Preparation and Characterization of Immunomodulatory Peptides from Spent Hen Muscle Proteins

by

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ABSTRACT

Each year the egg industry produces about 18 million spent hens in Canada. Since a viable market is not available, spent hens are a liability to the egg producers who have to pay the cost associated with disposal. The overall objective of this thesis was to prepare and characterize bioactive peptides with immunomodulatory properties from underutilized spent hens. We hypothesized that spent hens could be a good source of muscle proteins for preparing immunomodulatory peptides. Spent hen muscle proteins were hydrolyzed by nine proteases with degree of hydrolysis and the nitrogen recovery ranging from 11.2 to 32.7%, and from 44.4 to 86.5%, respectively; the molecular weight of all hydrolysates was less than 12kDa. In LPS-activated macrophage-like U937 cells, at a concentration of 500 μg/mL, Protease M and Protex 50FP hydrolysates inhibited the production of interleukin (IL)-6 and tumor necrosis factor (TNF)-α by 13% and 25%, 15% and 4%, respectively, in comparison to 28% and 57% inhibition by lactoferrin. The immunomodulatory properties of these two hydrolysates were further studied in healthy weanling Sprague-Dawley rats. Feeding at a dose of 5% (w/w diet based), only Protex 50FP hydrolysate exhibited a stimulatory effect on IL-10 (regulatory cytokine) in splenocytes stimulated *ex vivo* with mitogens (lipopolysaccharides, LPS; pokeweed mitogen, PWM; $p<0.05$). Meanwhile, no detrimental effect was observed in the young rodents at this dosage. The IL-10 stimulatory activity and *in vitro* IL-6 inhibitory activity suggests potential anti-inflammatory properties of this hydrolysate, which prompted us to test the hydrolysate in an inflammation-related disease model. In a hapten-induced colitis model, three weeks pretreatment of Protex 50FP hydrolysate attenuated the weight loss up to 48 hr post disease induction. On day 5 after

colitis treatment, this hydrolysate did not show any protective effect on colon damage and immune functions; but changes were observed on phenotype, including a higher proportion of T_H cells expressing CD25 (IL-2 receptor) in spleen and a lower proportion of T cells expressing CTLA-4 (activation marker) in mesenteric lymph nodes. To characterize the peptides with IL-6 inhibitory activity, these two hydrolysates (Protex 50FP and Protease M) were subjected to ultrafiltration, cation exchange chromatography and reverse-phase chromatography. Twenty five novel peptide sequences were identified from these two hydrolysates. This thesis reported for the first time the preparation and characterization of immunomodulatory peptides from spent hen muscle proteins. This study might lead to alternative method of spent hen uses for preparing "value-added" functional bioactive peptides.

PREFACE

This thesis contains original work done by Wenlin Yu and has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta. The concept of this thesis originated from my supervisor Dr. Jianping Wu and the research was funded by the grants from Alberta Livestock and Meat Agency (ALMA) and the Natural Sciences and Engineering Research Council of Canada (NSERC) discovery grant. The experimental protocol for the animal study was approved by the Animal Care and Use Committee at the University of Alberta (Protocol # 00000571) in accordance with the guidelines issued by the Canada Council on Animal Care.

The thesis consisted of seven chapters: Chapter 1 provides a general introduction on the context and the objectives of the thesis; Chapter 2 is a literature review on several topics relevant to this thesis, including food-derived bioactive peptides, immune and dietary nutrients with a focus on peptides, and current challenge and potential of bioactive peptides; Chapter 3 contains the work on hydrolysate preparation and characterization, and activity study using a cell model of endotoxin-activated macrophage; Chapter 4 further investigates immunomodulatory properties of the hydrolysate by feeding to healthy rats; Chapter 5 is about evaluating the potential of spent hen hydrolysate in a diseased model of colitis rats; Chapter 6 describes fractionation and characterization of peptides with IL-6 inhibitory activity from spent hen hydrolysate; the last Chapter 7 gives concluding remarks and discusses for future research direction.

Dr. Jianping Wu and Dr. Catherine J. Field greatly contributed to the experimental design, data interpretation, thesis preparation and edits. Dr. Vera C. Mazurak helped with the thesis preparation and edits. I was responsible for literature search relevant for the above studies, designing and performing laboratory experiments, data collection and analysis, and drafting the thesis. Ms. Susan Goruk has provided substantial guidance and assistance for the cell culture study and animal studies. Mrs. Nicole Coursen and Mrs. Kristina MacNaughton have provided technical assistance in animal studies including animal husbandry and tissue sampling upon termination. Mr. Gary Sedgwick performed the amino acid analysis of the enzymatic hydrolysates in Chapter 3. Dr. Keith Sharkey and Ms. Cathy MacNaughton helped to review the Standard Operating Procedure for inducing colitis in rats in Chapter 5. Dr. Richard Uwiera provided guidance for histological analysis of colon slides. And Mrs. Jing Zheng performed the mass spectrometry of peptide fractions in Chapter 6.

DEDICATION

Dedicated to my beloved parents,

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LIST OF ABBREVIATIONS

ACE: Angiotensin I converting enzyme

ACN: Acetonitrile

bLf: Bovine lactoferrin

BW: Body weight

CD (disease): Crohn's disease

CD (immune): Cluster of differentiation

ConA: Concanavalin A

CPPs: Caseinophosphopeptides

CRP: C-reactive protein

DC: Dendritic cells

DH: Degree of hydrolysis

DTH: delayed-type hypersensitivity

ELISA: Enzyme-linked immunosorbent assay

FI: Food intake

GALT: Gut-associated lymphoid tissue

GMP: Glycomacropeptide

HPLC: High-performance liquid chromatography

IBD: Inflammatory bowel diseases

IFN: Interferon

IL: Interleukin

LPS: Lipopolysaccharides

MAPK: Mitogen-activated protein kinases

MHC: Major histocompatibility complex

MLN: Mesenteric lymph node

MPO: Myeloperoxidase

NF-κB: Nuclear factor kappa B

NK: Natural killer (cell)

NO: Nitric oxide

NR: Nitrogen recovery

PMA: Phorbol 12-myristate 13-acetate

PMN: Polymorphonuclear leukocytes

PWM: Pokeweed mitogen

ROS: Reactive oxygen species

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TFA: Trifluoroacetic acid

TGF: Transforming growth factor

Th/T_H: Helper T (cell)

TLR: Toll-like receptor

TNBS: 2,4,6-Trinitrobenzenesulfonic acid

TNF: Tumor necrosis factor

Treg: Regulatory T (cell)

UC: Ulcerative colitis

CHAPTER 1 – General Introduction and Objectives

1.1. General introduction

There were 1,007 registered egg farms in Canada, generating $893.5 million in total farm cash receipts (AAFC, 2013b). In 2013, Canada exported 8.3 million kilograms of processed eggs worth approximately $21.1 million and 760,381 dozen shell eggs worth over $1.1 million (AAFC, 2013a). Spent hens are layers that are to be replaced usually after one laying cycles. It is estimated that each year the egg industry produces about 18 million spent hens in Canada and 150 million in North America (Newberry, Webster, Lewis, & Van Arnam, 1999). Although spent hens are processed for food and feed uses, the value of meat processed from spent hens is low due to its high cost of processing, a relatively low yield, and poor meat quality such as objectionable toughness and the presence of residual bone fragments (Kersey & Waldroup, 1998), when compared with high-meat-yielding broiler chickens. For this reason, euthanasia followed by burial, composting, or incineration are often used to dispose spent hens, which generates additional expenses to the producer and raises concerns over environmental impact and animal welfare (Freeman, Poore, Middleton, & Ferket, 2009; Newberry et al., 1999). Therefore, there is a demand for alternative way of utilization of spent hens.

Spent hen, as an inexpensive source of protein, can be utilized for production of bioactive peptides. It was reported that manually deboned spent hen meat contains 78.3% moisture, 3.7% fat and 15.3% protein (McIvor, Baccus-Taylor, & Comissiong, 2002). Bioactive peptides constitute an important category of health-promoting nutrients for uses as functional foods or nutraceuticals that convey physiological benefits beyond their basic nutritional values (Hettiarachchy, Sato, Marshall, & Kannan, 2011; Yoshinori Mine, Li-Chan, & Jiang, 2010). The growing awareness among consumers on the use of foods to promote well-being and to help reduce the risk of diseases has contributed to a worldwide growing market of functional foods and natural health products (AAFC, 2009, 2014).

Food-derived peptides have been reported to have various bioactivities such as antihypertensive, antimicrobial, antioxidant, mineral-binding, immunomodulatory, anticancer, opioid and cholesterol-lowering activities (Hartmann & Meisel, 2007; Korhonen & Pihlanto, 2006; Yamamoto, Ejiri, & Mizuno, 2003). Protein rich foods such as milk (Korhonen, 2009; Meisel, 2004), egg (Mine & Kovacs-Nolan, 2006; Wu & Majumder, 2010), meat (Udenigwe & Howard, 2013; Wu, Jahandideh, Yu, & Majumder, 2015), fish (Harnedy & FitzGerald, 2012; Kim & Wijesekara, 2010) and pulse (López-Barrios, Gutiérrez-Uribe, & Serna-Saldívar, 2014) are good candidates for production of bioactive peptides. Spent hen meat contains the characteristic muscle proteins including myosin, actin, titin, tropomyosin, troponin, glyceraldehyde phosphate dehydrogenase, myoglobin, collagen and elastin (Keeton & Eddy, 2004); from which many bioactive sequences have been identified with antihypertensive, antioxidant and antimicrobial activities (Udenigwe & Howard, 2013). To our knowledge, there has been no study on immunomodulatory peptides from chicken meat proteins. The potential of chicken as a raw material for immune functions can be traced back to the traditional use of chicken soup as a home remedy in treating common cold in many parts of the world (American College of Chest Physicians, 2000). Its medicinal benefit in mitigating inflammation was supported by new evidence of neutrophil chemotaxis inhibitory activity using homemade or commercial chicken soups (Rennard, Ertl, Gossman, Robbins, & Rennard, 2000). The active components for this effect are not known but could be due to the peptides released upon heating.

The immune system is vital to our health providing host defense against foreign organisms or substances. Improper immune responses (deficiency/overreaction) often result in common manifestations such as immunodeficiency, allergies and asthma, autoimmune diseases (Kindt, Goldsby, Osborne, & Kuby, 2007). The onset and progression of various chronic diseases has been associated with persist inflammation, such as in the case of metabolic disorders (Hotamisligil, 2006), atherosclerosis (Hansson, 2005; Libby, 2002), and some types of cancer (de Visser, Eichten, & Coussens, 2006). Abnormal levels of cytokines (a major marker of immune functions) have been reported in many inflammation-associated diseases. For example, elevated IL-6 levels have been found in

serum and synovial fluid of patients with rheumatoid arthritis; most studies in adult patients with inflammatory bowel disease have shown augmented IL-6 serum levels as well as a strong correlation between serum IL-6 levels and clinical activity of the disease (Neurath & Finotto, 2011). TNF-α levels correlate with the degree of adiposity and the associated insulin resistance; and circulating TNF-α levels were found to be elevated in sera of patients with chronic heart failure (Aggarwal, Gupta, & Kim, 2012). Targeting pro-inflammatory cytokines (IL-6, TNF-α) and/or its receptors has been suggested as a promising treatment strategy for these conditions (Aggarwal, Gupta, & Kim, 2012; Neurath & Finotto, 2011). On the other hand, cytokine IL-10 plays a central role in regulation of inflammatory responses to minimize host tissue injury; protective roles of IL-10 have been suggested in murine models of rheumatoid arthritis, allergy and colitis (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001). To date, food-derived peptides have been reported to exert various modulatory activities on production of cytokines as well as other immune indices, which will be reviewed in Chapter 2.

1.2. Objectives and hypotheses

The overall objective of this research was to prepare and characterize bioactive peptides with immunomodulatory properties from underutilized spent hens for development of "value-added" functional food products. We hypothesized that spent hen muscle proteins could be a good source of immunomodulatory peptides when released by enzymatic hydrolysis using a variety of commercially available food-grade proteases. The specific objectives of thesis are:

1) To prepare and characterize spent hen muscle protein hydrolysates with immunomodulatory properties.

2) To evaluate the safety and the *in vivo* immunomodulatory effects of spent hen hydrolysates in healthy young rodents.

3) To test the anti-inflammatory activity of spent hen muscle protein hydrolysate in a colitis model.

4) To purify and identify peptide sequences from the spent hen hydrolysates using liquid chromatography and LC-MS/MS.

1.3. Chapter format

The studies performed to achieve the specific objectives were organized in thesis chapters; brief descriptions of each experimental chapter were given as follows:

Chapter 3 describes the procedure of peptides preparation using the spent hen carcass as the starting material. Nine different food-grade commercial proteases were tested for their ability in generation of peptides with immunomodulatory properties. Derived hydrolysates were characterized for the protein content, nitrogen recovery, degree of hydrolysis, molecular weight profile, and amino acids composition. The effect of hydrolysates on immune function was tested in endotoxin-activated macrophages, a commonly used cell model of anti-inflammatory activity (Udenigwe & Aluko, 2012); the cytokine (IL-6, TNF-α) production and cell viability after peptide treatment were studied. Objective 1 is addressed in this chapter.

Chapter 4 examines the effects of two hydrolysates (that exhibited effects in cells) on immune function of healthy weanling Sprague-Dawley rats when fed in diet. The impact of dietary supplement of spent hen hydrolysate on proportion of immune cells in spleen, and mitogen-stimulated cytokine responses were studied; the body weight, food intake and organ characteristics were also recorded for safety evaluation. Objective 2 is addressed in this chapter.

Chapter 5 investigates the application/benefits of the immunomodulatory properties of a spent hen hydrolysate (prepared by Protex 50FP hydrolysis) in rats induced colitis by chemical reagent 2,4,6-trinitrobenzenesulfonic acid (TNBS), representing a model of Crohn's disease. The rats were monitored for body weight, food intake and distress and stool consistency. Colon damage was assessed upon termination. This study also investigates the impact of TNBS-induced colitis and the hydrolysate treatment on immune

cell phenotype and mitogen-stimulated cytokine production using immune cells isolated from rat spleen and mesenteric lymph nodes. Objective 3 is addressed in this chapter.

Chapter 6 studies the purification of peptides with IL-6 inhibitory activity. Peptides were purified by ultrafiltration, cation exchange chromatography and reverse-phase chromatography based on the structural characteristics including size (molecular weight), net charge and hydrophobicity. The fractions exhibiting strong IL-6 inhibitory activity were sequenced by mass spectrometry. Objective 4 is addressed in this chapter.

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CHAPTER 2 – Literature Review

2.1. Bioactive peptides from food proteins

Diet plays an important role in the maintenance and promotion of human health. In addition to providing us with macronutrient and micronutrient for energy and metabolism, the presence of bioactive components in diet are believed to exert beneficial effects for certain chronic diseases, such as cardiovascular diseases, cancers, and diabetes (Jew, AbuMweis, & Jones, 2009). In recent decades we have seen the disease pattern shifting from a dominance of acute, infectious diseases to chronic degenerative diseases (WHO, 2014). Comparing to pharmaceuticals, food-derived compounds have the advantage of a lower risk of negative side effects likely due to their naturally existing mechanism for metabolism.

Of various dietary compounds, bioactive peptides derived from dietary proteins represent an important category. These peptides remain inactive within the sequence of their parent proteins but can be released by digestion, fermentation or food processing (Korhonen & Pihlanto, 2006). Food-derived peptides may exert a wide range of physiological activities, such as antihypertensive, antimicrobial, antioxidant, immunomodulatory, opioid and anticancer activities, upon administration (Hartmann & Meisel, 2007; Korhonen & Pihlanto, 2006). Bioactive peptides derived from various food proteins and their respective activities are briefly reviewed in the following subsections (2.1.1-2.1.5). A critical review of immunomodulatory peptides is presented in section 2.2.4.

2.1.1. Antihypertensive peptides

Cardiovascular diseases (CVDs), accounting for 31% of all global death, are the leading cause of death in many countries according to the World Health Organization (WHO, 2015). Hypertension (high blood pressure) is a major risk factor in CVDs. Antihypertensive peptides are mainly angiotensin converting enzyme (ACE)-inhibitory peptides, as elevated ACE activity is associated with high blood pressure and high rate of vasoconstriction. Pharmacological ACE inhibitory drugs (e.g. Captopril, Enalapril) are

effective antihypertensive drugs but associated with side effects such as dry cough, hyperkalemia, fatigue, nausea, and renal failure (Sangole & Dadkar, 2010), which prompted research interests in searching for safer alternatives.

A great number of ACE-inhibitory peptides have been identified from various plant and animal protein sources, such as milk, egg, meat, fish and legumes (FitzGerald & Meisel, 2000; Iwaniak, Minkiewicz, & Darewicz, 2014). ACE-inhibitory activity was evaluated *in vitro* by IC_{50} (concentration required to decrease the ACE activity by 50%) values. ACE-inhibitory peptides were reported with IC_{50} values ranged from less than 1 μM to few hundreds of μM, in comparison to an IC_{50} value of ~0.006 μM of the reference drug Captopril (FitzGerald & Meisel, 2000). Although ACE-inhibitory peptides had weaker activities *in vitro* compared to Captopril, *in vivo* anti-hypertensive activities of certain ACE-inhibitory peptides were comparable to Captopril as tested in spontaneously hypertensive rat (SHR). VPP (IC_{50} = 9.0 μM) and IPP (IC_{50} = 5.0 μM) from milk casein fed to SHR at a dose of 0.6 and 0.3 mg/kg BW, respectively, reduced blood pressure by ~30 mmHg 4h or 8h after oral administration (Nakamura, Yamamoto, Sakai, & Takano, 1995). LKP from dried bonito (IC_{50} = 0.3 μM) fed to SHR at a dose of 9 mg/kg BW reduced systolic blood pressure by 15 mmHg 2h after oral administration (Fujita & Yoshikawa, 1999). Egg ovotransferrin derived peptide IRW (IC_{50} = 0.6 μM), when fed to SHRs at a dose of 15 mg/kg BW significantly reduced blood pressure by 40 mmHg in an 18 days of feeding (Majumder, Chakrabarti, Morton, et al., 2013). These peptides are effective in regulating blood pressure, yet did not produce any significant side effects as ACE inhibitor drugs; therefore they have great potential in utilization for nutraceutical/functional food development.

2.1.2. Antimicrobial peptides

Both animals and plants possess potent antimicrobial peptides as an important component of (innate) immune defenses. These peptides are usually short cationic amphipathic peptides with broad-spectrum activities against bacteria, fungi, viruses and protozoa. Once accumulated on the cell surface and inserted to the membrane, the antimicrobial peptides

act by either disrupting the integrity of the membrane, or translocate across the membrane to act on internal targets (Hancock & Sahl, 2006). Antimicrobial peptides have shown great potential as alternative to conventional antibiotics for treating infectious diseases, especially to antibiotic-resistant bacteria.

Antimicrobial peptides have been identified from enzymatic hydrolysates of food proteins especially the milk and egg proteins (Chan & Li-Chan, 2006). Milk and egg proteins are good source of anti-microbial peptides due to their naturally important feature in protecting the newborns or embryo against microbial threats. Lactoferricins (Lfcin) generated from milk lactoferrin by pepsin cleavage is one of the most studied. Lfcin is antibacterial on a wide range of Gram-negative and -positive bacteria likely by disintegrating the cytoplasmic membrane; and has been reported to be antifungal against *Candida albicans* and antiprotozoal in experimental murine toxoplasmosis (Farnaud & Evans, 2003; Orsi, 2004). In addition to Lfcin, a few antibacterial peptides have been identified from two major milk proteins, α_{s1}-casein (e.g. casecidin, isracidin), and α_{s2}-casein (e.g. Casocidin-I), exhibiting antimicrobial activity against various Gram-positive and -negative bacteria (Chan & Li-Chan, 2006; Korhonen & Pihlanto, 2006). Egg white lysozyme has been widely used as food-derived preservative agent with anti-microbial activity; lysozyme-derived peptide IVSDGNGMNAW was reported to be bactericidal against Gram-negative bacteria *Escherichia coli* (Yoshinori Mine, Ma, & Lauriau, 2004). Another peptide (OTAP-92) from egg antimicrobial protein ovotransferrin exhibited bactericidal activity against Gram-positive (*S. aureus*) and Gram-negative (*E. coli K-12*) bacteria (Kovacs-Nolan, Phillips, & Mine, 2005).

2.1.3. Antioxidant peptides

The balance between oxidants and antioxidants is critical for maintaining good health. Endogenous enzymatic antioxidants (e.g. superoxide dismutase, catalase, glutathione peroxidase) and nonenzymatic compounds (e.g. glutathione, selenium, α-tocopherol, vitamin C) help to protect tissues and organs from free radicals such as hydroxyl radicals (·OH), peroxyl radicals (·OOR), superoxide anion ($O_2^{-}\cdot$), and peroxynitrite ($ONOO^-$)

(Samaranayaka & Li-Chan, 2011). But the balance can be destabilized by the progression of age or other factors (e.g. pollution, fatigue, UV radiation, excessive caloric intake, smoking, etc.), resulting in excessive formation of free radicals (oxidative stress), which is suggested to involve in initiation/progression of many chronic diseases such as cardiovascular diseases, inflammation, cancer and neuropathies (Mayne, 2003).

Antioxidant peptides can be supplemented to the diet to provide extra protection against oxidative stress. Peptides were identified from various food protein sources (e.g. fish, milk, egg, soy) with anti-oxidative activities (Power, Jakeman, & FitzGerald, 2013; Samaranayaka & Li-Chan, 2011). Antioxidative activity was mostly studied by employing chemical assays such as oxygen radical absorbance capacity (ORAC), ferric ion reducing antioxidant power (FRAP), DPPH· scavenging method and the linoleic acid peroxidation system. The chemical-based methods are efficient and useful for screening and study the action mechanism of potent antioxidants. However, as antioxidant activity depends on not only the radical scavenging property but also the regulation of antioxidant/detoxifying enzymes, modulation of redox cell signaling and gene expression *in vivo*, it is necessary to move to cellular assays or animal models in order to get an overall picture of the antioxidant activity (López-Alarcón & Denicola, 2013). Compared to antioxidant activity tested by chemical assays, there are limited reports using cellular assays. A hepta-peptide HFGBPFH from marine blue mussel showed comparable antioxidative effects as α-tocopherol against t-BHP induced oxidative stress in cultured human lung fibroblasts (ATCC CCL-75) (Rajapakse, Mendis, Jung, Je, & Kim, 2005). Oligophosphopeptides (PPPs) from egg yolk phosvitin possess potent antioxidative activity against oxidative stress in human intestinal epithelial cells (Caco-2); cellular level of glutathione, activities of γ-glutamylcysteine synthetase, glutathione reductase, glutathione S-transferase and catalase were elevated by PPPs treatment (Katayama, Ishikawa, Fan, & Mine, 2007). A dipeptide (Pro-Arg) from salmon protamine could protect against H_2O_2-induced cellular oxidative damage and H_2O_2-induced cell death in human fetal lung diploid fibroblasts (MRC-5) (Wang, Zhu, Chen, Han, & Wang, 2009). Egg-derived tripeptides IRW and IQW exhibited antioxidant effects as shown by reduction of TNF-induced superoxide generation

in human endothelial cells (Majumder, Chakrabarti, Davidge, & Wu, 2013). It is expected that there will be more studies exploring antioxidant activities using the cellular assays.

2.1.4. Opioid peptides

Opioid peptides are opioid receptor ligands with agonistic or antagonistic activities. Opioid receptors are located in the nervous, endocrine, immune system and the gastrointestinal (GI) tract of mammals (Korhonen & Pihlanto, 2006); peptides with opioid activity could affect appetite, gastrointestinal motility and behavior (Zioudrou, Streaty, & Klee, 1979). Opioid peptides are mainly identified from milk proteins. There are milk-derived opioid agonists (casomorphins, α-lactorphin, β-lactorphin, serorphin) and opioid antagonists (casoxins, lactoferroxins) as well (Clare & Swaisgood, 2000; Korhonen & Pihlanto, 2006).

Casomorphins, the most studied opioid peptides, can be obtained from pepsin hydrolysis of β- or α_{S1}-casein. The peptide fragments can also be produced during cheese ripening and yogurt fermentation by proteolytic activity of bacteria (De Noni, Stuknytė, & Cattaneo, 2015; Hamel, Kielwein, & Teschemacher, 1985; Nguyen, Johnson, Busetti, & Solah, 2015). In rodents, casomorphins showed prominent effect on nociception (analgesia), as well as effects on spontaneous behavior and memory (Lister, Fletcher, Nobrega, & Remington, 2015). The health benefits for casomorphins are somehow controversial. Recent studies suggest the connection of bovine casomorphins with intensified symptoms of autism, schizophrenia, and allergy (Jarmołowska et al., 2013; Shah, Trivedi, Hodgson, & Deth, 2013). On the other hand, there are studies reporting beneficial effects of casomorphin on intestinal homeostasis, which is important in protection against damaging agents of the intestinal lumen (Plaisancié et al., 2013); anticarcinogenic effects of carsomorphins have also been reported on human breast cancer cell T47D and human colon cancer cell Caco-2 as reviewed by (Pepe, Tenore, Mastrocinque, Stusio, & Campiglia, 2013). Considering the broad expression of opioid receptor, the health benefits and related health issues for the use of opioid agonist/antagonist would require further investigations.

2.1.5. Anticancer peptides

With declined death toll from infectious diseases in the Western world, cancer has become a leading cause of death, ranked second only to the CVDs (WHO, 2014). Carcinogenesis is a multistage process derived from a combination of multiple heritable and environmental factors (Hernández-Ledesma, Hsieh, & de Lumen, 2009b). Although newly explored, anticancer peptides have been identified in soy, marine products and milk (Hernández-Ledesma et al., 2009b; Sah, Vasiljevic, McKechnie, & Donkor, 2015; Suarez-Jimenez, Burgos-Hernandez, & Ezquerra-Brauer, 2012).

The food-derived anticancer peptide lunasin was first discovered in soy and exists in wheat, barley and other seeds (Hernández-Ledesma et al., 2009b). Lunasin exhibited anticancer activity *in vitro* against chemical carcinogens and viral oncogenes induced transformation of mammalian (human, murine) cells; and inhibited tumorigenesis *in vivo* when applied topically in a mouse model of skin cancer (Galvez, Chen, Macasieb, & de Lumen, 2001; Lam, Galvez, & de Lumen, 2003). The anticancer activity of lunasin was attributed to its binding properties to the chromatin, which disturbed kinetochore formation leading to mitosis arrest and cell death (Galvez & de Lumen, 1999). Moreover, the anti-inflammatory and antioxidant properties of lunasin could complement its chemopreventive effects (Hernández-Ledesma, Hsieh, & de Lumen, 2009a). Other than lunasin and lunasin-derived peptides, anticancer activities have been reported in enzymatic hydrolysates of marine products (fish and seafood) with a focus on antiproliferative activity (Suarez-Jimenez et al., 2012). Two peptides purified from tuna dark muscle byproduct were found to inhibit the proliferation of human breast cancer cell line MCF-7 (Hsu, Li-Chan, & Jao, 2011). In milk, a few peptides were found to show anticancer activities, of which many exerted multiple bioactivities (Pepe et al., 2013; Sah et al., 2015). For example, casomorphins exert anticancer and opioid agonist activity, caseinphosphopeptides (CPPs) have anticancer and mineral-binding activity, and lactoferricin possesses anticancer, anti-microbial and immune regulatory activities (Pepe et al., 2013; Sah et al., 2015). These might suggest possible multifaceted strategies in search of anticancer peptides.

2.2. Immunomodulatory peptides

2.2.1. The immune system

The immune system is vital to our survival; it is one of the largest organs dispersing throughout the body to provide host defense against foreign organisms or substances (antigens). In vertebrates there are two arms of immunity, the innate immunity and adaptive immunity. Innate immunity constitutes the first line of defense, which includes barriers (skin, mucous membrane, and physiological barriers), phagocytic cells (blood monocyte, neutrophil, tissue macrophage) and pattern recognition molecules (lysozyme, complement components, Toll-like receptors (TLRs)). Adaptive immunity is characterized by antigen specificity, diversity, memory and self-nonself recognition. Lymphocytes bearing antigen receptors (T cells, B cells) are the central cells of adaptive immunity, interacting with the antigen-presenting cells to initiate effector responses. Effector T cells are T helper cells (T_H cells) and cytotoxic T lymphocytes (CTLs). Cytokine-secreting T_H cells help to activate B cells, T cytotoxic (T_C) cells, various other cells (e.g. macrophages), and as well help to maintain immune homeostasis; CTL has an important role in eliminating virus-infected cells and tumor cells. Effector B cells (plasma cell) secret antibodies, which has three major functions including opsonisation, complement activation, and antibody-dependent cell-mediated cytotoxicity. Innate immunity and adaptive immunity collaborate to form a complex network of cells and molecules to protect the body against antigens such as bacteria, viruses, or tumor cells. The communication and collaboration between innate and adaptive system are mainly through a group of chemical messengers called cytokines; and the antigen-presenting function is important in initiating and regulating the adaptive immune response (Kindt, Goldsby, Osborne, & Kuby, 2007). Figure 2.1 provides an overview of the immune system.

Organs of immune system are distinguished by function as the primary and secondary lymphoid organs. Primary lymphoid organs (thymus, bone marrow) are where lymphocytes mature; secondary lymphoid organs provide sites for mature lymphocytes to interact with antigen, including lymph nodes, the spleen, and various mucosa-associated

lymphoid tissues (MALT) (Kindt et al., 2007). The largest immune organ in our body is gut-associated lymphoid tissue (GALT) which is the part of the MALT lining the digestive tract. GALT is composed of tissues and cells such as Peyer's patches (PPs), lymphoid follicles, lamina propria, intestinal epithelial cells (IECs), intraepithelial lymphocytes (IELs), as well as mesenteric lymph nodes (MLNs). It is continuously exposed to a massive load of dietary antigen and commensal and pathogenic bacteria, therefore has evolved mechanisms to mount protective responses to pathogenic organisms while maintaining a state of tolerance to commensal bacteria and food antigens (Janeway, Travers, Walport, & Shlomchik, 2001; Wershil & Furuta, 2008). Other than its role in gut immunity, GALT also plays a critical role in the development of the systemic immune response, in which primed lymphocytes migrate from the intestine to other sites of the body to protect against immune challenges (Ruth & Field, 2013).

While a healthy immune system protects the host, improper response (deficiency/overreaction) from the immune system can result in immunodeficiency, allergies and asthma, autoimmune diseases (Kindt et al., 2007). In the case of inflammation, acute inflammation is a crucial host defense mechanism for combating infection and tissue repair; but the consequences of prolonged (chronic) or unregulated severe acute inflammation are not beneficial and would eventually resulting in severe tissue damage. Chronic inflammation has been associated to pathogenesis of some chronic diseases, such as metabolic disorders (Hotamisligil, 2006), atherosclerosis (Hansson, 2005; Libby, 2002), and cancer (de Visser, Eichten, & Coussens, 2006). For example, it now accepted that inflammation is a key feature of metabolic disorders including obesity and type 2 diabetes (Hotamisligil, 2006). Modern sedentary lifestyle and high caloric diets easily result in high rate of overweight and obesity. Enlarged adipocytes overexpress various inflammatory mediators (e.g. tumor necrosis factor-α as the most renowned) and facilitate macrophage infiltration into adipose tissue, which could reinforce the obesity-induced inflammatory responses. A nutritional surplus status also stresses the endoplasmic reticulum (ER) resulting in activation of principal inflammatory pathways (JNK–AP-1 and IKK–NF-κB). The above derived inflammatory signalling are rivals to insulin activation, when persist

would cause insulin resistance and impair insulin secretion, leading to type 2 diabetes (Hotamisligil, 2006).

Another example of immunopathology in chronic diseases is the inflammatory bowel diseases (IBD), which is gaining prevalence in developed countries and urbanized area. It is hypothesized that IBD arises from a disruption of mucosal immune homeostasis, where inappropriate and exaggerated immune response were launched towards normal constituents of the mucosal microflora in genetically susceptible individuals (Bouma & Strober, 2003; Xavier & Podolsky, 2007). Many immunoregulatory abnormalities are noted in IBD, including the ratio of proinflammatory (interleukin (IL)-12, interferon (IFN)-γ, IL-13, TNF-α, IL-6, IL-1β) to immunosuppressive (IL-10, transforming growth factor (TGF)-β) cytokines, selective activation of helper T cell subsets (a T_H1-type like cytokine profile in Crohn's disease and a T_H2 profile in ulcerative colitis), deficiency in regulatory T cell function, and aberrations in antigen presentation function (Bouma & Strober, 2003; Sartor, 1997). Adaptive immune response has been classically considered to play a major role in the pathogenesis of IBD, as it is the more proximate driver of tissue damage. Recent advances suggest equal importance of innate immune response, especially the epithelial barrier function; the defective barrier exposes lamina propria immune cells to the continual presence of resident luminal bacteria, bacterial products, or dietary antigens, which perpetuates the inflammatory cascade and can be the onset of IBD (Geremia, Biancheri, Allan, Corazza, & Di Sabatino, 2014).

As immune aberrations have been associated with the onset and progression of various diseases, it is therefore of great interest to modulate the immune responses and to re-establish immune homeostasis. Diet as one of the environmental factors exerts influences on the development and functioning of the immune system. Many dietary constituents have ever since been discovered to exhibit immune-modulating properties. Before reviewing the immunomodulatory properties of dietary components, it is important to understand the commonly used immune assays and biomarkers.

2.2.2. Assays and biomarkers for immune functions

There is no one overall measure of immune functions, but there is a wide range of techniques to assess various immune functions. Measurements can be made of immune response *in vitro* (i.e. treatment applied *in vitro*), of cell functions *ex vivo* (i.e. cells isolated following *in vivo* treatment and tested in primary culture), of indicators of immune function *in vivo* (e.g. measuring circulating factors), or of responses to an immunological challenge (e.g. vaccination, antigen inoculation) (Albers et al., 2005; Calder, 2007).

