ฤทธิ์ต้านการอักเสบของเพปไทด์จากขนไก่ปีนในเซลล์เพาะเลี้ยงแมคโครฟาจ RAW 264.7

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ANTI-INFLAMMATORY ACTIVITY OF PEPTIDE FROM CHICKEN FEATHER MEAL IN MACROPHAGE RAW 264.7

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รมย์ธีรา สุขบูรณ์ : ฤทธิ์ต้านการอักเสบของเพปไทด์จากขนไก่ป่นในเซลล์เพาะเลี้ยงแมคโครฟาจ RAW 264.7 (ANTI-INFLAMMATORY ACTIVITY OF PEPTIDE FROM CHICKEN FEATHER MEAL IN MACROPHAGE RAW 264.7) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.อภิชาติ กาญจนทัต, 69 หน้า.

กระบวนการอักเสบเป็นกระบวนตอบสนองของระบบภูมิคุ้มกันในร่างกายที่สำคัญต่อสิ่งกระตุ้นหรือสารที่อันตราย ซึ่งทำให้เกิดการเปลี่ยนแปลงของหลอดเลือด และยังนำไปสู่โรคต่างๆ เช่น โรคมะเร็ง โรคอัลไซเมอร์ และ โรคหัวใจ เป็นต้น อย่างไรก็ตามในปัจจุบันงานวิจัยที่เกี่ยวข้องกับการศึกษากลไกการออกฤทธิ์ต้านการอักเสบของเพปไทด์ยังมีไม่มากนัก ดังนั้นการศึกษาฤทธิ์ต้านการอักเสบของเพปไทด์จากธรรมชาติจึงเป็นสิ่งที่น่าสนใจ โดยงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาสภาวะที่เหมาะสมในการเตรียมเพปไทด์จากขนไก่ป่นที่มีฤทธิ์ต้านการอักเสบในเซลล์ Macrophage RAW 264.7 โดยใช้เอนไซม์โปรติเอสจากจุลินทรีย์ (อัลคาเลส ฟลาโวไซม์ และนิวเตรส) ความเข้มข้นตั้งแต่ 1% 2.5% และ 5% แล้วทำการทดสอบฤทธิ์ขจัดอนุมูลอิสระด้วยวิธีไนตริกออกไซด์ จากผลการศึกษาพบว่าเพปไทด์ที่ได้จากการย่อยด้วยเอนไซม์ฟลาโวไซม์เข้มข้น 1% แสดงค่าการยับยั้งการสร้างอนุมูลอิสระด้วยวิธีไนตริกออกไซด์ได้ดีที่สุดเมื่อเทียบกับเอนไซม์ตัวอื่นๆ โดยแสดงค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งได้ร้อยละ 50 (IC_{50}) เท่ากับ 5.48± 1.0 µg/ml คัดแยกเพปไทด์ตามขนาดโมเลกุลด้วยเทคนิคอัลตราฟิลเตรชัน 10 5 3 และ 0.65 กิโลดาลตันตามลำดับ พบว่าเพปไทด์ที่มีขนาดโมเลกุลต่ำกว่า 0.65 กิโลดาลตัน มีฤทธิ์ในการขจัดอนุมูลอิสระไนตริกออกไซด์ได้ดีที่สุด แล้วทำบริสุทธิ์โดยเทคนิคโครมาโตกราฟี และทำการตรวจสอบความเป็นพิษของเพปไทด์จากขนไก่ป่นด้วยวิธี MTT Assay และตรวจสอบผลการยับยั้งการสร้างไนตริกออกไซด์ในเซลล์ Macrophage 264.7 ที่ถูกกระตุ้นด้วยสารไลโปโพลิแซคคาไรด์ พบว่าเพปไทด์จากขนไก่ป่นไม่มีความเป็นพิษต่อเซลล์และมีฤทธิ์ในการยับยั้งการสร้างไนตริกออกไซด์ จากนั้นนำเพปไทด์ไปทำบริสุทธิ์ด้วยเทคนิคโครมาโตกราฟีของเหลวสมรรถนะสูงและนำเพปไทด์ที่ได้ไปพิสูจน์เอกลักษณ์ด้วยเทคนิคแมสสเปกโตรเมตรีพบเพปไทด์ทั้งหมด 5 สาย นอกจากนี้เพปไทด์จากขนไก่ป่นยังได้ทำการศึกษาผลการแสดงออกของยีนที่เกี่ยวของกับการอักเสบ พบว่าเพปไทด์จากขนไก่ป่นมีฤทธิ์ในการยับยั้งการแสดงออกของยีน inducible nitric oxide synthase (iNOS) Cyclooxygenase-2 (COX-2) Interleukin-6 (IL-6) และ Tumor necrosis factor alpha (TNF-α) ภายในเซลล์แมคโครฟาจ RAW 264.7 ด้วย จากผลการศึกษาดังกล่าวแสดงให้เห็นว่าฤทธิ์ต้านการอักเสบของเพปไทด์จากขนไก่ป่นสามารถนำไปประยุกต์ใช้ในอุตสาหกรรมทางการแพทย์ เภสัชกรรมและผลิตภัณฑ์เครื่องสำอางต่อไป

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ROMTEERA SUKABOON: ANTI-INFLAMMATORY ACTIVITY OF PEPTIDE FROM CHICKEN FEATHER MEAL IN MACROPHAGE RAW 264.7. ADVISOR: ASSOC. PROF. APHICHART KARNCHANATAT, Ph.D., 69 pp.

Inflammation is a defense mechanism in the body induced by foreign chemicals or pathogens. Thus, inflammation is one of the most critical factors implicated in vasodilation, carcinogenesis and other degenerative disorders. However, the study of the anti-inflammatory mechanism of peptides has been limited. So studies on anti-inflammatory peptides from natural sources are increasing in interest. The objective of this study, to investigate the optimal condition of enzymatic hydrolysis to prepare an anti-inflammatory peptide from chicken feather meal by using 3 types of microbial protease (Alcalase®, Flavourzyme®, and Neutrase®) in various concentrations (1% 2.5% and 5%). Peptide hydrolysate with 1% Flavourzyme® produced the best results for inhibition of NO production (IC_{50} 5.48±1.0 µg/ml) when compared with other proteases. These peptide fractions were fractionated by ultrafiltration with 10, 5, 3 and 0.65 kDa membranes and further analyzed for NO scavenging activity. Among the fractions, MW ≤ 0.65 kDa exhibited a high level of antioxidant activity toward the NO radical scavenging assay. The ≤ 0.65 kDa fractions were selected for further fractionation by gel filtration chromatography. The F2 fraction was the strongest in inhibiting nitric oxide production. After that, F2 fraction was selected to study the NO production and cytotoxicity test by MTT assay in LPS-stimulated macrophage RAW 264.7. The result shows that the peptide also inhibited NO production and wasn't toxic to macrophage RAW 264.7. The peptide fraction was further purified by HPLC and identification by mass spectrometry with amino acid sequence characterized into 4 peptides. In addition, the peptides are suppressed in the expression mRNA of iNOS, COX-2, IL-6 and TNF-α which are responsible for pro-inflammatory cytokine production. The results indicate that bioactive peptide from chicken feather meal can be developed to medical, pharmaceutical and cosmetic products.

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LIST OF ABBREVIATIONS

Abs	Absorbance
bp	Base pair
cDNA	Cpmplementary DNA
CO_2	Carbon dioxide
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle Medium
eNOS	Endothelial nitric oxide synthase
et al	and others
FBS	Fetal bovine serum
h	Hour
IC_{50}	Median inhibitory concentration, 50% maximum inhibition
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
kDa	Kilodalton
LPS	Lipopolysaccharide
mg	Milligram
min	Minutes
ml	Milliliter
mM	Millimolar
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NFκB	Nuclear factor-kappa B
ng	Nanogram

nm	Nanometer
nNOS	Neuron nitric oxide synthase
NO·	Nitric oxide
O2·-	Superoxide anion
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
psi	Pound per square inch
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Round per minute
RT	Reverse transcription
s	second
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TNF-α	Tumor necrosis factor alpha
UV	Ultraviolet
α	Alpha
β	Beta
ºC	Degree Celsius
γ	Gamma
µg	Microgram
µl	Microliter
µM	Micromolar
/	Per

%	Percentage
:	Ratio

CHAPTER I
INTRODUCTION

Inflammation is a host defense mechanisms which involves physiological and pathological process within an organism and which is induce by the invasion of pathogens or tissue injury caused by biological, chemical or physical damage (Je J. Y. and S. K. Kim, 2012). The activation of several immune cells (monocytes and macrophages) produces inflammation mediators such as nitric oxide (NO), cyclooxygenase-2 (COX-2), prostaglandins E_2 (PGE_2) and other pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β (Berenbaum, 2000; Fujiwara and Kobayashi, 2005; Je J.-Y. and S.-K. Kim, 2012; Lawrence *et al.*, 2002). The macrophage is a major participant which play an important role in the inflammation mechanism and which is initiated by bacterial lipopolysaccharide (LPS) that is thought to originate from outer membrane of gram-negative bacteria, IFN-γ and pro-inflammatory cytokines (Guha and Mackman, 2001). In addition, oxidative stress is involved in the pathogenesis of several health problems. The physical and biochemical process in human body may generate free radical as by-products which can help trigger a wide range of diseases (Reddy and Reddanna, 2009). The interaction between reactive nitrogen species (RNS) such as nitric oxide and reactive oxygen species (ROS) interaction toxic agents and is strongly linked with inflammation and cancer (Lee *et al.*, 2013). However, excessive of inflammatory mediators contribute to acute and chronic disease characterized by the uncontrolled production of pro-inflammatory cytokines such as cancer, coronary heart disease, rheumatoid arthritis, asthma and Alzheimer's (Dia *et al.*, 2009). During the inflammation process, inflammatory mediators such as NO and PGE_2 are produced via the oxidation of L-arginine by inducible nitric oxide synthase (iNOS) and the conversion of arachidonic acid by cyclooxygenase (COX-2). Moreover, NO is a key signaling biological molecule involved in the vasodilation and regulation of blood pressure neurotransmission and host immune defense (de Mejia and Dia, 2009). The production of pro-inflammatory cytokine and nitric oxide are closely correlated

to inflammation. Inflammation can be regulated by suppressing pro-inflammatory cytokine and nitric oxide production (Lee S.-J. *et al.*, 2012).

Protein hydrolysate may be derived from a purified protein source through heating with chemical or proteolytic enzyme into peptides. This study focused on enzymatic hydrolysis to prepare protein hydrolysate from chicken feather meal. The production of protein hydrolysate is a sequential action between endopeptidase and exopeptidase (Clemente, 2000; Manninen, 2009). The benefits of enzymatic hydrolysis include that it is not consumed during reaction, it has a high conversion yield, high selectivity, high specificity, soft condition, is nontoxic, and can be produced a large-scale production (Kristinsson and Rasco, 2000). There are several factors that influence in enzymatic hydrolysate such as pH, incubation time, temperature, enzyme and substrate (Salwanee *et al.*, 2013). In general, peptides consist of a complex mixture of free amino acid around 3-20 residues in each sequence and bioactivity is characteristic of the amino acid composition and sequence (Pihlanto-Leppälä, 2000). Moreover, the sequences of amino acid within peptide of their native proteins usually take on an inactive bioactive role while specific enzymatic hydrolysis under controlled condition can be enhance the potential bioactivity of peptide (Sarmadi and Ismail, 2010).

Currently, natural compounds with biological activity are increasing. The requirement of functional food or dietary supplements has increased significantly in the health improvements of the human body (Cian *et al.*, 2012). Peptides are known for being highly selective with excellent safety and efficacy among humans (Fosgerau and Hoffmann, 2015). Recently, there has been much pharmaceutical research addressing the utilization and focus on peptides from plants or animals due to their different health benefits and biological activities as sources of bioactive peptides for drug development (Vo *et al.*, 2013). Bioactive peptide has been found to possess anticancer, antimicrobial, hypocholesterolemic, antihyperterolemic, antihypertensive, antithrombotic and anti-inflammatory properties (Ahn *et al.*, 2012).

Poultry processing plants and the related industry is expanding in many countries, especially in Thailand. Arising from this, chicken feather meal is a major waste by-product and produced in large amounts annually (Gang *et al.*, 2013). Chicken feather meal represents around 5-7% of chicken body weight. However, the substantial accumulation of chicken feather meal may cause environmental problems and pollutions (Nomura *et al.*, 2005). Chicken feather meal is also an alternative value- added product used as animal feed, organic fertilizer and feed supplement due to its content of approximately 90% protein, as well as its disulphide bonds and cross-linkages (Tiwary and Gupta, 2013). The structure is mainly composed of pure keratin and important amino acids like cysteine arginine glycine and phenylalanine (Kumar *et al.*, 2012). Therefore, the objective of this study was to prepare peptides from chicken feather meal by enzymatic hydrolysis using microbial protease and to determine anti-inflammatory activity in the macrophage MACROPHAGE RAW 264.7 . Therefore, the bioactive peptide from chicken feather meal can also be further formulated for peptide drug in the pharmaceutical industry or as ingredient in cosmetic products in the global market.

CHAPTER II
LITURATURE REVIEW

2.1 Free radicals

Free radicals are any chemical species such as atom or molecule which contain one or several unpaired electron in its outer shell. This unpaired electron also pulse radicals unstable and highly reactive. The radicals can react with both of radical and non-radical molecules to create chain reaction by donate its unpaired electron to a non-radical molecule or seize an electron from another molecule in order to form a paired electron and more damaging species (Lopez-Torres *et al.*, 1991). There are several types of free radicals in living systems. Mostly, radicals are mainly derived from oxygen (reactive oxygen species/ROSs) such as superoxide anion $O_2^{\cdot -}$ and hydroxyl radicals $OH^{\cdot}$. Moreover, they are also derived from nitrogen (reactive nitrogen species/RNSs) such as nitric oxide $NO^{\cdot}$, nitrogen dioxide $NO_2^{\cdot}$ and nitrate radical $NO_3^{\cdot}$ (Table 2.1) (Halliwell, 2001). Free radicals are very short lived, with half-life in milli-, micro-, or nanoseconds, extremely unstable. The presences of free radicals are lead to overpower the radicals scavenging mechanism, oxidative stress and available to react with organic substrate including lipid, DNA and proteins to generate harmful products such as lipid peroxidation and other lipid adducts (Dhawan, 2014). The resulting in protein damage may cause loss of enzyme activity, while DNA damage can lead to mutagenesis and carcinogenesis (Davalos *et al.*, 2004; Wiseman and Halliwell, 1996). There are numerous studies suggested that free radicals are involved in the several human diseases, as well as in ageing, cancer and inflammation. Both of ROS and RNS also generate from either endogenous or exogenous source. For endogenous source, free radicals are generated from immune cell activation, inflammation, mental stress and infection. In contrast, exogenous of free radical mostly from air, water, pollution, cigarette smoke, alcohol and transition metals (Pham-Huy *et al.*, 2008).

Table 2.1 Different type of reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced in cell (Dhawan, 2014)

Reactive species	Free radicals	Formula
Reactive oxygen species (ROS)	Superoxide	$O_2^{\cdot -}$
	Hydroxyl	$OH\cdot$
	Peroxyl	$RO_2\cdot$
	Alkoxyl	$RO\cdot$
	Hydroperoxyl	$HO_2\cdot$
Reactive nitrogen species (RNS)	Nitric oxide	$NO\cdot$
	Nitrogen dioxide	$NO_2\cdot$

However, the link between free radical and inflammation are investigated. One of the chronic inflammatory diseases which involved in oxidative stress is rheumatoid arthritis. The pathological process of this disease is caused from reproduction of ROS and RNS in human body (Kavazis and Powers, 2013).

