ANTI-INFLAMMATORY PROPERTIES OF POTATO PROTEIN HYDROLYSATES IN CELLULAR AND ANIMAL MODELS

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Abstract

Inflammation is a series of non-specific immune responses and irregular inflammation can lead to injury and chronic diseases such as cardiovascular diseases. TNF-α is one of the primary pro-inflammatory cytokines mediating inflammatory responses including release of reactive oxygen species (ROS). In this study, potato protein hydrolysates produced with 8 proteases were evaluated for anti-inflammatory activities using mice ANA-1 and C57BL/6 mice primary cell models and inflammatory BALB/c mice model. The potato protein hydrolysates suppressed tumor necrosis factor (TNF)-α release and ROS secretion in the cells. The potato protein hydrolysate produced with ficin (H_{Fic}) exhibited a dosage-dependent pattern in inhibiting the release of TNF-α in both cell models. Intraperitoneal administration of H_{Fic} to BALB/c mice resulted in the dose-dependent reduction in the amount of cytokines including TNF-α, interleukin (IL)-6 and IL-12 in the mice serum and peritoneal fluid. These finding indicate that the potato protein hydrolysates possess physiological anti-inflammatory properties. The mechanism of anti-inflammatory activity was not clear, but the *in vitro* and physiological antioxidative activities of the hydrolysates suggest possible interaction with oxidative stress. H_{Fic} was found to be potentially stable to gastric proteases based on HPLC analysis, which would encourage future studies on oral delivery of the hydrolysates.

List of Abbreviations Used

ADAM17	A Disintegrin and Metalloproteinase
ANS	8-Anilino-1-naphthalene sulfonic acid
DAMP	Damage-associated molecular patterns
DD	Death domain
DTNB	5, 5′-dithiobis (2-nitrobenzoic acid)
DTT	Dithiothreitol
EDHF	Endothelium-dependent hyperpolarizing factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial NOS
FRAP	Ferric reducing antioxidant power
FBS	Fetal Bovine Serum
IFA	Inflammation
IFN-γ	Interferon-gamma
IKK	IκB kinase
iNOS	Inducible NOS
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cell
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
OPA	Ortho-phthalaldehyde
PAMP	Pathogen-associated molecular patterns
PAR	4-(2-Pyridylazo) resorcinol
RIP	Receptor-interacting protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TACE	Tumor necrosis factor alpha converting enzyme
TAK1	Transforming-growth factor-β-activated kinase 1
TLR	Toll-like receptor
TNFR	TNF-α receptor
TNF-α	Tumor necrosis factor-alpha
TRADD	TNFR1-associated death domain protein
TRAF2	TNFR-associated factor-2
VCAM-1	Vascular cell adhesion molecule-1

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Chapter 1 Introduction

Inflammation (IFA) is a series of non-specific immune responses induced by harmful stimulus, cell or tissue injury and pathogens present in body (Ferrero-Miliani et al., 2007). The inflammatory process involves a diverse range of biological activities that alter molecular and cellular responses to remove irritants and restore homeostasis (Ashley et al., 2012). Inflammation can be divided into two types: acute and chronic inflammation. Once induced by pathogens, acute inflammation can occur within minutes or days whereas chronic or prolonged inflammation lasts for longer duration. IFA can serve as a means of defense for the body but becomes problematic if not properly regulated. Inflammation, especially chronic inflammation, is often associated with ageing-related diseases and health conditions such as diabetes, stroke, oxidative stress, auto-inflammatory diseases (rheumatoid arthritis) and atherosclerosis (Galkina and Ley, 2009; Larsen et al., 2007; Lucas et al., 2006; [b]Kim et al., 2013; Ashley et al, 2012).

Chronic inflammation is mediated by a number of factors including cytokines. Cytokines are a group of small glycoproteins (molecular weight, 8 to 40 kDa) secreted by different cell types such as endothelial cell, fibroblasts and immune cells including macrophages, T- and B-lymphocytes (Niu et al., 2008; McKellar et al., 2009; Kubo & Kuroyanagi, 2005). By binding to specific receptors on cell membrane, cytokines mainly function as intracellular mediators to regulate host immune responses including

inflammation (Dinarello, 2000). According to resulting responses, cytokines can be divided into two groups: pro- and anti-inflammatory. Tumor necrosis factor (TNF)-α and interleukin (IL)-1 are the most widely investigated pro-inflammatory cytokines and blockage of these cytokines can attenuate tissue destruction, fever and stress of inflammation related diseases (Palladino et al., 2003). Moreover, anti-inflammatory cytokines such as IL-4, IL-10, IL-11 and IL-13 can reduce inflammatory responses by inhibiting pro-inflammatory cytokines synthesis and regulating polarized function of T helper cell (Dinarello, 2000; Opal & DePalo, 2000).

Inflammation, especially during chronic inflammatory diseases, is strongly associated with reactive oxygen species (ROS) and reactive nitrogen species (RNS) activities. ROS are highly biologically reactive species including superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\cdot$), which are synthesized as by-products of oxygen metabolism (Devasagayam et al., 2004). Normally, ROS function beneficially in response to pathogens and injured tissues; however, when produced in large amounts, they can cause oxidative damage to tissue proteins, lipids and DNA (Choi et al., 2013). Excessive production of ROS is mediated by NADPH oxidase (NOX) or generated on inner membrane of mitochondria where redundant electrons react with oxygen to form superoxide radical due to irregular metabolism of the electron transport chain (Choi et al., 2013; Lee and Yang, 2012). Besides, NOX is considered as a major source of ROS during inflammation since it is up-regulated by pro-inflammatory cytokines such as TNF-α and IL-1β (Lee and Yang, 2012).

Moreover, nitric oxide (NO) is a ROS/RNS produced during inflammation, but also acts as a biological messenger and in vascular homeostasis. Bioavailability of NO is regulated by nitric oxide synthase (NOS) and reaction with superoxide radical, which converts NO to highly reactive peroxynitrite ($ONOO^-$) and other toxic RNS (Hsieh et al., 2014). Inflammation and oxidative stress are tightly linked and several research efforts are directed at developing drugs and nutraceuticals for managing and treating inflammation and related health conditions.

Bioactive peptides have become the focus of several research investigations due to their demonstrated multifunctional properties that can be applied in human health promotion (Udenigwe & Aluko, 2012). These peptides are generated by food protein hydrolyzed with proteases or during gastric digestion. There is evidence that peptides present within some food protein hydrolysates possess antioxidative properties *in vitro*, in cellular and animal models (Sarmadi and Ismail, 2010) and anti-inflammatory activities in cell cultures (Chakrabarti et al., 2014). The connection between the antioxidative and anti-inflammatory activities of peptides and protein hydrolysates is unclear. Moreover, there is a dearth of information on the structural requirements of peptides for anti-inflammatory function, which makes it challenging to design potent peptides from agri-food resources for the formulation of functional food products. This study is a part of a research program that focuses on the development of anti-inflammatory peptides from potato proteins for functional food and nutraceutical applications.

Chapter 2 Literature Review

2.1. Inflammation

2.1.1 IFA pathogenesis

IFA is partly considered of the innate immune system and its purpose is to isolate or remove identified foreign irritants. It takes several steps to accomplish this goal: (1) recognition of pathogens or damaged tissue; (2) signal transduction; (3) release of pro-inflammatory cytokines; and (4) leukocyte adhesion and inflammatory effects. IFA is initiated by identifying pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are molecules shared by pathogens and in most cases they are carbohydrate, lipoprotein, and lipopolysaccharide (LPS) present on fungal or bacterial cell wall. Broadly speaking, most PAMPs can be found in non-pathogenic microbes; therefore, it is also called microbe-associated molecular patterns (Newton & Dixit, 2012). DAMPs are intracellular compounds released from damaged or death cells, such as nuclear or cytosolic proteins, they contribute to inflammation induction in the absence of pathogens (Rubartelli & Lotze, 2007). Both PAMPs and DAMPs can be detected by pattern-recognition receptors of innate immune cells (usually resident macrophage in injury or infected site), which can exist either in membrane-bound or cytoplasmic forms. Two representative pattern-recognition receptors are toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD-like) receptors, which can recognize extracellular and

intracellular pathogens respectively (Rebeccah et al., 2008; Ashley et al., 2012). TLR belongs to type I transmembrane protein containing a Leu-rich repeat. There are 11 members of TLR family, which are responsible for recognizing bacterial and PAMPs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) and endolysomes (TLR3, TLR7, TLR8, TLR9, and TLR10) outside the cell (Newton & Dixit, 2012). Stimulation of TLRs except for TLR3 recruits MyD88 (myeloid differentiation primary-response protein 88), which in turn activates TAK1 (transforming-growth factor-β-activated kinase). Active TAK1 phosphorylates IKK complex (IκB-kinase complex), which subsequently degrades inhibitor of nuclear factor-κB (IκB) and leads to translocation of nuclear factor (NF)-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) to the nucleus (Ashley et al., 2012). NF-κB binds to target genes and mediates the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8 (Tak & Firestein, 2001).

Another pathway to induce NF-κB activation is regulated by NOD-like receptors, which associates with caspase 1 enzyme to cleave pro-inflammatory cytokines IL-1β and IL-18 (Newton & Dixit, 2012). The released cytokines have multiple effects on local or adjacent cells. For example, increased TNF-α level leads to elevated vascular permeability, which enhances entry of immunoglobulin G and cells (especially leukocytes) into inflamed tissue (vasodilation) and can subsequently leads to fever (Janeway et al., 2001). Moreover, the major function of the cytokines is in leading leukocytes (mostly neutrophils and monocytes) recruitment towards the damaged sites

by chemotaxis. Moreover, mast cells around injury sites produce histamine, which causes vessel dilation and opening of endothelial cell line. Then, the migrated leukocyte "squeezes through" endothelial cells to reach inflamed tissue (extravasation) (Ashley et al., 2012). This process leads to certain inflammatory features such as swelling (increased permeability of vascular vessel and leakage of protein-rich exudate) and redness (hyperemia results from vasodilation to allow entry of more leukocytes). Moreover, the migrated neutrophils release toxic components such as ROS, RNS and proteases to damaged or infected areas, which can nonspecifically eliminate both pathogens and injury host cells. This process is called respiratory burst since it usually occurs fast and requires large amounts of oxygen and glucose (Ashley et al, 2012). If inflammatory reaction failed to remove these irritants or pathogens, prolonged non-specific inflammatory responses will damage tissue and eventually cause inflammation-related complications such as rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, psoriasis, pulmonary inflammation and cardiovascular diseases (Charbonneau et al., 2007; Trifilieff et al., 2002; Kawaguchi et al., 2005; Cesaro et al., 2009; Ridker & Morrow, 2003).

2.1.2 Inflammation in adaptive immunity

As mostly agreed, inflammation is part of the innate immune system; however, recent studies revealed adaptive immunity also participates in inflammatory process. Naive T

cell is differentiated in bone marrow and transfers to several effector and regulatory cells such as T helper cells (Th1 and Th2 cell) and regulatory cell (Tregs cell) (Ashley et al., 2012). Both Th1 and Th2 cells involve in host defense; Th1 cells function against intracellular pathogens while Th2 cells eliminates extracellular pathogens (Kato and Nariuchi, 2000; Damsker et al., 2010). Besides, Th1 cells mainly act by secreting pro-inflammatory interferon-gamma (IFN-γ), IL-2 and TNF-α, while Th2 cells release anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13 (Kato and Nariuchi, 2000; Damsker et al., 2010).

2.2 Mechanism of TNF-α activation and effects

The precursor of TNF-α (proTNF-α) is a 26-kDa homotrimer that exists on cell surfaces. proTNF-α can be cleaved by ADAM 17 to release soluble TNF-α (Silva et al., 2010). TNF-α primarily produced by macrophages and monocytes in innate immunity, chronically by T lymphocytes in adaptive immunity and sometimes by non-immune cells (fibroblasts, glial and granuloma cells) or even tumor cells (Silva et al., 2010). Besides, harmful stimuli such as UV light, X-radiation, heat, chemical or immunological stimulus can induce TNF-α production (Feldmann, 2001). Dysregulation or overproduction of TNF-α can lead to immune-mediated inflammatory diseases such as rheumatoid arthritis, Crohn's disease, psoriatic arthritis, ankylosing and other chronic inflammation (Silva et al., 2010). TNF-α induced pathogenesis is

initiated when it binds its receptors TNFR1 (p55) and TNFR2 (p75) (Feldmann, 2001). These receptors are membrane-attached glycoproteins found in all cell types although TNFR1 are widely studied and mainly responsible for pro-inflammatory effects (Palladino et al., 2003). TNF-α signaling transduction is initiated by the activation of death domain (DD), which is a cytoplasmic receptor domain present in only TNFR1 (Ashkenazi & Dixit, 1998). Receptor binding causes conformational change in TNFR1 and activates DD by removing death domain silencer (Eichholtz-Wirth et al., 2003). Activated DD recruits TNFR1-associated death domain protein (TRADD), a pivotal branching point in TNF-α signaling pathways. Thereafter, several pathways are induced to cause inflammatory responses: (1) For NF-κB pathway, TRADD attracts TNFR-associated factor-2 (TRAF2) and receptor-interacting protein (RIP) to form a complex, which in turn attaches to inhibitor of IκB kinase (IKK). Normally NF-κB is suppressed by inhibitor IκB; however, IκB is phosphorylated and eventually degraded leading to activation of NF-κB in the presence of IKK (Itani et al., 2002). (2) TRADD/TRAF2/RIP complex also activates mitogen-activated protein kinase (MAPK) pathway, which eventually induces c-Jun N-terminal kinase, p38 MAP kinase, and extracellular signal-regulated kinases activation (Kant et al., 2011). (3) Last and minor pathway is cellular apoptosis, induced by aggregation of Fas-associated death domain and caspase-3 to TRADD (Gaur, 2003; Wu & Zhou, 2010).

2.3 Roles of TNF-α on IFA and related diseases

TNF-α is a 158-amino acid residue cytokine belonging to type II transmembrane protein. It was first identified as endotoxin-induced glycoprotein that caused necrosis of a methylcholanthrene-treated mice sarcoma (Carswell, 1975). Later, TNF-α was linked to inflammatory disease as a stimulator of collagenase and prostaglandin E2, inhibiting bone formation and inducing bone resorption, and considered as major pro-inflammatory cytokine (Gilbert et al., 2000; Zhao et al., 2012). The release of TNF-α from its zymogen leads to a series of inflammatory responses including vascular endothelial cell interaction, leukocyte adhesion, cellular apoptosis and ROS production (Paola et al., 2013).

2.3.1 TNF-α contributes to oxidative stress via NOX activation

Oxidative stress is highly connected to inflammatory response (Packer and Sies, 2008; Skalicky et al., 2008). Cytokine-induced ROS are synthesized to mediate cellular signaling that destroy pathogens and damaged tissues (Zhang et al., 2009; Lee and Yang, 2012). Several pathways function in physiological ROS production: mitochondrial inner membrane, NOX and lipoxygenase. NOX activity is known to be the major source of ROS (mainly superoxide radical) in response to pro-inflammatory cytokines (Zhang et al., 2009; Lee and Yang, 2012). NOX is a membrane-bound enzyme present in both phagocytic and non-phagocytic cells. In resting state, part of

NOX remains soluble in the cytoplasm and assembles with the membrane-bound region once activated, thereafter it utilizes electron from NADPH to reduce molecular oxygen and produce superoxide anion radical (Cross & Segal, 2004 Lee and Yang, 2012). During inflammatory diseases, NOX expression is mediated by TNF-α through NF-κB activation (Zhang et al., 2009). TNF-α-mediated oxidative stress also contributes to cardiovascular diseases by enhancing oxidized low-density lipoprotein (LDL) level, thrombosis formation, myocardial cell damage and apoptosis (Tsutamoto et al., 2001; Pashkow, 2011). According to, TNF-α promotes the expression of NOX and its subunits such as p22phox, p47phox and p67phox in human coronary artery endothelial cells (Yoshida & Tsunawaki, 2008).