In vitro assays are commonly used in study of immunomodulatory activities. They are very useful tools to identify the potential effects of active components and to study the mechanisms of action. However, there are some drawbacks such as a relatively isolated environment from other cells or mediators, and an inconsistency on the bioavailability (form/concentration) of test component. *Ex vivo* assays shares the test methodologies with *in vitro* assays and can reflect the cellular response towards *in vivo* treatment. Commonly used *in vitro* or *ex vivo* immune assays include phagocytosis, respiratory (oxidative) burst, natural killer cell activity, lymphocyte proliferation, cytokine and antibody measurement, and cell surface expression of molecules. *Ex vivo* assays have advantages in standardizing conditions within and between experiments. However, the changes in cell's environment during isolation and subculture (e.g. cell-to-cell contact, hormone) can be a drawback. Therefore effects observed *ex vivo* on immune function changes may not necessarily be produced *in vivo*. *In vivo* assays can be used in combination with *ex vivo* assays to provide us a better understanding of the impact from treatment. The circulating concentration of immune mediators and the delayed-type hypersensitivity (DTH) response were two of the commonly used *in vivo* measures. The methodologies of the above mentioned immune assays are listed in Table 2.1. Other than these assays, IgA is a unique marker for mucosal immune system. Concentration of IgA in saliva, tears and intestinal mucosal surfaces can be tested by ELISA for assessing of immune function at the mucosal lining (Albers et al., 2005; Calder, 2007).

2.2.3. Immunomodulatory properties of food constituents

Many dietary nutrients have been reported to exhibit immune-modulating properties; these include probiotics and prebiotics, fatty acids, proteins and peptides, amino acids, flavonoids, β-glucans and micronutrients (i.e. minerals and vitamins) (Calder & Yaqoob, 2013). Some nutrients are essential for optimal functioning of immune systems. For example, micronutrients are required by humans in small amounts to orchestrate a range of physiological functions, including immune functions. It is now well accepted that a deficiency status of certain micronutrients (e.g. Selenium, Zinc, Vitamin A, Vitamin C and Vitamin E) compromise immune functions and increase the risk for infectious diseases. Various activities have been reported with different mechanisms for different micronutrients (Calder & Yaqoob, 2013; Field, Johnson, & Schley, 2002), which I will not be able to cover in this chapter. In addition, glutamine (Gln) and arginine (Arg), although categorized as dietary non-essential amino acids, are important "immunonutrients". Gln as the most abundant free amino acid in the human body is an important respiratory substrate and nitrogenous nutrient for cells of the immune system; Arg is a physiological substrate for the synthesis of polyamines (needed for cell proliferation) and nitric oxide (a mediator of immune responses), therefore essential to both innate and adaptive immune systems. Clinical data support the use of Gln or Arg supplement in patients under most immunosuppressive states associated with high rate of infection (e.g. burn injury, cancer, HIV infection), but evidence is lacking for healthy individuals (Field, Johnson, & Pratt, 2000).

Some nutrients preferably act as immunostimulants or immunosuppressors under specific mechanisms. For example, β-glucans are mainly immunostimulants. They contain pathogen-associated molecular patterns (PAMPs) that can be recognized by a variety of pattern-recognition receptors (PPRs, e.g. Dectin-1, TLR), leading to activation of nuclear factor (NF)-κB and p38 mitogen-activated protein kinases (MAPK). The PPRs binding ability was suggested to improve protection against infections and be beneficial when combined with cancer therapy (Murphy, Davis, & Carmichael, 2010; Savelkoul, Chanput, & Wichers, 2013)). On the other hand, flavonoids were known for their anti-inflammatory

effects. For example, quercetin, the most studied compound, has been reported for anti-inflammatory activities *in vitro* on innate immune system. Quercetin was found to decrease the LPS-induced oxidative burst (ROS and NO) and cytokine (IL-6, TNF-α) production in neutrophils and macrophages; while relatively limited effect was observed on the adaptive immune system. It is suggested that the antioxidant property of quercetin largely contributed to its anti-inflammatory activities by regulating the oxidative stress and the activation of transcription factors including NF-κB, activator protein (AP)-1 and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Ioannone, Miglio, Raguzzini, & Serafini, 2013).

More commonly, both immunostimulatory and immunosuppressive effects were observed for dietary nutrients, such as probiotics, fatty acids, proteins and peptides. Probiotics are specific bacterial strains with health promoting properties. The most commonly applied probiotic genera are *Lactobacilli* and *Bifidobacteria*; of those, some are known to interact with the host's immune system. For example, they can stimulate immune response against pathogen or foreign antigens, as well as activate regulatory mechanisms to avoid uncontrolled inflammation (Trejo & Sanz, 2013). To date, use of probiotics in treating infections, allergy, inflammatory bowel diseases (IBD) and autoimmune diseases have been investigated (Trejo & Sanz, 2013). Among all, IBDs such as Crohn's disease and ulcerative colitis are the most popular targets for probiotic intervention; and favourable results have been reported preventing recurrence of the disorders (Trejo & Sanz, 2013). It is suggested that probiotics exert immune modulation via interaction with IECs and dendritic cells (DC); maturation of DC to a phenotype of regulatory DC (rDC) promotes Treg differentiation and upregulates the production of regulatory cytokines IL-10 and TGF-β (Klaenhammer, Kleerebezem, Kopp, & Rescigno, 2012; Kwon et al., 2010). These properties are important for maintaining intestinal homeostasis.

Fatty acids are important dietary nutrients that can be obtained from the diet and some synthesized in the body from non-lipid precursors. Among different fatty acids, saturated and some *trans* fatty acids are generally considered pro-inflammatory; studies showed positive relationships between dietary saturated and *trans* fatty acids intake and inflammatory markers (IL-6, CRP, sE-selectin) (Calder, 2013). On the other hand, marine

n-3 fatty acids (EPA, 20:5n-3; DHA, 22:6n-3) are very potent at reducing inflammation as evident from cell and animal studies as well as clinical trials (Calder, 2013). Lower concentrations of inflammatory markers (CRP, IL-6, TNF, soluble adhesion molecules) and higher concentrations of anti-inflammatory cytokines (IL-10, TGF-β) were associated with EPA/DHA administration (Calder, 2013). Several possible mechanisms including effects on eicosanoid production, signal transduction, and gene expression have been proposed for their anti-inflammatory properties (Field et al., 2002). These anti-inflammatory effects are expected to be beneficial for diseases that involved inappropriately activated immune response; so far, most promising results are observed in rheumatoid arthritis, in which fish oil supplement help improve joint pathology and reduce use of non-steroidal anti-inflammatory drugs (Goldberg & Katz, 2007).

Some food proteins can act directly in their intact form to elicit various immunomodulatory effects, such as lactoferrin and κ-casein from milk (Cross & Gill, 2000; Mulder, Connellan, Oliver, Morris, & Stevenson, 2008; Puddu, Valenti, & Gessani, 2009) and lysozyme and ovotransferrin from egg (Mine & Kovacs-Nolan, 2006). But current research interest is more focused on peptides released from parent proteins by digestion, hydrolysis, fermentation and food processing. Numerous peptides with immunomodulatory properties will be reviewed in the following section.

2.2.4. Review of immunomodulatory peptides

A number of immunomodulatory peptides have been characterized from various food sources, including milk, egg, fish, soy and rice. Effects of peptides on different components of the immune system have been reported, such as phagocytosis activity, lymphocyte proliferation, antibody synthesis and cytokine regulation. There are peptides acting as immunostimulants enhancing the basal level of both innate and adaptive immune responses, which can be used as adjunct to chemotherapy or in treatment of persistent infections; and there are peptides suppressing immune responses specifically or nonspecifically, which can be used for control of graft rejection and autoimmune disorders. In recent years, with an observed steady increase in the incidence and prevalence of

chronic diseases that are associated with chronic inflammation, more research interests are direct to the immune-regulatory peptides.

2.2.4.1. Immunomodulatory peptides in cell models

Many protein hydrolysates have been prepared from animal or plant sources by enzymatic hydrolysis and tested *in vitro*; among them, only very few peptides have been identified and verified for their immunomodulatory activities. Food-derived immunostimulatory peptides were first characterized from human casein tryptic hydrolysate (Jolles et al., 1981), which inspired the exploration of milk proteins as a source of immunomodulatory peptides (Gill, Doull, Rutherfurd, & Cross, 2000; McCarthy, Mills, Ross, Fitzgerald, & Stanton, 2014). Milk proteins consist of two classes of proteins: caseins (αs1-casein, αs2-casein, β-casein and κ-casein) and whey proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulin, lactoferrin, etc), from which several bioactive peptide sequences have been identified. For example, caseinophosphopeptides (CPPs; f1-28 from β-casein and f1-32 from αs2-casein) exhibited stimulatory effects in murine and rabbit lymphocytes, improving cell proliferation, immunoglobulin secretion, and cytokine (IL-6) production (Hata, Ueda, & Otani, 1999; Otani, Watanabe, & Tashiro, 2001; Tobita, Kawahara, & Otani, 2006); β-casein-derived peptide fragment f(193-209) stimulated phagocytosis activity and MHC II expression of macrophages isolated from germ-free and human gut microbiota-associated mice (Sandre et al., 2001); β-lactoglobulin-derived hexapeptide VAGTWY f(15-20) induced murine splenocyte proliferation and enhanced cytokine (IFN-γ, IL-4) expression of resting or ConA-stimulated T helper cells (Jacquot, Gauthier, Drouin, & Boutin, 2010); α-lactalbumin-derived di-peptide YG f(18-19) or f(50-51) enhanced proliferation of human peripheral lymphocytes (Kayser & Meisel, 1996). In contrast, the opioid peptide casomorphin-7 exhibited inhibitory effects on proliferation of human lymphocytes from lamina propria (Elitsur & Luk, 1991). The well-known κ-casein-derived glycomacropeptide (GMP) demonstrated potent inhibition on proliferation of lymphocytes isolated from murine spleen or rabbit Peyer's patch (Otani & Monnai, 1995; Otani, Monnai, Kawasaki, Kawakami, & Tanimoto, 1995), but acted as a stimulant for macrophage proliferation and phagocytosis activity (Li & Mine, 2004). Lactoferricin B

(Lfcin), an active fragment of the multi-functional protein lactoferrin, was found to stimulate the release of neutrophil chemokine IL-8 and promote phagocytosis in human polymorphonuclear (PMN) leukocytes (Miyauchi et al., 1998; Shinoda et al., 1996), to enhance immunoglobulin production in cultured murine splenocytes and Peyer's patch cells (Miyauchi, Kaino, Shinoda, Fukuwatari, & Hayasawa, 1997), but to suppress the IL-6 response in LPS-activated monocytic cell line THP-1 (Mattsby-Baltzer et al., 1996).

Soy, rice and fish have also been used as sources of immunomodulatory peptides. The rice peptide Oryzatensin (GYPMYPLPR) stimulated phagocytosis and ROS production in human peripheral neutrophils (Takahashi, Moriguchi, Yoshikawa, & Sasaki, 1994); similarly, soy-derived Soymetide (MITLAIPVNKPGR) could enhance phagocytosis activity of human blood neutrophil (Tsuruki et al., 2003). By contrast, soy peptide lunasin and a tri-peptide VPY demonstrated inhibitory (anti-inflammatory) properties modulating endotoxin-induced inflammatory responses in macrophages. Lunasin decreased the ROS production, cytokine (TNF-α, IL-6, IL-1β) and nitric oxide, PGE_2 synthesis, transcription factor NF-κB activation, and inducible nitric oxide synthases (iNOS), cyclooxygenase (COX)-2 expressions in activated murine RAW264.7 macrophage cell line (de Mejia & Dia, 2009; Hernández-Ledesma et al., 2009a); a peptide VPY reduced TNF-α production in activated human THP-1 monocytic cell line (Kovacs-Nolan et al., 2012). The sequences and activities of food-derived peptides with reported *in vitro* immunomodulatory activities are summarized in Table 2.2.

2.2.4.2. Immunomodulatory peptides in animal models

Several peptides exhibiting immunomodulatory effects *in vitro* have been shown to have consistent activities in animals when fed in the diet. For example, β-casein (1-28), a caseinophosphopeptide, that stimulated immunoglobulin and cytokine production *in vitro* in lymphocytes, up-regulated systemic (serum) and secretary (intestinal, feces) IgA levels and splenic Th2 cytokines (IL5, IL-6) in mice (Otani, Kihara, & Park, 2000; Otani, Nakano, & Kawahara, 2003). Soymetide-4 (MITL) stimulated phagocytic activity in human peripheral neutrophils (*in vitro*) also acted as a stimulant for serum cytokine (TNF-α)

production when fed to mice (Tsuruki et al., 2003). Soy tri-peptide VPY suppressed TNF-α production in LPS-activated human monocytic cells; the anti-inflammatory potential of VPY was later proved to be beneficial in dextran sulfate sodium (DSS)-induced colitis in mice as evident from improved colon pathology and suppressed colonic gene expression of TNF-α and other pro-inflammatory cytokines (IL-6, IL-1β, IFN-γ and IL-17) (Kovacs-Nolan et al., 2012). Milk peptide GMP acted as an immunosuppressor *in vitro* inhibiting lymphocyte proliferation and blocking IL-1β action in murine and rabbit immune cells (Otani & Monnai, 1995; Otani et al., 1995); *in vivo,* GMP attenuated weight loss, anorexia and colonic damage in 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-induced rat colitis and ileitis models (Daddaoua et al., 2005; Requena et al., 2008) and inhibited IL-1β production in both intestine tissue of colitis rats and in plasma of healthy mice (Sawin et al., 2015).

Additionally, animal studies have been carried out to investigate immunomodulatory activities of protein hydrolysates prepared from milk, egg, soy and marine products. The immunostimulatory activities have been reported in healthy animals. For example, feeding Balb/c mice with egg yolk low lipid peptic digests for 5 days enhanced gut immunity as evident from a higher proportion of IgA+ B cells, and IL-4+, IL-10+, IFN-γ +, IL-12+ T cells in the intestine lamina propria, as well as improved phagocytic activity of peritoneal macrophages (Nelson, Katayama, Mine, Duarte, & Matar, 2007). Stimulatory activities on gut immunity were also reported in a commercial fish protein hydrolysate (Seacure®) (Duarte, Vinderola, Ritz, Perdigón, & Matar, 2006), pea protein thermolysin hydrolysate (Ndiaye, Vuong, Duarte, Aluko, & Matar, 2012), and milk peptides prepared by fermentation with kefir microflora (Vinderola et al., 2006). Another example is salmon-derived oligopeptides that exhibited stimulatory effects on systemic immunity in a 4-week feeding trial in mice: higher indices of lymphocyte proliferation, plaque-forming cell population, NK cell activity and percentage of CD4+ cells in spleen, as well as increased production of serum cytokines (IL-2, IFN-γ, IL-5, IL-6) (Yang et al., 2009). And oral administration of fish egg-derived peptides from pepsin hydrolysis was reported to up-regulate splenic NK cell activity, peritoneal macrophage phagocytic activity, and mucosal and serum IgA production in Balb/c mice (Chalamaiah et al., 2015).

Infection models can also be used to evaluate the immunostimulatory activity of peptides against immune challenges, and to examine the immunoregulatory activities towards inflammation. For example, administered prior to the infection, milk peptide fraction from *Lactobacillus helveticus* fermentation prevented the weight loss caused by enteral *E. coli* challenge; this protective effect was attributed to the immunostimulatory and immunoregulatory activities of the milk peptides, as a higher number of IgA+ B cells in lamina propria, enhanced total secretory and systemic IgA responses, and a lower level of pro-inflammatory cytokine interferon (IFN)-γ were observed in the peptide-treated group (LeBlanc, Fliss, & Matar, 2004). A whey peptide fraction increased immune vigilance towards *E. coli* infection in mice as indicated from stimulated secretion of serum IgA and TGF-β1 (a regulatory cytokine) (Saint-Sauveur, Gauthier, Boutin, Montoni, & Fliss, 2009). Protective immunostimulatory effects were also reported for a shark protein hydrolysate – a higher number of intestinal IgA+ cells, a higher level of serum TGF-β1 secretion, a lower level of IL-17 in serum, and a higher number of IL-10+, CTLA-4 (cytotoxic T-lymphocyte-associated protein-4)+ cells in intestine (Mallet et al., 2014).

Experimentally induced colitis models are widely used for evaluation of immunoregulatory activities of food-derived peptides on acute and chronic inflammation. Generally, colitis is induced in mice, rats, or piglets with chemical reagent DSS or TNBS. Soy di- and tri-peptides were found to ameliorate DSS-induced colitis in piglets; the peptide treatment inhibited the expression of inflammatory mediators (IFN-γ, IL1-β, TNF-α, RORC, and IL-17) and modulated FOXP3 expression in colon and ileum (Young, Ibuki, Nakamori, Fan, & Mine, 2012). Similarly, egg white peptides prepared by aminopeptidase of *Aspergillus* sp. origin down-regulated colonic expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ, IL-8, IL-17) and improved the pathological symptoms in a DSS model (Lee et al., 2009). A casein hydrolysate from *Lactobacillus delbrueckii* fermentation increased IL-10 while reduced IFN-γ secretions in intestine of TNBS-induced mouse colitis model (Turbay, de LeBlanc, Perdigón, de Giori, & Hebert, 2012). Table 2.3 summarized current reports of immunomodulatory peptides and protein hydrolysates tested in healthy or diseased animal models.

2.2.4.3. Clinical trials

Clinical trials are the necessary step following animal study for evaluating the efficacy and pharmacokinetics of immunomodulatory peptides for application as nutraceuticals and functional foods. There are a few pilot trials showing promising results from peptide treatment. For example, CPPs-formulated cakes increased fecal IgA levels in 7 healthy volunteers after 30 days of consumption in comparison to the placebo cakes (Kitamura & Otani, 2002). But a relatively small test population and the absence of control over the diet or lifestyle patterns weakened the study conclusion. A wheat gluten hydrolysate ("Glutamine Peptide, GP-1", Nisshin Pharma, Tokyo, Japan) has been tested in 9 healthy human subjects (5 subjects in test group, 4 subjects in control group). Oral intake of 3 g wheat protein hydrolysate per day for 6 days was found to increase *ex vivo* natural killer (NK) cell activity in test group (Horiguchi, Horiguchi, & Suzuki, 2005). But a small test population, the absence of placebo control, baseline information and diet control impaired the power of the study. Recently, a whey peptide supplement (MEIN™, Meiji Dairies Co., Tokyo, Japan) was tested in stable chronic obstructive pulmonary disease (COPD) patients for anti-inflammatory properties. Nutritional supplement containing whey peptides increased body weight and inhibited systemic inflammation (blood hsCRP, IL-6, IL-8, and TNF-α) in a 12-week trial in patients participating in a low-exercise program (Sugawara et al., 2012). This study has the strength with relatively large number of participants (36 participants), double-blinded randomization, comprehensive baseline information, adherence confirmation (diary), dietary intake assessment and exclusion of confounding factors (e.g. smoking, medication, condition change). However, it was not placebo-controlled, and the long-term effect was not investigated. The nutritional supplement contained n-3 fatty acids and vitamins in addition to whey peptides, and low-intensity exercise are required to obtain the beneficial effects; therefore the effect of whey peptides in this study could not be determined independent of other treatment. It is expected that more well designed double-blinded randomized clinical trials of immunomodulatory peptides will emerge to show the benefits.

2.2.4.4. Mechanism of action

The mechanisms by which the food-derived peptides exert their immunomodulatory effects are largely unknown. Some immunomodulatory peptides are multifunctional. It is suggested that opioid peptides might act on lymphocytes in a manner similar to that for cytokines through the opioid receptors (Bidlack, 2000; Moughan & Rutherfurd-Markwick, 2013); a good example is β-casomorphin and derived peptides. Additionally, some peptides may modulate the immune system indirectly through different activities. For example, ACE-inhibitory peptides blocks the conversion of angiotensin I to angiotensin II and prevent the cleavage of bradykinin; as both of bradykinin and angiotensin II are immune mediators, the ACE-inhibitory peptide could exert an effect on immune systems (Gill et al., 2000). Antioxidant peptides could help reduce oxidative stress and balance the production and elimination of reactive oxygen species (ROS); through the effect on ROS, which acts as both a key signaling molecule and a mediator of inflammation (Mittal, Siddiqui, Tran, Reddy, & Malik, 2014), peptides with antioxidant activities could exhibit immunomodulatory effects. The mineral-binding CPPs possess the ability to bind and solubilize minerals (e.g. Ca, Mg, Fe, Zn and Se) (R. J. FitzGerald, 1998); the calcium binding activity of CPPs could contribute to their mitogenic activity on lymphocytes, as calcium influx is important in triggering of lymphocyte stimulation (Isao Hata, Higashiyama, & Otani, 1998); additional beneficial effects of CPPs can be expected from the improved absorption of Fe, Zn and Se, of which a deficiency status could impair the immune system (Field et al., 2002).

The NF-κB/REL family of transcription factors has a central role in coordinating the expression of a wide variety of genes that control immune responses, including antimicrobial peptides, cytokines, chemokines, stress-response proteins and anti-apoptotic proteins (Li & Verma, 2002). Therefore it is expected that peptides exhibiting immunomodulatory properties may possess an effect on NF-κB activation. Effect of soy-derived lunasin and lunasin-like peptides on NF-κB signaling has been reported in endotoxin-activated macrophages, a commonly used *in vitro* cell model (de Mejia & Dia, 2009; Montoya-Rodríguez, de Mejía, Dia, Reyes-Moreno, & Milán-Carrillo, 2014); but this has not yet been established in other cell models.

As for the structure-function relationship, it has been suggested that certain structural motifs are important for immunomodulatory activity. The presence of arginine residue in the N- or C-terminal region of the peptide can be important structural entity to be recognized by specific membrane bound receptors (Paegelow & Werner, 1986); glutamine residues in peptides can substitute for the free amino acid glutamine, which is an important "immunonutrient" utilized at a high rate by immune cells, to exhibit non-specific immuostimulatory effects (Haque, Chand, & Kapila, 2008; Moughan & Rutherfurd-Markwick, 2013); and it was speculated that the positively charged region of the molecule can bind and activate the chemokine receptors on immune cell (e.g. lactoferricin) (Mercier, Gauthier, & Fliss, 2004; Vogel et al., 2002). Moreover, tryptophan has been increasingly recognized for its involvement in immune suppression; accumulation of tryptophan catabolites (most notably kynurenine) can modulate homeostasis of the immune system by inducing apoptosis of Th1 cells and stimulating the development of Treg cells (Zhang, Hu, Kovacs-Nolan, & Mine, 2014). More studies are required to confirm these hypotheses, and much remained to be explored in this area.

2.2.4.5. Summary of immunomodulatory peptides

In summary, food-derived peptides have been greatly explored for *in vitro* and *in vivo* immunomodulatory properties tested in various cell and animal models. However, current studies have certain limitations. First, there is a lack of subsequent study for the reported immunomodulatory peptides and hydrolysates. For example, with the majority of peptides and hydrolysates tested *in vitro* for immunomodulatory activities, only a few have been further studied *in vivo*; of the peptides and hydrolysates reported to be active in healthy animals, only a few have been verified for potential health benefits in a diseased model; compared to a large number of food-derived hydrolysates reported to exert immunomodulatory properties, limited number of active peptide sequences have been identified. To date, there are limited reports on clinical studies, regulatory mechanism and structure-function relationship of immunomodulatory peptides. In addition, the effects of peptide treatment on cell viability or animal health were missing in some studies, which should be carefully examined when evaluating the benefits. Moreover, diet used in animal

study should be carefully designed to produce consistent results. For example, the test diets should be balanced to be isocaloric and isonitrogenous; and a semi-purified diet has the advantage in reducing batch-to-batch variation when compared to the commonly used chow diet.

2.3. Bioactive peptides for natural health products

2.3.1. Current market opportunity

In the past few decades, consumers have switched from an emphasis on satisfying hunger to an emphasis on the promising use of foods to promote well-being and to help reduce the risk of disease. The global market for functional foods and natural health products (FFNHP) is estimated to reach approximately US$477 billion by 2015 and is expected to continue growing at an annual rate of 8% to 14%, outpacing that of the traditional processed food market (AAFC, 2014). Bioactive proteins and peptides are an important category within the nutraceuticals food sector currently valued at $75 billion per year (Mine, Li-Chan, & Jiang, 2010). The use of bioactive peptides in intervention of human diseases offers many advantages such as lack of adverse side effects, low health cost, and additional nutritional value as compared to the traditional pharmaceutical drugs. Nowadays, a number of the bioactive peptides are made commercially available in the form of functional foods or nutraceuticals carrying associated health claims. The milk-derived hypotensive peptides VPP and IPP are the first peptides that launched in the market in 1997, with a product name of 'Ameal S' by Calpis Co. Ltd, Japan, which now becomes one of the top brands focusing on blood pressure (Calpis, 2015). CPPs are marketed to improve the absorption of calcium and they have been commercialized for bone health with different brand names such as "Capolac" (Arla Foods, Denmark), "Tekkotsu Inryou" (Suntory, Japan), "Kotsu Kotsu calcium" (Asahi, Japan) and "CE90CPP" (DMV, Netherlands). Soy peptide bound phospholipids with cholesterol-lowering activity was enriched in soft drink by Kyowa Hakko, Japan with the brand name of "CholesterBlock". Glutamine Peptides with immunomostimulatory activity was commercialized as ingredients for natural health products by DMV international, Netherlands. There are many other examples of bioactive

peptides for application as innovative FFNHP products (Hartmann & Meisel, 2007; T. Shimizu & Hettiarachchy, 2012).

2.3.2. Potential challenges and future opportunities

A major challenge for the application of bioactive peptides is how to ensure an effective and convenient delivery of the peptides in the body. Development of food-derived peptides as enterally potent natural health products poses high requirements for stability against gastrointestinal proteases and delivery property to the cellular site of action. Bioavailability of peptides depends on the physicochemical properties of the peptides, such as molecular size, hydrophobicity, charge, and solubility (Power et al., 2013; Udenigwe & Aluko, 2012). In general, bioactive peptides are susceptible to gastrointestinal digestion, in which oligopeptides are hydrolyzed into smaller peptides by peptidases; di- and tri-peptides are digested by brush-border peptidases at the epithelial cells to produce amino acids (Mahato, Narang, Thoma, & Miller, 2003; Shimizu, 2004). Peptide absorption and transportation to the target site can also be challenging. Absorption of peptide mainly takes place in small intestine through four general mechanisms, including specific transport via PepT1 peptide transporter, paracellular passive diffusion, transcellular via endocytosis/exocytosis, and IgA/M-cell presentation to Peyer's patches/lymphoid tissue (Moughan & Rutherfurd-Markwick, 2013). Peptide transportation through PepT1 and transcytosis can be subjected to intracellular hydrolysis causing a major loss. And the absorbed peptides have to survive the sera enzymes and the hepatic first-pass metabolism.

Despite the challenges in peptide delivery, milk anti-hypertensive tripeptide IPP had been detected in circulation in a placebo-controlled crossover human clinic trial (Foltz et al., 2007). It is suggested that hydrophobicity and the presence of proline residue could protect the peptides from proteolysis (Korhonen & Pihlanto, 2006) and tri- and di-peptides have a better chance for absorption. β-casomorphins that are rich in proline have been detected in chyme of minipigs and human small intestine (Meisel, 1986; Svedberg, de Haas, Leimenstoll, Paul, & Teschemacher, 1985). For large peptides, it is suggested that paracellular passive diffusion can be an important absorption mechanism (Shimizu, 2004).

And lymphatic pathway provides an alternative route for bioactive peptides to enter the circulation and make their way to the target site of action after crossing the intestinal monolayers (Mahato et al., 2003). Additionally, some bioactive peptides could produce beneficial effects in gastrointestinal tract without being absorbed; these include the mineral-binding, hypocholesterolemic peptides, and immunomodulatory peptides which could exert effects through gut-associated lymphoid tissue (GALT). Moreover, research efforts focusing on new strategies to improve bioavailability such as chemical modification, formulation vehicles, use of enzyme inhibitors, absorption enhancers and mucosal adhesive polymers (Shaji & Patole, 2008) are expected to help solve the challenges.

2.4. General conclusions

While the field of bioactive peptide becomes an exciting and growing area of research, there are several issues that should be considered and addressed considering the target application for nutraceutical and functional foods (Figure 2.2). For many bioactivity studies of food-derived protein hydrolysates and peptides, much of the work is still at an early stage investigating the beneficial effects *in vitro*, with limited studies demonstrating the effects in animal models or clinical trials. Sufficient data from animal studies and clinical trials are important for evaluation of *in vivo* efficacy and toxicity/safety, and solid pharmacokinetic data will be needed to establish the proper dosage and frequency. Current research progress on different bioactivities is imbalanced, for certain bioactivities there are limited number of identified peptide sequences; characterization of individual component from complex peptide-rich hydrolysates is important for understanding the mechanism of action and function-structure relationship. For target application in the form of FFNHP, the effect of gastrointestinal digestion on peptide structural integrity and the taste and compatibility of peptides in the food matrix should be investigated. Despite a general absence of side effects, the allergenicity of food-derived peptides, the safety of long-term ingestion, and the risk of overdose should be carefully investigated to avoid potential adverse effects.

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Table 2.1 Immune assays for measurement of immune indices.

	Immune Assay	Cells	Technique	Description
In vitro & ex vivo	Phagocytosis	Neutrophils, monocytes	Flow cytometry	Substrates for phagocytosis include bacteria, sheep red blood cells, and yeast particles. Information can be acquired on the number of phagocytes (neutrophils and monocytes) involved in phagocytosis, as well as the level of activity.
	Respiratory (oxidative) burst	Neutrophils, monocytes	Flow cytometry	Stimuli to induce respiratory burst include bacteria and protein kinase C activators such as phorbol esters (e.g. PMA). Fluorescence probes (e.g. Dihydrorhodamine 123) can be used to detect production of oxygen metabolites.
	Natural killer (NK) cell activity	NK cells	Flow cytometry	To measure NK cell activity, NK-sensitive K562 target cells are briefly co-cultured with NK cells. Target cell lysis can be measured by the release of a pre-labelled fluorescent dye (e.g. ^{51}Cr), cytoplasmic enzymes or stained DNA in target cells.
	Cell proliferation	Lymphocytes	Cell counter, flow cytometry	Increase in number of lymphocyte in response to stimuli is studied. Mitogen (e.g. concanavalin A) is the most commonly used stimulus. Radioactively labelled thymidine or bromodeoxyuridine (Brd-U) (DNA incorperation) or fluoresence dyes such as Hoechst 33342 (DNA-binding) and the calcein (cytoplasmic dye) can be used to determine the proliferation.
	Production/mRNA expression of cytokine	T helper cells, macrophages	ELISA, RT-PCR	To assess the function of T helper cells, a cluster of cytokines should be tested to cover T_H1 (IFN-γ, IL-2), T_H2 (IL-5, IL-4, IL-13) and regulatory T cell (IL-10, TGF-β) functions. In analysis of innate immunity, macrophage-derived cytokines (TNF-α, IL-1β, IL-6, IL-10) can be measured.
	Production of antibody	B cells	ELISA	Antibody production can be measured without stimulation (spontaneous production) or be induced *ex vivo* by culture with B cell stimuli (e.g. pokeweed mitogen or lipopolysaccharides). The measurement can be made of a certain class or a particular isotype of antibodies.
	Cell surface expression of molecules	Any	Flow cytometry	Cell populations and subpopulations, and cell functions on bacterial recognition (e.g. toll-like receptors), antigen presentation (e.g., MHC molecules), and cellular activation (e.g. cytokine receptors, co-stimulatory factors) can be analyzed. So far, numerous surface markers have been identified for detection with monoclonal antibodies.
In vivo	Circulating concentration of immune mediators	N/A	ELISA	In animal or human studies, blood samples can be analyzed for circulating concentrations of immune mediators, such as acute-phase protein, cytokines, soluble cytokine receptors, antibodies and complement proteinsThese markers are ideal for analysis after immune challenges (e.g. vaccination) or during ongoing inflammatory response.
	DTH response	N/A	skin test	The DTH response represents an integrated cell-mediated immune response and works as a good indicator of the host defense capacity. Technically, the response is measured as the size of induration around the area of antigen application at a period of 24-72 hr. Antigens for immune challenge include bacterial and fungal products as well as chemicals.

Table 2.2 Identified peptides with *in vitro* immunomodulatory activities.

