and the volume was adjusted to 200 mL with deionized water. Serine was used as a standard. An aliquot of 400 μL of each sample, or serine standard, was mixed with 3 mL OPA reagent and mixed for 5 sec, the mixture was incubated for 2 min at room temperature and the absorbance was read at 340 nm. The DH was calculated according to **Eq. (3.2)**.

$$DH\ (\%) = [((B \times N_b)/M_P) \times (1/\alpha) \times (1/h_{tot})] \times 100 \qquad (3.2)$$

Where: B is the base consumption (mL), N_b is the normality of the base, α is the average degree of dissociation of the α-NH_2 groups, M_P is the mass of protein (g), and h_{tot} is the total number of peptide bonds in the protein substrate (8.0 mEq/g food protein).

3.4 Determination of protein content

The protein content was determined using the Bradford method (Bradford, 1976). Bovine serum albumin was used as the standard at concentrations of 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 μg/mL. Each sample (20 μL) was mixed with 200 μL of Bradford working buffer in a 96-well plate, allowed to stand for 2 min at room temperature, and then the absorbance was measured at a wavelength of 595 nm. The solubility was expressed as a percentage of total protein concentration.

3.5 Determination of ABTS radical scavenging assay

The ABTS radical scavenging assay is based on the ability of different substances to scavenge the ABTS radical cation ($ABTS^{\bullet+}$). The fractions were analyzed by the ABTS cation decolorization assay as described by Re et al. (1999). with some modifications. $ABTS^{\bullet+}$ was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and allowing the mixture to

stand in the dark at room temperature for 12 h before use or leaving the mixture for 4–16 h until the reaction was complete and the absorbance was stable. The $ABTS^{\bullet+}$ solution was diluted with distilled water to an absorbance of 0.7 ± 0.02 at 734 nm. The photometric assay was conducted using a 750 μL sample of the $ABTS^{\bullet+}$ solution and 25 μL of test samples (1:30); the solutions were vortexed for 5 sec and the absorbance was measured at 734 nm after standing for 10 min. L-ascorbic acid was used as a reference compound. The antioxidative activity of the tested samples was calculated by determining the decrease in absorbance at different concentrations using the following **Eq. (3.3)**:

$$\frac{[(Abs \text{ control} - Abs \text{ blank}) - (Abs \text{ sample} - Abs \text{ background})]}{(Abs \text{ control} - Abs \text{ blank})} \times 100 \qquad (3.3)$$

where *Abs* control is the A_{734} of the control (no sample), *Abs* sample is the A_{734} of the tested sample, *Abs* background is the A_{734} of the sample without the reagents, and *Abs* blank is the A_{734} of deionized water. The IC_{50} value (the concentration of sample that inhibited 50% of the ABTS radical scavenging) was calculated using GraphPad Prism version 6.01 for windows software (GraphPad Software Inc., California, USA).

3.6 Ultrafiltration

Ultrafiltration through a semipermeable membrane was used for separation into fractions based on molecular weight. Samples were fractionated according to molecular weight using four different molecular weight cut off (MWCO) membranes (10, 5, 3, and 0.65 kDa) (Pall Corporation, USA) and the ABTS radical-scavenging activity was calculated. The obtained protein hydrolysate fractions were stored at –20 °C until further use.

3.7 Reversed-phase high-performance liquid chromatography (RP-HPLC)

The peptide fraction from the ultrafiltration that exhibited the highest ABTS scavenging activity (MW < 0.65 kDa) was then filtered through a 0.45 μm filter (Whatman, GE, Buckinghamshire, UK) and separated using a RP-HPLC system with a C-18 column (Shimpak, 250 × 46 mm, Luna SU; Phenomenex, Torrance, CA, USA). A mobile phase of 0.1% TFA and 70% acetonitrile (ACN) containing 0.05%

TFA was used to separate the peptides, eluted with a rational gradient at a flow rate of 0.7 mL/min. The injection volume was 50 µL and the injected sample had a protein concentration of ~1.0 µg protein/mL. Peptides were detected by measuring the absorbance at 280 nm. Five subfractions, F_1, F_2, F_3, F_4, and F_5, were obtained, lyophilized, and their ABTS scavenging activity was determined.

3.8 Identification of peptides by mass spectrometry

The peptides isolated from RP-HPLC that showed the highest ABTS scavenging activity were identified by ESI-Q-TOF mass spectrometry. The ESI-Q-TOF mass spectrometry involved electrospray ionization and a quadrupole time of flight mass spectrometer. The ESI-Q-TOF mass spectrometer was calibrated using peptide chains in the mass range 50–25,000 m/z. Mascot were used to evaluate all collected ESI-Q-TOF mass spectrometry data. The Basic Local Alignment Search Tool (BLAST) was used to compare the obtained peptide sequence with a database.

3.9 Protective effect of the MW < 0.65 kDa fraction against oxidation-induced DNA damage

3.9.1 Preparation plasmid from *Escherichia coli* (*E. coli*)

E. coli culture strains containing pUC19, pBR322, or pKS plasmids were cultivated on LB agar, supplemented with ampicillin, overnight at 37 °C. Single colonies from the plated *E. coli* strains containing each plasmid were picked, inoculated into LB broth (5 mL) containing ampicillin, incubated for 12–16 h at 30 °C with shaking at 200 rpm, and harvested by centrifugation. Plasmid DNA purification was performed using the Spin clean Plasmid Miniprep Kit (Norgen Biotek Corp., Canada) as per the manufacturer's instructions, eluting the plasmid DNA from the spin column resin in 50 µL.

3.9.2 DNA damage assay

The ability of the MW < 0.65 kDa fraction to protect against DNA damage from hydroxyl radicals was evaluated using the pUC19, pBR322, and pKS plasmids in supercoiled forms and subjected to the Fenton reaction as described by Sheih et al. (2009). Oxidative damage can change the supercoiled form to open-circular or linear

forms. The reactions were composed of 3 μL of DNA (16.5 μg/mL pUC19, 2686 bp; 17.5 μg/mL pBR322, 4361 bp; and 18.8 μg/mL pKS, 2958 bp) and 4 μL of various concentrations of the MW < 0.65 kDa fraction. The reaction mixtures were incubated for 20 min at room temperature, and then 3 μL of 2 mM $FeSO_4$ and 4 μL of 0.06 mM H_2O_2 were added (to initiate the Fenton reaction) and the mixtures were incubated at 37 °C for 30 min. The plasmid DNA was then resolved into supercoiled, nicked, linear, or fragmented types by agarose gel electrophoresis, followed by staining and UV-transillumination to visualize the DNA bands, and sized by comparison with co-resolved DNA size ladders.

3.10 Determination of cellular antioxidant activity assay (CAA)

3.10.1 Cytotoxicity activity of the MW < 0.65 kDa fraction

The MW < 0.65 kDa fraction, which had ABTS scavenging activity, was assessed for *in vitro* cytotoxic activity toward the HT-29 (human colon carcinoma) cell line. The cell line was obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Thailand. The cell suspensions in complete medium [CM; RPMI with 10% (v/v) fetal calf serum (FCS)] were diluted and plated in 200 μL/wells in 96-well plates to a final A_{540} value of 1.0 (approximately 5×10^3 cells/well), and then incubated at 37 °C with 5% (v/v) CO_2 for 72 h. The cell culture medium was then replaced with fresh CM containing various concentrations of the MW < 0.65 kDa fraction and the mixture was incubated as above for 72 h. Next, 10 μL of 5 mg/mL MTT in normal saline solution was added to each well, mixed, and incubated as above for 4 h. The media was then removed and 150 μL/well of dimethyl sulfoxide was added to dissolve the insoluble purple formazan crystals before measuring the A_{540} using a microplate reader spectrophotometer. The A_{540} was taken to be directly proportional to the number of viable cells, and so the relative percentage cell viability was calculated from **Eq. (3.4)**;

$$\text{Cell survival (\%)} = (A_{540\text{-sample}} \times 100)/(A_{540\text{-control}}) \qquad (3.4)$$

Where the control (no sample) was set to be 100% cell survival. The IC_{50} value was determined from the data using version 6.01 of the GraphPad Prism software. All assays were performed in triplicate.

3.10.2 CAA

The *in vitro* cellular antioxidant activity of the peptide fraction was determined in HT-29 cells following the method of Wolfe and Liu (2007). Human colon cancer cells (HT29) were seeded at a density of 6×10^4 cells per well on a 96-well plate in culture medium and incubated for 24 h until the cells were 90% to 100% confluent. Confluence was confirmed using a microscope. The outer wells of the microplate were left empty to reduce any variation from the plate location. The growth medium was removed after confluence was achieved, and the cells were then washed with sterile 20 mM phosphate buffer saline (PBS; pH 7.4) to remove any non-adherent and dead cells. Next, 100 μL of different concentrations of the MW < 0.65 kDa fraction were added to each well, followed by 50 μL of 50 μM DCFH-DA probe solution. Then, cells were incubated for 1 h at 37 °C. Then, the treatment solutions were removed, and the cells were washed three times with 100 μL of PBS. Finally, 100 μL of 500 μM AAPH solution was added to each well, except for the blank and negative control wells. Fluorescence emitted at 528 nm upon excitation at 485 nm was measured every 5 min for 90 min at 37 ºC using a microplate reader. The percent reduction (or the CAA unit) was calculated as follows **Eq. (3.5)**:

$$\text{CAA Unit} = \%\text{reduction} = (1 - AUC_{sample}/AUC_{control}) \times 100 \qquad (3.5)$$

Where AUC_{sample} is the integrated area under the sample fluorescence versus time curve, and $AUC_{control}$ is the integrated area from the control curve of quercetin gathered from triplicate determinations of four separate experiments and reported as the mean ± standard error.

