การหาภาวะที่เหมาะสมโดยวิธีพื้นผิวตอบสนอง
สำหรับการเตรียมโปรตีนไฮโดรไลเสตด้วยโปรติเอส จี6 และฤทธิ์ขจัดอนุมูลอิสระจากขนไก่ปั่น

นางสาววลีพร พิบูลย์ผล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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OPTIMIZATION OF PREPARATION PROTEIN HYDROLYSATE BY PROTEASE G6 USING RESPONSE SURFACE METHODOLOGY AND FREE RADICAL SCAVENGING ACTIVITIES FROM FEATHER MEAL

Miss Waleeporn Pibulpol

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	OPTIMIZATION OF PREPARATION PROTEIN HYDROLYSATE BY PROTEASE G6 USING RESPONSE SURFACE METHODOLOGY AND FREE RADICAL SCAVENGING ACTIVITIES FROM FEATHER MEAL
By	Miss Waleeporn Pibulpol
Field of Study	Biotechnology
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วลีพร พิบูลย์ผล : การหาภาวะที่เหมาะสมโดยวิธีพื้นผิวตอบสนองสำหรับการเตรียมโปรตีนไฮโดรไลเสตด้วยโปรติเอส จี6 และฤทธิ์ขจัดอนุมูลอิสระจากขนไก่ปั่น (OPTIMIZATION OF PREPARATION PROTEIN HYDROLYSATE BY PROTEASE G6 USING RESPONSE SURFACE METHODOLOGY AND FREE RADICAL SCAVENGING ACTIVITIES FROM FEATHER MEAL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. อภิชาติ กาญจนทัต, 42 หน้า.

งานวิจัยนี้มีจุดประสงค์เพื่อหาค่าที่เหมาะที่สุดในการเตรียมโปรตีนไฮโดรไลเสตจากขนไก่ปั่นด้วยเอนไซม์โปรติเอส จี6 ออกแบบการทดลองโดยใช้วิธีพื้นผิวตอบสนองและการวางแผนส่วนประกอบจุดกลางเพื่อให้ได้ค่าที่เหมาะที่สุดในการเตรียมโปรตีนไฮโดรไลเสตจากขนไก่ปั่น โดยศึกษาปัจจัยที่มีผลต่อการเตรียมโปรตีนไฮโดรไลเสตสามประการคือ อุณหภูมิ (41.59 – 58.41 องศาเซลเซียส) เวลา (0:38 – 7:21 ชั่วโมง) และอัตราส่วนเอนไซม์ต่อสารตั้งต้น (0.8- 9.2) โดยมีระดับการย่อยสลายเป็นปัจจัยตอบสนอง ผลการทดลองพบว่าภาวะที่เหมาะที่สุดในการผลิตโปรตีนไฮโดรไลเสตจากขนไก่ปั่นคือ อุณหภูมิ 55 องศาเซลเซียส เวลานาการบ่ม 4:40 ชั่วโมง และอัตราส่วนเอนไซม์ต่อสารตั้งต้น 7.5 เท่า แบบจำลองพื้นผิวตอบสนองทำนายได้ว่าที่สภาวะนี้จะมีระดับการย่อยสลาย 53.97% จากนั้นแยกขนาดโปรตีนไฮโดรไลเสตที่ผลิตได้จากสภาวะที่เหมาะสมนี้ด้วยวิธีอัลตราฟิวเตรชั่น โดยใช้เมมเบรนขนาด 10, 5, 3 และ 0.65 กิโลดาลตัน และทดสอบฤทธิ์ในการต้านอนุมูลอิสระของ 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) และฤทธิ์ต้านอนุมูลอิสระประจุบวกของ 2,2’-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^{+\bullet}$) พบว่าโปรตีนที่มีน้ำหนักโมเลกุลน้อยกว่า 0.65 มีฤทธิ์ดีที่สุดโดยแสดงค่าความเข้มข้นต่ำสุดที่ยับยั้งได้ร้อยละ 50 (IC_{50}) เท่ากับ 16.96 ± 1.08 ไมโครกรัมต่อมิลลิลิตร และ 1.24 ± 0.56 ไมโครกรัมต่อมิลลิลิตร สำหรับฤทธิ์ในการต้าน $DPPH^{\bullet}$ และ $ABTS^{+\bullet}$ ตามลำดับ จากนั้นนำโปรตีนที่แยกส่วนแล้วนี้ไปทำให้บริสุทธิ์ด้วยเทคนิคโครมาโตกราฟความดันสูง สรุปได้จากกระบานการหาเงื่อนไขที่เหมาะที่สุดสามารถผลิตโปรตีนไฮโดรไลเสตจากขนไก่ปั่นได้โดยมีระดับการย่อยสลายสูง และสามารถนำไปประยุกต์ใช้ในการพัฒนาอุตสาหกรรมยาหรือ ใช้เป็นส่วนผสมในเครื่องสำอาง

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5772143023 : MAJOR BIOTECHNOLOGY
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WALEEPORN PIBULPOL: OPTIMIZATION OF PREPARATION PROTEIN HYDROLYSATE BY PROTEASE G6 USING RESPONSE SURFACE METHODOLOGY AND FREE RADICAL SCAVENGING ACTIVITIES FROM FEATHER MEAL. ADVISOR: ASSOC. PROF. APHICHART KARNCHANATAT, Ph.D., 42 pp.

The aim of this research was to study the optimal condition for chicken feather meal protein hydrolysate production using a microbial protease, Protease G6. The experimentation was conducted using Central Composite Design (CCD) and Response Surface Methodology (RSM). The three independent variables including temperature (°C), time and enzyme-substrate ratio were studied. Optimization process for obtaining high yield of chicken feather meal protein hydrolysate was performed using response surface methodology (RSM) by optimizing a combination of three independent variables namely, temperature (41.59 – 58.41°C), time (0:38 - 7:21 h) and enzyme-substrate ratio (0.8 – 9.2) with degree of hydrolysis (DH) as a response. The optimum hydrolysis conditions were obtained at temperature of 55°C, 4:40 h of incubation time and enzyme-substrate ration is 7.5. RSM generated model predicted that 53.97%of DH could be achieved at these conditions. Protein hydrolysate from optimal condition was separated by ultrafiltration with 10, 5, 3 and 0.65 kDa membranes and further analyzed for 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging activity and 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{+\bullet}$) scavenging activity. Among the fraction, protein with molecular weight less than 0.65 exhibited high activity (IC50 16.96 ± 1.08 μg/ml and 1.24 ± 0.56 μg/ml for $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ scavenging activity, respectively). This separated protein hydrolysate fraction was further purified by RT-HPLC. High yield of DH obtained from the optimization process could produce chicken feather meal protein hydrolysate with good for use as a source of peptide drugs that can be further developed in the pharmaceutical industry or an ingredient in cosmetic products in the global market.

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

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LIST OF ABBREVIATIONS

Abs	Absorbance
ABTS	2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BSA	Bovine serum albumin
CCD	Central composite design
DH	Degree of hydrolysis
DPPH	2,2-diphenyl-1-picrylhydrazyl
et al.	and others
h	Hour
H	hydrogen
H_2O	water
IC_{50}	median inhibitory concentration, 50% maximum inhibition
kDa	kilodalton
mg	milligram
min	minute
mM	millimolar
MW	molecular weight
NaCl	sodium chloride
nm	nanometre
O_2	oxygen
OD	Optical density
rpm	round per minute
RSM	Response surface methodology
UV	ultraviolet
°C	degree Celsius
µl	microlitre
µM	micromolar
/	per
%	percentage
:	ratio

Chapter 1
Introduction

Protein hydrolysis is widely used in food industry to modify physiochemical properties of protein, improve the nutritional quality of protein and produce bioactive peptide (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012). Protein hydrolysis involves the cleavage of peptide bonds and breakdown of protein into amino acid and short peptides by using acid, alkali or enzyme (Neklyudov, Ivankin, & Berdutina, 2000). Enzymatic protein hydrolysis using commercial food grade enzymes is more desirable and consistent due to the better control in term of hydrolysates properties (Šližyte, Daukšas, Falch, Storrø, & Rustad, 2005). Protein hydrolysate have been widely applied as ingredient in various industries, including pharmaceutical, nutraceuticals, cosmetic or animal nutrition. Several protein hydrolysates possess a range of dynamic physical, chemical, and functional properties. Moreover, bioactive peptide derived from a variety of protein source have been developed and applied as functional foods and nutraceuticals due to their biological properties such as antioxidant, antimicrobial activities and inhibition of angiotensin-I-converting enzyme (Jimeno, Faircloth, Sousa-Faro, Scheuer, & Rinehart, 2004; Mayer et al., 2005; Wijesekara & Kim, 2010).

Generally, there are several controlled variables during the hydrolysis process, such as temperature, time, pH level and enzyme concentration (See, Hoo, & Babji, 2011). To obtain the optimum hydrolysis conditions with the targeted responses, such as yield and degree of hydrolysis, optimization should be performed. Response surface methodology (RSM) is the one of most effective tools for optimizing the process when many factors and interactions affect the desired response (Molla & Hovannisyan, 2011). The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions. It normally uses an experimental design such as a central composite design (CCD) to fit a first- or second- order polynomial by least significant technique. The contour plots can be usefully employed to study the response surfaces and locate the optimum. One of the reasons for using RSM in the determination of hydrolysis conditions is that it

generates a mathematical model that accurately describes the overall processes with significant estimation ability (Wasswa, Tang, & Gu, 2008).