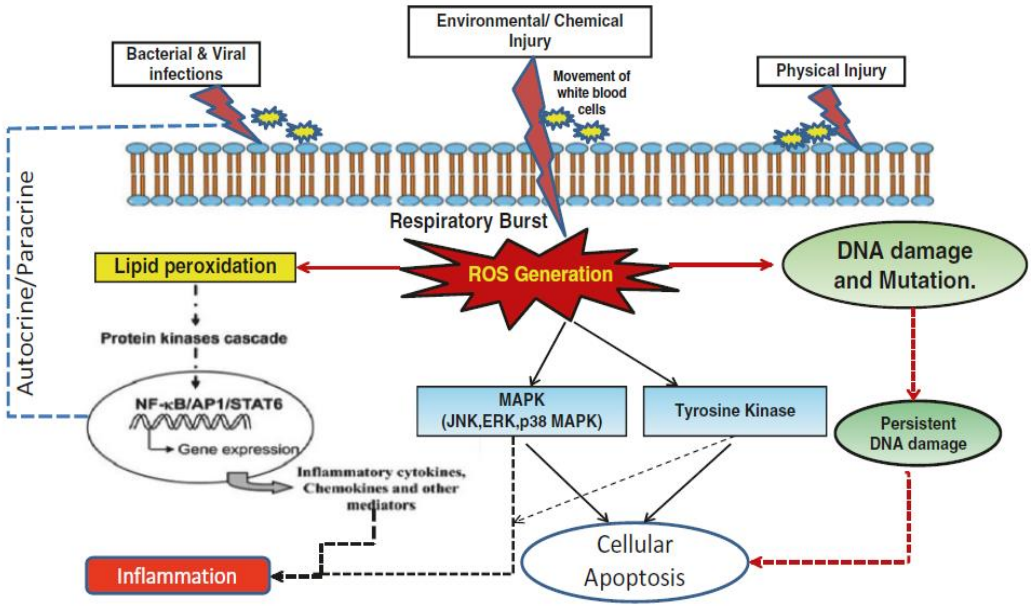


Figure 2.1 Role of ROS and RNS in tissue damage (Dhawan, 2014)

2.2 Inflammation

Inflammation is the complex process of the protective response of the body which is stimulated by microbial infection, chemical and physical damage (Abarikwu, 2014). The causes of inflammation are many and varied but most the common is that from the immune response to an infectious microorganism (Hoebe *et al.*, 2004). Inflammation can lead to several cardinal sign of pain, heat, redness, swelling and loss of function. The purpose of inflammation response is to eliminate the pathogenic insult and remove the injured tissue components. Excessive inflammation processes also lead to various diseases such as cancer, rheumatoid arthritis, artherosclerosis and asthma (Wang A.-Y. *et al.*, 2011). The inflammation mechanism can be classified into acute and chronic inflammation.

Acute inflammation is the immediate and early response to injury designed to deliver leukocytes to the sites of injury. Thus, invading microbes have been removed and necrotic tissues also broken down. During this process, the following events will occur:

2.2.1. Vascular change: revision in blood vessels resulting in increased blood flow (vasodilation) and structural change that plasma and protein leak (increased vascular permeability).

2.2.2 Cellular event: migration of the leukocyte from the microcirculation and accumulation in the focus of injury (cellular recruitment and activation).

Chronic inflammation is immortalized and prolongs the duration (weeks, months and years) in which the active inflammation tissue injury and healing proceed simultaneously. The causes of chronic inflammation are mostly from viral infection, persistent microbial infection, exposure to potentially toxic or chemical agents and autoimmune diseases. There is also tissue destruction which is directed by the inflammatory cell. Chronic inflammation also occurs following infiltration of the mononuclear cell (macrophage, lymphocytes and plasma cells), new vessel proliferation (angiogenesis) and fibrosis (Yu H. *et al.*, 2009).

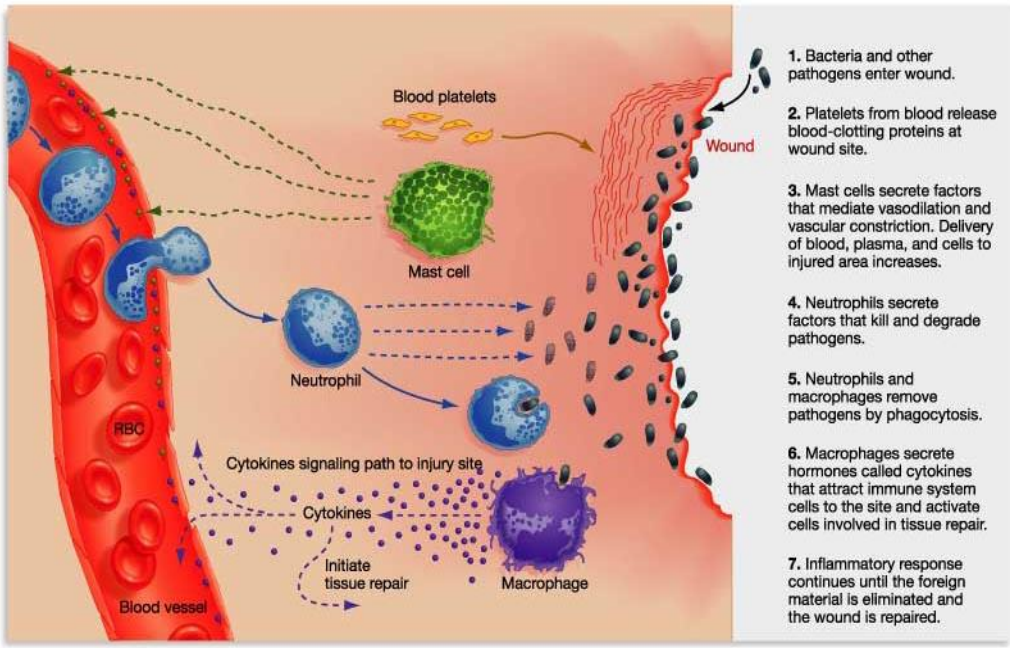


Figure 2.2 Step of inflammatory response against pathogens

2.3 Biological significance of nitric oxide

Nitric oxide is catalyzed by enzyme as nitric oxide synthase (NOS) which are present in the body. The synthesis of NO oxide is generated by conversion of L-arginine to L-citrulline in a highly control process. There are two cofactors have been found to be involved in this process which includes oxygen and NADPH (Omer *et al.*, 2012). In addition, there three isoforms of NOS have been identified as names which basis of their activities, following by neuronal NOS (nNOS), inducible NOS (iNOS) and endotherial NOS (eNOS)(Table 2.2) (Nathan and Xie, 1994). There are many chemical reactions which occur under biological conditions. The chemical reaction involving NO production is described below (Blough and Zafiriou, 1985):

1. Reactivity with oxygen to yield NO_2 gas or NO_2^- in solution:

$$2NO + O_2 \longrightarrow 2NO_2^-$$

2. Reactivity with superoxide anion (O_2^-) to yield the unstable intermediate peroxonitrite anion ^-OONO), which rearranges to form NO_3^-

$$NO + O_2^- \longrightarrow {}^-OONO \longrightarrow NO_3^-$$

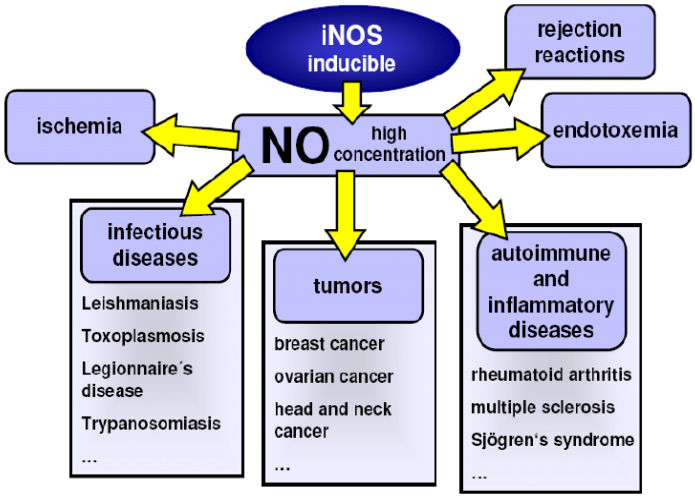


Figure 2.3 Assumption of iNOS to produce NO in various human diseases (Hemmrich *et al.*, 2003).

NO is diatomic free radical and chemically unstable, with extremely short life around 3-5 s in aqueous solution under physiological conditions of concentration, temperatire, pH and oxygen (Bryan and Grisham, 2007). NO is one of the chemical mediators in inflammation of the body which significant for its role inregulating vascular tone and in signaling neurotransmission (Moncada *et al.*, 1991). The high level production of nitric oxide is dependent on induction and expression of the inducible nitric oxide synthase (iNOS) expressed in macrophages. However, overproduction of nitric oxide may lead to circuratory shock, chronic inflammation and carcinogenesis (Fang, 1997). NO also has been implicated in different mechanisms of diseases such as artheroscleosism, asthma, neurologic disorder and septic shock (Barton *et al.*, 1998). The isoform of nitric oxide synthase and their physical functions are summarized in Figure 2.4.

Table 2.2 Classification of mammalian nitric oxide synthase

Isoform[a]	Typical localization	Cellular localization	Monomer molecular mass (kDa)
nNOS	Neuron	Soluble and particulate	-160
eNOS	Endothelial cells	Particulate	-135
iNOS	Macrophage	Soluble	-130

[a]nNOS, neuronal nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS

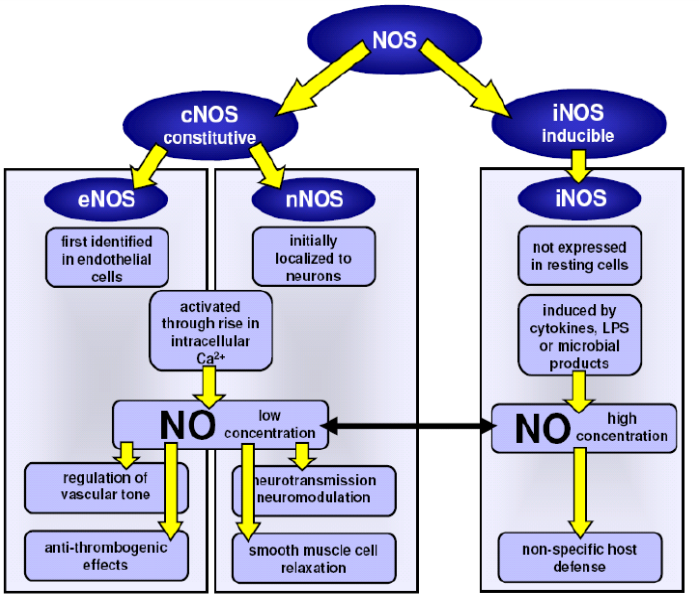


Figure 2.4 The isoform of nitric oxide synthase. Two cNOS enzyme (eNOS, nNOS) are contrasted by third, inducible NOS (iNOS) (Hemmrich *et al.*, 2003).

2.4 Role of nitric oxide physiological function and inflammation

All cells produce both reactive oxygen and nitrogen species (ROS/RNS) such as superoxide anion (O^{-}_{2}), hydrogen peroxide (H_2O_2), NO and peroxynitrite ($ONOO^{-}$) during normal respiration or activated macrophages. Moreover, ROS/RNS are primary members of cellular signaling pathways, particularly those leading to the inducible activation of nuclear factor kappa B (NF-KB). NF-KB is a transcription factor associated with the expression of various inflammatory genes including COX-2 and iNOS. Nitric oxide (NO) is a free radical involved in inflammation that is classified as a reactive nitrogen species. The role of nitric oxide is that of also regulating a variety of diseases and physiological functions of the body including blood pressure, neural signal transmission, and platelet function as well as playing a critical role in the anti-pathogen and tumoricidal response of the immune system (Liu *et al.*, 2008). However, during the inflammation process, in vivo formation of NO is a normal physiological function in the infection of tissues. Increased NO production levels may cause some adverse effects of NO such as the formation of the carcinogenic N-nitroso compound, deamination of DNA bases and mutagenesis, oxidation of DNA bases and tissue injury caused by potent oxidative agents (Liu *et al.*, 2008). Thus, production of NO via iNOS expression is also implicated as a deleterious agent in various pathophysiological conditions and tissue damage (Moncada *et al.*, 1991). Figure 2.9 shows the physiological and pathological roles of NO.

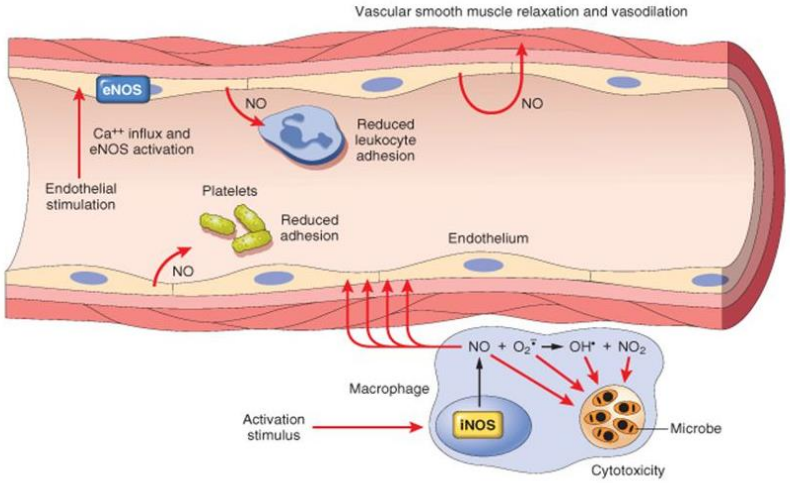


Figure 2.5 Function of nitric oxide (NO) in blood vessel and macrophage, produced by two NO synthase enzyme (eNOS and iNOS). (Kumar *et al.*, 2012)

2.5 Role of cytokines in inflammation process

Inflammation is mediated by a variety of soluble factors such as cytokines. Cytokines play a key role in cellular infiltrate, the state of cellular activation and the systemic response to inflammation. In addition, cytokines are associated with the extensive networks that correlate to synergistic as well as antagonistic interactions. There are several cytokines involved in the inflammatory process such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6).

2.5.1 Tumor necrosis factor alpha (TNF-α)

Tumor necrosis factor (also known as TNF-α) is a polypeptide cytokine related to systemic inflammation and in a member group of cytokines that stimulate the acute phase of inflammation reaction. TNF-α has been implicated in common biological properties and a diverse range of inflammatory infectious. The importance of TNF-α in inflammation is controlling disease activity in rheumatoid arthritis and other inflammatory conditions. TNF-α also induces apoptotic cell death, inhibits tumorigenesis and viral replication. Overproduction of TNF-α has been implicated in human diseases such as cancer (Sprague and Khalil, 2009).

TNF-α is one of the products produced by activated monocyte/macrophage lineage, with T lymphocytes, neutrophils, mast cells and endothelium. Regulation of the transcription factor NF-KB is a key component of TNF-α signal transduction. Physical stimuli such as ultraviolet light, X-radiation and heat are also responsible for TNF-α production and release. Normally, TNF-α is released from preformed storage by cleavage of the membrane TNF-α on the macrophage, neutrophils and activated T cells by a TNF-α converting enzyme (TACE/ADAM17) and the release of cytoplasmic granules from a mast cell and eosinophils (Feldmann and Maini, 2001). Large amounts of TNF-α are released in response to lipopolysaccharides and other bacterial products. Moreover, TNF-α can be induce by the expression of COX-2 which leads to vasodilation and thus increased blood flow.

2.5.2 Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is produced by important immune cells macrophages and monocytes at the inflammation site. IL-6 is a cytokine with redundancy and pleiotropic activity that contributes to the host defense against acute environmental stress. Moreover, the down regulation of IL-6 production leads to a pathological role in various auto immune and chronic inflammatory diseases. When acting on hepatocytes, IL-6 strongly induces broad spectrum acute phase proteins such as C-reactive proteins (CPR) in addition to reducing albumin, cytochrome P450, fibronectin and transferrin (Figure 2.6) CRP is the best biomarker of inflammation and its expression mainly depends on IL-6. IL-6 also promotes the differentiation of

osteoclasts and angiogenesis, the proliferation of keratinocytes and mesangial cells, and the growth of myeloma and plasmacytoma cells (Tanaka *et al.*, 2014)

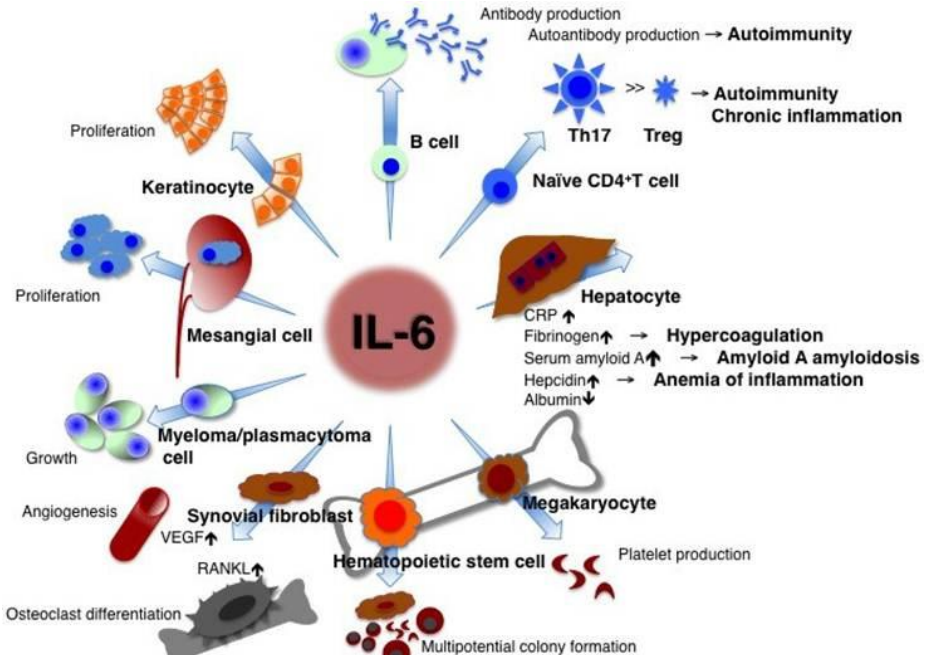


Figure 2.6 IL-6 has a pleiotropic effect which dysregulates persistent production and causes the onset and development of various autoimmune and chronic inflammatory diseases (Tanaka *et al.*, 2014).