3.11 Statistical analysis

Statistical analyzes in this experiment were performed in triplicate and the results are shown as the mean ± standard error. The analysis was performed using SPSS statistic software and IC_{50} values were calculated using GraphPad Prism Version 6.01 for Windows (GraphPad software Inc., San Diego, CA, USA).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Preliminary experiments

Before the optimum conditions for preparing protein hydrolysate from split gill mushroom was studied using RSM, some preliminary experiments with variations in temperature, pH, time, and E/S were performed. Each condition was selected from the activity of Alcalase. Which the optimal reaction conditions of Alcalase was selected the center point of central composite design (CCD). The hydrolysis was tested at different temperatures (45–60 °C) with a fixed pH and E/S ratio (pH 8 and 1% v/w E/S ratio), and the highest degree of protein hydrolysis occurred at 50 °C [**Figure 4.1(a)**]. Different pH values (7.5–9.0) were investigated using a hydrolysis time of 2 h, a hydrolysis temperature of 50 °C, and an E/S ratio of 1% v/w; the data indicated that maximum activity occurred at pH 8.0 [**Figure 4.1(b)**]. Different hydrolysis times (1–5 h) were investigated at pH 8, an E/S ratio of 1% v/w and a hydrolysis temperatures 50 ^{o}C; the highest degree of protein hydrolysis occurred at 2 h, after which the activity began to decrease [**Figure 4.1(c)**], which is similar to the typical hydrolysis curve reported by Kristinsson and Rasco (2000). Different substrate concentrations (1.0–3.0 v/w) were investigated at pH 8 and with a hydrolysis time of 2 h and a hydrolysis temperature 50 ^{o}C; the data indicated that the higher the enzyme to substrate ratio, the lower the DH [**Figure 4.1(d)**].

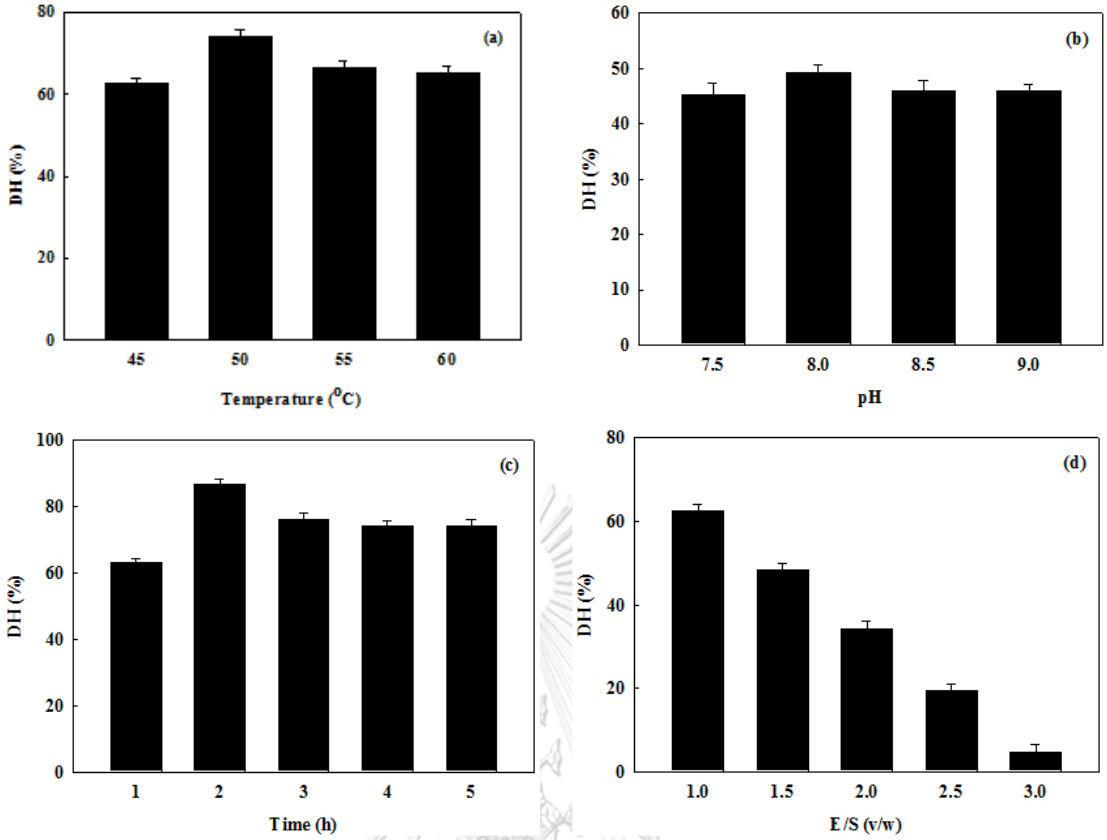

Figure 4.1 Preliminary study of the effect of varying different factors of the reaction on DH. (a) Temperature, (b) pH, (c) time, and (d) E/S ratio.

4.2 Optimization of split gill mushroom protein hydrolysis

We used RSM to determine the combination of hydrolysis parameters that maximized the antioxidant activity of split gill mushroom protein hydrolysate. Temperature, pH, time, and the E/S ratio were selected as the independent variables and analyzed according to the preliminary experiment results, and the ABTS radical-scavenging activity was selected as the dependent variable. The values of the selected factors are illustrated in **Table 4.1**. Each factor was varied at five levels and CCD was used to design experiments and for mathematical model building. The experimental design was constructed based on a 2^3 factorial consisting of 20 sets of experiments according to CCD with three independent variables: time (X_1, min); temperature (X_2, °C); and the E/S ratio (X_3, % v/w). However, to minimize error, all of the coefficients were considered in the design. The equation for the predicted model (based on the actual value) was used to estimate the DH and the ABTS radical-

scavenging activity, which are the response variables Y_1 and Y_2, respectively, of split gill mushroom protein hydrolysate.

Table 4.1 Actual levels of independent variables and the tested values for the response variables.

Run Number	Space Type	X_1	X_2	X_3	Factor			Y_1: DH (%)	Y_2: ABTS (IC_{50}; µg/mL)
					X_1: Time (min)	X_2: Temperature (°C)	X_3: E/S (%v/w)		
1	Center	0	0	0	120	50	4	73.247	0.292
2	Center	0	0	0	120	50	4	74.534	0.280
3	Center	0	0	0	120	50	4	69.927	0.277
4	Center	0	0	0	120	50	4	69.566	0.263
5	Center	0	0	0	120	50	4	70.226	0.309
6	Center	0	0	0	120	50	4	74.209	0.286
7	Axial	+1.68	0	0	220.8	50	4	97.836	0.274
8	Axial	0	0	-1.68	120	50	0.64	16.723	0.245
9	Axial	0	-1.68	0	120	41.6	4	88.187	0.318
10	Axial	-1.68	0	0	19.2	50	4	72.290	0.393
11	Axial	0	0	+1.68	120	50	7.36	116.243	0.331
12	Axial	0	+1.68	0	120	58.4	4	74.128	0.261
13	Factorial	-1	-1	-1	60	45	2	54.017	0.305
14	Factorial	+1	+1	-1	180	55	2	50.882	0.204
15	Factorial	-1	-1	1	60	45	6	104.32	0.368
16	Factorial	-1	+1	-1	60	55	2	48.077	0.249
17	Factorial	+1	-1	-1	180	45	2	40.175	0.317
18	Factorial	+1	+1	+1	180	55	6	113.721	0.257
19	Factorial	-1	+1	+1	60	55	6	80.214	0.416
20	Factorial	+1	-1	+1	180	45	6	127.966	0.265

The effects of the variables on the linear (X_1, X_2, X_3), quadratic (X_{12}, X_{22}, X_{13}), and interaction terms (X_1X_2, X_1X_3, X_2X_3) of the model were evaluated for adequacy, fitness, and significance by ANOVA (**Tables 4.2 and 4.3**). The model was significant ($p < 0.05$). The results showed that time, temperature, and the E/S ratio had a strong influence on the DH value (Y_1) and ABTS radical-scavenging activity (Y_2). High DH values were observed with increasing times but decreasing temperatures and E/S ratios. With increasing time, temperature, and E/S ratio, the

antioxidant activity was observed to increase (as shown in **Figure 4.2**). Similar results have been reported for hydrolysis conditions that also influenced the DH and antioxidant activity, which in turn were dependent on the characteristic composition and amino acid sequence of the derived peptides (Chen et al., 1996). The R^2 values in the present model for the DH value (Y_1) and ABTS scavenging activity (Y_2) were 0.9968 and 0.9722, respectively, which further indicates that the model adequately represented the relationships between the variables. The predicted R^2 values of 0.9343 and 0.9866 are in reasonable agreement with the adjusted determination coefficient values of 0.9471 and 0.9939. The Lack of Fit was not significant relative to the pure error. Non-significant Lack of Fit is good. The value for R^2 indicates that there is a good agreement between the experimental values and the predicted values obtained from the model. In general, the *p*-value determined the significance of each coefficient in the model.

