CHAPTER II

LITERATURE REVIEW

2.1 Inflammation

The development of an inflammatory response is an important mechanism by which an organism defends itself against pathogenic agents and initiates both structural and functional repair of damaged tissue. It is characterized by the movement of fluid, plasma proteins, and leukocytes into target tissues in response to injury, microbial invasion, foreign material, or antigens. The purpose of this response is to contain or destroy the pathogenic insult and to remove the tissue debris resulting from this response. Inflammatory response has been viewed as a continuum from the early stages of acute inflammatory to a more chronic inflammatory response followed by duration criteria (Sigal and Ron, 1994; Kindt et al., 2007).

2.1.1 Acute Inflammatory Response

An acute inflammatory response has a rapid onset and short duration. This is a complex cascade of non-specific events that provides early protection by restricting the tissue damage. The acute inflammatory response involves both localized and systemic responses. The cardinal signs of localized acute inflammatory response are swelling, redness, heat, and pain. Tissue macropharges recognize microbial products. The macrophages release cytokines and other inflammatory mediators such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α that cause vasodilation, increased vascular permeability and have chemotactic effects on monocytes and neutrophils. Monocytes and neutrophils are recruited to the site and there is accumulation of plasma fluid and proteins at the site causing edema.

Inflammatory mediators can activate mast cells to release further mediators that amplify the response. When the local production of cytokines is high enough, the cytokines then travel in the blood and affect other organs, particularly the brain. The clinical signs of systemic acute-phase response are fever, anorexia, somnolence, increased synthesis of hormones such as hydrocortisone, increased production of white blood cell number, and production of a large number of acute-phase proteins in the liver (Sigal and Ron, 1994; Wood, 2001; Kindt et al., 2007).

2.1.2 Chronic Inflammatory Response

Chronic inflammation develops because of the persistence of antigen. For example, some microorganisms have cell-wall components that enable them to resist the host defense mechanisms, resulting in significant tissue damage. Chronic inflammation also occurs in a number of autoimmune diseases, which self-antigens continually activate T cells (thymus derived cells). In addition, many conditions can lead to a chronic inflammatory state such as cancer and hypertension. The accumulation and activation of macrophages are hallmarks of chronic inflammation. Cytokines released by the chronically activated macrophages also stimulate fibroblast proliferation and collagen production (Goldsby et al., 2001; Wood, 2001).

2.2 Inflammatory Mediators

The inflammatory mediators (inflammatory markers) are soluble, diffusible molecules that act locally at the site of tissue damage and infection and at more distant sites (Štvrtinová, 1995). Inflammatory mediators are typically induced in response to inflammation. They include a variety of pro-inflammatory cytokines,

acute phase proteins, lipid mediators, proteases, plasma-, and cell-derived growth factors (Sigal and Ron, 1994).

Pro-inflammatory cytokines are cytokines produced predominantly by activated immune cells, such as monocytes and/or macrophages. These cytokines involved in the amplification of inflammatory process. They display pleiotropic functions in different cells and tissues. In spite of the fact that they interact differently in structural receptors, their activities overlap substantially. Pro-inflammatory cytokines encompass IL-1, IL-6, and TNF-α (Mantovani, 2000).

Acute phase proteins (APPs) or acute phase reactants (APRs) are the proteins whose concentrations rose or fell during the acute phase response. Those proteins which are up-regulated during inflammation are called positive acute phase proteins; those whose synthesis is diminished are termed negative acute phase proteins. APPs represent a complex, nonspecific, rapid response to many types of tissue damage. APPs serve to control damage, clean up debris, and start repair. These functions are accomplished by alterations in hepatic protein synthesis which accompany inflammation. In addition, pro-inflammatory mediators TNF-α, IL-1, and IL-6 are the major signals responsible for induction of the acute phase response. APPs such as complements, ceruloplasmin, fibrinogen, C-reactive protein (CRP), and serum amyloid A contribute to defense in several ways (Sigal and Ron, 1994; Kindt, 2007).