2.6 Prostaglandins

Prostaglandin is a product of the COX-2 pathway, generating inflammation by increasing vascular permeability, vasodilation and release of proinflammatory cytokines to the site of injury (Issekutz and Movat, 1982). Moreover, the level of prostaglandins is connected to the setup of chronic inflammation and cancer. Cyclooxygenase production is the first step in the formation of prostaglandins and thromboxane from free arachidonic acid (Kim G.-H., 2008). There are two isoforms: COX-1 and COX-2. COX-1 also constitutively expressed in all cell types and involved in normal kidney, gastrointestinal and reproductive functions (Smith and Langenbach, 2001). COX-2 may be expressed in some tissue, but is largely inducible by a wide variety of mitogens, hormones, cytokines and other stimuli. COX-2 is also

associated with inflammation and disease. In addition, macrophages secrete prostaglandins following stimulation with LPS, primarily due to the induced transcription of the COX-2 gene and production of COX-2 enzyme (Ishimura *et al.*, 2004). The gene encoding for the expression of COX-2 protein is controlled by NF-KB, which is a transcription factor influenced by oxidant status.

2.6.1 Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX) or prostaglandin H_2 synthase (PGHS) is a key enzyme in the synthesis of prostaglandins (PGs) from arachidonic acid (AA) (Abarikwu, 2015). However, COX-2 expression is basically absent in normal cell function. In contrast, COX-2 expression is widely induced by inflammatory stimuli in cells such as macrophages, endothelial cells and synoviocytes. Induction of COX-2 by stimuli is also associated with cell activation and inflammation disease. COX-2 plays an important role in agiogenesis, colon cancer and Alzheimer's disease. Investigating the mechanism of the COX-2 expression could lead to the discovery of a new drug for reducing inflammation without removing the protective PGs in the stomach and kidneys. Therefore, COX-2 inhibitors also have great and innovative potential in reducing inflammation.

2.7 Macrophage

One major type of macrophages is the mononuclear phagocyte of the immune system which are important cells that originate from bone marrow including blood monocytes and tissue macrophage. Macrophages play a critical role in the initiation, maintenance and resolution of inflammation. During the inflammation process and infection, macrophages act as phagocytic cells capable of targeting and ingesting invading organisms activated by lymphokines and different bacterial products. Lipopolysaccharides (LPS) are the residues of the outer membrane of gram-negative bacteria, which are the most effective activators of monocytes. LPS can activate several signal transduction pathways through the induction of G-proteins, cAMP dependent kinase, protein kinase C and JNK mitogen activated kinase (MAPK) families (Furukawa *et al.*, 1988).

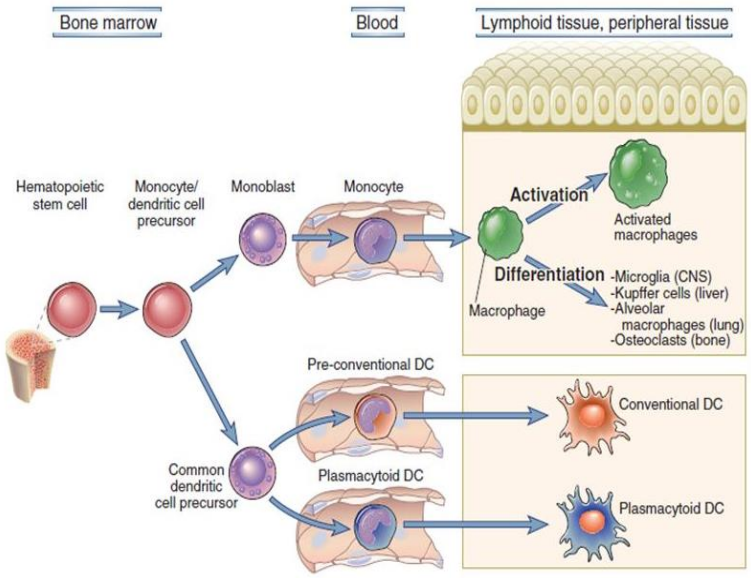


Figure 2.7 Maturation of mononuclear phagocyte develop from bone marrow (Abbas *et al.*, 2011).

Macrophages also produce a wide range of biologically active molecules that participate in the inflammatory process. Their function in inflammatory process is one of innate and adaptive immunity, playing a key role in the host defense mechanism. In innate immunity, the macrophage induces chemical mediators by releasing cytokine, which then leads to inflammation (Condeelis and Pollard, 2006).

2.8 Nitric oxide radical scavenging activity assay

Nitric oxide is one of the free radicals classified as reactive nitrogen species (RNS). Nitric oxide (NO) is produced from the conversion of amino acid L-arginine by endothelial cells, macrophages and neurons (Parul *et al.*, 2013). NO is an important signaling molecule in vasodilation, neurotransmission, and the host immune defense (Pacher *et al.*, 2007). There are three isoforms of the nitric oxide synthase (NOS): endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and indicuble nitric oxide synthase (iNOS). These are constitutively expressed and essential for maintaining tissue homeostasis (Jung *et al.*, 2007). iNOS is influenced in response to pro-inflammatory cytokine production including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and

several inflammatory responses (Ndiaye *et al.*, 2012). Determination of NO production can be carried out by various experimental methods. Mostly, in vitro nitric oxide radical scavenging activity is measured spectrophotometrically. When sodium nitroprusside is mixed with aqueous solution at physiological pH (7.2), nitric oxide (NO) is generated under aerobic conditions to produce nitrite and nitrate ion that can be estimated by the Griess reagent. The absorbance of the chromophore formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride is immediately measured at 540 nm (Menaga *et al.*, 2013).

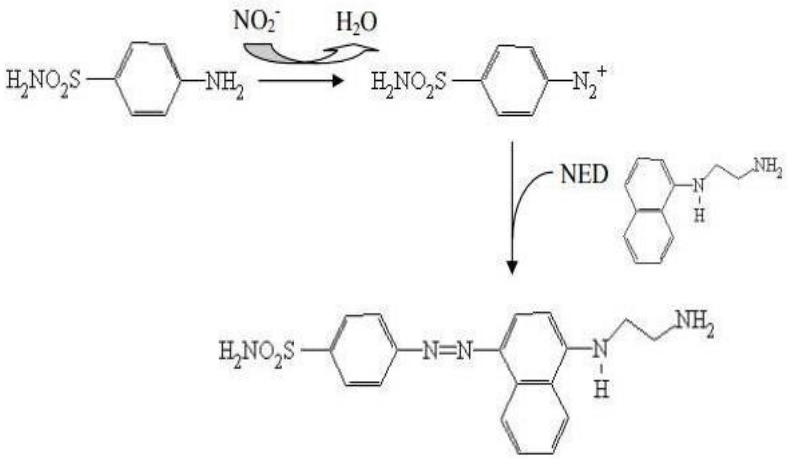


Figure 2.8 Chemical reactions involved in the determination of NO_2 using the Griess Reagent System.

2.9 Cytotoxic activity

Cytotoxic activity is an in vitro cytotoxicity test of the chemicals or unknown compounds affecting the normal functions of cells which are common to all cells. The toxicity of the compound can be evaluated by assessing the cellular damage. The normal experiment for the evaluation of cell death is that of MTT cytotoxicity assay (Figure 2.9). Other methods also widely use sensitive, quantitative and reliable colorimetric assays. MTT cytotoxicity assay is the measure of the viability of the proliferation and activation of cells (Senthilraja and Kathiresan, 2015). The assay is based on the capacity of the mitochondria dehydrogenase enzyme in the living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product which is insoluble in water. Living cells can reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple blue formation which precipitates in the cellular cytosol and can be dissolved after cell lysis, whereas cells that have died following toxic damage cannot transform MTT. This formation production is proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. The reaction is mediated by the dehydrogenase enzyme involved with the endoplasmic reticulum and the mitochondria (Fotakis and Timbrell, 2006).

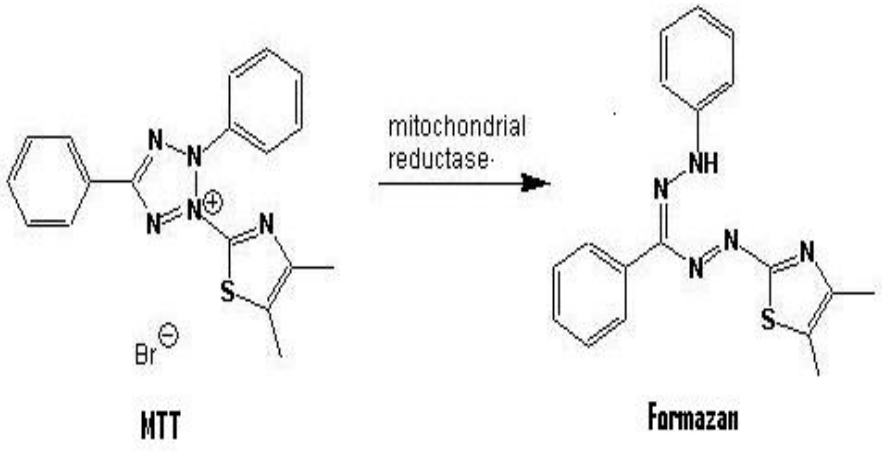


Figure 2.9 MTT assay (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide a yellow tetrazole), is reduced to purple formazan in living cells

2.10 Bioactive peptide

Currently, there is increasing utilization of natural proteins as sources of biologically active peptides with diverse heath benefits and these peptide can be generated by proteolytic enzyme treatment under specific conditions. Peptides are short polymers of amino acids linked by peptide bonds. Protein is the essential components of tissues in biological organisms and participates in a large number of physiological processes within the cells. In general, bioactive peptides also contain around 3-20 amino acid units (Pihlanto-Leppälä, 2000). Biologically active peptides, once liberated as dependent entities, act as potential metabolism modulators and control compounds with hormone-like activities. However, the bioactivities that have been reported for peptide include antihypertensive, antioxidant, anticancer, antimicrobial and opioid activities as well as immunomodulatory and cholesterol-lowering effects. In addition, peptides with anti-inflammatory effects have been less investigated (Montoya-Rodríguez *et al.*, 2014). The activity of bioactive peptides is dependent on their amino acid composition and sequence.

2.10.1 Anti-inflammatory peptide

The anti-inflammatory effect of peptides has been identified in a broad variety of natural sources. Tripeptide from salmon pectoral fin produced with low molecular weight, an anti-inflammatory peptide prepared from pepsin hydrolysate, has shown anti-inflammatory activity by suppressing the protein expression of inducible nitric oxide synthase, cyclooxygenase-2 and production of cytokines including tumor necrosis factor-α, interleukin-6 and -1β (Ahn *et al.*, 2012). Another anti-inflammatory peptide, protein hydrolysate from yellow field pea seeds was reported to significantly inhibit nitric oxide production by activating macrophages and inhibiting the secretion of pro-inflammatory cytokines, TNF-α and IL-6 respectively (Ndiaye *et al.*, 2012). The *Mytilus edulis* hydrolysates fraction was able to inhibit LPS-stimulated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein and gene expression. These peptide fractions also inhibited the translocation of NF-KB (nuclear factor-kappa B) through the prevention of IkBα (inhibitory factor kappa B alpha) phosphorylation(Kim Y.-S. *et al.*, 2016).

Table 2.3 Anti-inflammatory peptide from natural source

Source of bioactive peptide	Enzymatic hydrolysis	Result	Amino acid sequence	Reference
Pisum sativum L. (Yellow field pea seeds)	Thermolysin	Inhibited NO production and secretion of pro-inflammatory cytokines	-	(Ndiaye *et al.*, 2012)
Salmo salar (Salmon byproduct)	Pepsin	Inhibited nitric oxide production by 63.08% and Prostaglandins by 45.33%.	Pro-Ala-Try	(Ahn *et al.*, 2012)
Mytilus coruscus (Korean mussel)	Alcalase, Flavpurzyme, Neutrase, Protamex, and Papain	Flavourzyme hydrolysate inhibited nitric oxide production on macrophage	Gly-Val-Ser-Leu-Leu-Gln-Gln-Phe-Phe-Leu	(Kim E.-K. *et al.*, 2013)

Table 2.3 Anti-inflammatory peptide from natural source (continue)

Source of bioactive peptide	Enzymatic hydrolysis	Result	Amino acid sequence	Reference
Ruditapes philippinarum (Japanese carpet shell)	Alcalase, Flavpurzyme, Neutrase, Protamex, Papain, Pepsin, Trypsin and α-chymotrypsin	Alcalase hydrolysate inhibited LPS induced-NO production in RAW264.7	Gln-Cys-Gln-Gln-Ala-Val-Gln-Ser-Ala-Val	(Kim E.-K. *et al.*, 2013)
Tuna cooking juice	Orientase, flavourzyme and alcalase	Alcalase hydrolysate inhibited nitric oxide production on murine macrophage RAW264.7	Pro-Arg-Arg-Thr-Arg-Met-Met-Asn-Gly-Gly-Arg	(Cheng *et al.*, 2015)

2.10.2 Enzymatic hydrolysis

Enzymatic hydrolysis is an alternative method to the conversion and utilization of biomass into bioactive protein. Hydrolysis of the protein involves the digestion of peptide bonds to generate peptides of various sizes and amino acid composition. There are three types of hydrolysis: enzymatic, acid and alkali hydrolysis. Chemical hydrolysis is a complex process to regulate and reduce the functional quality of products (Radha *et al.*, 2008). Enzymatic hydrolysis is used to produce protein hydrolysate without disruption of amino acid without more severe treatment such as chemical and physical treatment (Clemente, 2000). However, much research has shown that pH and temperature also affect enzymatic hydrolysis. The products obtained from enzymatic hydrolysis treatment also have high functionality and nutritive values. Production of protein hydrolysate by use of digestive proteolytic enzymes from animals or from plants and microorganisms have been reported safe for human consumption (McCarthy *et al.*, 2013).

Table 2.4 Comparison of the chemical hydrolysis and enzymatic hydrolysis to produce protein hydrolysate (Kristinsson and Rasco, 2000; Sanmartín *et al.*, 2009).

Processing method	**Advantage**	**Disadvantage**
Chemical hydrolysis (acid and alkali)	-High protein recovery -Short processing time -Low processing cost	- Absence of homogeneous hydrolysates -Disturb functionality - Metal corrosion of equipment - Reaction is hard to control -Form toxic substance
Enzymatic hydrolysis (protease)	-Maintain functions and nutritive value of final hydrplysates -Produce homogeneous hydrolysate -Mild condition -Nontoxic and biodegradable	- High cost processing - Long processing time

2.10.3 Proteolytic enzyme

Proteolytic enzymes (Proteinases, peptidase or protease) are enzymes involved in proteolysis. This process is the digestion of protein into small polypeptides or free amino acids by reacting with peptide bonds. Proteolytic enzymes can be split into endopeptidase and exopeptidase. Exoprotease hydrolyzes the peptide bonds at the terminal peptide bond and releases amino acids from either the N or C terminal side. Endopeptidase breaks down the peptide bond with protein molecules at a random side (Zhuang *et al.*, 2009). Protease has been used in the production of diverse bioactive peptides from native protein.(Sun *et al.*, 2016) reported that egg ovomucin hydrolyzed by alcalase might exert anti-inflammatory activity. Flavourzyme hydrolysate from *Ruditapes philippinarum* exhibited a strong anti-inflammatory activity (Lee S. J. *et al.*, 2012). Many bioactive peptides have been experimentally produced under specific conditions by using different commercial proteases as shown in Table 2.5.