2.3.2 TNF-α and NO production

Nitric oxide (NO) is one of most important signaling molecules during immune and inflammatory responses. NO possesses anti-inflammatory effect in normal conditions, and is also considered pro-inflammatory when over-produced (Sharma et al., 2007). Synthesis of NO is catalyzed by NO synthase (NOS), which converts arginine and molecular oxygen to citrulline and NO (Coleman, 2001). NOS family can be divided into several groups: neuronal NOS, inducible NOS (iNOS), endothelial NOS (eNOS) and bacterial NOS (Guzik et al., 2003). As opposed to other NOSs, iNOS activation is independent of intracellular Ca^{2+} levels. iNOS only can be activated in response to

inflammation activators and usually leads to the production of large amounts of NO within a short period. Immune cells, mostly macrophages, are major sources of iNOS after induction by cytokines or bacterial cell wall LPS (Guzik et al., 2003; Bauer & Sotníková, 2010). iNOS expression decreases as a result of inhibition of downstream effectors of TNF-α (tyrosine kinases including NF-κB, MAPK and JAK) and vice versa (Poljakovic et al., 2003; Pautz et al., 2010; Iovine et al., 2008). The half-life of iNOS-derived NO is very short since iNOS and NOX are both induced by TNF-α through NF-κB pathway, and NOX-derived superoxide radical can react with NO to form peroxynitrite ($ONOO^-$). This reduces the bioavailability of NO while unstable ONOO- will further form other RNS (Zhang et al., 2009).

2.3.3 Effects of TNF-α on vascular endothelium

TNF-α affects vascular endothelial cells and leads to some principal features at inflamed sites: (1) Redness and warmth, which is due to increased blood flow after vessel expansion. The amounts of NO and prostacyclin are elevated after TNF-α release, and these molecules function as potent dilators promoting vasodilation for increased blood flow. (2) Leakage of plasma protein-rich fluid accounting for swelling; increased permeability results from the open junction between endothelial cells, and this is modulated by TNF-α-induced activation of myosin light chain kinase (Ye, 2006). (3) Besides, white blood cells are attracted to infected area and both leukocyte and

endothelium are activated to produce a group of mediators called adhesion molecules, which regulate interaction between endothelial cells and leukocytes (Bradley, 2008). Moreover, vascular cell adhesion molecule (VCAM)-1 and P-selectin are expressed on endothelial cell surfaces and bind with specific ligands on leukocyte membrane (Libby, 2006; Bradley, 2008).

Endothelium dysfunction refers to abnormal modulation on vasodilation and vasoconstriction, and is considered as major sign of cardiovascular disease pathogenesis, especially atherosclerosis (Galle et al., 2003). Many studies indicate that TNF-α contributes to endothelium dysfunction by regulating eNOS and endothelium-dependent hyperpolarizing factor (EDHF). TNF-α-treated endothelial cells showed increased iNOS mRNA expression but diminished eNOS (Xia et al., 2006; Goodwin et al., 2007; Galle et al., 2003). This can be explained by different activation mechanism between eNOS and iNOS. eNOS expression is triggered by increased intracellular Ca^{2+} levels, while iNOS can be directly induced by pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-1, or irritant-like LPS through NF-κB pathway (Bauer and Sotníková., 2010; Xie et al., 1994). Impaired eNOS expression leads to decrease in NO synthesis, which diminishes vasodilation and eventually promotes vessel blockage during atherosclerosis. Besides, studies on EDHF suggested that TNF-α can reduce EDHF-mediated vasodilation in both human and mice cell models although this topic still remains controversial (Park et al., 2008; Kessler et al., 1999).

2.3.4 IFA and TNF-α in atherosclerosis

Many studies have provided strong evidence to confirm the link between inflammation and cardiovascular diseases pathogenesis (Libby, 2006; Ridker & Morrow, 2003). In the case of atherosclerosis, IFA contributes to atherosclerotic lesion formation and plaque rupture (Libby, 2006). In an inflamed artery vessel, atherosclerosis is initiated by increasing leukocyte adhesion to endothelial cells, and VCAM-1 is secreted to facilitate migration (Bradley, 2008; Libby, 2006). VCAM-1 production can also be triggered by consumption of high-fat diet, which elevates the level of LDL, a lipoprotein complex that is commonly known as "bad cholesterol". LDL enables fat transportation to endothelial cells to meet cholesterol requirements. When LDL level exceeds the capacity of endothelial cells, it penetrates and gets trapped in subendothelial layer, where it becomes oxidized and eventually causes IFA (Hansson et al., 2006; Kleinbongard et al., 2010; Galkina & Ley, 2009). Once migrated to inflamed sites, monocytes differentiate into macrophages, which engulf the oxidized LDL; more cytokines (TNF-α, IFNγ) are subsequently produced, amplifying inflammatory responses at the sites.

Macrophage cell death results in the release of lipids that form a lipid core in intima (Hansson et al., 2006). The released lipid core attracts more macrophages until plaque is formed. Moreover, a fibrous cap layer consisting of collagen and elastin covers the plaque and prevent its contact with blood. There is no reduction in blood flow at the initial stage because enlarged elastic membrane enables the vessel to maintain normal

blood flow. However, with increased accumulation of oxidized LDL in intima, the plaques grow continuously and then ruptures, resulting in contact of blood and lipid, clothing and thrombus formation (Libby, 2006). Additionally, during inflammation, T-lymphocytes secrete cytokines (TNF-α, IL-1) that inhibit collagen secretion by stimulating macrophages to produce collagen-degrading enzyme. This thins the fibrous cap, which was originally used to protect the plaque from rupturing (Libby, 2006). Moreover, TNF-α-induced ROS production and diminished NO bioavailability may contribute to atherosclerosis pathogenesis. TNF-α also augments the risk of inflammatory-induced cardiovascular diseases and diabetes by causing endothelial and hepatic cells apoptosis (cell-programmed death) and vascular smooth muscle cell (VSMC) migration; TNF-α-facilitated apoptosis is also activated through the NF-κB pathway (Rastogi et al., 2012).

2.4. ADAM17 mediated release of TNF-α

ADAM17 (*a d*isintegrin *a*nd *m*etalloproteinase 17), also known as TNF-α-converting enzyme (TACE), is a zinc-dependent 824-amino acid residue enzyme that releases TNF-α from pro-TNF-α (Gooz, 2010). ADAM17 belongs to a subgroup of metzincin; the name "ADAM" was first mentioned by Wolfsberg et al. (1995) since the enzymes are assembled with disintegrin and metalloprotease domains. The structures of ADAM (including ADAM17) include a prodomain, Cys-rich domain, EGF-like domain,

transmembrane domain and cytoplasmic tail. ADAM functions in cell adhesion and proteolytic cleavage of cell surface receptors and signaling molecules (ectodomain sheddase) (Edwards et al., 2008). ADAM has been identified in several species from nematodes to humans, and are related to many human diseases due to their roles in ectodomain shedding and release of various growth factors, chemokines and mediators (Huxley-Jones et al., 2007). Particularly, overexpression of ADAM17 was reported to be associated with inflammatory disease such as rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, psoriasis and pulmonary inflammation (Charbonneau et al., 2007; Trifilieff et al., 2002; Kawaguchi et al., 2005; Cesaro et al., 2009).

Since ADAM17 is a metalloprotease, its enzymatic activity requires zinc, which is second most abundant trace element in the human body (Vallee, 1986). Normally, 10-15 mg zinc is needed per day for regular metabolism of a 70-kg male adult (McCance & Widdowson, 1942). About 300 metalloenzymes require zinc in their catalytic, co-catalytic and structure supporting sites (McCall et al., 2000). Zinc displays suitable properties for proteome-binding including: (1) flexible coordination geometry; most zinc metalloenzymes have slightly modified tetrahedral geometry, namely zinc center coordinates, with 3-4 protein side chains. Zinc also exists in trigonal bipyramidal or even octahedral complexes (McCall et al., 2000; Zhang et al., 2012). (2) Fast ligand exchange; the relatively rapid dissociation rate of products makes zinc an efficient enzyme co-factor (McCall et al., 2000). In addition, multiple proteomic ligands for zinc such as His, Asp, Glu and Cys, provide a diverse zinc-binding site in enzymes (Zhang

et al., 2012). (3) Low susceptibility to redox reactions; most transition metals such as iron and manganese have uncompleted *d* orbital and this leads to their multiple valence states (e.g. Fe^{2+} and Fe^{3+}). Compared to these transition metals, zinc has a filled *d* orbital (d^{10}) and only occur as a divalent cation (Zn^{2+}), which makes it redox-stable during metabolism (Tapiero et al., 2003). For ADAM17, zinc is present in its catalytic site and shares the same zinc-binding catalytic domain sequence with most metalloproteases (Seals, 2003). Zinc is ligated with 3 His residues and one liable water molecule, which is used during enzymatic hydrolysis (Seals, 2003).

2.5. Anti-TNF-α therapies

Due to the pro-inflammatory role of TNF-α signaling pathways, a number of therapeutic targets have been pursued towards the treatment and management of inflammatory diseases. These targets include TNF-α mRNA/protein, ADAM 17, p38 kinase and NF-κB. TNF-α inhibitors have been widely studied and a number of TNF-α blocking drugs have been approved by the U.S. Food and Drug Administration including Etanercept, Infliximab and Adalimumab (Palladino et al., 2003). These drugs normally contain human or animal-derived antibodies that can bind specifically to TNF-α and are usually taken by intravenous injection (Palladino et al., 2003). Moreover, ADAM 17 inhibitors, such as Marimastat, appear to be less efficient compared to other anti-TNF-α drugs. Animal experiment showed that Marimastat

impaired ADAM17-produced TNF-α; however, LPS-induced illness was slightly modulated (Rasmussen et al., 1997). Furthermore, for signaling pathway-blocking drugs, a p38 MAP kinase-inhibitor was found to reduce p38 kinase phosphorylation both *in vitro* and *in vivo* (Vanderkerken et al., 2007; Giafis et al., 2006). Lastly, NF-κB inhibitors usually are small molecules that can directly bind NF-κB subunit or function as protector of IκB, a natural inhibitor of NF-κB (Sheehan et al., 2002; Oka et al., 2007). Isohelenin is a cell permeable sesquiterpene lactone originally isolated from medical herb *Arnica montana* and reported to show anti-inflammatory property by reacting with p65 subunit of NF-κB (Sheehan et al., 2002; Palladino et al., 2003). Another natural NF-κB inhibiting compound, parthenolide, from feverfew (*Tanacetum parthenium*) was reported to inhibit IκB phosphorylation by IKK (Oka et al., 2007; Palladino et al., 2003). Moreover, a decapeptide (RDP58) has been developed to reduce generation of pro-inflammatory cytokines by directly interfering their mRNA translation (Travis et al., 2005; Palladino et al., 2003). These pathways have become important targets in developing anti-TNF-α compounds including nutraceuticals for managing inflammatory conditions and associated pathogenesis.

2.6. Anti-inflammatory peptides from food sources

Bioactive peptides discussed in this thesis are specific protein fragments generally consisted of 2 to 20 amino acids and have positive effects on metabolic processes

(Korhonen & Pihlanto, 2003; Udenigwe & Aluko, 2012). Studies on bioactive peptides have developed rapidly since the 1980s. Recent advances in bioinformatics and genomics have been used to study the structure and elucidate the metabolic functions of the peptides especially in disease states (Sharma et al., 2011; Udenigwe, 2014). Bioactivities of the food protein-derived peptides include opioid-like (opiate), mineral binding, immunomodulatory, antimicrobial, antioxidative, antithrombotic, anticancer, hypolipidaemic and antihypertensive activities (Sharma et al., 2011; Udenigwe & Aluko, 2012; Howard & Udenigwe, 2013). Bioactive peptides are inactive within the parent protein structure and must be released by proteolytic activity of enzymes in order to exert their bioactivity. Moreover, bioactive peptides can be produced during gastric digestion of proteins, microbial fermentation, food processing and Pressurization (Udenigwe & Aluko, 2012). With the development of combinatorial chemistry and metabolic biology, the scope of research and development of therapeutic peptides have become broader. There are various sources for bioactive peptides but recent studies have mostly focused on enzymatic hydrolysates of proteins from foods such as milk (caseins, whey proteins), plant-derived proteins (e.g. soy proteins), animal meat, fish and other marine proteins (Table 2.1).

A number of studies have reported the use of bioactive peptides present in food protein hydrolysates against inflammatory reactions, with the effects mostly occurring at the post-transcriptional stage (Table 2.1), similar to the properties of well-known anti-inflammatory drugs. There are a number of advantages and limitation of the use of

bioactive peptides in disease management. Compared to synthetic drugs, the peptides contain natural proteinogenic amino acid residues and thus have lower risk of showing negative side effects. However, several studies conducted with bioactive peptides assume the safety and did not evaluate possible toxicity. This needs to be revisited especially as substantial processing occurs in producing the peptides that may lead to derivatization and new compound formation (Udenigwe, 2014). Moreover, since proteins are extensively hydrolyzed to produce peptides, there is low risk of allergenicity as the epitopes will most likely be degraded. Furthermore, the bioactive peptides are cheap to produce from abundant protein-rich agri-food raw materials. Besides, finding proper peptide sources is also important in order to avoid competing with primary food proteins (Chakrabarti et al., 2014; Udenigwe, 2014). A recent review has provided a comprehensive discussion of other major challenges and prospects of using bioactive peptides in functional food formulation for health promotion (Udenigwe, 2014). In summary, there is a rapid growth of food peptide studies with emerging work recently focusing on elucidation of their pharmacological properties using cellular and animal disease models as well as in human subjects. The mechanism of the anti-inflammatory activity of peptides is not well understood. This information, if available, will help in developing more active peptides for use in maintaining optimum health conditions.

Table 2.1 Processing methods for producing food protein hydrolysates and their roles in modulating inflammatory reactions

Protein Source	Digestion method	Functions	Model	Ref.
Milk				
Milk casein	Combination of pepsin and corolase®	Down-regulated the transcription levels of TGF-β1, COX-2, and NF-κB	IEC-6 rat intestinal epithelial cells	Nielsen et al., 2012
Whey protein	Pressurization	Reduced LPS induced IL-8 secretion	CFTE29o- cystic fibrosis respiratory epithelium	Iskandar et al., 2013
Whey protein	High hyperbaric pressure treatment	Reduced IL-8 secretion and ROS generation	Caco-2 human colonic adenocarcinoma cell	Piccolomini et al., 2012
Lactoferrin	N/A	Diminished LPS-induced cytokine production in monocyte by interfering NF-κB pathway	THP-1 Human monocyte cells	Håversen et al., 2002
Lactoferrin	Acid hydrolysis	Attenuated IL-1β and IL-6 (destructive cytokines) and iNOS and TLR2, increase IL-4 and IL-10 (protective cytokines)	Human articular cartilage and synovium model	Yan et al., 2013
Lactoferrin	Acid-pepsin hydrolysis	Suppressed IL-1, iNOS, IL-6, and toll-like receptor-2 (TLR-2) and TLR-4.	Bovine coccygeal discs derived cells	Kim et al., 2013
Egg				
Ovotransferrin	Synthesized	Impaired TNF-α induced ICAM-1 and VCAM-1; Antioxidant	Human umbilical vein endothelial cells	Majumder et al., 2013
Soybean				
Soy protein	Alcalase hydrolysis	Inhibition on nitric oxide, iNOS, PGE, COX-2 and TNF-α	Murine macrophage cell line RAW 264.7	Vernaza et al., 2012
Soy protein	Fermentation	Depress inflammation through TGFβ pathway	MCF-7 Human breast cancer cell	Hwang et al., 2011
Lunasin	Solvent extraction	Inhibition on IL-6, IL-1β, NF-κB, NO and iNOS	Murine macrophage cell line RAW 264.7	De et al., 2009

TGF, tumor growth factor; COX, cyclooxygenase; NF, nuclear factor; IL, interleukin; iNOS, inducible nitric oxide synthase; TLR, toll-like receptor; TNF, tumor-necrosis factor; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; PG, prostaglandin; NO, nitric oxide

2.7 Potato proteins

Potato (*Solanum tuberosum*, Solanaceae) is one of the most widespread and popular crops in the world. The protein content of fresh potato tuber varies from 2-2.5%; the major proteins in the potato tuber include 40-kDa glycoproteins, patatin, 22-kDa complex protein group and proteinase inhibitor (Giuseppin et al., 2010). Patatin is the most abundant protein in potato tuber ranging from 20 to 40% of total protein content and present in almost all types of cultivated potato (Barta and Bartova, 2008). Patatin is a family of glycoproteins with molecular weight of 43 kDa and isoelectric point (pI) of 4.8-5.2 (Pots, 1999). Previous research has demonstrated that the patatin proteins possess antioxidative activity and that the activity was higher than those of the protein in onions, carrots and peppers (Al-Saikhan et al., 1995). Similarly Liu et al. (2003) reported that purified patatin exhibited *in vitro* antioxidant potential by scavenging dose-dependently free radicals (EC_{50} 0.582 mg/mL). Moreover, patatin is known to play a role in tuber self-defense mechanism by functioning as acyl hydrolase and transferase, which is expressed when invaded by pathogens or when the tuber gets damaged (Andrews et al., 1988). These findings indicate that potato proteins can be bioactive, and these activities can be further explored in human health promotion. Since the intact potato protein structure will be lost under physiological conditions (especially when consumed as a part of the diet), it is possible that the antioxidant activity shown by the proteins would be retained in the hydrolyzed peptide fragments.