2.2.1 Interleukin (IL)-6

IL-6 is a variably glycosylated protein with a molecular mass of 22-27 kilodalton depending on the cellular source and amount of post-translational modification. It is synthesized as a precursor protein of 212 amino acids, with a 28 amino acids signal sequence and a 184 amino acids mature segment. IL-6 is a member

of a family of cytokines that consists of leukemia inhibitory factor, IL-11, ciliary neurotropic factor, oncostatin M, and cardiotrophin 1. Their membership is based on similarities in helical protein structure and a shared receptor subunit (transmembrane glycoprotein 130) (Febbraio and Pedersen, 2002).

IL-6 is produced by many different cells, but the main sources in vivo are stimulated monocytes/macrophages, fibroblasts, and vascular endothelial cells. Other cells known to express IL-6 include keratinocytes, osteoblasts, T cells, bone marrow derived cells (B cells), neutrophils, eosinophils, mast cells, smooth muscle cells, and skeletal muscle cells. Recently, 10-35% of the body's basal circulating IL-6 is derived from adipose tissue (Febbraio and Pedersen, 2002). Typical stimuli for IL-6 production are IL-1, TNF-α, and bacterial endotoxin. Concentrations of circulating IL-6 are low in healthy humans, generally at levels of ≤ 4 pg/ml (Carey and Febbraio, 2004).

IL-6 acts on target cells via complex receptor system composing of IL-6 receptor, a ligand binding subunit. After IL-6 binds to IL-6 receptor, the complex induces the signal transduction receptor component. Regulators of IL-6 activity and IL-6 receptor have been reported to be an important signaling pathway intimately involved in the transition between the early and the late phase of the inflammatory response (Kim et al., 2009).

IL-6 is independently described by various groups as hepatocyte stimulating factor, hybridoma growth factor, interferon (IFN)-β2, and B cell-stimulating factor 2. IL-6 is multi-functional cytokine acting on many cells and tissues. One of the main effects of IL-6 is the induction of hepatic CRP production (Bastard et al., 2006). IL-6 enhances immunoglobulin secretion by B cells and

synergizes with IL-3 to support proliferation of neutrophil, monocyte, eosinophil, and megakaryocyte colonies. It inhibits the growth of fibroblasts and induces class I human leukocyte antigen (HLA) expression on their cell surfaces. In some systems, IL-6 can elicit the full APPs of liver cells cultured in vitro, and probably serves to amplify the APPs in vivo. Its effects on the APPs involve a complex interaction with several other proteins, notably IL-1 and TNF. IL-6 also augments natural killer cell (NK)-cell activity (Sigal and Ron, 1994). In addition, IL-6 is a possible mediator of metabolic processes (Febbraio and Pedersen, 2002).

2.2.2 C-Reactive Protein (CRP)

CRP is a complex molecule composed of 523 kilodalton subunits and has been classified as a member of the pentraxin family, which bound ligands in a calcium-dependent reaction. The ligands are a polysaccharide found on the surface of pneumococcal species and phosphorylcholine, which are present on the surface of many microbes. CRP bound to these ligands on the surface of a microbe promotes uptake by phagocytes and activates a complement-mediated attack on the microbe (Kindt, 2007).

CRP is predominantly made in the liver. It is secreted within 6 hours after initial tissue injury and continues to increase several hundred folds at least every 8 hours, reaching a peak after about 50 hours. CRP remains elevated during the acute-phase response, and returns to normal with restoration of tissue structure and function. The median normal concentration of CRP is 0.8 mg/l. It appears that approximately 90% of healthy individuals have CRP value less than 3 mg/l, and 99% have less than 12 mg/l. Elevated values are abnormal suggesting the presence of inflammation disease (Reeves, 2007).

An extensive body of epidemiologic and clinical trial evidence has been accumulated concerning CRP role in atherosclerosis. Additionally, the utility of CRP as a marker of cardiovascular risk stratification has continued to expand as improved methods of analysis have been developed. The original assays for CRP exhibited wide ranges of normality. Considerable intra-individual variability was problematic in analyzing the clinical relevance of relatively minor changes occurring over time. The subsequent availability of high-sensitivity assay is an automated blood test designed for greater accuracy in measuring low levels of CRP. High-sensitivity CRP (hs-CRP) has allowed the analysis of changes of hs-CRP within the normal range. Epidemiologic studies in primary prevention have demonstrated that a single measurement of hs-CRP is a strong predictor of future vascular events and is independent of the traditional cardiac risk factors (Heber, 2008). The concentrations of hs-CRP were classified according to the American Heart Association recommendation:

hs-CRP	< 1 mg/l	low cardiovascular risk
	1-3 mg/l	intermediate cardiovascular risk
	> 3 mg/l	high cardiovascular risk

If a level of hs-CRP >10 mg/l is identified, there should be a search initiated for an obvious source of infection or inflammation, which could obscure any prediction of coronary risk that might be attributed to the elevated level (Pearson et al., 2003).

