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ANTIOXIDANT ACTIVITY IN COOKED AND GASTROINTESTINAL ENZYME DIGESTED EGGS

by

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ABSTRACT

The avian egg is an excellent source of nutrients, and consists of components with beneficial properties but there is a limited knowledge on the effect of various cooking methods and gastrointestinal digestion on antioxidant activity of eggs. The present study was focused on the effect of cooking and simulated gastrointestinal digestion on antioxidant activity of eggs by 3 assays; Oxygen radical absorbance capacity (ORAC) assay, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ABTS decolorization assay, and 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) assay. The results suggest that fresh egg yolk have higher antioxidant activity than fresh egg white and whole eggs. Cooking reduced but simulated gastrointestinal digestion increased the antioxidant activity. Boiled egg white hydrolysate showed the highest antioxidant activity; a total of 63 peptides were identified, indicative of the formation of novel antioxidant peptides upon simulated gastrointestinal digestion. This study suggests the potential role of eggs as dietary source of antioxidants.

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LIST OF ABBREVIATIONS

ALA	-	α-lipoic acid
AAPH	-	2, 2'-azobis (2-amidino-propane)
ABTS	-	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
AGE	-	Advanced glycation end products
ALE	-	Advanced lipoxidation end products
AMD	-	Age related macular degeneration
ANOVA	-	Analysis of variance
CAT	-	Catalase
CRNI	-	Canadian Recommended Nutrient Intake
DHLA	-	Dihydrolipoic acid
DHA	-	Docosahexaenoic acid
DPPH	-	1, 1-Diphenyl-2-picryl-hydrazyl
EPA	-	Eicosapentaenoic acid
ESI	-	Electrospray ionization technique
GSH	-	Glutathione
GPx	-	Glutathione peroxidase
Gred	-	Glutathione reductase
GSSG	-	Oxidized glutathione
H_2O_2	-	Hydrogen peroxide
HNE	-	4-hydroxynonenal
$^{\bullet}OH$	-	Hydroxyl radical
$O_2^{\bullet -}$	-	Superoxide anion radical
$^1O_2$	-	Singlet oxygen
O_3	-	Ozone
$ONOO^-$	-	Peroxynitrite anion
ONOOH	-	Peroxynitrous acid
LOOH	-	Lipid hydroperoxide
$LOO^{\bullet}$	-	Lipid peroxyl radical
$L^{\bullet}$	-	Lipid radical
LPC	-	Lysophosphatidylcholine
LC-MS/MS	-	Liquid chromatography tandem mass spectrometry
MDA	-	Malondialdehyde
NADH	-	Nicotinamide adenine dinucleotide
NAD(P)H	-	Nicotinamide adenine dinucleotide phosphate
$NO^{\bullet}$	-	Nitric oxide
NO_2	-	Nitrogen dioxide
ORAC	-	Oxygen radical absorbance capacity
PC	-	Phosphatidylcholine
PE	-	Phosphatidylethanolamine
PPPs	-	Phosphopeptides
PUFA	-	Polyunsaturated fatty acids
Q-TOF	-	Quadrupole Time-of-Flight
RDA	-	Recommended Dietary Allowance
RMCD	-	Randomnly methylated β- cyclodextrin
RNS	-	Reactive nitrogen species
$RO^{\bullet}$	-	Alcoxyl radical
$ROO^{\bullet}$	-	Peroxyl radical
ROOH	-	Organic hydroperoxide
ROS	-	Reactive oxygen species

RP-HPLC	-	Reverse-phase-high-performance-liquid chromatography
SOD	-	Superoxide dismutase
SPH	-	Sphingomyelin
Srx	-	Sulfiredoxin
SR	-	Sarcoplasmic reticulum
TEAC	-	Trolox equivalent antioxidant capacity
TFA	-	Trifluoroacetic acid
T-OH	-	Vitamin E
$T\text{-}O^{\bullet}$	-	Vitamin E radical

The one letter and three letter codes of amino acids used in the text:

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

CHAPTER -1 LITERATURE REVIEW

1.1 OXIDATIVE SUBSTANCES/FREE RADICALS

Oxygen is essential for all aerobic organisms, but it can be a source of certain molecules capable of destroying cells (Haddad, 2002). As a result of essential biochemical reactions, certain highly reactive oxygen species (ROS) are continuously formed in the body (Serafini, 2006). These reactive oxygen species have a tendency to donate electrons to other substances, many of them are free radicals having one or more unpaired electrons and therefore unstable and highly reactive (Machlin & Bendich, 1987; Bagchi & Puri, 1998). The free radicals are also derived from nitrogen, known as reactive nitrogen species (RNS) (Espey et al., 2000; Moini, Packer, & Saris, 2002; Turrens, 2003). These ROS and RNS formed in the body function as signaling molecules and are well regulated in such a manner to maintain the homeostasis at the cellular level (Devasagayam et al., 2004; Valko et al., 2007). Apart from these endogenous factors, certain exogenous factors like tobacco smoke, certain pollutants, ozone, X-rays, toxic chemicals etc., could also lead to the formation of free radicals (Church & Pryor, 1985; Bagchi & Puri, 1998). The superoxide anion radical ($O_2^{\bullet -}$) formed from cellular metabolism or physical irradiation is considered as a primary ROS, which can further interact with other molecules to generate secondary ROS (Valko, Morris, & Cronin, 2005).The various ROS includes superoxide($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), which yields potent species like $^{\bullet}OH$, peroxyl radical ($ROO^{\bullet}$), organic hydroperoxide (ROOH), singlet oxygen ($^1O_2$), and ozone (O_3); while RNS consists of nitri c oxide ($NO^{\bullet}$), peroxynitrite ($ONOO^-$), peroxynitrous acid (ONOOH) and nitrogen dioxide (NO_2) (Devasagayam et al., 2004; Trachootham, Alexandre, & Huang, 2009). The etiology of many diseases as well as aging, have been associated with the excessive formation of free radicals and there is a surge of research in the areas related to prevention of diseases.

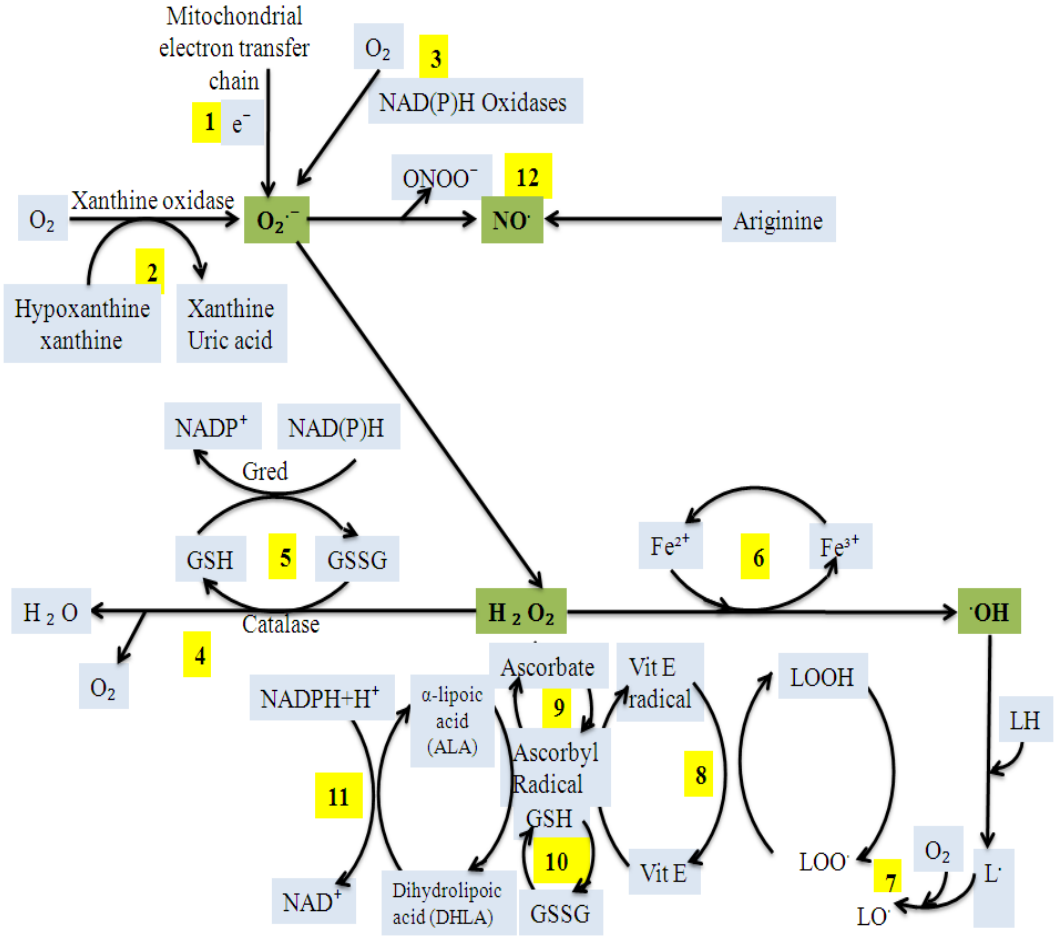

Figure 1.1: Various pathways of Reactive oxygen species (ROS) formation (Modified from (Valko et al., 2007; Trachootham et al., 2009))

1. The main free radical $O_2^{\bullet -}$, the precursor for the formation of H_2O_2, is formed mainly by mitochondrial electron transport chain, the endoplasmic reticulum system and the Nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase (NOX) complex (Dionisi, Galeotti, Terranova, & Azzi, 1975; Turrens, 1997; Liu, Fiskum, & Schubert, 2002; Trachootham et al., 2009).
2. The redox reactions in mitochondrial electron transport chain component, Complex I (NADH dehydrogenase) and Complex III (semi-ubiquinone) plays important roles in the non-enzymatic formation of superoxide (Turrens, 1997; Droge, 2002). It was reported that the electrons possess a greater tendency towards oxygen and forms $O_2^{\bullet -}$ rather than jumping to next electron carrier in the chain (Cadenas & Davies, 2000). In addition to the direct extra mitochondrial release of superoxide, the premature leakage of electrons generated during energy transduction in the mitochondria also forms $O_2^{\bullet -}$ rather than getting reduced to water (Muller, Liu, & Van Remmen, 2004).
3. The enzymatic reduction of molecular oxygen is also carried out by enzymes, NAD(P)H and xanthine oxidase, resulting in the formation of superoxide anion

radical ($O_2^{\bullet -}$) and it is then rapidly converted to hydrogen peroxide by the superoxide dismutase (SOD) (Turrens, 1997; Trachootham et al., 2009).

4. The H_2O_2 may convert back to $O_2^{\bullet -}$ or to water by enzyme catalase (Trachootham et al., 2009).
5. The enzyme glutathione peroxidase (GPx) also can act on H_2O_2 to form water (Cohen & Hochstein, 1963). For this reaction, enzyme GPx requires glutathione (GSH), which acts as antioxidant by donating the electron and then this oxidized glutathione (GSSG) is converted back to GSH by glutathione reductase (Gred), which in turn uses NAD(P)H as the electron donor.
6. In the presence of reduced transition metals (e.g., Fe^{2+}, Cu^{+} and others), the $O_2^{\bullet -}$ and H_2O_2 provides substrate for the highly reactive hydroxyl radical ($^{\bullet}OH$) (Turrens, 2003). Normally, there exists a strict physiological limit with in cell linked to an iron (and copper) redox couple, ensuring there is no free intracellular iron. However, during stress conditions, increased level of superoxide radicals leads to abnormal release of free iron from those iron containing molecules. Also, during disease conditions like hemochromatosis, b-thalassemia, and hemodialysis availability of free iron from erythrocytes destruction may lead to the formation of hydroxyl radicals, resulting in deleterious effects (Valko et al., 2005).
7. The hydroxyl radical, which is the neutral form of hydroxide ion, reacts with polyunsaturated fatty acids (LH) forming the carbon centered lipid radical ($L^{\bullet}$) and this will continue reacting with molecular oxygen to form lipid peroxyl radical ($LOO^{\bullet}$).
8. Within the membrane, the presence of antioxidants like reduced Vitamin E (T-OH) convert the $LOO^{\bullet}$ into lipid hydroperoxide (LOOH) and a Vitamin E radical ($T\text{-}O^{\bullet}$) (Gropper, Smith, & Groff, 2008).
9. The Vitamin E is then regenerated back from $T\text{-}O^{\bullet}$ by reduction using Vitamin C (the physiological form is ascorbate monoanion, $AscH^{-}$) leaving an ascorbyl radical ($Asc^{\bullet -}$).
10. The Vitamin E radical ($T\text{-}O^{\bullet}$) can also be regenerated by GSH and then the oxidized glutathione (GSSG) and the $Asc^{\bullet -}$ is converted back to GSH and $AscH^{-}$ by dihydrolipoic acid (DHLA).
11. DHLA is changed to α-lipoic acid (ALA), which is then reversed by the action of NAD(P)H. The ALA is a disulfide derivative of octanoic acid, and can cross blood brain barrier and be readily absorbed by the cells hence functions as ‘‘metabolic antioxidant’’. The ALA and DHLA can acts antioxidants in the hydrophilic as well as lipophilic conditions (Moini, Packer, & Saris, 2002). The ALA protects the lipid cell membranes and exhibits antioxidant properties by metal chelation and as a scavenger of ROS; while the reduced DHLA, can regenerate the vitamin E, C, and glutathione, thereby enhances the function of endogenous antioxidants (Farris, 2007). The other mechanism to remove lipid hydroperoxides (LOOH) is the GPx system, which converts LOOH to alcohols and dioxygen with the help of the antioxidant GSH. The LOOH reacts with Fe^{2+} and Fe^{3+} forming lipid alkoxyl radical ($LO^{\bullet}$) and $LOO^{\bullet}$ respectively. The lipid peroxyl radical ($LOO^{\bullet}$) can undergo cyclisation reactions to form endoperoxides, with the end products malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Martinez-Cayuela, 1995; Valko et al., 2007).
12. In addition, nitric oxide ($NO^{\bullet}$) formed from arginine by nitric oxide synthase (NOS) can react with $O_2^{\bullet -}$ to form peroxynitrite ($ONOO^{-}$), a very powerful oxidant (Poyton, Ball, & Castello, 2009; Trachootham et al., 2009).