2.10.3.1 Alcalase

Alcalase (E.C. 3.4.21.62) is an enzyme obtained from *Bacillus licheniformis* which contains several proteinases with various specificities. Its optimum pH for catalysis ranges from 6.5-8.5. Alcalase has been used widely to prepare soluble hydrolysates with the nutritional and functional properties of natural protein maintained, such as soy protein and fish protein (REBECA *et al.*, 1991) as well as to produce bioactive peptides.

2.10.3.2 Flavourzyme

Flavourzyme (E.C. 3.4.11.1) is a kind of compound enzyme composed of the incision enzyme and the circumscribed enzyme from *Aspergillus oryzae*. Flavourzyme also acts as exopeptidase and endoprotease. Enzyme preparation is numerous and used for protein hydrolysis in diverse industrial and research applications. (Doucet *et al.*, 2003; Klompong *et al.*, 2008).

2.10.3.3 Neutrase

Neutrase (E.C. 3.4.24.28) is a protease produced by *Bacillus amyloliquefaciens* which is extensively used to break protein down into peptide. The optimal working conditions are 45-55 ºC and pH 5.5-7.5. Neutrase is formulated in a way that provides the highest degree of safety during handling (Lassoued *et al.*, 2015).

Table 2.5 Proteolytic enzymes used to preparation of protein hydrolysate and peptide (Najafian and Babji, 2012).

Enzyme	**Type of protease**	**Source of origin**	**Condition**	
			pH	**Temperature (ºC)**
Alcalase	Endo-protease	*Bacillus licheniformis*	6.5-8.5	45-65
Flavourzyme	Endo-exo-peptidase	*Aspergillus oryzae*	5-7	90
Neutrase	Metallo-endo-protinase (Zn)	*Bacillus amyloliquefaciens*	5.5-7.5	45-55
Papain	Cysteine protease, endopeptidase	Carica papaya (papaya latex)	6-7	60-70
Pepsin	Acid protease and endo-peptidase	Porcine gastric mucosa	1-4	37-42

2.11 Chicken feather meal and feather meal

Enlargement of global poultry production has been accompanied by an increase in the amount of by products such as feather meal (Chaturvedi *et al.*, 2014). Chicken feather meals are bio-resources with high protein content of more than 750 g/kg of crude protein. In the past, chicken feather meal was generally used as animal feed because of its high protein content. In addition, keratin is the main component of feathers, comprising nearly 90% of the feather's weight. Feather keratin shows an elevated content of the amino acids such as glycine, alanine, serine, cysteine and valine (Grazziotin *et al.*, 2006). Accumulations of high amounts of chicken feather meal lead to environmental pollution. Today, environmental regulations and safety are of greater concern, restricting the methods in terms of processing feathers for animal meal or waste landfilling (Kwiatkowski *et al.*, 2013). Several pretreatment methods including chemical, enzymatic or biological treatment, have therefore been investigated in the past few years to improve the digestibility of feathers (Coward-Kelly *et al.*, 2006). (Taskin *et al.*, 2011) reported that protein hydrolysate from chicken feather was rich in protein, mineral and amino acids. However, the biological activity of chicken feather meal has been less investigated and evaluated. In contrast, there has been much research on the utilization of chicken feather meal (Table 2.6). So, chicken feather meal might act as a source of protein hydrolysate with novel anti-inflammatory activity.

Table 2.6 Utilization of chicken feather meal in many researched fields

Source	Biological activity	Result	References
Chicken feather meal	Antioxidant and antihypertensive	Feather hydrolysates displayed in *vitro* antioxidant properties and inhibit the angiotensin I-converting enzyme.	(Fontoura *et al.*, 2014)
Chicken feather meal	Bioenergy	Bioenergy from chicken feather meal are successfully replaces the formerly used coal burner.	(Kwiatkowski *et al.*, 2013)
Chicken feather meal	Biological resource	The by-product can be produce useful biotechnological products.	(Lasekan *et al.*, 2013)
Chicken feather meal	Keratinase production	chicken feather as a potential renewable raw material to produce keratinase	(Demir *et al.*, 2015)

CHAPTER III
EXPERIMENTAL

3.1 Biological materials

Chicken feather meal (CFM) in this study was supplied by Betagro Co., Ltd. (Thailand) and amino acid composition of chicken feather meal was determined according to the Association of Official Analytical Chemist method (AOAC 994.12).

3.2 Chemicals

Alcalase and Flavourzyme were purchased from Brentag (Mülheim, Germany). Neutrase was purchased from Novozymes (Bagsvaerd, Denmark). Acetic acid, ethanol and phosphoric acid were purchased from Merck (Gibbstown, NJ). Bovine serum albumin (BSA). curcumin from *Curcuma longa* (Turmeric), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), interferon-g from mouse (IFN-g), Lipopolysaccharide (LPS) from *Escherichia coli*, potassium persulfate, sodium nitrite, (1-Naphthyl)ethylenediamine (NED), sodium nitroprusside (SNP), sodium pyruvate, sulfanilamide, streptomycin sulfate were purchased from Sigma-Aldrich (St. Louis, MO).

3.3 Determination of amino acid content

Amino acid content of chicken feather meal was determined based on the standard AOAC 994.12 acid hydrolysis method. 1 g of chicken feather meal was dissolved in 5 ml of 6 N HCl in test tube, and placed in heating block at 110 ºC for 24 h. to liberate the amino acid. Then, the internal standard (10 ml 2.5 mM L-α-amino-n-butyric acid in 0.1 M HCl) was added, diluted with deionized water to 250 ml and placed in a heating block at 55 ºC for 10 min. Amino acid were determined by reverse-phase high performance liquid chromatography (RP-HPLC) analysis on a Hypersil GOLD column C_{18} (4.6 mm X 150 mm, 3µM), elute with sodium acetate buffer pH 4.90 and 60% acetonitrile, at flow rate of 0.3 ml/min.

3.4 Preparation of enzymatic hydrolysate from chicken feather meal

CFM was dried at 60 ºC overnight and ground pass through a 150 micron sieve. 5 g of CFM was mixed with 100 ml of phosphate buffer saline (20 mM phosphate buffer with 0.15 M NaCl pH 7.2). Then the suspensions were stirred overnight at 4 ºC overnight. Three type of microbial protease; Alcalase®, Flavoruzyme® and Neutrase® were hydrolyzed in various concentrations (0, 1, 2.5 and 5%). The optimum pH, temperature and characterization of various enzymes are summarized in Table 3.1. The reactions were carried out for 4 h with shaking (150 rpm) respectively. After hydrolysis, the mixtures were heated at 90 ºC for 10 min to inactive enzyme, and then centrifuged at 10,000 rpm for 15 min and collected the supernatant. The hydrolysates were stored in -20 ºC until use.

Table 3.1 Condition for enzymatic hydrolysate from chicken feather meal

Conditions	Alcalase	Flavourzyme	Neutrase
pH	8.0	7.0	7.0
Temperature (°C)	50	50	50
Time (h)	4	4	4

3.5 Protein Content Determination

To determine the protein concentration of CFM protein hydrolysate was measured according to Bradford's procedure (Bradford, 1976)), using bovine serum albumin (BSA) as the standard curve with various concentrations to construct the calibration curve. 20 µL of each sample were mixed with 200 µL Bradford working buffer into each of three well of a 96-well plate, and then incubate for 20 min. The absorbance was measured at 540 nm with a microplate reader spectrophotometer. The absorbance value was calculated to protein concentration using linear equation computed from standard curve.

3.6 Nitric oxide radical scavenging assay

Nitric oxide (NO) radical scavenging assay was slightly modified method from Chantaranothai et al., (2013). The peptide hydrolysates (5 µL) was mixed with 10 mM of sodium nitroprusside in a phosphate buffer saline pH 7.2 and incubate at room temperature for 150 min. Then 100 µL Griess reagent (0.33% sulfanilamide in 5% phosphoric acid) was added and incubated for 5 min. After that, 100 µL of *N*-naphthyl-ethyldiamine (NED) was added and further incubated for 30 min at room temperature. The absorbance was determined at 540 nm spectrometer. The percentage of scavenging activity (%) and IC_{50} were calculated. Curcumin was used as positive control. The percentage of scavenging activity was calculated using the following this equation.

$$\% \text{ inhibition} = \left[\frac{(\text{Abs control} - \text{Abs blank}) - (\text{Abs sample} - \text{Abs background})}{(\text{Abs control} - \text{Abs blank})} \right] \text{x } 100$$

3.7 Purification of protein hydrolysate from CFM

3.6.1 Molecular weight cut-off by ultrafiltration

The peptide hydrosate from CFM were fractionated through ultrafiltration (UF) membranes with a range of molecular weight cut-off (MWCO) of 10, 5, 3 and 0.65 kDa. Peptide hydrolysates were devided into five fraction including >10 kDa, 10-5 kDa, 5-3 kDa, 3-0.65 kDa and < 0.65 kDa. All fractions were collected to determine nitric oxide radical scavenging activity.

3.7.2 Gel filtration chromatography

The fraction was shown highest inhibit nitric oxide radical scavenging from molecular weight cut-off (≤ 0.65 kDa) by ultrafiltration was loaded onto a preparative Sephacryl S-200 gel filtration column chromatography (AKTA™ prime with Hitrap™, Amersham Biosciences), column (2.6 x 80 cm.) at flow rate 0.5 ml/min. Each fraction of sample was detected at 280 nm and assayed for nitric oxide radical scavenging.

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

The most effective fraction from gel filtration chromatography was further purified by reverse-phase high performance liquid chromatography on C_{18} column (250×4.6 mm, Luna 5U, Phenomenex, Torrance, CA, USA). The peptide was eluted with rational gradient of mobile phase A (0.1% v/v trifluoroacetic acid) and mobile phase B (70% (v/v) acetonitrile in 0.05% (v/v) trifluoroacetic acid) at flow rate 0.7 ml/min. The elution peak was detected at 280 nm. Chromatographic analyses were completed with ChromQuest Software (Thermo Sciencetific).

3.8 Identification of anti-inflammatory peptides

Amino acid sequences of the purified peptides were determined by LC-MS/MS Q-TOF mass spectrometer. The system consisted of liquid chromatography in combination with a quadrupole time to flight mass spectrometer (Q-TOF). LC-MS/MS data in mass range from 25 to 20,000 m/z. All of data were processed and analyzed by de novo sequencing which is to use the mass difference between two fragment ions to calculate the mass of an amino acid residue on the peptide chain.

3.9 Cell culture

The macrophage RAW 264.7 were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS, 100 U/ml penicillin-G, 0.4 mg/ml streptomycin sulfate, 1% (w/v) sodium pyruvate, and 1% (w/v) of HEPES at 37 ◦C in a humidified atmosphere with 5% (v/v) CO_2. For routine maintenance in culture (passage) (Saisavoey *et al.*, 2016), cells were seed in non-tissue culture treated dish at approximately 10% confluency and grown to approximately 80% confluency, which typically took 2 days. Old medium was aspirated, cells were gently rinsed with PBS, then dislodged by gently scraping with a rubber spatula and then harvest by centrifugation 15,000 x g for 5 min. PBS was removed, the cell pellet was resuspended in CM and the in CM and the viable and total cell density was evaluated by using Trypan blue dye exclusion on an improved Neubauer hemacytometer. The following equation was then used to calculate the viable cell numbers.

Total cell count (cells/ml) = the number of cells counted in 16-large squares x 2 x 10^4

For storage, cells were harvested and resuspended in cold freezing media (CM plus 10% (v/v) dimethyl sulfoxide) at 2 x 10^6 cells/ml, aliquoted at 1 ml per cryogenic tubes and transferred to -80 ºC immediately overnight and then into liquid nitrogen. To initiate cultures from frozen stocks the cells were thawed at 37 ºC, transferred into 10 ml of serum free DMEM media and centrifuged at 15,000 x g for 5 min. The cell pellet was resuspened in CM, plated and cultured as above except that after the cells had adhere (approximately 6 h), the old medium was replaced.

3.10 Pretreatment of macrophage RAW 264.7 Cells

The macrophage RAW 264.7 cells were seeded in 96-well plates at a density of 1x104 cells/well in 100 µl CM and incubated overnight at 37 ºC in 5% (v/v) CO2. After that the medium was replaced with a fresh supplemented without sample (negative control), various concentration of protein hydrolysate from CFM or parthenolide at 2.5 µg/ml was ultilized as a positive control and incubated for 1 h. Then, NO production was stimulated by the addition of 100 ng/ml of lipopolysaccharide (LPS) and incubated for 12 h.

3.11 MTT assay for measuring of cell proliferation

The cytotoxicity activity of CFM protein hydrolysate was determined by MTT assay with slightly modified according to Yang *et al.* (2014); (Chantaranothai *et al.* (2013)). The macrophage RAW 264.7 cells were plated at density of 1x104 in 96-well plate overnight. After overnight, cells were treated with sample in different concentration and LPS. Then 100 µl of 5 mg/ml MTT solution (in PBS) was added per well. After incubated at 37 ºC, 5% CO2 incubator for 4 h. Finally, dimethyl sulfoxide (DMSO) was added 100 µl/well in 96-well plates due to DMSO solubilizes the formed formazan crystals. The volume of formazan crystals was determined by measuring the absorbance at 540 nm.

3.12 Determination of nitric oxide (NO) production from macrophage RAW 264.7 cells

Nitric oxide production was determined by measure nitrite production in the culture medium according to the Griess reaction. The macrophage RAW 264.7 cells were incubated with samples in various concentrations and without sample. Fifty-microliter of sulfanilamide was added to 50 µl of the culture supernatant in a 96-well plate and incubate at room temperature for 10 min. after that, Fifty-microliter of NED solution was added and further incubate for 10 min, then absorbance at 540 was measured using microplate reader.

3.13 Total RNA Isolation from macrophage RAW 264.7 Cells

To study about the mechanism of action on cytokine release, the assay for mRNA expression were carried out. Total RNA was isolated from macrophage RAW 264.7 cells which treated with solvent (negative control), CFM protein hydrolysate in various concentrations and 2.5 µg/ml of parthenolide (positive control). Cells were stimulated with 100 ng/ml of LPS. After 12 h incubation, to harvest the total RNA by using the MaterPure™ Complete DNA & RNA Purification Kit (Epicentre) were used according to the manufacturer's instruction. The RNA concentration was measured by using Nanodrop 2000 UV-vis Spectrometer (Thermo Fisher Scientific, Inc., USA).

3.14 Detection of iNOS, IL-6, TNF-α, COX-1 and COX-2 mRNA by Reverse transcription polymerase chain reaction (RT-PCR) analysis

A content of 1 µg of total RNA from each condition was reverse-transcription became single-stranded complementary DNA (cDNA) was synthesis by using Precision™ nanoscript 2 Reverse Transcription kit (PrimerDesign Co., Ltd. UK) according to manufacturer's protocol. Total volume of cDNA synthesis were consisting of up to 5 µg of total RNA, 1 µl of RT primer and RNAse/DNAse free water were adjusted to final volume (10 µl). After that incubate at 65 ºC for 5 min, then immediately cool in ice water bath for 1 min. After that, nanoscript2 4X buffer 5 µl, dNTP mix 10 mM, RNAse/DNAse free water 3 µl and 1 µl of nanoscript2 enzyme were added. The mixture was gently mixed and reaction was performed at 42 ◦C for 20 min, 75 ◦C for 10 min. cDNA was used as a template for PCR.

Then PCR analysed were performed on aliquots of the cDNA preparation to detect inducible nitric oxide synthase (iNOS), Interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), Cycloxygenase-1 (COX-1), Cycloxygenase-2 (COX-2) and beta-actin (β-actin). The PCR primer of each gene in this study are listed below in Table 3.2 denaturation step at 95 ºC for 3 min, 30 s at 95 ºC for denaturation, annealing step of each primer were shown in table 3.2 for 1 min, and extension at 72 ºC for 1 min. After amplification, the RT-PCR reaction product were separated with 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide for 10 min. Then, visualized under a UV light using gel documentation system (Biorad, Hercules, CA).