2.3 Inflammation and Diabetes Mellitus

The relationship between DM and inflammation is supported by the demonstration that inflammatory mediators are strong predictors of development of type 2 DM. Pradhan et al. (2001) designed a 4-year follow-up prospective study in 27,628 women. The subjects were free of diagnosed DM, cardiovascular disease, and cancer. The result showed that 188 women had developed DM. The baseline IL-6 and hs-CRP levels of developing diabetes subjects were significantly higher than matched 362 disease-free controls. The results agreed with study of Duncan et al. (2003). They established that DM occurred in 581 subjects during 9 years follow-up period of 10,275 without DM case-cohort study. The baseline inflammatory mediator (IL-6, hs-CRP, sialic acid, orosomucoid) levels of those were significantly increased when compared to 572 noncases. Consistently, Tan et al. (2003) designed a prospective study in 2,900 subjects without DM. The subjects were underwent a 75-g oral glucose tolerance test (OGTT). A total 288 subjects with impaired glucose tolerance (IGT) underwent repeat OGTT. After 2 years, plasma hs-CRP was measured from their stored baseline samples and from 228 subjects with non-IGT matched for age and body mass index (BMI). The result found that plasma hs-CRP of the subjects with IGT was significantly greater than the controls.

There was a study of 2,052 initially nondiabetic men aged 45-74 years. The subjects were followed up for an average of 7.2 years. The result showed that DM occurred in 101 subjects during the follow-up period. Men with hs-CRP levels in the highest quartile (hs-CRP $\geq$ 2.91 mg/l) had a 2.7 times higher risk of developing diabetes, compared with men in the lowest quartile (hs-CRP $\leq$ 0.67 mg/l) (Thorand et al., 2003). Likewise, Spranger et al. (2003) designed the case-control study of 27,548

free of type 2 DM subjects. During a 2.3 year follow-up period, they found that 192 cases had developed type 2 DM. The baseline IL-6 and TNF-α levels of the subjects with incident type 2 DM were elevated when compared to 384 non-disease-developing control subjects. The reports were similar to the case-control study of Hu et al. (2004). They studied in 32,826, free of DM subjects, cardiovascular disease, and cancer. They revealed that 737 cases had developed type 2 DM. The baseline TNF-α receptor 2, IL-6, and hs-CRP levels of the developed DM subjects were significantly higher than the 785 control cases.

The relationship between DM and inflammation is also supported by the numerous studies that plasma IL-6 and hs-CRP are elevated in individuals with DM. Pickup et al. (1997) showed that IL-6 in subjects with more than two features of metabolic syndrome had greater inflammation (increased serum hs-CRP and IL-6 levels) when compared to those who had fewer than two features. Similarly, Ford (1999) performed that the hs-CRP concentration of participants with a BMI of 25 to ≥ 40 kg/m^2 were elevated when compared to participants with a BMI < 25 kg/m^2. The individuals without diabetes or with impaired fasting glucose had lower plasma hs-CRP concentrations when compared to newly or previously diagnosed diabetes individuals. The results were consistent with study of King et al. (2003). They found that the diabetic patients who had elevated hemoglobin A1c (HbA1c) levels ($\geq 9.0\%$) had significantly higher percentage of elevated hs-CRP than individuals with low HbA1c levels ($< 7\%$). Consistently, Pickup et al. (2000) reported that plasma IL-6 and TNF-α of type 2 DM subjects were significantly increased compared to normal subjects when stimulating blood cells of those with lipopolysaccharide (LPS).