Chicken feather meal is a byproduct of the poultry industry. Due to its low price, traditional culture and religious reasons, Poultry is the main protein source in South East Asia. In 2015, 1.2 million metric tons of chicken were produced in Thailand. Approximately 70% of production was domestic consumption, whereas 30% was exported. Moreover, domestic consumption of chicken meat is expected to increase by 4-5% in 2017, from around 670 million tons in 2016 (*Factsheet poultry sector in Thailand*, 2016). Feathers represent 5-7% of body weight of living bird or chicken. Millions of tons of feather are generated every year. If they are land filled or burnt, these feathers may prove detrimental to the environment. Chicken feather meal is rich in the protein content of keratin. However, these proteins are non-consumable due to the absence of enzyme keratinase in human and also in animal body. Some enzymes from microorganisms can digest and break down keratin in feathers into small peptides. These peptides are interesting as new source of antioxidant peptide.

The objective of this study was to apply RSM for optimizing the production of chicken feather meals hydrolysates using a microbial protease, G6. We speculate whether chicken feather meal can serve as a new source of protein hydrolysate, which may be suitable for use as a source of peptide drugs that can be further developed in the pharmaceutical industry or as an ingredient in cosmetic products in the global market.

Chapter 2

Literature review

2.1 Free Radical

Free radicals are molecules with unpaired electrons in their atomic orbital. They were unstable and highly reactive. In human body, free radicals are produced from cellular respiration and other metabolism. Free radical also damage biomolecule such as carbohydrates, lipids, protein and DNA. Damage in macromolecule leads to cell damage and homeostatic disruption (Lobo, Patil, Phatak, & Chandra, 2010). The most two important radicals in living body are reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Dröge, 2002)

Oxygen is the terminal oxidant in aerobic respiration which gains 18 ATP of energy more than anaerobic respiration. However, oxygen present in cells also leads to redox reaction that damages other critical molecules including carbohydrate lipid proteins and DNA. ROS may be expressed in various form such as super superoxide ($^{\bullet}O_2^-$), hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$), nitric oxide radicals ($^{\bullet}NO$), nitrogen dioxide ($^{\bullet}NO_2$), and peroxynitrite ($^{\bullet}ONOO^-$). Because of their benefits, ROS may be generated deliberately by cells. A low concentration of ROS plays a useful role in defence against inflection reagent, in cellular signaling and in induction of a mitogenic response. ROS is mainly generated from the electron leakage from the electron transport chain in mitochondria (Datta, Sinha, & Chattopadhyay, 2000; Dröge, 2002; Valko et al., 2007).

The Nitric oxide radical ($NO^{\bullet}$) is generated by specific nitric oxide synthases (NOSs), which metabolize arginine to citrulline with the formation of $NO^{\bullet}$ *via* a five electron oxidative reaction. $NO^{\bullet}$ acts as an important signaling molecule in physiological processes, including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation (Valko et al., 2007).

2.2 Antioxidant

An antioxidant is a molecule that can donate an electron to a free radical and change this free radical to take a stable from. The redox reaction reduces oxidative stresses and reduces harmful from free radical molecules. Many antioxidants are found in nature such as ascorbic acid, flavonoid, curcumin, among other.

2.2.1 Antioxidant protein and peptide

Several studies have demonstrated the ability of proteins to inhibit lipid oxidation in food. Proteins originating from milk, blood plasma, and soy protein all have been shown to exhibit antioxidant activity in muscle foods (Elias, Kellerby, & Decker, 2008). Many protein antioxidant mechanisms are dependent on amino acids composition (e.g. metal chelation, free radical scavenging, hydroperoxide reduction, aldehyde adduction). However, the antioxidant activity of these amino acids residues is limited by the tertiary structure of the polypeptide, since many amino acids with antioxidant potential can be buried within the protein core where they are inaccessible to prooxidants (Elias et al., 2008).

2.3 Antioxidant determination

2.3.1 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) assay

DPPH$^{\bullet}$ scavenging assay is the oldest indirect method for determining antioxidant activity. It was firstly suggested in1950s originally to discover H-donors in natural materials. Later, the test was quantified to determine the antioxidant potential of both individual phenolics and food as well as of biologically relevant samples.

The DPPH radical is one of the few stable organic nitrogen radicals and is deep purple in colour. In DPPH assay, the antioxidants are able to reduce the stable radical DPPH to the yellow coloured diphenyl-picrylhydrazine. This method is based on the reduction of DPPH in alcoholic solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H in the reaction. This assay is based on the measurement of the reducing ability of

antioxidants toward DPPH$^{\bullet}$. This ability can be evaluated by measuring the decrease in its absorbance. It is a stable free radical showing a maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donor substrate such as an antioxidant, the radicals are scavenged and the absorbance is reduced (Gulcin, 2012).

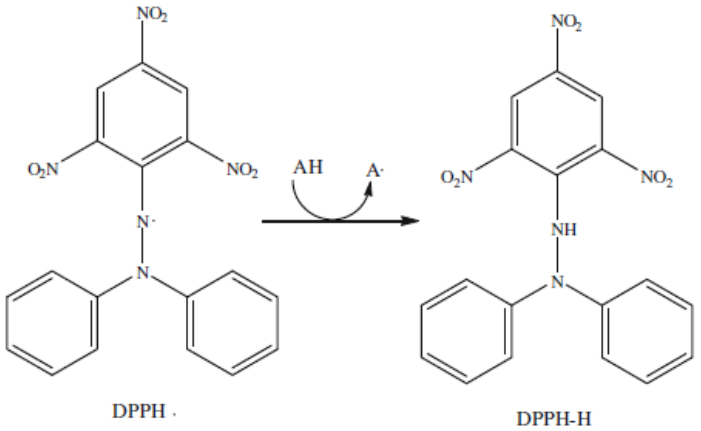


Figure 2.1 DPPH radicals scavenging effect by an antioxidant (AH) (Gulcin, 2012)

2.3.2 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{+\bullet}$) assay

In this assay, ABTS is oxidized by oxidants to its radical cation, $ABTS^{+\bullet}$, which is intensely coloured, and antioxidant capacity is measured as the ability of test compounds to decrease the colour reacting directly with the ABTS radical. $ABTS^{+\bullet}$ is applicable for both lipophilic and hydrophilic compounds.

Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to measure the total antioxidant activity of pure substances, aqueous mixtures and beverages. A more appropriate format for the assay is a decolourization technique in which the radical is generated directly in a stable form prior to the reaction with putative antioxidants. $ABTS^{+\bullet}$ has absorption maxima in aqueous media (414, 734 and 815 nm) and in ethanolic media (414, 730 and 873) (Gulcin, 2012)

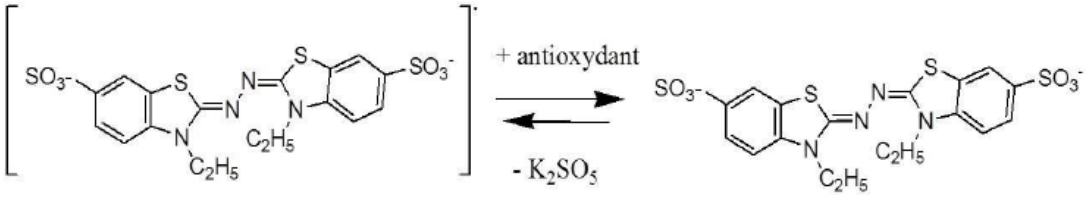


Figure 2.2 Reaction of the ABTS radical in the presence of the antioxidant compound during the ABTS assay. (El Rayess, Barbar, Wilson, & Bouajila, 2014)

2.4 Protein Hydrolysate

Protein hydrolysate is derived from digesting and breaking down proteins or polypeptides into short peptides or free amino acids using a chemical reagent or enzyme. Digestion using chemical reagent is less costly, however, it is hard to control and has limited usage in pharmacy and food. Protein hydrolysate from chemical digestion is inconsistent in quality. On the other hand, digestion of protein by enzyme can control the quality of the product because proteases digest protein at the polypeptide bond. Hydrolysed proteins have higher solubility and changed character due to their group being more carboxylic.

Table 2.1 Common used in producing protein hydrolysate

Enzyme	Type of enzyme	Condition	
		pH	Temperature (°C)
Alacase®	Alkaline serine endopeptidase	6.5-8.5	60
Papain	cysteine protease	6.0-7.0	65
Trypsin	a serine protease that specifically cleaves between lysine (K) and arginine (R) residues	7.5-8.5	37
Protease G6	Alkaline serine endopeptidase	7.0-10.0	55-70
Flavourzyme®	Endoprotease/exopeptidase	5.5-7.5	50-55

2.5 Degree of hydrolysis

Degree of hydrolysis is defined as the percentage of cleavage peptide bond from total peptide bond (Adler-Nissen, 1979). It is a key parameter in understanding and interpreting the effects and extent of hydrolytic process of proteins and is useful to establish the relationships between proteolysis and the improvement of the functional, bioactive and sensory properties of these biomolecules. Several methods of monitoring the DH during protein hydrolysis have been proposed in the literature. These methods are based on four principles: the protons release after peptide bond breaking, the changes in the freezing point of the protein solution by osmometry, released soluble nitrogen content and determining the free amino acid released (Morais et al., 2013; Nielsen, Petersen, & Dambmann, 2001).