Table 3.2 The PCR primer in this study

Primer	**Sequence (5' to 3')**	**Annealing temperature (ºC)**	**Base pair (bp)**
β-actin forward	ACCAACTGGGACGACATGGAGGA	55	380
β-actin reward	GTGGTGGTGAAGCTGTAGCC		
iNOS forward	CCATCATGGACCACCACACA	65	423
iNOS reward	CCATGCAGACAACCTTGGTG		
IL-6 forward	CATGTTCTCTGGGAAATCGTGG	50	417
IL-6 reward	AACGCACTAGGTTTGCCGAGTA		
TNF-α forward	CCTGTAGCCCACGTCGTAGC	50	375
TNF-α reward	TTGACCTCAGCGCTGAGTTG		
COX-1 forward	AGTGCGGTCCAACCTTATCC	63	382
COX-1 reward	CCGCAGGTGATACTGTCGTT		
COX-2 forward	GGAGAGACTATCAAGATAGT	50	861
COX-2 reward	ATGGTCAGTAGACTTTTACA		

The PCR mixture was consisted of cDNA 1 µl, RNase free water 10.5 µl, PCR reagent (Pcrbiosystem, United Kingdom) 12.5 µl, 10 µM forward primer 1 µl and 10 µM reverse primer 1 µl. The PCR Reaction was carried out in a volume of 25 µl (final volume) containing. Amplification was completed by using a PCR thermal cycler (Bio-Rad, Laboratories, Inc., USA) for PCR with following condition: initial denaturation step at 95 ºC for 3 min, 30 s at 95 ºC for denaturation, annealing step of each primer were shown in table 3.2 for 1 min, and extension at 72 ºC for 1 min. After amplification, the RT-PCR reaction product were separated with 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide for 10 min. Then, visualized under a UV light using gel documentation system (Biorad, Hercules, CA).

3.15 Statistical analysis

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

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Amino acid composition of chicken feather meal

The amino acid composition of chicken feather meal is shown in Table 4.1. The amino acid composition of protein material plays a significant role in various physiological and biological activities. According to the result, chicken feather meal was rich in 18 types of amino acid including alanine, arginine, glycine, aspatic acid, valine, cysteine, glutamic acid, leucine, isoleucine, histidine, threonine, proline, lysine, methionine, serine, phenylalanine, tyrosine and tryptophan. In addition, anti-inflammatory peptide from *Mytilus coruscus* which consists of glycine, valine, serline, leucine, glutamine and phenylalanine has shown anti-inflammatory activity (Kim E.-K. *et al.*, 2013). In addition, Lee S. J. *et al.* (2012) reported that peptide consisting of glycine, cysteine, alanine, valine and serine can exhibit nitric oxide production. There is a previous study reporting that glycine, cysteine and histidine exhibited anti-inflammatory activity by inhibiting the NF-KB signaling pathway (Hasegawa *et al.*, 2012).

Table 4. 1 Amino acid profile of chicken feather meal (mg/100 mg)

Amino acids	Results (%)
Alanine (Ala)	3.80
Arginine (Arg)	6.30
Glycine (Gly)	6.82
Aspatic acid (Asp)	5.70
Valine (Val)	5.85
Cysteine (Cys)	2.90
Glutamic acid (Glu)	10.6
Leucine (Leu)	6.46
Isoleucine (Ile)	3.94
Histidine (His)	0.59
Threonine (Thr)	3.96
Proline (Pro)	8.37
Lysine (Lys)	1.45
Methionine (Met)	0.67
Hydroxyproline (Hyp)	Not detected
Serine (Ser)	7.84
Phenylalanine (Phe)	4.03
Hydroxylysine (Hyl)	Not detected
Tyrosine (Tyr)	1.10
Tryptophan (Trp)	0.20

4.2 In vitro nitric oxide radical scavenging assay

Nitric oxide is a reactive radical involved in the inflammatory process in living organisms. The NO scavenging activity may help prevent the chain reaction that promotes excessive generation of NO causing damage to human health (Yu M. *et al.*, 2014). The crude protein hydrolysate of chicken feather meal from various types of protease were subjected to testing of their anti-inflammatory activity by using nitric oxide radical scavenging assay. The inhibition results in Table 4.2 suggest that the 1% flavourzyme hydrolysate (5.49±0.97 µg/ml) exhibited the highest nitric oxide radical scavenging activity when compared with half maximal inhibitory concentration (IC_{50}). The 1% neutrase hydrolysate had shown weak NO scavenging activity (175.70±3.96 µg/ml). These results were comparable with curcumin as the standard for inhibiting this reaction (positive control). Therefore, 1% flavourzyme hydrolysate was more effective than curcumin. In addition, there has been some research about protein hydrolysate which has anti-inflammatory activity that has reported that flavourzyme hydrolysate from *Mytilus coruscus* has a high capability of inhibiting nitric oxide production in macrophage RAW 264.7 cells (Kim E.-K. *et al.*, 2013). The results for 1% flavourzyme hydrolysate concurred with previous study. Therefore, the 1% flavourzyme hydrolysate responsible for anti-inflammatory was purified for further study.

Table 4. 2 Nitric oxide radical scavenging activity of crude peptide from chicken feather meal

Enzymes	NO radical scavenging activity IC_{50} (µg/mL)			
	Control	**1%**	**2.5%**	**5%**
Alcalase	134 ± 4.72^{e}	80.59 ± 5.40^{d}	29.97 ± 1.81^{a}	40.23 ± 0.78^{b}
Flavourzyme	158.37 ± 1.50^{E}	5.49 ± 0.97^{A}	82.07 ± 6.96^{D}	68.68 ± 3.57^{C}
Neutrase	175.70 ± 3.96^{ee}	133.03 ± 5.40^{dd}	36.99 ± 0.43^{aa}	78.71 ± 3.91^{cc}

*All data are shown as mean±standard deviation of triplicate (the result show significant difference of $p\leq0.05$)

** curcumin as a positive control with IC_{50} = 60.53±3.18 µg/ml

4.3 Fractionation of 1% Flavourzyme hydrolysate by Ultrafiltration

For further study, 1% Flavourzyme hydrolysate was filtered through different molecular weight cutoff (MWCO) membranes: 10, 5, 3 and 0.65 kDa membrane filters (Pellicon XL filter, Merck). Then, each fraction was subjected to examination for nitric oxide radical scavenging activity. The results of each fraction are shown in Table 4.3. The anti-inflammatory activity of the fraction ≤ 0.65 kDa size molecular weight exhibited the most effective nitric oxide radical scavenging activity compared with the others (3.64±0.27 µg/ml). It was reported that the biological activity of hydrolysates depend on the molecular weight of each peptide (Wang A.-Y. *et al.*, 2011). An array of anti-inflammatory peptides with low molecular weights fractionated by ultrafiltration were effective in interaction with the internal barrier to promote the biological effect (Ahn *et al.*, 2015). So, low molecular weight peptides are more effective than high molecular weight peptides. Thus, this fraction was selected for further study.

Table 4. 3 Nitric oxide radical scavenging activity of peptide fraction from ultrafiltration

Molecular weight (kDa)	NO radical scavenging activity IC_{50} (µg/mL)
> 10 kDa	21.87 ± 0.70^{d}
5-10 kDa	10.42 ± 0.67^{c}
3-5 kDa	7.17 ± 0.47^{b}
0.65-3 kDa	5.82 ± 0.56^{ab}
<0.65 kDa	3.64 ± 0.27^{a}

*All data are shown as mean±standard deviation of triplicate (the result show significant difference of $p\leq0.05$)

** curcumin as a positive control with IC_{50} = 60.53±3.18 µg/ml

4.4 Sephacryl S-200 gel filtration chromatography of 1% flavourzyme hydrolysate

The anti-inflammatory peptide fraction ≤ 0.65 kDa was subjected to the Sephacryl S-200 column, and the active peaks were separated into four peaks (F1, F2, F3 and F4), as shown in Figure 4.1. The four fractions were evaluated for nitric oxide radical scavenging activity. The results of each fraction are shown in Table 4.4. The F2 fractions exhibited the highest nitric oxide radical scavenging activity among all fractions with IC_{50} values of 34.60±3.45 µg/ml. The IC_{50} of nitric oxide radical scavenging activity for peptide fractions F3 and F4 could not be calculated due to their low concentrations of protein. Then the fraction F2 was further purified and studied.

Table 4. 4 Nitric oxide radical scavenging activity of peptide fraction from Sephacryl S-200 gel filtration chromatography

Nitric oxide radical scavenging activity IC_{50} (µg/ml)			
Fraction 1	**Fraction 2**	**Fraction 3**	**Fraction 4**
73.49±2.77[b]	34.60±3.45[a]	>51.47	>33.71

*All data are shown as mean±standard deviation of triplicate (the result show significant difference of $p \leq 0.05$)

** curcumin as a positive control with IC_{50} = 60.53±3.18 µg/ml

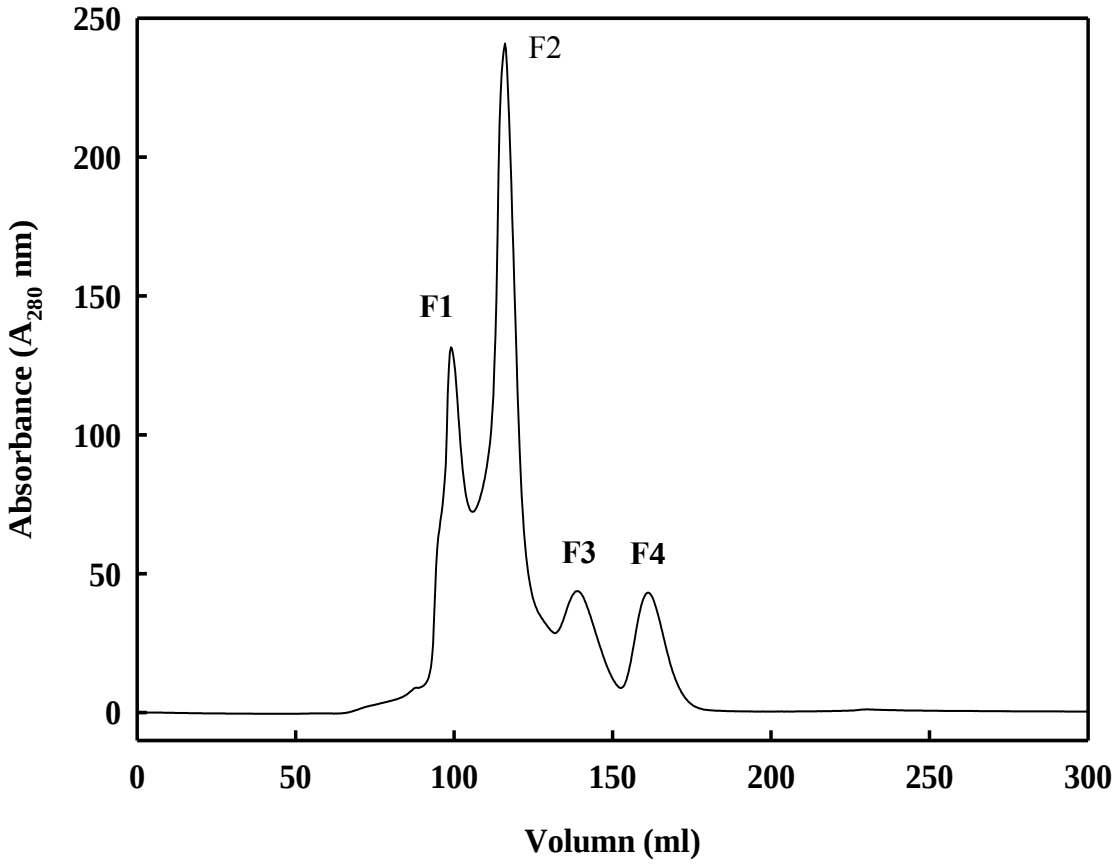


Figure 4. 1 Gel filtration chromatogram of 1% flavourzyme hydrolysate from chicken feather meal (MW < 0.65 kDa) from Sephacryl S-200 gel filtration chromatography

4.5 MTT assay for measuring of cells proliferation

The fraction F2 from gel filtration chromatography was measured cells proliferation of Macrophage RAW 264.7 cells using MTT assay. Mostly, MTT assay is mainly use as determined cells viability, cells proliferation and cytotoxicity of drug, herbal extract and bioactivity compound (Han *et al.*, 2010). The results are given in Figure 4.2. The F2 fraction did not exhibit any cytotoxic effect on macrophage RAW 264.7 cells within the range of concentration up to 40 µg/ml. These studies confirm that 1% flavourzyme hydrolysate was not cytotoxic to macrophage RAW 264.7 cells.

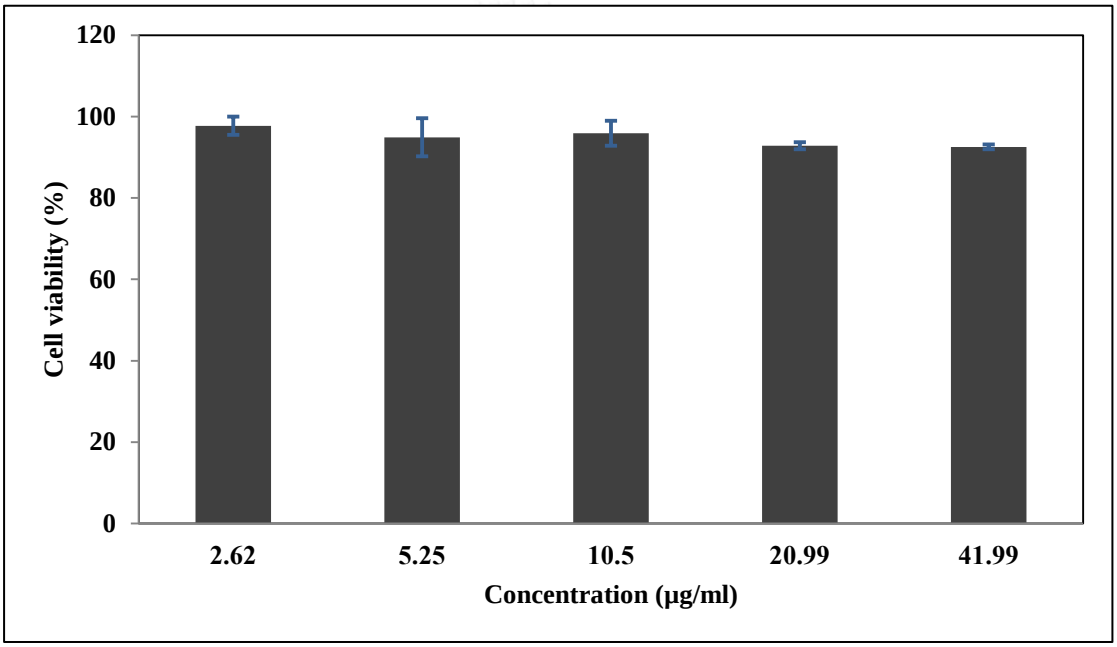


Figure 4. 2 Cell viability of macrophage RAW 264.7 cells in the presence of the F2 fraction, which show no toxicity against the growth of the macrophage.

4.6 Determination of nitric oxide (NO) production from Macrophage RAW 264.7

Nitric oxide (NO) plays an important role in the inflammatory mechanism, immune response and is mainly released from macrophage cells. The treatment of macrophage RAW 264.7 cells with LPS activation resulted in increased NO production in the culture medium. Then the F2 fraction was added into the culture medium to LPS activation in normalized NO production. As can be seen from the results in Figure 4.3, the F2 fraction from gel filtration chromatography was observed to exhibit LPS-induced NO production from macrophage RAW 264.7.