Table 4.2 Analysis of variance for the DH by the response surface quadratic model.

Source	Sum of square	Degree of freedom	Mean square	F-value	*p*-value	
Model	13975.48	9	1552.83	347.17	<0.0001[a]	**significant**
X_1-Time	580.95	1	580.95	129.88	<0.0001[a]	
X_2-Temp	239.81	1	239.81	53.62	<0.0001[a]	
X_3-E/S	11741.54	1	11741.54	2625.06	<0.0001[a]	
X_1X_2	87.83	1	87.83	19.64	0.0013[a]	
X_1X_3	581.23	1	581.23	129.95	<0.0001[a]	
X_2X_3	232.40	1	232.40	51.96	<0.0001[a]	
X_1^2	296.05	1	296.05	66.19	<0.0001[a]	
X_2^2	143.08	1	143.08	31.99	0.0002[a]	
X_3^2	59.98	1	59.98	13.41	0.0044[a]	
Residual	44.73	10	4.47			
Lack of Fit	18.52	5	3.70	0.71	0.6438	**not significant**
Pure Error	26.21	5	5.24			
Cor Total	14020.21	19				
Std. Dev. = 2.11, Mean = 75.82, R^2 = 0.9968, Adjusted R^2 = 0.9939, Predicted R^2 = 0.9866						

Table 4.3 Analysis of variance (ANOVA) for ABTS scavenging activity by response surface quadratic model.

Source	Sum of square	Degree of freedom	Mean square	F-value	*p*-value	
Model	0.0493	9	0.0055	38.82	<0.0001[a]	**significant**
X_1-Time	0.0180	1	0.0180	127.32	<0.0001[a]	
X_2-Temp	0.0037	1	0.0037	26.26	0.0004[a]	
X_3-E/S	0.0103	1	0.0103	73.29	<0.0001[a]	
X_1X_2	0.0016	1	0.0016	11.32	0.0072[a]	
X_1X_3	0.0066	1	0.066	46.49	<0.0001[a]	
X_2X_3	0.0055	1	0.055	38.73	<0.0001[a]	
X_1^2	0.0036	1	0.0036	25.52	0.0005[a]	
X_2^2	8.055E-07	1	8.055E-07	0.01	0.9412	
X_3^2	1.247E-06	1	1.247E-06	0.01	0.9269	
Residual	0.0014	10	0.0001			
Lack of Fit	0.0002	5	0.0000	0.18	0.9596	**not significant**
Pure Error	0.0012	5	0.0002			
Cor Total	0.0507	19				
Std. Dev. = 0.0119, Mean = 0.2955, R^2 = 0.9722, Adjusted R^2 = 0.9471, Predicted R^2 = 0.9343						

Note: a indicates identified variables with a significant effect on the response ($p < 0.05$)

$$\mathbf{Y_1 = 360.32 - 1.03x_1 - 12.63x_2 + 37.18x_3 + 0.01x_1x_2 + 0.07x_1x_3 - 0.54x_2x_3 + 0.13x_2^2 - 0.51x_3^2} \quad \textbf{(4.1)}$$

$$\mathbf{Y_2 = 0.68 + 0.002x_1 - 0.009x_2 - 0.088x_3 - 4.708e\text{-}05x_1x_2 - 0.0003x_1x_3 + 0.003x_2x_3 + 4.396e\text{-}06x_1^2} \quad \textbf{(4.2)}$$

Where; Y_1, Y_2, X_1, X_2, and X_3 are DH (%), ABTS (μg/mL; IC_{50}), time (min), temperature (°C), and E/S ratio (% v/w), respectively.

Validation of the statistical model and regression equation were conducted using the optimal values for the three factors (161.41 min, temperature 55 °C, and E/S ratio 2% v/w). Under these optimized conditions, the predicted responses for the DH and ABTS values were 48.01% and 0.208 μg/mL, respectively, while the observed experimental values were 45.63% and 0.205 μg/mL, respectively. These results confirmed the validity of the model and that the experimental designs used in this work were appropriate for predicting the optimized conditions.

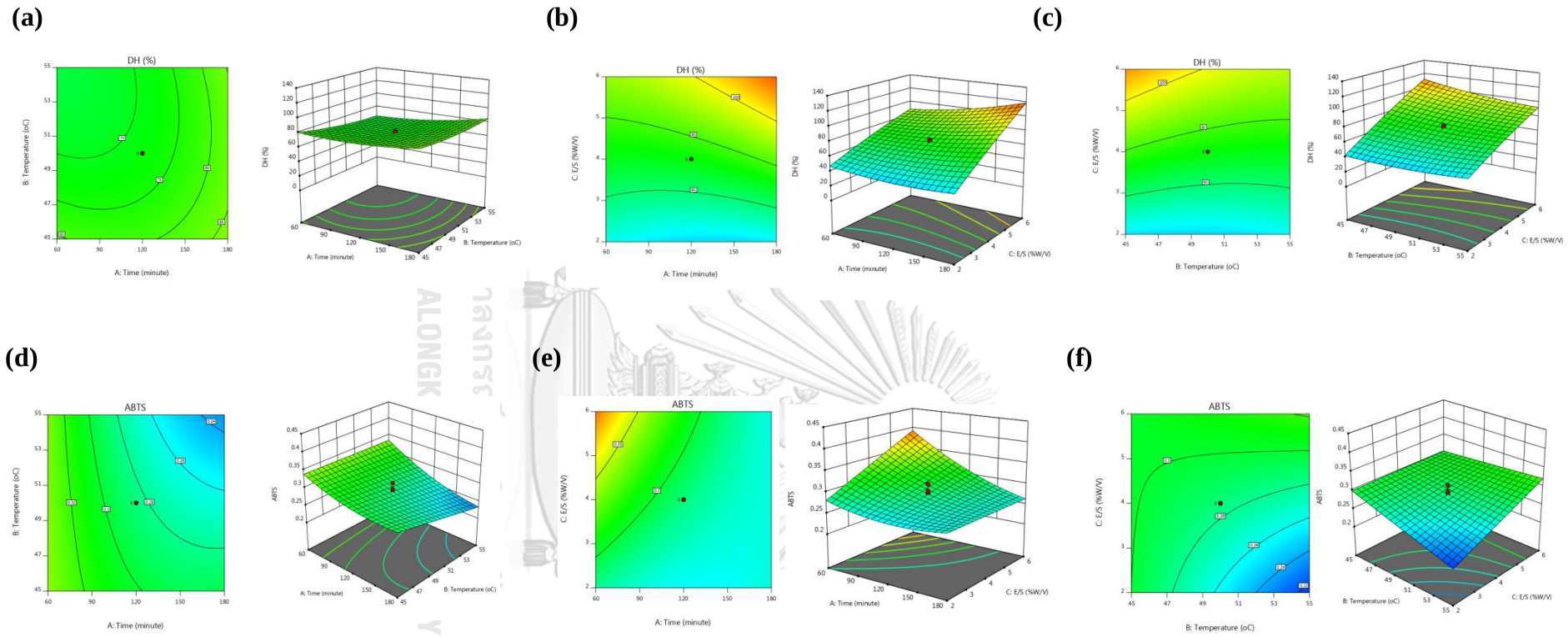

Figure 4.2 Contour plot of time and temperature (a), time and E/S ratio (b), and temperature and E/S (c) on the DH; and contour plot of time and temperature (e), time and E/S ratio (d), and temperature and E/S ratio (f) on the ABTS radical-scavenging activity

4.3 ABTS radical-scavenging activity after size fractionation of the crude protein hydrolysate by ultrafiltration

Ultrafiltration membranes were used to perform peptide separation according to molecular weight. The split gill mushroom protein hydrolysate was fractioned using two different MWCO membranes for MWs of 10, 5, 3, and 0.65 kDa. These membranes allowed the separation of six different fractions of split gill mushroom protein hydrolysate. The smallest size fraction, MW < 0.65 kDa, clearly had the highest ABTS radical-scavenging activity compared with the other fractions (**Table 4.4**), with an IC_{50} of 0.026 ± 0.0004 μg/mL, but the activity was still lower than for the positive control, ascorbic acid, which had an IC_{50} value of 96.27 ± 0.42 μg/mL. Many researchers have reported that low molecular weight peptides had high antioxidant activity (Wang et al., 2007); peptides with a molecular weight of 1–3 kDa (Kim et al., 2007) and < 3.5 kDa (Bougatef et al., 2009; Sila & Bougatef, 2016) have been reported to have high antioxidant activity. Ultrafiltrated Pacific hake protein hydrolysate with a size of 1–3 kDa had the greatest ABTS radical-scavenging activity of all tested fractions; the total amino acid compositions of the crude Pacific hake protein hydrolysate and the ultrafiltration fractions were relatively similar (Samaranayaka et al., 2010) . In addition, oligopeptides with sizes < 1400 Da have been found to be major contributors to ABTS radical-scavenging activity with little dependence on the peptide composition (Cheung et al., 2012).