Two well-known methods of determining the DH base on measuring the free amino acids released are the trinitro-benzene-sulfonic acid (TNBS) method and the o-phthaldialdehyde (OPA) method. In the first, the TBNS reagent reacts with primary amino groups to form coloured trinitrophenyl-amino acid derivatives which can determined by measuring the absorbance at 340 nm. The OPA reaction consists of two steps: (1) the OPA reagent reacts with the thiol group in the reaction buffer, and (2) the OPA-thiol intermediate reacts with the amino group of proteins to form 1-alkylthio-2-alkyl substituted isoindoles which also can be quantified by measuring their absorbance at 340 nm (Mulcahy, Fargier-Lagrange, Mulvihill, & O'Mahony, 2017). In comparison, the OPA method is more rapid, more stable, and less toxic than the TBNS method (Nielsen et al., 2001).

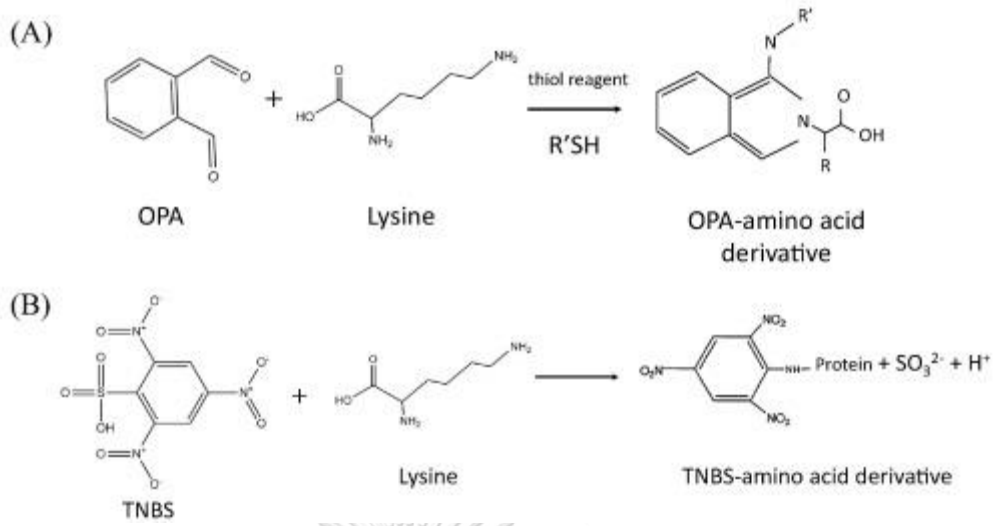


Figure 2.3 The reaction of (A) *ortho*-phthaldialdehyde (OPA) and (B) trinitro-benzene-sulfonic acid (TNBS) with the amino acid lysine.

2.6 Response Surface Methodology (RSM)

RSM is a useful statistical technique for optimization first present by Box and Wison in 1951. This technique studies relationship between independent variable and response to find maximum or minimum response value. The purpose of considering a model is threefold (Khuri & Mukhopadhyay, 2010):

1) To establish a relationship between y and $x_1, x_2, \ldots, x_k$ that can be used to predict response values for given settings of the control variables.
2) To determine, through hypothesis testing, significance of the factors whose levels are represented by $x_1, x_2, \ldots, x_k$
3) To determine the optimum settings of $x_1, x_2, \ldots, x_k$ that result in the maximum (or minimum) response over a certain region of interest.

If each independent variable does not have an interaction effect, the regression equation is called the first-order model.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_i x_i + \varepsilon \qquad (1)$$

If interaction effect between each independent variable exists, the secondary-order model will show.

$$y = \beta_0 + {}_{i=1}\Sigma^{k}\, \beta_i x_i + {}_{i=1}\Sigma^{k}\, \beta_i x_{ii}^{2} + {}_{i<}\Sigma_{j=1}\Sigma^{k}\, \beta_{ij} x_i x_j + \varepsilon \qquad (2)$$

The general scenario is as follows. The response is a quantitative continuous variable (e.g., yield, purity, cost), and the mean response is a smooth but unknown function of the levels of *p* factors (e.g., temperature, pressure), and the levels are real valued and accurately controllable. The mean response, when plotted as a function of the treatment combinations, is a surface in p+1 dimensions, called the *response surface* (Dean, Voss, & Draguljić, 2017). Figure 2.1 shows a response surface for two factors.

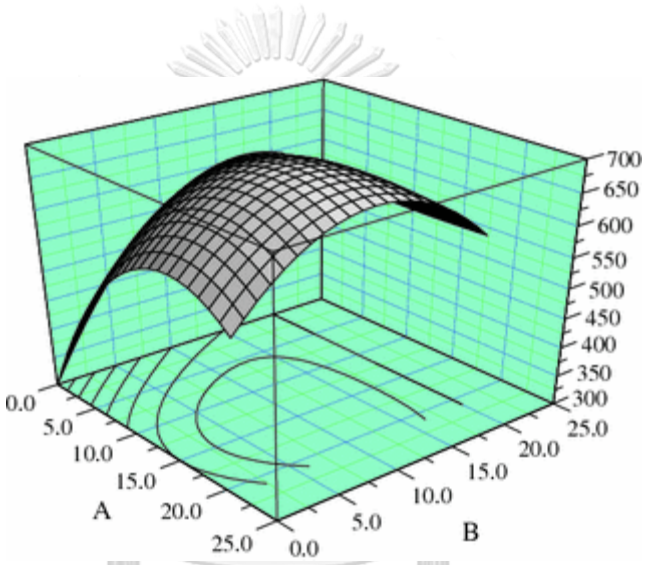


Figure 2.4 Response surface for two factors

2.6.1 Central composite design (CCD)

Central composite design (Box & Wilson, 1951) is one of the most common experimental designs for estimating a full second-order polynomial regression model for optimization. CCD requires a minimum number of three parts of experiments for modeling: a two level full or fractional factorial, axial points, and center points.

The number of all experiments (n) can be indicated from follow equation:

$$n = 2^k + 2k + m \qquad (3)$$

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 number of axial

runs is 2k. Central composite design according in the three factors is shown in Table 2.2

Table 2.2 Central composite design in three factors

	factor		
	1	2	3
Full factorial	+1	+1	+1
	+1	+1	-1
	+1	-1	+1
	+1	-1	-1
	-1	+1	+1
	-1	+1	-1
	-1	-1	+1
	-1	-1	-1
Axial points	+α	0	0
	-α	0	0
	0	+α	0
	0	-α	0
	0	0	+α
	0	0	-α
Center points	0	0	0
	0	0	0
	0	0	0
	0	0	0

The value of α can be calculated, $\alpha = 2^{k/4}$. The values of α for 2-5 independent variables are shown in table 2.3.

Table 2.3 value of α for 2-5 independent variables

Number of independent variable (k)	2	3	4	5
Number of experiment	9	15	25	43
Value of α	1.4142	1.6818	2	2.3784

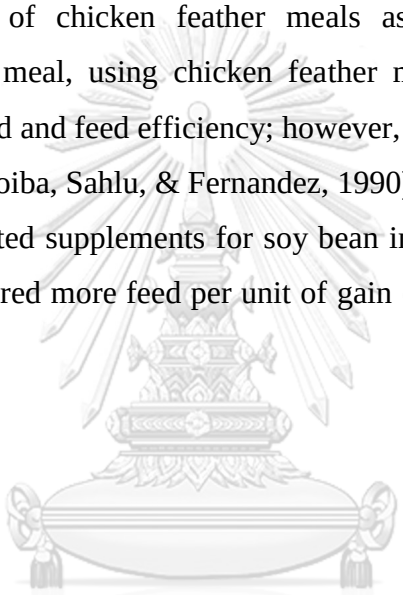
RSM is commonly used for studies concerning the optimization of protein hydrolysate preparation. Some previous studies are summarized and reported in table 2.4.