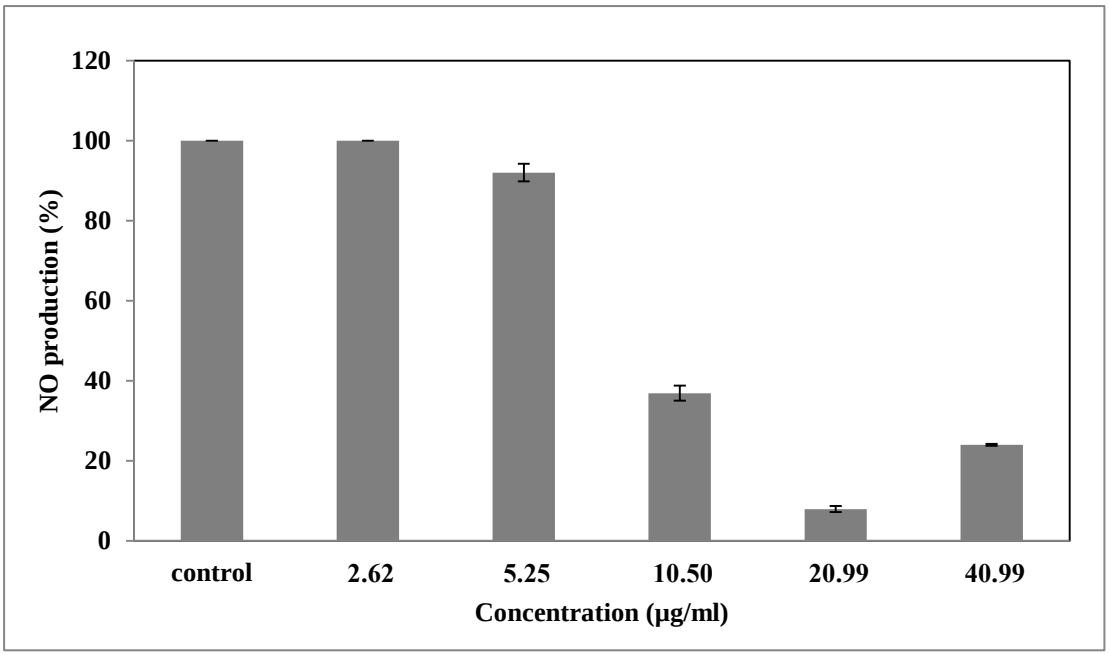


Figure 4. 3 Inhibitory effect of 1% Flavourzyme protein hydrolysate from chicken feather meal on LPS-induced Macrophage RAW 264.7 cell.

4.7 Purification of 1% flavourzyme hydrolysate by RP-HPLC

Throughout the purification process, the potent fraction F2 obtained using gel filtration chromatography showed the highest nitric oxide radical scavenging activity. These fractions were further separated by reverse- phase HPLC on a Shimpack C-18 column (250x46 mm) using a gradient of trifluoroacetic acid (TFA) 0.1% and acetonitrile containing a trifluoroacetic acid 0.05% solvent system at a flow rate of 0.7 ml/min. Elution was detected at 280 nm. In this purification step, peptides were isolated into five fractions: F2-1, F2-2, F2-3, F2-4 and F2-5. The RP-HPLC chromatogram is shown in Figure 4.4. From the result, the nitric oxide radical scavenging activities of F2-1, F2-2, F2-3 and F2-5 reached 16.79±1.47, 15.34± 0.66, 17.43±0.39 and 33.99±1.93 µg/ml, but F2-4 had a low concentration of protein so the IC_{50} value of nitric oxide radical scavenging activity could not be calculated. The fractions which showed nitric oxide radical scavenging activities were subjected to further identification of the amino acid sequence by LC-MS/MS Q-TOF mass spectrometry.

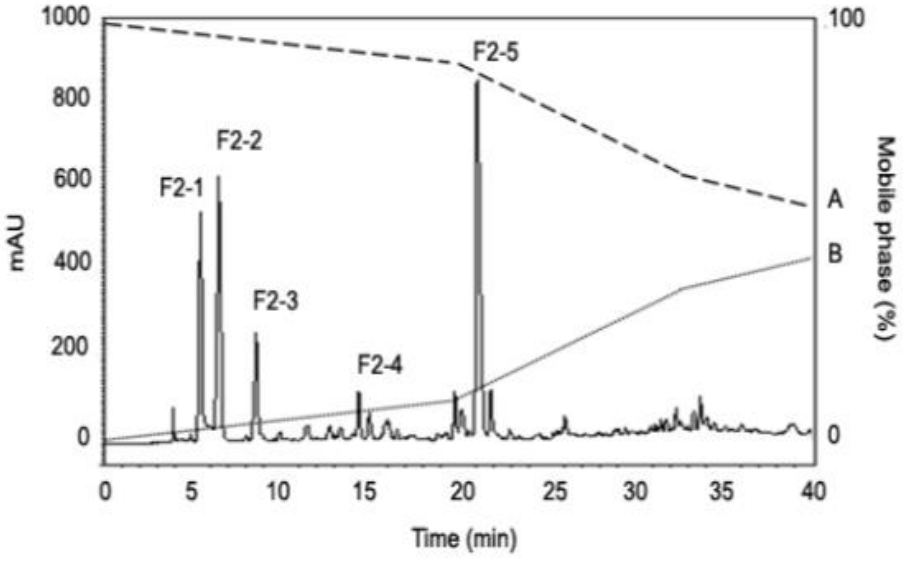


Figure 4. 4 RP-HPLC Chromatogram of 1% flavourzyme hydrolysate from chicken feather meal on a Luna C_{18} (4.6 mm x 250 mm) column.

4.8 Identification of anti-inflammatory peptide

The mass spectrometry technique has widely emerged as a technology of choice with high throughput and sensitivity to the analysis of amino acid sequence and peptide identification. The peptide fractions from RP-HPLC which had nitric oxide radical scavenging activity (F2-1, F2-2, F2-3 and F2-5) were further identified by LC-MS/MS Q-TOF mass spectrometers for the characterization of peptides. Normally, there are two methods to analyze peptide interpretations: the database search and de novo sequencing (Frank *et al.*, 2007; Li and Deinzer, 2007). The results in this experiment were identified by de novo sequencing in fractions F2-1, F2-3 and F2-5 and F2-2 and were analyzed by the MASCOT database program. From purification by RP-HPLC, 1% flavourzyme hydrolysate was separated into five fractions which were analyzed by LC/MS/MS, and only four fractions inhibited nitric oxide production. The amino acid sequences and molecular masses of fractions F2-1, F2-2, F2-3 and F2-5 are shown in Table 4.5, and identified in four peptide chains. Each sequence contained around 9-13 amino acid residues. Fraction 1 (F2-1) was identified as SNPSVAGVR, Fraction 2 (F2-2) as SLFLHTHSIVADK, Fraction 3 (F2-3) as AVLKKKVTSTFGR and Fraction 4 (F2-5) as LSPWPVKGV. Regarding the relationship between the properties and anti-inflammatory activity of amino acids, all of the peptide sequences contained mainly amino acids as the anti-inflammatory of Gly (G) and His (H). Thus, there are some hydrophilic aminos: Ala (A) Phe (F), Leu (L), Pro (P) and Trp (W). These have also been reported to have high antioxidant activity (Bougatef *et al.*, 2010; Mendis *et al.*, 2005; Sacchetti *et al.*, 2008). The anti-inflammatory activity of glycine and histidine has been reported by Hasegawa *et al (Hasegawa et al., 2012)* in their study on glycine, histidine and cysteine. It has been suggested that glycine, histidine and cysteine also acts as an anti-inflammatory by reducing NF-KB activation and inhibiting the expression of IL-6 in human coronary arterial endothelial cells.

Table 4. 5 Peptide sequence of 1% flavourzyme hydrolysate from chicken feather meal identified by Q-TOF LC/MS/MS

Fractions	Amino acid sequence	Protein name	Organism	Accession number
F2-1	SNPSVAGVR	putative E3 ubiquitin-protein ligase SH3RF2	*Gallus gallus*	XP_015157384.1
		putative E3 ubiquitin-protein ligase SH3RF2 isoform X2	*Gallus gallus*	XP_015157384.1
		putative E3 ubiquitin-protein ligase SH3RF2 isoform X1	*Gallus gallus*	XP_414662.3
		PRELI domain containing protein 3B [Gallus gallus]	*Gallus gallus*	NP_001026037.1
		hypothetical protein RCJMB04_17b4, partial	*Gallus gallus*	CAG32065.1
F2-2	SLFLHTHSIVADK	Lactate dehydrogenase	*Phodilus badius*	GI629677270
F-2-3	AVLKKKVTSTFGR	cystine/glutamate transporter	*Gallus gallus*	XP_426289.3
		dynein heavy chain 1, axonemal	*Gallus gallus*	XP_015148334.1
		unconventional myosin-XVI isoform X2	*Gallus gallus*	XP_004938593.1
		unconventional myosin-XVI isoform X1	*Gallus gallus*	XP_416950.3
		transcription initiation factor TFIID subunit 9B	*Gallus gallus*	NP_001264725.1

Table 4.5 Peptide sequence of 1% flavourzyme hydrolysate from chicken feather meal identified by Q-TOF LC/MS/MS

Fractions	Amino acid sequence	Protein name	Organism	Accession number
F2-5	LSPWPVKGV	zinc finger matrin-type protein 1-like isoform X1	*Gallus gallus*	XP_004936323.1
		neogenin isoform X6	*Gallus gallus*	XP_015134538.1
		neogenin isoform X5	*Gallus gallus*	XP_015134537.1
		neogenin isoform X4	*Gallus gallus*	XP_015134536.1
		neogenin isoform X3	*Gallus gallus*	XP_015134535.1

4.9 Anti-inflammatory effect of the F2 Fraction in LPS induced Macrophage RAW 264.7 cells

NO and pro-inflammatory cytokine plays a critical role in the physiology and pathology of diverse tissues including the immune system of the body. The F2 fraction was tested for nitric oxide radical scavenging activity and inhibitory effect against NO production on LPS-induced macrophage RAW 264.7 cells and was shown to have the highest activity against NO production with an IC_{50} value of 34.60±3.45 µg/ml. In order to investigate the mechanism of purified F2 fraction on LPS-induced NO production and inflammatory cytokines such as IL-6, TNF-α and COX-2 expression which are known to be secreted by LPS-induced macrophage RAW 264.7 cells by RT-PCR, it was compared with budesonide (positive control). The results showed that unstimulated macrophage RAW 264.7 cells iNOS, Cox-2, TNF-α and IL-6 were undetectable. However, iNOS, Cox-2, TNF-α and IL-6 were expressed at the mRNA level by LPS and the F2 fraction significantly inhibited these expressions. When the F2 fraction was compared with budesonide as a positive control, it was found that both the F2 fraction and budesonide strongly inhibited the LPS mediating increased incellular iNOS, IL-6 and TNF-α mRNA level (Figure 4.5). Nevertheless, the fraction F2 did not affect the expression of COX-1, for maintaining normal physiological functions, and β-actin, the house keeping gene. Inducible nitric oxide synthase (iNOS) is involved in the response to various pro-inflammatory cytokines including interferon-$^{\gamma}$ (INF-$^{\gamma}$), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), and mediates several inflammatory responses (Jung *et al.*, 2007). Therefore, the results also agreed with the results of the inhibitory effect of nitric oxide production. Moreover, it has been reported that the inducible isoforms of NOS and COX-2 are involved in the production of large amounts of NO and prostaglandin E_2 (PGE_2), respectively (Lappas *et al.*, 2002; Wang S. Y. *et al.*, 2008). The activation of nuclear factor kappaB (NF-KB) is responsible for the expression of iNOS and COX-2 (Brown *et al.*, 2008; Park *et al.*, 2006).

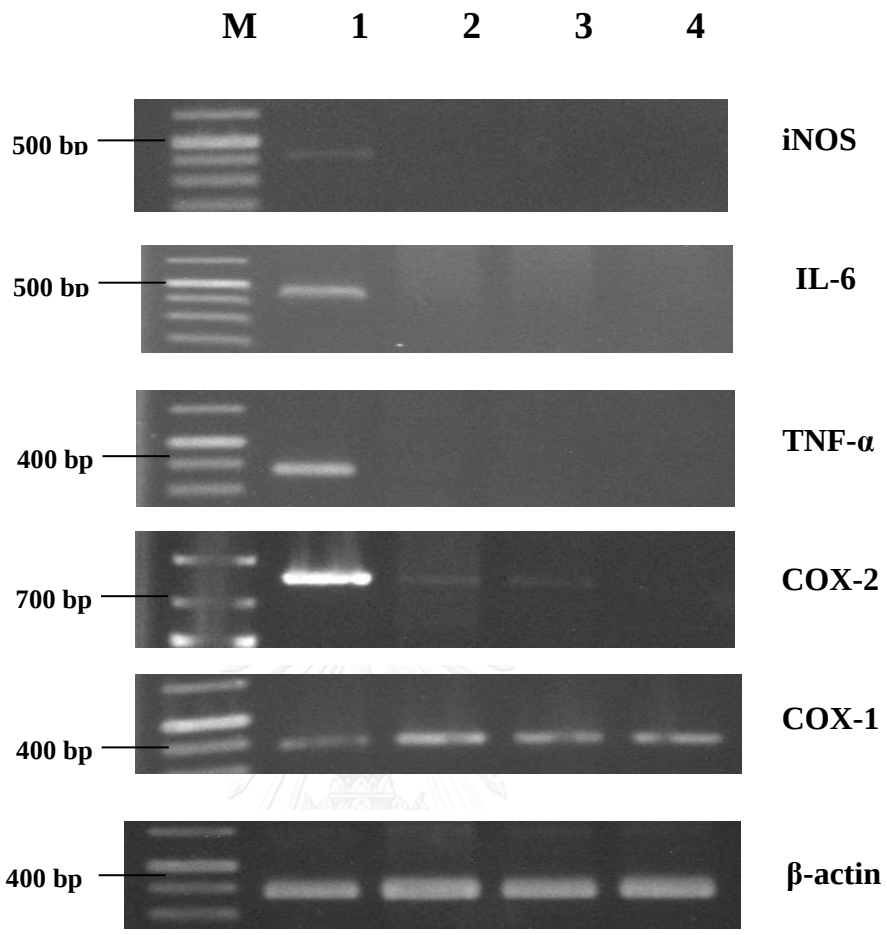


Figure 4.5 The effect of the F2 fraction on LPS induced iNOS (423 bp), IL-6 (417 bp), COX-1 (382 bp), COX-2 (861 bp) and TNF-α (375 bp) expression in macrophage RAW 264.7 cells. For all panels: Lane 1: LPS, Lane 2: LPS + F2 fraction (40 µg/ml), Lane 3: LPS+budesonide (positive control), Lane 4: no addition and Lane M: 1000 bp

CHAPTER V
CONCLUSION

In conclusion, the present study demonstrated chicken feather meal peptide produced by microbial enzyme which had anti-inflammatory activity was evaluated by nitric oxide radical scavenging assay. The results showed that 1% flavourzyme hydrolysate had the highest nitric oxide radical scavenging activity. Then, the peptide was fractionated by ultrafiltration indicating that the lowest molecular weight peptides (MW $\leq$ 0.65 kDa) were effective in their nitric oxide radical scavenging activity. The F2 fraction from Sephacryl S-200 gel filtration chromatography was not toxic and helped prevent NO production in macrophage RAW 264.7 cell-induced by LPS. The anti-inflammatory peptide was further purified by RP-HPLC and the amino acid sequence identified in 4 chains: SNPSVAGVR, SLFLHTHSIVADK, AVLKKKVTSTFGR and LSPWPVKGV. The peptide sequence contained amino acids with anti-inflammatory activity especially glycine and histidine. Moreover, the F2 fraction is a potent inhibitor of LPS-induced NO, PGE_2, TNF-α and IL-6 production at mRNA level which is a pro-inflammatory cytokine. These results suggest that chicken feather meal hydrolysate could be used as a natural anti-inflammatory in functional food or pharmaceutical products.

REFERENCES

Abarikwu, S. O. (2014). Kolaviron, a natural flavonoid from the seeds of Garcinia kola, reduces LPS-induced inflammation in macrophages by combined inhibition of IL-6 secretion, and inflammatory transcription factors, ERK1/2, NF-κB, p38, Akt, pc-JUN and JNK. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1840*(7), 2373-2381.

Abarikwu, S. O. (2015). Anti-inflammatory effects of kolaviron modulate the expressions of inflammatory marker genes, inhibit transcription factors ERK1/2, p-JNK, NF-κB, and activate Akt expressions in the 93RS2 Sertoli cell lines. *Molecular and cellular biochemistry, 401*(1-2), 197-208.

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2011). *Cellular and molecular immunology*: Elsevier Health Sciences.

Ahn, C.-B., Cho, Y.-S., & Je, J.-Y. (2015). Purification and anti-inflammatory action of tripeptide from salmon pectoral fin byproduct protein hydrolysate. *Food chemistry, 168*, 151-156.

Ahn, C.-B., Je, J.-Y., & Cho, Y.-S. (2012). Antioxidant and anti-inflammatory peptide fraction from salmon byproduct protein hydrolysates by peptic hydrolysis. *Food research international, 49*(1), 92-98.

Barton, M., Haudenschild, C. C., d'Uscio, L. V., Shaw, S., Münter, K., & Lüscher, T. F. (1998). Endothelin ETA receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. *Proceedings of the National Academy of Sciences, 95*(24), 14367-14372.