The half life period of the free radicals vary, hydroxyl radical ($^{\bullet}OH$) is highly reactive with a very short *in vivo* half life of 10^{-9}s. Alcoxyl radical ($RO^{\bullet}$) has a half life of 10^{-6} s, singlet oxygen $^1O_2$ with 10^{-5} s; while peroxynitrite anion ($ONOO^-$) has a half life of 0.05-1 s, peroxyl radical ($ROO^{\bullet}$) with 7 s, nitric oxide ($^{\bullet}NO$) a half life of 1- 10 days (Bergendi & Bene, 1999).

1.1.1 Free radicals and oxidative damage

The ROS have beneficial effects when produced in a steady state concentration. It plays important roles in cellular response; especially responses against infectious agents, thereby provides protection against pathogens. The neutrophils, known as phagocytic cells when stimulated by pathogens, recognizes the foreign material and start a cascade of reactions called a respiratory burst. NAD(P)H oxidase, the vital component of host defense present in the neutrophils produces $O_2^{\bullet-}$, which result in the invaders destruction (Decoursey & Ligeti, 2005). The $O_2^{\bullet-}$ and related ROS also regulates ventilation, controls erythropoietin production, smooth muscle relaxation, neuromuscular signal transduction, and enhances immune functions (Adler, Yin, Tew, & Ronai, 1999; Droge, 2002). Among the various RNS, the $NO^{\bullet}$ generated in biological tissues have important role in physiological processes, acts on cardiovascular, nervous and endocrine systems by regulation of blood pressure and vascular tone, signal transmission by the nerves and the neuroendocrine activity, and also contributes to defence mechanisms, relaxation of smooth muscles and regulation of the immune system (Bergendi & Bene, 1999). Hence, when present in normal concentration, the free radicals or their derivatives are involved in the regulation of various functions and enhancement signal transduction and thereby involved in establishing a redox homeostasis (Droge, 2002).

The various harmful effects induced by the free radicals in the biological system are termed as oxidative stress and nitrosative stress (Turrens, 2003; Dalle-Donne et al., 2005; Poyton et al., 2009). This occurs as a consequence of imbalance between the producing and scavenging of ROS and RNS or due to deficiency of antioxidants in the system. The regulation of balance in the concentration of free radical production and their rates of removal by various antioxidants is termed redox homeostasis (Dorge, 2002).

The reactive species at elevated level under pathophysiological conditions lead to oxidative stress, which in turn alters cell function and damage the cells, ultimately results in cell death (Sies, 1997; Droge, 2002). The increased production of ROS in the cell results either from mitochondrial electron transport or by extra stimulation of reduced form of nicotinamide adenine dinucleotide phosphate ($NADP^+$) formed during oxidative stress.

The free radicals act as the mediators to damage the cell components: nucleic acids, lipids, polysaccharides and protein. The ROS mainly attacks nucleic acids and alters the bases and the deoxyribose sugars resulting in nucleic acid destruction, leading to conformational changes in the DNA. The oxidative protein damage also decreases the efficiency of the DNA polymerase and repair enzymes (Wiseman & Halliwell, 1996; Dizdaroglu, Jaruga, Birincioglu, & Rodriguez, 2002; Cadet, Douki, Gasparutto, & Ravanat, 2003; Cooke, Evans, Dizdaroglu, & Lunec, 2003; Cadet, Douki, & Ravanat, 2011). Free radical also targets lipids causing peroxidation of the membrane structures and thus changes the permeability (Cejas et al., 2004; Vera-Ramirez et al., 2011). The ROS oxidizes the monosaccharides and reacts with polysaccharides and can induce depolymerization (Martinez-Cayuela, 1995; Wiseman & Halliwell, 1996; Poyton et al., 2009). Oxidation of proteins subsequently increases its hydrophobicity and sensitivity to proteolysis and ROS reaction with amino acids results in cross linking and aggregation

(Castro, Demicheli, Tórtora, & Radi, 2011; Grimm, Hoehn, Davies, & Grune, 2011; Quiney, Finnegan, Groeger, & Cotter, 2011).

1.1.2 Free radicals in diseases and ageing

Free radical's roles in the pathology of certain human diseases were validated using biomarkers of oxidative damage. Mutation of the genetic material is the initial step in the etiology of carcinogenesis. The free radical induced nuclear DNA damage leads to the breakage of the DNA strands, alteration of purine, pyrimidine or deoxyribose, and may lead to cross linking of the DNA, which in turn results in either inhibition or stimulation of pathways associated with signal transcription, altering the replication process (Marnett, 1999). The malondialdehyde (MDA), which is formed due to lipid peroxidation process, reacts with nucleic acid bases to form mutagenic multiple adducts. There is a dose dependent effect that exists between the oxidative stress and the etiology of disease, ranging from tumors, mutation, and finally to the initiation of apoptosis or necrosis (Feig, Reid, & Loeb, 1994). There are reports stating that there is an increased occurrence of colorectal and lung cancer due to the iron induced oxidative stress (Stayner, Dankovic, & Lemen, 1996; Valko, Morris, Mazur, Rapta, & Bilton, 2001). Recent reports states the effect of both intracellular and extracellular oxidative stress on the pathogenesis of breast cancer (Vera-Ramirez et al., 2011). The increased ROS generation in the cancer cells enhances genetic instability by promoting irregular signaling pathways which results in the abnormal proliferation of neoplastic cells. They may lead to change in the growth factors, receptor mechanisms, error in the signals to the nuclear membrane, which might affect the entire cell cycle process, alter drug sensitivity and develop drug resistance (Pelicano, Carney, & Huang, 2004; Wu, 2006; Valko et al., 2007).

The free radicals can stimulate the disease conditions in which cell injury is involved, including those affecting multi organs, as well as inflammatory immune responses (Cross et al., 1987). Oxidatant-mediated lung injury can lead to necrosis with subcellular disintegration, cytoplasmic swelling, membrane rupture and random cell death or apoptosis with hetero-chromatization and fragmentation, mitochondrial dysfunction, membrane blebbing and apoptotic bodies' formation and finally cell suicide and dismantling (Haddad, 2002).

Free radicals can be instrumental for cardiovascular tissue injury, leading to various cardiovascular diseases, such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy, and congestive heart failure (Hoeschen, 1997; Parthasarathy, Khan-Merchant, Penumetcha, & Santanam, 2001; Bassenge, Schneider, & Daiber, 2005). Increased production of superoxide radical, hydroxyl radical, and nitric oxide affects the cardiac and vascular myocytes; brings drastic changes in the subcellular organelles, and promotes the sarcoplasmic reticular (SR) Ca^{2+} release by the interaction with the cardiac and skeletal SR Ca^{2+} release channels (ryanodine receptors) leading to a critical Ca^{2+} overload, which results in myocardial dysfunction (Stoyanovsky, Murphy, Anno, Kim, & Salama, 1997; Dhalla, Temsah, & Netticadan, 2000). Endothelial dysfunction acts as the key variable in pathology and complications of atherosclerosis and it eventually lead to congestive heart failure (Bonetti, Lerman, & Lerman, 2003; Davignon & Ganz, 2004). Oxidative modification of LDL also plays an important role in the progression of artherosclerosis (Witztum & Steinberg, 1991; Steinberg, 2009); oxidization of lipids yields products including aldehydes which react with lysines and tyrosines of the apo-lipoprotein B-100, altering their functions; even minimal modification of LDL could result in pro-atherogenic effects (Berliner et al., 1990; Berliner & Watson, 2005). The involvement of the oxidized LDL in foam cell formation,

endothelial cell damage and inflammation, plaque formation and rupture and further complications leading to thrombosis, infarction and ischemia was reported (Niki, 2011). Furthermore, the oxidation of the cardioprotective high density lipoprotein (HDL) affects its inherent anti-atherogenic properties (Shao, Oda, Oram, & Heinecke, 2009). White *et al.* (2010) stated the relation between the decrease in the estrogen level and the oxidative stress in old women. This study reported an increased NOS production of superoxide radicals in the body, which in turn results in vasodilation due to decrease in NO and thus an increased risk of cardiovascular diseases.

The pathogenesis of rheumatoid arthritis is associated mainly with the free radicals as it directly damages the articular constituents or indirectly acts by generating irregular induction of redox sensitive signaling pathways at inflammatory locations like joints and the tissues around the joints (Hadjigogos, 2003). Imbalance in the free radical and antioxidant levels lead to an adverse effect on vascular permeability, smooth muscle contraction, and excessive mucus secretion in the respiratory pathways, thus aggravating an asthmatic condition (Nadeem, Masood, & Siddiqui, 2008).

Other clinical manifestations like diabetes have complications associated with oxidative stress, as hyperglycemia stimulates the formation of ROS from oxidative phosphorylation, glucose autooxidation, and by triggering superoxide over production, activation of poly (ADP-ribose) polymerase and depletes NAD^+ concentration hence slow down the rate of glycolysis (Giugliano, Ceriello, & Paolisso, 1996; Ceriello, 2003). Also, it has been reported that the diabetes alters the mitochondrial site of superoxide formation from complex I and the ubiquinone–complex III interface to complex II (Nishikawa et al., 2000). Studies have proven that the production of reactive oxygen species reduce both enzymatic and non enzymatic antioxidants, leading to accumulation of free radicals persuading further cell damage (Valko et al., 2007). Reports also suggest the potential role of oxidative stress in the dysfunction of pancreatic beta cells and endothelium (Ceriello & Motz, 2004).

Several neurodegenerative diseases results from oxidative stress, as the most susceptible organ to oxidative injury is the brain, due to its increase demand for oxygen, large amount of polyunsaturated fatty acids, and the presence of the transition metals and comparatively low antioxidant capacity (Noseworthy & Bray, 1998). Tretter *et al.* (2004) reported the effect of oxidative stress in the pathogenesis of Parkinson's disease are the free radicals; it contributes a series of incidences, leading to the degeneration of the cells of substantia nigra that produce the neurotransmitter called as dopamine, resulting with the disease symptoms.

The role of free radicals in the aging process was first explained by Denham Harman, in 1956. It is a well established fact that species with long life span have more competent antioxidant mechanisms (Perez-Campo, Lopez-Torres, Cadenas, Rojas, & Barja, 1998). The plausible explanation for the aging process is associated with the ROS induced mitochondrial damage (Cadenas & Davies, 2000; Raha & Robinson, 2000; Barja, 2004). Mitochondrial oxidative damage advances with more mitochondrial ROS production, coupled with decline in mitochondrial function as well as the oxidative damage imparted to the DNA, proteins, and lipids (Lapointe & Hekimi, 2010). It was reported that along with the decline in repair activity, the extensive destruction of mitochondrial DNA will finally kills the mitochondria, leading to cell death (Gredilla, 2011). The recent research showed that of Vitamin E supplementation prevents the hippocampus and frontal cortex mitochondrial damage in aged rats (Navarro, Bandez, Lopez-Cepero, Gómez, & Boveris, 2011).

1.2 ANTIOXIDANTS

Antioxidants are molecules that protect biological systems either by inhibiting or preventing the oxidation of substrate by free radicals (Serafini, 2006). Enzymatic antioxidants include the most important intracellular superoxide dismutase (SOD), glutathione peroxidase (GPx), which protects against low levels of oxidative stress and catalase (CAT), and the non enzymatic antioxidants like ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavanoids (Perez-Campo, Lopez-Torres, Rojas, Cadenas, & Barja, 1994; Thannickal & Fanburg, 2000; Nordberg & Arnér, 2001; Valko et al., 2006). Antioxidants protect cells not only by scavenging the deleterious free radicals, but also regulating the gene expression by modulating the signal pathways, regulating normal cell cycle, restraining the neoplastic cell proliferation, hindering tumor invasion and angiogenesis, activating the immune system, reducing inflammatory oxidative conditions, and thereby promoting immunity (Matés, Pérez-Gómez, & De Castro, 1999; Valko et al., 2007).