Table 2.4 Optimization of protein hydrolysis using RSM

Substrate	Enzyme	Parameter	DH	Reference
Fish waste	Papain	Enzyme concentration Temp Time	83.83%	(พีริยา ไรเกษ, โอรส รักชาติ, เหรียญทอง สิงห์จานุสงค์, & ปวีนา น้อยทัพ, 2554)
Visceral waste proteins of Catla	Alacase®	Time Temperature pH Enzyme/substrate	50%	(Bhaskar, Benila, Radha, & Lalitha, 2008)
muscle of grass carp	Papain	Enzyme substrate ratio Time temperature	29.86%	(Ren et al., 2008)
Shrimp waste	Alacase®	Temperature pH Enzyme/substrate ratio Time	33.13%	(Dey & Dora, 2014)
Bovine plasma protein	Alacase®	Temperature Time pH	20.73%	(Seo et al., 2015)
Porcine liver	Alacase®	Enzyme/substarte pH temperature	24.12%	(Yu & Tan, 2017)
sea urchin gonad	Papain	Temperature pH Enzyme/substrate ratio Enzyme concentration	27.69%	(Zhou et al., 2012)
Common Cuttlefish	Trypsin	Time Temperature Enzyme activity	21%	(Kechaou, Bergé, Jaouen, & Ben Amar, 2013)

2.7 Chicken Feather Meals

Feather is a major waste from poultry industry since they are approximately 5-7% of living chicken body weight (Brandelli, Sala, & Kalil, 2015). Feather can be an important protein source of livestock because of their high protein content of more than 80%. However, feather in nature state are poorly digested in most animal. A lot of researches are focus on hydrolysis feather to obtain more digestible protein (El Boushy, van der Poel, & Walraven, 1990; Latshaw, Musharaf, & Retrum, 1994; Pedersen, Yu, Plumstead, & Dalasgaard, 2012). Several research studied have investigated the used of chicken feather meals as a supplement in livestock. Compared to soybean meal, using chicken feather meal as a supplement gives a similar rate of milk yield and feed efficiency; however, it also gives a lower content of milk protein (Lu, Potchoiba, Sahlu, & Fernandez, 1990). Feather meals and hair meals can be used as substituted supplements for soy bean in growing-finishing beef cattle with on effect but required more feed per unit of gain (Wray, Beeson, Perry, Mohler, & Baugh, 1979).



Chapter 3
Methodology

3.1 Preparation of protein hydrolysate

The chicken feather meal was provided by Betagro Group. It was sieved through a 150 µm sieve plate. Sieved feather meal was hydrolysed with protease G6 in various enzyme-substrate ratio, temperature, and time. A central composite design (CCD) with three factors was applied as shown in table 3.1. Degree of hydrolysis (DH) was selected as the response of independent variable.

Table 3.1 Independent factors and their coded and actual levels used in RSM studies for optimizing hydrolysis conditions using protease G6

Variables	Code level				
	-α	-1	0	+1	+α
Enzyme-substrate ratio (v/w): X_1	0.8	2.5	5.0	7.5	9.4
Temperature (°C): X_2	41.6	45	50	55	58.4
Time (h): X_3	0:38	2:00	4:00	6:00	7:21

3.2 degree of hydrolysis

Degree of hydrolysis was determined using method described (Nielsen et al., 2001). This method based on an absorbance shift of o-phthaldaldehyde (OPA) after reaction with free amino acid.

OPA solution was prepared by dissolved 7.620 g di-Na-tetraborate and 200 mg sodium-dodecyl-sulfate (SDS) in 150 ml deionized water, mixing the solution with 4 ml of ethanol that contained 160 mg o-phthaldaldehyde (OPA), adding 176 mg of dithiothreitol (DTT), finally, deionized water was added to 200 ml.

To determined degree of hydrolysate, 400 µl of sample was mixed with with 3 ml OPA solution. After incubated in room temperature for 2 min, absorbance at 340

nm of the mixture was determined. 100 mg/L serine was used as standard. Degree of hydrolysate was calculated using equation (1):

$$\text{Degree of hydrolysate} = h/h_{total} \times 100\% \quad (1)$$

Where h is the digested peptide bonds and h_{total} is total peptide bonds in protein. The digested peptide (h) was calculated using equation (2):

$$h = \text{serine-}NH_2 - \beta/\alpha \quad (2)$$

Where β and α are constant depended on each protein. The serine-NH_2 was calculated using equation (3):

$$\text{serine-}NH_2 = (OD_{sample} - OD_{blank}) / (OD_{standard} - OD_{blank}) \times 0.9516 \times 0.1 \times 100/X \times P \quad (3)$$

Where OD_{sample} is an absorbance at 340 nm of the mixture sample and OPA solution. OD_{blank} is an absorbance at 340 of mixture water and OPA solution. $OD_{standard}$ is an absorbance of mixture serine and OPA solution. X is mass of protein sample in gram. P is percentage of protein in sample.

3.3 Determination of protein concentration

Concentration of protein hydrolysate was determined by Bradford's assay. 60 µl of protein hydrolysate were mixed with 600 µl of Bradford working buffer. Bovine serum albumin was used as the standard for constructing calibration curve. This assay is based on an absorbance shift of Coomassie Brillant Blue G-250 after binding to protein.

3.4 Determination of antioxidant activities

3.4.1 DPPH radical scavenging activities

DPPH radical scavenging activities were determined using method described. Following the reaction between antioxidants and DPPH radicals, the DPPH radicals changed form to DPPH. The activities can be determined based on the absorbance shift of DPPH after reduced. In this research, 80 µl of protein solution in various

concentrations was mixed with 320 µl of DPPH radical solution. After incubated in dark for 30 min, the mixture was centrifuged at 10,000 rpm for 3 min. Absorbance at 517 nm was measured. The %inhibition and IC50 were calculated.

3.4.2 ABTS radical scavenging activities

ABTS radicals solution was generated by mixing 7 mM ABTS with 2.45 mM potassium persulfate in equal volume and kept in dark for 12 h. The solution was diluted to obtain 0.7 ± 02 units of an absorbance at 734 nm.

The ABTS radical scavenging activities were determined by mixing 10 µl of protein solution in various conditions and 450 µl of ABTS radical solution. After being kept in the dark for 15 min, absorbance at 734 nm was measured. The %inhibition and IC_{50} were calculated.

The percentage of radical scavenging was calculated as follow:

$$((Abs_{control} - Abs_{blank}) - (Abs_{sample} - Abs_{background})) \times 100 / (Abs_{control} - Abs_{blank})$$

Where Abscontrol is the absorbance of the control (no sample), Abssample is the absorbance of the chicken feather meals hydrolysate,. Absbackground is the absorbance of the sample, and Absblank is the absorbance of deionized water.

3.5 isolation and purification

3.5.1 Ultrafiltration

Protein hydrolysate was separated by ultrafiltration through semipermeable membranes that can separate protein by their molecular weight at 10, 5, 3, and 0.65 kDa. Each protein molecular weight cut off was collected and determined antioxidant activities.

3.5.2 reverse-phase high-performance liquid chromatography (RP-HPLC)

The protein hydrolysates from ultrafiltration were purification using reversed-phased high-performance liquid chromatography (RT-HPLC) on a Luna C18 column (4.6 mm × 250 mm). The peptide was eluted with rational gradient of mobile phase B

(0.1% trifluoroacetic acid, TFA) and mobile phase C (70% acetonitrile, ACN, in 0.05% trifluoroacetic acid) at flow rate of 0.7 ml/min. The protein peaks eluted were monitored at 280 nm.

3.7 Statistical analysis

Statistical analyzes in this experiment were done for triplicate and results were shown as 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 experiment

Before the optimum condition of preparing protein hydrolysate from chicken feather meal using RSM was studied, some preliminary experiments with different level of enzyme, substrate, enzyme-substrate ratio and time were tried (Table 4.1 – 4.2). Each condition was selected from center point and axial point of CCD. The first run was assumed as center point. Run number 2 and 3 were selected as enzyme level was fixed and substrate level was changed. Run number 4 and 5 were the experiment that changed both enzyme level and substrate level. Run number 6 and 7 were selected as substrate level was fixed and enzyme level was changed. Finally, run number 8 to 12 were tested the effect of incubation time.

The highest DH was from run number 3 with enzyme-substrate ratio is 7.69 and follow by run number 4 and number 6 with enzyme-substrate ratio is 5.50 and 6.13, respectively. Statistical analysis showed that, there was no significant different between degree of hydrolysis of run number 3, 4 and 6 (Table 4.1). The optimum enzyme-substrate ratio was expected to be between 5.50 and 7.69. The enzyme-substrate ratio of 0.8, 2.5, 5.0, 7.5 and 9.2 that selected for RSM are cover the expected area.

In comparison of different hydrolysis time, run 12 that 5 h of incubation time reveal highest degree of hydrolysis. Degree of hydrolysis of run 11 with incubation time 4 h and run 10 with incubation time 3 h were slightly lower than that of run 12 (table 4.2). Statistical analysis showed that, there was no significant different between degree of hydrolysis of run number 10, 11 and 12. The optimum hydrolysis time was expected to be between 3 and 6 h. The hydrolysis time of 0:38, 2:00, 4:00, 6:00 and 7:21 h that selected for RSM are cover the expected area.

Table 4.1 Preliminary experiment with different enzyme-substrate ratio and their DH

Run	Enzyme (ml)	Substrate (g)	E/S (w/v)	Time (h)	DH
1	1.00	0.30	3.33	2	22.6 ± 1.85^{c}
2	1.00	0.47	2.13	2	$17.52 \pm 1.91^{b,c}$
3	1.00	0.13	7.69	2	45.75 ± 12.23^{d}
4	1.21	0.22	5.50	2	44.24 ± 2.49^{d}
5	0.43	0.39	1.10	2	$10.66 \pm 1.88^{a,b}$
6	1.84	0.30	6.13	2	39.06 ± 2.77^{d}
7	0.15	0.30	0.50	2	4.65 ± 1.57^{a}

[a], [b], [c], [d] Statistic analysis by SPSS variance (ANOVA) with post hoc comparison (one-way) by using Duncan's multiple range test. The same of superscripts alphabet are not significantly different at p-value < 0.05.