Protein Source	Parent protein	Peptide Name/Sequence	Cell tested in	Immunomodulatory effects	Reference
milk	β-casein	VEPIPY (54-59)	Murine peritoneal macrophages	↑ macrophage phagocytosis	Parker et al. (1984)
		Caseinophosphopeptide (CPP)/ 28-aa (1-28, 4P)	Murine splenocytes, rabbit Peyer's patch cells	↑ proliferation and immunoglobulin production of splenocytes; ↑ proliferation and IL-6 expression on CD19+ (B) cells;	Hata et al. (1999); Tobita et al. (2006); Otani et al. (2001)
		YGEGEPVLGPVRGPFPIIV (193-209)	Murine marrow macrophages	↑ macrophage phagocytosis, MHC II expression	Sandre et al. (2001)
		β-casomorphin-7 / YPFPGPI (60-66)	Human lamina propria lymphocytes	↓ lymphocyte proliferation	Elitsur & Luk (1991)
	αs2-casein	Caseinophosphopeptide (CPP)/ 32-aa (1-32, 5P)	Murine splenocytes, rabbit Peyer's patch cells	↑ proliferation and immunoglobulin production of splenocytes	Hata et al. (1999)
	κ-casein	Glycomacropeptide (GMP) / 64-aa structure (106-169)	Murine spleen lymphocytes, rabbit Peyer's patch cells, human U937 cell line	↓ lymphocyte (B and T cells) proliferation; block IL-1 action by receptor binding; ↑ proliferation, phagocysis of macrophage	Otani et al. (1995); Otani & Monnai (1995); Li & Mine (2004)
	β-lactoglobulin	VAGTWY (15-20)	Murine splenocytes	↑splenocyte proliferation, cytokine (IL-4, IFN-γ) production	Jacquot et al. (2010)
		LIVTQTMK (1-8), YLLF (102-105)	Murine splenocytes	↓ splenocyte proliferation	Jacquot et al. (2010)
	α-lactalbumin	YG (18-19, 50-51)	Human peripheral lymphocytes	↑ lymphocyte proliferation	Kayser & Meisel (1996)
	Proline-rich polypeptides	(VE)SYVPLFP	Mouse splenocytes	↑ splenic antibody response	Janusz et al. (1987)
	Lactoferrin	Lactoferricin (Lfcin) / 25-aa structure (17-41)	Human neutrophils, murine spleen lymphocytes, rabbit Peyer's patch cells, human THP-1 cell line	↑ phagocytosis, IL-8 production of neutrophil; ↑ immunoglobulin (IgM, IgG and IgA) production in splenocytes; ↓ IL-6 response in monocytic cells	Shinoda et al. (1996); Miyauchi et al. (1998); Miyauchi et al. (1997); Mattsby-Baltzer et al. (1996)
Soy	β-Conglycinin	Soymetide / MITLAIPVNKPGR	Human neutrophil	↑ phagocytosis of neutrophil	Tsuruki et al. (2003)
	Glycinin A	(HC)QRPR	Human neutrophil	↑ phagocytosis of macrophage and neutrophils	Yoshikawa et al. (1993)
	N/A	VPY	Human THP-1 cell line	↓ TNF-α production in monocytic cells	Kovacs-Nolan et al. (2012)
	Soy albumin (GM2S-1)	Lunasin / 43-aa structure	Murine RAW264.7 cell line	↓ Pro-inflammatory markers (IL-6, IL-1β, TNF-α, NO, ROS, PGE2) and NF-κB activation in macrophage	de Mejia & Dia (2009); Hernández-Ledesma et al. (2009a)
Rice	N/A	Oryzatensin / GYPMYPLPR	Human peripheral leukocytes	↑ phagocytosis of neutrophil; ↑ ROS production in peripheral leukocytes	Takahashi et al. (1994)
Fish	N/A	PRRTRMMNGGR, MGPAMMRTMPG	Murine RAW264.7 cell line	↓ cytokine (TNF-α, IFN-γ, IL-2) production in macrophage	Cheng et al. (2015)

Table 2.3 Immunomodulatory peptides and protein hydrolysates tested in animal models.

Source	Peptide Name/Sequence OR Hydrolysate preparation	Animal model tested in	Immunomodulatory effects	Effective Dose and Duration	Reference
Milk	(VE)SYVPLFP	Mouse	↑ splenic antibody response to foreign erythrocyte antigens	1.0 and 10 μg, single dose	Janusz et al. (1987)
	CPPs/β-casein (1-28, 4P), αs2-casein (1-32, 5P)	Mouse	↑ Serum and intestinal antigen-specific IgA; ↑ splenic IgA, IL-5 and IL-6 production	0.1% to 1.0% of diet weight, 63 days; 0.1% of diet weight, 21 or 35 days.	Otani et al. (2000); Otani et al. (2003)
	Milk peptide fraction from *Lactobacillus helveticus* fermentation	Mouse infection	Protect against *E. coli* O157:H7 challenge; ↑ IgA+ B cells in lamina propria, secretory and systemic IgA response; ↑ Th2 (IL-4) ↓ Th1 (IFN-γ) response towards infection	50 μg per day, various treatment period (2-17 days)	LeBlanc et al. (2004)
	Whey peptide basic fraction (pH>7) from trypsin-chymotrypsin digestion	Mouse infection	↑ Serum IgA, TGF-β1 secretion in response to E. coli O157:H7 infection	3mg per day, 7 days	Saint-Sauveur et al. (2009)
	Casein hydrolysate fermented by *Lactobacillus delbrueckii*	Mouse colitis	Ameliorate TNBS-induced colitis; ↑ IL-10 ↓ IFN-γ in intestine	150 μg per day, 10 days	Turbay et al. (2012)
	Glycomacropeptide (GMP)	Rat colitis	Ameliorate TNBS-induced colitis; ↓ colonic iNOS and IL-1β expression	500 mg/kg BW per day, 9 days	Daddaoua et al. (2005)
		Rat ileitis	Ameliorate TNBS-induced ileitis; ↓ intestinal inflammatory mediators (MPO, iNOS, IL-1β, TNF-α, IL-17)	500 mg/kg BW per day, 7 days	Requena et al. (2008)
		Mouse	↓ Plasma cytokine (IFN-γ, TNF-α, IL-1β and IL-2)	20% of diet weight, from weanling through 8-9 wk of age	Sawin et al. (2015)
Soy	Soymetide-4	Mouse	↑ Serum TNF-α production	300 nmol, single dose	Tsuruki et al. (2003)
	VPY	Mouse colitis	Ameliorate DSS-induced colitis; ↓ colonic expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ, IL-17)	100 mg/kg BW per day, 14 days	Kovacs-Nolan et al. (2012)
	Commercial soy di- and tripeptides ("HINUTE-AM")	Porcine colitis	Ameliorate DSS-induced colitis; ↓ expression of inflammatory mediators (IFN-γ, IL1-β, TNF-α, RORC, and IL-17) in ileum and colon; modulate FOXP3 expression	250 mg/kg BW per day, 5 days	Young et al. (2012)
Egg	Egg yolk low lipid peptic digests	Mouse	↑ Phagocytic activity of peritoneal macrophages; ↑ IgA+, IL-4+, IL-10+, IFN-γ+,IL-12+ cells in lamina propria	~121.5 μg per day, 5 days	Nelson et al. (2007)
	Commercially prepared egg white peptides	Porcine colitis	Ameliorate DSS-induced colitis; ↓ colonic expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ, IL-8, IL-17)	150 mg/kg BW per day, 5 days	Lee et al. (2009)
Marine products	Salmon-derived oligopeptides	Mouse	↑ Lymphocyte proliferation, number of plaque-forming cells, NK cell activity, percentage of CD4+ cells in spleen; ↑ serum cytokine (IL-2, IFN-γ, IL-5, IL-6) production	0.22 g/kg BW per day, 4 weeks	Yang et al. (2009b)
	Fish egg-dervied peptides by pepsin hydrolysis	Mouse	↑ Splenic NK cell activity, macrophage phagocytosis; ↑ mucosal and serum IgA	0.25-1 g/kg BW per day, 45 days	Chalamaiah et al. (2014)
	Shark protein hydrolysate by trypsin-chymotrypsin hydrolysis (PeptiBal™)	Mouse infection	↑ IgA+, IL-10+, CTLA-4+ cells in intestine; ↑ TGF-β ↓ IL-17 in response to *E. coli* H10407 challenge	4.5 mg/kg BW per day, 7 days	Mallet et al. (2014)

Figure 2.1 Overview of immune system.

Figure was drawn according to Kindt et al. (2007).

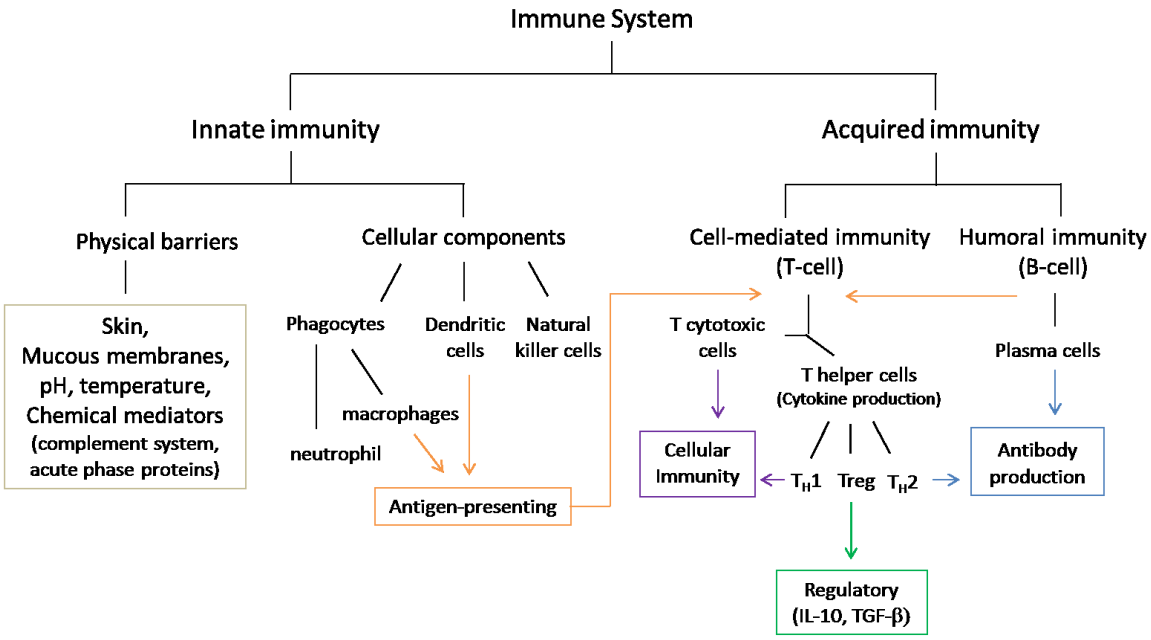
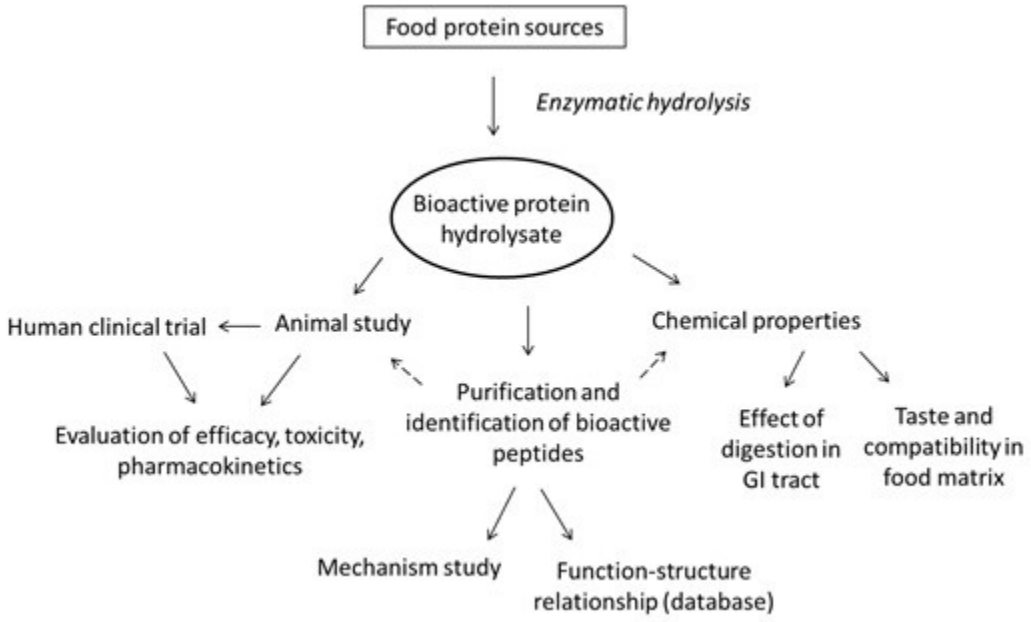


Figure 2.2 Issues to be addressed for applications of bioactive peptides in the form of functional foods and natural health products (FFNHP).



CHAPTER 3 – Enzymatic Preparation and Characterization of Hydrolysates with Immunomodulatory Properties from Spent Hen Muscle Proteins

3.1. Introduction

In the egg industry, when the laying hens reach the end of their productive life they are seen as a by-product that requires disposal. It is estimated that the egg industry in North America produces about 150 million spent hens each year (Newberry, Webster, Lewis, & Van Arnam, 1999). Although spent hens are processed for food and feed uses, the value of meat processed from spent hens is low due to its high cost of processing, a relatively low yield, and poor meat quality such as objectionable toughness and the presence of residual bone fragments (Kersey & Waldroup, 1998). For this reason, euthanasia followed by burial, composting, or incineration are often used to dispose spent hens, which raises additional expenses to the producer and environmental concerns (Freeman, Poore, Middleton, & Ferket, 2009). Therefore, there is a need to explore alternative way of utilization of spent hens.

Spent hen carcasses, similar to other poultry products, consist of mainly three components, fat, protein and moisture. It was reported that manually deboned spent hen meat contains 78.3% moisture, 3.7% fat and 15.3% protein (McIvor, Baccus-Taylor, & Comissiong, 2002). As an inexpensive and easy-accessible source of protein, spent hen can be utilized for production of bioactive peptides. Bioactive peptides are latent within their parent sequences but can be released upon enzymatic hydrolysis or food processing such as fermentation (Yoshikawa et al., 2000). These peptides have physiological benefits beyond their basic nutritional functions, and can be used as functional foods (Korhonen, 2009). Indeed, several angiotensin-converting enzyme (ACE)-inhibitory peptides have been isolated and identified from enzymatic hydrolyzed chicken meat proteins or bone collagen extract (Fujita, Yokoyama, & Yoshikawa, 2000; Saiga et al., 2003; Saiga et al., 2008). However there has been no study on immunomodulatory peptides generated from chicken meat proteins. The potential of chicken as a raw material for immune functions can be

traced back to the traditional use of chicken soup as a home remedy in treating common cold in many parts of the world. Its medicinal benefit in mitigating inflammation was supported by new evidence of neutrophil chemotaxis inhibitory activity using homemade or commercial chicken soups (Rennard, Ertl, Gossman, Robbins, & Rennard, 2000). The active components for this effect are not known but could be due to the peptides released upon heating.

As many of the chronic diseases, including metabolic disorders (Hotamisligil, 2006), atherosclerosis (Hansson, 2005; Libby, 2002), and cancer (de Visser, Eichten, & Coussens, 2006), are associated with chronic inflammatory conditions, research interests are directed to the discovery of anti-inflammatory peptides. Inflammation is a complex response to local injury or infection; and it involves various immune-system cells and numerous mediators (Kindt, Goldsby, Osborne, & Kuby, 2007). The objective of this study was to test the feasibility of utilizing spent hen carcass for production of immunomodulatory peptides for regulating inflammatory responses. In this study, peptides were prepared enzymatically using commercially available enzymes and their respective hydrolysates were characterized on degree of hydrolysis, protein content, nitrogen recovery, molecular weight distribution, and amino acid composition. The immunomodulatory activity was tested by endotoxin-induced cytokine response in macrophages, a commonly reported *in vitro* immune cell model for testing food-derived anti-inflammatory peptides (Udenigwe & Aluko, 2012). Bovine lactoferrin (bLf), a well-recognized immunomodulatory protein naturally existing in milk (Actor, Hwang, & Kruzel, 2009; Comstock, Reznikov, Contractor, & Donovan, 2014), was used as the positive control in this study; bLf exhibited anti-inflammatory effects in a human monocytic cell line THP-1 suppressing LPS-induced pro-inflammatory cytokine (interleukin (IL)-6, tumor necrosis factor (TNF)-α) productions (Håversen, Ohlsson, Hahn-Zoric, Hanson, & Mattsby-Baltzer, 2002; Mattsby-Baltzer et al., 1996). The effect of hydrolysates on cell viability was further assessed for those exhibiting bioactivity *in vitro*.

3.2. Material and methods

3.2.1. Chemicals and reagents

Alcalase 2.4 L was obtained from Novozymes (Franklinton, NC, USA). Protease M and Protease S were obtained from Amano Enzyme Inc. (Nagoya, Japan). Protex 6L, Protex 26L and Protex 50FP were obtained from Genencor International Inc. (Rochester, NY, USA). Pepsin from porcine gastric mucosa, thermolysin from *Bacillus thermoproteolyticus rokko*, trysin from porcine pancreas, Methanesulfonic acid solution, 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS), Cytochrome C from equine heart, aprotinin from bovine lung, glycine, $(glycine)_3$, Vitamin $B_{12,}$ Phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharides (LPS, *E. coli* strain *0111:B4*) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Precision Plus Protein™ Unstained Standards (10-250kD) was purchased from Bio-Rad (Hercules, CA, USA). RPMI-1640 culture media, fetal calf serum, antimycotic–antibiotic solution (10,000 µg/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate, and 25 µg/ml amphotericin B) and HEPES were purchased from Invitrogen (Burlington, Ontario, Canada). Human IL-6 ELISA Ready-SET-Go!® was purchased from eBioscience (San Diego, CA, USA). Cell proliferation reagent WST-1 was purchased from Roche (Laval, QC, Canada). Lactoferrin was purchased from Beyond A Century, Inc. (Greenville, ME, USA).

3.2.2. Preparation of hydrolysates from spent hen muscle proteins

Spent laying hen carcasses were purchased from local supermarket. Chicken meat was excised manually, trimmed of external fat and heavy connective tissue. Meat was homogenized with distilled water (1:4; w/v) using a Waring heavy duty blender (Waring Commercial, Torrington, CT, USA). Homogenized meat slurry was filtered through a metal fine sieve (1 mm mesh) to remove excess connective tissues. Further, pH was adjusted to 5.0 followed by centrifugation (10,000g x 25 min, 4°C; Beckman Coulter Inc., Fullerton, CA, USA), and the precipitate was harvested and freeze-dried for enzymatic hydrolysis.

The precipitate was mixed with distilled water at 4% (w/v) and hydrolyzed in a jacketed beaker with circulating water bath for temperature control; the pH was maintained constant using a Titrando instrument (Metrohm, Herisan, Switzerland). After pre-heating to 90°C for 10 min (to denature the proteins), the slurry was adjusted to working condition based on suppliers' recommendation (temperature, pH): Alcalase (50°C, 8.0), Protex 6L (60°C, 9.5), Protease S (70°C, 8.0), thermolysin (60°C, 8.0), trypsin (40°C, 7.0), Protease M (45°C, 5.0), pepsin (37°C, 2.0), Protex 50FP (50°C, 3.0), Protex 26L (55°C, 3.0). The enzyme was added at a level of 4% (w/w of substrate protein content) for a 3 hr hydrolysis. Then the hydrolysis was terminated by heating to 95°C for 15 min. Digested slurry was centrifuged (10,000g x 25 min, 4°C) and the supernatant further filtered through a Whatman #1 filter paper (Whatman Inc., Clifton, NJ, USA) to remove undigested proteins and excessive fat. The filtrate was collected and freeze-dried for analysis. All samples were prepared in duplicate.

3.2.3. Analysis of degree of hydrolysis

The degree of hydrolysis (DH), defined as the percentage of cleaved peptide bonds (of total peptide bonds present), was measured by TNBS (2,4,6-Trinitrobenzenesulfonic acid solution) method reported by Adler-Nissen (1979). Samples for total amino groups quantification were pre-treated with methanesulfonic acid (4 M, 115°C, 24 hr) in a Pico-tag working station (Waters, Milford, MA, USA) for a complete cleavage of peptide bonds (Simpson, Neuberger, & Liu, 1976).

The degree of hydrolysis (DH) is calculated using the formula reported by Baek & Cadwallader (1995). The content of free amino acids (primary amines) was expressed as leucine equivalents (calculated from the standard curve). $DH = (L_s-L_o)/(L_{max}-L_o) \times 100\%$, where L_s represents the amount of primary amines released by hydrolysis in sample; L_o be the amount of primary amines in original sample without enzymatic hydrolysis; and L_{max} be the maximum amount of primary amines in sample after acid hydrolysis.

3.2.4. Analysis of molecular weight distribution profile

Molecular weight distribution was studied by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion chromatography. The protein standard (10-250 kDa) and SDS treated hydrolysate samples were loaded to a 10-20% Tris–HCl precast gel (Bio-Rad, Hercules, CA, USA) and ran at constant voltage (200V) in an electrophoresis apparatus (Mini-PROTEAN Tetra cell attached to PowerPac Basic power supply; Bio-Rad, Hercules, CA, USA). Size-exclusion chromatography was run to examine the molecular weight distribution below 10 kDa. Samples were dissolved in 30% aqueous acetonitrile containing 0.1% trifluoroacetic acid (TFA) and loaded onto a Superdex peptide 10/300 GL column coupled with an AKTA explorer 10XT fast protein liquid chromatography system (GE Healthcare, Uppsala, Sweden). Fractions were eluted under isocratic condition and monitored by a UV detector at 215 nm. Five marker molecules (Cytochrome C, 12,384 Da; Aprotinin, 6,512 Da; Vitamin B12, 1,355 Da; (Gly)3, 189 Da; Gly, 75 Da) were run under identical conditions to plot the standard curve; and the molecular mass was calculated according to Irvine (1994).

3.2.5. Analysis of protein content and nitrogen recovery

The nitrogen content was determined using a Leco TrueSpec CN (carbon-nitrogen) elemental analyzer (Leco Corporation, St. Joseph, MI, USA). The protein content was calculated from nitrogen content using a conversion factor of 5.8 (Sosulski & Imafidon, 1990). The nitrogen recovery (NR) from hydrolysis was calculated as the percentage ratio of total nitrogen content in the hydrolysate (excluding undigested proteins in the precipitate removed during preparation) to that of the initial substrate.

3.2.6. Analysis of amino acid composition

Free and total amino acid compositions were analyzed based on a method reported by Sedgwick, Fenton, & Thompson (1991). Briefly, the separation and quantification of amino acids was accomplished on a Varian 5000 high-performance liquid chromatography (HPLC) equipped with a Supelcosil 3 micron LC-18 reverse phase column (Supelco,

Mississauga, ON, Canada) and a Fluorichrom detector (Varian, Palo Alto, CA, USA). Ethanolamine and β-aminobutyric acid were used as internal standards. Quantification was carried out using standard calibration curves.

3.2.7. Monocyte culture and activation

U937 monocytic cell line purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) were grown in RPMI 1640 medium supplemented with 10% (v/v) heat inactivated fetal calf serum and 1% (v/v) antibiotics in a humidified incubator (37°C, 5%CO_2). Cell density was maintained between 1 x 10^5 and 2 x 10^6 cells per mL for up to 20 passages. For cytokine assay, cells were transformed to macrophage-like cells by PMA followed by LPS activation to initiate cytokine cascade as reported with modifications (Okoko & Oruambo, 2009). Briefly, cells were seeded in a 24-well plate at a concentration of 5 x 10^4 cells per mL with PMA (40 ng/mL) for 24 hr. The second day, samples (500 µg/mL) were added to the cells 30 min prior to the addition of LPS (1 µg/mL); and the cells were kept for another 48 hr incubation. Equal amount of medium was added to replace the sample volume in the control; and lactoferrin was added as the positive control. In the end, the plates were centrifuged (460g x 5min, 4°C) and the supernatant were collected and stored at -80°C until the cytokines were measured.

3.2.8. Enzyme-linked immunosorbent assay (ELISA)

IL-6 and TNF-α concentrations in cell culture supernatant were measured using commercial enzyme-linked immunosorbent assay (ELISA) kit as per manufacturer's instructions. Samples were measured in duplicate and read at 450 nm on a SpectraMax 190 microtitier plate reader (Molecular Devices, CA, USA). Any values below the detection range (15.6-1000 pg/mL) were assigned half of the value of the lowest standard; dilution was performed for samples with values above the detection range. The coefficient of variance between duplicate samples was ≤10%. Cytokine inhibition percentage was calculated as: (Cc-Cs)/Cc x 100%, where Cc represents cytokine concentration in supernatant of the control cells; and Cs represents cytokine concentration in supernatant of cells treated with lactoferrin or the spent hen hydrolysates.

3.2.9. Cell viability study

Cell viability was estimated using the WST-1 assay as per manufacturer's instructions. Briefly, cells were seeded at 5 x 10^4 cells per mL in a flat bottom 96-well cell culture plate and incubated with PMA (24 hr, 40 ng/mL) followed by the addition of protein hydrolysate solutions (250-1000 µg/mL) with or without LPS (1 µg/mL) for another 2 days. Culture medium was added to the wells as the control. Then the plate was centrifuged (460g x 5 min, 4°C) and the supernatant was replaced with 100 µL fresh medium and 10 µL WST-1 proliferation reagent. The plate was incubated at 37°C for another 2 hr and read at 440 nm on the SpectraMax 190 microtiter plate reader (Molecular Devices, CA, USA). Samples were tested in quadruplicate.

3.2.10. Statistical analysis

Statistical analysis was conducted using the SAS software (Version 9.3; SAS Institute, Cary, NC, USA). All data that was non-parametric was log-transformed prior to statistical analysis. Data were reported with means and standard error of the mean (SEM). Data was analyzed by one-way ANOVA with Duncan's multiple range test and correlation analysis by Pearson's correlation calculation; statistical significance was reported at $P<0.05$.

3.3. Results and discussion

3.3.1. Preparation of spent hen hydrolysates

Laying hens were genetically selected to have smaller body weights, which have limited meat yield compared with broilers (commercial meat breeds). In the present study, we recovered about 40% (by weight) meat from the spent hen carcass by manual deboning, which is consistent with a previous report of 34.4 ~ 43.7% meat recovery from spent hens (McIvor et al., 2002). A meat yield of 60% was reported for female broilers (Moran & Bilgili, 1990). Meat proteins are generally classified into three groups based on their function and solubility, which are sarcoplasmic proteins (~29%), myofibrillar proteins (~61%) and stroma proteins (~10%) (Keeton & Eddy, 2004). Since acid precipitation at pH

5.0 was used to remove the heme pigment in meat slurry, about 27% protein loss was observed due probably to the loss of water-soluble sarcoplasmic proteins (mainly myoglobin and glycolytic enzymes). After removing pigment, spent hen protein extracts were enzymatically hydrolyzed to prepare spent hen hydrolysates. Degree of hydrolysis (DH) and molecular weight profile were determined as they are useful parameters for monitoring peptide bonds cleavage and the extent of protein degradation.

DH values of spent hen hydrolysates ranged from 11.2% to 32.7% (Figure 3.1A) depended on the enzyme used. Molecular weight distribution of hydrolysates was studied by SDS-PAGE; as shown in Figure 3.1B, large meat protein molecules were broken down into smaller molecules. The original meat sample contained a number of bands with molecular weights ranging from 15 to 250 kDa, which included major meat proteins actinin (95 to 105 kDa), actin (46 kDa), α-tropomyosin (36 kDa) and light myosin chains as previous reported (Kurozawa, Park, & Hubinger, 2008). The extent of hydrolysis was observed from loss or decreased intensity of large molecular weight bands and the appearance of a diffusing band with a molecular weight below 10 kDa in the hydrolysate loaded lanes. Size-exclusion chromatography further confirmed that all hydrolysates were composed of molecules less than 12 kDa (Figure 3.1C); Protease M hydrolysate contained mainly small peptides below 1 kDa.

3.3.2. Hydrolysis yield and amino acid compositions of spent hen hydrolysates

The protein content of the hydrolysates ranged from 70% (Alcalase hydrolysate) to 83% (trypsin hydrolysate; Figure 3.2). Nitrogen recovery (NR), as an index of hydrolysis yield, varied among different samples (Figure 3.2). Thermolysin hydrolysis had the highest NR of 86%, followed by Alcalase and Protex 26L; trypsin hydrolysis had the lowest NR of 44%. Statistical analysis of DH and NR results found a positive correlation between them ($r=0.508$, $n=18$, $P<0.05$). Generally, hydrolysates with higher DH values had a higher NR; for example, Alcalase hydrolysate had a high DH value and a high NR, trypsin hydrolysate had the lowest DH value and the lowest NR. A previous study on enzymatic hydrolysis of

fish meat proteins reported correlations between DH and NR when hydrolyzed with Alcalase and Neutrase (Benjakul & Morrissey, 1997).

The total amino acids composition was not affected before and after hydrolysis with different enzymes. Chicken meat and its hydrolysates contained high amount of Glx (glutamic acid + glutamine) and Asx (aspartic acid + asparagine). This has been reported in chicken and spent hen meat (Keeton & Eddy, 2004; Kersey, Parsons, Dale, Marr, & Waldroup, 1997). On the other hand, enzymatic hydrolysis greatly increased the content of free amino acids as compared to the meat sample without digestion. Free amino acids composition varied among different hydrolysate samples. Protease M, Protex 26L and Protex 50FP hydrolysates had notably higher free amino acid contents compared to the others; additionally, the hydrolysates had higher percentages of aromatic and hydrophobic amino acids but a lower percentage of positively charged amino acids when compared to others (Table 3.1).

3.3.3. Effects of spent hen hydrolysates on macrophage-like cell line

After differentiation by PMA pre-treatment, the human monocytic cell line U937 represents an experimental model to study macrophage function. Macrophage provides an important front line in host defense against infection; upon activation, it exhibits phagocytic activity, antimicrobial and cytotoxic activities, functions on antigen processing and presentation, and secretes a variety of cytokines (Kindt, Goldsby, Osborne, & Kuby, 2007). In macrophages, LPS activates the transcription factor NF-κB through a TLR4-mediated signaling pathway (Tak & Firestein, 2001; Takeda & Akira, 2004); the classically activated macrophages secretes a variety of pro-inflammatory cytokines including IL-1, IL-6 and TNF-α (Mosser, 2003).

The production of IL-6 was significantly increased to an average of 972±26 pg/mL with LPS stimulation in the macrophage-like U937 cells, compared with a concentration below detection range (15.6 pg/mL) in the absence of LPS. Adding Protease M and Protex 50FP hydrolysates, at a final concentration of 500 μg/mL significantly reduced IL-6 production by 13% and 15%, respectively ($p<0.05$, Figure 3.3A) in LPS stimulated cells; as compared

to a 28% inhibition by lactoferrin (a well-established anti-inflammatory protein) at the same concentration. IL-6 productions from cells treated with other spent hen hydrolysates were not affected (Figure 3.3A). Similarly, the production of TNF-α was also significantly increased upon LPS stimulation (from a concentration below detection range (15.6 pg/mL) to an average of 613±17 pg/mL). Incubation with Protease M hydrolysate (500 μg/mL) for 48 hr inhibited TNF-α production by 25% ($p<0.001$), as compared to a 57% inhibition by bLf (Figure 3.3B). The effects were greater than those observed on IL-6 production. Protex 50FP hydrolysate, which suppressed IL-6 production in the cells, did not have an effect on TNF-α production; on the other hand, Protease S hydrolysate that did not exhibit effect on IL-6 enhanced TNF-α production in cells (Figure 3.3B).

Cell viability/metabolism (as defined by a decrease in WST-1 absorbance) was not affected by hydrolysates at concentrations up to 1000 μg/mL in PMA differentiated U937 cells (Figure 3.4A). Adding LPS alone to the differentiated cells significantly reduced cell viability/metabolism; adding LPS along with hydrolysates did not further suppress the cell viability rather improved cell viability (increased absorbance readings) at a low concentration of 250 μg/mL for Protex 50FP hydrolysate (Figure 3.4B), suggesting a possible anti-apoptosis effect (Xaus et al., 2000). This possible anti-apoptosis effect did not significantly decrease at the concentration of 500 μg/mL. Therefore, the observed IL-6 and TNF-α inhibitory activity from hydrolysates at 500 μg/mL were unlikely the results of the hydrolysate having a toxic effect on cell metabolism and viability.

The cytokines IL-6 and TNF-α are generally considered cytokines involved in the inflammatory response. TNF-α is reported to induce fever, stimulate prostaglandin E_2 (PGE_2) and IL-6 synthesis, and shares the inflammatory property with IL-6 in inducing acute-phase proteins synthesis in liver; IL-6, in addition to the effect on acute-phase proteins, exerts effects on adaptive immunity, acting on maturation and activation of B cells and T cells (Feghali & Wright, 1997). IL-6 and TNF-α are elevated in most inflammatory states (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011) and elevated systemic concentrations are associated with a variety of chronic diseases such as diabetes, rheumatoid arthritis and colitis (Aggarwal, Gupta, & Kim, 2012; Neurath & Finotto, 2011).

Therapeutic blockade of IL-6 or TNF-α has been found to be effective in treating inflammation-related diseases (Aggarwal et al., 2012; Scheller et al., 2011).

Anti-inflammatory activities on macrophage-derived pro-inflammatory cytokines have been reported in many other food-derived peptides. In a murine macrophage cell line RAW264.7, yak casein Alcalase hydrolysate inhibited IL-6 and TNF-α production by about 61% and 45%, respectively, at a concentration of 500 μg/mL (Mao, Cheng, Wang, & Wu, 2011); a peptide fraction (1000–2000 Da) from salmon pepsin hydrolysate inhibited IL-6 and TNF-α production by about 50% and 34%, respectively, at a concentration of 200 μg/mL (Ahn, Je, & Cho, 2012). Spent hen muscle protein hydrolysates showed relatively weaker activity than previously reported hydrolysates but the use of different cell lines might be at least partially responsible for this difference.

3.4. Conclusions

A protocol to prepare spent hen muscle protein hydrolysates was developed. The hydrolysates were characterized for their degree of hydrolysis (11.2 to 32.7%), protein content (70 to 83%), nitrogen recovery (44.4 to 86.5%), molecular weight distribution profile (< 12 kDa), and amino acid composition. For the first time we showed anti-inflammatory activities of hydrolysates prepared from spent hen muscle proteins. In human macrophage-like cells, spent hen hydrolysate prepared from Protex 50FP inhibited the production of IL-6 with LPS stimulation; spent hen hydrolysate prepared with Protease M exhibited suppressive effect on the production of pro-inflammatory cytokines IL-6 and TNF-α. Spent hen muscle protein hydrolysate showed comparably activity to bovine lactoferrin, a well-established immunomodulatory molecule, indicating the potential of spent hen muscle protein hydrolysate as functional food ingredients against chronic inflammation and the related diseases.