Table 4.4 ABTS scavenging activity (as IC_{50} values) of the five fractions (10, 5, 3, and 0.65 kDa MWCO membrane-filtered fractions) of the split gill mushroom protein hydrolysate derived from Alcalase hydrolysis.

Molecular weight (kDa)	**ABTS scavenging activity (IC_{50}; μg/mL)**
Crude protein	0.435 ± 0.003^{a}
> 10	0.369 ± 0.005^{b}
5–10	0.206 ± 0.007^{c}
3–5	0.183 ± 0.003^{d}
0.65–3	0.103 ± 0.001^{e}
< 0.65	0.026 ± 0.0004^{f}
Ascorbic acid	96.27 ± 0.42

The results are presented as the mean ± SE (n = 3). Means with a different superscript within a column are significantly different ($p < 0.05$).

4.4 ABTS radical-scavenging activity of the MW < 0.65 kDa fraction after RP-HPLC fractionation ($F_{1–5}$)

RP-HPLC is an effective technique to purify and quantify peptides in a mixture, based on hydrophobic characteristics. The retention time (RT) can be used to qualitatively analyze the isolated peptide and can be adjusted by changing the ratio of methanol or acetonitrile in the mobile phase, and the peak area can be used for quantitative analysis of the isolated peptides. The MW < 0.65 kDa fraction was further fractionated by RP-HPLC on a C_{18} column. The chromatogram of the obtained peptides (as A_{280} nm values) is shown in **Figure 4.3**. There were five principal peaks, with many minor peaks, and the major peaks were collected as five subfractions. The retention times of the subfractions corresponded to the position of the peptides from the hydrolysate. In the chromatograms from the RP-HPLC, the hydrophilic and more polar peptides had shorter retention times, and the hydrophobic and less polar peptides had longer retention times. The F_4 subfraction, which had the maximum ABTS radical-scavenging activity was selected for further analysis by Q-TOF-ESI-MS/MS.

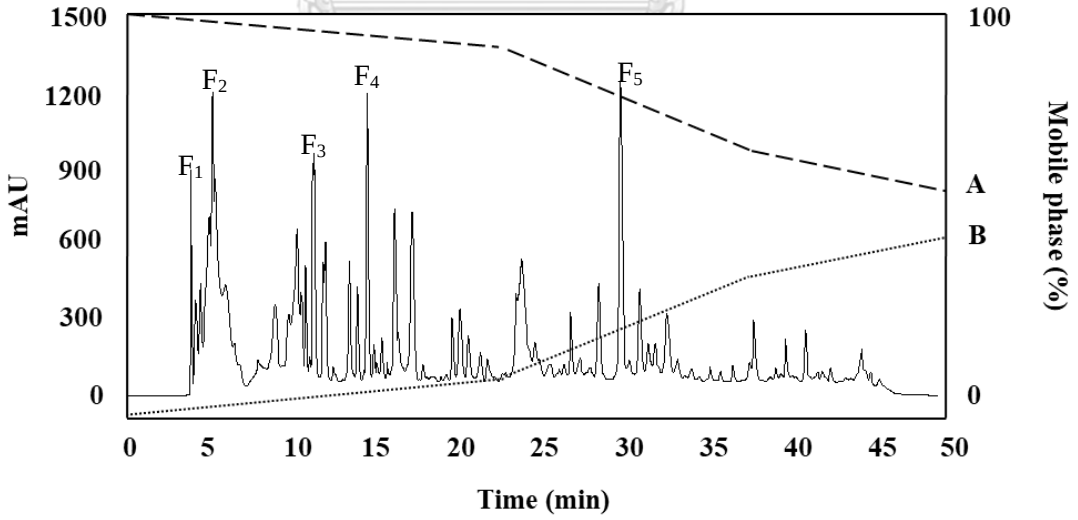

Figure 4.3 RP-HPLC chromatography of the MW < 0.65 kDa fraction.
(A = 0.1% Trifluoroacetic acid (TFA), B = 70% acetonitrile + 0.05% TFA)

4.5 Identification of peptides by Q-TOF-ESI-MS/MS

The F_4 sub-fraction of the split gill mushroom protein hydrolysate was subjected to Q-TOF-ESI-MS/MS analysis to identify the principal peptides. The raw MS/MS data were submitted for a database search against the SwissPlot database using the Mascot database. The amino acid sequences of five peptides were obtained: Met-Tyr-Ser-Glu-Lys-His-Gly-Ser-Gly-Gly-Thr (MYSEKHGSGGT; 1,153.23 Da); Pro-Gly-Thr-Arg-Gly-Ala-Ile-Ala-Ala-Ser-Ser-Pro-Gln-Val (PGTRGAIAASSPQV; 1,311.44Da); Met-Val-Ser-Thr-Leu-Ala-Val-Leu-Gly-Ile-Arg-Glu-Pro (MVSTLAVLGIREP; 1,385.67 Da); Glu-Lys-Glu-Ala-Ala-Glu-Leu-Gly-Lys-Gly-Ser-Phe (EKEAAELGKGSF; 1,265.37 Da); and Met-Ser-Val-Thr-Leu-Leu-Leu-Leu-Phe-Ile-Ser-Leu-Val-Trp-Val-Thr-Ile-Ser-Gly-Leu-Asn (MSVTLLLLFISLVWVTISGLN; 2,319.84 Da). The detected molecular masses of the peptides agreed well with the theoretical masses calculated from the sequence.

All fragments were aligned to those homologs available in the BLAST. The longest peptide sequence, MSVTLLLLFISLVWVTISGLN, revealed 100% amino acid sequence identity to the NADH dehydrogenase subunit 3 and 71.43% similarity to the pri3a-like protein (both from *S. commune*). The peptides MYSEKHGSGGT, PGTRGAIAASSPQV, and MVSTLAVLGIREP (m/z = 1152.49, 1310.69, and 1384.77, respectively) were similar to a polysaccharide lyase family 8 protein, a HD2 mating type protein, and a glycosyltransferase family 69 protein, respectively (all from *S. commune*), suggesting that this protein could be a member of the transport/signaling family of proteins. Accordingly, this peptide can replace polymorphic regions in a protein, and may be derived from divergent subunit proteins.

Generally, the ability of a peptide to stabilize free radicals is because of its ability to donate electrons to the free radicals or absorb electrons from the free radicals to reduce the reactivity of the free radicals (Ren et al., 2008). Amino acids, such as Tyr, Phe, Trp, Met, Cys, and His have been reported to be important for the antioxidant activity of peptides because of their structures: Tyr and Phe have aromatic groups that can serve as hydrogen donors; Trp has an indole group that can act as a hydrogen donor; Met is prone to oxidation to methionine sulfoxide; Cys can donate the sulfur hydrogen and can directly interact with radicals; His contains an imidazole

ring that may be involved in hydrogen donation and lipid radical trapping. In addition, Gly, Asp, and Glu are able to quench unpaired electrons or radicals by supporting protons. Furthermore, peptides containing hydrophobic amino acids may contribute to inhibition of lipid peroxidation because the hydrophobic amino acids can increase the solubility of the peptides in the lipid system, and thereby facilitate better interactions with the radical species (Qian et al., 2008).

4.6 Protective effect of the MW < 0.65 kDa fraction on oxidation-induced DNA damage

Plasmid DNA, namely, pBR322, pKS, and pUC19, were used to measure oxidation-induced DNA damage to verify the protective effect of the MW < 0.65 kDa fraction. DNA damage caused by hydroxyl radicals, through the Fenton reaction, changes supercoiled DNA into linear and open circular DNA. **Figure 4.4(a)** shows the capability of the MW < 0.65 kDa fraction to protect pBR322 plasmid DNA. The supercoiled DNA was completely induced to linear DNA as a result of the hydroxyl radical damage (Lanes 4–10). The MW < 0.65 kDa fraction at concentrations of 0.55, 0.27, 0.14, and 0.07 μg/mL (Lanes 4–7) protected against oxidation-induced DNA damage. This result was similar to that for the pUC19 plasmid DNA [**Figure 4.4(b)**], in which the amount of supercoiled DNA in pUC19 was decreased and was induced to form linear DNA.

The protective effect of the MW < 0.65 kDa fraction on the pKS plasmid DNA is shown in **Figure 4.4(c)**. The MW < 0.65 kDa fraction at concentrations of 0.55, 0.27, and 0.14 μg/mL (Lanes 4–6) protected against oxidation-induced DNA damage. The supercoiled DNA form was completely converted to the linear DNA form Sheih et al. (2009) reported that the purified peptide from algae protein waste hydrolysate protected PET-28a DNA from oxidation-induced damage. The supercoiled DNA was clearly converted to open circular DNA after hydroxyl radical damage through the Fenton reaction (Fe^{2+} + H_2O_2) and increasing concentrations of peptide from 10.6 to 84.9 μM exhibited increasing protective capacity. In addition, Zhang et al. (2014) have reported that peptides from sweet potato protein hydrolysates protected the plasmid pBR322 from oxidative damage. The protective effect of the peptide increased with increasing peptide concentrations; concentrations of 1, 2.5, 5, and 10 mg/mL had protective effects of 28.52%, 36.99%, 50.11%, and 61.33%, respectively.

The data from the present study demonstrated that the MW < 0.65 kDa fraction exhibited OH^-scavenging activity and Fe^{2+}chelating ability.