1.2.1 Sources of antioxidants

The antioxidant properties of the natural sources were attributed during the increased free radical production by either reducing or scavenging the reactive species, quenching singlet oxygen, or by chelating with pro-oxidant metals (Pratt, 1992). Antioxidants are present in various natural sources like plants, animals, microbes, etc. Naturally occurring antioxidants generally originate from plant based ingredients like fruits, vegetables, cereals, and nuts. However, animals also forms source of antioxidants, for example, muscle tissues with carnosine, a dipeptide with metal a chelating and free radical scavenging property (Shahidi, 2000). Certain vitamins, minerals, and enzymes also serve as an antioxidant.

1.2.1.1 Antioxidants from plants sources

Plants, such as fruits, vegetables, oil seeds, nuts, cereals, spices, herbs, grains, etc., are a natural source of many antioxidants. The phytochemicals possess certain biological activities, mainly by their antioxidative properties (Peterson, 2001). Shahidi *et al*. (2000) reported that tocols exhibiting similar Vitamin E antioxidant activity occur widely in plant tissues and in edible oils. Kalt (2005) stated that vitamin C, carotenoids, and phenolics form the rich source of antioxidants in fruits and vegetables, while tocopherols and tocotrienols are the phytochemical antioxidants present mainly in nuts and grains. There were early reports on the antioxidant activity of soyabean and soyabean derived oils (Hayes, Bookwalter, & Bagley, 1977), and the increased presence of the polyphenols was responsible for the desired antioxidative property (Chen, Muramoto, Yamauchi, & Nokihara, 1996).

Increased presence of phenolic compounds in vegetable oils, for example in olive oil, adds to its increased antioxidant activity (Papadopoulos & Boskou, 1991; Baldioli, Servili, Perretti, & Montedoro, 1996; Owen et al., 2000; Visioli, Poli, & Gall, 2002). The presence of flavonoids and other phenolics in fruits and berries possess a remarkably improved role in scavenging free radicals (Kahkonen et al., 1999).

Anthocyanin rich fruits and vegetables, like blue berries, sweet cherries, and red onion cales have high antioxidative activities (Velioglu, Mazza, Gao, & Oomah, 1998). Wang *et al.* (2000) reported that berry crops posses' high ascorbic acid levels. They exhibit

antioxidant activities against superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen and thus contribute to the increased protective activity of fruit crops.

Cereals, like oats, contain phenolic compounds and a series of cinnamic acid conjugates called avenanthramides, which possess antioxidant activity (Dimberg, Theander, & Lingnert, 1993; Zielinski & Kozlowska, 2000; Peterson, 2001; Peterson, Hahn, & Emmons, 2002). The presence of ferulic acid, which forms the major phenolic acid in rye and wheat also show antioxidant activity (Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002).

Studies have shown that the culinary and medicinal herbs possess antioxidant property and there exists a positive correlation between the phenolic content and radical scavenging property; even though *Cantharanthus roseus* showed the greatest antioxidant activity among medicinal herbs, the culinary herbs *Poliomintha longiflora*, *Origanum* × *majoricum*, and *O. vulgare* ssp. *hirtum* showed much higher antioxidant activity than medicinal herbs (Zheng & Wang, 2001).

1.2.1.2 Antioxidants from animal sources

Based on the origin of the food from biological tissues, there exists antioxidative functional variation as free radical scavengers, metal chelators, singlet oxygen quenchers, and antioxidant enzymes (Kitts & Weiler, 2003; Ribaya-Mercado & Blumberg, 2004; Descalzo & Sancho, 2008; Korhonen, 2009). The milk protein casein and casein-derived peptides exhibit antioxidant properties by inhibiting enzymatic and nonenzymatic lipid peroxidation (Rival, Boeriu, & Wichers, 2001). Caesinophosphopeptides derived from tryptin digestion of milk protein casein possess both hydrophilic and lipophilic antioxidant activity due to the metal chelating and free radical scavenging property (Kitts & Weiler, 2003; Díaz & Decker, 2004). Pihlanto (2006) also reported that the peptides derived from the digestion of milk protein showed antioxidant activity.

Carnosine is a naturally occurring histidine containing dipeptide found in the skeletal muscles of vertebrates. It's a potent hydrophilic antioxidant that scavenges singlet oxygen and free radicals *in vitro* (Boldyrev, Dupin, Bunin, Babizhaev, & Severin, 1987; Boldyrev, Koldobski, Kurella, Maltseva, & Stvolinski, 1993; Kang et al., 2002). The presence of carnosine and anserine in the chicken essence and meat contributes to antioxidative property (Wu, Pan, Chang, & Shiau, 2005; Intarapichet & Maikhunthod, 2005). Studies on the antistress effect of chicken essence in mice also indicated the role of carnosine and anserine as antioxidants (Kurihara et al., 2006). The increase in plasma level of carnosine after beef consumption showed its bioavailability as an potent antioxidant (Park, Volpe, & Decker, 2005).The hydrolysates obtained from porcine myofibrillar proteins after treatment with protease (papain or actinase E) exhibited antioxidant activity (Saiga, Tanabe, & Nishimura, 2003). Liu *et al.* (2009) reported that porcine plasma protein hydrolysates exhibits antioxidant activity. Antioxidant proteins and associated peptides derived from eggs are listed in Table 1.1.

1.2.1.3 Antioxidants from fish/marine sources

Hoki (*Johnius belengerii*) skin gelatin trypsin hydrolysate exhibited high antioxidant activity by scavenging superoxide radicals (Mendis, Rajapakse, & Kim, 2005). The hydrolysates of skin gelatin obtained from the Jumbo flying squid (*Dosidicus eschrichitii* Steenstrup) was studied for the antioxidant activity and those treated with properase E and pepsin showed the potent radical scavenging property (Lin & Li, 2006). Klompong *et*

al. (2009) reported that peptides derived from yellow stripe trevally (*Selaroides leptolepsis)* could serve as an alternative for natural antioxidants. A novel antioxidative peptide identified as Leu-Val-Gly-Asp-Glu-Gln-Ala-Val-Pro-Ala-Val-Cys-Val-Pro (1.59 kDa), obtained by *in vitro* gastro intestinal digests of sea mussel (*Mytilus coruscus*) was reported to possess antioxidative activity higher than that of ascorbic acid and the alpha-tocopherol against polyunsaturated fatty acids (PUFA) (Jung et al., 2007). Suetsuna (2000) isolated antioxidative peptides from the muscles of prawn (*penaeus japonicas)* and identified amino acid structures as Ile-Lys-Lys, Phe-Lys-Lys, and Phe-Ile-Lys-Lys. Purified dark muscle peptides obtained from bigeye tuna (*Thunnus obesus*) have protective activity on free radical-mediated oxidative systems (Je, Qian, Lee, Byun, & Kim, 2008). Hydroxyl radical scavenging activity and linoleic acid peroxidation inhibiting activity of the purified peptides from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate showed its potential antioxidant property (Je, Park, & Kim, 2005).

1.2.1.4 Antioxidants from microbial sources

Many studies were conducted in order to find out the antioxidant activity of the substances derived from various fungi. The edible beefsteak fungus (*Fistulina hepatica*) derived lyophilized aqueous extracts exhibited a concentration dependent antioxidant activity and displayed ability to act as superoxide radical scavenger and XO inhibitor, explaining its potential use as an easily assessable natural antioxidant (Ribeiro, Valentão, Baptista, Seabra, & Andrade, 2007). Another fungus, *Inonotus obliquus* (persoon) has been studied and identified seven phenolic components with antioxidant activity. Among other medicinal fungi (*Agaricus blazei Mycelia*, *Ganoderma lucidum* and *Phellinus linteus*), persoon showed the most potent activity in terms of both superoxide and hydroxyl radicals scavenging properties (Nakajima, Sato, & Konishi, 2007).

1.3 EFFECT OF COOKING/PROCESSING ON ANTIOXIDANT ACTIVITY OF FOOD

The role of the proper diet in human health has been studied over the decades and many reports have proven the antioxidant properties of food. However, it is relevant to consider the effect of food processing on the beneficial properties of food. Nicoli *et al.* (1999) reported that most of the developments in the food processing have promoted the nutritional studies pertaining to that food, in order to ensure that the availability of the beneficial properties stay intact. The food processing may not always affect the antioxidant activity; naturally occurring antioxidant concentration sometimes remain unchanged or the loss of natural antioxidants will be balanced by the simultaneous formation of novel or improved compounds. However, the possible outcome on the changes in overall antioxidant activity includes the loss of naturally occurring compounds, formation of novel compounds possessing antioxidant or pro-oxidant activities, and the interactions among various compounds present in the food, for example lipids and natural antioxidants, as well as lipids and Maillard reaction products (Nicoli, Anese, & Parpinel, 1999).

Kalt (2005) reported that the domestic, as well as commercial level of processing affects the structural integrity of food. Various methods like maceration, heating, and other separation steps may result in oxidation, thermal deprivation, oozing, and other events; which eventually reduces antioxidants in processed food in comparison to fresh foods. Processing procedures cause the changes in certain antioxidants like carotenoids and thereby convert it to more bioavailable active antioxidant form (e.g. *trans*-isomers of

lycopene in tomato converts to *cis*-isomers) to improve gastrointestinal absorption (Shi & Maguer, 2000).

The effect of cooking allocation of antioxidants components in vegetables was investigated both qualitatively and quantitatively with an emphasis on the phenolics, ascorbic acid, as well as carotenoids (Zhang & Hamauzu, 2004). It was reported that the total antioxidant activity, as well as phenolic antioxidant activity, decreased during conventional and microwave cooking (Zhang *et al.* 2004). Zhang *et al.* (2004) thus concluded that there is a heavy loss of antioxidant activity during the cooking process. The radical scavenging activity of the water soluble components were studied in mushrooms (*Psalliota campestris*), onions (*Allium cepa*), white cabbage (*Brassica oleracea var. alba*), and yellow bell peppers (*Capsicum annuum*). It was found that the mushrooms subjected to thermal treatment possess greater antioxidant activity, suggestive of a thermolabile component as the major component responsible for antioxidant activity. Onions and white cabbage were relatively insensitive to thermal treatment and it was reported that there was a partial increase in activity of white cabbage juice (Racchi et al., 2002).

Assessment of the antioxidant activity of vegetables based on the storage, processing, and cooking of peas showed a statistically significant difference in the antioxidant activity, ranging from fresh peas with greater activity followed by frozen, and then canned and jarred peas with the lowest antioxidant activity (Hunter & Fletcher, 2002). Fresh spinach showed that the highest level of antioxidant activity, followed by frozen leaf, frozen chopped, and then canned products (Hunter & Fletcher, 2002). A study examining microwave cooking of peas showed no significant loss of water or lipid soluble antioxidant activities; it was also found that boiling resulted in a small loss of both water and lipid soluble antioxidant activities, but overcooking resulted in a greater reduction in the water soluble antioxidant activity (Hunter & Fletcher, 2002). Microwave cooking of spinach had no significant effect in the water and lipid soluble antioxidants, but it was reported that there was a large increase in the small lipid soluble antioxidant activity, which the study concluded to be due to the further disruption of the cellular components and the subsequent release of more carotenoids compounds (Hunter & Fletcher, 2002). Due to cooking an increased loss in the water soluble antioxidant activity was observed in green leafy vegetables (Hunter & Fletcher, 2002; Ismail, Marjan, & Foong, 2004; Kuti & Konuru, 2004; Oboh, 2005).

1.4 ANTIOXIDANT ACTIVITY OF EGGS

Avian egg is an excellent source of nutrients, containing high quality proteins, lipids, such as triacylglycerols, phospholipids and cholesterol, minerals and vitamins, mainly E, A, B_{12}, B_2 and folate (Herron& Fernandez, 2004; Kovacs-Nolan, Phillips, & Mine, 2005; Surai & Sparks, 2001). The egg shell, including the shell membranes between the albumen and the inner shell surface forms 9.5 % of the whole egg, while the egg white forms 63% and the yolk constitutes 27.5% (Cotterill & Geiger, 1977; Li-Chan, Powrie, & Nakai, 1995). Egg shell forms a rich source of inorganic salts, mainly calcium carbonate, and traces of magnesium carbonate and tricalcium phosphate (Li-Chan et al., 1995; Mine, 2002). Approximately 75% of an egg is composed of water, proteins and lipids contribute 12 % each, and rest is of the egg is comprised of carbohydrates and minerals (Burley & Vadehra, 1989; Li-Chan et al., 1995). Thus, eggs can have an important role in the human diet as a balanced source of essential amino acids and fatty acids and are cost effective when added to a diet (Fisinin, Papazyan, & Surai, 2008).