Table 4.2 Preliminary experiments with different hydrolysis time and their DH

Run	Enzyme (ml)	Substrate (g)	E/S (w/v)	Time (h)	DH
8	1	0.3	3.33	1	15.26 ± 0.66^{a}
9	1	0.3	3.33	2	18.31 ± 0.56^{a}
10	1	0.3	3.33	3	23.19 ± 3.03^{b}
11	1	0.3	3.33	4	23.94 ± 1.77^{b}
12	1	0.3	3.33	5	26.86 ± 0.90^{b}

[a], [b] Statistic analysis by SPSS variance (ANOVA) with post hoc comparison (one-way) by using Duncan's multiple range test. The same of superscripts alphabet are not significantly different at p-value < 0.05.

4.1 Optimization of protein hydrolysis

The influent of temperature (X_1), hydrolysis time (X_2), and enzyme-substrate ratio (X_3) on the hydrolysis by protease G6 was determined as mention earlier. The observed values for degree of hydrolysis at different combinations of the independent variables are presented in Table 4.3.

The highest degree of hydrolysis (53.69%) was reveal in run 8 and the lowest degree of hydrolysis (10.40)

Table 4.3 Actual levels of independent variables along with the observed values for the response variable, degree of hydrolysis (DH)

Run	Code level			Actual levels			Response
	Temperature (°C)	Time (h)	E/S (v/w)	Temperature (°C)	Time (h)	E/S (v/w)	DH
	X_1	X_2	X_3	X_1	X_2	X_3	Y
1	-1	-1	-1	45.00	2:00	2.50	16.79
2	+1	-1	-1	55.00	2:00	2.50	26.59
3	-1	+1	-1	45.00	6:00	2.50	31.11
4	+1	+1	-1	55.00	6:00	2.50	31.49
5	-1	-1	+1	45.00	2:00	7.50	43.55
6	-1	-1	+1	55.00	2:00	7.50	50.01
7	-1	+1	+1	45.00	6:00	7.50	52.15
8	+1	+1	+1	55.00	6:00	7.50	53.69
9	-α	0	0	41.60	4:00	5.00	35.77
10	+α	0	0	58.40	4:00	5.00	44.05
11	0	-α	0	50.00	0:38	5.00	19.19
12	0	+α	0	50.00	7:21	5.00	42.74
13	0	0	-α	50.00	4:00	0.80	10.40
14	0	0	+α	50.00	4:00	9.20	51.55
15	0	0	0	50.00	4:00	5.00	41.50
16	0	0	0	50.00	4:00	5.00	40.08
17	0	0	0	50.00	4:00	5.00	44.57
18	0	0	0	50.00	4:00	5.00	40.65
19	0	0	0	50.00	4:00	5.00	47.29
20	0	0	0	50.00	4:00	5.00	43.48

An overall second polynomial equation by multiple regression analysis was developed as given below:

$$Y = - 91.65860 + 1.54193 X_1 + 18.88240 X_2 + 11.69970 X_3 - 0.179289 X_1X_2 - 0.021831 X_1X_3 - 0.173538 X_2X_3 - 0.002454 X_1^2 - 0.805879 X_2^2 - 0.515067 X_3^2$$

Where, Y, X_1, X_2 and X_3 are DH, temperature (°C), time (h) and enzyme-substrate ratio (g), respectively. The results were analyzed by using analysis of variance (ANOVA) with statistically significant checked by F-test as shown in Table 4.3.

In table 4.3, the meaning of each column was explained:

Source:	A meaningful name for the rows.
Sum of square:	Sum of the squared differences between the overall average and the amount of variation explained by that rows source.
df:	Degrees of Freedom: The number of estimated parameters used to compute the source's sum of squares.
Mean Square:	The sum of squares divided by the degrees of freedom. Also called variance.
F-value:	Test for comparing the source's mean square to the residual mean square.
p-value:	The probability of finding the observed, or more extreme, results when the null hypothesis (H0) of a study question is true. If the p-value is very small (less than 0.05 by default) then the source has tested significant.

And each row was explained:

Model:	The model row shows how much variation in the response is explained by the model along with the over-all model test for significance.
Residual:	The residual row shows how much variation in the response is still unexplained.

Lack of Fit:	The amount the model predictions miss the observations.
Pure Error:	The amount of difference between replicate runs.
Cor Total:	The amount of variation around the mean of the observations. The model explains part of it, the residual explains the rest.

The independent variables time and enzyme-substrate ratio had strongly significant effect on degree of hydrolysis (p-value ≤ 0.01). The independent variable temperature also had significant effect on degree of hydrolysis (p-value ≤ 0.05), but its effect was less than that of time and enzyme-substrate ratio. The interactions between the different factors did not significantly influence hydrolysis (p-value ≥ 0.1).

This response surface quadratic model is significant with an F-value of 20.67. There is only a 0.01% chance that an F-value this large could occur due to noise. The Lack of Fit F-value of 2.87 implies the Lack of Fit is not significant relative to the pure error. There is a 13.58% chance that a Lack of Fit F-value this large could occur due to noise.

The similar result was shown in previous study (Mokrejs, Svoboda, Hrncirik, Janacova, & Vasek, 2011), in which poultry feathers were processed through a two-stage alkaline-enzymatic hydrolysis. In the first stage, feathers were mixed with a 0.1 or 0.3% KOH water solution in a 1:50 ratio and were incubated at 70°C for 24 h. After adjusting pH to 9, the effects of proteolytic enzyme additions (1–5%), time (4–8 h) and temperature (50–70°C) were studied in second stage processing. Level of proteolytic enzyme additions and time were significant effect on the percentage of degraded feathers in environment of both 0.1 and 0.3 KOH. The hydrolysis time is significant effect in environment of 0.1 KOH but is not significant effect in environment of 0.3 KOH. The interactions between the different factors did not significant influence hydrolysis.

Table 4.4 Statistic analysis for the response surface quadratic model obtained from RSM design

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2690.74	9	298.97	20.67	< 0.0001	significant
X_1-temp	75.49	1	75.49	5.22	0.0454	
X_2-time	370.20	1	370.20	25.59	0.0005	
X_3-E/S	1936.66	1	1936.66	133.89	< 0.0001	
X_1X_2	25.72	1	25.72	1.78	0.2120	
X_1X_3	0.5957	1	0.5957	0.0412	0.8432	
X_2X_3	6.02	1	6.02	0.4164	0.5333	
X_1^2	0.0542	1	0.0542	0.0038	0.9524	
X_2^2	149.75	1	149.75	10.35	0.0092	
X_3^2	149.34	1	149.34	10.32	0.0093	
Residual	144.65	10	14.46			
Lack of Fit	107.30	5	21.46	2.87	0.1358	not significant
Pure Error	37.35	5	7.47			
Cor Total	2835.39	19				

As shown in table 4.5, the total determination coefficient, $R^2 = 0.9490$, implies that the regression models explained the reaction well. Adjusted R^2 was 0.9031, which indicates that the model explains 90.32% of the variation in the data, and that the experiment error was very small. However, the Predicted R^2 of 0.6934 was not as close to the adjusted R^2 of 0.9031 as one might normally expect. This may indicate a large block effect or a possible problem with model or data.

The model was considered adequate with an adequate precision value measuring the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 14.894 indicates an adequate signal. This model can be used to navigate the design space.

Table 4.5 Fit Statistic analysis for model

Std. Dev.	3.80	R^2	0.9490
Mean	38.33	Adjusted R^2	0.9031
C.V. %	9.92	Predicted R^2	0.6934
		Adeq Precision	14.8942

The normal probability plot, Figure 4.1, show the distribution of residual values - defined as the difference between the predicted and observed - forms a straight line, and residual values are normally distributed on both sides of line indicating that the experiment points are reasonably aligned with the predicted value.

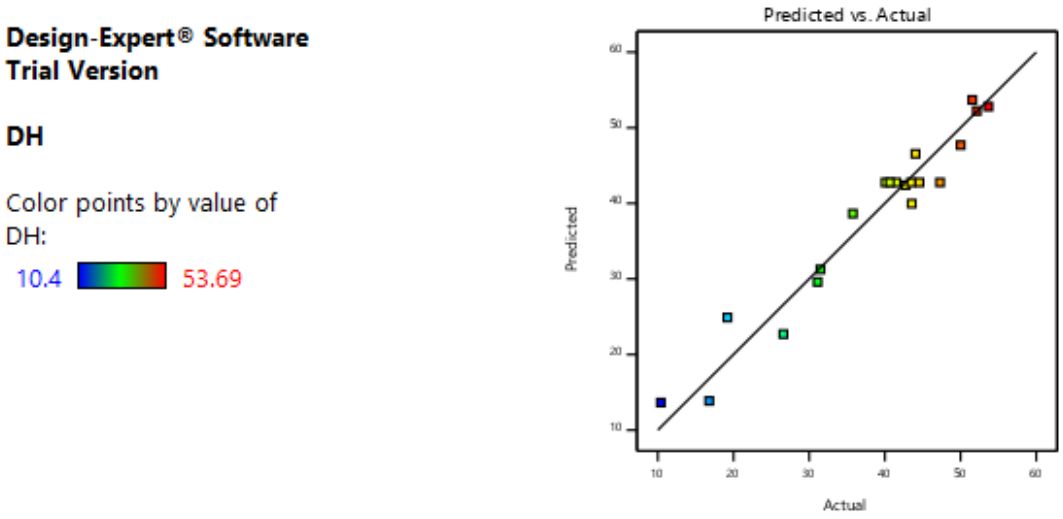


Figure 4.1 Relationship between the observed and predicted values of the degree of hydrolysis.