Berenbaum, F. (2000). Proinflammatory cytokines, prostaglandins, and the chondrocyte: mechanisms of intracellular activation. *Joint Bone Spine, 67*(6), 561-564.

Blough, N. V., & Zafiriou, O. C. (1985). Reaction of superoxide with nitric oxide to form peroxonitrite in alkaline aqueous solution. *Inorganic Chemistry, 24*(22), 3502-3504.

Bougatef, A., Nedjar-Arroume, N., Manni, L., Ravallec, R., Barkia, A., Guillochon, D., & Nasri, M. (2010). Purification and identification of novel antioxidant

peptides from enzymatic hydrolysates of sardinelle (Sardinellaaurita) by-products proteins. *Food chemistry, 118*(3), 559-565.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry, 72*(1-2), 248-254.

Brown, C. M., Cruz, C. D. D., Yang, E., & Wise, P. M. (2008). Inducible nitric oxide synthase and estradiol exhibit complementary neuroprotective roles after ischemic brain injury. *Experimental neurology, 210*(2), 782-787.

Bryan, N. S., & Grisham, M. B. (2007). Methods to detect nitric oxide and its metabolites in biological samples. *Free Radical Biology and Medicine, 43*(5), 645-657.

Chantaranothai, C., Palaga, T., Karnchanatat, A., & Sangvanich, P. (2013). Inhibition of nitric oxide production in the macrophage-like RAW 264.7 cell line by protein from the rhizomes of Zingiberaceae plants. *Preparative Biochemistry and Biotechnology, 43*(1), 60-78.

Chaturvedi, V., Bhange, K., Bhatt, R., & Verma, P. (2014). Production of kertinases using chicken feathers as substrate by a novel multifunctional strain of Pseudomonas stutzeri and its dehairing application. *Biocatalysis and Agricultural Biotechnology, 3*(2), 167-174.

Cheng, M.-L., Wang, H.-C., Hsu, K.-C., & Hwang, J.-S. (2015). Anti-inflammatory peptides from enzymatic hydrolysates of tuna cooking juice. *Food and Agricultural Immunology, 26*(6), 770-781.

Cian, R. E., López-Posadas, R., Drago, S. R., de Medina, F. S., & Martínez-Augustin, O. (2012). A Porphyra columbina hydrolysate upregulates IL-10 production in rat macrophages and lymphocytes through an NF-κB, and p38 and JNK dependent mechanism. *Food chemistry, 134*(4), 1982-1990.

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science & Technology, 11*(7), 254-262.

Condeelis, J., & Pollard, J. W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell, 124*(2), 263-266.

Coward-Kelly, G., Chang, V. S., Agbogbo, F. K., & Holtzapple, M. T. (2006). Lime treatment of keratinous materials for the generation of highly digestible animal feed: 1. Chicken feathers. *Bioresource technology, 97*(11), 1337-1343.

Davalos, A., Miguel, M., Bartolome, B., & Lopez-Fandino, R. (2004). Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection®, 67*(9), 1939-1944.

de Mejia, E. G., & Dia, V. P. (2009). Lunasin and lunasin-like peptides inhibit inflammation through suppression of NF-κB pathway in the macrophage. *Peptides, 30*(12), 2388-2398.

Demir, T., Hameş, E. E., Öncel, S. S., & Vardar-Sukan, F. (2015). An optimization approach to scale up keratinase production by Streptomyces sp. 2M21 by utilizing chicken feather. *International Biodeterioration & Biodegradation, 103*, 134-140.

Dhawan, V. (2014). Reactive Oxygen and Nitrogen Species: General Considerations *Studies on Respiratory Disorders* (pp. 27-47): Springer.

Dia, V. P., Wang, W., Oh, V., De Lumen, B., & De Mejia, E. G. (2009). Isolation, purification and characterisation of lunasin from defatted soybean flour and in vitro evaluation of its anti-inflammatory activity. *Food chemistry, 114*(1), 108-115.

Doucet, D., Otter, D. E., Gauthier, S. F., & Foegeding, E. A. (2003). Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. *Journal of Agricultural and Food Chemistry, 51*(21), 6300-6308.

Fang, F. C. (1997). Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *Journal of Clinical Investigation, 99*(12), 2818.

Feldmann, M., & Maini, R. N. (2001). Anti-TNFα therapy of rheumatoid arthritis: what have we learned? *Annual review of immunology, 19*(1), 163-196.

Fontoura, R., Daroit, D. J., Correa, A. P., Meira, S. M., Mosquera, M., & Brandelli, A. (2014). Production of feather hydrolysates with antioxidant, angiotensin-I converting enzyme-and dipeptidyl peptidase-IV-inhibitory activities. *New biotechnology, 31*(5), 506-513.

Fosgerau, K., & Hoffmann, T. (2015). Peptide therapeutics: current status and future directions. *Drug discovery today, 20*(1), 122-128.

Fotakis, G., & Timbrell, J. A. (2006). In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology letters, 160*(2), 171-177.

Frank, A. M., Bandeira, N., Shen, Z., Tanner, S., Briggs, S. P., Smith, R. D., & Pevzner, P. A. (2007). Clustering millions of tandem mass spectra. *Journal of proteome research, 7*(01), 113-122.

Fujiwara, N., & Kobayashi, K. (2005). Macrophages in inflammation. *Current Drug Targets-Inflammation & Allergy, 4*(3), 281-286.

Furukawa, S., Matsubara, T., Jujoh, K., Yone, K., Sugawara, T., Sasai, K., . . . Yabuta, K. (1988). Peripheral blood monocyte/macrophages and serum tumor necrosis factor in Kawasaki disease. *Clinical immunology and immunopathology, 48*(2), 247-251.

Gang, G., Jie, C., Jun-gao, W., Qiu-xia, H., & Ke-chun, L. (2013). A two-step biotechnological process for improving nutrition value of feather meal by Bacillus licheniformis S6. *Journal of Northeast Agricultural University (English Edition), 20*(3), 71-77.

Grazziotin, A., Pimentel, F., De Jong, E., & Brandelli, A. (2006). Nutritional improvement of feather protein by treatment with microbial keratinase. *Animal Feed Science and Technology, 126*(1), 135-144.

Guha, M., & Mackman, N. (2001). LPS induction of gene expression in human monocytes. *Cellular signalling, 13*(2), 85-94.

Halliwell, B. (2001). Role of free radicals in the neurodegenerative diseases. *Drugs & aging, 18*(9), 685-716.

Han, M., Li, J., Tan, Q., Sun, Y., & Wang, Y. (2010). Limitations of the use of MTT assay for screening in drug discovery. *J Chin Pharmaceu Sci, 19*, 195-200.

Hasegawa, S., Ichiyama, T., Sonaka, I., Ohsaki, A., Okada, S., Wakiguchi, H., . . . Furukawa, S. (2012). Cysteine, histidine and glycine exhibit anti-inflammatory effects in human coronary arterial endothelial cells. *Clinical & Experimental Immunology, 167*(2), 269-274.

Hemmrich, K., Suschek, C. V., Lerzynski, G., & Kolb-Bachofen, V. (2003). iNOS activity is essential for endothelial stress gene expression protecting against oxidative damage. *Journal of Applied Physiology, 95*(5), 1937-1946.

Hoebe, K., Janssen, E., & Beutler, B. (2004). The interface between innate and adaptive immunity. *Nature immunology, 5*(10), 971-974.

Ishimura, N., Bronk, S. F., & Gores, G. J. (2004). Inducible nitric oxide synthase upregulates cyclooxygenase-2 in mouse cholangiocytes promoting cell growth. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 287*(1), G88-G95.

Issekutz, A., & Movat, H. (1982). The effect of vasodilator prostaglandins on polymorphonuclear leukocyte infiltration and vascular injury. *The American journal of pathology, 107*(3), 300.

Je, J.-Y., & Kim, S.-K. (2012). 7 Chitosan as Potential Marine Nutraceutical. *Advances in food and nutrition research, 65*, 121.

Je, J. Y., & Kim, S. K. (2012). Chitosan as potential marine nutraceutical. *Adv Food Nutr Res, 65*, 121-135. doi: 10.1016/B978-0-12-416003-3.00007-X

Jung, K. H., Ha, E., Kim, M. J., Won, H. J., Zheng, L. T., Kim, H. K., . . . Yim, S. V. (2007). Suppressive effects of nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression by Citrus reticulata extract in RAW 264.7 macrophage cells. *Food Chem Toxicol, 45*(8), 1545-1550. doi: 10.1016/j.fct.2007.02.017

Kavazis, A. N., & Powers, S. K. (2013). Impact of Exercise, Reactive Oxygen and Reactive Nitrogen Species on Tumor Growth *Exercise, Energy Balance, and Cancer* (pp. 7-20): Springer.

Kim, E.-K., Kim, Y.-S., Hwang, J.-W., Kang, S. H., Choi, D.-K., Lee, K.-H., . . . Park, P.-J. (2013). Purification of a novel nitric oxide inhibitory peptide derived from enzymatic hydrolysates of Mytilus coruscus. *Fish & shellfish immunology, 34*(6), 1416-1420.

Kim, G.-H. (2008). Renal effects of prostaglandins and cyclooxygenase-2 inhibitors. *Electrolyte & Blood Pressure, 6*(1), 35-41.

Kim, Y.-S., Ahn, C.-B., & Je, J.-Y. (2016). Anti-inflammatory action of high molecular weight Mytilus edulis hydrolysates fraction in LPS-induced

RAW264. 7 macrophage via NF-κB and MAPK pathways. *Food chemistry, 202*, 9-14.

Klompong, V., Benjakul, S., Kantachote, D., Hayes, K. D., & Shahidi, F. (2008). Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. *International journal of food science & technology, 43*(6), 1019-1026.

Kristinsson, H. G., & Rasco, B. A. (2000). Fish protein hydrolysates: production, biochemical, and functional properties. *Critical reviews in food science and nutrition, 40*(1), 43-81.

Kumar, D., Lavanya, S., Priya, S., Rebecca, A. I. N., Balakumaran, M., & Kalaichelvan, P. (2012). Production of feather protein concentrate from feathers by in vitro enzymatic treatment, its biochemical characterization and antioxidant nature. *Middle-East J. Sci. Res, 11*(7), 881-886.

Kwiatkowski, K., Krzysztoforski, J., Bajer, K., & Dudyński, M. (2013). Bioenergy from feathers gasification–Efficiency and performance analysis. *Biomass and Bioenergy, 59*, 402-411.

Lappas, M., Permezel, M., Georgiou, H. M., & Rice, G. E. (2002). Nuclear factor kappa B regulation of proinflammatory cytokines in human gestational tissues in vitro. *Biology of Reproduction, 67*(2), 668-673.

Lasekan, A., Bakar, F. A., & Hashim, D. (2013). Potential of chicken by-products as sources of useful biological resources. *Waste management, 33*(3), 552-565.

Lassoued, I., Mora, L., Nasri, R., Aydi, M., Toldrá, F., Aristoy, M.-C., . . . Nasri, M. (2015). Characterization, antioxidative and ACE inhibitory properties of hydrolysates obtained from thornback ray (Raja clavata) muscle. *Journal of proteomics, 128*, 458-468.

Lawrence, T., Willoughby, D. A., & Gilroy, D. W. (2002). Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nature Reviews Immunology, 2*(10), 787-795.

Lee, S.-J., Cheong, S. H., Kim, Y.-S., Hwang, J.-W., Kwon, H.-J., Kang, S.-H., . . . Park, P.-J. (2013). Antioxidant activity of a novel synthetic hexa-peptide derived from an enzymatic hydrolysate of duck skin by-products. *Food and chemical toxicology, 62*, 276-280.

Lee, S.-J., Kim, E.-K., Kim, Y.-S., Hwang, J.-W., Lee, K. H., Choi, D.-K., . . . Park, P.-J. (2012). Purification and characterization of a nitric oxide inhibitory peptide from Ruditapes philippinarum. *Food and chemical toxicology, 50*(5), 1660-1666.

Li, H.-J., & Deinzer, M. L. (2007). Tandem mass spectrometry for sequencing proanthocyanidins. *Analytical chemistry, 79*(4), 1739-1748.

Liu, J., Wang, Z.-T., & Ge, B.-X. (2008). Andrograpanin, isolated from Andrographis paniculata, exhibits anti-inflammatory property in lipopolysaccharide-induced macrophage cells through down-regulating the p38 MAPKs signaling pathways. *International immunopharmacology, 8*(7), 951-958.

Lopez-Torres, M., Pérez-Campo, R., & de Quiroga, G. B. (1991). Effect of natural ageing and antioxidant inhibition on liver antioxidant enzymes, glutathione system, peroxidation, and oxygen consumption inRana perezi. *Journal of Comparative Physiology B, 160*(6), 655-661.

Manninen, A. H. (2009). Protein hydrolysates in sports nutrition. *Nutrition & metabolism, 6*(1), 1.

McCarthy, A. L., O'Callaghan, Y. C., & O'Brien, N. M. (2013). Protein hydrolysates from agricultural crops—bioactivity and potential for functional food development. *Agriculture, 3*(1), 112-130.

Menaga, D., Rajakumar, S., & Ayyasamy, P. (2013). Free radical scavenging activity of methanolic extract of pleurotus florida Mushroom. *Int J Pharm Pharm Sci, 5*(4), 601-606.

Mendis, E., Rajapakse, N., & Kim, S.-K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *Journal of agricultural and food chemistry, 53*(3), 581-587.

Moncada, S., Palmer, R., & Higgs, E. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological reviews, 43*(2), 109-142.

Montoya-Rodríguez, A., Mejía, E. G., Dia, V. P., Reyes-Moreno, C., & Milán-Carrillo, J. (2014). Extrusion improved the anti-inflammatory effect of amaranth (Amaranthus hypochondriacus) hydrolysates in LPS-induced human THP-1 macrophage-like and mouse RAW 264.7 macrophages by preventing

activation of NF-κB signaling. *Molecular nutrition & food research, 58*(5), 1028-1041.

Najafian, L., & Babji, A. S. (2012). A review of fish-derived antioxidant and antimicrobial peptides: their production, assessment, and applications. *Peptides, 33*(1), 178-185.

Nathan, C., & Xie, Q.-w. (1994). Nitric oxide synthases: roles, tolls, and controls. *Cell, 78*(6), 915-918.

Ndiaye, F., Vuong, T., Duarte, J., Aluko, R. E., & Matar, C. (2012). Anti-oxidant, anti-inflammatory and immunomodulating properties of an enzymatic protein hydrolysate from yellow field pea seeds. *European journal of nutrition, 51*(1), 29-37.

Nomura, Y., Aihara, M., Nakajima, D., Kenjou, S., Tsukuda, M., & Tsuda, Y. (2005). Process for producing solubilized keratin: Google Patents.

Omer, N., Rohilla, A., Rohilla, S., & Kushnoor, A. (2012). Nitric oxide: role in human biology. *Int J Pharm Sci Drug Res, 4*(2), 105-109.

Pacher, P., Beckman, J. S., & Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiological reviews, 87*(1), 315-424.

Park, E.-M., Cho, S., Frys, K. A., Glickstein, S. B., Zhou, P., Anrather, J., . . . Iadecola, C. (2006). Inducible nitric oxide synthase contributes to gender differences in ischemic brain injury. *Journal of Cerebral Blood Flow & Metabolism, 26*(3), 392-401.

Parul, R., Saha, P., & Kundu, S. K. (2013). In Vitro Nitric Oxide Scavenging Activity Of Methanol Extracts Of Three Bangladeshi Medicinal Plants. *The Pharma Innovation, 1*(12).

Pham-Huy, L. A., He, H., & Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *Int J Biomed Sci, 4*(2), 89-96.

Pihlanto-Leppälä, A. (2000). Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends in Food Science & Technology, 11*(9), 347-356.

Radha, C., Kumar, P. R., & Prakash, V. (2008). Preparation and characterization of a protein hydrolysate from an oilseed flour mixture. *Food chemistry, 106*(3), 1166-1174.

REBECA, B. D., Pena-Vera, M., & Diaz-Castaneda, M. (1991). Production of fish protein hydrolysates with bacterial proteases; yield and nutritional value. *Journal of Food Science, 56*(2), 309-314.

Reddy, D. B., & Reddanna, P. (2009). Chebulagic acid (CA) attenuates LPS-induced inflammation by suppressing NF-κB and MAPK activation in RAW 264.7 macrophages. *Biochemical and Biophysical Research Communications, 381*(1), 112-117.