Table 1 Composition of bovine milk

Component	Average percentage	Range for average percentage
Water	86.6	85.4-87.7
Fat	4.1	3.4-5.1
Non-fat solids		
Protein	3.6	3.3-3.9
Lactose	5.0	4.9-5.0
Ash	0.7	0.68-0.74

Source: Swaisgood, 1996

2.4 Whey Protein

Whey protein is a protein complex derived from milk. Milk is the secreted fluid of the mammary glands of female mammals. It contains nearly all the nutrients necessary to sustain life. The average composition of milk with respect to major classes of compounds and the range of average values for milks of western cattle are shown in **Table 1** (Swaisgood, 1996).

Milk contains 30-36 g/l of total protein, and it rates very high in nutritive quality. Milk proteins are classified as either caseins or whey proteins. Casein and whey protein comprises 80% and 20% respectively of the bovine milk proteins (**Table 2**) (Schaafsma and Steijns, 2000). In addition, amino acid composition of the total protein, casein, and whey protein of bovine milk are presented in **Table 3** (Belitz, 2009).

Traditionally, whey was regarded in the dairy industry as an undesirable by-product. The discovery of whey protein was a potentially valuable source of nutrients that stimulates interest in the development of commercially viable processes to convert liquid whey into viable animal and human food products. Whey protein is now regarded as a co-product of cheese and casein manufacture, and the objective of the industry is to apply the best technologies available, which allow the profitable processing of large volumes of high quality whey into products that can be advantageously used in a broad range of food, feed and industrial applications (Mulvihill and Grufferty, 1997).

Whey is the serum liquid portion remaining after removal of fat and casein from milk during the manufacture of cheeses and casein. After processing occurs, casein is the protein responsible for making curds, while whey remains in an aqueous environment. Liquid whey processed in advance technologies including ultrafiltration, microfiltration, reverse osmosis, and ion-exchange have resulted in development of several different finished whey protein products. The whey protein products consist of whey protein concentrate (WPC), whey protein isolate (WPI), and whey protein hydrolysate (WPH). Each whey product varies in the amount of protein, carbohydrates, immunoglobulins (Ig), lactose, minerals and fat in the finished product. These variables are important factors in the selection of whey fractions for specific nutritional applications (Marshall, 2004). **Table 4** describes the various whey protein products availability.

Table 2 Concentrations of the major proteins in milk

Protein	Concentration (g/l)	Approximate percentage of total protein	
Caseins	24-28		80
α-caseins	15-19	42	
β-caseins	9-11	25	
κ-caseins	3-4	9	
γ-caseins	1-2	4	
Whey proteins	5-7		20
β-LG	2-4	9	
α-LA	1-1.5	4	
Proteose-peptones	0.6-1.8	4	
BSA	0.1-0.4	1	
Ig	0.6-1.0	2	
LF	0.02-0.2		
lactoperoxidase	0.03		
Total		100	100

g/l = gram per liter; α = alpha; β = beta; γ = gamma; κ = kappa; β-LG =beta lactoglobulins; α-LA =alpha lactalbumin; BSA = bovine serum albumin; Ig = Immunoglobulins; LF = lactoferrin
Source: Swaisgood, 1996; Schaafsma and Steijns, 2000

Table 3 Amino acid contents of total protein, casein, and whey protein of bovine milk[1]

Amino acid	Total protein	Casein	Whey protein
Alanine	3.7	3.1	5.5
Arginine	3.6	4.1	3.3
Aspartic acid	8.2	7.0	11.0
Cystine	0.8	0.3	3.0
Glutamic acid	22.8	23.4	15.5
Glycine	2.2	2.1	3.5
Histidine	2.8	3.0	2.4
Isoleucine	6.2	5.7	7.0
Leucine	10.4	10.5	11.8
Lysine	8.3	8.2	9.6
Methionine	2.9	3.0	2.4
Phenylalanine	5.3	5.1	4.2
Proline	10.2	12.0	4.4
Serine	5.8	5.5	5.5
Threonine	4.8	4.4	8.5
Tryptophan	1.5	1.5	2.1
Tyrosine	5.4	6.1	4.2
Valine	6.8	7.0	7.5

[1]: g amino acid per 100 g protein
Source: Belitz, 2009

Table 4 Types of commercially available whey proteins

Product	Protein concentration	Nutritional content
WPI	90-95%	Little if any
WPC	• Range from 25-89% • Most commonly available as 80%	• Some fat, lactose and minerals • As protein concentration increases, fat, lactose and mineral content decreases
WPH	• Variable • Hydrolysis used to cleave peptide bonds • Larger proteins become smaller peptide fractions with non-hydrolyzed	Varies with protein concentration