The prediction value of degree of hydrolysis based on temperature, time, and enzyme-substrate ratio was illustrated (figure 4.2, 4.3 and 4.4) for studying the interactions among the three factors in order to determine the optimum condition for maximum degree of hydrolysis.

After overall analysis it was found that optimum degree of hydrolysis occurred at 55°C, 4:40 h of incubation time and enzyme-substrate ration is 7.5, and the prediction value of degree of hydrolysis was 53.97%. To confirm the validity of statistical strategies, three additional verification experiments at optimum condition were conducted. The error of the response was calculated between the observed and predicted values. The observed value of degree of hydrolysis at optimum condition is 50.96%, so, the error of the response was 5.58%.

These results demonstrated that the model were suitable for hydrolysis of feather meals using protease G6. The condition of 55°C, 4:40 h of incubation time and 7.5 enzyme-substrate ratios were chosen as optimal conditions owing to the highest degree of hydrolysis.

This model indicates a short hydrolysis time compare to previous study (Kshetri et al., 2017), which showed the optimum condition for preparing feather protein hydrolysate using a multifaceted keratinolytic bacterium *Chryseobacterium sediminis* RCM-SSR-7 was 5% (w/v) feather concentration, pH 7.5, 30°C and 84 h incubation time.

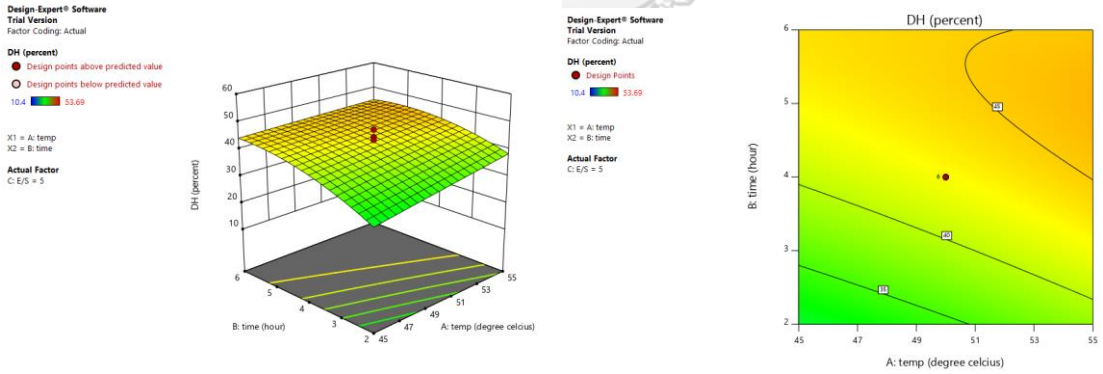


Figure 4.2 The 3D response surface graph and the contour plot for the effect of temperature (A) and time (B) on the degree of hydrolysis during hydrolysis of chicken feather meals with protease G6.

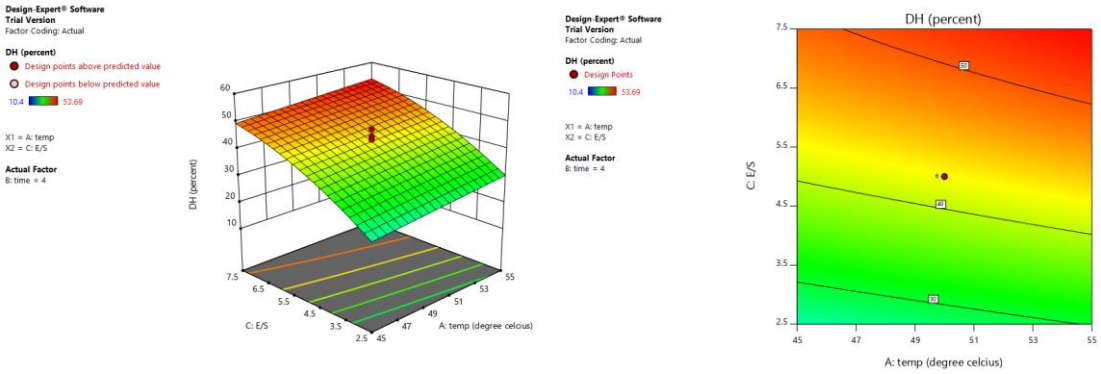


Figure 4.3 The 3D response surface graph and the contour plot for the effect of temperature (A) and enzyme and substrate ratio (C) on the degree of hydrolysis during hydrolysis of chicken feather meals with protease G6.

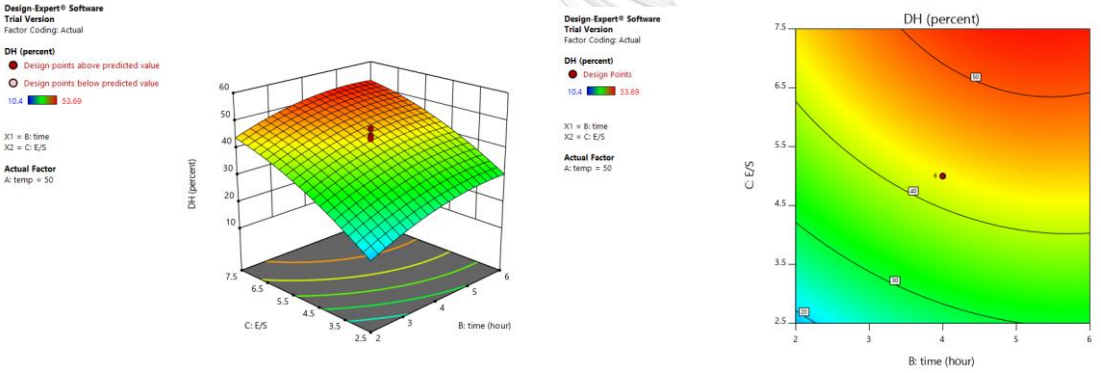


Figure 4.4 The 3D response surface graph and the contour plot for the effect of time (A) and enzyme and substrate ratio (C) on the degree of hydrolysis during hydrolysis of chicken feather meals with protease G6.

4.2 The antioxidant activity of protein hydrolysate

The protein concentration of each hydrolysates samples were determined using Bradfrod's assay. The activities to scavenging the DPPH radical were investigated. As the results shown in table 4.6, the DPPH radical scavenging activities fluctuated with the changes in the degree of hydrolysis values.

Table 4.6 Protein concentration and DPPH radical scavenging activities.

Run	Temperature (°C)	Time (h)	E/S (v/w)	degree of hydrolysis	Protein concentration (µg/ml)	%inhibition of DPPH
1	45.00	2:00	2.50	16.79	181.52	49.66
2	55.00	2:00	2.50	26.59	200.13	38.16
3	45.00	6:00	2.50	31.11	198.84	31.02
4	55.00	6:00	2.50	31.49	190.39	38.92
5	45.00	2:00	7.50	43.55	170.39	57.62
6	55.00	2:00	7.50	50.01	175.14	48.02
7	45.00	6:00	7.50	52.15	163.98	40.55
8	55.00	6:00	7.50	53.69	169.17	51.06
9	41.60	4:00	5.00	35.77	154.04	53.24
10	58.40	4:00	5.00	44.05	176.13	51.41
11	50.00	0:38	5.00	19.19	139.32	24.59
12	50.00	7:21	5.00	42.74	138.03	20.81
13	50.00	4:00	0.80	10.40	177.68	59.63
14	50.00	4:00	9.20	51.55	153.30	33.07
15	50.00	4:00	5.00	41.50	194.78	51.01
16	50.00	4:00	5.00	40.08	197.56	43.46
17	50.00	4:00	5.00	44.57	201.03	44.63
18	50.00	4:00	5.00	40.65	196.72	47.66
19	50.00	4:00	5.00	47.29	199.24	44.36
20	50.00	4:00	5.00	43.48	201.30	42.24
Optimum	55.00	4:40	7.50	50.96	203.18	54.52

An overall second polynomial equation by multiple regression analysis was developed as given below:

$$Y_2 = 472.29605 - 15.42094\ X_1 - 12.19572\ X_2 - 4.66589\ X_3 + 0.493875\ X_1X_2 + 0.045100\ X_1X_3 + 0.096250\ X_2X_3 + 0.131355\ X_1^2 - 1.79754\ X_2^2 + 0.187422\ X_3^2$$

Where, Y_2, X_1, X_2 and X_3 are %inhibition, temperature (°C), time (h) and enzyme-substrate ratio (g), respectively. The 3D response surface graph and the contour plot of the effect of the independent variables on percent inhibition of protein hydrolysate against DPPH radical were showed in figure 4.5 - 4.7. However, the regression equation and the graph plot are not reliable since the model F-value of 2.16 implies the model is not significant relative to the noise and the Lack of Fit F-value of 12.15 implies the Lack of Fit is significant. That mean there is a 12.37% chance that an F-value this large could occur due to noise and only a 0.80% chance that a Lack of Fit F-value this large could occur due to noise (Table 4.7).