If bioavailable, these peptides can exhibit physiological antioxidant effects and also modulate other abnormal process related to oxidative stress such as inflammation.

2.8. Objectives and hypothesis

Since potato proteins demonstrated *in vitro* antioxidative activities, it is hypothesized that enzymatic potato protein hydrolysates will exhibit antioxidative and anti-inflammatory activities in cell culture and animal model of inflammation. It is also hypothesized that the zinc-chelating activity of potato protein hydrolysates will lead to zinc chelation, cellular ADAM17 inhibition and reduced TNF-α activation.

The objectives of this project were to (1) hydrolyze potato proteins using food-grade and gastric proteases, (2) evaluate the antioxidant and zinc-chelating activities of the protein hydrolysates, (3) evaluate the anti-inflammatory and antioxidative activities of the hydrolysates by measuring ADAM17 activity, TNF-α and other inflammatory and oxidative biomarkers, and (4) evaluate the anti-inflammatory and antioxidative activities of the hydrolysates in a mice inflammation model.

Chapter 3 Methodology

3.1. Materials and reagents

Russet potatoes were purchased from a local store. Pepsin from porcine gastric mucosa (P7125), papain from papaya latex (P3375), thermolysin from *Bacillus thermoproteolyticus rokko* (88303), bromelain from pineapple stem (B4882), pancreatin from porcine pancreas (P7545), Alcalase® from Bacillus licheniformis (P4860), ficin from fig tree latex (F4165), 8-anilino-1-naphthalene sulfonic acid (A1028), 5,5′-dithiobis (2-nitrobenzoic acid) (D8130), glycine (G8898), trifluoracetic acid (T62200), phosphoric acid (P5811), RPMI-1640 medium (R8758), methanol (34860), glutathione (G4251) were purchased from Sigma-Aldrich. Ammonium thiocyanate (A709-500), ferrous chloride (I90-500), Tris-base (BP152-1), acetonitrile (A996-4), monobasic sodium phosphate (BP329-1), dibasic sodium phosphate (S374-500) and ethanol (A995-4) were purchased from Fisher Scientific.

3.2. Preparation of the potato protein hydrolysates

3.2.1 Potato protein isolation

Potato extract was prepared according to the procedure reported by Strætkvern and Schwarz (2012). Peeled and sliced tubers were weighed and homogenized using a food blender with 5 mM sodium bisulfite at 1:4 ratio (w/v). The homogenate was filtered

through cheesecloth and centrifuged at 3,000 × g for 10 min. The resulting supernatant was used for protein isolation. Potato protein isolation was conducted based on the isoelectric point of patatin (pI 4.8-5.5). The potato extract was adjusted to pH 5.0 using 1 M HCl and left for 15 min at room temperature followed by centrifugation at 3000 × g for 20 min. The resulting precipitate was collected, freeze-dried and stored at -20°C until further processing (Giuseppin et al., 2010).

3.2.2 Hydrolysis of isolated proteins

Lyophilized potato proteins were dissolved in deionized water at 5% (w/v) and hydrolyzed with pepsin, papain, thermolysin, bromelain, Alcalase®, pancreatin and ficin at E/S ratio of 1:100. The reaction was conducted in a shaking water bath for 5 h at optimum temperature and pH values (37°C, pH 2.0 for pepsin; 65°C, pH 7.0 for papain; 65°C, pH 8.0 for thermolysin; 37°C, pH 6.5 for bromelain; 52°C, pH 8.0 for Alcalase®; 40°C, pH 7.5 for pancreatin; 37°C, pH 7.0 for ficin). Simulated gastric digestion of the proteins was also conducted by first hydrolyzing with pepsin for 1 h and then with pancreatin for 3 h under the optimum conditions of the proteases. The protease specificities are presented in Table 3.1. Thereafter, hydrolysis was terminated by heating the mixture at 90°C for 15 min. The reaction mixtures were cooled to room temperature, followed by centrifugation at 15,000 × g for 20 min to remove all insoluble matter, and freeze drying to obtain the hydrolysates powders. Yield of protein

hydrolysate was calculated as mass of potato protein hydrolysate/isolated protein × 100%. Lyophilized hydrolysates were labeled as H_{Pep} (pepsin), H_{Pap} (papain), H_{Ther} (thermolysin), H_{Bro} (bromelain), H_{Alc} (Alcalase®), H_{Panc} (pancreatin), H_{Fic} (ficin) and $H_{Pep+Panc}$ (pepsin+pancreatin). The sample preparation steps are summarized in Figure 3.1.

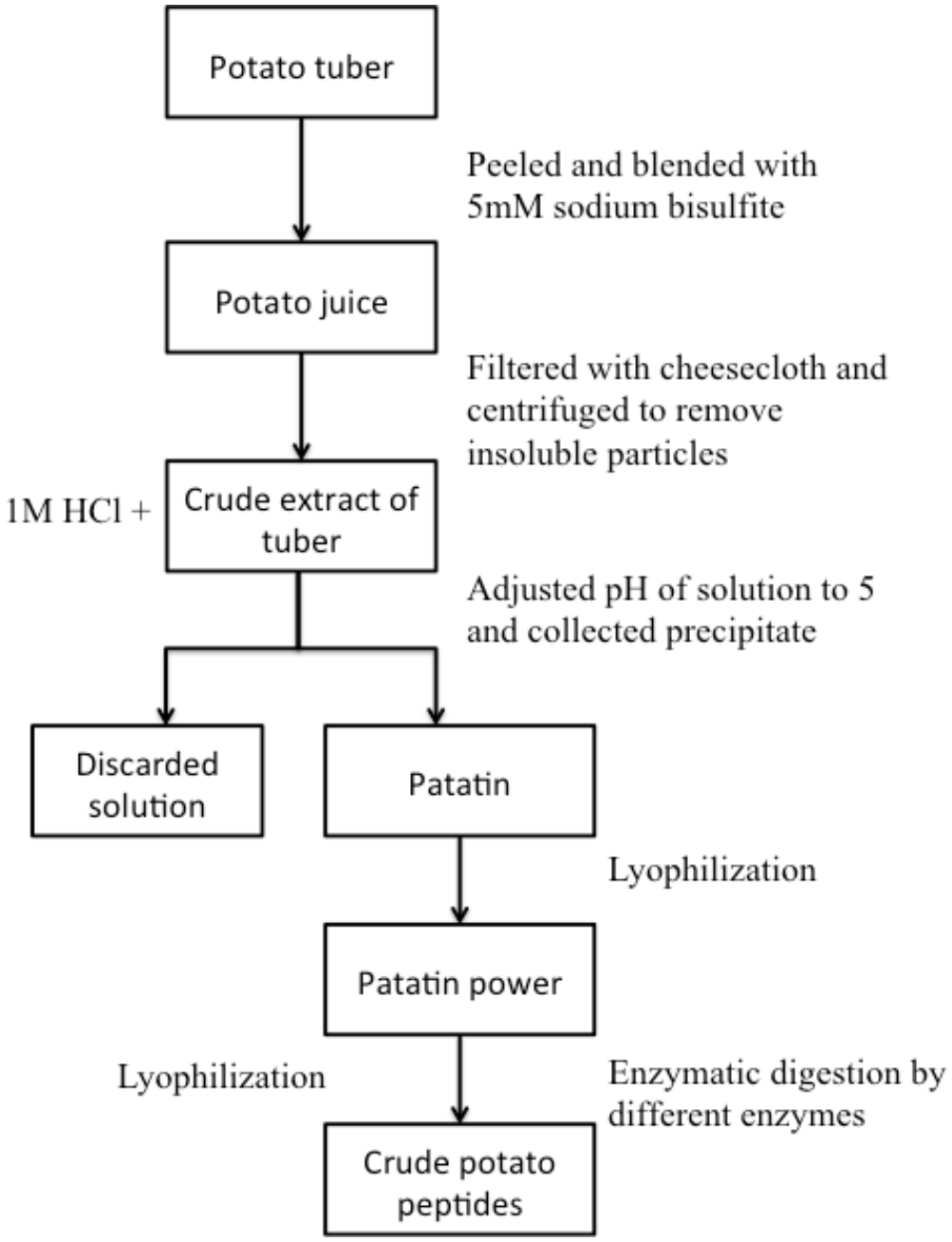

Figure 3.1. Flowchart for potato protein isolation and enzymatic hydrolysis

Table 3.1 Preferential cleavage sites of the proteases used in potato protein hydrolysis

			Cleavage preference			
Enzyme	**Type**	**Source**	**P3**	**P2**	**P1**	**P1'**
Pepsin	Endopeptidase	Stomach	Xaa	Xaa	Hydrophobic	Hydrophobic
Papain	Endo- and exopeptidases	Papaya	Xaa	Hydrophobic	Arg, Lys	Val
Thermolysin	Endopeptidase	*Bacillus thermoproteolyticus*	Xaa	Xaa	Xaa	Leu, Phe
Bromelain	Endopeptidase	Pineapple	Xaa	Xaa	Lys, Ala, Tyr	Xaa
Alcalase	Endoprotease	*Bacillus subtilis*	Broadly-specific enzyme			
Pancreatin	Endo- and exopeptidases	Pancreas	Broadly-specific enzyme			
Ficin	Endopeptidase	Fig	Xaa	Hydrophobic	Gly, Ser, Glu, Tyr	Xaa

Hydrophobic: hydrophobic amino acid including Ala, Val, Leu, Ile, Phe, Trp and Tyr
Xaa indicates any amino acid

3.3. Zinc chelation assay

High zinc-affinity hydrolysates were determined based on a previously reported method (Jakob et al., 2000). Dye 4-(2-pyridylazo) resorcinol (PAR) can be used to measure free zinc since it can form coordinates with metal ions through its pyridine nitrogen resulting in the formation of orange color (Karipcin & Kabalcilar, 2007). Zinc-reconstituted hydrolysates were prepared by dissolving the potato protein hydrolysates samples in 1.5 mL of 40 mM HEPES-KOH buffer (pH 7.5) with 2 mM dithiothreitol (DTT) and 50 μM $ZnSO_4$. Final concentration of hydrolysate was 2.5 mg/mL. PAR (15 μL of 10 mM, in HEPES-KOH buffer) was added to the zinc-reconstituted peptide solution to bind with free zinc. Thereafter, absorbance (A) was measured at 500 nm and percentage of zinc chelation was calculated as $[(A_{blank}-A_{sample})/A_{blank}] \times 100$.

3.4. Ferric reducing antioxidant power (FRAP) assay

FRAP is a colorimetric method to assess reducing power of antioxidants based on the reduction of ferric to ferrous ions and the formation of a blue complex, ferrous-tripyridyltriazine (Singh & Singh, 2008). *In vitro* FRAP assay was conducted as reported by Pownall et al. (2010). The potato protein hydrolysate samples were prepared in 0.2 M phosphate buffer (pH 6.6) at a concentration of 4 mg/mL; 250 μL of the sample was mixed with equal volumes of 1% potassium ferricyanide solution and

buffer. The mixture was heated at 50°C for 20 min. Thereafter, 250 μL of 10% trichloroacetic acid was added followed by vortex mixing. A 250 μL aliquot of the above mixture was then mixed with 0.1% ferric chloride and 200 μL distilled water followed by 10 min incubation at room temperature. Absorbance was then measured at 700 nm. A standard curve was plotted by using ferrous chloride and % ferric ion reduced was calculated as: (equivalent reduced ferrous ion/total ferric ion) × 100.

3.5. Cell culture assays

3.5.1 Mouse macrophage cell line

Mouse macrophage (ANA-1) cell was provided by Dr. Jude Uzonna's Host-Pathogen Interaction lab, Department of Immunology, University of Manitoba. Cells were passaged and maintained in completed RPMI medium containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin and 0.1% 2-mercaptoethanol. The cells were grown to confluence at 37°C and 5% CO_2 incubator. Harvested ANA-1 cells were suspended in complete RPMI medium and plated in 24-well plate at 1×10^6 cells/well (250 μL), incubated with equal volume of the potato protein hydrolysates at 37°C and 5% CO_2 for 24 h, followed by the addition of 500 μL of 2 μg/mL LPS and incubate at the same condition for another 12 h. Thereafter, the cell supernatants were collected and store at -20°C. LPS and samples

were dissolved in complete culture medium and final sample concentrations of 1, 5, 50 and 125 μg/mL were used in the experiments.

3.5.2 Human monocyte-derived macrophage

Human monocyte (THP-1) cell was provided by Dr. Jude Uzonna's Host-Pathogen Interaction lab, Department of Immunology, University of Manitoba. Cells were cultivated in completed RPMI medium to confluence. The cells were dispensed at 3×10^6 cells in 5 mL petri dish and differentiated into macrophage by transferring the cells into 4 mL differentiation medium containing 5% FBS, 0.5% sodium pyruvate and 3μL of 200 μg/mL phorbol myristate acetate. The cells were incubated at 37°C and 5% CO_2, and replaced with completed medium that contains only 5% FBS and 0.5% sodium pyruvate on day 2. On day 3, the THP-1 cells were harvested and seeded in 96-well microplate at 1×10^5 cells/well with 100 μL serum-free RPMI medium (no FBS). Serum starvation was allowed for 4 h at 37°C followed by addition of 100 uL of the hydrolysates samples at final concentrations of 1, 5, 50 and 125 μg/mL, and incubation for 12 h. This was followed by the addition of 50 μL LPS (1 μg/mL, in complete medium). The cells were then washed twice with ice-cold 1× PBS followed by the addition of 10 μL lysate buffer (1% NP-40 and 150 mM NaCl in 50 mM Tris buffer, pH 8.0) containing 1% sodium orthovanadate and 1%

phenylmethylsulfonyl fluoride. The cells were lysed on ice for 10 min, then scraped from the plates and transferred into microfuge tubes for further analysis.

3.5.3 Femoral bone marrow-derived macrophage

Bone marrow derived macrophage cells of 8 week-old C57BL/6 mice were supplied by Dr. Jude Uzonna's Host-Pathogen Interaction lab, Department of Immunology, University of Manitoba. Femurs and tibias were collected from terminated mice and stored in ice-cold incomplete RPMI medium (without 10% FBS). The bones were placed on sterilized 35 mL petri dish containing 5 mL cold 1× PBS; the skin, muscle tissues and epiphyses of the bone were removed. The bone marrow was repeatedly washed using a 1 mL-syringe filled with incomplete RPMI medium with 27-gauge needle until bone turned light pink or white color. The collected bone aspirate was centrifuged at 1200 rpm for 5 min and the supernatant and red blood cells was decanted, while the primary cells were suspended in incomplete RPMI medium for cell counting. The cells were then centrifuged and resuspended at 5×10^5 cells in 10 mL conditioned medium complete RPMI medium containing 30% L929-sup (supernatant of L929 cell culture medium containing macrophage stimulating factor) and incubated at 37°C and 5% CO_2. On day 3, additional 7 mL complete medium was added and on day 6, the cells were harvested. Non-adherent cells were washed out with 1× PBS and treated with

the potato protein hydrolysate in 24-well plate as previously described for ANA-1 cells (see section 3.5.1).

3.5.4 Cells counting

Cell number was determined using a hemocytometer. A 10 μL cell suspension was mixed with equal volume of trypan blue, which can stain dead cells. A cover slip was placed on a slide and 10μl of the mixture was added to the edge of the cover slip. When counting chamber was fully covered with cell suspension, unstained cell number was recorded by viewing under a microscope. Cell concentration was calculated as: (number of cells counted in chamber/chamber number) × 10^4 × dilution factor.

3.6. Sandwich enzyme-linked immunosorbent assay (ELISA)

Immunological techniques such as western blot, ELISA and fluorescent marker are popular methods used to identify or quantify protein levels in cell cultures because the antibodies can specifically bind to the target proteins (Berg et al., 2002). ELISA is most convenient and rapid method; it allows antigen identification and quantification at the same time and has detection limit around 10^{-9} g of protein (Berg et al., 2002). In sandwich ELISA, as the name suggests, the target protein (or antigen) is wrapped between two antibodies (capture and detection antibodies). Since capture antibodies are

coated to microplate, unbound antigens can be washed off and only target antigen would stay. Simultaneously, capture antibodies are conjugated with an enzyme that can cleave a chromogenic or fluorogenic substrate. When unbound enzyme is removed, the amount of hydrolyzed substrate is proportional to the antigen level.