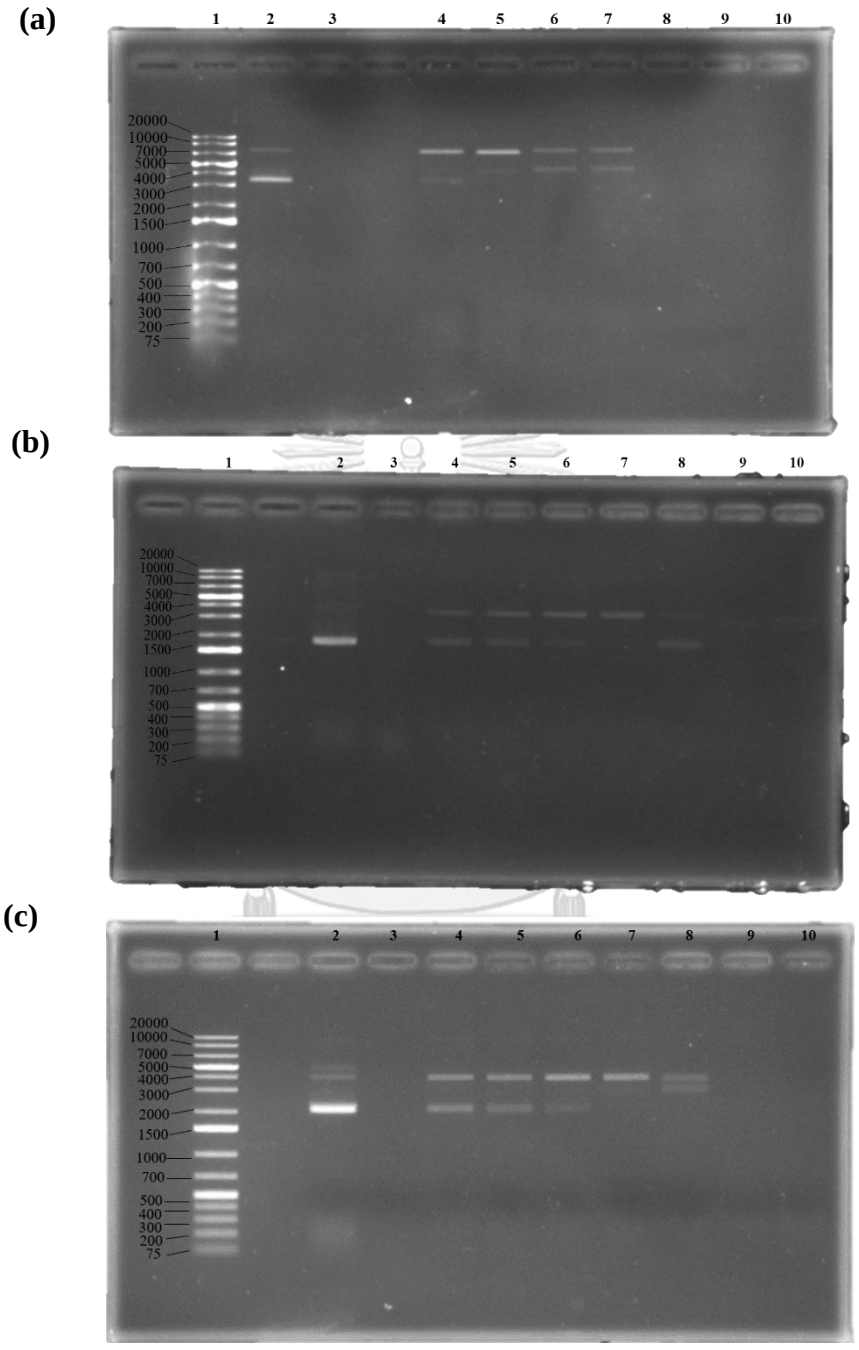

Figure 4.4 Protective effect of the MW < 0.65 kDa fraction on hydroxyl radical-induced oxidation of (a) pBR322 (4,361 bp; 17.5 μg/mL), (b) pUC19 (2686 bp; 16.5 μg/mL), and (c) pKS (2958 bp; 18.8 μg/mL) plasmid DNA. Lane 1: Ladder 1 kb, Lane 2: plasmid DNA, Lane 3: plasmid DNA with $FeSO_4$ and H_2O_2treatment (as DNA damage control), Lanes 4–10: plasmid DNA with $FeSO_4$ and H_2O_2 treatment in the presence of the MW < 0.65 kDa fraction at concentrations of 0.55, 0.27, 0.14, 0.07, 0.03, 0.01 and 0.005 μg protein/mL. Gels shown are representative of those seen from three independent trials.

4.7 CAA results using the HT29 cell line

The CAA reflects changes in the absorption, metabolism, and distribution of antioxidants at the cellular level. The CAA method has a shorter experimental time and simpler measurement process than animal experiments. CAA is based on the study of the anticancer mechanism of antioxidants, and liver cancer is used as the test cell type. Because the MW < 0.65 kDa fraction had high ABTS radical-scavenging activity, only this fraction was analyzed in the CAA. The cytotoxic effect of several different concentrations of the MW < 0.65 kDa fraction on the proliferation of HT-29 cells was investigated using the MTT method. From the results of the MTT cell viability assay shown in **Figure 4.5(a)**, the majority of cells were still viable at sample concentrations of 0.00781 and 0.45 μg/mL.

DCFH-DA is widely used to measure oxidative stress in cells because of the high sensitivity of this fluorescence-based assay. DCFH-DA penetrates into cells and is hydrolyzed to DCFH by intracellular esterases. The presence of ROS can oxidize DCFH to form DCF, a fluorescent product. However, pretreatment with the MW < 0.65 kDa fraction decreased the DCF fluorescent intensity. The MW < 0.65 kDa fraction showed comparable DCF fluorescent intensity ($p > 0.05$) at concentrations up to 0.45 μg/mL [**Figure 4.5(b)**]. This result suggested that the MW < 0.65 kDa fraction had a cellular radical scavenging effect.

It has been reported that hydrolyzed food proteins from various sources have exhibited remarkable antioxidant activity (Chanput et al., 2009). In the CAA, high lipid solubility may be important for antioxidant effects as peptides with high lipid solubility are able to bind to the cell membrane and/or pass through it. Therefore, peptides with high lipid solubility may act to prevent peroxyl radical-mediated cell membrane oxidation and/or inhibit DCFH intracellular oxidation (Wolfe & Liu, 2007). Zhang et al. (2010) have reported that a purified peptide isolated from rice endosperm could scavenge intracellular ROS in mouse macrophage RAW 264.7 cells. Liu et al. (2010) have reported that three purified peptides from water buffalo horn protected rat cerebral microvascular endothelial cells against H_2O_2-induced injury. The authors suggested that Tyr, Gly, and Cys residues and Phe-Pro-Pro may play important roles in scavenging radicals through direct electron transfer. It was also

suggested that hydrophobic amino acids might increase the affinity and reactivity of peptides toward the cell membrane in rat cerebral microvascular endothelial cells, leading to better antioxidant activity.