Although hydrolysates with high degree of hydrolysis, which containing more low-molecular-weight peptides, should have higher antioxidant activity, theoretically, similar result was report earlier (Chabeaud, Dutournie, Guerard, Vandanjon, & Bourseau, 2009; Yu & Tan, 2017).

Sarmadi and ismail (Sarmadi & Ismail, 2010) posited that overall antioxidant activity was more affected by the integrative of many factors, such as operational conditions, protease type, DH, specific hydrolysate amino acid sequence, peptide structure, and peptide concentration, more than the individual action of peptides or amino acid. Moreover, the enzyme specificity affects the amount and composition of free amino acid and peptides as well as their amino acid sequence, subsequently influencing the molecular size, hydrophobicity, and antioxidant activity of the hydrolysates. Protein hydrolysate from optimum condition exhibited high antioxidant activity at 54.52%. This value is high enough to warrant further study.

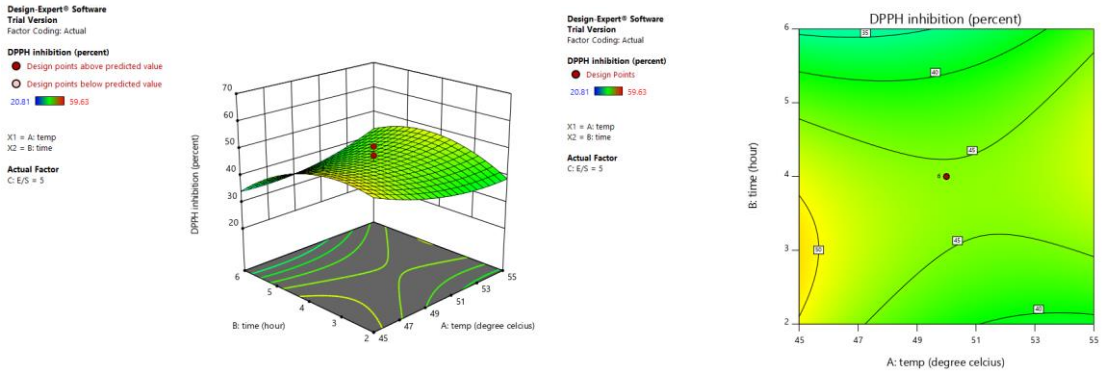


Figure 4.5 The 3D response surface graph and the contour plot for the effect of temperature (A) and time (B) on the %inhibition against DPPH radicals of the protein hydrolysate.

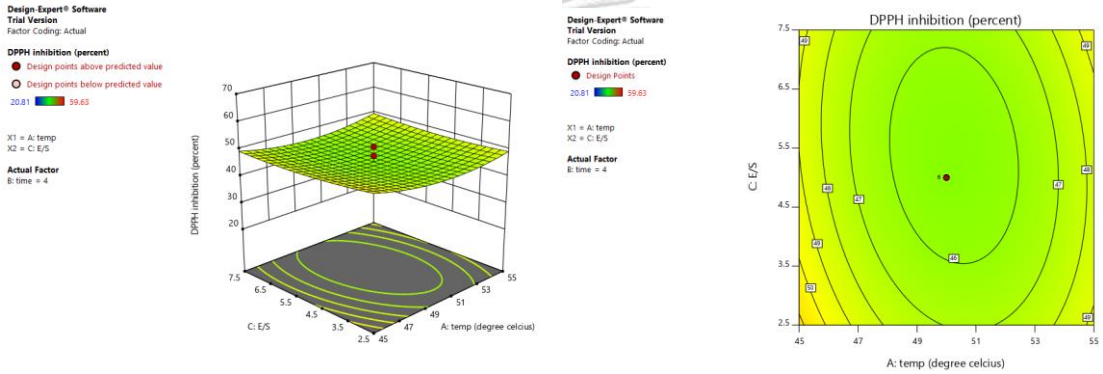


Figure 4.6 The 3D response surface graph and the contour plot for the effect of temperature (A) and enzyme-substrate ratio (C) on the %inhibition against DPPH radicals of the protein hydrolysate.

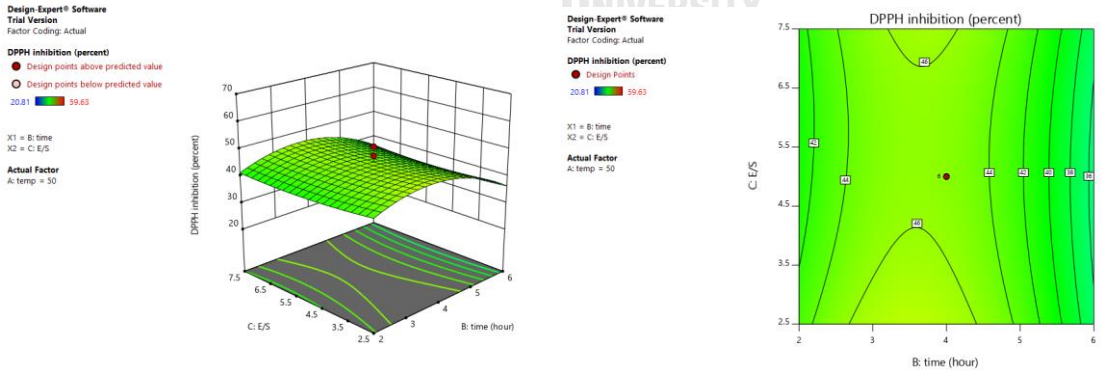


Figure 4.7 The 3D response surface graph and the contour plot for the effect of time (B) and enzyme-substrate ratio (C) on the %inhibition against DPPH radicals of the protein hydrolysate.

Table 4.7 Statistic analysis for the response surface quadratic model obtained from RSM design

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1322.90	9	146.99	2.16	0.1237	not significant
X_1-temp	2.44	1	2.44	0.0357	0.8539	
X_2-time	107.23	1	107.23	1.57	0.2384	
X_3-E/S	1.96	1	1.96	0.0288	0.8686	
X_1X_2	195.13	1	195.13	2.86	0.1216	
X_1X_3	2.54	1	2.54	0.0373	0.8508	
X_2X_3	1.85	1	1.85	0.0272	0.8724	
X_1^2	155.41	1	155.41	2.28	0.1621	
X_2^2	745.04	1	745.04	10.92	0.0079	
X_3^2	19.77	1	19.77	0.2899	0.6020	
Residual	682.01	10	68.20			
Lack of Fit	630.17	5	126.03	12.15	0.0080	significant
Pure Error	51.85	5	10.37			
Cor Total	2004.92	19				

Protein hydrolysate from optimum condition was selected for further investigates. It was separated by ultrafiltration through semipermeable membranes that can separate protein by their molecular weight at 10, 5, 3, and 0.65 kDa. Each protein molecular weight cut off was collected and determined antioxidant activities. The DPPH radical scavenging activities and ABTS radical cation scavenging activities of each molecular weight cut off protein were shown in table 4.8.

The result showed that the smaller peptide has higher antioxidant activities. The lowest IC_{50} of DPPH radical scavenging activities and ABTS radical cation scavenging activities (16.96 ± 1.08 and 1.24 ± 0.56, respectively) were found in the peptide with a molecular weight size smaller than 0.65 kDa. This result concurs with the previous results reported (Ajibola, Fashakin, Fagbemi, & Aluko, 2011), which

showed that low molecular weight peptide fractions had higher DPPH radicals scavenging activities than high molecular weight peptides.

All fractions showed higher activities to scavenge ABTS radical cation than DPPH radical. This result may due to Tyr/Trp-containing dipeptides in protein hydrolysate since Tyr/Trp-containing dipeptides exhibited very weak DPPH scavenging activities but showed very strong ABTS radical cation activities (Zheng, Lin, Su, Zhao, & Zhao, 2015).

Table 4.8 Antioxidant activities of each molecular weight cut off protein.

	DPPH	ABTS
	IC_{50} (µg protein/ml)	IC_{50} (µg protein/ml)
crude	157.60 ± 17.55	31.04 ± 13.66
larger than 10 kDa	125.04 ± 28.88^{c}	24.06 ± 1.49^{D}
5-10 kDa	53.79 ± 3.78^{b}	10.94 ± 1.11^{C}
3-5 kDa	46.59 ± 3.38^{b}	6.94 ± 1.55^{B}
0.65-3 kDa	21.27 ± 3.25^{a}	2.70 ± 0.37^{A}
smaller than 0.65 kDa	16.96 ± 1.08^{a}	1.24 ± 0.56^{A}
ascorbic acid	15.28 ± 1.81	25.75 ± 0.81

^{a}, ^{b}, ^{c}, ^{A}, ^{B}, ^{C}, ^{D} Statistic analysis by SPSS variance (ANOVA) with post hoc comparison (one-way) by using Duncan's multiple range test. The same of superscripts alphabet are not significantly different at p-value < 0.05.