Eggs serve as an excellent source of protein, which is present in the egg white and the yolk, with a limited amount of protein in egg shell and membrane. Ovalbumin is a glycoprotein that forms the major portion constituting 54-58% (w/w) of the total egg white; consists of sequence with 386 amino acids and with a molecular mass of 45 kilo Dalton (kDa) (Li-Chan et al., 1995; Huntington & Stein, 2001; López-Expósito et al., 2008). Second major protein is ovotransferrin (12-14 %, w/w) consists of sequence with 686 amino acid residues and with molecular mass of 78 kDa. It is a disulfide rich single chain glycoprotein and belongs to transferrin family with strong iron binding capacity (Li-Chan et al., 1995; Williams, Elleman, Kingston, Wilkins, & Kuhn, 1982). Ovomucoid, a serine protease inhibitor is another major egg white protein (Kato et al., 1987). Other components include lyzozyme, avidin, cystatin, ovoinhibitor, ovostatin, ovoglycoprotein, ovoflavoprotein, and G2 and G3 globulin are found in the egg white and contain minor levels of carbohydrates, minerals and lipids (Li-Chan et al., 1995; Mine, 2002). The egg yolk forms 36% of the weight of fresh whole avian egg (Anton, 2007). The egg yolk protein consists of spovitellenin, phosvitin, α and β lipovitellin apoproteins, α livetin (serum albumin), β livetin (α2 glycoprotein), γ livetin (γ globulin) and traces of biotin binding protein (Li-Chan et al., 1995; Mine, 2002). The key portion of yolk lipids exists in the form of lipoproteins. The lipids are made up of triglycerol, phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, sphingomyelin, and cholesterol (Li-Chan et al., 1995; Mine, 2002). Carotenoids are natural pigments present, giving the yellow pigmentation to the yolk, and include mainly carotene and xanthophylls (lutein, cryptoxanthin and zeaxanthin) (Anton, 2007). The composition, physiochemical properties and biological activities of egg white and yolk was shown in Table 1.2 and 1.3 respectively.

1.4.1 Inherent antioxidants in eggs

Beyond the role as a major nutritional source, egg components especially protein and the egg derived peptides possess certain bioactivities. Ovalbumin, the major egg white protein has potential scavenging effect on the hydroxyl and superoxide radicals and hence exists as a natural source of nontoxic antioxidant (Xu, Shangguan, Wang, & Chen, 2007). Earlier reports show the protective effect on lipid peroxidation and antioxidant activity of ovalbumin-polysaccharide conjugates (Nakamura, Kato, & Kobayashi, 1992).Studies conducted on the ovalbumin hydrolysates showed the antioxidant activity of the egg white derived peptides; a significant reduction in ROS production and subsequent age related damage in the serum and liver of aged mice (Xu, Shangguan, Wang, & Chen, 2007). Graszkiewicz *et al.* 2007 reported that the egg white protein precipitate obtained as byproduct from industrial isolation of cystatin and lysozyme when hydrolysed with trypsin yielded bioactive peptides with free radical scavenging property. The enzymatic hydrolysates of duck egg white showed inhibitory activity on lipid peroxidation, scavenging of superoxide radicals and strong iron chelating effect (Yi-Chao, His-Shan, Cheng-Taung, & Fu-Yuan, 2009). The pepsin hydrolysates of crude egg white produced the peptide Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu with strong antihypertensive as well as high radical scavenging activity *in vitro* (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004).

Ovotransferrin, the second major protein in the egg is a disulfide rich glycoprotein, capable to induce intracellular oxidative response, and involved in redox linked signals and oxidative stress (Ibrahim & Hoq, 2007). It is a metal ion binding protein from the transferrin family that can reversely bind with iron and other metal ions, including toxic metals (Guérin-Dubiard, Castellani, & Anton, 2007). It has been reported that the radical

scavenging activity of ovotransferrin is specific to the superoxide anion (Ibrahim & Hoq, 2007). The cysteines holding the two sensitive disulfide domains make it a protein that responds to redox homeostasis (Ibrahim, Haraguchi, & Aoki, 2006). During embryonic development the ovotransferrin serves to prevent oxidative damage and thus play an important role in the defense system (Ibrahim & Hoq, 2007).

Lysozyme, the defensin present in egg white, provides protection against the acute and chronic oxidant injury. Lysozymes bind with the advanced glycation end products (AGE) that produces free radicals and thus suppresses the reactive oxygen species and the oxidative stress genes. This also helps to elevate level of antioxidant reserves in transgenic mice (Liu et al., 2006). The pro-oxidant derivatives formed from protein and fat rich diets like AGE or advanced lipoxidation end products (ALE) contributes to the extra oxidant load in the body (Miyata, Kurokawa, & Vanypersele, 2000; Goldberg et al., 2004). Lysozymes enhance the removal of AGEs by serving as an opsonizing factor and subsequent detoxification (Mitsuhashi, Li, Fishbane, & Vlassara, 1997).

Chicken egg white cystatin, a small protein of approximately 13 kDa molecular weight, is a potent competitive inhibitor of cysteine proteinases (Colella, Sakaguchi, Nagase, & Bird, 1989). Vray *et al.* (2002) suggested cystatin has immunomodulatory properties by stimulating the synthesis of $NO^{\bullet}$ production in interferon γ –activated murine macrophages. As it was reported that modulated high level of $NO^{\bullet}$ provide protection without inducing damage to the cell (Joshi & Ponthier, 1999); it also provides protection against free radicals by $NO^{\bullet}$ induced gene up regulation of protective proteins and prevents H_2O_2 induced toxicity (Kim, Bergonia, & Lancaster, 1995). Recent research postulates the role of $NO^{\bullet}$ in eliciting the adaptive response to oxidative stress as it stimulates the $NO^{\bullet}$-mediated sulfiredoxin (Srx) up-regulation transcription factor/ Srx antioxidant pathway in the macrophages (Abbas et al., 2011). Hence the role of cystatin in inducing antioxidant activity along with immunomodulatory cannot be denied.

Frenkel *et al.* (1987) reported the possible role of chicken ovoinhibitor in preventing the ROS formation by polymorphonuclear leukocytes during inflammatory response. The egg yolk contains significant amount of unsaturated fatty acids and iron, which are susceptible to lipid oxidation (Hartmann & Wilhelmson, 2001), but the presence of antioxidants prevent the oxidization in the egg itself (Yamamoto et al., 1990).The egg yolk protein hydrolysates also showed antioxidative effect by preventing the oxidation of cookies with linoleic acid and inhibiting the lipid oxidation of beef and fatty tuna homogenates (Sakanaka, Tachibana, Ishihara, & Raj Juneja, 2004; Sakanaka & Tachibana, 2006).

Egg yolk phosphoglycoprotein, phosvitin with strong cation binding ability, was reported to inhibit oxidative reactions, especially to inhibit Fe^{2+}-catalyzed phospholipid oxidation. Phosvitin serves as potentially natural antioxidant in eggs (Lu & Baker, 1986; Lu & Baker, 1987; Guérin-Dubiard et al., 2007). Ishikawa *et al.* (2004) reported that egg yolk phosvitin have antioxidative properties against iron-catalyzed hydroxyl radical formation, as well as protective properties on genetic material against oxidative damage induced by Fe^{2+} and H_2O_2 and are suggested to be used in iron medicated oxidative stress related pathological conditions like colorectal cancer. Studies conducted on the mouse dorsal homogenate for ultra violet light induced lipid peroxidation in the presence of iron ions, suggested that egg yolk phosvitin has a protective effect against the formation of free radicals (Ishikawa, Ohtsuki, Tomita, Arihara, & Itoh, 2005).

Egg yolk phospholipids like sphingomyelin (SPH), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) exhibit antioxidant activity in a refined salmon oil model system, and also demonstrated that the presence of nitrogen improved the antioxidant activity of phospholipids (King et al., 1992). Choline and the ethanolamine with two functional groups a basic amino group and an alcoholic hydroxy group was present in the side chain moieties of phospholipids; which might have contributed to the inhibition of free radicals (Saito & Ishihara, 1997). Sugino *et al*. (1997) reported the antioxidant activity of phospholipids in the egg yolk and also suggested that the antioxidant property is influenced by the degree of saturation of the fatty acyl chain.

The egg normally contains 200 to 300 µg of carotenoids dispersed in the lipid matrix of the egg yolk, which improves their bioavailability (Handelman, Nightingale, Lichtenstein, Schaefer, & Blumberg, 1999). The incorporation of the natural carotenoids in the layer diet helps the transfer of those pigments and hence imparts the yellow pigmentation of the egg yolk (Karadas, Grammenidis, Surai, Acamovic, & Sparks, 2006). Lutein and zeaxanthin reacts with singlet oxygen generated in water phase and function as antioxidants; they accumulate in the macular surface membranes of the retina (Herron & Fernandez, 2004; Ribaya-Mercado & Blumberg, 2004). They decrease the oxidation rate, minimizing oxygen permeability through the membrane, thereby reducing damage and protecting the retina from increased oxidative metabolism (Herron & Fernandez, 2004). Lutein exhibits radical scavenger properties against peroxynitrite formed from nitric oxide and superoxide *in vivo* (Panasenko, Sharov, Briviba, & Sies, 2000).

The presence of the vitamins E, A along with the minerals such as selenium also improves the antioxidant activity of the eggs (Burton, Cheeseman, Doba, Ingold, & Slater, 1983; Sparks, 2006; Fisinin et al., 2008). Selenium functions as an antioxidant nutrient and present in antioxidant enzymes, such as glutathione peroxidases and thioredoxin (Burk, 2002; Weiss & Landauer, 2003). The antioxidants derived from egg were shown in Table 1.3.

1.4.2 Enriched antioxidants in eggs

Food derived antioxidants can modulate free radical to a balanced state and reduce oxidative stress. During the last few decades, research has been focused on enhancing the nutritional quality of the egg by enriching eggs with n-3 fatty acids, like docosahexaenoic acid (DHA, 22:6n-3), vitamin E, carotenoids and minerals such as selenium and iodine.

Lewis *et al.* (2000) reported that the n-3 PUFA enriched eggs improved the n-3 status in the Canadian consumers; and suggested the use of n-3 PUFA enriched eggs as a source to meet the Canadian Recommended Nutrient Intake (CRNI) recommendations. A comparison of the nutrients showing antioxidant property in enriched eggs and table eggs is illustrated in Table 1.4.

1.4.2.1 n-3 enriched eggs

The diet rich in n-3 polyunsaturated fatty acids was reported for their possible role in reducing the risk of fatal ischemic heart disease in older adults (Hu et al., 1999; Lemaitre et al., 2003; Albert et al., 2005). Among the n-3 fatty acids, the alpha linolenic acid (ALA) serves as a precursor for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); these n-3 fatty acids are not produced by the body hence it has to be supplemented by diet (Covington, 2004). The n-3 fatty acids was also reported to have potent hypotriglyceridemic property as it reduces the plasma triglyceride levels (Rambjør, Wålen, Windsor, & Harris, 1996). The dietary supplementation of EPA/DHA

along with antioxidants helps to improve the health of schizophrenic patients (Arvindakshan, Ghate, Ranjekar, Evans, & Mahadik, 2003). Studies conducted in diabetic patients with a combined treatment of antidiabetic drug and n-3 fatty acids showed decrease in the lipid peroxidation as well as increase in GPx activity (Kesavulu, Kameswararao, Apparao, Kumar, & Harinarayan, 2002). Among functional foods, enriched eggs serve as an ideal delivery source for n-3 fatty acids (Surai, Speake, & Sparks, 2001). The fatty acid profile of the egg can be manipulated through changes of the hen diet by directly feeding fish oil or indirectly by incorporating the n-3 PUFA precursor in the form of flax seeds, linseeds or oils from these seeds (Sparks, 2006).

1.4.2.2 Vitamin E enriched eggs

Vitamin E functions as a primary antioxidant, as it is involved in breaking the chain in the free radical reaction (Burton et al., 1983). Several animal model studies have shown the cancer preventing effects of Vitamin E on skin, oral cavity and mammary gland (Shklar, 1982; Perchellet, Owen, Posey, Orten, & Schneider, 1985; Kline, Yu, & Sanders, 2004). Dietary supplementations of micronutrient antioxidants, like vitamin E, have an effect on the lung function (Britton et al., 1995). Meluzzi *et al.* (2000) reported that a formulated diet with dietary supplements increases the demand of designer eggs with enriched vitamin E.