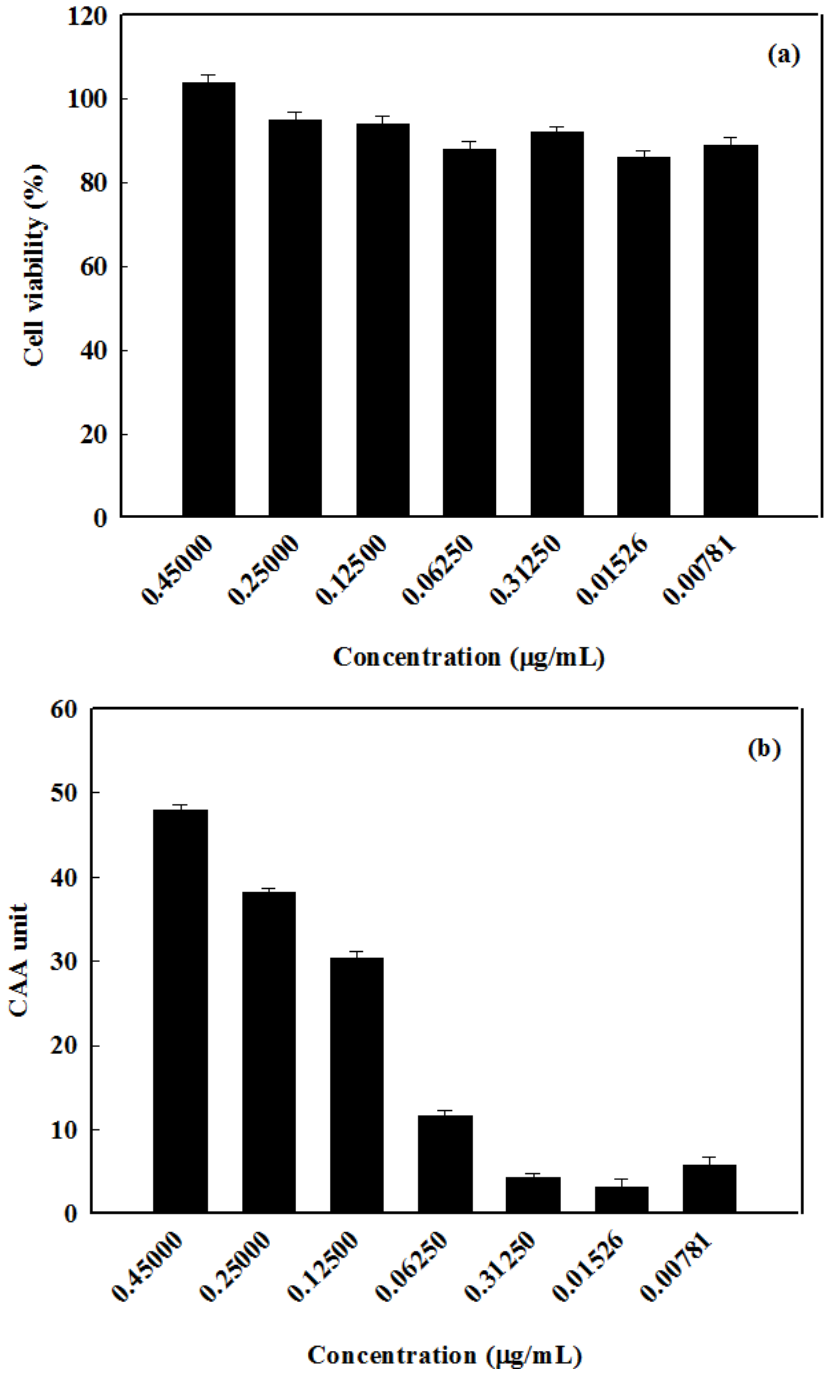

Figure 4.5 (a) Viability of HT-29 cells after treatment with various concentrations of the MW < 0.65 kDa fraction for 72 h, (b) Cellular antioxidant activity of the MW < 0.65 kDa fraction. Graph represents the mean ± SEM (n = 3).

CHAPTER 5
CONCLUSION

The hydrolysates of split gill mushroom protein hydrolyzed by Alcalase were found to possess antioxidant activity. RSM was used to improve the DH and ABTS-radical scavenging activity of these hydrolysates. The model was validated and shown to be statistically adequate and accurate in predicting the response. The optimum conditions obtained from experiments were: time 161.41 min; temperature 55 °C; and E/S ratio 2% v/w. There was no correlation between the DH and the ABTS radical-scavenging activity; the ABTS radical-scavenging activity depended upon the molecular weight, amino acid composition, and amino acid sequence. The MW < 0.65 kDa fraction had the highest scavenging ability. Further fractionation by RP-HPLC yielded five main fractions ($F_{1–5}$) of which fraction F_4 was the most active in the ABTS assay. In addition, the MW < 0.65 kDa fraction demonstrated protection against free-radical damage to DNA and exhibited a cytoprotective effect in the CAA, which was dependent on the peptide concentration. These results suggest that the protein hydrolysate from the split gill mushroom could be useful as a natural source of bioactive compounds and as a material in the development of health-promoting functional foods. The hydrolysate also has a potential use against the proliferation of cancer because of the antioxidant ability.

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APPENDIX A

Buffer and reagents preparation

1. 20 mM Phosphate buffer, pH 7.2

KH_2PO_4	2.721 g
K_2HPO_4	3.483 g
NaCl	8.766 g

Adjusted volume to 1 L with deionized water and adjusted the pH to 7.2 with 1 M NaOH or 1 M HCl. Storage at 4 ˚C until use.

2. Bradford solution and protocol

2.1 Bradford Stock Solution, 650 mL

95% Ethanol	100 mL
88% Phosphoric acid	200 mL
Serva Blue G Dye	350 mg

Stable indefinitely at room temperature

2.2 Bradford Working Buffer, 500 mL

Deionized water	425 mL
95% Ethanol	15 mL
88% Phosphoric acid	30 mL
Bradford Stock Solution	30 mL

Note: before using, Bradford working solution must be filtrated through the Whatman No.1 paper. Bradford working solution is kept in a brown glass bottle at room temperature. Usable for several weeks, but may need to be refiltered.

2.3 1mg/mL bovine serum albumin (BSA), 10 mL

BSA	10 mg

10 mg BSA was dissolved by 10 mL deionized water and mix well for standard curve protein concentration. Storage at 4 ˚C until use.

3. OPA solution, 200 mL

Step 1	Sodium tetraborate decahydrate	7.62 g
	Sodium dodecyl sulfate (SDS)	200 mg
	Dissolve in 150 mL of deionized water	
Step 2	Phthaldialdehyde (OPA)	160 mg
	Dissolve in 4 mL ethanol	
Step 3	Transfers solution from step 2 to solution from step 1	
Step 4	Add 176 mg Dithiothreitol (DTT) in solution from step 3.	
	Add deionized water for making solution up to 200 mL.	

4. DPPH solution

0.1M DPPH	0.004g
Adjusted volume to 100 mL with methanol	

5. ABTS solution

5.1 7mM ABTS (solution A)

ABTS	0.096 g
Dissolve in 25 mL with deionized water	

5.2 2.45 mM potassium persulphate (solution B)

Potassium persulphate	0.016 g
Dissolve in 25 mL with deionized water	

5.3 ABTS solution

Mix solution A and solution B in the dark room for 12 – 16 hours. Before using, the $ABTS^{\bullet+}$ solution was diluted with distilled water to an absorbance of 0.7 ± 0.02 at 734 nm.

6. Mobile phase in RT-HPLC analysis

6.1 Eluent A: 0.1% trifluoroacetic acid (TFA), 1000 mL

Double deionized water	999 mL
TFA	1 mL
Mix and filtrate through a cellulose acetate membrane**.**	

6.2 Eluent B: 70% acetonitrile containing 0.05% TFA, 1000 mL

Step 1 Filtrate 350 mL acetonitrile through PTFE membrane

Step 2: Add 75 µL of TFA in 150 mL double deionized water. Then, filtrate though a cellulose acetate membrane. Add this solution into filtrated acetonitrile. Both of eluent, before using, must be degassing by sonication 15 minutes.

7. LB agar for *E. coil*

Peptone	1 g
Yeast extract	0.5 g
NaCl	1 g
Agar power	2 g
Dissolve in 100 mL with deionized water then sterilization at 121 ^{0}C, 15 min.	

8. LB Broth for *E. coil*

Peptone	1 g
Yeast extract	0.5 g
NaCl	1 g

Dissolve in 100 mL with deionized water then sterilization at 121 ^{0}C, 15 min.

9. DNA damage

9.1 2 mM $FeSO_4$

$FeSO_4 \cdot 7\ H_2O$	0.0278 g

Dissolve in 50 mL with deionized water.

9.2 30 % H_2O_2

APPENDIX B

Standard cure for determine the protein concentration by Bradford method

Bovine serum albumin (BSA) protein derived from cows, is used as a protein concentration standard in experiments. The standard curve remains linear from about 2.5 µg to 15 µg of BSA were prepared following

µg Protein (µg)	Standard solution (1 mg/mL BSA)	Experiment Buffer (µg)	Bradford Reagent (mL)	A_{595}
0	0	100	1	0.000
2.5	2.5	97.5	1	0.067
5	5	95	1	0.110
7.5	7.5	92.5	1	0.168
10	10	90	1	0.225
12.5	12.5	87.5	1	0.278
15	15	85	1	0.338
17.5	17.5	82.5	1	0.367
20	20	80	1	0.428

Absorbance at 595 nm was plot as y-axis and BSA protein concentration was plot as x-axis.

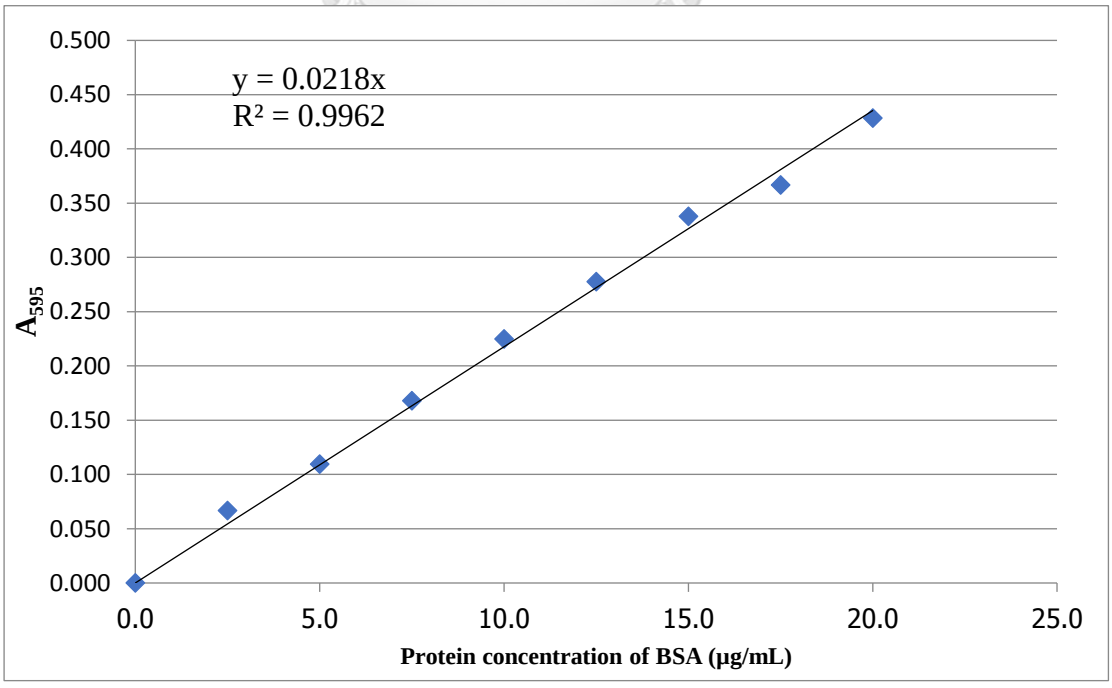

Figure 1B Standard curve of BSA concentration at the absorbance of 595 nm

APPENDIX C
Amino acid abbreviations and structures

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

APPENDIX D
Mass spectrum analysis

MS/MS Fragmentation of MYSEKHGSGGT

Found in AIA82936.1 in NCBIprot, asparaginase-like protein, partial [*Beauveria bassiana*]