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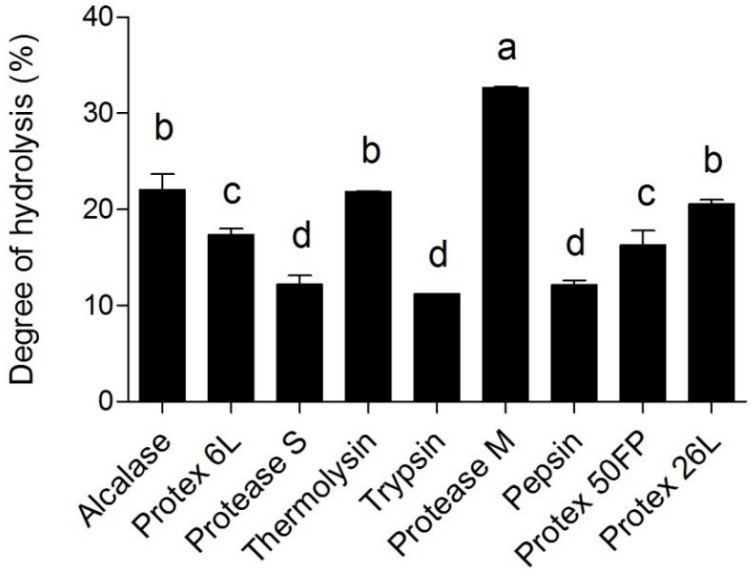
Table 3.1 Free amino acid composition of spent hen protein hydrolysates and unhydrolyzed meat protein sample (nmol/mg of dry weight). Values were expressed as mean (n=4 per group). Within a row, means without a common superscript (a-g) differ ($P < 0.05$). Positively charged amino acids: arginine, histidine, lysine; acidic amino acids: aspartic acid, glutamic acid; aromatic amino acids: phenylalanine, tryptophan, tyrosine; hydrophobic amino acids: alanine, valine, isoleucine, leucine, phenylalanine, tryptophan, tyrosine, methionine.

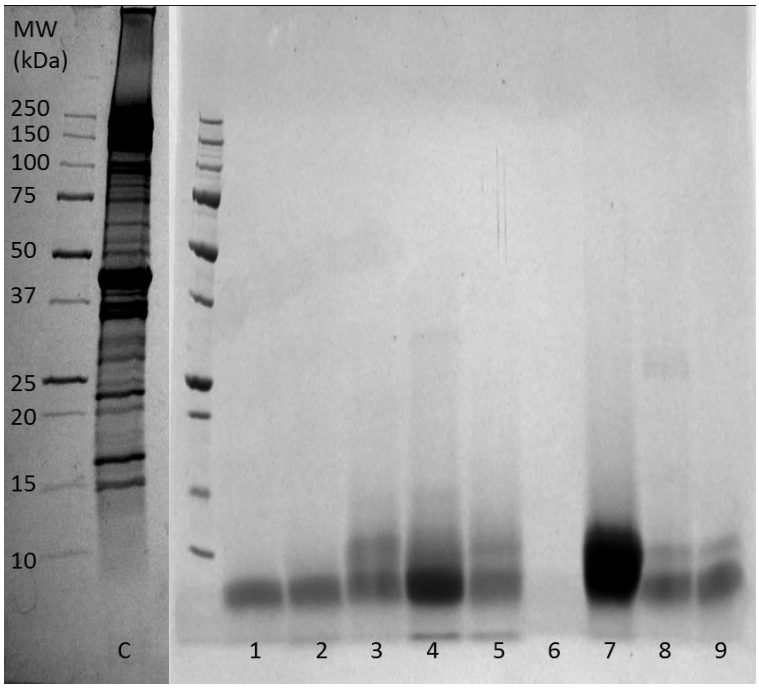
Amino acid	Control	Alcalase	Protex 6L	Protease S	Thermolysin	Trypsin	Protease M	Pepsin	Protex 50FP	Protex 26L	Average SEM
Asp	2.0^{c}	2.4^{c}	2.6^{c}	4.0^{c}	2.5^{c}	3.1^{c}	19.1^{a}	11.0^{b}	18.5^{a}	21.7^{a}	1.2
Glu	3.5^{b}	7.6^{b}	4.0^{b}	5.8^{b}	3.9^{b}	4.9^{b}	35.0^{a}	7.1^{b}	32.1^{a}	39.1^{a}	2.3
Asn	0.5^{e}	2.0^{cd}	1.8^{cd}	0.8^{de}	0.3^{e}	0.3^{e}	14.8^{a}	0.9^{de}	2.9^{c}	5.3^{b}	0.4
Ser	2.8^{e}	5.0^{cd}	4.6^{cde}	$5.2c^{d}$	3.6^{de}	3.6^{de}	25.5^{a}	4.3^{cde}	5.8^{c}	8.0^{b}	0.5
Gln	2.5^{d}	0.1^{f}	1.6^{de}	1.9^{de}	1.0^{ef}	2.3^{de}	25.2^{a}	0.0^{f}	4.1^{c}	5.8^{b}	0.5
His	1.2^{c}	1.1^{c}	1.4^{c}	1.4^{c}	0.3^{d}	1.6^{c}	16.3^{a}	1.4^{c}	2.5^{b}	2.6^{b}	0.3
Gly	2.1^{c}	3.7^{b}	2.9^{bc}	3.9^{b}	2.1^{c}	3.1^{bc}	16.4^{a}	4.1^{b}	3.9^{b}	4.1^{b}	0.5
Thr	1.9^{c}	2.7^{c}	2.7^{c}	2.9^{c}	2.2^{c}	2.4^{c}	32.8^{a}	2.9^{c}	4.4^{b}	5.4^{b}	0.3
Arg	9.5^{e}	10.7^{de}	10.6^{de}	16.1^{de}	10.3^{de}	21.1^{cd}	69.6^{a}	11.9^{de}	42.3^{c}	30.2^{b}	3.5
Ala	4.8^{d}	9.6^{d}	6.6^{d}	6.9^{d}	5.1^{d}	6.3^{d}	41.0^{a}	7.1^{d}	15.5^{c}	25.3^{b}	1.9
Tyr	1.6^{e}	3.7^{de}	2.2^{de}	2.2^{de}	2.5^{de}	3.9^{de}	28.2^{a}	5.8^{d}	12.2^{c}	18.2^{b}	1.2
Trp	0.5^{e}	3.4^{cd}	4.8^{c}	1.6^{de}	3.8^{c}	7.9^{b}	11.4^{a}	2.8^{cd}	7.9^{b}	8.7^{b}	0.6
Met	1.4^{e}	6.8^{d}	5.8^{de}	3.1^{de}	3.4^{de}	4.0^{de}	29.4^{a}	4.5^{de}	13.6^{c}	19.0^{b}	1.4
Val	1.9^{d}	4.4^{bcd}	3.5^{cd}	4.5^{bcd}	6.4^{bcd}	4.0^{cd}	34.1^{a}	4.0^{cd}	7.5^{bc}	8.8^{b}	1.4
Phe	1.8^{e}	3.0^{e}	3.2^{e}	3.0^{e}	5.4^{de}	5.6^{de}	41.7^{a}	9.9^{d}	18.7^{c}	29.3^{b}	1.9
Ile	1.4^{b}	2.0^{b}	5.0^{b}	3.3^{b}	5.8^{b}	2.3^{b}	38.7^{a}	2.6^{b}	5.3^{b}	4.7^{b}	1.6
Leu	3.1^{b}	6.6^{b}	8.4^{b}	7.1^{b}	10.7^{b}	10.8^{b}	57.9^{a}	11.1^{b}	52.9^{a}	57.0^{a}	3.7
Lys	2.3^{g}	23.3^{f}	34.3^{ef}	69.6^{b}	69.3^{b}	50.8^{cde}	45.0^{de}	95.1^{a}	66.5^{bc}	54.9^{bcd}	5.7
Total	44.7^{c}	98.3^{cd}	106.0^{cd}	143.3^{c}	138.8^{c}	137.4^{c}	582.1^{a}	186.4^{c}	316.6^{b}	348.0^{b}	28.5
% Positively charged	29.0^{d}	35.4^{c}	43.5^{b}	60.6^{a}	57.9^{a}	54.9^{a}	22.3^{e}	56.9^{a}	35.0^{c}	25.2^{de}	1.8
% Acidic	12.4^{b}	10.4^{bc}	6.1^{d}	6.8^{d}	4.6^{d}	5.9^{d}	9.2^{c}	9.8^{c}	16.0^{a}	17.4^{a}	0.8
% Aromatic	8.4^{d}	10.3^{cd}	9.9^{cd}	4.8^{e}	8.4^{d}	12.3^{bc}	14.0^{ab}	10.1^{cd}	12.3^{bc}	16.2^{a}	1.0
% Hydrophobic	36.6^{cd}	40.4^{bc}	37.8^{bcd}	22.0^{f}	30.9^{e}	33.5^{e}	48.2^{a}	26.0^{f}	42.4^{b}	49.2^{a}	1.7

Figure 3.1 Degree of hydrolysis (A), SDS-PAGE (B), and gel filtration chromatograms (C) of spent hen hydrolysates prepared with nine different proteases. Bars represent mean values ± SEM (n=4 per treatment); bars that do not share a common letter (a-d) differ ($P < 0.05$). On SDS-PAGE gel, C - Control (unhydrolyzed spent hen meat protein sample); Lane 1 – 9: 1 - Alcalase, 2 - Protex 6L, 3 - Protease S, 4 - Thermolysin, 5 - Trypsin, 6 - Protease M, 7 - Pepsin, 8 - Protex 50FP, 9 - Protex 26L.

(A)



(B)


MW
(kDa)
250
150
100
75
50
37
25
20
15
10
C
1
2
3
4
5
6
7
8
9

(C)

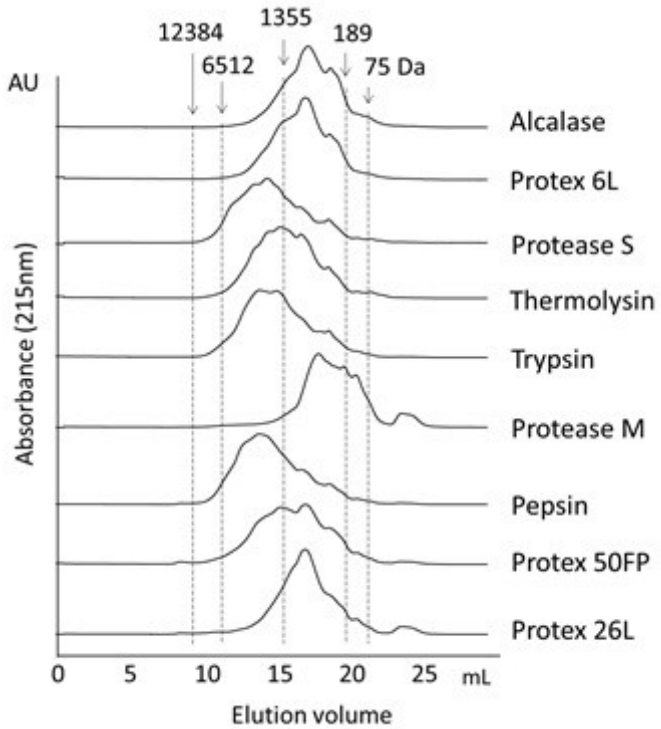

12384
6512
1355
189
75 Da
AU
Alcalase
Protex 6L
Protease S
Thermolysin
Trypsin
Protease M
Pepsin
Protex 50FP
Protex 26L
Absorbance (215nm)
0
5
10
15
20
25
mL
Elution volume

Figure 3.2 Hydrolysis nitrogen recovery and protein content of different spent hen hydrolysates. Bars represent mean values ± SEM (n=4 per treatment); bars that do not share a common letter differ (P<0.05). Lowercase letters denote differences in nitrogen recovery, whereas uppercase letters represent differences in protein content of the hydrolysates.

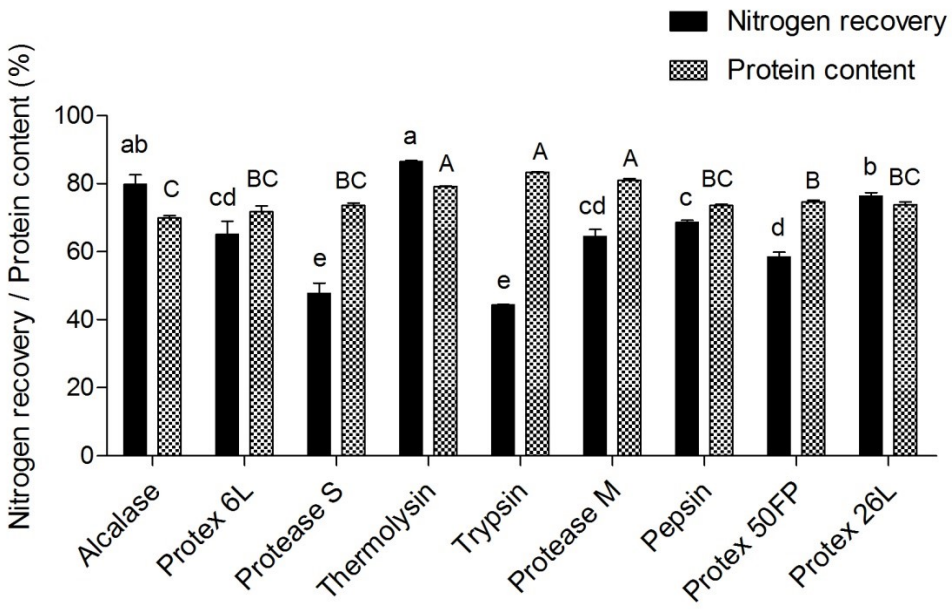
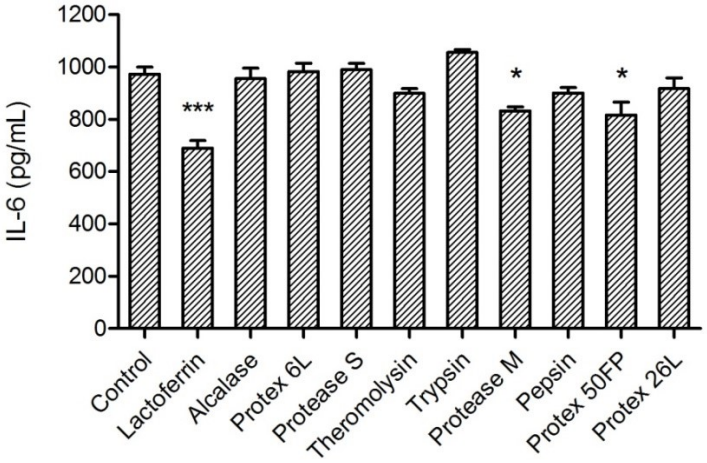


Figure 3.3 Effect of spent hen hydrolysates on IL-6 and TNF production of LPS-stimulated U937 cells. Hydrolysate was added at a concentration of 500 μg/mL and incubated for 48 hr. Bars represent mean values ± SEM (n=6 per treatment). * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ compared to the control.

(A)



(B)

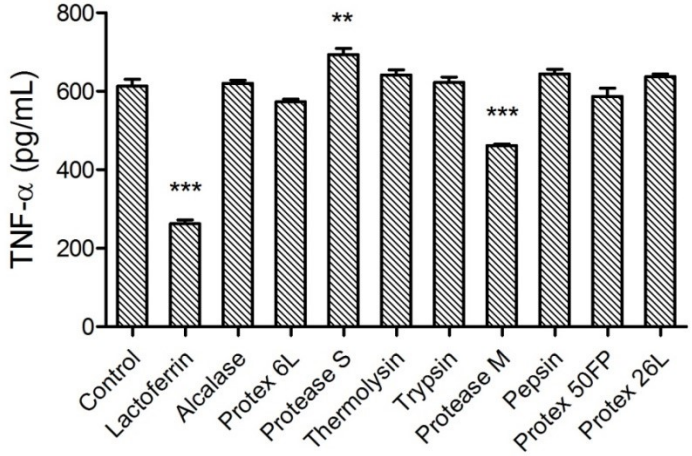
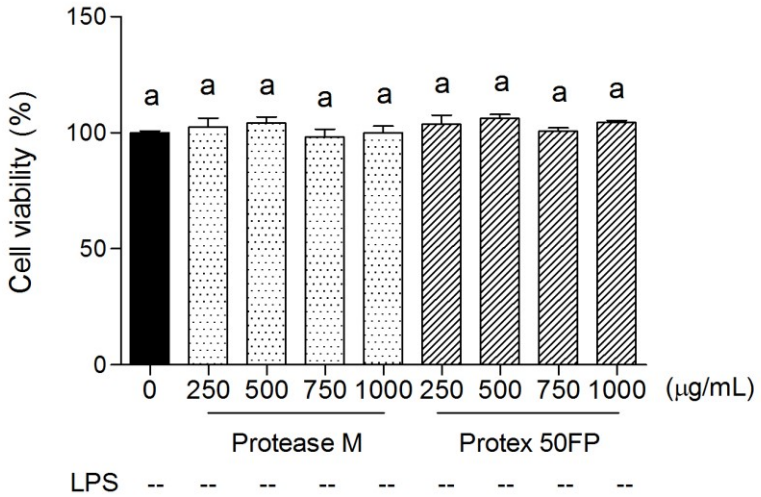
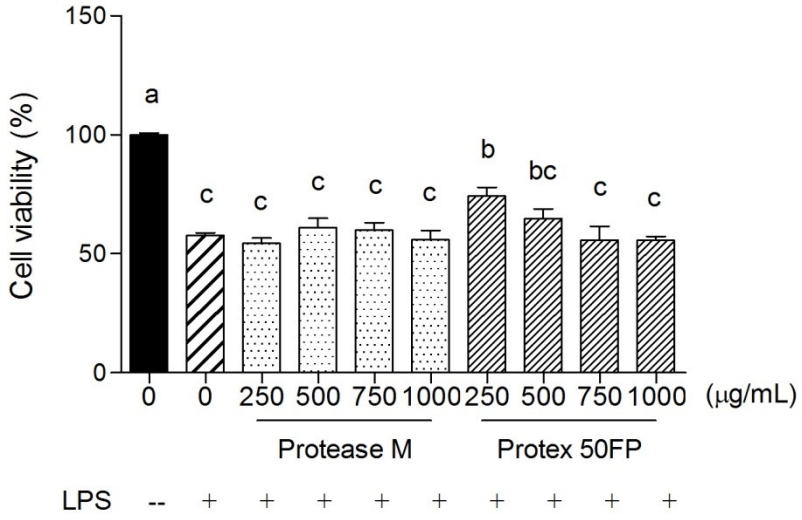


Figure 3.4 Effect of hydrolysate incubation on WST-1 cell viability in the absence (A) and presence (B) of LPS. Bars represent mean values ± SEM (n=4 per treatment); bars that do not share a common letter (a-c) differ ($P < 0.05$).

(A)



(B)



CHAPTER 4 – Spent hen muscle protein hydrolysate exhibits immunomodulatory effects in healthy Sprague-Dawley rats

4.1. Introduction

Each year about 150 million spent hens have to be disposed by the egg industry in North America (Newberry, Webster, Lewis, & Van Arnam, 1999). Although spent hen is traditionally processed for food and feed uses, the value of meat processed from spent layers is low due to the high cost of processing, a relatively low yield, and poor quality such as objectionable toughness and the presence of residual bone fragments (Kersey & Waldroup, 1998). In addition to paying the cost for disposal, there are environmental concerns on spent hen disposal by burial, composting or incineration due to associated carbon footprints and the risk of ground water contamination (Freeman, Poore, Middleton, & Ferket, 2009).

Alternatively, spent hen could be utilized as a source of bioactive peptides. It is now well recognized that dietary proteins are precursors of bioactive peptides, which once being released could exert physiological activities on cardiovascular, nervous, gastrointestinal and immune systems of the body (Hartmann & Meisel, 2007; Korhonen & Pihlanto, 2006). Spent hen meat contains the characteristic muscle proteins including myosin, actin, titin, tropomyosin, troponin, glyceraldehyde phosphate dehydrogenase, myoglobin, collagen and elastin (Keeton & Eddy, 2004); from which many bioactive sequences have been identified with antihypertensive, antioxidant and antimicrobial activities (Udenigwe & Howard, 2013). In our previous study (Chapter 3), spent hen muscle proteins digested with the enzyme Protease M or Protex 50FP have yielded hydrolysates with inhibitory activities on macrophage-produced pro-inflammatory cytokines (IL-6, TNF-α). Beneficial effects have been demonstrated *in vitro* in an endotoxin-stimulated macrophage model; therefore further examination of spent hen hydrolysates *in vivo* is warranted.

To evaluate the immunomodulatory effects using animal models, peptides or hydrolysates have been incorporated into the diet; body fluids or immune cells have been isolated from

organs of peptide-fed animals and assessed for various immune indices such as lymphocyte proliferation, cell surface expression of molecules, cytokine and antibody productions, and phagocytosis activities (Calder & Kew, 2002). Many of the food-derived peptides exhibiting *in vitro* immunomodulatory activities have exhibited efficacy in animal experiments. For example, caseinophosphopeptide β-casein (1-28) stimulated immunoglobulin and cytokine production *in vitro* in lymphocytes (Hata, Ueda, & Otani, 1999); when fed to mice, it up-regulated systemic (serum) and secretary (intestinal, feces) IgA levels and splenic Th2 cytokines (IL5, IL-6) (Otani, Nakano, & Kawahara, 2003). An enzymatic hydrolysate of pea proteins (PPH) exhibited anti-inflammatory effect *in vitro* in activated macrophages inhibiting the production of NO, TNF-α and IL-6; oral administration of PPH stimulated phagocytic activity of peritoneal macrophages and the gut mucosa immune response (elevated number of IgA+, IL-4+, IL-10+ and IFN-γ+ cells) in mice (Ndiaye, Vuong, Duarte, Aluko, & Matar, 2012). Milk peptide glycomacropeptide (GMP), which inhibited proliferation of mouse splenocytes and rabbit Peyer's patch cells *in vitro* (Otani & Hata, 1995), lowered concentrations of plasma cytokines (IFN-γ, TNF-α, IL-1β and IL-2) in mice (Sawin et al., 2015) and enhanced FoxP3 (Treg marker) expression in splenocytes of healthy Wistar rats (Requena et al., 2010).

As indicated from these studies, spent hen peptides exhibiting anti-inflammatory activity *in vitro* could exert immunomodulatory effects *in vivo* when fed to animals. The objective of this work was to evaluate the safety (based on organ measurements) and effects of feeding two spent hen hydrolysates on immune function of healthy young rodents, by measuring phenotype and cytokine response in isolated splenocytes (representing systemic immunity).

4.2. Material and methods

4.2.1. Reagents

Protease M was obtained from Amano Enzyme Inc. (Nagoya, Japan); Protex 50FP was obtained from Genencor International Inc. (Rochester, NY, USA). Lipopolysaccharides (LPS, *E. coli* strain *0111:B4*), concanavalin A (ConA), pokeweed mitogen (PWM) were purchased from Sigma-Aldrich (Oakville, ON, Canada). DuoSet® ELISA Development

Systems for interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), IL-10, IL-2 and interferon-γ (IFN-γ) were purchased from R&D Systems (Minneapolis, MN, USA). Fluorescent pre-labeled monoclonal antibodies were purchased from eBioscience (CD3, CD4, CD28, CD80; San Diego, CA, USA) and BioLegend (CD8, OX62, CD25, CD11b/c; Vineland, ON, Canada) except for OX12 purchased from AbD Serotec (Raleigh, NC, USA) and OX6 from BD Biosciences Pharmingen (Mississauga, ON, Canada). Basal mix (with fat source omitted) and high protein casein were purchased from Harlan Teklad (Madison, WI, USA).

4.2.2. Preparation of spent hen hydrolysates

Spent hen hydrolysates were prepared as previously described (Chapter 3). Briefly, spent hens were deboned manually to excise meat. Chicken meat was homogenized with distilled water (1:4; w/v) and the excess connective tissues were removed. The meat slurry was pH to 5.0 to remove the heme pigment by centrifugation (10,000 g x 25 min, 4°C; Beckman Coulter Inc., Fullerton, CA, USA); the precipitate (crude proteins) was harvested for enzymatic hydrolysis. The meat proteins (5%, w/v mixed with distilled water) were digested by proteases (4%, w/w of protein substrate) in a jacketed beaker with circulating water bath; pH was maintained constant using a Titrando instrument (Metrohm, Herisan, Switzerland). Meat slurry was pre-heated (90°C, 10 min) to denature the proteins and adjusted to working conditions based on suppliers' recommendation (Protease M, 45°C, pH=5.0; Protex 50FP, 50°C, pH=3.0) for a 3 hr digestion. The hydrolysis was terminated by heating at 95°C for 15 min. After centrifugation (10,000 g x 25 min, 4°C) the supernatant was collected and freeze-dried for diet preparation.

4.2.3. Animals and diets

Male weanling Sprague-Dawley rats were obtained from Biosciences Animal Service (University of Alberta, Edmonton, AB, Canada). All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the Faculty of Agricultural, Life and Environmental Sciences Animal Ethics Committee at the University of Alberta. All rats were housed two per cage in a temperature

and humidity controlled room with a 14L: 10D light-dark cycle. Rats were given free access to water and were fed *ad libitum* a nutritionally complete semi-purified diet for 3 weeks. Body weight and feed intake were recorded three times a week and rats were visually monitored for health. At the end of the feeding period, rats were killed by CO_2 asphyxiation. Body weight, liver and spleen weights and intestine length were recorded and the spleens were removed under aseptic conditions.

The nutritionally adequate basal diet contained the components as previously reported with slight modification (Field, Toyomizu, & Clandinin, 1989) as follows (g/kg of diet): high protein casein, 270; cornstarch, 200; cellulose, 50; dextrose, 210 g; fat, 200; choline, 1.4; inositol, 6.25; L-methionine, 2.5; AOAC vitamin mix, 10; Bernhart-Tomarelli mineral mix, 50; fat, 200g. The 200 g (per kg diet) fat contained 86 g of canola tallow, 69.5 g of sunflower oil, 40 g of olive oil, 4 g of flax oil, and 0.5 g of ARASCO; mixed to provide a ratio of polyunsaturated to saturated fatty acids (P/S ratio) of 0.4. The experimental diets were prepared by supplementing the basal diet with the addition of muscle protein hydrolysates or high protein casein. In a dose-repose pilot study, the two spent hen hydrolysates were each added at four concentrations (0.5%, 1%, 2% and 5%; w/w diet based) to determine the effective dose. The diets were made isocaloric and isonitrogenous by supplementing bovine casein to all the diets to ensure a total of 5% (w/w) added proteins. The control diet contained 5% (w/w) added bovine casein. 18 weanling rats were randomized to one of the nine diet groups in the pilot study. As an effective dose was determined at 2% (w/w) of Protex 50FP hydrolysate, a follow-up feeding experiment with enlarged group size (n=8/group) was carried out to test the effect of Protex 50FP hydrolysate at a low dose of 2% (w/w) and a high dose of 5% (w/w).

4.2.4. Isolation of splenocytes and primary culture conditions

At necropsy, spleens were removed and placed in sterile Krebs-Ringer-HEPES buffer (pH 7.4) supplemented with 0.5% (w/v) bovine serum albumin and splenocytes were isolated as previously reported (Ruth, Proctor, & Field, 2009). Isolated splenocytes were resuspended in the culture media, counted on a haemocytometer and again resuspended to be seeded in 4 mL sterile polystyrene tubes as previously described. Splenocytes were

cultured in replicates without (unstimulated) or with mitogen (ConA, 5 μg/mL; LPS, 15 μg/mL; or PWM, 55 μg/mL). Supernatant was removed at 24 hr, 48 hr and 72 hr and stored at -80°C until cytokine analysis.

4.2.5. Phenotype analysis

Freshly isolated immune cells were analyzed using a direct four-colour labelled immunofluorescence assay as previously reported with modifications (Ruth et al., 2009). Immune cells were incubated with pre-labelled monoclonal antibodies to identify and quantify the phenotypes. The following antibody combinations were used: CD3/CD4/CD8, CD28/CD25/CD4/CD8, OX12/CD4/CD8, OX6/CD11b/c, and OX62/CD80/OX6. After the final wash, cells were fixed in phosphate buffered saline containing 1% (w/v) paraformaldehyde, and the proportion of positive cells for each marker was determined according to the relative fluorescence intensity by flow cytometry (LSR-Fortessa; BD Biosciences, Mississauga, ON, Canada) using the Kaluza software (Beckman Coulter Inc., Mississauga, ON, Canada).

4.2.6. Cytokine production

Supernatants of splenocytes were measured for IL-1β (31.2-2000 pg/mL), TNF-α (31.2-2000 pg/mL), IFN-γ (31.2-2000 pg/mL), IL-6 (78-5000 pg/mL), IL-10 (15.6-1000 pg/mL) and IL-2 (23.4-1500 pg/mL) production with R&D Systems DuoSet® ELISA kits according to the manufacturer's instructions. Samples were measured in duplicates at 450 nm in a SpectraMax 190 microtiter plate reader (Molecular Devices, CA, USA) and compared to a standard curve within the concentrations described above. Any values below the detection range were assigned half of the value of the lowest standard. The average of the duplicate data with a coefficient of variance ≤10% was used for statistical analysis.

4.2.7. Statistical analysis

Statistical analysis was conducted using the SAS software (Version 9.4; SAS Institute, Cary, NC, USA). All data that was non-parametric was log-transformed prior to statistical

analysis. Results were analyzed by one-way analysis of variance (ANOVA) with Duncan's multiple range test, and presented as means ± the standard error of the mean (SEM). Regression analysis was performed for data from dose-repose pilot study. Splenocyte cytokine production measured at multiple time points were analyzed using a mixed model two-way ANOVA.

4.3. Results

Pilot data informed the hydrolysate and the dose used in this study (Appendix I).

4.3.1. Body weight, food intake and organ characteristics

Consistent with the pilot study, feeding Protex 50FP hydrolysate did not exert any detrimental effects on food intake, body weight or organ measures (liver weight, intestine length) at either of the doses fed (Table 4.1).

4.3.2. Immune cell phenotype in spleen

Rats fed the high dose of 5% (w/w) had a lower proportion of OX6+ (cells expressing major histocompatability complex, MHC class II molecule) cells and a higher proportion of CD11b/c+ (macrophages, granulocytes and dendritic cells) cells in spleen compared with rats fed by the casein-supplemented control diet ($p<0.05$, Table 4.2). There was no significant effect of supplementing with the hydrolysate at a low dose of 2% (w/w). No significant difference was observed amongst experimental groups in the proportion of total T cells (CD3+), T cell subsets including T helper cells (CD3+CD4+) and T cytotoxic cells (CD3+CD8+), T cells subsets expressing IL-2 receptor (CD25+) and co-stimulatory factor (CD28+), total B cells (OX12+), dendritic cells (OX62+), and antigen presenting cells expressing MHC II and co-stimulatory factor (OX6+CD80+) (Table 4.2).

4.3.3. Cytokine production

Supplementing the diet with Protex 50FP hydrolysate at the high dose of 5% (w/w) exerted stimulatory effects on splenic IL-10 production in response to mitogen stimulation; two-

way ANOVA analysis suggested significant effects of diet treatment on IL-10, resulted in a higher production of IL-10 at 72 hr with LPS ($p<0.01$, Figure 4.1A); and at 24 and 48 hr with PWM ($p<0.01$, Figure 4.1B). After incubation with LPS, IL-10 production did not differ significantly at the studied time points (24, 48, 72 hr). IL-10 production peaked at 48 hr post-stimulation with PWM, for which the 72 hr result was not shown. Splenocytes isolated from rats fed a low dose (2%, w/w) diet did not significantly differ from the control in mitogen stimulated production of IL-10 (Figure 4.1).

Feeding the hydrolysate diet did not affect the production of the other cytokines (IL-1, IL-6, TNF-α, IL-2, and IFN-γ) in response to mitogens (LPS, PWM or ConA) as suggested by two-way ANOVA analysis (Table 4.3). For these cytokines the production peaked at 24 hr or 48 hr after mitogen stimulation, therefore the results of 72 hr incubation were not shown. Although the productions of IL-1β and TNF-α were not significantly different at 24 hr and 48 hr after PWM stimulation, the 24-48 hr cytokine incremental increase (cytokine formed at 48 h minus cytokine formed at 24 hr) was significantly less than that by cells from the control and low dose fed rats ($p<0.05$) (Figure 4.2).

4.4. Discussion

The effect of spent hen muscle protein hydrolysates on phenotype and cytokine response of spleen immune cells of healthy weanling Sprague-Dawley rats was studied. Weanling Sprague-Dawley rats have newly developed immune system that are more susceptible to immune challenges (Holsapple, West, & Landreth, 2003). In current study, we did not observe any detrimental effects from hydrolysate feeding based on organ characteristics in young healthy rats. The absence of adverse side effects is an important feature of protein hydrolysates/peptides or other natural products as compared to pharmaceutical drugs.

Our results showed that Protex 50FP hydrolysate exerted immune modulating effects on proportion of immune cells in spleen at a dose of 5% (w/w, diet based) addition, including a lower proportion of antigen presenting cells (OX6+) and a higher proportion of macrophages, granulocytes and dendritic cells (CD11b/c+) in spleen. Antigen presenting cells are dendritic cells, activated macrophages and mature B cells expressing MHC II

molecule, which are essential for T helper cell activation (Kindt, Goldsby, Osborne, & Kuby, 2007). As a higher proportion of CD11b/c+ cells (dendritic cells and macrophages) was observed from hydrolysate treatment (Table 4.2), the lower proportion of OX6+ cells was likely due to a lower proportion of mature B cells rather than other antigen presenting cells.