Three pro-inflammatory cytokines, TNF-α, IL-6 and IL-12, were detected in the cell cultures using this method. Coating buffer (pH 9.5) was prepared by dissolving 3.56 g Na_2CO_3 and 8.4 g $NaHCO_3$ in 1 L deionized water; washing buffer consisted of 25 mL of 10% Tween and 500 mL of 10× PBS in 5 L of deionized water; 400 mL blocking buffer was made of 5% non-sterile FBS and 10% of 10× PBS. The capture antibody was loaded to ELISA microplate: rabbit anti-murine TNF-α (Biolegend, 100 μg/mL), anti-mouse IL-6 (Biolegend, 500 μg/mL) and IL-12 (p70, BD Pharmingen™, 2 mg/mL) were dissolved in the coating buffer at final concentrations of 0.5 μg/mL, 1.25 μg/mL and 2 μg/mL, and 50 μL of reconstituted capture antibodies transferred into a microplate, sealed with preservative film and incubated at 4°C overnight. For blocking, the plate was tapped gently to remove coating buffer and washed 5 times with washing buffer. of Blocking buffer (100 μL) was added into each well to block non-specific antibody binding sites followed by incubation at 37°C for 2 h. Thereafter, the standard or samples (cell culture supernatant) were added, washed with washing buffer, followed by dilution of 50 μL cell supernatant and standard antibodies in blocking buffer to 20 ng/μL for murine TNF-α (PepoTech Cat.315-01A), 10 ng/μL for IL-6 (BD Biosciences, Cat.51-26536E) and 20 ng/μL for IL-12 (p70, BD optEIA™,

Cat.51-26191E). The plate was then sealed and incubated at 4°C overnight. Then, the samples were washed out followed by the addition of 50 µL of detection antibodies, biotin anti-mouse TNF-α (Biolegend Cat.506311), biotin anti-mouse IL-6 (Biolegend, Cat.504602) and rat anti-mouse IL-12 (p40/p70, BD Pharmingen™, Cat.554476) at final concentration of 1 µg/mL. The plate were then incubated for 2 h at 37°C and 50 µL of conjugated enzyme, avidin (eBioscience, Cat.18-4100; diluted in blocking buffer at ratio of 1:500) was added to each well, followed by incubation for 30 min at 37°C. ABTS substrate [2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonate, 50 µL] was added and absorbance measured at 405 nm.

3.7. Nitrite measurement

As earlier discussed, NO is generated as toxic agent during inflammation and usually converted to nitrite in cell culture medium (Laurent et al., 1996). The level of nitrite was determined in the cell cultures using the Griess reagent (Green et al., 1982). In the assay, nitrite reacts with sulfanilamide and N-napthylethylenediamine to form azo dye, the red purple color that can be measured by spectrophotometry at 540 nm. The method reported by Rebelo et al. (2014) was adapted with modification; solution A was prepared by dissolving sulfanilamide (1%) in 2.5% H_3PO_4 (Sigma, S9251) and solution B contained 0.1% of N-napthylethylenediamine dihydrochloride in 2.5% H_3PO_4. The working reagent was prepared fresh before the assay by mixing solution A

and B at 1:1 ratio. The cell culture supernatant (50 µL, spiked with 15.625 µM $NaNO_2$) and 50 µL of working reagent were mixed in a microplate and absorbance measured at 540 nm after 10 min incubation at room temperature. A standard curve was prepared with 0.0078-0.5 mM $NaNO_2$ in complete RPMI medium.

3.8. ADAM17 enzymatic activity

THP-1 cell lysate (2 µL) was mixed with 48 µL 25 mM Tris-HCl pH 8.0 and the mixture was incubated at 37°C for 5 min. After incubation, 50 µL of 20 µM fluorogenic ADAM17 substrate (R&D, Cat. ES003) diluted in Hanks'Balanced Salt solution (HBSS, BioWhittaker®, Cat. 10-527F) was added and fluorescence was measured at excitation wavelength of 320 nm and emission wavelength of 405 nm. The fluorescence intensity as a result of the enzymatic release of a fluorogenic product was used as indication of ADAM17 activity.

3.9. Preliminary animal experiment

Female mice BALB/c (8 weeks old) were generously provided by Dr. Jude Uzonna's Host-Pathogen Interaction lab, Department of Immunology, University of Manitoba. The mice were maintained at the Central Animal Care Service in ventilated cages at 21°C and 55% humidity, and fed with regular diet for 1 week. The 3 treatment groups were control (PBS), sample 1 (received 100 µg H_{Fic}/mouse) and sample 1 (received

300 μg H_{Fic}/mouse), with 2 mice in each group. The lyophilized ficin-produced potato protein hydrolysate (H_{Ficn}) was dissolved in 1× PBS (pH 7.4) and administered to the mice through intraperitoneal injection. After 6 h, the mice were injected with LPS at 5 mg/kg to induce inflammation. The mice were terminated 6 h after injection, and the blood and peritoneal fluid were collected. The blood samples were then centrifuged at 5000 rpm for 5 min to remove blood cell and the supernatant (serum) was collected. All the samples were stored at -20°C until further analyses. The animal experiments were conducted following the animal experiment guideline stipulated by the Canadian Centre for Animal Care. The cytokines TNF-α, IL-6 and IL-12, as well as FRAP were measured in the mice serum and peritoneal fluid as earlier discussed in sections 3.6 and 3.4, respectively.

3.10. Peptide characterization

3.10.1 Degree of hydrolysis (DH)

The degree of potato protein hydrolysis was determined by measuring the amount of free amino nitrogen released by the breakage of peptide bonds during hydrolysis (Nielsen et al., 2001). Ortho-phthalaldehyde (OPA) reacts with amino group in amines and free amino acids in the presence of reducing agent dithiothreitol (DTT) to form isoindole derivatives, which can be quantified under UV radiation. The samples were prepared at 1 mg/mL (or 0.1 mg/mL serine standard); 400 μL of the hydrolysate

was mixed with 3 mL of a working reagent containing 0.69 mM sodium dodecyl sulfate (Fisher, Cat.BP166-100), 1.19 mM OPA (ICN Biomedicals Inc. Cat.102648), 1.27 mM DTT (Fisher, Cat.C4H10O2S2) and 20 mM sodium tetraborate (Fisher, BP175-500). The absorbance of the mixture was measured at 340 nm after incubation for 10 min at room temperature. The degree of hydrolysis was calculated as previously reported (Nielsen et al., 2001).

3.10.2 Surface hydrophobicity (S_o)

8-Anilino-1-naphthalene sulfonic acid (ANS) is a hydrophobic fluorescent molecule that has high emission when combined with nonpolar parts of proteins (Ali et al., 1999). SH was determined following a method reported by Horax et al. (2004) with modifications. Hydrolysates (1 mg/mL stock solution) was directly diluted in a microplate with 0.01 M phosphate buffer (pH 7.0) to 9.4 to 150 ng/mL. Thereafter, 100 µL of 0.04 mM ANS was added to each well followed by gently mixing and measurement of fluorescence at excitation wavelength of 390 nm and emission wavelength of 470 nm. The slope of fluorescence verse hydrolysate concentration plot represent the S_o.

3.10.3 Sulfhydryl (SH) group determination

Sulfhydryl group of proteins can impact antioxidative activities by donating electrons

and quenching free radicals. In this assay, sulfhydryl group can reduce disulfide bond of 5,5′- dithiobis(2-nitrobenzoic acid (DTNB) resulting in the formation of 3-thio-6-nitrobenzoate (TNB), which gives yellow color in alkaline condition (Ellman, 1959). The colorimetric SH detection method was modified from a previous report (Udenigwe et al., 2014). The hydrolysates (0.25 mL of 10mg/mL) were mixed with 1 mL of 0.1 M Tris-glycine buffer (pH 8.0) containing 10 M urea. The mixture was incubated at 40°C for 30 min and 30 μL of 4 mg/mL DTNB (in 0.1 M Tris-glycine buffer, pH 8.0) was added. The mixture was incubated at room temperature for 10 min and absorbance measured at 412 nm.

3.10.4 Reversed-phase HPLC analysis

Reversed-phase high performance liquid chromatography (RP-HPLC) is a liquid chromatography technique that uses hydrophobic stationary phase. Hydrophobic compounds can easily attach to the stationary phase leading to longer retention period; as a result, each compound elutes at different time and are then quantified (Nic et al., 1997). Linear gradient mobile phase was used as earlier described (Udenigwe et al., 2013). Hydrolysates were prepared in mobile phase A (0.05% trifluoracetic acid in water) at 5 mg/mL; 20μl of the sample was injected into a Waters 1525 Binary HPLC system equipped with C18 column (250×4.6 mm, 5 μm). The sample was then eluted from the column using a linear gradient of 0 to 66%

mobile phase B (0.05% trifluoracetic acid in acetonitrile) for first 45 min, raised to 100% B for another 5 min and then equilibrated to 0% B for 5 min. Elution was monitored with a Waters 2998 photodiode array detector at 280 nm.

3.10.5 Amino acid analysis

The amino acid profile of H_{fic} was analyzed at the SPARC Center, The Sick Kids Hospital, Toronto, Canada after hydrolysis with 6 N HCl and pre-column derivatization with phenylisothiocyanate followed by reverse-phase HPLC with a Waters Pico-Tag System, and reported as percent amino acid composition.

3.11. Statistics analysis

Data collected in each assay were repeated in triplicate or quadruple and expressed as mean with standard deviation (GraphPad Prism version 6.0c). Difference among each treatment were analyzed by running one-way analysis of variance using least significant difference (LSD) test with Minitab Express version 1.1.0.

Chapter 4 Results

4.1. Zinc chelating assay

Proteome-based matrix has been considered a potential metal chelator and applied in varied fields like heavy metal elimination and enhancement of mineral absorption (Bhattacharyya et al., 1998; Ashmead, 2012). Among the amino acids, sulfhydryl group-containing cysteine plays multiple roles in biochemical reactions including redox reaction, metal chelation and electron donation (Giles et al., 2003). Cysteine can bind metals such as iron, zinc, copper and cadmium by functioning as mono- or bidentate ligands (Strop et al., 2001; Ogata et al., 2002). For potato protein, every 173 g potato (baked) contains 0.05 g Cys amino acid and this number will be relatively higher in raw potato (ESHA Research, USA; Hughes, 1958). In this assay, zinc chelating activities of the potato protein hydrolysates were measured by using *in vitro* colorimetric method, which was firstly developed by Hunt et al. (1989) and modified from Jakob et al. (2000). Results in Figure 4.1 indicate that the potato protein hydrolystates exhibited relatively low zinc chelating activity compared to EDTA, which had 3-fold higher activity at 8 μg/mL than the hydrolysates. Besides, although most of the samples showed similar zinc chelating power, H_{Pep} tended to chelate more zinc whereas $H_{Pep+Panc}$ had the lowest activity. Therefore, H_{Pep} was selected as best candidate for ADAM17 inhibition assay in inflammatory cell culture based on the mechanism that binding the zinc co-factor would suppress ADAM 17 activity.

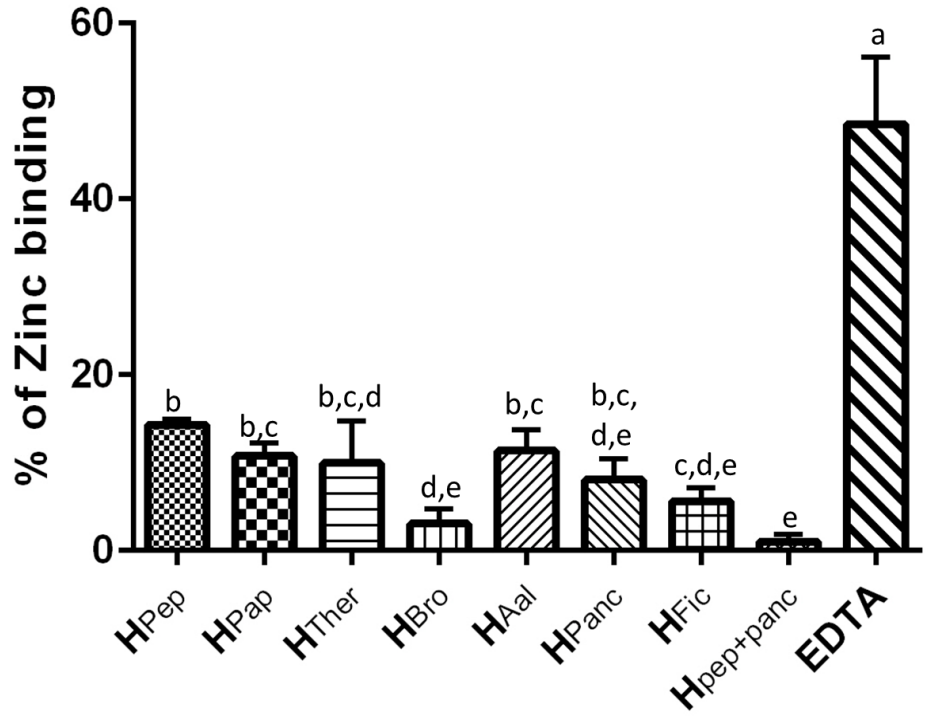

Figure 4.1 Zinc chelating activities of the potato protein hydrolysates. Unbound zinc reacts with colorimetric dye 4-(2-Pyridylazo) resorcinol and absorbance was measured at 500 nm. Hydrolysate final concentration, 2.5 mg/ml, EDTA: 8 µg/ml (n= 3, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

4.2. *In vitro* FRAP assay

ROS are produced during inflammation and regulated by NOX, which is up regulated by TNF-α through the NF-κB pathway (Zhang et al., 2009). ROS in low concentration is necessary for mediating cellular signaling, apoptosis and gene expression, but will damages cellular molecules when overproduced (Lü et al., 2010). ROS can be neutralized by antioxidants through different mechanisms such as reduction and scavenging. Ferric reducing antioxidant power is one of most popular antioxidant assays. By reducing ferric ion to ferrous ion and reacting with colorimetric reagent, % of reduced ferric was measured as FRAP value. As shown in Figure 4.2, results from this study indicate that all the potato protein hydrolysates possess ferric reducing potential, and can be antioxidative. Compared to equal amount of glutathione (GSH), the hydrolysates gave moderate FRAP values with H_{Panc}, H_{Fic} and $H_{Pep+Panc}$ showing the best reducing power and H_{Ther} exhibiting the lowest FRAP. Although the activities are mostly similar, the hydrolysates resulting from ficin and the gastric enzymes are candidates for further evaluation. *In vitro* FRAP assay is an indirect measurement of antioxidative potential and does not provide evidence of physiological role of the hydrolysates in controlling oxidative stress. Therefore, cellular studies were performed to evaluate the bioactive properties of the potato protein hydrolysates in modulating inflammation and associated oxidative stress.