Match to Query 73: 1151.544748 from (576.779650,2+) intensity (8852.0000) index (45)

Title: Cmpd 46, +MSn(576.7797), 18.2 min

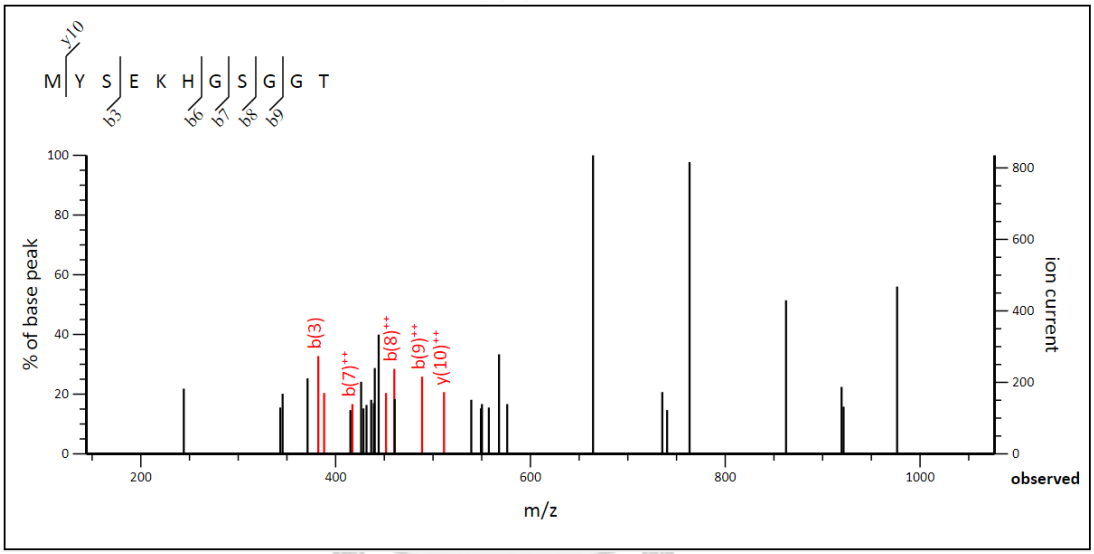

Figure 1D Identification of the peptide, MYSEKHGSGGT

(Met - Tyr - Ser - Glu - Lys - His - Gly - Ser - Gly - Gly - Thr)

MS/MS Fragmentation of PGTRGAIAASSPQV
Found in ODO08660.1 in NCBIprot, hypothetical protein L198_00392 [*Tsuchiyaea wingfieldii* CBS 7118]

Match to Query 86: 1311.578568 from (656.796560,2+) intensity (2200.0000) index (15)
Title: Cmpd 16, +MSn(656.7966), 15.1 min

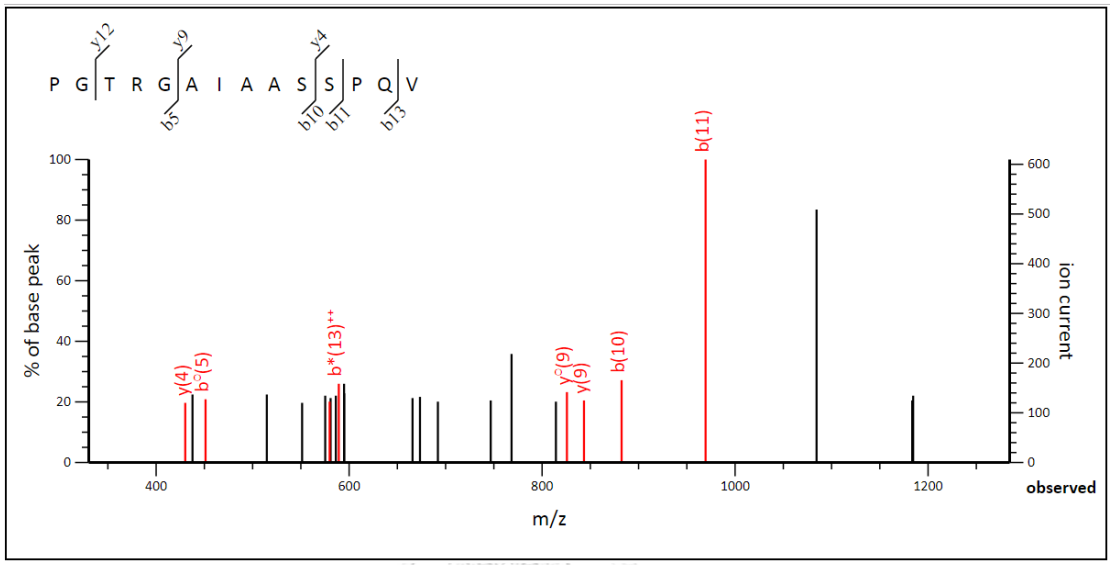

Figure 2D Identification of the peptide, PGTRGAIAASSPQV (Pro - Gly - Thr - Arg - Gly - Ala - Ile - Ala - Ala - Ser - Ser - Pro - Gln - Val), by mass spectrometry.

MS/MS Fragmentation of MVSTLAVLGIREP
Found in CCX05399.1 in NCBIprot, Protein of unknown function [*Pyronema omphalodes* CBS 100304]

Match to Query 90: 1384.594002 from (462.538610,3+) intensity (5048.0000) index (43)
Title: Cmpd 44, +MSn(462.5386), 18.2 min

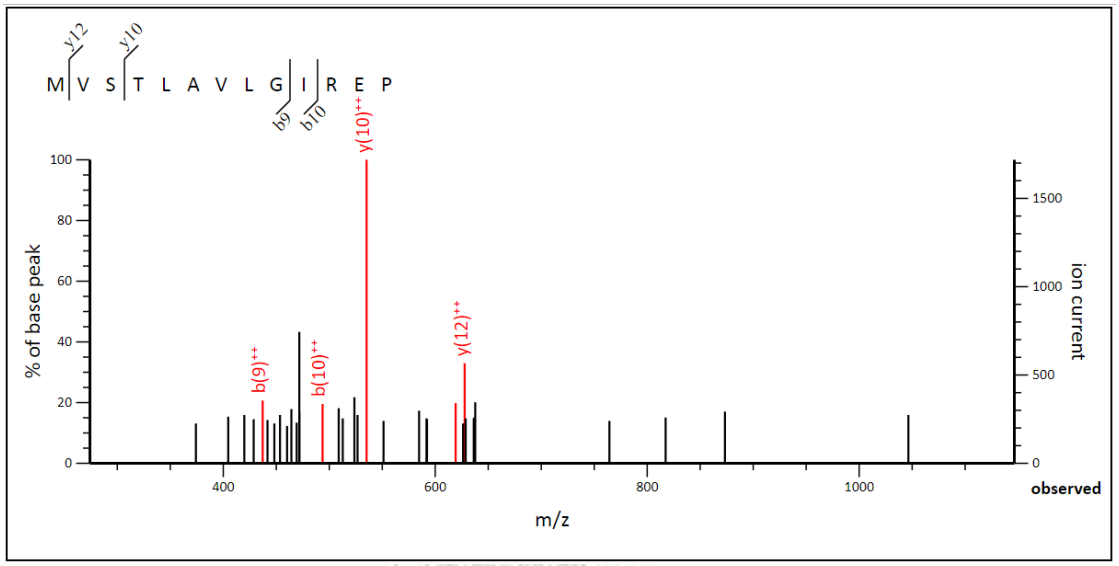

Figure 3D Identification of the peptide, MVSTLAVLGIREP (Met - Val - Ser - Thr - Leu - Ala - Val - Leu - Gly - Ile - Arg - Glu - Pro), by mass spectrometry.