Despite a higher proportion of macrophages (CD11b/c+) in spleen from hydrolysate feeding, the production of pro-inflammatory cytokine (IL-6, TNF-α, IL-1β) did not differ among experimental groups with mitogen (LPS, PWM) stimulation. And a lower incremental increase of IL-1β and TNF-α from 24 to 48 hr in hydrolysate fed group (Figure 4.2) suggested a regulatory-effect on cytokine production from 24 to 48 hr by hydrolysate feeding.

Feeding with Protex 50FP hydrolysate at a dose of 5% (w/w, diet based) addition resulted in higher IL-10 productions in isolated splenocytes when stimulated with LPS (activates macrophages and B cells, representing an infection challenge; Mogensen, 2009) and PWM (polyclonal T and B cell mitogen; Gallart, Angel De La Fuente, Josep Barcelo, & Alberola-Ila, 1997) (Figure 4.1). IL-10 is mainly produced by T helper cells and innate immune cells including macrophages and dendritic cells (Saraiva & O'Garra, 2010). The stimulatory effect of the hydrolysate on IL-10 was likely due to a higher proportion of macrophages and dendritic cells (CD11b/c+) or possibly an effect on B cells activating T helper cell for IL-10 production.

IL-10 is an important regulatory cytokine, which has a central function to limit and ultimately terminate inflammatory response. IL-10 suppresses T helper cell responses (Th1 and Th2) and limits the innate immune factor from macrophages and dendritic cells (Saraiva & O'Garra, 2010). IL-10 is important in preventing inflammatory and autoimmune pathologies (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001) and has been reported in many studies of food-derived protein/peptides with immunomodulatory properties. Feeding with bovine lactoferrin (a well-known immunomodulatory protein; Takakura, Wakabayashi, Yamauchi, & Takase, 2006), or a kefir fraction containing milk

peptides (Vinderola et al., 2006) has been reported to yield higher IL-10 production in healthy Balb/c mice. Casein-peptide QEPLV could protect BALB/c mice from LPS challenge, in which higher production of IL-10 was observed in serum helping to limit LPS-induced inflammation (Zhou et al., 2014). A shark-derived protein hydrolysate increased intestinal IL-10 secretion, which turned out to protect the mice against inflammatory reaction induced by E. coli infection (Mallet et al., 2014). And a β-casein hydrolysate protected mice against trinitrobenzene sulfonic acid (TNBS)-induced colitis, in which elevated IL-10 was observed in the intestine regulating TNBS-induced inflammation (Turbay, de LeBlanc, Perdigón, de Giori, & Hebert, 2012). The IL-10 stimulatory effects observed in this study from hydrolysate feeding could be beneficial regulating inflammatory conditions.

In summary, for the first time we reported immunomodulatory properties of feeding meat-derived peptides to healthy rodents. When supplemented to the diet of weanling Sprague-Dawley rats, at a dose of 5%w/w the hydrolysate exerted a stimulatory effect on mitogen stimulated IL-10 response. Additionally, modulatory effect on proportion of immune cells isolated from spleen was observed from the peptide treatment, resulting in a higher proportion of macrophage and dendritic cells and a lower proportion of antigen presenting cells. Our results suggest possible application of the spent hen hydrolysate in regulating inflammation-related chronic diseases.

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Table 4.1 Effect of Protex 50FP hydrolysate diet on food intake, body weight, spleen and liver weight and intestine length of Sprague-Dawley rats. Values were expressed as mean±SEM, n=8 per group. Control, 5% w/w casein; Low dose, 2% w/w Protex 50FP hydrolysate + 3% w/w casein ; High dose, 5% w/w Protex 50FP hydrolysate. *Food intake was calculated as the total amount of food intake per cage in three weeks.

	Diet group		
	Control	Low dose	High dose
Food intake* (g)	680±9	664±23	654±12
Initial body weight (g)	68±1	69±2	68±1
Terminal body weight (g)	255±3	250±5	247±4
Liver weight (g)	14±0	14±1	13±0
Spleen weight (g)	0.8±0.0	0.8±0.0	0.8±0.0
Intestine length (cm)	124±2	124±2	121±1

Table 4.2 Phenotype analysis of freshly isolated splenocytes of Sprague-Dawley rats fed by two doses of Protex 50FP spent hen hydrolysate. Values were percentage proportion of the total gated cells as determined by immunofluorescence, n=6/group, expressed as mean±SEM. Control, 5% w/w casein; Low dose, 2% w/w Protex 50FP hydrolysate + 3% w/w casein ; High dose, 5% w/w Protex 50FP hydrolysate. NS, not significant. Within a row, means without a common superscript (a-b) differ ($p<0.05$).

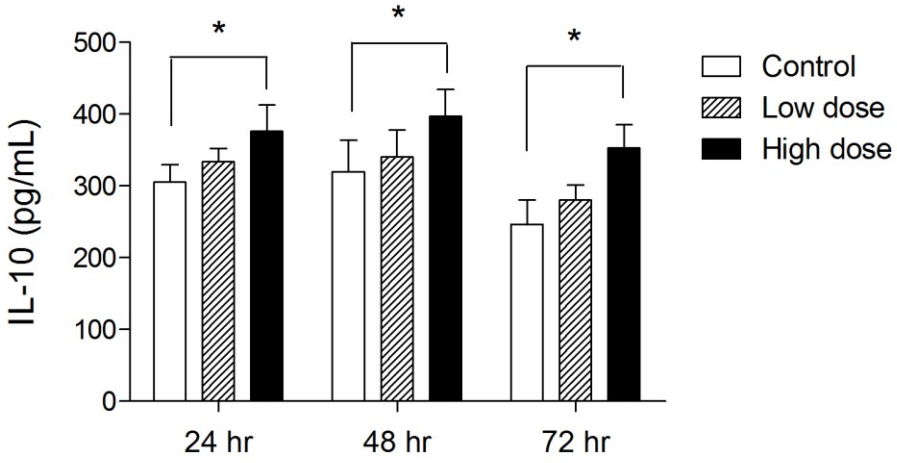
	Diet group			Significance,
	Control	Low dose	High dose	$P \leq$
CD3+	29. 6±0.6	30.6±1.0	31.5±0.8	NS
CD3+CD4+	16.5±0.4	17.2±1.0	17.9±0.8	NS
CD3+CD8+	11.2±0.3	12.2±0.4	12.6±0.5	NS
CD4+CD25+	6.9±0.8	5.8±0.5	6.1±0.5	NS
CD8+CD25+	2.2±0.3	1.8±0.2	2.1±0.2	NS
CD4+ CD28+	18.9±0.6	20.6±1.4	19.6±0.7	NS
CD8+ CD28+	12.8±0.5	11.7±1.2	12.7±0.5	NS
OX12+	51.7±1.2	54.0±1.0	50.9±0.6	NS
CD11b/c+	14.5±0.7 a	14.4±0.8 a	18.9±1.7 b	0.04
OX6+	46.7±1.2 a	48.1±1.1 a	43.3±0.5 b	0.02
OX6+CD80+	2.6±0.2	2.5±0.1	2.6±0.1	NS
OX62+	6.3±0.3	5.8±0.2	5.9±0.4	NS

Table 4.3 Cytokine production by mitogen-stimulated splenocytes. Values are means±SEM, in pg/mL. n=8/group. Control, 5% w/w casein; Low dose, 2% w/w Protex 50FP hydrolysate + 3% w/w casein ; High dose, 5% w/w Protex 50FP hydrolysate. NS, not significant.

		24 hr			48 hr			Significance	
Mitogen	Cytokine	Control	Low dose	High dose	Control	Low dose	High dose	Treatment	Period
LPS	IL-1	165±14	160±9	183±17	275±39	298±24	341±41	NS	<0.001
	IL-6	1047±82	1060±68	1153±134	1291±192	1405±181	1386±143	NS	0.02
	TNF-α	520±36	520±55	615±52	800±84	1011±121	1088±140	NS	<0.001
ConA	IL-2	4633±395	4262±479	4054±313	6421±552	6905±533	6204±474	NS	<0.001
	IFN-γ	1571±252	1183±252	1061±272	830±168	666±107	621±116	NS	0.001
	TNF-α	410±41	446±42	443±39	485±56	525±50	544±56	NS	0.04
PWM	IL-1	144±13	156±14	178±22	216±16	207±18	209±30	NS	<0.01
	IL-6	1230±113	1475±118	1491±159	1154±113	1141±83	1153±96	NS	0.01
	TNF-α	484±42	485±39	596±60	638±73	574±73	592±77	NS	NS
	IL-2	760±34	699±63	656±57	1195±253	1231±202	1276±170	NS	<0.001
	IFN-γ	764±91	754±175	619±232	794±115	840±130	667±123	NS	NS

Figure 4.1 IL-10 productions in isolated splenocytes stimulated with A) LPS and B) PWM for 24 hr, 48 hr and 72 hr. Bars represent mean values ± SEM, n=8/group. Control, 5% w/w casein; Low dose, 2% w/w Protex 50FP hydrolysate + 3% w/w casein ; High dose, 5% w/w Protex 50FP hydrolysate. * indicates $p<0.05$.

(A)



(B)

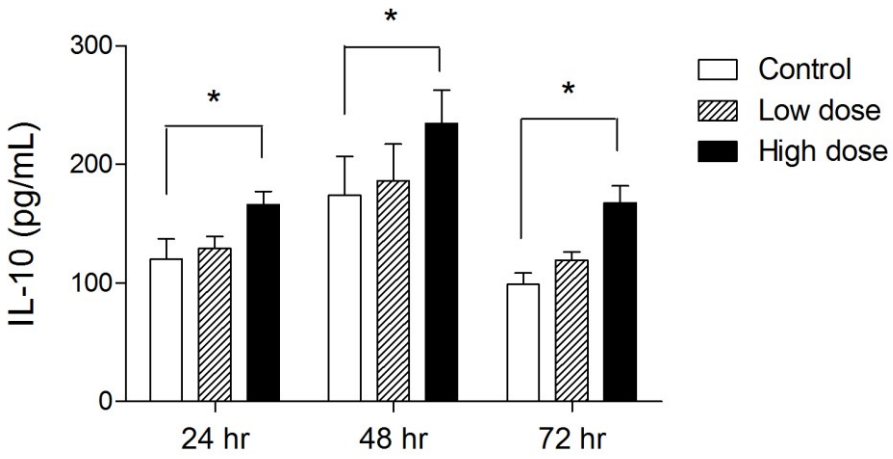
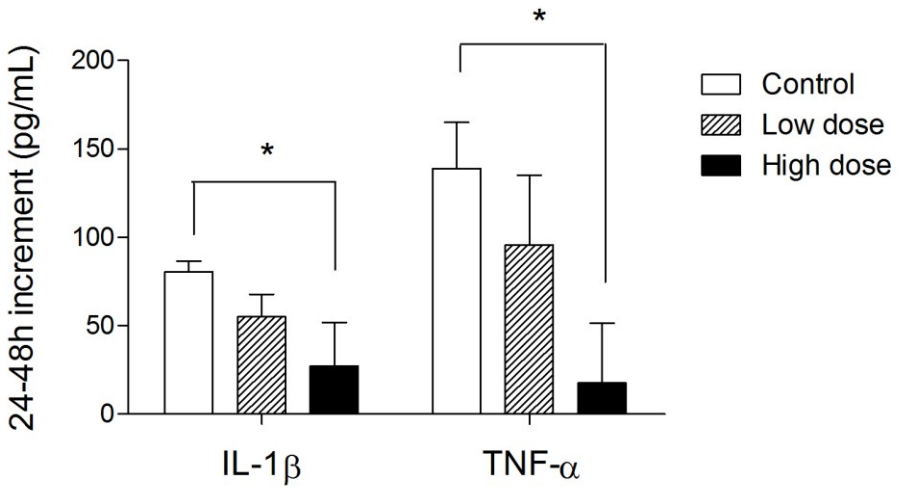


Figure 4.2 24-48 hr increment of cytokine (IL-1β and TNF-α) productions in isolated splenocytes after PWM stimulation of rats fed the experimental diets. Bars represent mean values ± SEM, n=8/group. Control, 5% w/w casein; Low dose, 2% w/w Protex 50FP hydrolysate + 3% w/w casein; High dose, 5% w/w Protex 50FP hydrolysate. * indicates $p<0.05$ compared to the control.



4.6. Appendix I: Supplementary information

A dose-response pilot study was performed to determine the hydrolysate and the dose to use in the feeding trial. The hydrolysates were supplemented to the diets at various doses (0.5%, 1%, 2%, 5%; w/w diet based). The diets were made isocaloric and isonitrogenous by supplementing bovine casein (4.5%, 4%, 3%, 0%; w/w diet based) to all the diets to ensure a total of 5% (w/w) added proteins. The control diet contained 5% (w/w) added bovine casein.

No detrimental effect was observed on food intake, body weight or organ measurements (liver weight, intestine length) in the rats when the Protex 50FP and Protease M hydrolysates were added to the diets at various doses up to 5% (w/w diet based; Table 4.6.1).

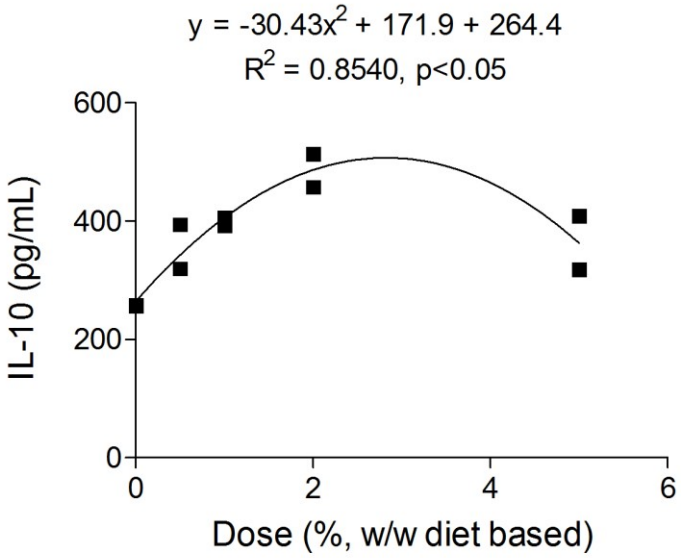
Regression analysis of cytokine response indicated a quadratic polynomial trend of splenic IL-10 productions in response to increased doses of Protex 50FP hydrolysate when stimulated for 48 hr by LPS ($p<0.05$, Figure 4.6.1); increasing IL-10 production was observed with increasing doses of hydrolysate from 0.5% to 2% (w/w diet based). No dose relationship was observed for the other cytokines (IL-1β, IL-6, TNF-α, IL-2, IFN-γ) studied in the pilot study by dietary supplement of Proxtex 50FP hydrolysate. Feeding different doses of Protease M hydrolysate did not significantly affect immune cell phenotype and mitogen-stimulated cytokine production *ex vivo* in this study.

Based on these, Protex 50FP hydrolysate at doses of 2% (low dose) and 5% (high dose) w/w of diet were selected for feeding in the second study, respectively.

Supplementary Table 4.1 Effect of Protease M and Protex 50FP hydrolysate on food intake, body weight, spleen and liver weight and intestine length of Sprague-Dawley rats. Values were expressed as mean±SEM, n=2 per group. *Food intake was calculated as the total amount of food intake per cage in three weeks.

	Control	Protease M				Protex 50FP			
		0.5%	1%	2%	5%	0.5%	1%	2%	5%
Food intake* (g)	633	579	580	532	583	624	578	610	570
Initial body weight (g)	74±4	70±3	72±4	69±1	72±1	66±4	65±4	74±3	66±1
Terminal body weight (g)	259±19	254±8	261±1	244±12	250±10	247±4	240±4	266±16	234±7
Liver weight (g)	15±2	14±1	15±1	13±2	14±2	14±1	14±1	14±1	13±2
Spleen weight (g)	1.0±0.0	1.1±0.1	1.2±0.2	0.9±0.1	0.9±0.2	0.9±0.1	1.0±0.1	0.9±0.1	0.9±0.0
Intestine length (cm)	113±3	119±3	105±5	108±3	111±2	106±2	112±2	114±2	106±3

Supplementary Figure 4.1 Quadratic polynomial trends of splenic IL-10 productions in response to doses of Protex 50FP hydrolysate in diet (dose-response pilot); cells were stimulated with lipopolysaccharide (LPS) for 48 hr. R2=0.8540. Data were graphed as replicates and the trend line plotted, n=2/group.



CHAPTER 5 – Altered Immune Responses in Trinitrobenzenesulfonic Acid Solution (TNBS)-Induced Colitis Rats and the Effects of Feeding a Spent Hen Hydrolysate

5.1. Introduction

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the gastrointestinal tract; ulcerative colitis (UC) and Crohn's disease (CD) are the two main forms. IBD affects 1 in every 150 Canadians, ranking Canada's incidence the highest worldwide. The economic costs for IBD are conservatively estimated at $2.8 billion in 2012 (CCFC, 2012). IBD severely impacts quality of life through ongoing debilitating symptoms, which limits the ability to work, the career choices, and raises financial and psychological issues such as social stigma (CCFC, 2012). Dysregulated immune responses (driven by genetic and environmental factors) towards commensal bacteria play an important role in IBD pathogenesis (Xavier & Podolsky, 2007). Current treatment of IBD involves the administration of immunosuppressors (such as corticosteroids, azathioprine), 5-aminosalicylic acid (5-ASA), or antibodies specific for target inflammatory molecules (Hanauer, 2006). However, these treatments are not devoid of severe side effects, therefore continuous research efforts were employed in search of novel therapy.

Food and nutrition as a key environmental factor has a significant impact on the development and progression of IBD. Patients with IBD have increased protein needs to compensate for losses from inflammatory conditions and for recovery from surgery; however, reduced oral intake and malabsorption of nutrients are usually observed in IBD (Eiden, 2003). It is estimated that up to 85% of hospitalized IBD patients have protein energy malnutrition (Han, Burke, Baldassano, Rombeau, & Lichtenstein, 1999). Protein hydrolysates prepared by enzymatic digestion are traditionally included in enteral nutrition therapy for IBD as they are easily digestible, hypoallergenic nutrients (Clemente, 2000; Dieleman & Heizer, 1998; Royall et al., 1994). Additionally, protein hydrolysates containing various peptides are reported to confer many biological activities *in vitro* and *in vivo* beyond basic nutritional functions, such as antihypertensive, anti-

inflammatory/oxidative stress, antimicrobial, immunomodulatory and anticancer as reviewed by (Udenigwe & Aluko, 2012). Several peptides with anti-inflammatory/oxidative stress properties have been reported to alleviate inflammatory bowel conditions in animal models of colitis (Zhang, Hu, Kovacs-Nolan, & Mine, 2014). For example, egg white peptides and soy-derived peptides attenuated dextran sodium sulfate (DSS)-induced clinical symptoms, helped to restore gut barrier function and reduced local expression of pro-inflammatory cytokines in piglets (Lee et al., 2009; Young, Ibuki, Nakamori, Fan, & Mine, 2012); bovine milk-derived glycomacropeptide (GMP) or β-casein peptides helped to induce a faster recovery from trinitrobenzene sulfonic acid (TNBS)-induced colon damage, and reduce local expression of pro-inflammatory cytokines in murine models (Daddaoua et al., 2005; Turbay, de LeBlanc, Perdigón, de Giori, & Hebert, 2012).

The Protex 50FP spent hen hydrolysate prepared by enzymatic hydrolysis of chicken muscle proteins has been found to exert anti-inflammatory activities in our previous experiments (Chapter 3 & 4). It inhibited interleukin (IL)-6 (generally considered a pro-inflammatory cytokine) production *in vitro* in LPS-activated U937 cells, and when supplemented in diet, modulated splenocytes for a higher IL-10 (regulatory cytokine) production with *ex vivo* mitogen stimulation. Current studies suggest a pathological role of IL-6 in IBD, likely through the anti-apoptotic signaling on T cells (Bouma & Strober, 2003; Mudter & Neurath, 2007). A protective role of IL-10 in IBD has been demonstrated in experimental models acting through regulatory T cell and anti-inflammatory cytokine TGF-β (Bouma & Strober, 2003; Fuss, Boirivant, Lacy, & Strober, 2002). Therefore we hypothesized that the peptides could be beneficial for gut inflammatory conditions. In this study, the effect of the peptides was studied in rats using the model of TNBS-induced colitis as originally reported by Morris, et al. (1989). TNBS acts as a hapten (couples to mucosal proteins) to elicit a Th1 type immunologic responses and a severe transmural inflammation that shares many of the histopathological features of human Crohn's disease. The TNBS-induced colon damage was evaluated macroscopically and histologically, with colon tissues measured for MPO activity. To understand the potential beneficial effect of

peptides on underlying immunological pathways, phenotypes and cytokine profile of immune cells isolated from the mesenteric lymph nodes (MLN) and spleen were analyzed.

5.2. Materials and Methods

5.2.1. Reagents

Protex 50FP was obtained from Genencor International Inc. (Rochester, NY, USA). 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS), Lipopolysaccharides (LPS, *E. coli* strain *0111:B4*), concanavalin A (ConA), pokeweed mitogen (PWM) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Rat DuoSet® ELISA development kits were purchased from R&D Systems (Minneapolis, MN, USA). Fluorescent pre-labeled monoclonal antibodies were purchased from eBioscience (CD3, CD4, CD28, CD27, CD80; San Diego, CA, USA), BioLegend (CD8, CD25, CD152, CD71, OX62, CD11b/c; Vineland, ON, Canada) AbD Serotec (OX12; Raleigh, NC, USA) and BD Biosciences Pharmingen (OX6; Mississauga, ON, Canada). The AIN-76A Western Diet was purchased from TestDiet Inc. (Richmond, IN, USA). High protein casein was purchased from Harlan Teklad (Madison, WI, USA).

5.2.2. Preparation of spent hen hydrolysate

Spent hen hydrolysate was prepared as previously reported (Chapter 3). Briefly, manually deboned chicken meat was homogenized with distilled water (1:4; w/v) followed by a pH adjustment to 5.0. Crude proteins precipitated by centrifugation (10,000 g x 25 min, 4°C) was freeze-dried and mixed with distilled water at 5% (w/v) to prepared protein slurry. The slurry was hydrolyzed by Protex 50FP added at 4% w/w of dry protein weight for 3 hr (pH=3.0, 50°C). Hydrolysis was terminated by heating at 95°C for 15 min. The slurry was centrifuged (10,000 g x 25 min, 4°C) and the supernatant was collected and freeze-dried for inclusion in the diet preparation.

5.2.3. Animals

Male weanling Sprague-Dawley rats were obtained from Biosciences Animal Service (University of Alberta, Edmonton, AB, Canada). All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the Faculty of Agricultural, Life and Environmental Sciences Animal Ethics Committee at the University of Alberta. All rats were housed two per cage in a temperature and humidity controlled room with a 12L: 12D light-dark cycle. Rats were given free access to water and were provided the experimental diets *ad libitum*.

5.2.4. Induction of colitis and experimental design

The animal diets were prepared from a basal diet resembling the North American/European high-fat diet with regard to the macronutrient contents (TestDiet®5342 - AIN-76A Western Diet), which contains 51% carbohydrates, 20% fat and 7% fiber (w/w). The experimental diets were supplemented with 5% (w/w) extra protein (casein for the control diet while spent hen hydrolysate for the peptide diet) to the basal diet. The rats were randomly assigned to 3 groups (n=8) and balanced for body weights; the reference and the colitis control groups were fed the control diet (bovine casein) without or with TNBS treatment, while the colitis peptide groups were fed on spent hen hydrolysate with TNBS treatment (all diets are prepared isocalorically and isonitrogenically with an equally added 5% protein, w/w, diet based). The experimental design is shown in Figure 5.1. Following 3 weeks of feeding, the rats were fasted overnight for an intrarectal administration of TNBS to induce colitis as originally described by Morris *et al.* (1989). Rats were anesthetized with isoflurane, and given 300 µL of 70 or 80 mg/mL TNBS (dissolved in 50% ethanol) using a pediatric feeding tube inserted to the colon 7cm from the anus for 2 groups, or placebo treated with 300 µL of saline for the non-colitic reference group. All the rats continued to be fed the experimental diets for 5 days until termination. The rats were monitored daily for body weight (BW), food intake, distress and stool consistency. At day 5, rats were killed by CO_2 asphyxiation and tissues were harvested and processed accordingly.

5.2.5. Colon sampling and examination

Colon was removed, cut longitudinally, gently rinsed in ice-cold saline, blotted dry and placed on an ice-cold plate. The length and weight of colon were measured. Representative photos were taken for each group to record the severity and extent of colon damage; colons were examined for adhesion, obstruction, thickening and necrosis (Daddaoua et al., 2005). Representative colon specimens with gross macroscopic damage were fixed in 4% buffered paraformaldehyde for histological assessment of colonic damage. Tissues were dehydrated with ethanol and xylene in a tissue processor (Leica TP 1020, Leica Microsystems, Wetzlar, Germany), selected for cross-sections and embedded in paraffin (Leica EG1160). Sections were cut at 5 µm on a rotary microtome (Leica 2125) and stained with haematoxylin and eosin according to standard protocols. Stained slides were examined under microscope for loss of mucosal architecture, cellular infiltration, muscle thickening, goblet cell depletion and crypt abscess formation as reported by (Santiago, Pagán, Isidro, & Appleyard, 2007).

5.2.6. MPO measurement

Pieces of inflamed colon were collected and snap-frozen in liquid nitrogen for measurement of myeloperoxidase (MPO) activity according to Martin *et al.* (2006) with slight modification. Briefly, about 100 mg tissue was weighed and homogenized (FastPrep®-24, MP Biomedicals, Solon, OH) in 10 volumes of ice-cold saline, followed by a second homogenization in 10 volumes of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyl-trimethylammonium bromide (HETAB) and 10 mM ethylenediaminetetraacetic acid (EDTA, disodium salt dihydrate). After three cycles of freezing/thawing, the homogenate was centrifuged and the supernatant was collected. For each sample, 10 µL of the supernatant was added to a 96-well EIA plate (Corning 9017), 100 µL of the substrate mixture (1.6 mM tetramethyl benzidine (TMB) and 0.003% H_2O_2 in 50 mM phosphate citrate buffer (pH 5.0) mixed in equal volumes) was added to each well to start the reaction. Changes in absorbance at 650 nm at 1 min intervals were recorded in a spectrophotometer for 5 min at 25°C. The absorbance readings were plotted

in an X-Y chart against time, from which a linear trend line was drawn to calculate the slope (ΔAb/min). MPO activity (1 U) was defined as the amount of enzyme present to catalyze the TMB color reaction that produced a change in absorbance (ΔAb) of 1.0 unit per minute at 25°C; results were expressed as U $x10^3$/mg tissue.

5.2.7. Assessment of immune functions

At necropsy, spleens and MLN were removed aseptically and placed in sterile Krebs-Ringer-HEPES buffer (pH 7.4) supplemented with 0.5% (w/v) bovine serum albumin. Immune cells were isolated as previously reported (Biondo, Goruk, Ruth, O'Connell, & Field, 2008). Briefly, spleen and MLN tissues were pushed through a sterile nylon mesh screen (100 μm) to separate immune cells and red blood cells from connective tissue and fat. Spleen cells were additionally treated to remove the erythrocytes using a lysis buffer.

Freshly isolated immune cells from spleen and MLN were studied for cell phenotypes by a direct immunofluorescence assay as previously reported (Ruth, Proctor, & Field, 2009) Immune cells were incubated with pre-labelled monoclonal antibodies to identify and quantify the phenotypes. The following antibody combinations were used: CD27/CD3/CD4/CD8, CD25/CD3/CD4/CD8, CD28/CD152/CD4/CD8, CD71/OX12/CD4/CD8, CD80/CD11b/c, and CD3/CD161/OX62. Cells were fixed in phosphate buffered saline containing 1% (w/v) paraformaldehyde, and the proportion of positive cells for each marker was determined according to the relative fluorescence intensity by flow cytometry (LSR-Fortessa; BD Biosciences, Mississauga, ON, Canada) using the Kaluza software (Beckman Coulter Inc., Mississauga, ON, Canada).

Immune cells from spleen and MLN were resuspended in culture media, counted on a haemocytometer and seeded in 4 mL sterile polystyrene tubes at a concentration of 1.25×10^6 cells/mL. Splenocytes were cultured without mitogen (unstimulated) or in the presence of ConA (5 μg/mL), LPS (15 μg/mL), or PWM (55 μg/mL); immune cells from MLN were cultured without mitogen (unstimulated) or in the presence of ConA (5 μg/mL). Supernatant was removed at 24 hr (spleen) or 48 hr (spleen, MLN) for maximum stimulation and stored at -80°C until cytokine analysis. The concentration of cytokines IL-

6 (31.25-4000 pg/mL), TNF-α (15.6-2000 pg/mL), IL-10 (15.6-2000 pg/mL), IFN-γ (19.5-2500 pg/mL) and IL-2 (62.5-8000 pg/mL) were measured using ELISA kits as per manufacturer's instructions. Samples were diluted when necessary and values below the detection range were assigned half the value of the minimum detectable limit.

5.2.8. Statistical analysis

Statistical analysis was conducted using the SAS software (Version 9.4; SAS Institute, Cary, NC, USA). All datasets were blocked by TNBS dose/kill day. Splenocyte cytokine production measured at 24 hr and 48 hr were analyzed using a mixed model two-way ANOVA. All other datasets were analyzed by one-way ANOVA. Differences between groups were determined by Duncan's multiple range test ($p<0.05$). All data that was non-parametric was log-transformed prior to statistical analysis. Results were presented as means ± the standard error of the mean (SEM).

5.3. Results

5.3.1. Characteristics of TNBS-induced colitis and effect of spent hen hydrolysate

In this study, initial body weights were not different among 3 groups. Feeding a diet with spent hen hydrolysate for 3 weeks prior to colitis induction did not affect daily food intake (22±1g in control diet groups vs. 21±1g in peptide diet group, at day 21) and weight gain (Table 5.1). The intrarectal administration of TNBS resulted in intestinal inflammation as indicated from reduced food intake, weight loss and colon damage. In the replicate of rats treated with 80mg/mL of TNBS, one rat from the colitis control group died at day5 post chemical treatment and was excluded from the study. No mortality was reported for the second replicate treated with the lower dose (70mg/mL of TNBS). One rat from the colitis control group did not respond to TNBS treatment to exhibit clinical manifestation of colitis, therefore was excluded from the study.

The reference group gained weight steadily during the study; on the other hand, both TNBS-treated colitis groups experienced significant weight loss, in particular at 24 and 48 hr post-procedure (Figure 5.2). Colitis rats fed the peptide had less weight losses at 24 hr

($p<0.001$) and 48 hr ($p<0.05$) compared to the colitis control (Figure 5.2). Food intake in both colitis groups were lower from day1 through day 5 compared to the reference ($p<0.001$); no significant difference was found between the colitis control and colitis peptide groups (Figure 5.3).

The colitis groups had higher colon weight to length ratios (indicator of colon inflammation, $p<0.01$), higher colonic MPO activities (marker of neutrophil infiltration, $p<0.01$), and slightly lower numbers of immune cells in spleen than that of the reference (Table 5.1). Peptide feeding did not alter these parameters in TNBS treated rats. Spleen weights at termination were not significant different among experimental groups (Table 5.1).

Macroscopic inspection of distal colon revealed that there was no sign of inflammation in the reference group. In colitis groups, the distal colon showed a flaccid appearance with ulceration and bowel wall thickening in comparison to an elastic and thin colon wall in healthy reference (Figure 5.4). There was no significant difference on visually assessed macroscopic damage between the colitis control group and the colitis peptide group. These observations were in alignment with histological examination, showing normal features of colon structure in the reference group while extensive inflammation through all layers of the colon in both TNBS-treated colitis groups (Figure 5.5). TNBS-treated groups displayed mucosal epithelia erosions, muscle thickening, depletion of goblet cells and significant immune cell infiltration (granulocytes, monocytes and lymphocytes) in the lamina propria, submucosa and muscularis externa; peptide feeding did not improve the histological symptoms associated with inflammation.

5.3.2. Effect on immune cell phenotype

For analysis of immune cells in spleen, lymphocytes, monocytes and granulocytes were gated separately and combined as shown in Figure 5.6. A higher proportion of granulocytes (gate G, $p<0.001$), a lower proportion of lymphocytes (gate L, $p<0.001$), and a lower proportion of live cells (gate A, $p<0.05$) were observed in colitis groups compared to the reference in forward scatter (FSC) vs. side scatter (SSC) density plot (Figure 5.6).

Phenotype analysis of all live immune cells (gate A) showed that there were a higher proportion of CD11b/c+ cells (macrophage, granulocytes and dendritic cells) ($p<0.001$), and lower proportions of CD3+ (T cells) and OX12+ (B cells) ($p<0.05$) in colitis groups (Table 5.2). Despite a slightly lower proportion of T cells, a higher proportion of helper T cells expressing IL-2 receptor (CD4+CD25+) was found in colitis groups ($p<0.05$). This was also observed for other stimulatory and activation markers. The proportion of lymphocytes expressing co-stimulatory factor (CD28, CD152) or the transferrin receptor (CD71) were higher in TNBS-induced colitis group than the reference group ($p<0.05$). The proportion of antigen presenting cells expressing co-stimulatory factor (CD80+, CD11b/c+CD80+) were higher in colitis groups ($p<0.001$). Among those tested, proportions of cells expressing TNF-α receptor (CD27) was not different among the groups; and changes on T helper cells (CD3+CD4+), T cytotoxic cells (CD3+CD8+), dendritic cells (OX62+) and NK cells (CD3-OX62-CD161+) were not significant among experimental groups (Table 5.2).

Phenotype analysis of lymphocytes (gate L) suggested that the proportion of total CD3+ (T cells) and OX12+ (B cells) were not affected by disease treatment (Table 5.3). A higher expression of CD80+ (co-stimulatory factor) was observed on monocytes in consistency with a higher proportion of macrophages and dendritic cells (monocytes expressing CD11b/c+) (Table 5.3). And a higher proportion of helper T cells expressing IL-2 receptor (CD4+CD25+) was confirmed in lymphocytes in colitis groups (Table 5.3).