1.4.2.3 Carotenoid enriched eggs

Carotenoids react with singlet oxygen and function as antioxidants (Hiramatsu, Yoshikawa, & Inoue, 1997; Paiva & Russell, 1999). Natural carotenoids present in the eggs include lutein and zeaxanthin (Handelman, Nightingale, Lichtenstein, Schaefer, & Blumberg, 1999). Carotenoids derived maternally help the developing embryo to maintain redox homeostasis during the embryonic development and the initial days post hatching (Costantini & Moller, 2008). These oxygenated carotenoids, lutein and zeaxanthin, play an important role in the maintenance of normal vision and reduce the risk of progressive eye condition called age related macular degeneration (AMD) (Moeller, Jacques, & Blumberg, 2000; Richer et al., 2004). Also these carotenoids can absorb the ultraviolet light and protect the lens of the eye from oxidative damage (Goodrow et al., 2006). The avian egg consists of readily available lutein and zeaxanthin and reports suggested that increased intake of eggs resulted in increased circulatory concentration of carotenoids (Krinsky, Landrum, & Bone, 2003; Goodrow et al., 2006). Leeson and Caston (2004) enhanced the lutein level in egg yolk by dietary supplementation. Increased bioavailability was reported from lutein enriched eggs than from other sources such as lutein, lutein ester supplements, and spinach (Chung, Rasmussen, & Johnson, 2004).

Surai *et al.* (2000) reported that the consumption of designer eggs enriched with vitamin E, lutein, and DHA increased significantly the plasma levels of all the enriched compounds (1.88 fold increase of lutein content). A recent study showed an enhanced serum lutein level following the intake of n-3 fatty acid enriched eggs and organic eggs (Burns-Whitmore et al., 2010).

1.4.2.4 Selenium enriched eggs

Selenium (Se) level in the body is associated with the many physiological functions, as well as the maintenance of the immune status of the body. Selenium deficiency contributes to the development of various disease conditions; while an increased Se level

in the body has anti-carcinogenic effect and has a vital protective role against free radical induced diseases (Fisinin et al., 2008). Selenium enriched eggs contain up to 30 µg Se per egg, making these enriched eggs capable of providing 50% of Se Recommended Dietary Allowance **(**RDA) (Fisinin et al., 2008; Fisinin, Papazyan, & Surai, 2009). Eggs enriched with Se have a protective role against oxidative stress in the body as it increases the level of Se-dependent glutathione peroxidase (Se GSH-Px), a potent antioxidative enzyme (Surai, 2000). A direct link between the scarcity of dietary Se and oxidative stress was even reported (Sakuma, Matsuoka, Honda, Matsumoto, & Endo, 2008). Apart from this, Se plays an important role in the process of detoxification of xenobiotics, as well as some toxic metals (Bourre & Galea, 2006).

1.4.2.5 Iodine enriched eggs

Iodine assists in antioxidant activity and iodide, as a primitive antioxidant, is involved in many physiological functions (Venturi & Venturi, 1999; Venturi et al., 2000; Venturi & Venturi, 2007). Recent studies showed that hen dietary supplementation may improve iodine level in eggs; and consumption of iodine enriched eggs may help to solve the iodine deficiency (Bourre & Galea, 2006; Charoensiriwatana, Srijantr, Teeyapant, & Wongvilairattana, 2010). Iodine enriched eggs help to meet the dietary requirements for iodine. Bourre and Galea (2006) reported that designer eggs provide additional RDA amounts of n-3 fatty acid ALA, DHA, vitamin D, vitamin E, folic acid, lutein, zeaxanthin, and minerals like iodine and selenium. Research in the evaluation of iodine enriched egg consumption confirms the significant increase in bioavailability of iodine in the consumers (Charoensiriwatana, Srijantr, Teeyapant, & Wongvilairattana, 2010).

1.4.3 Effects of cooking and preparation on antioxidants in eggs

Cooking causes temperature-time related alternation in the physical and chemical property of the food, resulting in variation in the moisture, flavor, colour, texture, fat percent, and the overall nutrient level (Collison, 1993). Earlier reports showed an increased antioxidant activity in heated skim milk and suggested that heating has exposed sulfhydryl groups from cysteine (Taylor & Richardson, 1980). Elias *et al.* (2007) reported thermal processing of β lactoglobulin at 95^{o}C for 15 min exhibited high peroxyl radical scavenging capacity and lipid oxidation inhibiting property, despite the decrease in the iron chelation property and free sulfhydryl concentration. The general antioxidant activity of proteins was dependent on their structure and the exposure of amino acids increases the antioxidant activity (Levine, Mosoni, Berlett, & Stadtman, 1996). The structural disruption improves the accessibility of the amino acid residues for radical scavenging (Elias, McClements, & Decker, 2007); also synergistically influences the activities of amino acids such as tyrosine, tryptophan, phenylalanine and sulfur-containing cysteine, methionine from which hydrogen is easily abstracted as well as chelation of endogenous transition metals (Elias, McClements, & Decker, 2007; Elias, Kellerby, & Decker, 2008). Thus, the alterations due to cooking and processing may influence the total antioxidant activity of the egg proteins. Ovotransferrin was most thermolabile protein of all egg white proteins (Watanabe, Nakamura, Xu, & Shimoyamada, 2000). Castellani *et al.* (2004) reported that thermal treatments at 60 ^{o}C will not alter the iron binding capacity of phosvitin and food processing treatments with 90^{o}C for 1h will not change its antioxidant property. Processing egg yolk phosvitin at high temperature, such as autoclaving, decreases the antioxidant activity, while induction of a Maillard reaction, using a polysaccharide conjugate, maintain the inhibitory capacity of iron-catalyzed lipid oxidation (Nakamura, Ogawa, Nakai, Kato, & Kitts, 1998).

Studies on ovalbumin derived peptides exhibited antioxidant activities. Ovalbumin hydrolysates significantly prevented the decrease of the superoxide dismutase (SOD) activity in aged mice model dose-dependently (Xu et al., 2007). It was also noticed that the hydrolysates could reduce lipid peroxidation in a linolenic acid model system better than the control (Xu et al., 2007). Jing *et al.* (2009) evaluated the effect of chemical modification by Maillard reaction on the antioxidant activity of egg proteins. Incorporation of Maillard reaction products could improve the functional property of the egg proteins, as the heated protein sugar mixtures exhibited increased scavenging activity towards the DPPH radicals (Sun et al., 2006; Jing, Yap, Wong, & Kitts, 2009).

Delipidated egg yolk protein is a major by-product after lecithin extraction in the processing industry, and this upon enzymatic digestion using alcalase and protease N produces egg yolk peptides with antioxidative stress properties (Young, Fan, & Mine, 2010). The egg yolk peptides help to boost the GSH level in red blood cells and increase other antioxidant enzyme activities, especially catalase and glutathione S-transferase activities. The egg yolk peptides also reduce the oxidation of protein and lipid in the intestinal tract of piglets subjected to intraperitoneal infusions of hydrogen peroxide (Young, Fan, & Mine, 2010). It was concluded that the peptides derived from egg yolk could reduce oxidative stress, especially intestinal stress (Young, Fan, & Mine, 2010). The phosphopeptides (PPPs) prepared from egg yolk phosvitin, using enzyme trypsin showed strong antioxidant activity in Caco-2, the human intestinal epithelial cells (Katayama, Ishikawa, Fan, & Mine, 2007). In another study, H_2O_2 induced IL-8 secretion from Caco-2 cells was inhibited by PPPs, but the phosvitin was not able to perform the protective activity. This indicates the bioactivity of the phosvitin was improved upon enzymatic digestion (Katayama, Xu, Fan, & Mine, 2006).

Several studies have been conducted to determine the antioxidant activity of eggs, but there exists a paucity of information on the affect of different cooking methods along with simulated gastrointestinal digestion. Hence the specific objective of this research were

- To determine the effect of domestic cooking methods, including boiling and frying on the antioxidative activity of egg samples.
- To determine the affect of simulated gastrointestinal digestion on the antioxidant activity of cooked eggs.
- To purify the egg protein hydrolysates by sequential chromatographic separations.
- To characterise the peptide sequences derived from most potent fractions with antioxidant activity.

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Table 1.1 Antioxidant proteins and associated peptides derived from egg.

Egg components	**Enzyme treatment/ Preparation**	**Identified peptides**	**References**
Ovalbumin	Trypsin, Pepsin, Ovalbumin-polysaccharide conjugate	Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu	Nakamura, Kato, & Kobayashi, 1992; Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004; Graszkiewicz, Zelazko, Trziszka, & Polanowski, 2007; Xu, Shangguan, Wang, & Chen, 2007.
Ovotransferrin	Thermolysin, thermolysin–pepsin	Trp-Asn-Ile-Pro, Gly-Trp-Asn-Ile	Huang, Majumder, & Wu, 2010; Shen et al., 2010.
Lysozyme	Alcalase		Liu et al., 2006; You, Udenigwe, Aluko, & Wu, 2010
Ovoinhibitor			Frenkel, Chrzan, Ryan, Wiesner, & Troll, 1987
Cystatin			Colella,Sakaguchi,Nagase,& Bird, 1989
Phosvitin	Trypsin, Phosvitin-polysaccharide conjugate		Nakamura, Ogawa, Nakai, Kato, & Kitts, 1998; Ibrahim & Hoq, 2007; Xu, Katayama, & Mine, 2007
Egg yolk Phospholipids			Sugino et al., 1997
Egg yolk protein	Proteinase		Sakanaka, Tachibana, Ishihara, & Raj Juneja, 2004; Sakanaka & Tachibana, 2006
Lecithin free egg yolk protein	Alcalase	Leu-Met-Ser-Tyr-Met-Trp-Ser-Thr-Ser-Met, Leu-Glu-Leu-His-Lys-Leu-Arg-	Park, Jung, Nam, Shahidi, & Kim, 2001

		Ser-Ser-His-Trp-Phe-Ser-Arg-Arg.	
Carotenoids (Lutein and zeaxanthin)			Handelman, Nightingale, Lichtenstein, Schaefer, & Blumberg, 1999; Nelson, Bernstein, Schmidt, Von Tress, & Askew, 2003; Karadas, Grammenidis, Surai, Acamovic, & Sparks, 2006.
Egg shell membrane protein	Alcalase		Huang, Zhou, Ma, Cai, & Li, 2010.

Table 1.2 Composition, physiochemical properties, and biological activities of major egg white proteins (Li-Chan et al., 1995; Mine, 2002; Kovacs-Nolan, Phillips, & Mine, 2005; Miguel & Aleixandre, 2006).

Egg White Proteins (relative %,w/w)	**Molecular Weight (kDa)**	**Isoelectric point**	**Physio chemical property**	**Biological activity**
Ovalbumin (54)	44.5	4.5	Phospho glyco protein (Ibrahim, 1997)	Immunomodulatory activity due to release of alpha tumor necrosis factor (TNF) (Fan, Subramaniam, Weiss, & Monnier, 2003) Antibacterial activity exhibited by ovalbumin derived peptides (Pellegrini, Hulsmeier, Hunziker, & Thomas, 2004) Vasorelaxing activity due to chymotrypsin digestion derived peptide, ovokinin (Matoba, Usui, Fujita, & Yoshikawa, 1999) Antihypertensive property (Matoba et al., 1999)
Ovotransferrin (12)	77.7	6.1	Metal binding monomeric glycoprotein (Guerin-	Antioxidant activity due to the metal chelating property (Ibrahim & Hoq, 2007) Antibacterial activity, by altering

			Dubiard et al., 2007)	permeability of bacterial membranes, and subsequent changes in electrical potential (Aguilera, Quiros, & Fierro, 2003) Antiviral (Giansanti et al., 2002), Antifungal (Valenti, Visca, Antonini, & Orsi, 1985) , Immunomodulatory activity (Xie, Huff, Huff, Balog, & Rath, 2002) Antihypertensive property (Miguel & Aleixandre, 2006; Miguel et al., 2007)
Ovomucoid (11)	28	4.1	glycoprotein , cross-linked by intra domain disulfide bonds (Kato, Schrode, Kohr, & Laskowski Jr, 1987)	Immunomodulatory activity by inducing T cell secretion of cytokines Serine protease inhibitor (Kato et al., 1987) Target delivery of drug, act as biospecific ligand (Plate, Valuev, Sytov, & Valuev, 2002)
Ovomucin (3.5)	5.5-8.3 x 10^3	4.5-5.0	Glycosylated glycoprotein (Itoh, Miyazaki, Sugawara, & Adachi, 1987) Provides viscosity to the egg white (Tsuge, Shimoyamada, & Watanabe, 1997)	Immunomodulators, stimulates macrophages *in vitro* (Tanizaki, Tanaka, Iwata, & Kato, 1997) Antimicrobial and antiviral (Tsuge, Shimoyamada, & Watanabe, 1996; Tsuge et al., 1997) Antiadhesive, antitumor property (Watanabe, Tsuge, Shimoyamada, Ogama, & Ebina, 1998)
Lysozyme (3.4)	14.3	10.7	Mucopeptide, N-acetylmuramyl hydrolase (Salton,	Suppresses the ROS and oxidative stress genes (Liu et al., 2006) Antimicrobial activity, bacteriolytic activity by hydrolyzing the linkage between N-acetylmuraminic acid and N-