4.3 Reverse-phase high-performance liquid chromatography (RP-HPLC)

Protein hydrolysate with molecular weight less than 0.65 kDa was further purified using reverse-phase high-performance liquid chromatography (RP-HPLC) on Luna C_{18} column. The elution peaks were monitored at 280 nm. The result was shown in figure 4.8.

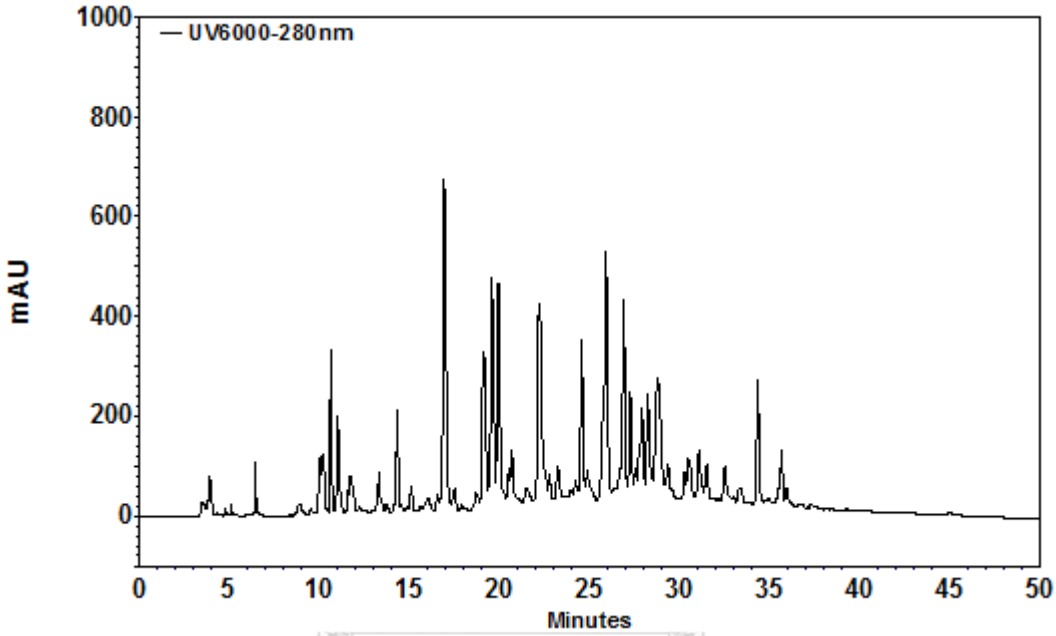
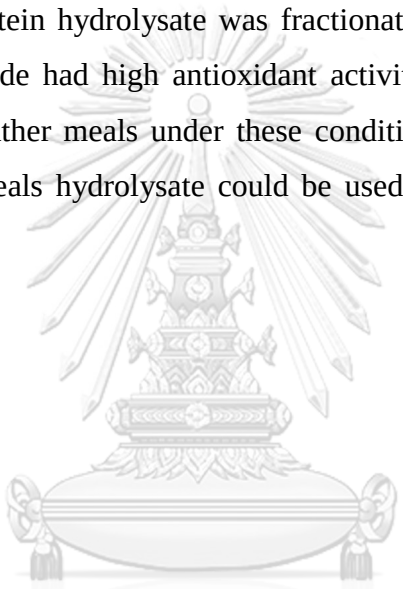


Figure 4.8 reverse-phase high-performance liquid chromatography (RP-HPLC) of Protein hydrolysate with molecular weight less than 0.65 kDa

Chapter 5
Conclusion

Chicken feather meals can be hydrolyzed for more antioxidant activity. To obtain highest degree of hydrolysis, the response surface methodology was applied. As the results showed that the optimal condition of preparing protein hydrolysate from chicken feather meals was 55°C, 4:40 h of incubation time and a 7.5 enzyme-substrate ratio. The prediction value of the degree of hydrolysis at optimal condition was 53.97%. After protein hydrolysate was fractionated by ultrafiltration, the small molecular weight peptide had high antioxidant activity. Therefore, we successfully hydrolyzed chicken feather meals under these conditions. These result also suggest that chicken feather meals hydrolysate could be used as source of new antioxidant peptide.



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APPENDIX

Appendix A

Buffer and reagents preparation

20 mM Phosphate buffer, pH 7.2 (1000 mL)

20 mM KH_2PO_4	2.721 g
20 mM K_2HPO_4	3.484 g
150 mM NaCl	8.766 g

Dissolve in 1000 mL deionized water and adjust pH to 7.2 by 6 N NaOH

OPA solution

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	Transfer 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.

Bradford solution

Bradford stock solution

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

Bradford working buffer

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

Bradford working buffer was kept in a brown glass bottle at room temperature and filtered through the Whatman No.1 paper before used.

DPPH solution

100 µM DPPH (MW = 394.32) 0.004 g
Dissolve in 100 mL Methanol

ABTS solution

Solution A: 7 mM ABTS (MW = 548.68) 0.096 g
Dissolve in 25 mL deionized water

Solution B: 2.45 mM potassium persulfate (MW = 270.32) 0.0166 g
Dissolve in 25 mL deionized water

Mix solution A and solution B and keep on dark for 12 hours before using and dilute with deionized water to obtain an absorbance value of 0.7 ± 0.02 at 734

Mobile phase in RT-HPLC analysis

Eluent A: 0.1% trifluoroacetic acid (TFA)

Double deionized water 999 ml

TFA 1 ml

Filtration through a cellulose acetate membrane

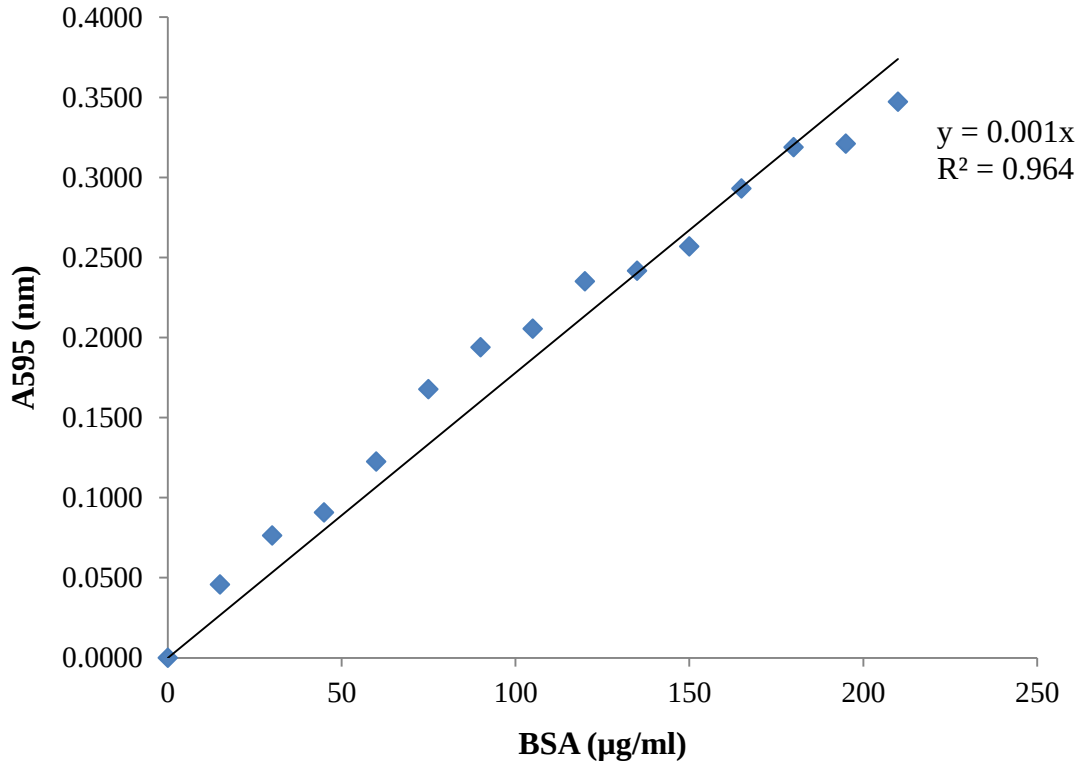
Eluent B: 70% acetonitrile containing 0.05% TFA

Step 1: Filter 350 ml acetronitrile through PTFE membrane

Step 2: Add 75 µl of TFA in 150 ml double deionized water. Then, filter through a cellulose acetate membrane. Add this solution into filtrate acetonitrile.

Appendix B

Standard curve of protein determination by Bradford method



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Pibulpol, W., Sangtanoo, P., Chimpibul, W., and Karnchanatat, A. Optimization of Preparing Protein Hydrolysate from Chicken Feather Meal by Protease G6 using Response Surface Methodology (RSM). In "The International Conference on Food and Applied Bioscience" 1-2 February 2018, The Empress Chiang Mai Hotel, Chiang Mai, Thailand (Proceeding book)