While multiple differences observed from disease treatment in spleen-derived immune cells, no significant effect was observed in immune cells isolated from MLN. As listed in Table 5.4, proportions of major lymphocytes including T cells (CD3+) and its subsets (CD3+CD4+, CD3+CD8+) and B cells (OX12+) were not different amongst experimental groups. No difference was observed on stimulatory and activation markers including CD25 (IL-2 receptor), CD27 (TNF-alpha receptor), CD71 (transferrin receptor), CD28 (co-stimulatory factor) and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4, activation marker).

Peptide treatment had minimal effect on the changes induced by disease treatment, but did present in a higher proportion of helper T cells expressing IL-2 receptor (CD4+CD25+) in lymphocytes of colitis rats fed the hydrolysate ($p<0.05$) compared to the colitis control (Table 5.3) or a lower proportion of T cells expressing CTLA-4 in MLN from peptide treatment ($p<0.05$; Table 5.4).

5.3.3. Effect on cytokine production

TNBS-induced colitis had an influence on splenic cytokine production in response to mitogens (Table 5.5). There were lower productions of T cell cytokines (IL-2 and IFN-γ) with ConA stimulation but higher productions of the macrophage-produced cytokine (TNF-α) with LPS stimulation in both colitis groups compared to the reference ($p<0.05$). Splenocytes from colitis groups produced less IL-6 and IL-2 with PWM stimulation; the differences reaching significance when comparing the reference and colitis peptide group ($p<0.05$). In immune cells from MLN, lower productions of TNF-α and IFN-γ with ConA stimulation were observed in peptide colitis group compared to the reference ($p<0.05$, Table 5.6).

5.4. Discussion

TNBS induced colitis in rat is characterized by body weight loss, anorexia, macroscopic and histological colon damage. These have been well documented in the use of this chemical induced colitis model (Morris et al., 1989). Our results suggests one time administration of TNBS likely induced an acute injury rather than a chronic inflammation in distal colon with significant infiltration of granulocytes, especially neutrophils, as indicated from greatly elevated colonic MPO activity. In murine acute TNBS colitis, elevated concentration of macrophage-derived/Th1 cytokine IL-12 and IFN-γ, Th17 cytokine IL-17, and chemokine MCP-1α in serum were reported (Alex et al., 2009). In colon, mRNA expression of pro-inflamamtory cytokines TNF-α, IFN-γ, IL-2, IL-12, and IL-18 peaked on day 2 or 3 after single TNBS administration (Hollenbach et al., 2005). This cytokine pattern is in agreement with the excessive Th1 response in Crohn's disease,

which is associated with increased secretion of IL-12, IFN-γ and/or TNF-α (Bouma & Strober, 2003).

The major benefit from the peptide supplement was attenuated body weight loss at 24 hr and 48 hr after colitis induction, suggesting a protective effect on the damage to the colon or the inflammatory response (Figure 5.2). This effect was greater within 24 hr as body weight changes during 24-48 hr were not significantly different between the colitis peptide and colitis control groups (6.3±0.9g in colitis control vs. 6.8±1.8g in colitis peptide group). At a very early stage within the first 24 hr, the reduced impact of TNBS could be attributed to a better gut barrier function. Better gut barrier function could be beneficial limiting TNBS exposure to mucosal proteins and deterring activation of antigen-specific T cells; therefore delays the onset of a T_H1 inflammatory response (characterized by IFN-γ and IL-17 secretion) and the resulted macrophage activation and neutrophil recruitment (Kish, Volokh, Baldwin, & Fairchild, 2011). Unfortunately, these hypotheses cannot be confirmed in this study as this difference between the peptide treated groups was no longer observed at day5 when the tissues were collected for analysis.

Disease treatment had an impact on proportions of immune cells, especially in spleen; a marked higher proportion of granulocytes (Figure 5.6), macrophages and dendritic cells (monocytes expressing CD11b/c+, Table 5.3) were observed in spleen of colitis rats. The higher proportion of granulocytes in spleen (representing systemic immunity) might suggest a systemic inflammation occurred in TNBS-induced colitis. In TNBS model, ethanol solution (in which TNBS dissolved in) functioned as gut "barrier breaker", and TNBS-induced inflammation further damaged the gut integrity. Therefore an increase of bacteria load in the peripheral circulation could be expected. Increased LPS plasma level has been reported in TNBS-model as well as in IBD patients who suffered from impaired gut barrier function (Alhouayek, Lambert, Delzenne, Cani, & Muccioli, 2011). As a result of systemic inflammation, there were higher proportions of lymphocytes expressing activations marker (CD25), and higher proportions of macrophages and dendritic cells expressing co-stimulatory factor CD80 (Table 5.3).

Despite the inflammatory conditions in distal colon, minimal influence on immune cell phenotypes was observed in MLN from TNBS treatment compared to the spleen. In the gut-associated lymphatic tissue, antigen exposure was presented to lymphocytes which migrate to mesenteric lymph nodes; activated lymphocytes then migrate from the intestine to the peripheral circulation (Janeway, Travers, Walport, & Shlomchik, 2001). The absence of difference on phenotypes of MLN cells between reference and colitis groups might suggest a recovery stage with reduced local antigen exposure from the previous induced colon damage.

Consistent with the changes observed on immune cell phenotypes in spleen, changes were observed on splenic cytokine productions in response to mitogens. A higher splenic TNF-α production in response to LPS were observed in colitis groups, while other macrophage-derived cytokine (IL-6, IL-10) remained unaffected. TNF-α favors neutrophil recruitment in acute inflammation, whereas IL-6 mediates the transition from neutrophil to monocyte recruitment (Kaplanski, 2003). The higher TNF-α production observed in TNBS colitis likely contributed to the higher granulocyte proportion in spleen as observed in the flow cytometry plot. Although a Th1-type inflammation and elevated IFN-γ production/expression have been reported in serum and colon of TNBS-treated mice (Alex et al., 2009; Hollenbach et al., 2005), a higher T cell cytokine (IL-2 and IFN-γ) production in colitis groups was not observed in ConA (T cell mitogen; Pang et al., 2012) stimulated splenocytes compared to the reference; rather, the production of IL-2 and IFN-γ were lower in colitis groups (Table 5.4) likely due to a lower proportion of T cells with the infiltration of granulocytes. It is reported that TNBS colitis caused a Th1 response that was limited to the colon, in which the Th1 cytokine mRNA levels were not increased in T cells isolated from MLN and spleen tissue (Ruyssers et al., 2009). Additionally, a higher proportion of activated T cells, as indicated from the higher expression of activation markers (CD25), could further reduce the IL-2 production (Hwang, Hong, & Glimcher, 2005), impairing T cell proliferation ability. Similarly, a lower production of IL-2 was observed in colitis groups in PWM (T and B cell mitogen; Gallart, et al., 1997)-stimulated splenocytes. As disease treatment did not alter major T cell population and activation

markers in MLN, it is no surprising that the IL-2 production in ConA-stimulated MLN was not different among experiment groups (Table 5.5).

At day 5, peptide supplement exhibited limited effect on cell phenotypes or cytokine response as compared to the colitis control and the reference group. A higher expression of CD25 (subunit of IL-2 receptor) of T helper cells was observed in spleen from peptide treatment. IL-2 receptor is important for the delivery of IL-2 signal, which is implicated in the differentiation and homeostasis of both the effector and regulatory T cells (Létourneau, Krieg, Pantaleo, & Boyman, 2009). Other than the effect on CD25, a lower proportion of T lymphocytes expressing CTLA-4 (CD152) was observed in CD4 and CD8 cells in MLN. CTLA-4 is not detectable in naive T cells but is rapidly upregulated upon T cell activation (to arrest the activation) and can be detected at cell surface until 24-48 hr after activation (Chambers, 2001). However, the role of CD25 and CTLA-4 on colitis condition has not been clearly established in the literature.

In summary, in this study TNBS-induced acute colitis was successfully established in Sprague-Dawley rats, characterized by a systemic inflammation and neutrophil predominant inflammation. The peptide supplement showed beneficial effects on body weight loss in the 24 hr immediately after the chemical was applied. At day 5, limited effect was observed from peptide treatment on colon damage and immune functions; a higher proportion of T helper cells expressing CD25 (activation marker, IL-2 receptor) in spleen and a lower proportion of T lymphocytes expressing CTLA-4 (activation marker) in MLN were reported. These effects might worth further investigations at an early end point in this TNBS model.

5.5. References

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Table 5.1 Body weight (BW), spleen weight, splenocytes population, macroscopic score, colon ratio and MPO activity of untreated and TNBS-treated rats fed or not fed with peptides. Values are mean values ± SEM. ND, not detected. Numbers with different letters (a-b) were significantly different ($P<0.05$) within each column.

	BW (day 0, g)	BW (day 5, g)	Spleen weight (g)	Splenocytes/g spleen ($x10^6$)	Colon weight:length ratio (mg/cm)	MPO (Ux10^3/mg tissue)
Reference (n=8)	207.4±7.2	268.7±9.2 a	0.85±0.04 a	607±63	83.1±2.6 a	3.8±0.9 a
TNBS control (n=6)	213.4±9.9	216.1±12.4 b	0.89±0.07 b	454±47	431.5±85.6 b	68.2±12.4 b
TNBS peptide (n=8)	214.6±5.4	216.3±8.9 b	0.89±0.06 b	439±68	446.5±57.2 b	61.2±15.3 b

Table 5.2 Splenocytes phenotypes of untreated and TNBS-treated rats fed or not fed with peptides. Values were percentage proportion of the total gated cells (Gate A) as determined by immunofluorescence. Values are mean values ± SEM. Numbers with different letters (a-b) differ significantly within each row ($p<0.05$).

	Reference (n=8)	Colitis control (n=6)	Colitis peptide (n=8)
CD3+	32±1 a	30±2 ab	28±1 b
CD3+CD4+	19±1	18±1	16±1
CD3+CD8+	9.0±1	7.7±1	7.9±1
CD25+	9±1 a	11±1 ab	12±1 b
CD4+CD25+	6.3±0.3 a	7.7±0.6 ab	9.0±0.6 b
CD8+CD25+	1.8±0.1	1.8±0.1	2.3±0.3
CD27+	30±1	30±1	30±1
CD4+CD27+	17±1	19±1	18±1
CD8+CD27+	8.9±1.0	7.8±0.8	8.5±0.6
CD28+	48±1 a	54±2 b	55±2 b
CD4+CD28+	24±1	27±1	26±1
CD8+CD28+	9.3±0.7	9.1±0.7	8.8±0.6
CD152+	4.7±0.5 a	8.8±1.8 b	12.4±2.6 b
CD4+CD152+	2.5±0.3 a	4.6±1.2 ab	5.7±1.3 b
CD8+CD152+	1.2±0.1 a	1.9±0.6 ab	2.7±0.5 b
CD71+	36±1 a	41±2 b	42±1 b
CD4+CD71+	15±1	17±2	18±1
CD8+CD71+	3.4±0.3	3.3±0.2	3.5±0.3
OX12+	36±1 a	33±2 ab	30±2 b
OX12+CD71+	22±0	21±1	21±1
CD80+	10±1 a	17±2 b	17±1 b
CD11b/c+	9.8±0.7 a	20±4 b	23±1 b
CD11b/c+CD80+	6.0±0.4 a	11±2 b	12±1 b
OX62+	2.2±0.3	2.5±0.3	3.2±0.4
CD3-OX62-CD161+	5.4±0.4	4.9±0.5	5.1±0.5

Table 5.3 Splenocyte phenotypes of untreated and TNBS-treated rats fed or not fed with peptides in individual gates. Phenotype values were percentage proportion of the total gated cells in each region as determined by immunofluorescence. Values are mean values ± SEM. Numbers with different letters (a-c) differ significantly within each row ($p<0.05$).

Gate		Reference	TNBS control	TNBS peptide
G (Granulocytes)	CD71+	90±1	91±2	88±3
	CD11b/c+	62±2 a	84±4 b	90±1 b
M (Monocytes)	CD71+	86±1	80±3	81±2
	CD11b/c+	33±2 a	45±6 b	43±2 b
	CD80+	30±2 a	46±5 b	43±2 b
L (Lymphocytes)	CD3+	32±1	33±1	31±2
	CD3+CD4+	18±1	20±1	19±1
	CD3+CD8+	9.0±0.9	8.3±0.9	8.7±0.8
	CD25+	3.9±0.3 a	5.0±0.6 ab	6.6±1.0 b
	CD4+CD25+	2.2±0.2 a	3.2±0.4 b	4.2±0.4 c
	CD8+CD25+	0.6±0.0 a	0.7±0.1 ab	1.1±0.2 b
	CD27+	34±1	34±1	34±2
	CD28+	38±1	39±1	39±2
	OX12+	30±1	29±1	28±2
	CD71+	21±1	20±2	20±1

Table 5.4 Phenotypes of immune cells in MLN of untreated and TNBS-treated rats fed or not fed with peptides. Values were percentage proportion of the total gated cells as determined by immunofluorescence. NS, not significant. Values are mean values ± SEM. Numbers with different superscript letters (a-b) differ ($P<0.05$) within each row.

	Reference (n=8)	Colitis control (n=6)	Colitis peptide (n=8)
CD3+	66±2	64±2	63±2
CD3+CD4+	44±1	44±1	44±1
CD3+CD8+	17±2	16±1	16±1
CD25+	14±1	13±1	13±1
CD4+CD25+	10±1	10±1	11±1
CD8+CD25+	2.0±0.1	1.9±0.1	2.0±0.1
CD27+	62±2	62±2	62±1
CD4+CD27+	41±1	42±1	43±1
CD8+CD27+	15±2	15±1	15±1
CD28+	67±2	67±2	66±1
CD4+CD28+	44±1	46±1	47±1
CD8+CD28+	19±1	18±2	17±1
CD152+	8.6±0.6 a	7.5±1.1 a	5.1±0.4 b
CD4+CD152+	5.4±0.4 a	5.0±0.7 a	3.5±0.2 b
CD8+CD152+	2.5±0.2 a	2.1±0.3 a	1.5±0.2 b
CD71+	20±1	20±1	18±1
CD4+CD71+	8.5±0.7	8.6±0.6	8.6±0.3
CD8+CD71+	2.8±0.2	2.6±0.2	2.5±0.1
OX12+	24±2	24±2	21±2
OX12+CD71+	11±1	10±1	9.1±0.5
CD11b/c+	2.5±0.4	2.6±0.4	2.7±0.3

Table 5.5 Cytokine production by mitogen-stimulated splenocytes. Values are means±SEM, in pg/mL. NS, not significant. Numbers with different letters (a-b) were significantly different (P<0.05) within each row of cytokine production at 24 hr; numbers with different letters (A-B) were significantly different (P<0.05) within each row of cytokine production at 48 hr. n=8/reference, n=6/colitis control, n=8/colitis peptide.

		24 hr			48 hr			Significance	
Mitogen	Cytokine	Reference	Colitis control	Colitis peptide	Reference	Colitis control	Colitis peptide	Treatment	Period
LPS	IL6	242±36	311±55	296±49	279±24	354±37	370±53	NS	NS
	TNF-α	339±30 a	695±130 b	758±93 b	612±71 A	1028±191 B	982±141 B	<0.001	0.004
	IL10	89±6	90±14	95±13	215±26	298±30	212±83	NS	<0.0001
ConA	IL2	2327±390	1611±354	1822±143	2491±353 A	1549±327 B	1471±168 B	0.01	NS
	IFN-γ	167±49	89±47	119±30	184±62 A	66±31 B	46±8 B	0.02	NS
PWM	IL6	224±23 a	208±29 ab	146±20 b	208±20	170±24	163±22	0.02	NS
	TNF-α	415±69	437±90	482±60	455±66	555±32	616±109	NS	NS
	IL10	114±8	104±3	90±13	102±7	82±7	97±22	NS	NS
	IL2	327±47 a	198±63 ab	126±18 b	416±62 A	290±84 AB	224±20 B	<0.001	0.03
	IFN-γ	135±40	91±20	70±26	85±28	84±22	106±42	NS	NS

Table 5.6 Cytokine production at 48 hr by ConA-stimulated immune cells from MLN. Values are means±SEM, in pg/mL. NS, not significant. Numbers with different letters (a-b) differ within each row ($P<0.05$).

	Treatment		
Cytokine	Reference (n=8)	Colitis control (n=6)	Colitis peptide (n=8)
TNF-α	227±22 a	192±19 ab	153±24 b
IL-2	10135±751	10692±1543	9866±1348
IFN-γ	1162±139 a	871±274 ab	490±120 b

Figure 5.1 Experimental design.

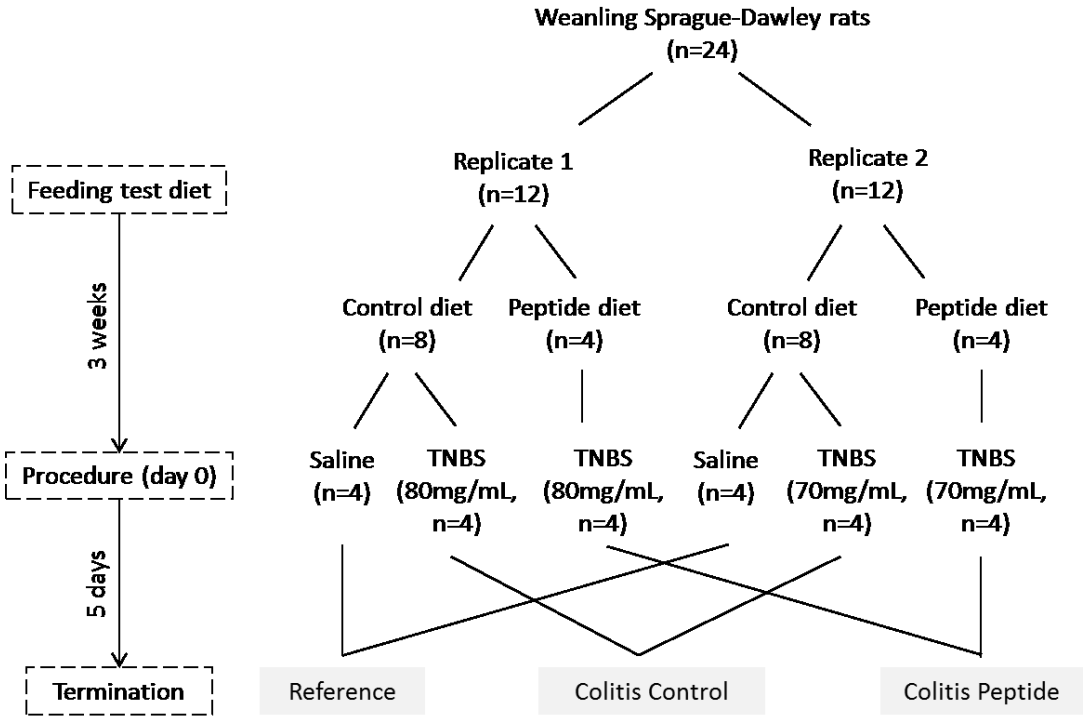


Figure 5.2 Post-procedure body weight changes of untreated and TNBS-treated rats fed or not fed with peptides from day 1 to day 5. Markers represent mean values ± SEM; *Denotes statistical significance at $p<0.05$ as compared to colitis control.

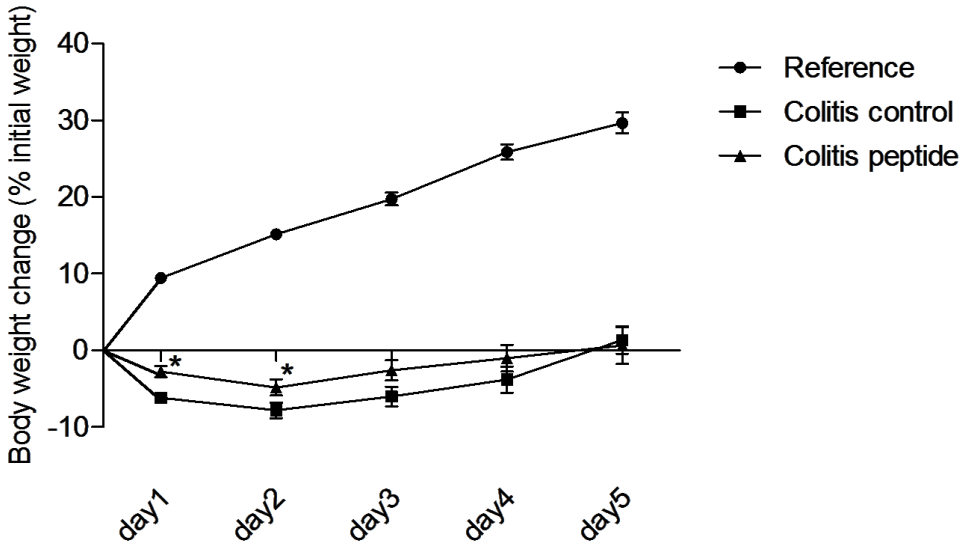


Figure5.3 Food intake of untreated and TNBS-treated rats fed and those not fed with peptides from day 1 to day 5. Markers represent mean values ± SEM.

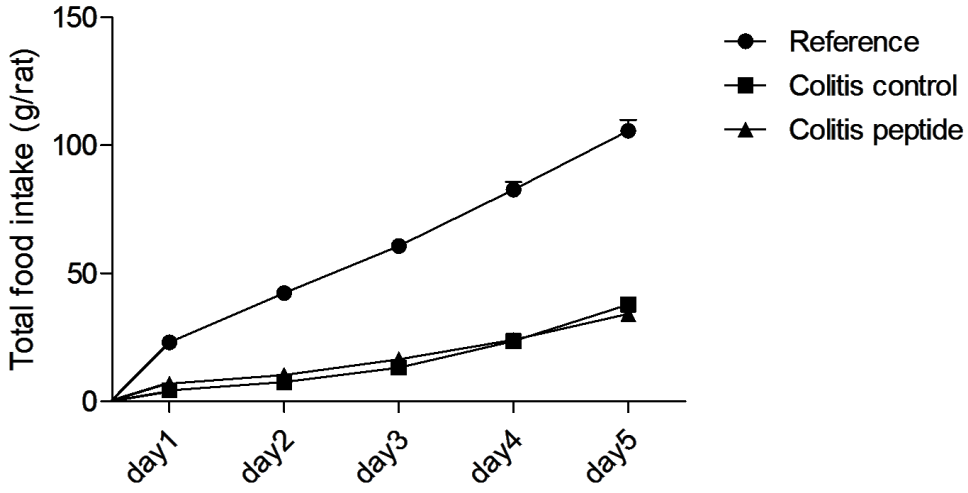


Figure 5.4 Representative photos of rat colon. Left – TNBS-induced colitis in colon with flaccid appearance with ulceration and bowel wall thickening; Right – healthy reference elastic and thin colon wall.

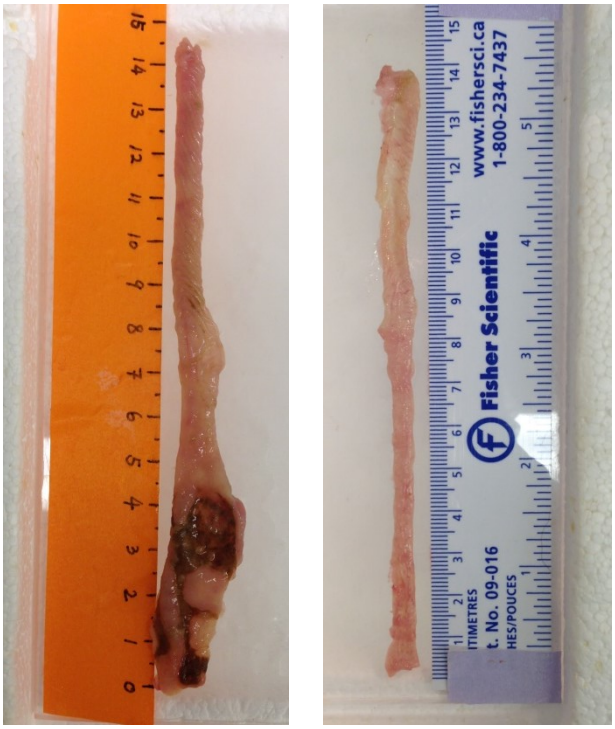


Figure 5.5 Rat colon segments after haematoxylin and eosin stain: reference (a,c), treated with TNBS (b, e) and peptide diet (c, f). Infiltration of immune cells and architecture alterations were observed in TNBS-induced colitis groups. M, mucosa; SM, submucosa; Mu, muscular layer.

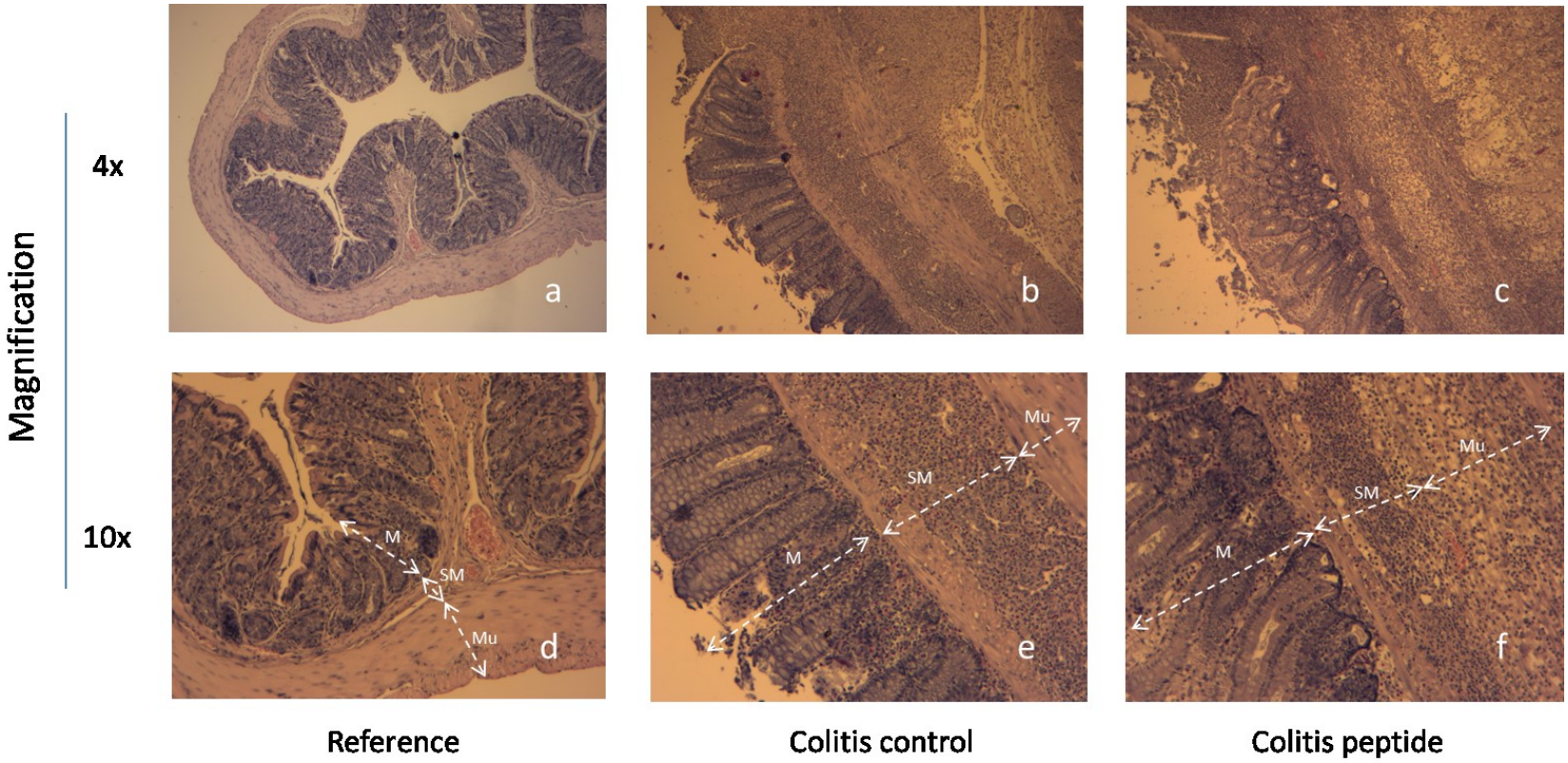
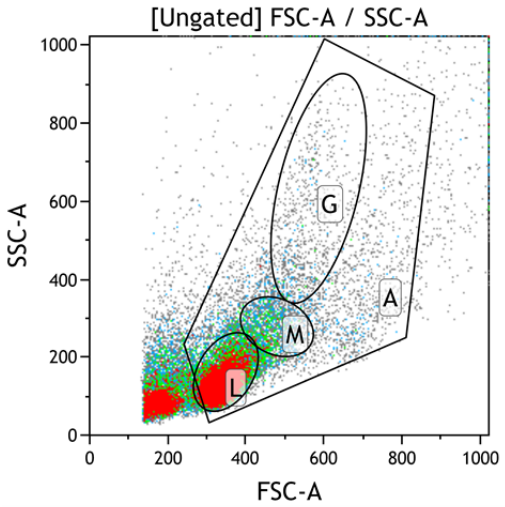


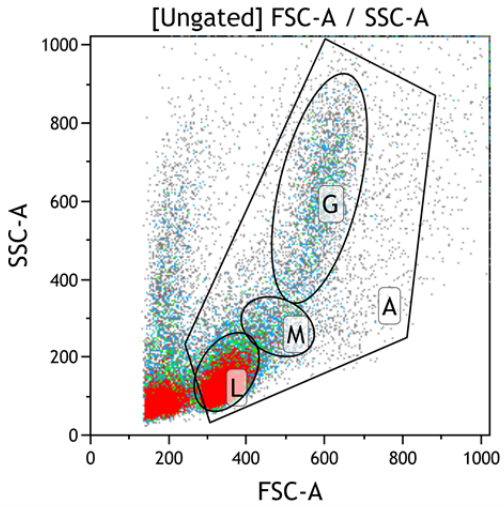
Figure 5.6 Gates and regions of splenocytes in density plot by flow cytometry. The lymphocytes, monocytes and granulocytes were gated as region "L", region "M" and region "G", respectively. A wider gate was drawn to include all the three populations of immune cells in spleen (region "A"). Cell counts were expressed as percentage proportion of all ungated cells as determined by flow cytometry. FSC, forward scatter; SSC, side scatter. * indicates $p<0.05$, *** indicates $p<0.001$ compared to the healthy reference.

Reference



Gate	Cell count
L	54±1
M	5.3±0.1
G	3.2±0.2
A	72±1

TNBS colitis



	Cell count	
Gate	Colitis control	Colitis peptide
L	46±3 ***	45±1 ***
M	5.7±0.5	5.4±0.4
G	8.1±1.4 ***	10.3±0.7 ***
A	69±1 *	70±1 *

CHAPTER 6 – Purification and Identification of Immunomodulatory Peptides from Enzymatic Hydrolysates of Spent Hen Muscle Proteins

6.1. Introduction

Food protein derived bioactive peptides are specific fragments that may exert beneficial physiological activities by acting on cardiovascular, nervous, gastrointestinal and immune systems (Hartmann & Meisel, 2007). Immunomodulatory peptides have been reported from various food sources including milk, egg, plant (soy, wheat, rice), and marine products (Chapter 2). The potential of meat proteins as a source of immunomodulatory peptides has not been explored, with major research work carried out on antihypertensive and antioxidant activities (Arihara, 2006; Ryan Thomas, Ross Paul, Bolton, Fitzgerald F., & Stanton, 2011; Udenigwe & Howard, 2013).

Theoretically peptides can be released from dietary proteins during gastrointestinal digestion, but the amount is generally too low to induce significant therapeutic effects *in vivo*. Enzymatic hydrolysis and separation technologies can be used to produce bioactive peptides on a commercial scale (Udenigwe & Aluko, 2012). In Chapter 3, several hydrolysates were prepared from spent hen carcass, a by-product from the egg industry, by enzymatic hydrolysis and evaluated for anti-inflammatory properties. The two spent hen hydrolysates, Protease M and Protex 50FP, exhibiting IL-6 inhibitory activity were used in this study for purification and identification of IL-6 inhibitory peptides. Generally considered a pro-inflammatory cytokine in chronic inflammation (Gabay, 2006), elevated IL-6 level has been associated with a variety of chronic diseases such as type I diabetes, rheumatoid arthritis and colitis, and blockade of IL-6 signaling was found to be effective in treating chronic inflammation related diseases (Feghali & Wright, 1997; Neurath & Finotto, 2011)

Various strategies have been employed in peptide purification; the mostly commonly used are ultrafiltration, size-exclusion chromatography, reverse-phase chromatography and ion exchange chromatography (Udenigwe & Aluko, 2012). These purification strategies were

developed to separate peptides based on structural characteristics such as size, net charge and hydrophobicity, which are important for inducing the physiological activities. For purification of immunomodulatory peptides, cation exchange chromatography, gel filtration chromatography and reverse-phase chromatography were used in a study of Alaska pollock frame (APF) hydrolysates with lymphocyte proliferation stimulatory activity (Hou et al., 2012); peptides with nitric oxide inhibitory activity in RAW264.7 cells was purified from enzymatic hydrolysates of clam (*Ruditapes philippinarumby*) hydrolysaste by ultrafiltration and multi-step reverse-phase chromatography (Lee et al., 2012). In this study, we applied ultrafiltration, cation-exchange chromatography, reverse-phase chromatography and LC-MS for purification and identification of peptides with IL-6 inhibitory activity.