			1957)	acetylglucosamine of peptidoglycan, the structural component of bacterial cell walls (Salton, 1957; Banks, Board, & Sparks, 1986) Antiviral activity, reportedly associated with its charge (Oevermann, Engels, Thomas, & Pellegrini, 2003) Immune-modulating and immune-stimulating agent, Enhances immunoglobulin productivity (Sava, Benetti, Ceschia, & Pacor, 1989)
Ovoinhibitor (1.5)	46.5*	5.1	serine proteinase inhibitor (Tomimatsu, Clary, & Bartulovich, 1966; Davis, Zahnley, & Donovan, 1969)	Antioxidant activity as well as Anti inflammatory activity inhibits formation of active oxygen species by polymorphonuclear leucocytes (Frenkel, Chrzan, Ryan, Wiesner, & Troll, 1987) Antiviral activity (Yolken, Willoughby, Wee, Miskuff, & Vonderfecht, 1987)
Ovomacro-globulin/ovostatin (0.5)	650**	4.5	glycoprotein with four subunits joined in pairs by disulfide bonds (Kitamoto, Nakashima, & Ikai, 1982)	Antimicrobial property due to proteinase inhibitory action Inhibits serine, cysteine,thiol and metallo protease inhibits kinin generating proteases (Kitamoto et al., 1982; Molla, Matsumura, Yamamoto, Okamura, & Maeda, 1987; Wu et al., 2001)
Cystatin (0.05)	13***	5.1	Inhibits most cysteine proteases (Kato et al., 2000)	Antimicrobial (Korant, Brzin, & Turk, 1985) Antitumor activity (Abrahamson, Alvarez-Fernandez, & Nathanson, 2003) Immunomodulatory activity, stimulates cytokines (Vray, Hartmann, & Hoebeke, 2002; Abrahamson et al., 2003) Prevents bone degeneration (Abrahamson et al., 2003)

Avidin (0.05)	68.3	10	Tetrameric glycoprotein, high affinity with biotin (Laitinen et al., 2002)	Antimicrobial activity, inhibits growth of biotin requiring bacteria (Korpela, Salonen, Kuusela, Sarvas, & Vaheri, 1984) Drug delivery, due to strong biotin binding and signal amplification property (Penichet, Kang, Pardridge, Morrison, & Shin, 1999)

* (Tomimatsu et al., 1966) ** (Donovan, Mapes, Davis, & Hamburg, 1969), ***(Colella, Sakaguchi, Nagase, & Bird, 1989)

Table 1.3. Composition, physiochemical properties and biological activities of major egg yolk components (Kovacs-Nolan et al., 2005).

Egg yolk components	**Physiochemical property**	**Biological activity**
Immunoglobulin (Ig)Y	Similar in function to Ig G (Carlander, Kollberg, Wejaker, & Larsson, 2000)	Antimicrobial, antiadhesive activity , antitumor activity (Carlander et al., 2000)
Phosvitin	Highly phosphorylated protein (Ishikawa, Ohtsuki, Tomita, Arihara, & Itoh, 2005)	Antioxidant activity (Lu & Baker, 1986; Lu & Baker, 1987; Guérin-Dubiard et al., 2007), Antibacterial activity (Khan et al., 2000), Increases calcium solubility (Jiang & Mine, 2001)
Sialyloligosaccharides and sialyglycopeptides		Antiadhesive property (Sugita-Konishi et al., 2002)
Yolk lipids, Lipoproteins		Antioxidant activity (Yamamoto, Sogo, Iwao, & Miyamoto, 1990; Sugino et al., 1997) Immunomodulatory activity, Antibacterial activity (Brady, Gaines, Fenelon, Mcpartlin, & O'Farrelly, 2002)
Phospholipids		Functions in brain development (Masuda, Kokubu, Yamashita, Ikeda, & Inoue, 1998) Reduces serum cholesterol (Masuda et al., 1998) Antioxidant activity (King, Boyd, & Sheldon, 1992)

Cholesterol		Normal component of cell membranes (Makrides, Hawkes, Neumann, & Gibson, 2002)
Fatty acids		Antibacterial activity (Brady et al., 2003)

Table 1.4 Comparison of antioxidants in the designer eggs and table eggs (Adapted from (Surai & Sparks, 2001))

Nutrient in the enriched egg	**Amount in enriched egg (mg)**	**Amount in table eggs (mg)**	**% Recommended dietary allowances (RDA)**
Vitamin E	19.3	0.72	150
DHA	209	32.4	100
Selenium	0.032	0.004	50
Lutein	1.91	0.12	Not known
Iodine	0.093.57 - .097.76*	0.0312**	150

*(Charoensiriwatana et al., 2010) ** (Travnicek, Kroupova, Herzig, & Kursa, 2006)

CHAPTER-2 EFFECT OF COOKING AND SIMULATED DIGESTION ON THE TOTAL ANTIOXIDANT ACTIVITY OF EGGS

2.1 INTRODUCTION:

Oxidation of the biomolecules occurs continuously within the body due to the formation of free radicals during normal metabolic reactions involved in the respiratory chain, degradation of lipids, the catecholamine response under stress, and inflammatory responses or from external sources such as radiations, cigarette smoking, air pollutants and industrial chemicals (Bagchi & Puri, 1998). The free radicals formed in the body are regulated by the antioxidant defenses in the body to maintain a balance in the redox homeostasis (Valko et al., 2007). When free radical formation exceeds the protective capacity of the antioxidant defense system it may lead to serious diseases, including cancer, atherosclerosis, malaria, and rheumatoid arthritis and neurodegenerative diseases (Aruoma, 1998). The antioxidative compounds can either prevent the harmful effects of free radicals or protect the biological system from the excessive damage induced by the free radicals (Arnao, 2000). Various endogenous antioxidants in the body such as superoxide dismutase (SOD), glutathione (reduced; GSH), GSH peroxidases, glutathione reductase, catalase, as well as exogenous source of antioxidants derived from vegetables, fruits, herbs, spices, cereals, nuts, meat, fish and eggs, constitute the principal antioxidant defense system in the body (Fang, Yang, & Wu, 2002; Pokorný, Yanishlieva, & Gordon, 2001). *Dietary antioxidants that occur naturally surpass the use of their synthetic alternatives, because of the protective effects and reduction in side effects.*

Antioxidant activity from many plant food commodities has been extensively studied (Nicoli, Anese, & Parpinel, 1999). Previous studies shows the presence of tocols with vitamin E like property in certain plant tissues and edible oils (Peterson, 2001), vitamin C, carotenoids, and phenolics in fruits and vegetables (Kalt, 2005), polyphenols in soyabean and soyabean derived oils (Hayes, Bookwalter, & Bagley, 1977), tocopherols and tocotrienols in nuts and grains (Kalt, 2005), phenolic components in cereals like oats and herbs posses antioxidant activity (Peterson, 2001; Peterson, 2001; Zheng & Wang, 2001). On the other hand, antioxidants from animal food commodities are less studied. Several well-known antioxidants from animal food products are carnosine (Shahidi, 2000), milk protein casein (Rival, Boeriu, & Wichers, 2001) and fish muscle derived peptides (Je, Qian, Lee, Byun, & Kim, 2008. The avian egg is considered as an excellent dietary source of nutrients, includes proteins, lipids, vitamins, minerals, embryonic growth factors, and various components to protect from pathogens (Kovacs-Nolan, Phillips, & Mine, 2005). Studies on egg revealed the presence of biological components with antioxidant activities (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004). Several egg white protein, ovalbumin (Nakamura, Kato, & Kobayashi, 1992), ovotransferrin (Ibrahim & Hoq, 2007), lyzozyme (Mitsuhashi, Li, Fishbane, & Vlassara, 1997), phovitin (Lu & Baker, 1986), were reported to have antioxidant activities. Egg yolk contains various antioxidants, such as phospholipids (Lu & Baker, 1986;

Sugino et al., 1997), carotenoids such as lutein and zeaxanthin (Lu & Baker, 1986; Ribaya-Mercado & Blumberg, 2004; Sugino et al., 1997), and free aromatic amino acids (Nimalaratne et al., 2011).

Cooking or food processing are known to affect antioxidants from fruits and vegetables by either increasing or decreasing the antioxidant activity (Nicoli, Anese, & Parpinel, 1999). Antioxidant peptides from animal proteins such as milk proteins (Rival, Boeriu, & Wichers, 2001), fish muscle derived peptides (Je, Qian, Lee, Byun, & Kim, 2008), as well as egg proteins (Park, Jung, Nam, Shahidi, & Kim, 2001; Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004; Sakanaka, Tachibana, Ishihara, & Raj Juneja, 2004; Xu, Shangguan, Wang, & Chen, 2007; Xu, Katayama, & Mine, 2007; Huang, Majumder, & Wu, 2010) were reported. As a protein rich food commodity, release of peptides in the human gut might further breakdown to antioxidant peptides that impart to human health. However, there is limited knowledge on the effect of cooking and gastrointestinal digestion on the antioxidant activity of eggs. The objectives of this study were to determine the effects of cooking methods and simulated gastrointestinal digestion on the antioxidant activity of eggs.

Spurred by various reports on the release of bioactive peptides from the parent protein upon action of the digestive enzymes increased the interest to study the effect of cooking and enzyme treatment on egg samples. Moreover food derived antioxidants pave the way for potential therapy against diseases ranging from aging to cancer and coronary heart disease by mitigating oxidative damage with related health impacts (Kalt, 2005).

2.2 MATERIALS AND METHODS:

2.2.1 Materials

Fresh white-shell eggs were obtained from Poultry Research Centre of the University of Alberta (Edmonton, AB, Canada). The enzymes, pepsin (porcine gastric mucosa) and pancreatin (porcine pancreas), were purchased from Sigma-Aldrich (Oakville, ON, Canada). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was obtained from Acros-Organics (Morris Plains, NJ, USA) and AAPH (2, 2'-azobis (2-amidino-propane) dihydrochloride and fluorescein (FL) (Na salt) were obtained from Aldrich (Milwankee, WI, USA). L-Tryptophan was obtained from Sigma-Aldrich (Oakville, ON, Canada). Randomnly methylated β- cyclodextrin (RMCD) (Trappsol) (pharmacy grade) was obtained from Cyclodextrin Technologies Development Inc. (High Springs, FL, USA).

2.2.2 Preparation of egg samples

For preparing fresh egg samples, egg white was separated manually from egg yolk; whole egg was prepared by homogenization after breaking. For preparing boiled eggs, fresh eggs were placed in a saucepan with water one inch above the shell and then boiled for 10 min. After boiling, the eggs were placed under

running water for 5 min, peeled, and then each egg white and yolk was separated. Boiled whole eggs were prepared from homogenizing boiled egg whites and egg yolks. For preparing fried whole eggs samples, separated egg white or egg yolk and/or homogenized whole egg, were transferred to preheated frying pan (350^{o}F) cooked each side for 40 s. All the samples were freeze dried for further analysis.

2.2.3 Preparation of egg hydrolysates

Freeze-dried egg samples were mixed with distilled water to 5% slurry (w/v, dry weight) and were kept in the water bath at 80°C for 15 min with continuous shaking. The temperature was adjusted to 37°C by adding ice cubes into the water bath, and the pH of the slurry was adjusted to 2 with 1 N HCl. After stabilized, pepsin (2% w/w of protein) was added to initiate digestion and the conditions were maintained constantly for a period of 3 h. Then the pH of the slurry was adjusted to 7.0 and pancreatin (2%, w/w of protein) was added to initiate another 3 h of digestion. The digestion was terminated by increasing the temperature to 95°C for 15 min, and centrifuged at 10,000 x g for 25 min. The supernatant was collected, freeze dried, and stored for further analysis. The digestion was carried out using Titrando (Metrohm, Herisan, Switzerland) and a circulating water bath was used for maintaining constant temperature.

2.2.4 Optimization of solvent concentration and extraction time

Freeze dried egg yolk samples (50 mg) were extracted with 10 mL of hexane/dichloromethane (1:1) vortexed for 1 h at room temperature at 600 rpm, followed by centrifugation at 3000 rpm for 5 min. The hexane/dichloromethane layer was collected, and dried under nitrogen to prepare the lipophilic fraction. The residues were dried, and extracted at various solvent concentrations (20, 40, 60, 80 % ethanol and absolute alcohol for 1 h) and extraction time (0.5, 1, 2, 4, 6, 8 and 24 h) with on an orbital shaker at 600 rpm. The extracted samples were then centrifuged at 3000 rpm for 5 min and the supernatants were collected for antioxidant analysis.

2.2.5 Measurement of antioxidant activity

All the freeze dried egg yolk or whole egg samples were extracted with1 mL of hexane/dichloromethane (1:1) followed by centrifugation 600 rpm and H/D layer was collected, and was evaporated under nitrogen to prepare the lipophilic fraction. The residue was dried and extracted with 80 % ethanol for 1 h with on an orbital shaker at 600 rpm. The extracted samples were then centrifuged at 3000 rpm for 5 min and the supernatants were collected for hydrophilic antioxidant analysis.