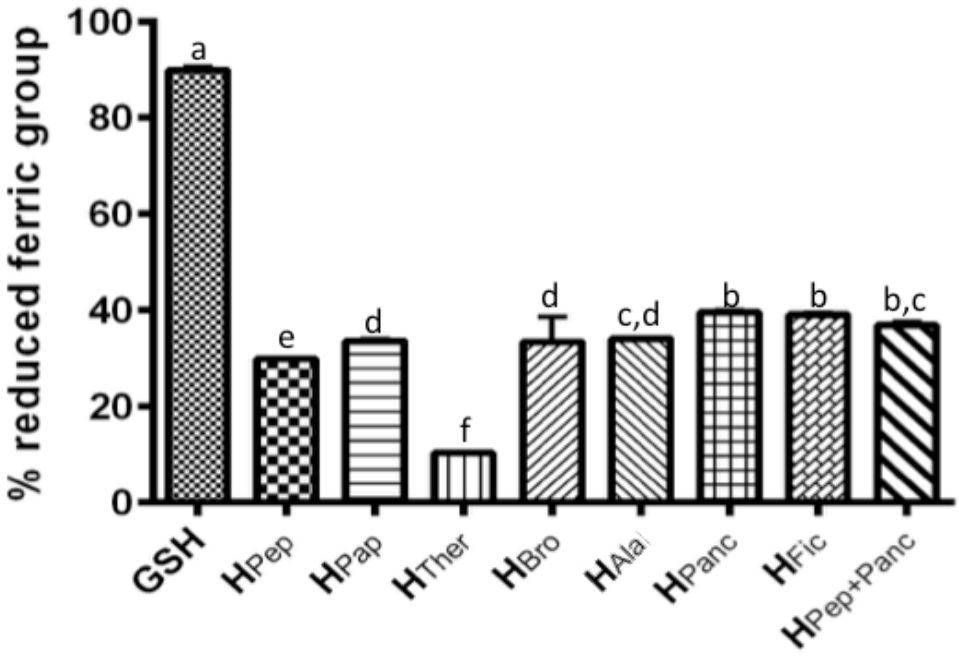

Figure 4.2 ***In vitro*** **ferric reducing antioxidant power (FRAP) values (% of reduced ferric ion) for potato protein hydrolysates. Reduced ferric (ferrous) ion formed blue ferrous tripyridyltriazine complex that was quantified at 700 nm. Glutathione (GSH) and hydrolysates: 1 mg/mL (n=3, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.**

4.3. Anti-inflammatory activity of potato protein hydrolysates

4.3.1 TNF-α inhibition in mouse ANA-1 cell model

To confirm anti-inflammatory function of potato protein hydrolysates in cellular model, TNF-α level in ANA-1 cell supernatant was tested in ELISA assay. ANA-1 cell line was first isolated from bone marrow of C57BL/6 mouse infected with J2 recombinant retrovirus (Cox et al., 1989). As shown in Figure 4.3, treatment of the ANA-1 cells with LPS induced >6-fold increase in TNF-α levels compared to the normal (no LPS) cells. When the cells were treated with the potato protein hydrolysates under the inflammatory condition (with LPS), the samples presented different extents of suppression of TNF-α release in the cells with >50% reduction in the cytokine level in some sample treatments. These promising activities indicate that the potato-derived samples can be explored for possible use as anti-inflammatory agents in food. Meanwhile, H_{Pep}, best zinc-chelating hydrolysate, showed the least inhibition of TNF-α release. This suggests that chelating the ADAM17 enzyme co-factor is likely not the (major) anti-inflammatory mechanism of the hydrolysates since H_{Pep} was not the best sample in reducing the release of the pro-inflammatory cytokine. The potato protein hydrolysates produced with ficin, bromelain, Alcalase®, pancreatin and pepsin-pancreatin expressed relatively strong activity in reducing the LPS-induced cellular TNF-α release, corresponding to the samples with the best *in vitro* FRAP values.

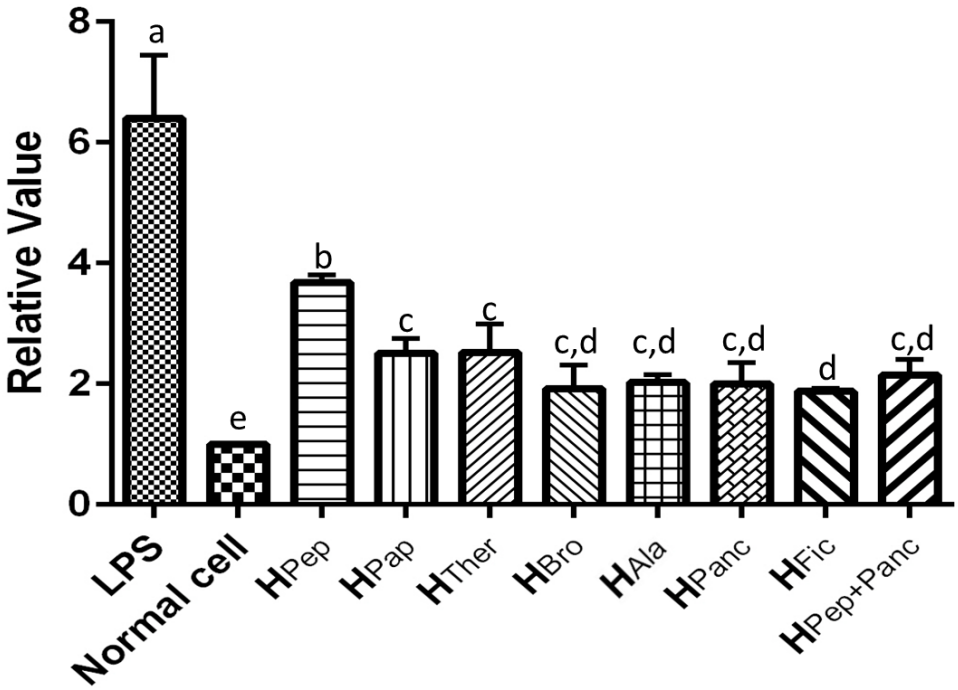

Figure 4.3 TNF-α relative values in ANA-1 cell culture. Cells were pretreated with 125 µg/mL potato protein hydrolysate for 24 h followed by 12 h treatment with 1 µg/mL LPS at 37°C, 5% CO2. LPS: 24 h incubation without samples and induced by 12 h LPS. Normal cell: not treated with hydrolysates or LPS. TNF-α level was measured by ELISA and expressed as relative value compared to normal cell (n=4. Mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

4.3.2 TNF-α inhibition in mouse primary cell model

Although cell lines have advantages of fast growth and unlimited extension, they have some drawbacks: inherent immortalization of cell line leads to modification of genotypes and phenotypes, and makes the cells lose certain specific functions (Pan et al., 2009). Therefore, subsequent primary cell screening was conduct in C57BL/6 mouse to confirm anti-inflammatory ability of the potato protein hydrolysates. Bone marrow-derived monocyte enters the blood and differentiates into macrophage in the tissue (Zhang et al., 2008). In this assay, isolated monocyte cells were cultivated and differentiated with L929 cell culture supernatant, which contains macrophage-stimulating factor. As shown in Figure 4.4, treatment of the cells with LPS resulted in >20-fold elevation in the amounts of secreted TNF-α, demonstrating the activation of intense inflammatory reactions in the primary cells compared to the ANA-1 cell line. Subsequent treatment of the cells with the hydrolysates resulted in potent inhibition on TNF-α release with $H_{Pep+Panc}$ exhibiting the best effect with the lowest secreted TNF-α level similar to the level in the normal (no LPS-treated) cells. There was not observed relationship between the activities observed for the hydrolysates in the primary cells and cell lines, although pepsin appeared to have produced the least active sample for both assays. The confirmation of anti-inflammatory activity in the primary cells provide evidence of the potential activity of the hydrolysates in regulating inflammation and associated oxidative stress under physiological condition.

From *in vitro* screening, the potato protein hydrolysates H_{Pep}, H_{Panc}, H_{Fic} and $H_{Pep+Panc}$ demonstrated potential anti-inflammatory activities based on zinc chelation and antioxidative properties. Thereafter, the zinc-chelating activity of H_{Pep} was not sufficient in making the sample the most active in suppressing pro-inflammatory TNF-α release in both cell line primary cell cultures. Besides, H_{Fic} demonstrated substantial *in vitro* antioxidative activity and inhibition of NO production, the key redox mediators of inflammatory responses. Although the gastric enzymes exhibited pronounced anti-inflammatory and antioxidative activities, the ficin hydrolysates was selected for further studies since ficin is a specific protease that cleaves protein substrates at Gly, Ser, Glu or Tyr residues at the N-terminal. This provides the advantage of defined peptide characterization compared to the products of the broadly-specific gastric digestion with pepsin-pancreatin combination (Table 3.1). In Figure 4.5, H_{Fic} demonstrated suppression of TNF-α level the primary cell model at 1-125 μg/mL. The highest concentration appeared to have induced a more prominent cytokine reduction, but the activity observed for the different concentrations were not statistically different. The particularly high activity of H_{Fic} in reducing LPS-induced TNF-α by >50%, even at very low concentration (1 μg/mL), enhances thet prospects of the potato protein hydrolysates for further evaluation as anti-inflammatory food ingredient.

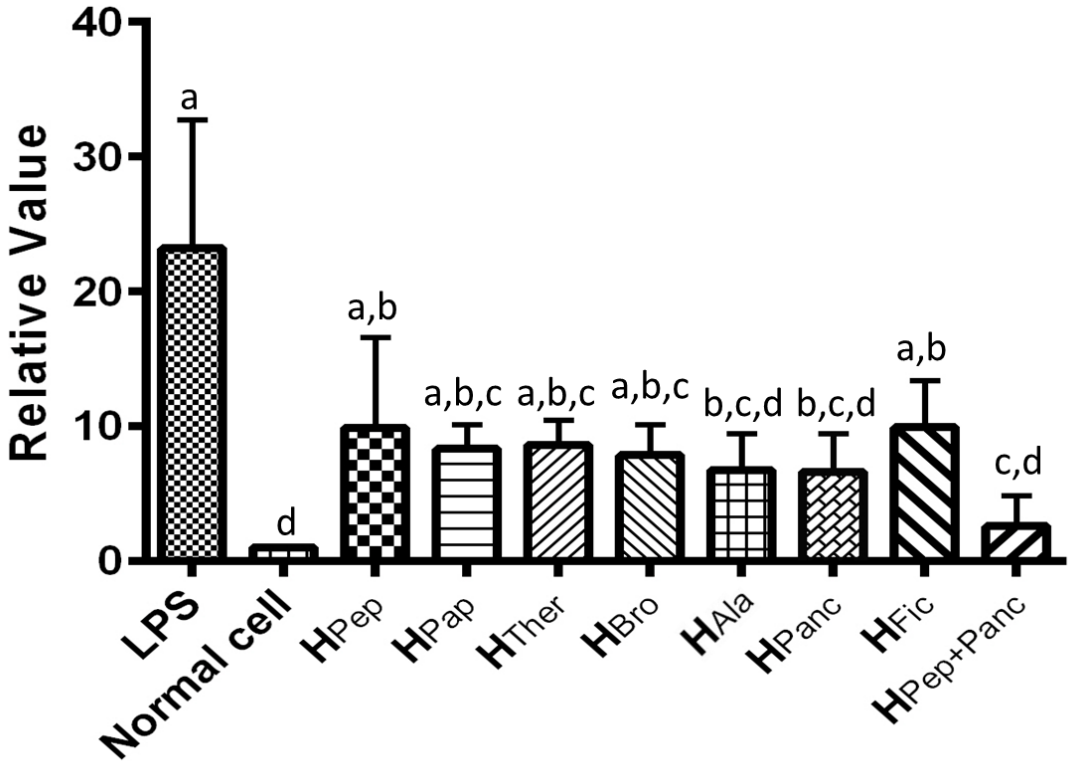

Figure 4.4 Relative TNF-α levels in C57BL/6 mice primary cell culture supernatant. Cell was derived from mouse femoral bone marrow and differentiated from monocyte to macrophage. Cells were pretreated with 125 μg/mL potato protein hydrolysate for 24 h followed by 12 h treatment with 1 μg/mL LPS at 37°C, 5% CO_2. LPS: 24 h incubation without hydrolysate and induced by 12 h LPS. Normal cell: not treated with samples or LPS. TNF-α level was measured by ELISA assay and expressed as relative value compared to normal cell (n=4, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

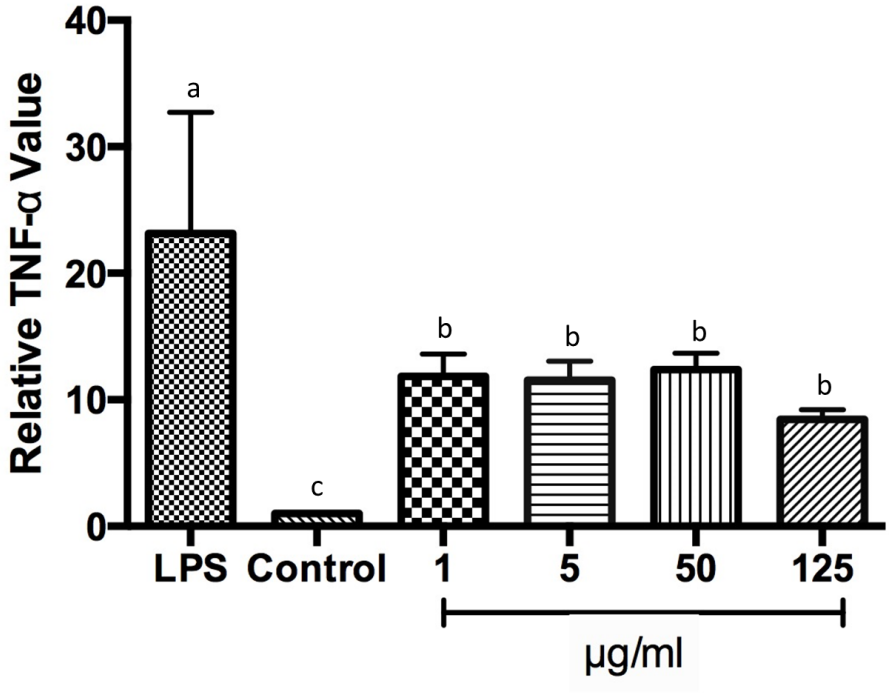

Figure 4.5 TNF-α relative values in the primary cell models. Mice primary cells were treated with H_{Fic} at different concentrations. Cells pretreated with 125ug/ml hydrolysate for 24h then 12h 1 μg/ml LPS incubation. LPS: 24h incubation without hydrolysate and induced by 12h LPS. Normal cell: neither treated by samples or LPS. TNF-α level was measured by ELISA assay and expressed as relative value compared to normal cell (n=4, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$).

4.3.3 Nitrite detection

During inflammation, NO produced through iNOS activation is a major contributor of toxic agent and biomarker of inflammation. iNOS mRNA expression is upregulated by TNF-α leading to elevated NO, which is unstable and normally measured in the form of nitrite and nitrate (Poljakovic et al., 2003; Pautz et al., 2010). NO can exhibit anti- and pro-inflammatory properties under different cellular conditions. At low levels, NO derived from eNOS functions in reducing inflammatory responses such as cytokines synthesis, leukocyte adhesion and adhesion molecule production, whereas at high levels due to iNOS activity, it acts as a toxic agent (Cirino et al., 2003; Guzik et al., 2003). In this study, nitrite was detected in the ANA-1 and primary cell cultures by reacting the cell supernatants with Griess reagent and results demonstrated in Figure 4.6. The potato protein hydrolysates presented varying suppressing effects on cellular nitrite, which indicates modulatory effects on inflammation. Thermolysin-produced H_{Ther} was observed to possess the best NO inhibitory hydrolysate and even reduced nitrite to a level similar to the normal (no LPS) ANA-1 cells. NO has a short active period since it reacts with other ROS (superoxide radical) to form peroxynitrite, $ONOO^-$ (Zhang et al., 2009). Hence the ROS level can be directly affected by the antioxidative hydrolysates during inflammation, subsequently influencing the NO level. As shown in Figure 4.2 and 4.6, nitric oxide inhibitory properties of most hydrolysates were positively associated with their antioxidant activities confirming this interference. Due to prolonged inflammation induction in cellular model (12h) it would not be

possible to directly relate the nitrite levels and TNF-α suppressing activities in the inflammatory cells. Besides, nitrite level in the normal and LPS-treated primary cells were similar (data not shown), possibly due to expected depletion of NO during cell cultivation time.