Sacchetti, G., Di Mattia, C., Pittia, P., & Martino, G. (2008). Application of a radical scavenging activity test to measure the total antioxidant activity of poultry meat. *Meat science, 80*(4), 1081-1085.

Saisavoey, T., Sangtanoo, P., Reamtong, O., & Karnchanatat, A. (2016). Antioxidant and Anti-Inflammatory Effects of Defatted Rice Bran (Oryza Sativa L.) Protein Hydrolysates on Raw 264.7 Macrophage Cells. *Journal of Food Biochemistry*.

Salwanee, S., Wan Aida, W., Mamot, S., & Maskat, M. Y. (2013). Effects of enzyme concentration, temperature, ph and time on the degree of hydrolysis of protein extract from viscera of tuna (euthynnus affinis) by using alcalase. *Sains Malaysiana, 42*(3), 279-287.

Sanmartín, E., Arboleya, J. C., Villamiel, M., & Moreno, F. J. (2009). Recent advances in the recovery and improvement of functional proteins from fish processing by-products: Use of protein glycation as an alternative method. *Comprehensive Reviews in Food Science and Food Safety, 8*(4), 332-344.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: a review. *Peptides, 31*(10), 1949-1956.

Senthilraja, P., & Kathiresan, K. (2015). In vitro cytotoxicity MTT assay in Vero, HepG2 and MCF-7 cell lines study of Marine Yeast.

Smith, W. L., & Langenbach, R. (2001). Why there are two cyclooxygenase isozymes. *The Journal of clinical investigation, 107*(12), 1491-1495.

Sprague, A. H., & Khalil, R. A. (2009). Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochemical pharmacology, 78*(6), 539-552.

Sun, X., Chakrabarti, S., Fang, J., Yin, Y., & Wu, J. (2016). Low-molecular-weight fractions of Alcalase hydrolyzed egg ovomucin extract exert anti-inflammatory activity in human dermal fibroblasts through the inhibition of tumor necrosis factor–mediated nuclear factor κB pathway. *Nutrition Research, 36*(7), 648-657.

Tanaka, T., Narazaki, M., & Kishimoto, T. (2014). IL-6 in inflammation, immunity, and disease. *Cold Spring Harbor perspectives in biology, 6*(10), a016295.

Taskin, M., Sisman, T., Erdal, S., & Kurbanoglu, E. B. (2011). Use of waste chicken feathers as peptone for production of carotenoids in submerged culture of Rhodotorula glutinis MT-5. *European Food Research and Technology, 233*(4), 657-665.

Tiwary, E., & Gupta, R. (2013). Rapid conversion of chicken feather to feather meal using dimeric keratinase from Bacillus licheniformis ER-15. *Journal of Bioprocessing & Biotechniques, 2012*.

Vo, T.-S., Ryu, B., & Kim, S.-K. (2013). Purification of novel anti-inflammatory peptides from enzymatic hydrolysate of the edible microalgal Spirulina maxima. *Journal of Functional Foods, 5*(3), 1336-1346.

Wang, A.-Y., Zhou, M.-Y., & Lin, W.-C. (2011). Antioxidative and anti-inflammatory properties of Citrus sulcata extracts. *Food chemistry, 124*(3), 958-963.

Wang, S. Y., Lan, X. Y., Xiao, J. H., Yang, J. C., Kao, Y. T., & Chang, S. T. (2008). Antiinflammatory activity of Lindera erythrocarpa fruits. *Phytotherapy Research, 22*(2), 213-216.

Wiseman, H., & Halliwell, B. (1996). Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochemical Journal, 313*(Pt 1), 17.

Yang, X., Kang, M.-C., Li, Y., Kim, E.-A., Kang, S.-M., & Jeon, Y.-J. (2014). Anti-inflammatory activity of questinol isolated from marine-derived fungus Eurotium amstelodami in lipopolysaccharide-stimulated RAW 264.7 macrophages. *J. Microbiol. Biotechnol, 24*(10), 1346-1353.

Yu, H., Pardoll, D., & Jove, R. (2009). STATs in cancer inflammation and immunity: a leading role for STAT3. *Nature Reviews Cancer, 9*(11), 798-809.

Yu, M., Lamattina, L., Spoel, S. H., & Loake, G. J. (2014). Nitric oxide function in plant biology: a redox cue in deconvolution. *New Phytologist, 202*(4), 1142-1156.

Zhuang, Y., Hou, H., Zhao, X., Zhang, Z., & Li, B. (2009). Effects of collagen and collagen hydrolysate from jellyfish (Rhopilema esculentum) on mice skin photoaging induced by UV irradiation. *Journal of Food Science, 74*(6), H183-H188.

APPENDIX

APPENDIX A

Preparation of 20 mM PBS with 0.15 M NaCl 1 L

KH_2PO_4	2.72 g
K_2HPO_4	3.48 g
NaCl	8.77 g

Adjusted pH to 7.2 with 1M KOH and adjusted volume to 1 L with deionized water.

Preparation of Bradford solution

Bradford Stock Solution 300 ml

95% ethanol	100 ml
88% phosphoric acid	200 ml
Serva Blue G	350 mg

Bradford Working buffer 500 ml

95% ethanol	15 ml
88% phosphoric acid	30 ml
Bradford stock solution	30 ml
Deionized water	425 ml

Preparation of solution for nitric oxide radical scavenging assay

10 mM sodium nitroprusside (SNP) in PBS pH 7.2

0.29 g of sodium nitroprusside was dissolve in 100 ml PBS pH 7.2

0.33% (w/v) sulfanilamide solution

0.33 g of sulfanilamide was dissolved in 5% phosphoric acid, and adjusted total volume to 100 ml by deionized water.

0.1% (w/v) N- (1-Napthyl) ethylenediamine dihydrochloride (NED)

0.1 g of NED was dissolved in 100 ml

APPENDIX B

Media: Complete medium

DMEM	100%
Fetal Bovine Serum (FBS)	10%

MTT solution

5 mg/ml MTT solution

MTT	5 mg
Deionized water	1 ml

LPS 1 µg/ml

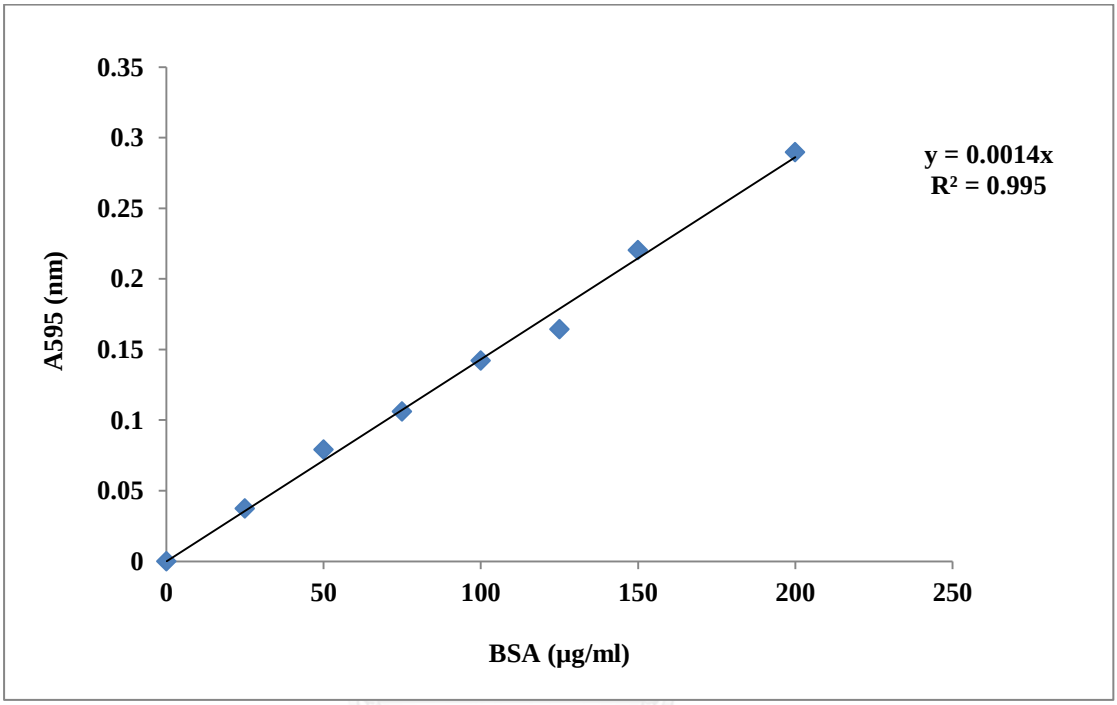
LPS 500 µg/ml	4 µl
DMEM	2 µl

TE buffer

50X Tris-acetic acid EDTA (TAE) buffer solution

APPENDIX C

Standard curve of protein determination by Bradford method



APPENDIX D

Amino acid abbreviations

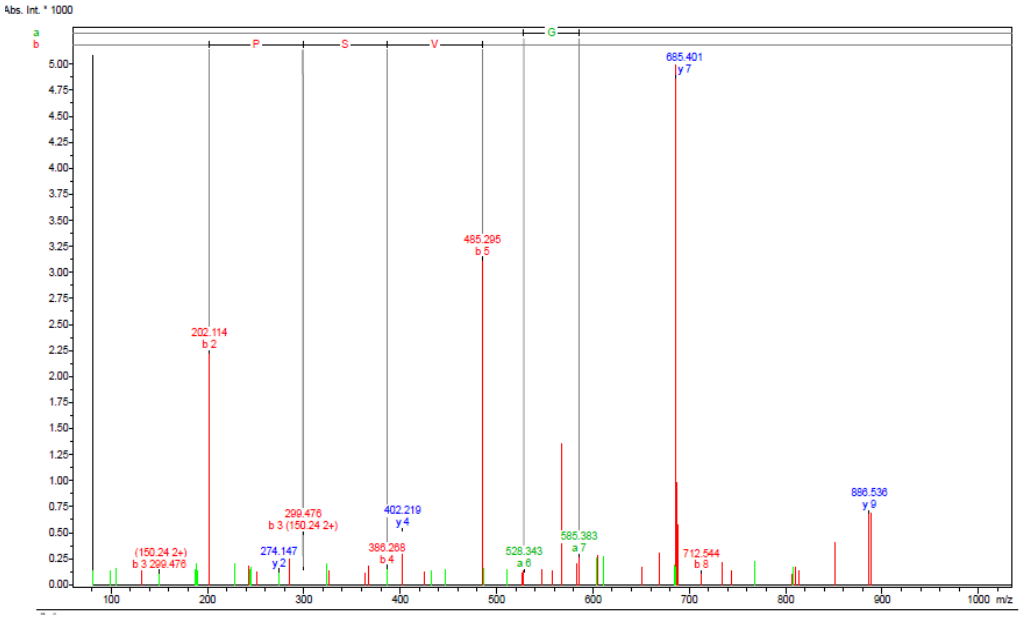
Amino acid	Three-letter	One-letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic-acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr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

APPENDIX E

Sequence F2-1 SNPSVAGVR

Parentmass 886.536

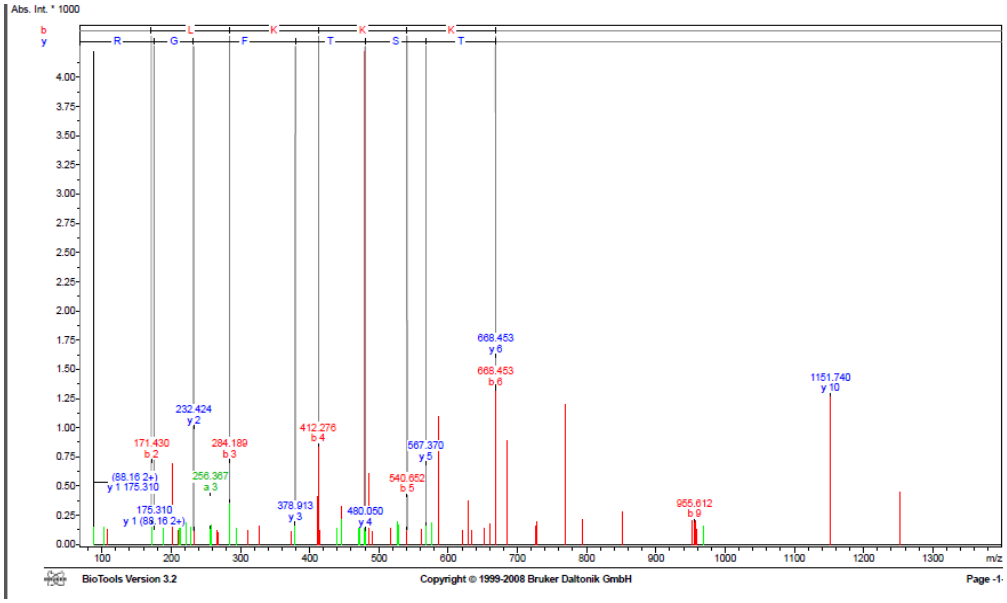
Mass error 0.061 MH+ (mono): 886.475 MH+ (avq): 886.975
Threshold (a.i): 0.000 Tolerance (Da): 0.500 Number of peak: 60
Above Threshold: 60 Assigned Peaks: 11 Not assigned Peaks: 49



Sequence F2-3 AVLKKKVTSTFGR

Parentmass 1434.917

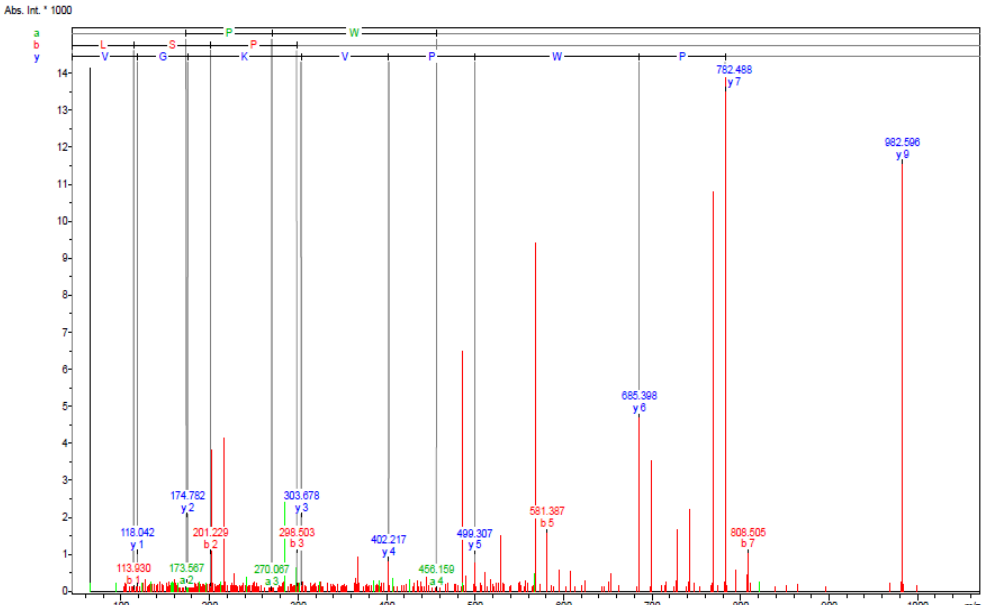
Mass error 0.037 MH+ (mono): 1434.880 MH+ (avq): 1436.737
Threshold (a.i): 0.000 Tolerance (Da): 0.500 Number of peak: 72
Above Threshold: 72 Assigned Peaks: 13 Not assigned Peaks: 59



Sequence F2-5 LSPWPVKGV

Parentmass 886.536

Mass error 0.061 MH+ (mono): 886.475 MH+ (avq): 886.975
Threshold (a.i): 0.000 Tolerance (Da): 0.500 Number of peak: 60
Above Threshold: 60 Assigned Peaks: 11 Not assigned Peaks: 49



VITA

Miss Romteera Sukaboon was born on March 3, 1992 in Pattani, Thailand. She got a Bacherlor's Degree of Science from School of Cosmetic science from Mae Fah Luang University, Thailand in 2013. She was further studied to the Master Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University in 2014.

Academic presentation;

1) Sukaboon, R., Sangtanoo, P., Saisavoey, T., and Karnchanatat, A. Inhibitory activity against nitric oxide production in macrophage RAW 264.7 by peptide hydrolysate derived from chicken feather meal. In "The 5th International Biochemistry and Molecular Biology Conference" 26-27 May 2016, B.P. Samila Beach Hotel, Bangkok Thailand (Proceeding book)