6.2. Materials and methods

6.2.1. Materials

Protease M was obtained from Amano Enzyme Inc. (Nagoya, Japan), and Protex 50FP was obtained from Genencor International Inc. (Rochester, NY, USA). U937 cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Ammonium acetate, ammonium carbonate, sodium phosphate and trifluoroacetic acid (TFA), phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharides (LPS, *E. coli* strain *0111:B4*) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). HPLC-grade acetonitrile was purchased from Fisher Scientific (Ottawa, ON, Canada). RPMI-1640 culture media, fetal calf serum and antimycotic–antibiotic solution were purchased from Invitrogen (Burlington, ON, Canada). Human IL-6 ELISA Ready-SET-Go!® was purchased from eBiosciences (San Diego, CA, USA).

6.2.2. Preparation of spent hen hydrolysates

Spent hen hydrolysates were prepared as described in Chapter 3. Briefly, crude muscle proteins were prepared from homogenized meat slurry by isoelectric precipitation (pH adjusted to 5.0) to remove the heme pigment. The muscle proteins were digested at a

concentration of 5% (w/v, dry weight). Hydrolysis was performed for 3 hr under constant temperature and pH (50°C and pH 3.0 for Protex 50FP, or 45°C and pH 5.0 for Protease M). Both enzymes were added at a level of 4% (w/w, dry weight) of crude muscle proteins. Hydrolysis was terminated by heating and pH adjustment. After centrifugation, the supernatants were filtered to remove excessive lipids and undigested proteins.

6.2.3. *In vitro* immunomodulatory activity

Freeze-dried peptide samples were tested for IL-6 inhibitory activities *in vitro* in human monocytic cell line U937 as described in Chapter 3. Briefly, cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat inactivated fetal calf serum and 1% (v/v) antibiotics in a humidified incubator (37°C, 5% CO_2). Cells were seeded at 5 x 10^4 cells per mL in 24-well cell culture plates and incubated with PMA for 24 hr. After differentiation, sterile sample solutions or phosphate buffered saline (control) were added to the wells 30 min prior to the addition of LPS for 48 hr. At the end of the experiment, the plates were centrifuged and supernatants were collected for cytokine analysis. Samples were tested in triplicate in repeated trials.

Concentrations of IL-6 were determined using a human IL-6 ELISA kit (7.8 – 500 pg/mL). The assay was performed as per manufacturer's instructions. All samples were tested in duplicate and the absorbance was read at 450 nm on a SpectraMax 190 microtitier plate reader (Molecular Devices, CA, USA). Coefficient of variance between duplicate was ≤10%. IL-6 inhibitory activity was expressed as inhibition percentage of IL-6 production calculated as: (Cc-Cp)/Cc x 100%, where Cc represents IL-6 concentration in supernatant of the control cells, and Cp represents IL-6 concentration in supernatant of cells treated with peptide samples.

6.2.4. Ultrafiltration and cation exchange chromatography

The two spent hen hydrolysates were purified as previously reported with slight modifications (Majumder & Wu, 2011). The hydrolysates were first passed through a 3 kDa molecular weight cutoff (MWCO) membrane (Millipore, Billerica, MA) in an Amicon

8400 UltraFiltration Cell (Millipore Corp., Bedford, MA, USA). Derived permeates were loaded onto a HiPreP 16/10 SP FF cation exchange column coupled with an ÄKTA explorer 10XT system (GE Healthcare, Uppsala, Sweden). After sample injection, the column was washed with 2 column volume (CV) of 10 mM ammonium acetate (pH 4) at a flow rate of 5 mL/min, and then eluted with 0.5 M ammonium carbonate (pH 8.8) by gradients up to 35% in 6CV. The elution was monitored at 280 nm; fractions were collected for activity test based on peak separation.

6.2.5. Purification by reverse phase chromatography

Reverse-phase liquid chromatography was performed in a Waters 600 HPLC system (Waters Inc., Milford, MA. USA) as previously reported with modifications (Majumder & Wu, 2011). Elutions were all monitored by Waters 2998 photodiode array at 220 nm.

Protease M hydrolysate was first loaded to a Waters Xbridge Prep C_{18} column (5 μm, 10 x 150 mm; Waters Associates, Milford, MA, USA) and eluted with 0.1% TFA in water (solvent A_1) and 0.1% TFA in acetonitrile (ACN, solvent B_1) by gradient from 1% to 30% B_1 over 50 min at a flow rate of 5 mL/min. Fractions were collected at 3 min interval and tested in cells (Step I). The active fractions were further loaded to the column but eluted with sodium phosphate buffers (20 mM sodium phosphate buffer with 0.05% TFA in water (solvent A_2) and 20 mM sodium phosphate buffer with 0.05% TFA in 80% acetonitrile (solvent B_2)) at a flow rate of 3 mL/min by gradients up to 50% B_2. Fractions were collected based on peak separation and were desalted using Waters Sep-Pak tC18 cartridges (900 mg sorbent) prior to the *in vitro* activity test (Step II). Fraction HA-9 was further loaded to a Waters Xbridge C18 column (5 μm, 3 x 250 mm) and eluted with the initial H_2O-ACN solvent system (A_1, B_1) at a low flow rate of 0.5 mL/min by gradients up to 50% B_1 for purification (Step III).

Protex 50FP hydrolysate was first loaded to Waters Sep-Pak tC_{18} cartridges (10g sorbent) and eluted by buffer B_1 (0.1% TFA in ACN) at 0%, 10%, 20% and 50% (Step I). The most active eluent was then purified by RP-HPLC following a procedure similar with Protease M hydrolysate. The sample was first loaded to a Waters Xbridge Prep C_{18} column (5 μm,

10 x 150 mm) and eluted with 20 mM sodium phosphate buffers (A_2, B_2) by gradients up to 50% B_2 at a flow rate of 3 mL/min. Fractions were collected at 1.5 min interval and were desalted using Waters Sep-Pak tC_{18} cartridges (900 mg sorbent) prior to the *in vitro* activity test (Step II). The active fractions were then loaded to a Waters Xbridge C_{18} column (5 µm, 3 x 250 mm) and eluted with the H_2O-ACN solvent system (A_1, B_1) at a flow rate of 0.5 mL/min by gradients up to 50% B_1 (Step III).

6.2.6. Analysis of peptide sequences by LC-MS/MS

The most active fractions were sent for sequence analysis using a Waters (Micromass) Q-TOF Premier (Waters Inc., Milford, MA. USA) paired with a Waters Atlantis dC_{18} (75 µm × 150 mm, 3 µm) UPLC column (Milford, MA) as previously reported (Majumder & Wu, 2011) with slight modification. Briefly, samples were eluted using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). At a constant flow rate of 0.35 µL/mL, peptides were separated with the following gradients: starting with 1% solvent B and raised to 15%B in 5 min, to 45%B in 35 min, to 95%B in 5 min and held for 5 min to elute all the peptides. Ionisation was performed by electrospray ionization technique (ESI) with a nano-Lockspray ionization source in a positive ion mode. A MS/MS full-scan was performed for each sample with acquisition ranges of 100–1000 in MS mode and 50-1500 in MS/MS mode. Instrumental control and data analysis were performed using MassLynx software (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K.). Obtained MS/MS data were analyzed by proteomic software Mascot (version 2.2; Matrix science) and Peaks Viewer 5.2 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) in combination with manual de novo sequencing to process the MS/MS for sequence information. The sequences were searched in NCBI protein database to match parent protein. Peptide sequences were selected based on spectra intensity to be chemically synthesized (GenScript Corp., Piscataway, NJ, USA) for verification of bioactivity in cells.

6.2.7. Statistical analysis

Statistical analysis was conducted using the SAS software (Version 9.4; SAS Institute, Cary, NC, USA). Data were presented as the mean and standard error of the mean (SEM).

The data were analyzed by one way analysis of variance (ANOVA) with Duncan's multiple range test. Significance of differences was reported at a 5% level ($p<0.05$).

6.3. Results and discussion

6.3.1. Ultrafiltration and cation exchange chromatography

The two enzymatic hydrolysates prepared from spent hen muscle proteins by Protease M and Protex 50FP have been found to inhibit IL-6 production *in vitro* in LPS-stimulated U937 macrophage-like cells. At a concentration of 500 µg/mL, Protease M and Protex 50FP hydrolysates reduced IL-6 production by 13% and 15%, respectively (Chapter 3).

At the same concentration, the permeate of Protease M and Protex 50FP hydrolysates obtained from 3 kDa ultrafiltration reduced IL-6 response by 14% and 7%, respectively. Ultrafiltration helped to enrich oligopeptides in samples despite a slightly weaker activity was found in Protex 50FP permeate. Many immunomodulatory peptides has been identified with a molecular size less than 30 amino acid residues (Gill, Doull, Rutherfurd, & Cross, 2000; McCarthy, Mills, Ross, Fitzgerald, & Stanton, 2014); therefore it was expected that no significant loss of immunomodulatory peptides occurred during ultrafiltration.

Ion exchange chromatography produced 3 fractions for Protease M hydrolysate and 4 fractions for Protex 50FP hydrolysate (Figure 6.1). Results from *in vitro* study indicated that peptide fractions of both hydrolysates collected from ion exchange chromatography failed to exhibit stronger IL-6 inhibitory activity comparing to the permeates. Rather, stimulatory effects were observed in the positively charged fractions (FA-2, FA-3; FB-2, FB-3, FB-4). For now, the structure-function relationship of immunomodulatory peptides remains largely unknown, but there are some evidence suggesting a positive correlation between immunostimulating activities and positively charged molecules. In a study of immunomodulatory peptide lactoferricin, it was suggested that the positively charged region of the molecule can bind and activate the chemokine receptors on immune cell (Vogel et al., 2002); Kong et al. (2008) prepared soy protein hydrolysates with various

enzymes and determined the positively charged peptide contents through cation exchange chromatography; in this study they observed a positive correlation between the immunomodulating activity on lymphocyte proliferation and the content of positively charged peptides. Our results are consistent with these studies suggesting a stimulating effect of positively charged peptides on cytokine response of macrophage-like cells.

6.3.2. Purification by reverse-phase chromatography

As cation exchange chromatography could not separate peptides for stronger IL-6 inhibitory activity, permeate from ultrafiltration was alternatively loaded to the reverse-phase chromatography. Peptides from Protease M hydrolysis were separated into 9 fractions (HA-1 to HA-9) using the Prep C_{18} column. The fractions exhibited significantly enhanced IL-6 inhibitory activity, among which HA-5, HA-8 and HA-9 inhibited IL-6 production by 20 %, 18% and 70%, respectively, at a concentration of 250 µg/mL (Figure 6.2).

The 2nd step purification with 20 mM sodium phosphate buffer was effective for further separation of IL-6 inhibitory peptides. At a concentration of 100 µg/mL, derived fractions HA-5-8 and HA-8-12 exhibited strong activity at a concentration of 100 µg/mL, inhibiting IL-6 production by 58% and 63%, respectively (Figure 6.3). Sodium phosphate buffer changed the eluent pH (from a pH of 1.8-2.0 in step I to a pH of 7.0), which changed the degree of analyte ionization and therefore the polarity and retention time based on the proximity of eluent pH to the pKa of analyte (Taylor, 2014). Changes on the retention time of ionizable peptides allow further separation of the active peptides.

Fractionation of HA-9 fractions produced 5 fractions; HA-9-4 had the best IL-6 inhibitory activity, inhibiting IL-6 production by 40% and 50% at 50 and 100 µg/mL, respectively. The purification process helped to reduce the complexity of the fraction but did not enhance the activity of fraction HA-9, which exhibited slightly higher IL-6 inhibitory activity of 46% and 59% at 50 and 100 µg/mL, respectively (Figure 6.3). Similar results were observed on fraction A8-12, for which after purification the derived fractions

exhibited weaker activities (*data not shown*). These might suggest a synergistic effect and the existence of several different peptides with IL-6 inhibitory activity.

Permeate from Protex 50FP hydrolysate was alternatively processed with Sep-Pak tC18 cartridge as the first step purification. Peptide separation by C18/tC18 cartridge has been reported for use on relative complex substrates such as plasma (Matsui, Imamura, & Oka, 2004), protein digest (Ohtsuru, Horio, & Masui, 2000; Pihlanto, Akkanen, & Korhonen, 2008; Yamamoto, Maeno, & Takano, 1999). Similar with RP-HPLC, peptides were separated based on the nature of polarity/hydrophobicity. Peptide separation using the Sep-Pak tC18 cartridge was found to be efficient and effective as shown in the chromatogram by loading to the Prep C18 column (Figure 6.4). Peptide fractions eluted with higher percentage of organic (non-polar) solvent (B_1) that are more hydrophobic exhibited stronger IL-6 inhibitory activity; fraction eluted with 50% B_1 (HB-50) inhibited IL-6 production by 78% at a concentration of 500 μg/mL as compared with a 57% and 19% suppression from 20%B1 (HB-20) and 10%B1 (HB-10) elute (Figure 6.4). Interestingly, similar results were observed on Protease M hydrolysate, in which late fractions (HA-9, HA-5-8, HA-8-12) tend to exhibit stronger IL-6 inhibitory activities. Hydrophobic peptide fractions of cheese slurry (Lee, Kim, Shin, Nam, & Woo, 1995), ginseng extract (Kim et al., 2003) and soy protein digest (Kim et al., 2000) have been reported for high inhibitory activity on proliferation ([^{3}H]Thymidine uptake) of a mouse macrophage-like cell line P388D1. Our results are consistent with their works suggesting strong IL-6 inhibitory activity of the hydrophobic fractions in human macrophage-like cells. In another study, hydrophobicity was positively correlated with the stimulatory effect of milk peptides on proliferation of murine splenocytes (Jacquot, Gauthier, Drouin, & Boutin, 2010); the difference might be explained by different cell types and cell to cell interactions of splenocytes.

The 2nd step purification of fraction HB-50 yielded fractions HB-50-12, HB-50-13 and HB-50-14 exhibiting stronger IL-6 inhibitory activity comparing to HB-50, suppressing IL-6 production by 62%, 62% and 77%, respectively, at a concentration of 250 μg/mL (Figure 6.5); at the same concentration, HB-50 inhibited IL-6 production by 60%. As

peptide separation carried out in tC18 cartridge allowed an efficient preparation of active peptides at a relatively high amount, fraction HB-50-12 (relatively pure) and HB-50-14 (strong activity) were selected for the 3rd step purification. Following an effective purification with the sodium phosphate buffer solvent system, the 3rd step purification was able to further separate peptides and enhance activity. HB-50-12-2 (52% at 100 μg/mL) showed slightly higher activity than HB-50-12 (43% at 100 μg/mL), HB-50-14-3 exhibited stronger activity than HB-50-14, which suppressed IL-6 production by 58% at 100 μg/mL (Figure 6.6).

After purification, peptide fractions exhibited greatly improved IL-6 inhibitory activities compared to the whole hydrolysates. At a concentration of 100 μg/mL, peptide fractions could induce over 50% of IL-6 inhibition in LPS-activated U937 human macrophage-like cells, these include fraction HA-5-8 (58%), HA-8-12 (63%), HA-9-4 (50%), HB-50-12-2 (52%) and HB-50-14-3 (>58%).

6.3.3. Peptide identification by mass spectrometry

The active peptide fractions HA-5-8, HA-9-4, HB-50-12-2, HB-50-14-1, HB-50-14-3, HB-50-14-8 were selected for LC-MS/MS peptide sequencing analysis. Representative MS spectra and MS/MS spectra for manual de novo sequencing of each of the hydrolysate were present in Figure 6.7 and Figure 6.8. Several peptide sequences were identified from these fractions as fragment of various muscle proteins as listed in Table 6.1. The peptides were released by enzymatic hydrolysis from muscle proteins such as myosin (light chain, heavy chain), actin, titin, troponin C, collagen, glyceraldehyde-3-phosphate dehydrogenase (G3PD) and β-enolase. These peptides varied in molecular size (5-17 amino acids), amino acids composition and polarity.

Of a few selectively synthesized peptides (based on spectra intensity), 8 peptides exhibited moderate to strong IL-6 inhibitory activities when tested at a concentration of 100 or 250 μg/mL as shown in Table 6.2. Hepta-peptide FLWGKSY from myomesin had the best inhibitory activity reducing 79% of IL-6 production at a concentration of 100 μg/mL. Peptides SFMNVKHWPW and AFMNVKHWPW are both fragments of myosin proteins

(*gallus gallus*) and inhibited over 50% of IL-6 production at a concentration of 100 μg/mL. Moreover, tri-peptide WPW identified from another fraction showed similar activity with the above two peptides and could be the core motif responsible for the IL-6 inhibitory activity. Our results suggested that the presence of tryptophan (W) residue in peptide sequence might be important for the IL-6 inhibitory activity. This is consistent with recent evidence indicating a role of tryptophan on immune suppression (Zhang, Hu, Kovacs-Nolan, & Mine, 2014). Additionally, the proline residue in peptide sequence might confer unique conformational constraints on the peptide chain for protection against proteolysis in the gastro-intestinal tract (Vanhoof, Goossens, De Meester, Hendriks, & Scharpé, 1995).

IL-6 inhibitory effect, as a measure of anti-inflammatory property, has been reported from other food-derived protein and peptides. Soy peptide lunasin, a well-known anti-cancer peptide, was found to inhibit 50% of IL-6 production at a concentration of 2 μM (~10 μg/mL) using murine macrophage (RAW264.7) (de Mejia & Dia, 2009). In RAW264.7, a pea protein hydrolysate (mainly consist of peptides with molecular weight < 3 kDa) exhibited strong IL-6 inhibitory activity inhibiting 50% of the IL-6 production at a concentration of 6.3-12.6 μg/mL (Ndiaye, Vuong, Duarte, Aluko, & Matar, 2012), a salmon peptide fraction SPHF1 containing 1000–2000 Da molecular weight peptides inhibited IL-6 production by ~25% at a concentration of 100 μg/mL (Ahn, Je, & Cho, 2012), and a salmon-derived tri-peptide Pro-Ala-Tyr inhibited ~33% of IL-6 production at 500 μM (~175 μg/mL) (Ahn, Cho, & Je, 2015). In a human monocytic cell line (THP-1), bovine milk protein lactoferrin was reported to inhibit 45% of IL-6 production at a concentration of 50 μg/mL, and its fragment (lactoferricin B) showed stronger activity inhibiting IL-6 production by 64% at 0.5 μg/mL (Mattsby-Baltzer et al., 1996). In this study, the purified fractions and peptides identified from chicken muscle proteins exhibited comparable IL-6 inhibitory activity to those reported by other research groups.

6.4. Conclusion

In this study, various techniques were tested for purification of IL-6 inhibitory peptides. Ultrafiltration with a 3 kDa molecular weight cutoff membrane to remove high-molecular

weight components helped to enrich oligopeptides without sacrificing the bioactivity. Cation exchange chromatography could not separate peptides for better IL-6 inhibitory activity but produced fractions with stimulatory activity, which might be attributed to the positive charge carried by the peptides. Reverse-phase chromatography turned out to be a useful tool for purification of IL-6 inhibitory peptides, with the activity greatly improved from 14% (500 μg/mL) to 63% (HA-8-12, 100 ug/mL) of Protease M hydrolysate, and from 7% (500 μg/mL) to 48% (HB-50-14-3, 50 ug/mL) of Protex 50FP hydrolysate. The effectivity of reverse-phase chromatography observed in this study is associated with peptide hydrophobicity that might pose an influence on IL-6 production in macrophages. For the first time, we reported several novel peptide sequences from major muscle proteins. Of them, many exhibited verified IL-6 inhibitory activities that are comparable to the work of other research group. As of now there is limited report on the function-structure relationship of immunomodulatory peptides (Chapter 2); this increases the difficulty for efficient and strategic preparation/purification/identification of immunomodulatory peptides. Our findings suggested that tryptophan residue in sequence might contribute to the IL-6 inhibitory activity in macrophages. Future research efforts will be directed to explore the underlying regulatory mechanism of these peptides and their potentials on other immune indices.

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Table 6.1 Peptide sequences identified in fractions with IL-6 inhibitory activity from spent hen hydrolysates by LC-MS/MS.

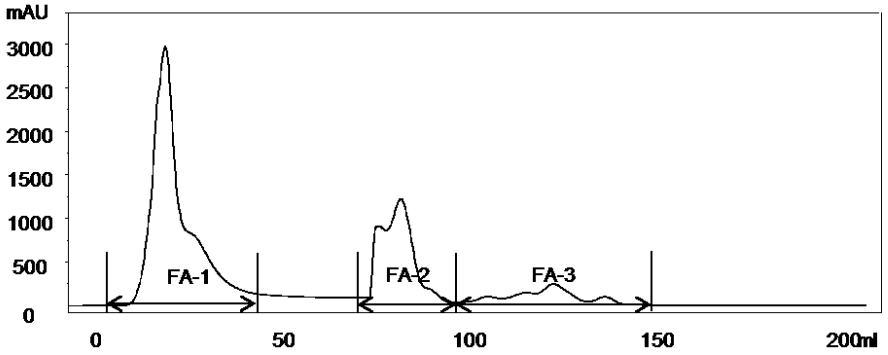
Fraction	Peptide sequence	Observed m/z	Calculated mass	Length (amino acids)	Parent protein
HA-5-8	FIGVL	548.34	547.34	5	Myosin
	NPLLEAFGNAK	587.32	1172.62	11	Myosin
	PGTPGPIG	695.35	694.37	8	Collagen
	DKEGNGTVM(+16)GAELRHVL	614.66	1840.91	17	Myosin
HA-9-4	EGIEW	633.33	632.29	5	Myosin
	ELGEIL	673.39	672.38	6	Troponin C
	INDPFID	833.43	832.4	7	Glyceraldehyde-3-phosphate dehydrogenase (G3PD)
	ETVIGLY	794.46	793.43	7	Myosin
HB-50-12-2	WPW	488.22	487.21	3	Myosin
	IIGMGPF	734.44	733.39	7	Titin
	VWIGGSIL	844.51	843.49	8	Actin
	SVWIGGSIL	931.51	930.52	9	Actin
	DQIISANPLLEAFGNAK	901.52	1799.95	17	Myosin
	AAADGPLKGILGYT	673.9	1345.73	14	G3PD
HB-50-14-1	AGLLGLL	656.42	655.43	7	Myosin
	FLWGKSY	900.48	899.46	7	M-protein, striated muscle (Myomesin)
	TAIYKLTGAVMHYGNLK	627.37	1879.01	17	Myosin
HB-50-14-3	SFMNVKHWPW	666.36	1330.64	10	Myosin
	AFMNVKHWPW	658.37	1314.64	10	Myosin
	FMVLPVGAASFHDA	731.41	1460.72	14	β-enolase
	FKGIGWSPLGSLD	688.9	1375.72	13	Nebulin
	TFLPMLQHIS	593.86	1185.63	10	Myosin
	ASLSTFQQMWITK	770.94	1539.76	13	Actin
HB-50-14-8	QIISANPLLEAFGNAK	843.46	1684.91	16	Myosin
	GLLGLL	585.43	584.4	6	Myosin
	WFTVL/WFTVI	665.41	664.37	5	Myosin-binding protein
	IIGWL/LLGWI	601.42	600.37	5	Myosin/Filamin

Table 6.2 IL-6 inhibitory activity of chemically synthesized peptides identified from spent hen hydrolysates.

* Peptides were not tested at 250 µg/ml due to low solubility in phosphate buffered saline.

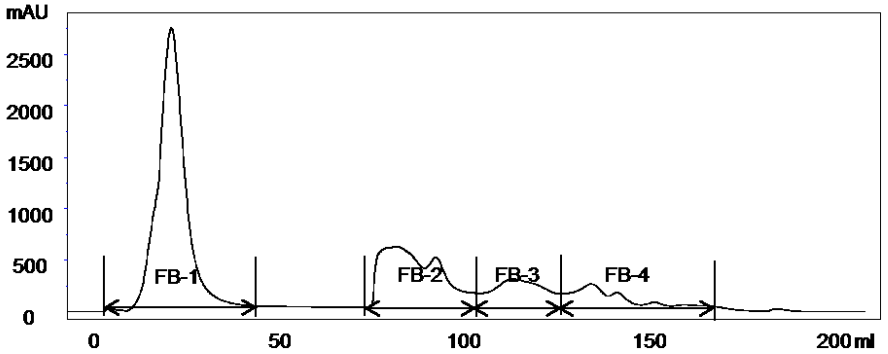
	IL-6 inhibitory activity (% inhibition)	
Peptide Sequence	100 µg/ml	250 µg/ml
FIGVL	13	38
TFLPMLQHIS	17	40
AGLLGLL*	21	N/A
ASLSTFQQMWITK*	38	N/A
AFMNVKHWPW	55	82
SFMNVKHWPW	69	86
WPW	64	83
FLWGKSY*	79	N/A

Figure 6.1 Cation exchange chromatogram of hydrolysate permeates and IL-6 inhibitory activity of derived fractions at the concentration of 500 µg/mL. * Negative values represent a stimulatory effect on IL-6 production. Numbers without a common superscript (a-b) differ ($P < 0.05$).



(A)

Sample	IL-6 inhibitory activity (%) *
Protease M (A) permeate	11.2±3.3
FA-1	-7.8±5.4 [a]
FA-2	-37.4±8.4 [b]
FA-3	-44.5±6.8 [b]



(B)

Sample	IL-6 inhibitory activity (%)
Protex 50FP (B) permeate	7.2±0.9
FB-1	-4.8±6.7
FB-2	-12.2±3.6
FB-3	-16.44±2.6
FB-4	-19.08±0.9

Figure 6.2 RP-HPLC chromatogram of Protease M hydrolysate permeate (Step I) and the IL-6 inhibitory activity of eluted fractions at 250 µg/mL. Bars that do not share a common letter (a-e) differ ($P < 0.05$).

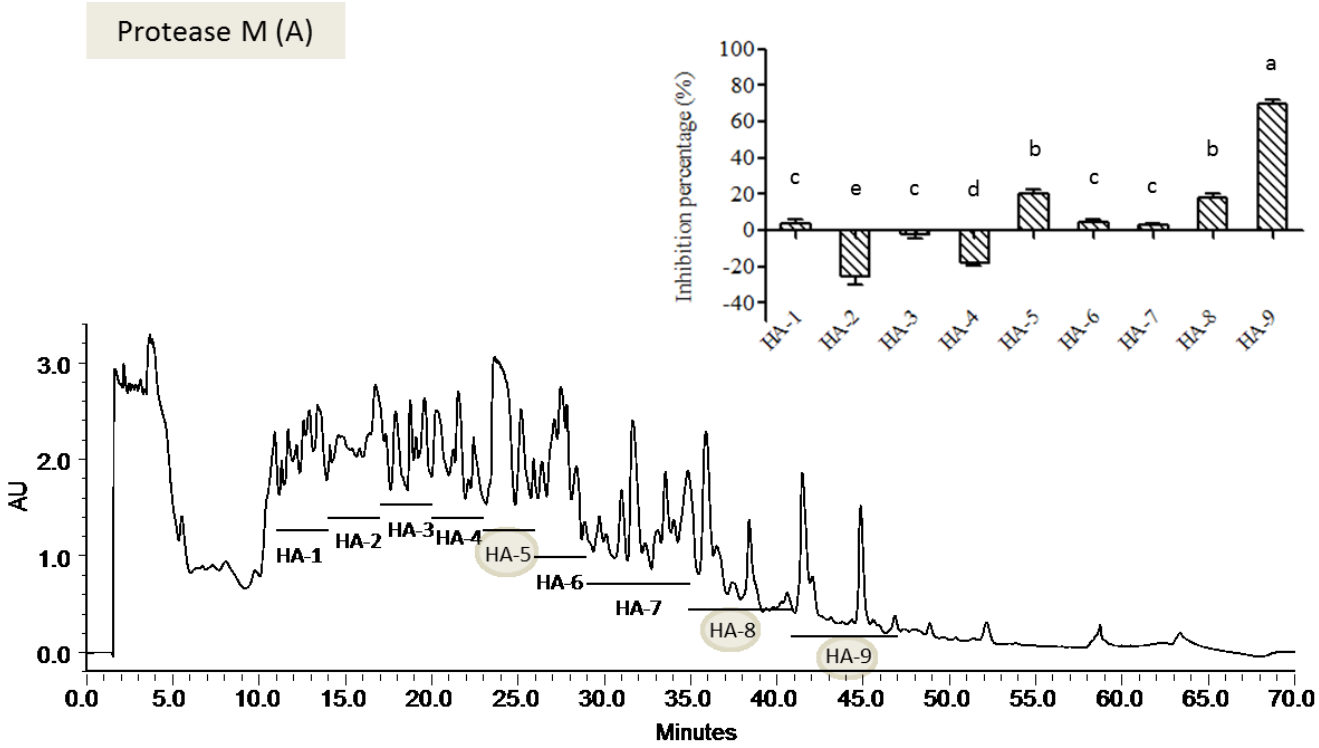


Figure 6.3 Purification chromatogram of fractions HA-5 and HA-8 (Step II), and HA-9 (step III) and the IL-6 inhibitory activity. Bars that do not share a common letter (a-f) differ ($P < 0.05$).

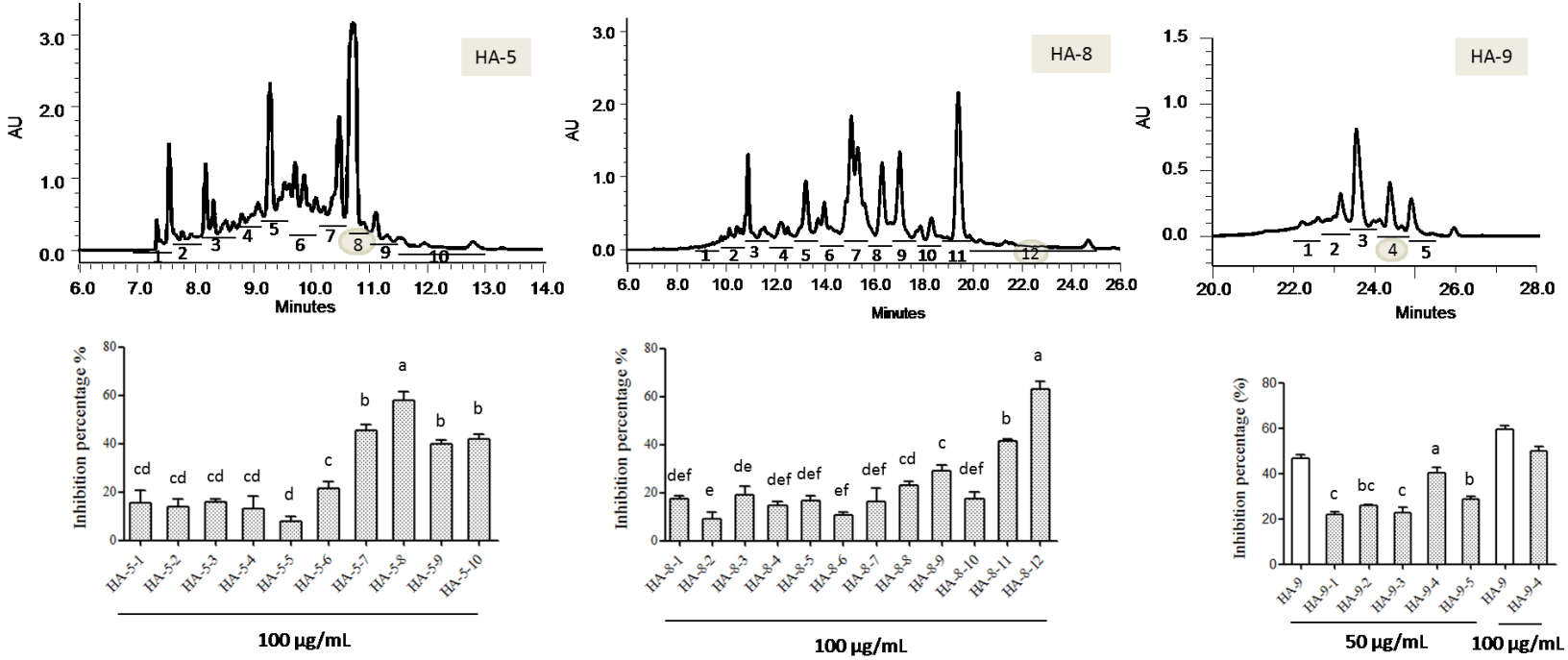


Figure 6.4 RP-HPLC chromatogram of cartridge eluted Protex 50FP hydrolysate fractions (Step I) and the IL-6 inhibitory activity at a concentration of 500 µg/mL. Bars that do not share a common letter (a-c) differ ($P < 0.05$).