MS/MS Fragmentation of EKEAAELGKGSF
Found in AGH88286.1 in NCBIprot, translation elongation factor 1-alpha, partial [*Diaporthe cf. nobilis* RG-2013]

Match to Query 85: 1265.688928 from (633.851740,2+) intensity (68108.0000) index (19)
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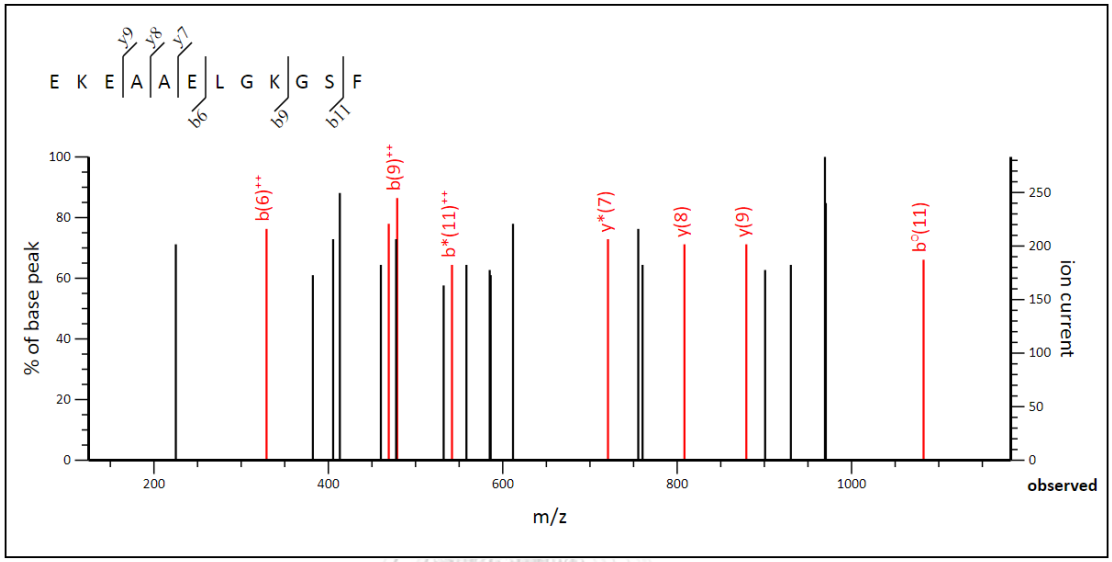

Figure 4D Identification of the peptide, EKEAAELGKGSF (Glu - Lys - Glu - Ala - Ala - Glu - Leu - Gly - Lys - Gly - Ser - Phe), by mass spectrometry.

MS/MS Fragmentation of MSVTLLLLFISLVWVTISGLN
Found in KTB32328.1 in NCBIprot, hypothetical protein WG66_15093 [*Moniliophthora roreri*]

Match to Query 95: 2317.202262 from(773.408030,3+) intensity(1760.0000) index(3)
Title: Cmpd 4, +MSn(773.4080), 12.1 min

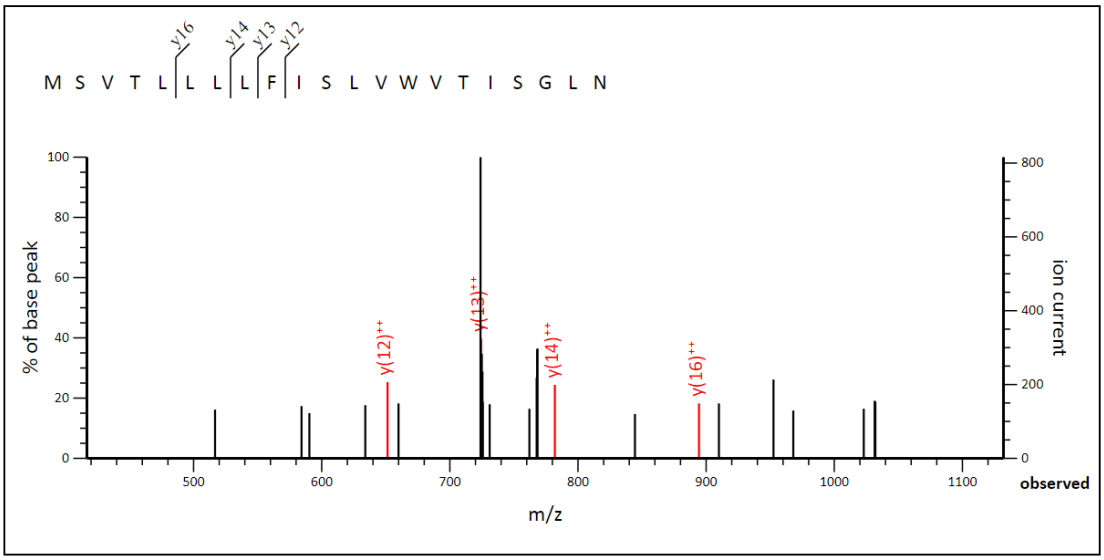

Figure 5D Identification of the peptide, MSVTLLLLFISLVWVTISGLN (Met - Ser - Val - Thr - Leu - Leu - Leu - Leu - Phe - Ile - Ser - Leu - Val - Trp - Val - Thr - Ile - Ser - Gly - Leu – Asn), by mass spectrometry.

APPENDIX E

Table E Peptide sequences identification by Q-TOF mass spectrometry that were taken from the RP-HPLC

Sequence	Organism	Mass	Query cover (%)	Identity (%)	Accession
1. MYSEKHGSGGT	polysaccharide lyase family 8 protein [*Schizophyllum commune* H4-8]	1152.49	81	100	XP_003031096.1
	hypothetical protein SCHCODRAFT_46713 [*Schizophyllum commune* H4-8]	1152.49	72	100	XP_003037321.1
	hypothetical protein SCHCODRAFT_57923 [*Schizophyllum commune* H4-8]	1152.49	54	100	XP_003030052.1
	expressed protein [*Schizophyllum commune* H4-8]	1152.49	81	100	XP_003030568.1
	expressed protein [*Schizophyllum commune* H4-8]	1152.49	36	100	XP_003032145.1
2. PGTRGAIAASSPQV	HD2 mating type protein [*Schizophyllum commune* H4-8]	1310.69	35	100	XP_003037495.1
	homeodomain protein [*Schizophyllum commune*]	1310.69	35	100	AAB41340.1
	expressed protein [*Schizophyllum commune* H4-8]	1310.69	35	100	XP_003031208.1
	hypothetical protein SCHCODRAFT_106188 [*Schizophyllum commune* H4-8]	1310.69	85	100	XP_003036279.1
	hypothetical protein SCHCODRAFT_114167 [*Schizophyllum commune* H4-8]	1310.69	92	100	XP_003026798.1
3. MVSTLAVLGIREP	expressed protein [*Schizophyllum commune* H4-8]	1384.77	69	100	XP_003037545.1
	glycosyltransferase family 69 protein [*Schizophyllum commune* H4-8]	1384.77	46	100	XP_003031540.1
	expressed protein [*Schizophyllum commune* H4-8]	1384.77	46	100	XP_003028527.1
	hypothetical protein SCHCODRAFT_256094 [*Schizophyllum commune* H4-8]	1384.77	76	100	XP_003035142.1
	hypothetical protein SCHCODRAFT_68324 [*Schizophyllum commune* H4-8]	1384.77	100	100	XP_003030843.1
4. EKEAAELGKGSF	translation elongation factor 1a [*Schizophyllum commune* H4-8]	1264.63	100	100	XP_003037261.1
	hypothetical protein SCHCODRAFT_74346 [*Schizophyllum commune* H4-8]	1264.63	75	100	XP_003035885.1
	expressed protein [*Schizophyllum commune* H4-8]	1264.63	41	100	XP_003038025.1
	hypothetical protein SCHCODRAFT_108645 [*Schizophyllum commune* H4-8]	1264.63	66	100	XP_003031577.1
	expressed protein [*Schizophyllum commune* H4-8]	1264.63	41	100	XP_003038706.1
5. MSVTLLLLFISLVWVTISGLN	hypothetical protein SCHCODRAFT_72473 [*Schizophyllum commune* H4-8]	2318.33	71	100	XP_003038273.1
	NADH dehydrogenase subunit 3 [*Schizophyllum commune*]	2318.33	80	100	NP_150127.1
	hypothetical protein SCHCODRAFT_106236 [*Schizophyllum commune* H4-8]	2318.33	90	100	XP_003036308.1
	expressed protein [*Schizophyllum commune* H4-8]	2318.33	57	66.67	XP_003028253.1
	pri3a-like protein [*Schizophyllum commune* H4-8]	2318.33	33	71.43	XP_003033809.1

VITA

NAME	Aunchalee Wongaem
DATE OF BIRTH	15 November 1992
PLACE OF BIRTH	Lopburi
INSTITUTIONS ATTENDED	Program in Biotechnology, Faculty of Science, Chulalongkorn University.
HOME ADDRESS	138/18 Bang Krathuek, Samphran District, Nakhon Pathom, 73110.
PUBLICATION	Academic Presentation; Wongaem, A., Sangtanoo, P., Karnchanatat, A. Optimization of Preparing Protein Hydrolysate from Split Gill Mushroom (Schizophyllum commune) by Alcalase Using Response Surface Methodology (RSM). In "The Pure and Applied Chemistry International Conference (PACCON 2019)". 7-8 February 2019, Bangkok International Trade & Exhibition Centre (BITEC), Bangna, Thailand. (proceeding).
AWARD RECEIVED	won an Outstanding Student Poster Award at the Pure and Applied Chemistry International Conference (PACCON) 2019 held on February 7-8, 2019 at the Bangkok International Trade & Exhibition Centre (BITEC).