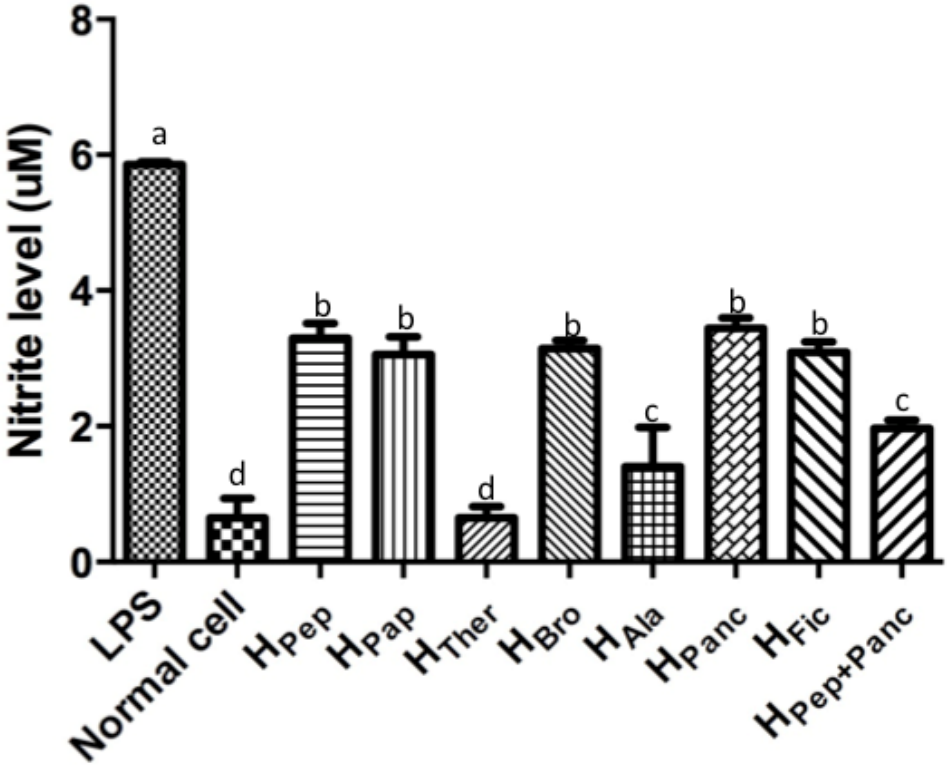

Figure 4.6 Nitrite level in ANA-1 cell culture. Cells were pretreated with 125 µg/mL potato protein hydrolysate for 24 h followed by 12 h treatment with 1 µg/mL LPS. LPS: 24 h incubation without hydrolysate and induced by 12 h LPS. Normal cell: not treated with sample or LPS. Nitrite level was measured using Griess reagent (n=4, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

4.4. ADAM17 enzymatic activity

Zinc-dependent ADAM17 is the enzyme that catalyzes the cleavage of transmembrane pro-TNF-α to release active TNF-α (Müller et al., 2009). By reacting with a fluorogenic substrate that resembles its cleavage site, enzymatic activities of membrane-bound ADAM17 on the lysed cells were measured. As shown in Figure 4.7, H_{Fic} treated ANA-1 and THP-1 cells under inflammatory conditions showed similar ADAM17 activities as the LPS only-treated cells. Moreover, the strong zinc-chelating property of EDTA did not promote cellular ADAM17 inhibition in the human THP-1 cell lysate (Figure 4.7B). According to Doedens et al. (2003), cellular stimulus, such as phorbol myristate acetate, used to differentiate THP-1 monocyte to macrophage can increase ADAM17 activity via inducing ROS such as superoxide radical production. This may have contributed to the total ADAM17 activity observed in the THP-1 cells. Besides, ADAM17 activity assays have been conducted with living cells in culture plates (Göoz et al., 2006). This method was not adopted, as the fluorogenic substrate would compete with pro-TNF-α for ADAM17, making it difficult to accurately quantify total enzymatic activity. Based on the results, the anti-inflammatory property observed for H_{Fic} in the cells is not related to ADAM17 inhibition both by co-factor zinc chelation or direct enzyme inhibition.

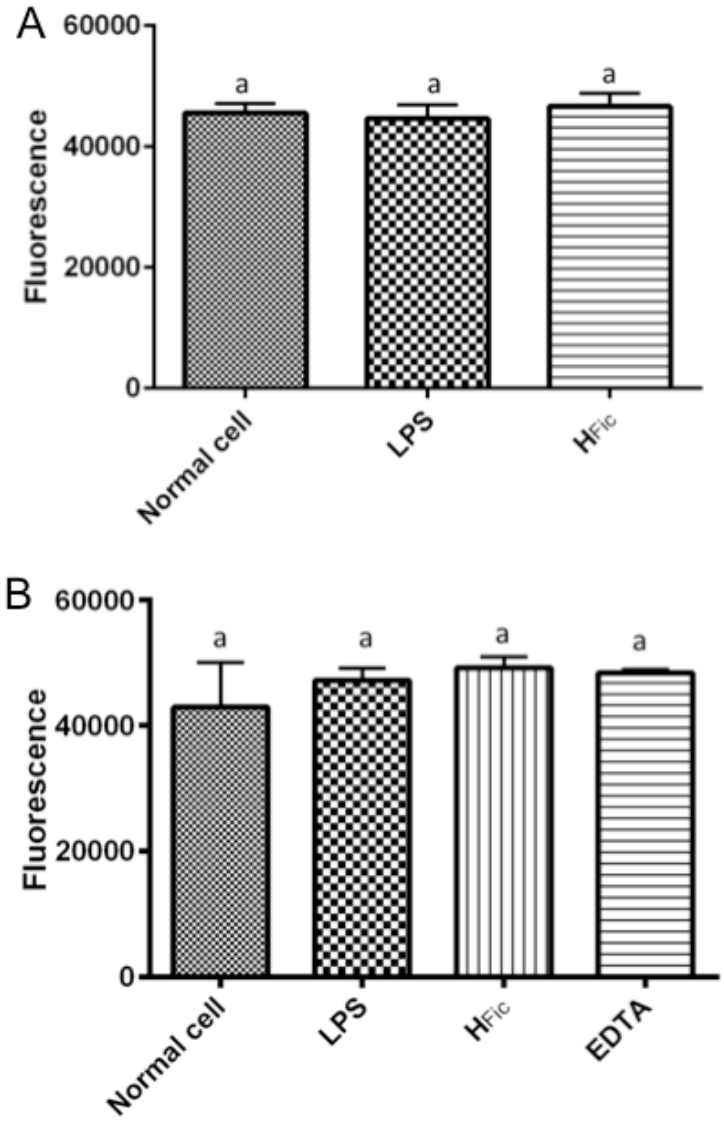

Figure 4.7 ADAM17 enzymatic activities in ANA-1 (A) and THP-1 cell lysates (B). HFic treated cells were lysed and mixed with fluorogenic ADAM17 substrate and read fluorescence at 320 nm excitation and 405 nm emission. (n=4, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

4.5. Physiological effects of H_{Fic} in inflammatory mice

The anti-inflammatory property of potato protein hydrolysates H_{Fic} was further evaluated in BALB/c mice with LPS-induced inflammation. The level of the three pro-inflammatory cytokines: TNF-α, IL-6 and IL-12 were measured in the serum and peritoneal fluid of the mice by ELISA. Prior to termination, the LPS-treated mice were observed to be extremely ill whereas the H_{Fic}-treated mice behaved normally. As shown in Figure 4.8, the hydrolysates significantly inhibited pro-inflammatory IL-6 secretion in the serum and peritoneal fluids of the mice. These effects were dose-dependent and the serum IL-6 level was particularly decreased to zero level when the mice received H_{Fic} at 300 µg/mouse (Figure 4.8A). Similarly, the higher sample dosage suppressed the levels of IL-12 (p70) in the serum and peritoneal fluid, and the sample was active at 3-times lower dosage only in the peritoneal fluid (Figure 4.9). This is because the physiological IL-12 level is higher in the serum compared to the peritoneal fluid, making it more challenging to lower the cytokine level in the former. Unexpectedly, the amount of TNF-α in the mice serum and peritoneal fluid were below ELISA limit of detection, which is 4 pg/mL for the BioLegend anti-mouse TNF-α kit. According to Blanqué et al. (1998), TNF-α level in mice serum after 50-300 µg/mouse LPS sensitization for 6 h ranged from 0-150 pg/mL, which were higher than both LPS dosage and ELISA detection limit in this study. Similarly, the nitrite level in mice serum and peritoneal fluid were lower than the detection limit even after spiking with the standard. As earlier discussed, this can be attributed to the short life span of NO due

to derivatization with ROS to form other reactive species.

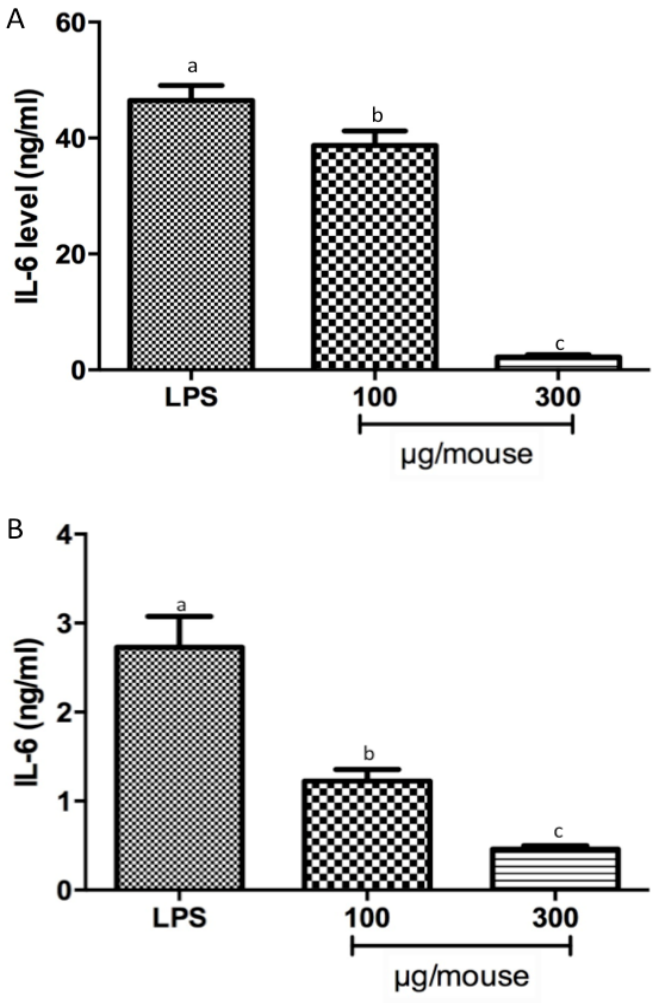

Figure 4.8 Pro-inflammatory cytokine IL-6 level in H_{Fic} treated BALB/c mice serum (A) and peritoneal fluid samples (B). 8 weeks old mice were fed with regular diet and injected with H_{Fic} samples in 1× PBS and followed by 5 mg/kg LPS induction after 6h. Serum samples were collected. (n=4, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

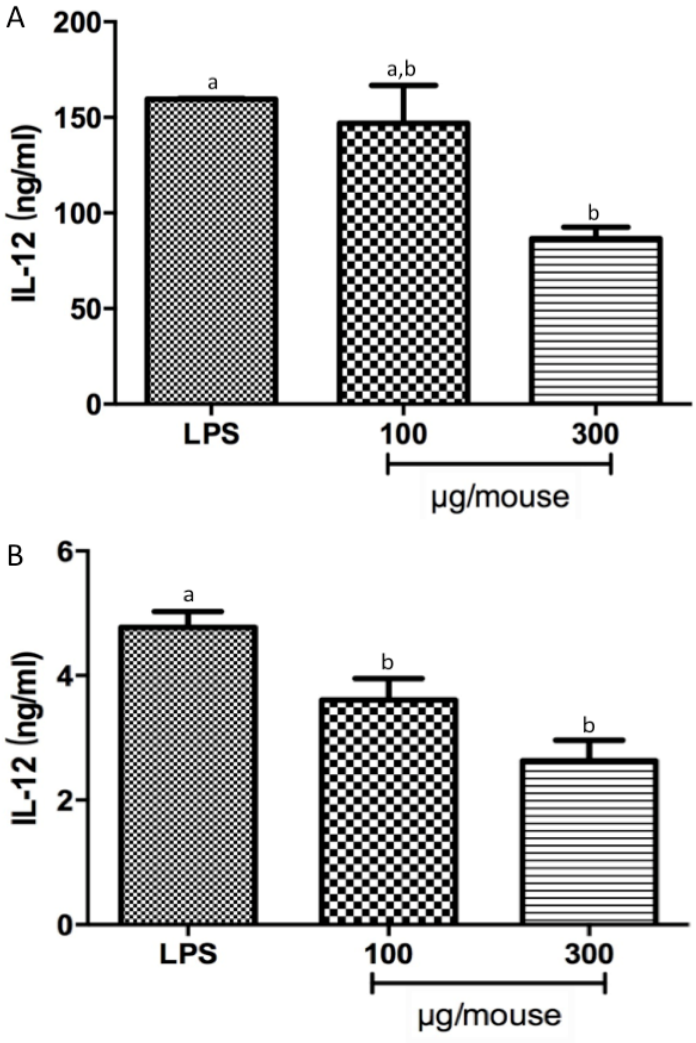

Figure 4.9 Pro-inflammatory cytokine IL-12 (p70) level in H_{Fic} treated BALB/c mice serum (A) and peritoneal fluid samples (B). 8 weeks old mice were fed with regular diet and injected with H_{Fic} samples in 1× PBS and followed by 5mg/kg LPS induction after 6h. Serum samples were collected. (n=4, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

Oxidative stress is well known to contribute to pathogenesis of inflammatory reactions. The ability of the physiological fluids to reduce ferric ion to ferrous ion (FRAP value) can be used as a direct indication of physiological oxidative (redox) state during inflammation. In this study, treatment of the BALB/c mice with H_{Fic} during inflammation resulted in significantly ($p<0.05$) elevated serum FRAP values represented as higher % reduced ferric ion compared to the serum of the LPS-only treated mice (Figure 4.10). The positive effect of H_{Fic} on the mice serum FRAP was not dose-dependent, and indicates a healthier redox state of the animals relative to the LPS-only inflammatory condition. However, FRAP (for LPS-only and H_{Fic} treatment) was not detectable in the mice peritoneal fluid, which is not a primary inflammation reaction site. The positive redox effect of the potato protein hydrolysates could be attributed to (1) its effect in inhibiting inflammation, thus suppressing oxidative stress and conserving the physiological antioxidants in the serum (e.g. glutathione), or (2) its direct antioxidative effect within the serum, since the hydrolysates exhibited high FRAP *in vitro* (Figure 4.2).

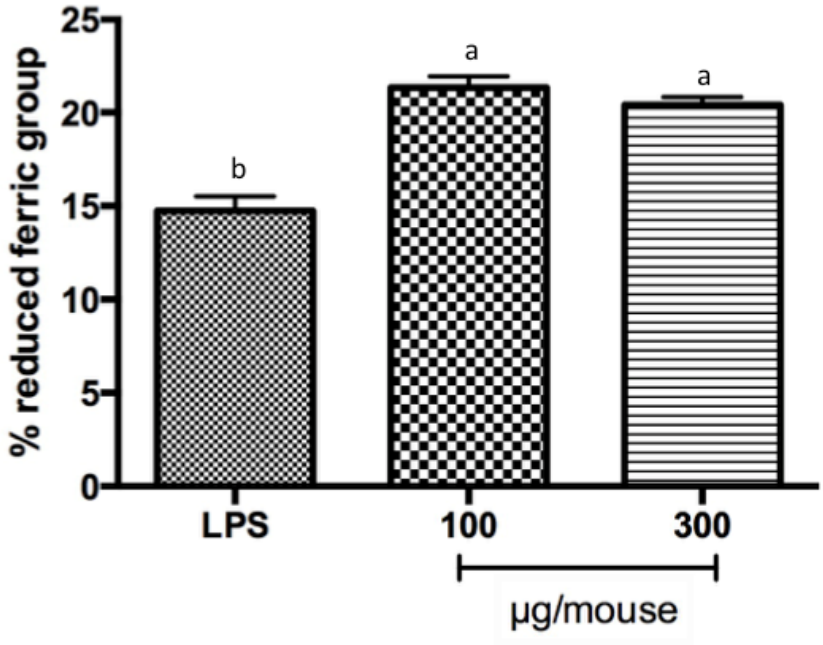

Figure 4.10 Ferric reducing antioxidant power (FRAP) values in H_{Fic} treated BALB/c mice serum. Reduced ferric (ferrous) ion formed blue ferrous tripyridyltriazine complex and can be detected at wavelength 700 nm. FRAP value was expressed as % of reduced ferric ion (n=3, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

4.6. Characterization of the potato protein hydrolysates

4.6.1 Peptide yield and degree of hydrolysis (DH)

As shown in Figure 4.11, the yields of the protein hydrolysates varied from 16% to 40%. As bioactive peptides are cryptic fragments of proteins that hide within the parent structure, the DH of the peptide bonds determines the type and amounts of peptides released from the proteins and possibly their functions (Udenigwe & Aluko, 2012). The higher the DH, the smaller the peptide chain length. It is thought that peptides with low molecular sizes are most likely to exhibit physiological bioactivity (depending on their sequence) as the small sizes would allow for accessibility to target ligands; moreover, smaller peptides (e.g. di- and tripeptides) are likely to be transported through intestinal peptide transporters, which would enhance their bioavailability in target tissues (Udenigwe & Aluko, 2012). As shown in Figure 4.12, the use of different proteases in hydrolyzing potato proteins resulted in different DH. Surprisingly, highly specific proteases, bromelain and ficin, gave the highest DH whereas, as expected, pepsin gave the lowest DH, compared to the activities of broadly-specific Alcalase® and pancreatin. The specificity of the enzymatic cleavage (Table 3.1) is expected to help in predicting DH patterns, but factors such as the enzyme-protein interactions and reaction conditions can impact the extent of peptide bond hydrolysis. DH was inversely correlated with zinc chelating activity (Figure 4.1) indicating that larger peptides may form more complexes with the metal, but no pattern was observed in antioxidative and anti-inflammatory activities.