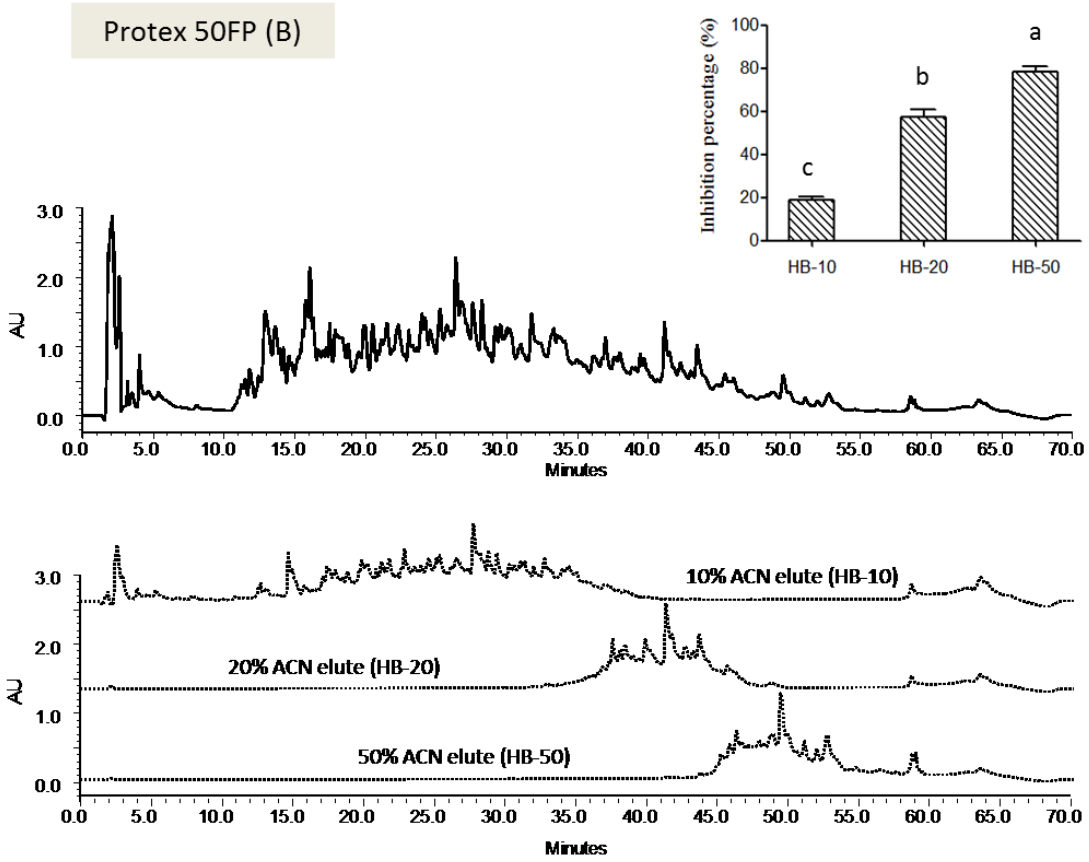


Figure 6.5 Purification chromatogram of fraction HB-50 with sodium phosphate buffer solvents (Step II) and the IL-6 inhibitory activity of the fractions at a concentration of 250 µg/mL. Bars that do not share a common letter (a-e) differ ($P < 0.05$).

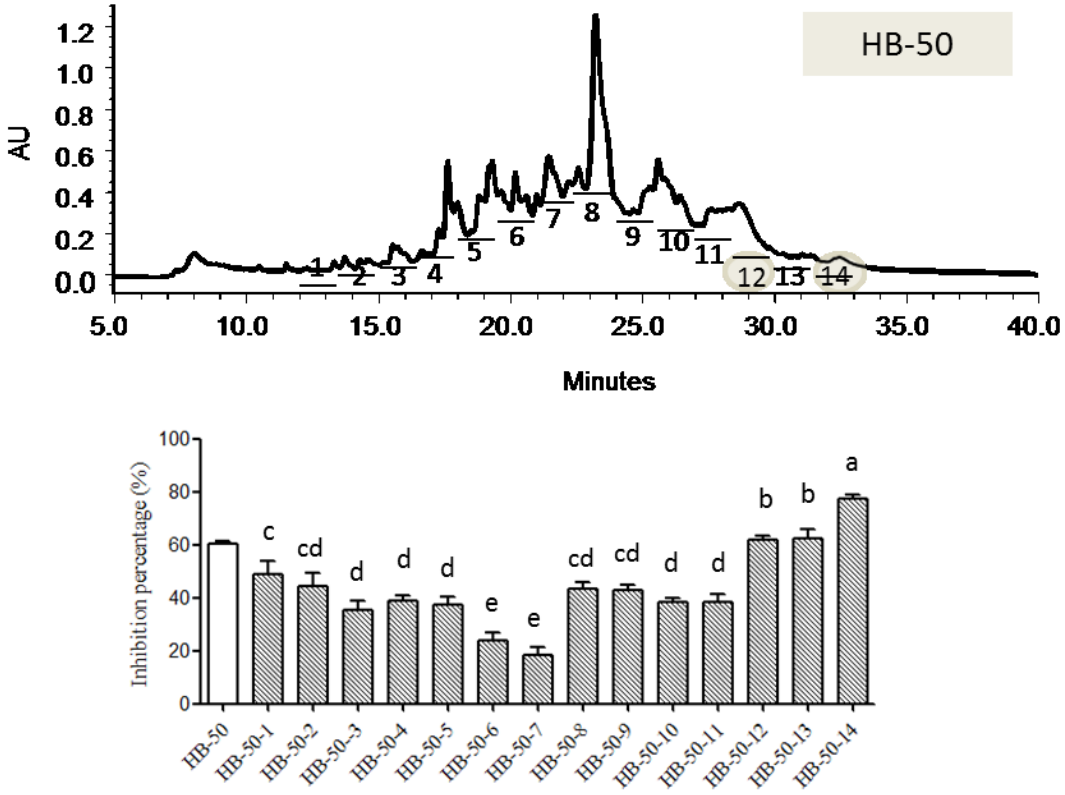


Figure 6.6 Purification chromatogram of fraction HB-50-12 and HB-50-14 by reverse-phase chromatography (Step III) and the IL-6 inhibitory activity of the eluted fractions. Bars that do not share a common letter (a-f) differ ($P < 0.05$).

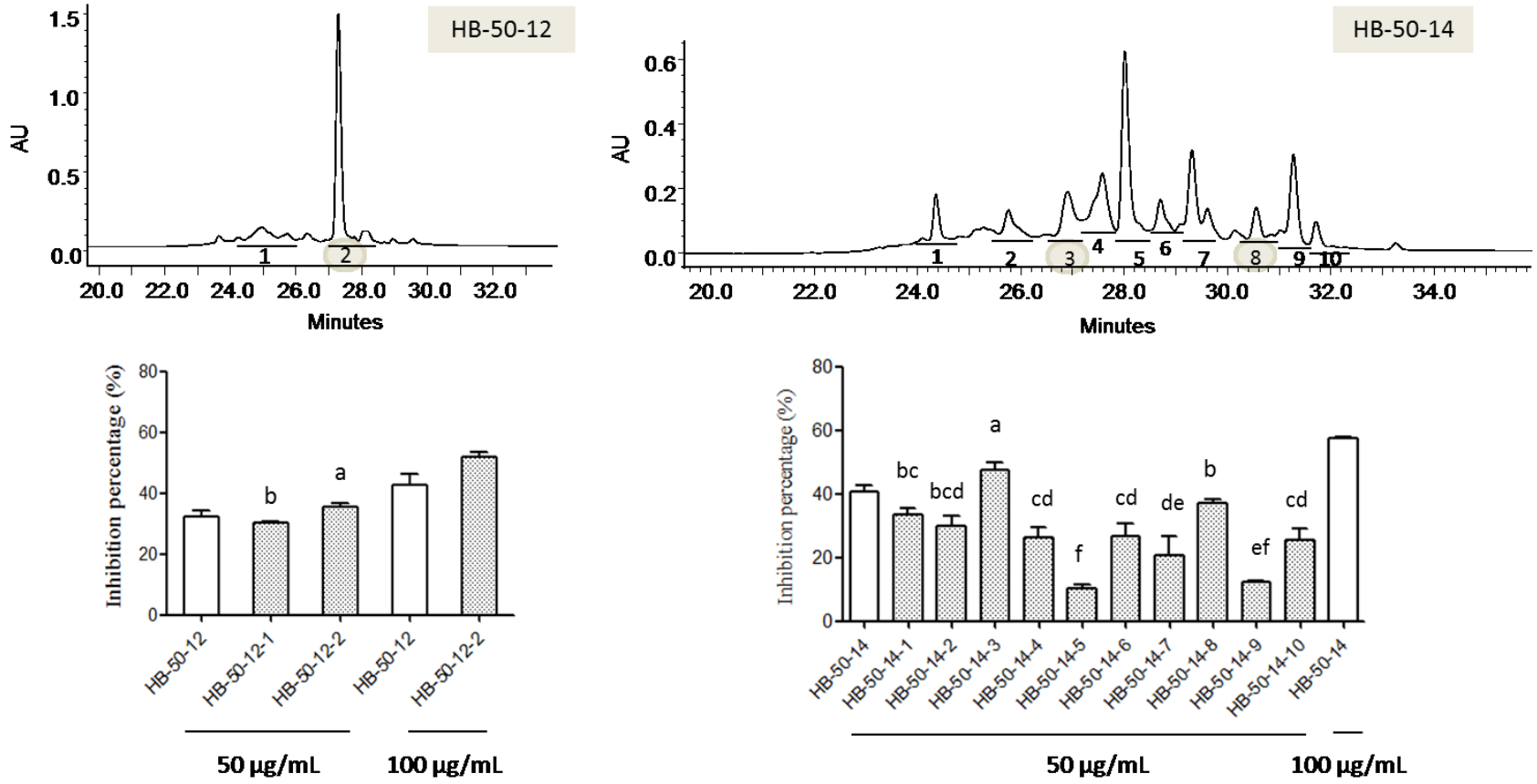


Figure 6.7 MS spectra of fraction HA-9-4 from Protease M hydrolysate and de novo sequencing of two representative peptides EGIEW (m/z 633.33) and INDPFID (m/z 833.44) using their MS-MS spectra.

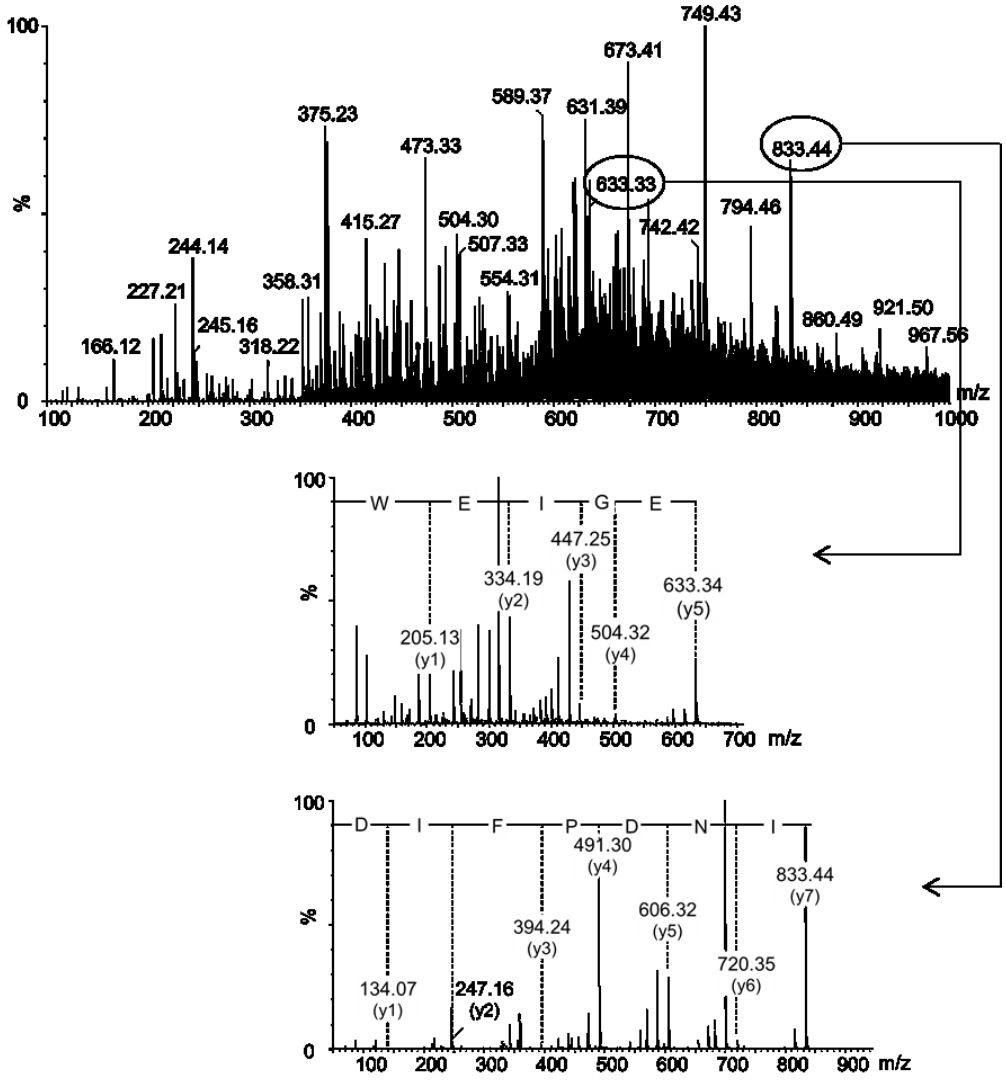
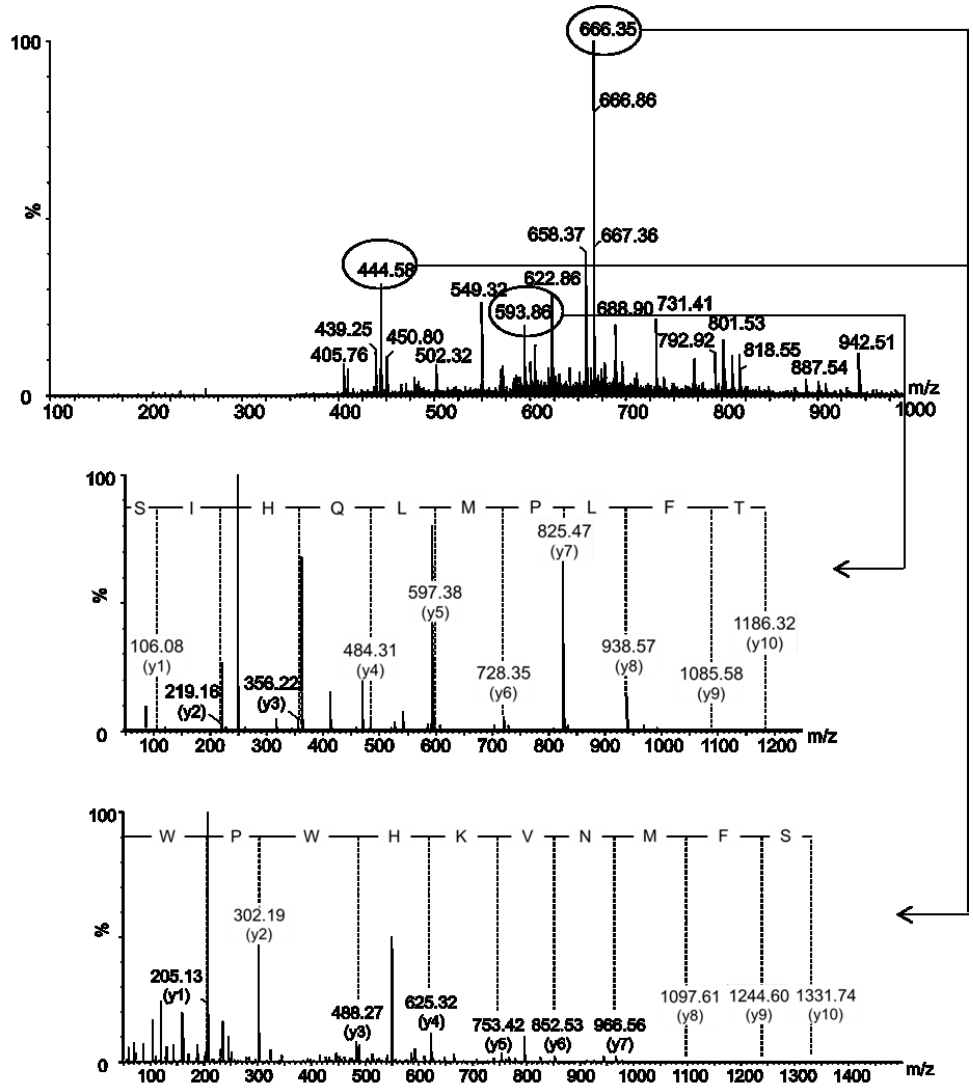


Figure 6.8 MS spectra of fraction HB-50-14-3 from Protex 50FP hydrolysate and de novo sequencing of two representative peptides TFLPMLQHIS (m/z 593.86) and SFMNVKHWPW (m/z 666.35, m/z 444.58) using their MS-MS spectra.



CHAPTER 7 – General Summary and Discussion

7.1. Key findings of the present study

The overall purpose of this research was to produce bioactive peptides with immunomodulatory properties from underutilized spent hens for development of "value-added" functional food products. The key findings of this thesis are outlined below:

1) The first objective of this thesis research is to prepare and characterize spent hen muscle protein hydrolysates with immune functional properties.

Manual deboning recovered about 40% meat from the spent hen carcasses, from which ~73% protein was extracted. Enzymatic hydrolysis with various proteases produced hydrolysates with the protein content ranged from 70% to 83% and the nitrogen recovery from 44.4% to 86.5%. Hydrolysates contains oligopeptides with molecular weight < 12 kDa and degree of hydrolysis ranged from 11.2% to 32.7%. Amino acid composition analysis suggested that hydrolysates prepared by Protease M, Protex 26L and Protex 50FP had the highest total content of free amino acids. Tested in endotoxin-activated macrophage-like cells, hydrolysates prepared by Protease M and Protex 50FP exhibited anti-inflammatory activities inhibiting 13% of IL-6 and 25% of TNF-α, and inhibiting 15% of IL-6 production, respectively, at a concentration of 500 μg/mL (Chapter 3). Spent hen muscle protein hydrolysate showed comparable activity to bovine lactoferrin, a well-established immunomodulatory molecule, indicating the potential of spent hen muscle protein hydrolysate for regulation of macrophage inflammation.

2) The second objective is to evaluate the safety and the in vivo immunomodulatory effects of spent hen hydrolysates in healthy young rodents.

Oral administration of spent hen hydrolysate at doses up to 5% (w/w diet based) addition did not produce any detrimental effects in young healthy rats; no change was observed on the organ characteristics including body weight, food intake, spleen and liver weights, and intestine length. The absence of adverse side effects is an important feature of protein

hydrolysates/peptides or other natural products as compared to pharmaceutical drugs. At a dose of 5% (w/w) the Protex 50FP hydrolysate exhibited a stimulatory effect *ex vivo* on mitogen (LPS, PWM) stimulated IL-10 response. In addition to the IL-10 stimulatory effect, Protex 50FP hydrolysate exerted modulatory effect on immune cells isolated from spleen, resulted in a higher proportion of cells expressing CD11b/c (macrophages, dendritic cells) and a lower proportion of cells expressing OX6 (antigen presenting cells). For the first time we reported immunomodulatory effects of meat-derived peptides in healthy rodents (Chapter 4). The IL-10 stimulatory activity reported in this chapter in combination with the *in vitro* IL-6 inhibitory activity observed in Chapter 3 suggest potential anti-inflammatory properties of this hydrolysate for application inflammation-related chronic disease.

3) The third objective is to test the anti-inflammatory activity of spent hen muscle protein hydrolysate in a colitis model.

A pathological role of IL-6 has been suggested in IBD, likely through the anti-apoptotic signaling on T cells (Bouma & Strober, 2003; Mudter & Neurath, 2007); and a protective role of IL-10 in IBD has been demonstrated in experimental models acting through regulatory T cell and anti-inflammatory cytokine TGF-β (Bouma & Strober, 2003; Fuss, Boirivant, Lacy, & Strober, 2002). Therefore we hypothesized that the Protex 50FP hydrolysate exhibiting regulatory effects on IL-6 and IL-10 could exert beneficial effects in a colitis model. In this study, TNBS-induced colitis model was selected, which was reported to elicit Th1 type immunologic responses sharing many features of the Crohn's disease (Kiesler, Fuss, & Strober, 2015). Three weeks feeding of the peptide diet prior to the onset of colitis exhibited clinical effects attenuating the weight loss immediately after the chemical was applied. However, this beneficial effect did not last till day 5 when tissues were collected. Therefore the regulatory effects cannot be further investigated in this study (Chapter 5).

Tissue analysis suggested the rats developed acute colitis from single administration of TNBS, characterized by a systemic and neutrophil predominant inflammation. Phenotype

analysis of the immune cells in spleen indicated that the colitis group had a higher proportion of granulocytes, and a lower proportion of lymphocytes (as a result of the infiltration of granulocytes). Despite a lower proportion of T cells, the colitis groups had a higher proportion of helper T cells expressing IL-2 receptor (CD4+CD25+). And a higher proportion of monocytes (macrophage, dendritic cells) expressing co-stimulatory markers CD80+ was found in colitis groups. In consistency with the findings in phenotype study, a higher production of macrophage-derived cytokine TNF-α and lower productions of T cell-derived cytokine IL-2 and IFN-γ were found *ex vivo* in response to LPS and ConA stimulation, respectively. Yet no significant effect on cytokine production was observed from peptide treatment at this time point (day 5). A significantly higher expression of CD25 (activation marker, IL-2 receptor) in spleen and a lower expression of CD154 (CTLA-4; activation marker) in MLN was found from peptide treatment. However, the effect of IL-2 receptor and CTLA-4 in gut inflammatory condition has not been clearly established (Chapter 5).

4) The last objective is to purify and identify peptide sequences from the spent hen hydrolysates using liquid chromatography and LC-MS/MS.

Various techniques were tested for purification of IL-6 inhibitory peptides from the two spent hen hydrolysates. Ultrafiltration with a 3 kDa molecular weight cutoff membrane separated high-molecular weight molecules (in retentate) and oligopeptides (in permeate); the IL-6 inhibitory activity of the permeates did not differ significantly compared to the non-treated hydrolysates. Purification by cation exchange chromatography based on the net charge of the peptides did not enhance the IL-6 inhibitory activity. Reverse-phase chromatography that separate peptides based on hydrophobicity was found to be effective for purification of peptides with IL-6 inhibitory activity. After purification, the activity improved from a 14% inhibition at 500 µg/mL to a 63% inhibition at 100 µg/mL (HA-8-12) of Protease M hydrolysate; and from a 7% inhibition at 500 µg/mL to a 48% inhibition at 50 µg/mL (HB-50-14-3) of Protex 50FP hydrolysate. The purified fractions and the peptides synthesized therefrom exhibited strong IL-6 inhibitory activity comparable to the work of other research groups. For the first time, we reported several novel peptide

sequences from major muscle proteins (Chapter 6). To date, there is limited report on function-structure relationship of immunomodulatory peptides; our findings suggested that Tryptophan residue in peptide sequence might contribute to the IL-6 inhibitory activity in macrophages.

7.2. General discussion

It is estimated that each year the egg industry produces about 18 million spent hens in Canada and 150 million in North America (Newberry, Webster, Lewis, & Van Arnam, 1999). Spent hens are layers that are to be replaced usually after one laying cycle; although spent hens are processed for food and feed uses, the value of meat processed from spent hens is low due to its high cost of processing, a relatively low yield, and poor meat quality such as objectionable toughness and the presence of residual bone fragments (Kersey & Waldroup, 1998), when compared with high-meat-yielding broiler chickens. Since a viable market is no longer available, spent hens become a liability to the egg producers who have to pay the cost associated with disposal. Euthanasia followed by burial, composting, or incineration are often used to dispose spent hens, which raises concerns over environmental impact and animal welfare (Freeman, Poore, Middleton, & Ferket, 2009; Newberry et al., 1999). Therefore, it is of great interest to explore alternative uses of spent hens. Spent hen, as an inexpensive source of protein, can be utilized for production of bioactive peptides. Bioactive peptides constitute an important category of health-promoting nutrients for uses as functional foods or nutraceuticals that convey physiological benefits beyond their basic nutritional values (Hettiarachchy, Sato, Marshall, & Kannan, 2011; Mine, Li-Chan, & Jiang, 2010). In this thesis, we developed a protocol for producing immunomodulatory peptides from spent hen muscle proteins.

The immune system, as one of the largest organs dispersing throughout our body, is vital to our health providing host defense against foreign organisms or substances (antigens). Improper immune responses (deficiency/overreaction) often result in common manifestations such as immunodeficiency, allergies and asthma, autoimmune diseases (Kindt, Goldsby, Osborne, & Kuby, 2007). The onset and progression of various chronic

diseases has been associated with persist inflammation, such as in the case of metabolic disorders (Hotamisligil, 2006), atherosclerosis (Hansson, 2005; Libby, 2002), and cancer (de Visser, Eichten, & Coussens, 2006). A number of pharmaceuticals are commercially available for the treatment of inflammatory diseases targeting cyclooxygenase (COX) proinflammatory enzymes, but are often associated with undesirable gastrointestinal and cardiovascular side effects (Kulkarni, 2006). Food-derived immunomodulatory peptides have been explored to provide safe alternatives of the synthetic drugs. A number of immunomodulatory peptides have been characterized from various food sources, including milk, egg, fish, soy and rice (Chapter 2). However, the muscle proteins were rarely explored for generation of immunomodulatory peptides. This is the first study reporting *in vitro* (in cells) and *in vivo* (in rodents) immunomodulatory properties of muscle protein-derived peptides.

Spent hen hydrolysates exhibited inhibitory activity *in vitro* on macrophage-derived cytokine IL-6 and TNF-α, which are generally considered pro-inflammatory cytokines. TNF-α induces fever, prostaglandin E2 (PGE2) and IL-6 synthesis, and shares the inflammatory property with IL-6 inducing acute-phase proteins synthesis in liver; IL-6, in addition, acting on maturation and activation of B cells and T cells (Feghali & Wright, 1997). Elevated systemic concentrations of IL-6 and TNF-α are associated with a variety of chronic diseases such as diabetes, rheumatoid arthritis and colitis (Aggarwal, Gupta, & Kim, 2012; Neurath & Finotto, 2011); and therapeutic blockade of IL-6 or TNF-α has been found to be effective in treating inflammation-related diseases (Aggarwal et al., 2012; Scheller et al., 2011). Additionally, the stimulatory activity of the hydrolysate on splenic IL-10 production observed in the feeding trial can be beneficial for inflammatory conditions. IL-10, as an important regulatory cytokine, controls the inflammatory response and is essential for the homeostasis of the immune system (Sanjabi, Zenewicz, Kamanaka, & Flavell, 2009). Furthermore, the hydrolysates exerted regulatory effects in the absence of detrimental effects *in vitro* and *in vivo* by oral administration. These results established that spent hen muscle proteins, as proteins from other food sources, have the potential for producing peptides for anti-inflammatory applications.

It should be noted that there are challenges and limitations of this thesis research. In screening the hydrolysates for immunomodulatory activities, cytokine (IL-6 and TNF-α) production in endotoxin-activated macrophages is the only immune index studied. As there is no overall measurement of immune function, it would worth exploring the effect of the hydrolysates on other aspects of immune functions. Food-derived peptides have been reported to show various immunomodulatory activities using primary cultures, such as murine peripheral macrophages, murine splenocytes, rabbit Peyer's patch cells, and human blood neutrophils; many biomarkers can be tested using these cells, including oxidative stress, lymphocyte proliferation, helper T cell-derived cytokine, and antibody production (Chapter 2, Table 2.1). Similarly, the purification strategies was developed using the biomarker of IL-6 inhibitory activity, it would be interesting to verify whether this purification strategy based on hydrophobicity would be as effective on other immune biomarkers. Additionally, the regulatory mechanism has not been explored in this study. Generally, a common pathway was reported for production of pro-inflammatory cytokines in endotoxin-activated macrophages. LPS activates macrophage through toll-like receptor 4 (TLR4) to induce the activation of the transcription factors nuclear factor (NF)-κB and activator protein (AP)-1 (Figure 7.1; Rahman & MacNee, 1998; Takeda & Akira, 2004). This pathway should be tested in future experiments (Requena et al., 2009).

In this study, Protease M hydrolysate exhibited potent inhibitory effects *in vitro* on macrophage-derived cytokines but was not effective when fed to rats. This was likely due to a relatively isolated environment in cultured immortal cell lines (lack of cell to cell interaction and absent of immune factors), and the bioavailability of peptides in target cells. A model of simulated *in vitro* gastrointestinal digestion (e.g. TNO's intestinal model (TIM); Nimalaratne, Savard, Gauthier, Schieber, & Wu, 2015) could be useful to study the changes of peptides during digestion, which can help partly to link the *in vitro* results to those observed in the animal study. Moreover, the *in vivo* immunomodulatory activities should be further examined in other lymphoid organ, such as the gut-associated lymphoid tissue (GALT). GALT is the most important site for interactions with diet components, and has been reported for distinctive characteristics from the rest of the peripheral lymphoid system (Janeway, Travers, Walport, & Shlomchik, 2001); therefore it would worth to study

the effect of peptides on GALT in immune cells from Peyer's patch or intestinal lamina propria (MacDonald, 2003; Mallet et al., 2014; Stillie, Bell, & Field, 2005), as well as on the intestinal barrier function (Ruth & Field, 2013). The study of peptide treatment on gut immunity might help to explain the results obtained from the colitis model.

The peptide characterization work in this thesis follows the conventional strategy. Using the conventional strategy, protein substrates are non-selectively hydrolyzed to produce peptides with targeted bioactivity; derived hydrolysates exhibiting bioactivity are then purified to identify bioactive peptide sequences. This is an expensive and less predictable procedure compared to the newly developed "targeted strategy", which involves the use of bioinformatics technology, such as the quantitative structure–activity relationship modelling (QSAR) and *in silico* digestion (Majumder & Wu, 2010; Wu, Aluko, & Nakai, 2006). However, the conventional strategy is necessary for initialization of targeted peptide production with immunomodulatory activities - the complexity of the immune system and the span of immune indices pose further challenges for building a database of peptides exhibiting immunomodulatory properties. A growing body of reports on novel peptide sequences and function-structure relationship could contribute to the immunomodulatory peptide database and the establishment of targeted strategy.

7.3. References

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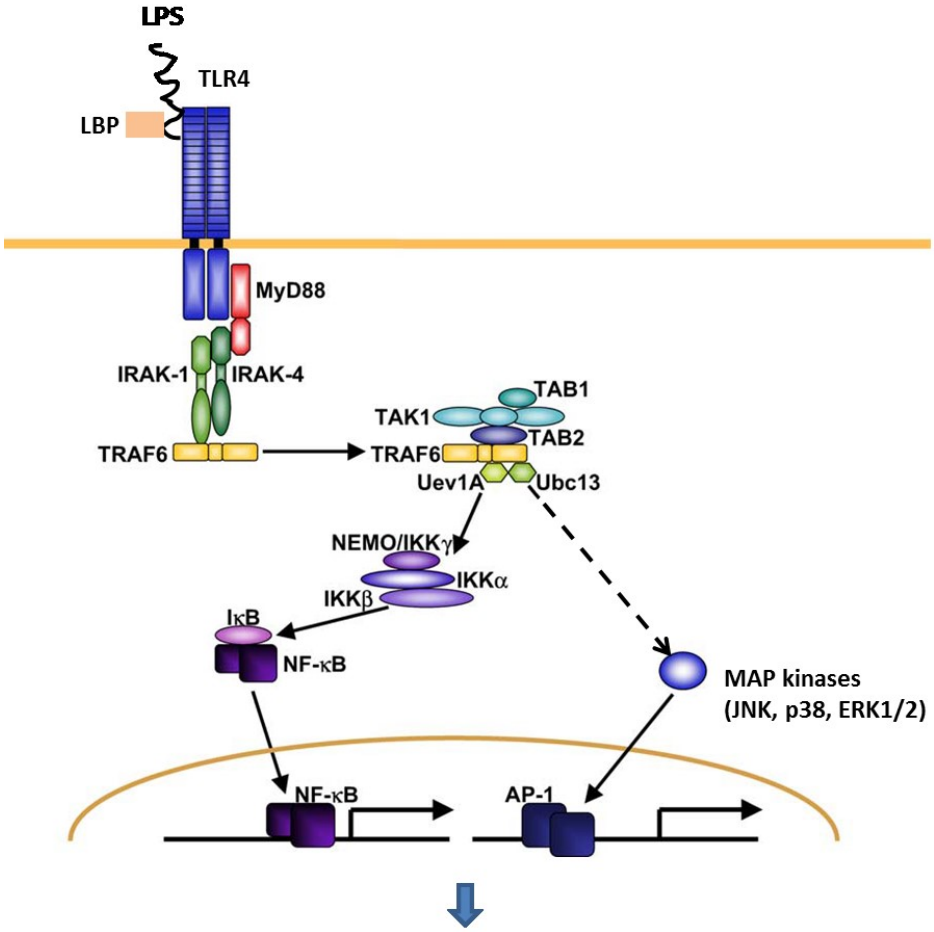
Figure 7.1 TLR-mediated MyD88-dependent signaling pathway in LPS activated macrophages.

LBP binds to LPS to activate macrophage through TLR4 mediated MyD88-dependent signaling pathway. Upon stimulation, association of IRAK-1 and MyD88 leads to phosphorylation of IRAK-1 by recruited IRAK-4. Phosphorylated IRAK-1, together with TRAF6, dissociates from the receptor. Dissociated TRAF6 forms a large complex with TAK1, TAB1, TAB2, Ubc13 and Uev1A, which induces the activation of TAK1. Activated TAK1 phosphorylates the IKK complex and thereby induces the activation of the transcription factors NF-κB. MAP kinases and transcription factors AP-1 were activated as a secondary consequence of the initial wave of NF-κB induction. Activation of NF-κB/AP-1 leads to gene expression of inflammatory proteins including cytokines, chemokines, adhesion molecules and inflammatory mediators.

LBP, lipopolysaccharides binding protein; TLR4, toll-like receptor 4; MyD88, Myeloid differentiation primary response gene; IRAK, interleukin-1 receptor-associated kinase; TRAF, TNF receptor associated factors; TAB, TGF-β activated kinase; NEMO, NF-κB essential modulator; IKK, IκB kinases; MAP kinase, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; AP-1, activator protein-1; iNOS, inducible nitric oxide synthase ; COX-2, cyclooxygenase-2; CRP, C-reactive protein.

Figure modified from (Rahman & MacNee, 1998; Takeda & Akira, 2004; Sharif, et al., 2007).


LPS
TLR4
LBP
MyD88
IRAK-1
IRAK-4
TRAF6
TAB1
TAK1
TAB2
TRAF6
Uev1A
Ubc13
NEMO/IKKγ
IKKα
IKKβ
IκB
NF-κB
MAP kinases
(JNK, p38, ERK1/2)
NF-κB
AP-1
Gene expression of
inflammatory proteins:
cytokines, chemokines,
adhesion molecules,
Inflammatory mediators
(iNOS, COX-2, CRP)

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