WPI = whey protein isolate; WPC = whey protein concentrate; WPH = whey protein hydrolysate
Source: Marshall, 2004

Table 5 Components found in whey protein

Whey components	% of whey protein	Benefit
β-LG	50-55%	Source of essential and branched chain amino acid (BCAA)
α-LA	20-25%	• Primary protein found in human breast milk • Source of essential amino acid and BCAA
Ig	10-15%	• Primary protein found in colostrum • Immune modulating benefits
GMP	10-15%	Source of BCAA
BSA	5-10%	Source of essential amino acid
LF	1-2%	• Antioxidant • Antibacterial, antiviral and antifungal • Control of the immune response during infection and inflammation
Lactoperoxidase	0.5%	Inhibits growth of bacteria

β-LG = beta lactoglobulins; α-LA = alpha lactalbumin; Ig = Immunoglobulins; GMP = glycomacropeptide; BSA = bovine serum albumin; LF = lactoferrin; BCAA = branched chain amino acid
Source: Marshall, 2004; Schaafsma and Steijns, 2000

The major proteins in whey include β-LG, α-LA, and BSA. The minor proteins comprise protease-peptone and GMP. A number of biologically active proteins include Ig, LF, and lactoperoxidase (Mulvihill and Grufferty, 1997). These proteins have been implicated in a number of biological effects observed in human and animal studies, such as antioxidant and inhibition of bacteria growth immune modulation (Marshall, 2004). **Table 5** summarizes the components found in whey protein.

Whey proteins are rich in cysteine and glutamic acid. This suggests that ingestion of whey proteins may contribute to increase the level of free cysteine and consequent production of glutathione (GSH). GSH is the tripeptide γ-glutamylcysteinylglycine. It has several important functions. It is a reductant which conjugated to drugs to make them more water soluble. It is involved in transport of amino acids across cell membranes. It is a part of some leukotriene structure, and it is a cofactor for some enzymatic reactions. GSH is synthesized form cysteine, glutamate and glycine by which cysteine incorporation is the rate-limiting step for its synthesis (Devlin, 2002; Sardesai, 2003; Madureira et al., 2007). Moreover, glutamine is partially oxidized in enterocytes to supply energy and precursor molecules for synthesis of pyrimidines and purines. Enterocytes, lymphocytes and marcrophages use glutamine as their major fuel source to ensure a continuous supply of the precursor molecules required for synthesis of pyrimidines and purines that these rapidly diving cells need for the synthesis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) (Harris and Crabb, 2002).

2.5 Whey Protein and Immune System

The relationship between whey protein and immune sytem is supported by the numerous studies. The studies showed that whey protein affects both specific and non-specific immune systems such as anti-inflammatory, lymphocyte proliferation, cytokine secretion, phagocytosis, and antibody response.

In vitro studies, Cross and Gill (1999) studied the effect of modified whey protein concentrate (mWPC) on murine immune function. The mWPC derived as a by-product from the commercial manufacture of cheese. The result showed that mWPC suppressed T and B lymphocyte proliferation and suppressed secretion of interferon (IFN)-γ and IL-4 to mitogens in a dose-dependent fashion. The immunosuppressive activity of mWPC would be beneficial for adult patients with chronic inflammatory disease of the digestive tract, particularly those with a lymphocyte-based action, such as atopic reactions and celiac disease. The effect of whey protein on lymphocytes proliferation in murine spleen was reported by Mercier et al. (2004). They demonstrated that microfiltrered-whey protein isolates (MF-WPI) and their enzymatic digest could stimulate lymphocytes proliferation. This suggested that whey protein contain some immunomodulating peptides, which can be released by enzymatic digestion.

Consistenly, Saint-Sauveur et al. (2007) determined effect of WPI, enzymatic digest, and peptide fractions on immunomodulatory of murine splenocytes. This study showed that enzymatic digest and peptide fractions stimulated the secretion of T helper (Th) 1 cytokines (IL-2 and/or IFN-γ). The peptide fractions with acidic mainly stimulated the secretion of Th1 cytokines. This result suggested that these peptides released from whey proteins by intestinal proteases might shift the

immune system towards a Th1 response and might play a valuable role in fighting infections.