The antioxidant activity was determined using three different methods: oxygen radical absorbance capacity (ORAC) assay, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ABTS decolorization assay, and 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) assay with slight modifications.

For the lipophilic antioxidant assays, the dried hexane/dichloromethane extract was dissolved in 250 µL of acetone and then diluted with 750 µL of a 7 % RMCD solution (50% acetone/50% water, v/v). The 7 % RMCD acts as a water solubility enhancer for lipophilic antioxidants (Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002), and was used for dissolving Trolox standards, as well as used the blank. All further dilution was made with the 7% RMCD solution.

For the hydrophilic antioxidant assay, any further dilutions of the hydrophilic fraction were made with phosphate buffer (75 mM, pH 7.4). All samples were extracted in duplicate and assayed in triplicate.

2.2.5.1 Oxygen radical absorbance capacity (ORAC) assay

ORAC was measured using the method explained by Davalos et al., (2004), with slight modifications for estimating the antioxidant activity. Fluorescein was used as fluorescent probe. 80 mM AAPH and 200 nM fluorescein in 75 mM phosphate buffer at pH 7.4 were prepared. 100 µL of Trolox standard solutions, at final concentrations of 1 to 8 µM, were placed in a 96 well microplate, followed by addition of 50 µl of the fluorescein solution. The mixture was preincubated for 15 min at 37^{o}C. 50 µL of AAPH was added rapidly using a multichannel pipette. The microplate was immediately placed in a Fluoroskan Ascent microplate reader with 485-P excitation and 538-P emission filters and the fluorescence recorded every minute for 100 min. Reaction mixtures were prepared in duplicate and the readings were recorded for three individual runs for each sample. All readings were recorded using Fluoroskan Ascent software. The area under the curve of fluorescence decay (AUC) was calculated using Graphpad prism software (trial version). After the fluorescence measurements, readings were normalized to that of a blank curve (no antioxidant). The following equation was used for the calculation of area under the fluorescence decay curve (AUC) using the normalized curves.

$$AUC = 1 + \sum_{i=0}^{i=100} f_i / f_0$$

f_0 is the initial fluorescence reading at the time of 0 min; f_i is the fluorescence reading at time i.

Using the difference between the blank AUC with that of the sample, the net AUC for each sample was calculated. Regression equations between AUC and antioxidant concentrations were calculated for all the samples. The ORAC value was calculated by dividing the slope of sample regression curve by the slope of Trolox regression curve. The final ORAC values were expressed as µmol of Trolox equivalent/mg of sample.

2.2.5.2 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ABTS decolorization assay

$ABTS^{\bullet+}$ decolorization assay was based on Strljbe, Haenen, Berg, & Bast (1997) with slight modifications. ABTS radical cation was generated by mixing 7 mM ABTS and 2.45 mM potassium persulfate and diluted 13 fold with an assay buffer (3 mM phosphate buffer at pH 7.5 containing 150 mM NaCl for hydrophilic ABTS or 95% ethanol for lipophilic ABTS) immediately before use. For each run, 20 µL of sample and 80 µL of phosphate buffer or 95% ethanol were placed in wells of a 96-well microplate, followed by addition of 100 µL of the ABTS radical solution. Absorbance was monitored at 734 nm after 5 min of incubation at 37 ^{o}C. A Trolox regression equation between absorbance and Trolox concentrations was calculated and used to calculate the Trolox equivalent antioxidant capacity (TEAC) value for all the samples. The TEAC value is expressed as µmol of Trolox equivalent/mg of sample.

2.2.5.3 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) assay

DPPH radical scavenging capacity forms the basis for DPPH antioxidant assay (Bersuder, Hole, & Smith, 1998). 20 µL of antioxidant and 80 µL of water for hydrophilic DPPH or 95% ethanol for lipophilic DPPH were placed in the wells of 96-well microplate, followed by addition of 100 µL of 0.2 mM DPPH in 95% ethanol solution. Absorbance was monitored at 517 nm after 45 min of incubation at 37 ^{o}C. A Trolox regression equation between absorbance and the standard (Trolox) concentrations was calculated and the DPPH radical scavenging activity was estimated for all the peptides. The results were expressed as µmol of Trolox equivalent/mg of sample.

2.2.6. Statistical analysis

All analysis were performed in triplicates and comparisons among the treatment groups were carried out by one-way analysis of variance (ANOVA), grouped by Duncan's multiple range test and Tukey's studentized range test using Statistical Analysis System Software, SAS version 9.0 (SAS Institute, Cary, NC). Groups were considered to be significantly significant when $P \leq 0.05$ and results were reported as mean ± SEM.

2.3 RESULTS AND DISCUSSION:

2.3.1 Effect of solvent concentration and extraction time on the antioxidant activity

The antioxidant activity of the lipophilic extract is 0.028 ± 0.05 µ mol TE/mg. Table 2.1 illustrates the effect of solvent concentrations on the scavenging property. Ethanol was used due to its nontoxic nature and environment friendly properties (Arnold & Choudhury, 1962; Wu, Duckett, Neel, Fontenot, & Clapham, 2008; Jang & Xu, 2009). A gradual increase in the antioxidant activity was observed up to 80% and there was a decline at 100% ethanol. A similar trend was reported when ethanol was used beyond 70% for the antioxidant activity of

extracts of Jerusalem Artichoke (Ling-Ling, Hai-Ying, Han, & Tao, 2009). The ethanol concentration influences the properties of the components by increasing the solvent to solid ratio and thereby increases the rate of diffusion of the compounds from the solid to the solvent (Cacace & Mazza, 2003). The presence of diverse compounds with different polarity might have contributed to the altered antioxidant property of the hydrophilic fraction of egg yolk samples. Our study showed extraction at 80% ethanol concentration has the highest antioxidant activity. Extraction time had significant effect on the antioxidant activity while the activity was not increased at prolonged extraction time. Studies on ethanolic extracts of defatted borage (Borago officinalis L.) seeds in a meat model system showed neither short (15 min) nor long (105 min) extraction times are suitable for the optimum antioxidant activity and reported a maximum free radical scavenging activity at 62 min (Wettasinghe & Shahidi, 1999). Our results showed the optimum time was 1 h (Table 2.2). The decrease in the antioxidant activity noticed after 1h may be because of the oxidation of the antioxidative compounds due to the increased oxygen exposure over the time (Chirinos, Rogez, Campos, Pedreschi, & Larondelle, 2007). As reported by Chew et al. (2011), the time of extraction plays an important role in the reduction of energy as well as extraction process; hence it is well recommended to select least time with maximum extraction. In the study, 80% ethanol was chosen as the solvent for extraction and 1 h as the extraction time.

Based on the solubility of antioxidants, they were grouped as hydrophilic antioxidants, for example, vitamin C, and lipophilic compounds, such as vitamin E and carotenoids (Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002). Hydrophilic antioxidants circulate in the body, while lipophilic antioxidants can penetrate the lipoprotein cell membrane with increased bioavailability and serve as an *in vivo* free radicals chain breaking antioxidant (Burton, Cheeseman, Doba, Ingold, & Slater, 1983). It is difficult to determine the exact amount of lipophilic components in food, as the antioxidants components were of chemical diversity and were differentially localized. **In eggs, the functional property is contributed by peptides derived from egg white proteins, as well as certain components in the egg yolk like phosvitin, carotenoids, phospholipids, etc.** Therefore, extraction of the lipophilic and hydrophilic fractions helps to determine the total antioxidant activity of the egg sample.

2.3.2 Effect of cooking and simulated digestion on the antioxidants

Effects of cooking methods on the antioxidant activity of eggs were determined using hydrophilic and lipophilic ORAC assays (Table 2.3). Among the egg white samples, the fresh samples showed higher antioxidant activity than the fried samples. But the fresh and boiled egg white samples did not show significant difference. The water-soluble amino acids and proteins possess the antioxidant activity by their metal chelating property (Lu & Baker, 1986), and may contribute to the antioxidant activity of the fresh egg samples. Wu et al. (2008) reported that cooking can alter the proteins, denature and degrade or reduce the antioxidant activity of compounds, especially the hydrophilic compounds. The digested egg

white samples exhibited much higher ($P < 0.05$) antioxidant activity than the undigested ones (Table 2.3). This is due to the release of peptides and amino acids during digestion. Amino acids can act as primary antioxidants, possess synergistic action (Flaczyk, Amarowicz, & Korczak, 2003), and the increased radical scavenging activity after digestion results from the breakdown of protein into peptides and free amino acids (Sakanaka, Tachibana, Ishihara, & Raj Juneja, 2004). Amino acids' antioxidative property is due to the reaction of amino or sulfur groups with the lipid peroxides in the free radical chain reaction, resulting in the formation of less reactive byproducts (Pokorný, Yanishlieva & Gordon, 2001). A positive correlation exists between the amount of peptides and the antioxidant activity (Wu, Chen, & Shiau, 2003).

Fresh egg yolk showed higher antioxidant activity than fresh egg white. The higher antioxidant activity of the egg yolk may be due to the presence of natural antioxidants present in the fresh sample. The egg yolk is a rich source of unsaturated fatty acids and iron (Hartmann & Wilhelmson, 2001), and in order to prevent the lipid peroxidation there exist an antioxidant system within the egg yolk (Yamamoto, Sogo, Iwao, & Miyamoto, 1990). The presence of egg yolk components like phosvitin, egg yolk phospholipids such as sphingomyelin, lysophosphatidylcholine, phosphatidyl choline, phosphatidylethanolamine, carotenoids like lutein and zeaxanthin with reported antioxidant activity contributes to the overall radical scavenging activity of the egg yolk samples (King, Boyd, & Sheldon, 1992; Ribaya-Mercado & Blumberg, 2004; Guérin-Dubiard, Castellani, & Anton, 2007). It was also noticed that cooking reduced the antioxidant activity, which might be due to the destruction or degradation of the antioxidant components during cooking. Simulated gastrointestinal digestion led to significant increase in the antioxidant activity. The boiled egg yolk samples treated with pepsin followed by pancreatin showed higher antioxidant activity than the other treated groups. These results suggest the release of antioxidant peptides or amino acids in the body during digestion.

Antioxidant activity of the fresh whole egg samples was much lower than the fresh egg yolk. This may be due to either an inefficient extraction of antioxidants from whole egg using one solvent, or the total antioxidant activity was masked by the interaction between proteins and carotenoids, similar to the masked effect was reported for the interaction between proteins and tea flavanoids (Arts et al., 2002). Interestingly, our results showed that antioxidant activity of whole egg samples increased after cooking; this may be due to decreased protein and carotenoid interaction during cooking, leading to improved extraction of carotenoids from the samples. Possible synergistic or addictive antioxidant activity was not observed in fresh whole egg samples and the decrease observed in the homogenized whole egg samples might be due to the interaction between the components present in the egg white and egg yolk, thereby reducing the free radical scavenging property. Similarly, simulated gastrointestinal digestion of whole egg samples also increased the antioxidant activity in a similar trend as above.

DPPH radical scavenging activity and ABTS assay showed similar trends as that of ORAC (Tables 2.4 and 2.5). DPPH is a very strong chromogen and the presence of the antioxidants and an electron or hydrogen donor in a sample, results in the discoloration of the radical chromogen (Arnao, 2000); except in egg yolk samples this activity was not reduced by cooking and was significantly increased upon digestion of cooked eggs. ABTS assays showed slight difference in the activity among fresh and cooked egg white samples, as well as whole egg samples, but not in egg yolk samples**.** But it was noticed that boiled samples treated with pepsin and pancreatin showed significant higher antioxidant activity than the fried pepsin and pancreatin treated samples. Among the whole egg samples, boiled samples showed no different from the fried samples (Table 2.4).

The present study showed the presence of antioxidants in eggs, and the antioxidant activity increased upon simulated digestion. All the assays showed an increase in antioxidant activity subjected to digestion; these findings coincide with other observations on the increased antioxidant activity of peptides derived from egg yolk (Young, Fan, & Mine, 2010; Xu, Katayama, & Mine, 2007; Katayama, Ishikawa, Fan, & Mine, 2007) and egg white (Davalos et al., 2004). Thus, this study shows the potential role of egg in the diet as a source of antioxidants that might contribute to the prospective benefits of egg consumption.

2.4 CONCLUSIONS:

Antioxidants are present both in egg white and egg yolk; fresh egg yolk shows higher antioxidant activity than the fresh egg white and the whole egg samples. The antioxidant activity of the egg samples tested by different assays resulted in similar trends on the effect of cooking and simulated gastrointestinal digestion. Cooking reduced antioxidant activity of egg yolk more than egg white and whole egg. Simulated gastrointestinal digestion increased significantly the antioxidant activity of all egg samples, which indicated the contribution of released peptides and amino acids. Insight of this study, further investigation into the identification of novel antioxidant components released could be of considerable interest.