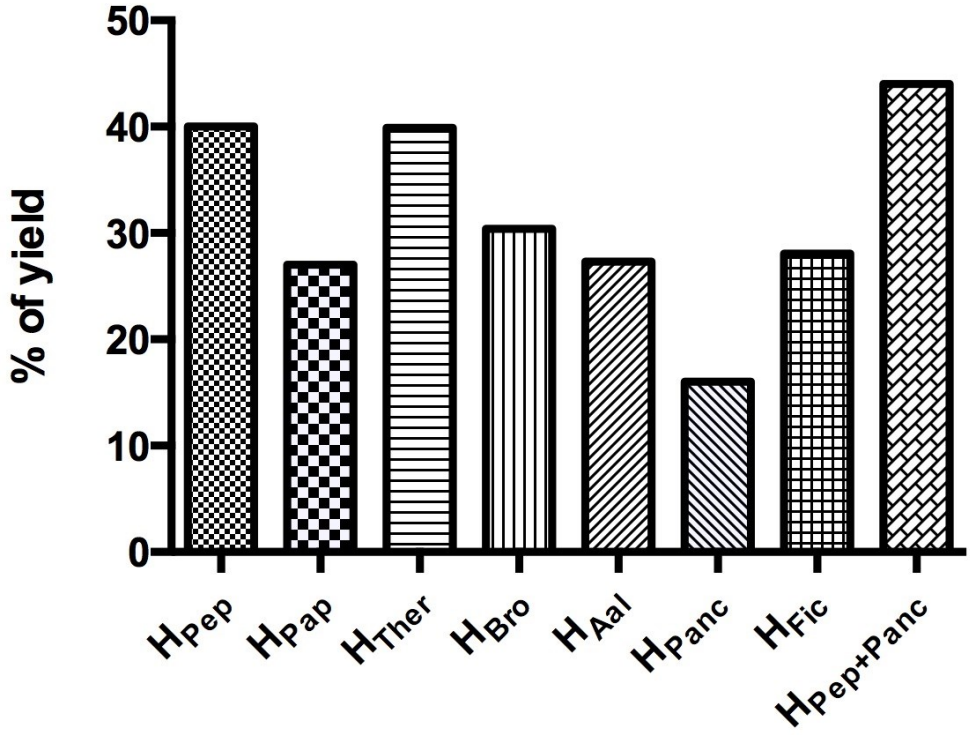

Figure 4.11 Yield (%) of potato protein hydrolysates obtained from the study.

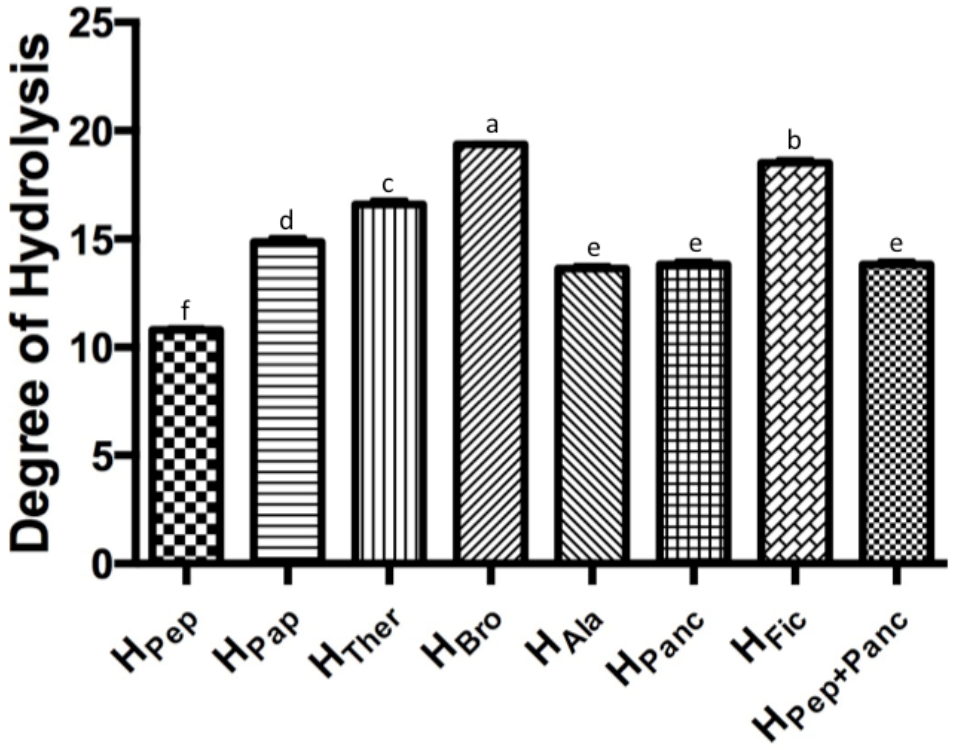

Figure 4.12 Degree of potato protein hydrolysis (%). Hydrolysates reacted with OPA reagent and absorbance were measured at 340 nm (n=3, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

4.6.2 Surface hydrophobicity and HPLC profiles of the hydrolysates

Surface hydrophobicity is attributed to hydrophobic amino acid residues of peptides and the value varies in different protein hydrolysates depending on the proteolytic enzymatic activities and degree of hydrolysis (Paraman et al., 2007). This surface property is important when the peptides interact with target molecules, and thus can influence bioactivity of the peptides. Surface hydrophobicity is thought inversely related to DH. As shown in Figure 4.13, this relationship is mostly applicable to the potato protein hydrolysates except for $H_{Bro,}$ which exhibited high surface hydrophobicity even with the highest DH. The flexibility of peptides contributes to their surface hydrophobicity because higher flexibility would lead to exposure of non-polar amino acid residues from the molecular core to the surface (Kato et al., 1985). Moreover, increased hydrophobicity of antioxidants can enhance their interactions with hydrophobic target biomolecules such as lipids; this increases their proximity for exertion of antioxidative activity (Laguerre et al., 2010; Pownall et al., 2010). In this study, surface hydrophobicity of the potato protein hydrolysates was not correlated with the *in vitro* and cellular antioxidative and anti-inflammatory activities, except for isolated cases. Based on HPLC profiles (Figure 4.14), the gastric enzyme-generated hydrolysates gave the highest content of hydrophobic peptides (with higher retention time). The hydrophobicity of individual peptides could not be related to the surface hydrophobicity of the hydrolysates as peptide molecular interactions in solution (e.g. aggregation) can affect the latter property.

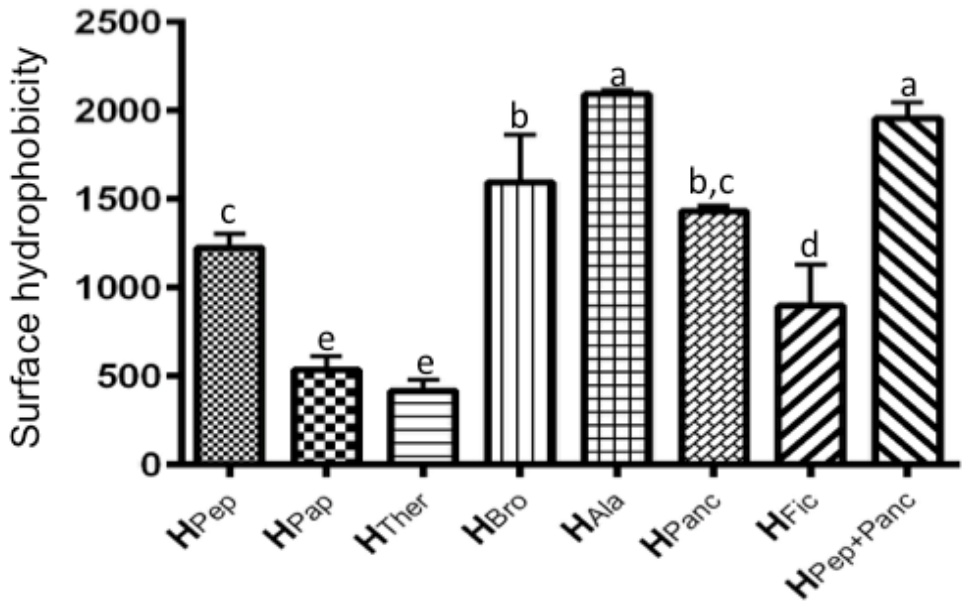

Figure 4.13 Surface hydrophobicity of the potato protein hydrolysates. The hydrolysates reacted with a hydrophobic probe and fluorescence was measured at excitation and emission wavelengths of 390 nm and 470 nm, respectively (n=3, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

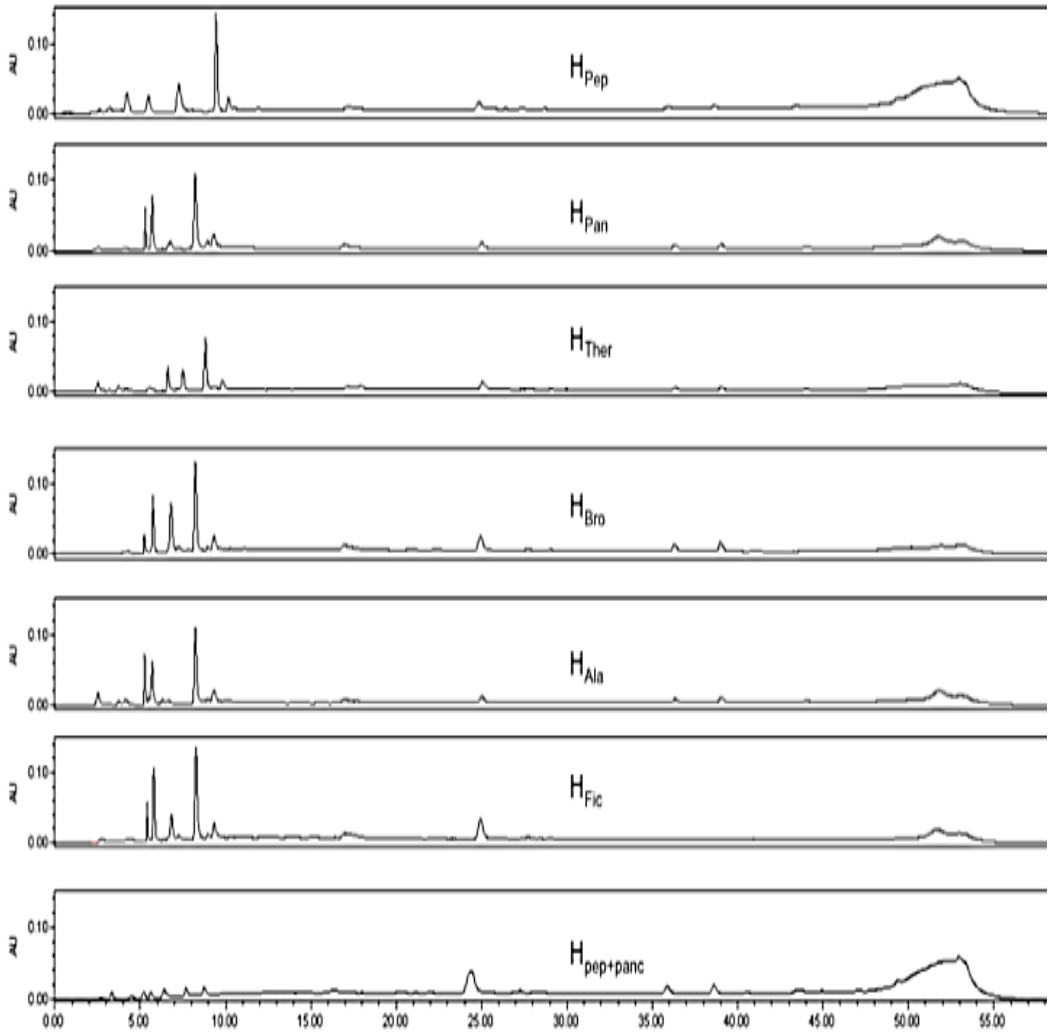

Figure 4.14 Reversed-phase HPLC profile for potato hydrolysates. Samples were eluted by gradient mobile phase consist of reagent A and B. Mobile phase A (0.05% trifluoracetic acid in water) decreased from 100% to 34% at 45min and mobile phase B (0.05% trifluoracetic acid in acetonitrile) increased from 66% to 100% at 50min, then equilibrated to 0% for 5min. Absorbance was measured at 280 nm.

4.6.3 Sulfhydryl content of the hydrolysates

The mechanisms of protein and peptide-based antioxidative activity include free radical scavenging, chelating of pro-oxidative metals (such as ferric and copper ion) and reduction of peroxides (Elias et al., 2008). As free radical scavengers, peptides have to be more redox active than oxidative tissues or molecules (such as lipids) and not produce another free radicals or final products that would promote oxidation. Peptides containing nucleophilic sulfhydryl side chain of cysteine and amino acid residues with aromatic rings such as phenylalanine, tryptophan and tyrosine are more likely to be oxidized than other amino acids leading to the formation of stable products (Elias et al., 2008). The sulfhydryl group (-SH) of cysteine is susceptible to oxidation to form the stable disulfide cystine (-SS-). Therefore, the amount of reactive sulfhydryl group in peptides can relate to their antioxidative potential. As shown in Figure 4.15, the potato protein hydrolysates were found to have different sulfhydryl contents. $H_{Pep+panc}$ and H_{Ther} displayed the highest sulfhydryl content compared to other hydrolysates. The sulfhydryl content was not correlated with *in vitro* FRAP and zinc chelation or cellular anti-inflammatory activities of the samples, suggesting that sulfhydryl group may not be the decisive factor in predicting bioactivities, especially antioxidative property. However, sulfhydryl content was positively related to the hydrolysates' ability to inhibit cellular ROS production. Hydrolysates with the lowest and highest sulfhydryl contents exhibited the weakest and strongest activities in suppressing inflammation-related cellular NO production (Figures 4.15 and 4.6).

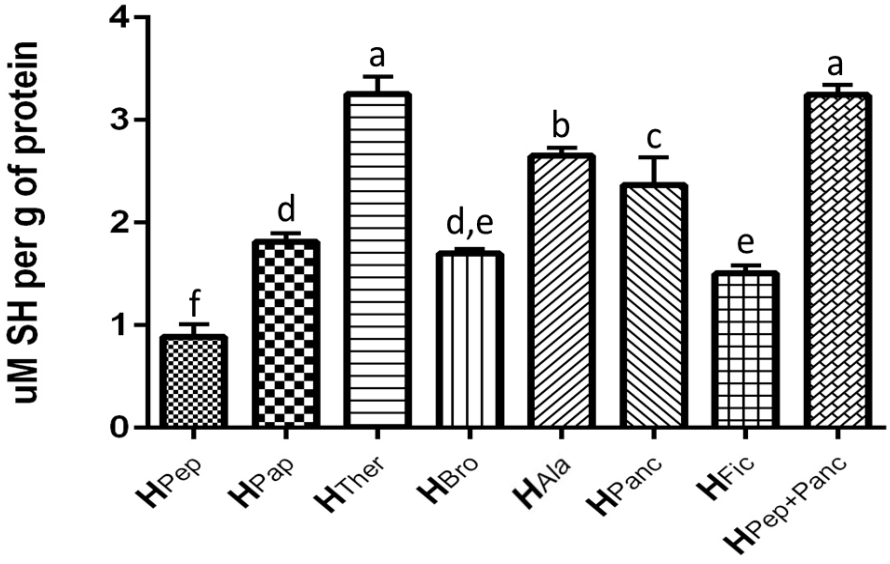

Figure 4.15 Sulfhydryl group determination assay was conducted by mixing hydrolysates with colorimetric reagent DTNB and reading absorbance at 412 nm (n=3, mean+SEM); bars with different letters represent statistically different mean values, $p<0.05$.