The effect of WPC on specific antibody responses in mice was studied by Low et al. (2003). The mice underwent orally or parenterally antigens, including influenza vaccine, diphtheria, tetanus toxoids, poliomyelitis vaccine, ovalbumin, and cholera toxin sub-unit. The result found that WPC-fed mice produced elevated levels of antigen-specific intestinal tract and serum antibodies against all testes antigens, compared to mice that were fed a standard diet. This indicated that WPC could increase humoral immune responsiveness to T-dependent vaccine antigens. WPC may be an adjunct dietary treatment to boost post-vaccination responses in humans, particularly in children and elderly.

The results were consistent with study of Rutherfurd-Markwick and Gill (2005a). They studied the effect of IMUCARE WPC, cheese WPC, and soybean protein on immune function in mice for 8 weeks. The study found that splenic lymphocytes derived from mice fed with IMUCARE WPC were significantly elevated proliferative responses to mitogen, compared with soy-fed mice. In addition, mice fed with whey protein tended to exhibit elevated mean intestinal tract antibody responses to orally administered ovalbumin and cholera toxin antigens, whereas soybean protein did not affect antibody responses. Similarly, Rutherfurd-Markwick and Gill (2005b) investigated the effect of IMUCARE WPC on immune system in mice. The mice were fed with MMP-based diets (nutritionally balance for young children) with or without IMUCARE WPC supplemented at 10.5g/100g of diet for 4 weeks. The result indicated that splenic lymphoproliferative response, blood and peritoneal leukocyte

phagocytic activity of mice fed MMP supplemented with IMUCARE WPC were elevated compared with either mice fed with MMP alone or control mice.

Grey et al. (2003) studied the effect of whey protein supplementation on glutathione levels in stable patients with cystic fibrosis (an inflammation diease). Twenty-one patients were randomly assigned to take 10 g of whey protein isolate twice a day or casein placebo for 3 months. The result found that the lymphocyte GSH levels were significantly increased from baseline in the supplemented group. This result showed that whey protein supplementation could increase GSH levels in cystic fibrosis. Moreover, there was a study about the effect of whey protein supplementation on cytokine secretion in children with atopic asthma (a Th 2 cytokine disease) (Lothian et al., 2006). The atopic asthma patients consumed 10 g of whey protein supplement twice daily. Eleven children underwent spirometry, methacholine provocation testing. Blood samples were analysed for serum IgE and lymphocyte GSH before and after 1 month of supplementation. The result found that IgE was significantly decreased following supplementation but no significant changes were found in lymphocyte GSH or FEV_1 (forced expired volume in 1 second).

The relationship between whey protein and immune sytem is supported by the demonstration that whey protein can be reduced the inflammatory mediators. Oben et al. (2008) determined the effect of oral proteolytic enzyme system on whey protein concentrate metabolism in healthy males. The subjects took 50 g WPC containing either 2.5 g or 5 g of a proteolytic enzyme from food grade *Aspergillus niger* and *Aspergillus oryzae* as test group. The subjects were received 50 g WPC as control group. Blood samples were collected for amino acid and CRP analyses (monitor differences in WPC absorption) and 24-hour urine was collected for total

nitrogen analysis. In the test group showed amino acids levels, nitrogen excretion was significantly increased and CRP level was significantly decreased compared to the control group. These results indicated that WPC supplemention with protease enzyme can increase WPC absorption rate.

There is a study about the effect of whey protein component on inflammation. Lactoferrin (LF) is a component of whey protein. It is an iron-binding glycoprotein and a non-enzymatic antioxidant (Marshall, 2004). Mattsby-Baltzer et al. (1996) found that the human LF, bovine LF, and lactoferricin B (a bactericidal pepsin-derived fragment of bovine LF) can suppress the LPS (lipopolysaccharide)-induced IL-6 from monocytic cell line (THP-1). Likewise, Håversen et al. (2002) reported that LF down regulated cytokine secretion of human monocytic cells when stimulate with LPS. The results suggested that LF had an anti-inflammatory activity.