4.6.4 Estimated gastric stability and amino acid profile of H_{Fic}

Gastric stability is one of the very important factors to consider when developing peptides intended for oral intake. Unstable peptides can lose their bioactive functions by structural and constitutive modifications during the process of gastric processing, and this is a big challenge in the use of bioactive peptide as functional foods (Udenigwe, 2014). Since the potato protein hydrolysates generated with ficin showed potent physiological effects in modulating inflammatory reactions in cell culture and mice, its gastric stability was estimated in vitro to evaluate its possible use as orally active anti-inflammatory agent. As shown in Figure 4.16, hydrolysates H_{Fic} appeared to have maintained its contents after treatments with pepsin and then pancreatin, based on the HPLC profiles before and after the simulated gastric digestion. Although promising, this finding does not guarantee retention of activity after oral intake as the peptide HPLC peaks are not well resolved especially in the hydrophobic region (higher retention time), and there could be more complex interactions in the gastric tract than during simulated *in vitro* processing. The delivery of bioactive peptides to target tissues remains challenge since peptides can be hydrolyzed and metabolized prior to exerting their activities.

Amino acid analysis indicates that H_{Fic} contains different molar% of the 20 proteinogenic amino acids with low contents of sulfhydryl-containing cysteine and aromatic tryptophan and high content of hydrophobic leucine (Table 4.1). Based on this composition, the hydrolysates is not particularly very hydrophobic as the molar ratio of

the total hydrophobic/hydrophilic amino acid residues was calculated to be 1.05:1.

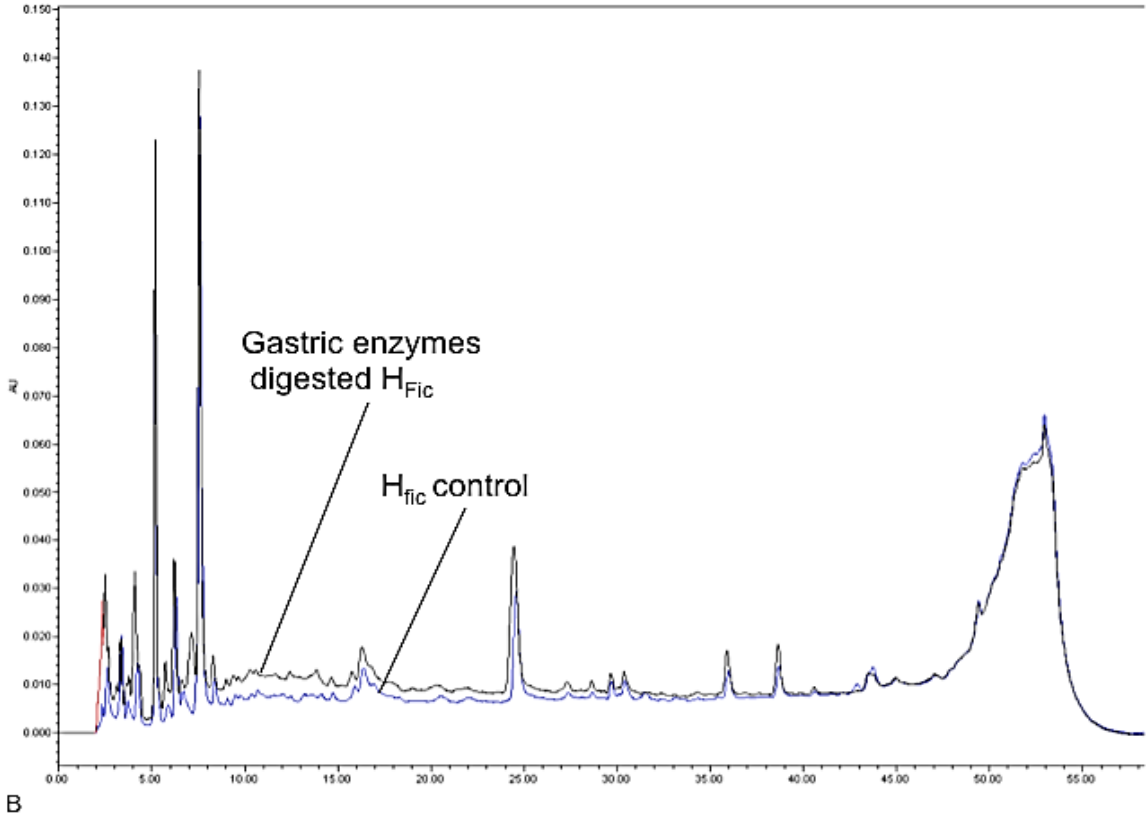

Figure 4.16 HPLC profiles of gastric digested H_{Fic} and H_{Fic} control. Samples were eluted by gradient mobile phase consist of reagent A and B. Mobile phase A (0.05% trifluoracetic acid in water) decreased from 100% to 34% at 45min and mobile phase B (0.05% trifluoracetic acid in acetonitrile) increased from 66% to 100% at 50min, then equilibrated to 0% for 5 min. Absorbance was measured at 280 nm.

Table 4.1 Amino acid composition (molar%) of potato protein hydrolysates produced with ficin (H_{Fic})

Amino acid	Molar%
Asx	12.22
Glx	13.56
Serine	5.27
Glycine	4.21
Histidine	2.19
Arginine	5.90
Threonine	5.14
Alanine	5.12
Proline	4.80
Tyrosine	3.94
Valine	6.20
Methionine	2.51
Isoleucine	5.03
Leucine	9.58
Phenylalanine	5.56
Lysine	7.76
Cysteine	0.87
Tryptophan	0.15

Asx = Aspartic acid + Asparagine; Glx = Glutamic acid + Glutamine

Chapter 5 Discussion and conclusion

The purpose of this study was to evaluate the anti-inflammatory and antioxidative activities of hydrolysates obtained with different proteases from potato proteins. *In vitro* antioxidative activities and inhibition of cellular TNF-α secretion and NO production were considered as the major parameters for screening, whereas zinc chelating activities was used to measure possible effects in inhibiting zinc-dependent TNF-α releasing enzyme, ADAM17. Based on previous studies, TNF-α functions as major pro-inflammatory cytokine mediating a series of inflammatory responses and related diseases through the NF-κB pathway (Silva et al., 2010). Results in this study indicate that the potato protein hydrolysates possess TNF-α inhibitory activities in mouse ANA-1 and primary cell models. Particularly, H_{Fic} and $H_{Pep+panc}$ exhibited substantial suppression of TNF-α secretion in ANA-1 and primary cell models, respectively. A number of enzymatic hydrolysates and peptides derived from milk, egg and soybean proteins have shown anti-inflammatory activities related to the NF-κB pathway (Haversen et al., 2002; Vernaza et al., 2012; Majumder et al., 2013; Iskandar et al., 2013). This study is the first to demonstrate the anti-inflammatory activities of hydrolysates derived from potato proteins. Despite the promising activities, the current literature does not provide clear mechanisms of the hydrolysates' functions, especially as it relates to their structures and composition. In this study, it was hypothesized that the potato protein hydrolysates that bind zinc can chelate the divalent metal

co-factor and inhibit the enzymatic activity of ADAM17 and subsequently inhibit TNF-α activation. This hypothesis was not supported by the findings from the cellular studies, as ADAM17 activity remained the same in the inflammatory hydrolysate-treated and normal cells. In fact, previous studies reported that supplemented zinc could reduce the occurrence of inflammation; conversely, zinc deficiency was proposed to promote inflammation by enhancing pro-inflammatory cytokine gene expression and secretion (Prasad et al., 2004; Haase et al., 2007; Foster & Samman, 2012). Cytokines secretion and respiratory burst are major features of inflammation. In human subjects, oral ingestion of zinc supplement attenuated occurrence of plasma lipid peroxidation, and isolated monocytes from the subjects showed decreased pro-inflammatory cytokines (TNF-α and interleukins) mRNA expression (Prasad et al., 2004; Foster & Samman, 2012). Zinc-chelating hydrolysates developed from this study were produced for oral ingestion and can function as non-specific chelator. This may diminish plasma zinc level potentially leading to increased cytokines and inflammatory responses. Besides TNF-α is not the only transmembrane proteins cleaved by ADAM17, which has very board substrates range including receptors (neurotrophin, growth hormone, IL-1, IL-6 and TNF-α receptors), ligands (delta-like ligand-1, kit ligand 1- and -2), cell adhesion molecules (L-selectin, VCAM-1) and precursors (pro-amphiregulin, pro- epiregulin and pro-HB-EGF) (Edwards et al., 2008). Therefore, suppression of ADAM17 activity may affect various TNF-α related molecules and pathway, which can lead to positive or negative effects on

the inflammatory process. Therefore, zinc chelation and ADAM17 inhibitory mechanism could not be used to explain the anti-inflammatory property of the potato protein hydrolysates. There is a possibility that the peptides within the hydrolysates permeated into cells to directly inhibit inflammatory signaling molecules. Besides, the anti-inflammatory sites of action of the ficin-hydrolyzed proteins could be located in the extracellular area, where the peptides can binds with receptors PAMPs and DAMPs such as toll-like and NOD-like receptors. These possible mechanisms need to be evaluated in subsequent studies.

Oxidative stress reported to promote inflammation related diseases through activation of NF-κB pathway (Rahman & Adcock, 2006). Therefore antioxidative effect can be one possible mechanism of reducing the damages induced by inflammatory reactions although the detailed mechanism remains unclear. Results of FRAP assays in both *in vitro* and animal models indicated all potato protein hydrolysates, especially ficin derived hydrolysate can potentially impair oxidative stress by their reducing activities.

The nitrite assay demonstrated that the potato protein hydrolysates suppressed NO production during cellular inflammation. NO is a vital gaseous mediator that can diffuse through cell membranes and react with ROS; it also regulates tissue metabolism by inducing vasodilation and leukocytes adhesion during inflammation (Sharma et al., 2007). NO exhibits both pro- and anti-inflammatory properties under different conditions. Previous studies reported that eNOS is mostly responsible for

regular NO synthesis up to physiological concentration range of 100 pM to 5 nM; at this time, NO mainly performs anti-inflammatory functions (Cirino et al., 2003; Hall & Garthwaite, 2009). However, during inflammation, cytokine-induced iNOS activity dramatically increases NO production and causes a series of inflammatory responses (Guzik et al., 2003). Importantly, the life span of iNOS derived NO is very short since it reacts with NOX-produced ROS, and the inflammatory induction time in this study was too long to detect accurate nitrite levels. This could be the explanation of the similar levels of nitrite observed in this study for the primary cells under normal and inflammatory conditions, and the undetectable nitrite levels in the animal serum and peritoneal fluid. Consequently, the cellular nitrite level provided evidence of the beneficial roles of the potato protein hydrolysates, but is not enough parameter for determining if the hydrolysates were exclusively anti-inflammatory or antioxidative or both.

Although the hydrolysates generated with gastric proteases demonstrated promising anti-inflammatory and antioxidative activities in the cell cultures, $H_{Pep+panc}$ is a product of multiple enzymatic activities (pepsin, amylase, lipase, trypsin, chymotrypsin, elastase, amino- and carboxypeptidases). This increases the difficulty of peptide characterization and structure predications, which are crucial for understanding structure-function relationships and in designing more active peptides for functional food and nutraceutical development. Moreover, multiple enzyme hydrolysis increases chance of producing free amino acids, which are less likely to exhibit bioactivity. Since

H_{Fic} possesses *in vitro* zinc chelating and antioxidative activities, and also exhibited pronounced inhibition of cellular TNF-α secretion and NO production, it was chosen for further analysis in mice model of inflammation. The hydrolysates was generated with highly specific protease (ficin), which provides a better platform for subsequent *in silico* analysis and peptide characterization.

In the BALB/c mice experiment, the mice that received different doses of H_{Fic} through intraperitoneal injection had significantly lower levels of pro-inflammatory cytokines IL-6 and IL-12 (p70) in the serum and intraperitoneal fluid especially at the higher dose, whereas TNF-α and inflammation-mediated NO levels were not detectable in the samples. This could be due to the fact that the interleukins are downstream indicators of inflammation compared to TNF-α, which could be have been depleted in the fluids after exerting its activity, or may just be present in undetectable amounts. As earlier discussed, the NO levels are difficult to detect after prolonged duration due to its short physiological life span. Despite the strong physiological evidence, the mechanism of the anti-inflammatory activity of H_{Fic} still remains unclear.

The bioactivities of protein hydrolysates and peptides have been demonstrated to be dependent on the degree of hydrolysis (Udenigwe & Aluko, 2012), which in turn determines their surface properties. These relationships were not observed in the cellular anti-inflammatory assays, and this could be due to the complex interactions of the hydrolysates constituents with the cell matrices, and the likelihood of further proteolytic processing of the peptides within the cell cultures. Several studies have

reported anti-inflammatory activities for low molecular weight peptides derived from food proteins (Hwang et al., 2011; Nielsen et al., 2012; Vernaza et al., 2012; Majumder et al., 2013; Iskandar et al., 2013). However, a high molecular weight peptide, lunasin, derived from soybean was reported to down-regulate the production of IL-6, IL-1β, NF-κB, iNOS and NO, indicating anti-inflammatory function (De Mejia & Dia, 2009) Besides, other factors such as the peptide sequence and amino acid compositions (not evaluated for all the hydrolysates in this study) can also determine bioactivity of protein hydrolysates (Udenigwe & Aluko, 2012). Regarding the latter, the difference in sulfhydryl content (indicating the amount of free cysteine residue) of the potato protein hydrolysates may have impacted their ability to suppress cellular NO production (and not *in vitro* FRAP). This activity could be due to electron donation by the sulfhydryl group leading to its dimerization (Elias et al., 2008), although this mechanism would be more relevant in free radical quenching than ROS scavenging. Therefore, the structure-function relationships of antioxidative and anti-inflammatory peptides and protein hydrolysates appear complex especially within physiological matrices.

Therapeutic application of anti-inflammatory hydrolysates can be limited by physiological bioavailability, which refers to the ability of peptide to maintain their functions after gastric digestion and interaction with biological targets. For the purpose of developing functional food products, nutraceutical or food additives, gastric stability of peptides is a primary requirement and is thought to impede rapid development of

physiologically active peptide products (Udenigwe, 2014). As demonstrated in the simulated gastric digestion study, H_{Fic} appears stable to gastric enzyme processing based on the HPLC peptide fingerprints. However, this property should be further evaluated especially for the hydrophobic peptides in the hydrolysates using more advanced techniques with higher resolving power (e,g, LC-MS or 2D gel electrophoresis). Several methods were reported to enhance gastric stability of these bioactive peptides. Encapsulation is a new technique used to protect bioactive peptides from digestion and deliver them to target sites (Hu et al., 2012). It is thought that small-size capsules formed from hydrophobic matrices are easier to be absorbed in intestinal layer (Cabuka et al., 2014). Besides, production of bioactive peptides using gastric enzymes would release peptides having better resistance to further digestion (Segura-Campos et al., 2011).

Another aspect of bioavailability refers to interaction between protein hydrolysates and biological targets. Normally, intestinal peptide transportors can only transport di- or tripeptides; however, depending on peptide composition, there are other alterative absorption routes (Segura-Campos et al., 2011). High hydrophobicity can promote the hydrolysates' interaction with non-polar tissues such as adipocytes and in permeating through highly lipophilic interior of cell membranes, where they can function in inhibiting lipid peroxidation. Therefore, the surface properties of the hydrolysates play an important role in increasing peptide bioavailability. Moreover, the cysteine content of peptides is liable to oxidation potentially leading to antioxidant activities. The

hydrophobicity and sulfhydryl group content of H_{Fic} are not particularly high to be considered responsible for the observed antioxidative and anti-inflammatory activities in this study. The amount of the amino acid residues contributing to the structural properties and bioactivity can be increased by further fractionation and purification of the protein hydrolysates (Udenigwe & Aluko, 2012).

In conclusion, potato protein hydrolysates produced with enzymes ficin (H_{Fic}) and pepsin/pancreatin combination ($H_{Pep+panc}$) demonstrated prominent activities in reducing inflammatory events in cultured mice primary cells and cell line. These findings will serve as evidence for future studies in our lab on the development of effective potato protein-derived peptides that can be used against inflammation and oxidative stress. Future directions of this project include (1) bioassay-guided purification of H_{Fic} to isolate pure peptides and identify structural basis of the anti-inflammatory activities, (2) study of the physiological stability and bioavailability of peptides in HFic to confirm that the peptides would reach their target tissues, and if not bioavailable, (3) molecular encapsulation of the hydrolysates, for increased stability and bioavailability of the constituent peptides.

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