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Table 2.1 Optimization of extraction conditions for determining hydrophilic ORAC (H-ORAC) of fresh egg yolk using different solvent concentrations.

Extraction solvent (Ethanol %)	**H-ORAC** (µ mol TE/ mg)	**Total ORAC** (µ mol TE/ mg)
Phosphate buffer (pH 7.5)	0.012 ± 0.04^{c}	0.031 ± 0.05^{d}
20	0.003 ± 0.03^{d}	0.040 ± 0.07^{c}
40	0.047 ± 0.04^{b}	0.075 ± 0.09^{b}
60	0.043 ± 0.03^{b}	0.071 ± 0.04^{b}
80	0.066 ± 0.02^{a}	0.094 ± 0.02^{a}
100	0.045 ± 0.04^{b}	0.073 ± 0.05^{b}

*The total antioxidant activity was calculated as the sum of H-ORAC and the lipophilic ORAC (L-ORAC) values. The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Duncan's multiple range test; different letters (a, b, c, d) denotes significant difference with the treatment groups ($P < 0.05$).

Table 2.2 Optimization of the extraction conditions for determining hydrophilic ORAC (H-ORAC) of fresh egg yolk using different time of extraction.

Extraction time (h)	**H-ORAC** (µ mol TE/ mg)	**Total ORAC** (µ mol TE/ mg)
0.5	0.042 ± 0.04^{d}	0.070 ± 0.07^{d}
1	0.067 ± 0.03^{a}	0.095 ± 0.05^{a}
2	0.043 ± 0.04^{d}	0.071 ± 0.09^{d}
4	0.044 ± 0.03^{d}	0.072 ± 0.04^{d}
6	0.059 ± 0.02^{b}	0.087 ± 0.02^{b}
8	0.048 ± 0.04^{c}	0.076 ± 0.05^{c}
24	0.057 ± 0.04^{b}	0.085 ± 0.05^{b}

*The total antioxidant activity was calculated as the sum of H-ORAC and the lipophilic ORAC (L-ORAC) values. The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Duncan's multiple range test; different letters (a, b, c, d) denotes significant difference with the treatment groups ($P < 0.05$).

Table 2.3 Total antioxidant (lipophilic and hydrophilic) activity of the egg samples, using ORAC assay.

Samples	**H-ORAC** (μ mol TE/ mg)	**L-ORAC** (μ mol TE/mg)	**Total ORAC** (μ mol TE/mg)
Egg white			
Fresh - No enzyme	0.058 ± 0.32^{i}	-	0.058 ± 0.32^{l}
Boiled - No enzyme	$0.056 \pm 0.12^{i, j}$	-	0.056 ± 0.12^{l}
Pepsin	$0.129 \pm 0.01^{c,d}$	-	0.129 ± 0.01^{g}
Pepsin+ Pancreatin	0.197 ± 0.10^{a}	-	0.197 ± 0.10^{a}
Fried - No enzyme	0.052 ± 0.04^{j}	-	0.052 ± 0.04^{m}
Pepsin	0.115 ± 0.03^{e}	-	0.115 ± 0.03^{h}
Pepsin+Pancreatin	0.151 ± 0.04^{b}	-	0.151 ± 0.04^{e}
Egg yolk			
Fresh - No enzyme	0.065 ± 0.04^{h}	0.027 ± 0.05^{e}	0.092 ± 0.08^{i}
Boiled - No enzyme	0.059 ± 0.14^{i}	0.020 ± 0.07^{f}	0.079 ± 0.12^{j}
Pepsin	0.117 ± 0.05^{e}	0.030 ± 0.04^{d}	0.147 ± 0.09^{f}
Pepsin+Pancreatin	0.120 ± 0.09^{e}	0.059 ± 0.12^{a}	0.179 ± 0.08^{c}
Fried - No enzyme	$0.055 \pm 0.02^{i, j}$	0.021 ± 0.09^{f}	0.076 ± 0.02^{j}
Pepsin	0.102 ± 0.13^{g}	0.031 ± 0.04^{d}	0.133 ± 0.18^{f}
Pepsin+ Pancreatin	0.105 ± 0.07^{g}	0.061 ± 0.12^{a}	0.166 ± 0.04^{d}
Whole egg			
Fresh - No enzyme	0.038 ± 0.05^{k}	0.026 ± 0.12^{e}	0.064 ± 0.07^{k}
Boiled - No enzyme	$0.055 \pm 0.11^{i,j}$	0.023 ± 0.07^{f}	0.078 ± 0.04^{j}
Pepsin	0.142 ± 0.04^{c}	0.022 ± 0.12^{f}	0.164 ± 0.09^{d}
Pepsin+Pancreatin	$0.129 \pm 0.07^{c, d}$	0.052 ± 0.03^{b}	0.181 ± 0.13^{b}

Fried - No enzyme	0.052 ± 0.04^{j}	0.025 ± 0.09^{e}	0.077 ± 0.02^{j}
Pepsin	0.111 ± 0.05^{f}	0.018 ± 0.03^{g}	0.129 ± 0.06^{g}
Pepsin+ Pancreatin	0.120 ± 0.10^{e}	0.042 ± 0.02^{c}	0.164 ± 0.13^{d}

*The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Tukey's studentized range test; alphabets denotes significant difference with the treatment groups ($P < 0.05$). Data represent mean ± SEM; n=3

Table 2.4 Total antioxidant (lipophilic and hydrophilic) activity of the egg samples, using DPPH assay.

Samples	**H-DPPH** (μ mol TE/mg)	**L-DPPH** (μ mol TE/mg)	**Total DPPH** (μ mol TE/ mg)
Egg white			
Fresh - No enzyme	0.019 ± 0.09^{j}	-	0.019 ± 0.09^{i}
Boiled - No enzyme	0.023 ± 0.04^{i}	-	0.023 ± 0.04^{g}
Pepsin	0.045 ± 0.12^{e}	-	0.045 ± 0.12^{e}
Pepsin+ Pancreatin	0.058 ± 0.09^{d}	-	0.058 ± 0.09^{d}
Fried - No enzyme	0.026 ± 0.19^{h}	-	$0.026 \pm 0.19^{f, g}$
Pepsin	0.056 ± 0.03^{d}	-	0.056 ± 0.03^{d}
Pepsin+ Pancreatin	$0.053 \pm 0.07^{d,e}$	-	0.053 ± 0.07^{d}
Egg yolk			
Fresh - No enzyme	0.017 ± 0.02^{k}	0.004 ± 0.002^{c}	0.021 ± 0.02^{h}
Boiled - No enzyme	0.017 ± 0.01^{k}	0.001 ± 0.001^{d}	0.018 ± 0.07^{i}
Pepsin	0.035 ± 0.02^{f}	0.010 ± 0.021^{b}	0.045 ± 0.04^{e}
Pepsin+Pancreatin	0.046 ± 0.02^{e}	$0.011 \pm .001^{a}$	0.057 ± 0.02^{d}
Fried - No enzyme	0.019 ± 0.02^{j}	$0.001 \pm .004^{d}$	0.020 ± 0.09^{h}
Pepsin	0.020 ± 0.02^{i}	0.003 ± 0.002^{d}	0.023 ± 0.14^{g}
Pepsin+Pancreatin	0.028 ± 0.02^{g}	0.002 ± 0.001^{e}	0.030 ± 0.22^{f}
Whole egg			
Fresh - No enzyme	0.016 ± 0.02^{k}	0.002 ± 0.01^{e}	0.018 ± 0.05^{i}
Boiled - No enzyme	0.025 ± 0.02^{h}	0.002 ± 0.003^{e}	$0.027 \pm 0.17^{f, g}$
Pepsin	0.068 ± 0.02^{c}	0.001 ± 0.011^{d}	0.069 ± 0.28^{c}
Pepsin+ Pancreatin	0.077 ± 0.02^{a}	0.001 ± 0.009^{d}	0.078 ± 0.04^{a}
Fried - No enzyme	0.023 ± 0.02^{i}	0.002 ± 0.001^{e}	$0.025 \pm 0.07^{f, g}$
Pepsin	$0.052 \pm 0.02^{d,e}$	0.004 ± 0.002^{c}	0.056 ± 0.33^{d}
Pepsin+Pancreatin	0.069 ± 0.02^{b}	0.004 ± 0.01^{c}	0.073 ± 0.21^{b}

*The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Tukey's studentized range test; alphabets denotes significant difference with the treatment groups (P <0.05). Data represent mean ± SEM; n=3

Table 2.5 Total antioxidant (lipophilic and hydrophilic) activity of the egg samples, using ABTS assays.

Samples	**H-ABTS** (µ mol TE/ mg)	**L-ABTS** (µ mol TE/mg)	**Total ABTS** (µ mol TE/mg)
Egg white			
Fresh - No enzyme	0.049 ± 0.05^{i}	-	$0.049 \pm 0.05^{l,m}$
Boiled - No enzyme	0.051 ± 0.11^{i}	-	$0.051 \pm 0.11^{k,l}$
Pepsin	0.103 ± 0.07^{c}	-	0.103 ± 0.07^{f}
Pepsin+Pancreatin	0.116 ± 0.20^{b}	-	0.116 ± 0.20^{c}
Fried - No enzyme	0.045 ± 0.04^{j}	-	0.045 ± 0.04^{m}
Pepsin	0.086 ± 0.15^{d}	-	0.086 ± 0.15^{h}
Pepsin+Pancreatin	0.126 ± 0.03^{a}	-	0.126 ± 0.03^{a}
Egg yolk			
Fresh - No enzyme	0.050 ± 0.09^{i}	$0.034 \pm 0.01^{c,d}$	0.084 ± 0.11^{h}
Boiled - No enzyme	0.018 ± 0.02^{n}	0.029 ± 0.07^{e}	0.047 ± 0.08^{m}
Pepsin	0.061 ± 0.06^{h}	0.046 ± 0.01^{a}	0.107 ± 0.02^{e}
Pepsin +Pancreatin	0.032 ± 0.03^{m}	0.096 ± 0.05^{g}	0.128 ± 0.03^{a}
Fried - No enzyme	0.039 ± 0.02^{l}	0.028 ± 0.07^{e}	0.067 ± 0.09^{j}
Pepsin	0.067 ± 0.10^{g}	0.032 ± 0.11^{d}	0.099 ± 0.13^{g}
Pepsin+Pancreatin	0.062 ± 0.02^{h}	0.046 ± 0.02^{a}	0.108 ± 0.04^{e}

Whole egg			
Fresh - No enzyme	0.044 ± 0.02^{k}	0.035 ± 0.04^{c}	0.079 ± 0.06^{k}
Boiled - No enzyme	0.044 ± 0.02^{k}	0.040 ± 0.04^{b}	0.084 ± 0.06^{h}
Pepsin	$0.069 \pm 0.05^{f,g}$	0.028 ± 0.02^{e}	0.097 ± 0.07^{g}
Pepsin+Pancreatin	0.076 ± 0.09^{e}	0.045 ± 0.04^{a}	0.121 ± 0.14^{b}
Fried - No enzyme	0.039 ± 0.02^{l}	0.029 ± 0.03^{e}	0.068 ± 0.05^{j}
Pepsin	0.070 ± 0.12^{f}	0.036 ± 0.03^{c}	0.106 ± 0.32^{e}
Pepsin+Pancreatin	0.071 ± 0.06^{f}	0.040 ± 0.01^{b}	0.111 ± 0.07^{d}

*The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Tukey's studentized range test; superscripts of alphabets denotes significant difference with the treatment groups ($P < 0.05$). Data represent mean ± SEM; n=3

CHAPTER-3 PURIFICATION AND CHARACTERISATION OF ANTIOXIDANT PEPTIDES DERIVED FROM BOILED EGG WHITE ENZYMATIC HYDROLYSATE

3.1 INTRODUCTION:

Free radicals may exhibit physiologic roles by functioning as signaling and regulatory molecules that are involved in the signal transduction, gene transcription, cellular regulation, and also pathogen destruction (Lander, 1997; McCord, 2000; Zheng & Storz, 2000), or pathologic roles in causing mammalian cell damage and pathogenesis of chronic diseases (Fridovich, 1999; McCord, 2000). Free radicals also exert deleterious impact on food, and are the major cause for the quality deterioration through lipid peroxidation and protein oxidation (Coupland & McClements, 1996; Elias, Kellerby, & Decker, 2008). Antioxidants play an important role in providing protection against the free radical induced oxidation (Elias et al., 2008). The antioxidant enzymes like superoxide dismutase, glutathione peroxidases, glutathione reductase, catalase and antioxidant nutrients like Vitamin E forms an important line of defense against free radicals (Fang, Yang, & Wu, 2002).

The potential role of the dietary protein in disease prevention is of greater interest these days. The possible capability of a protein as an efficient food antioxidant additive is attributed by various mechanisms including inactivation, reduction and removal of free radicals, chelation of transition metals and physical alteration of food particles (Amarowicz, 2008; Elias et al., 2008). Upon ingestion of proteins, a cascade of degradation occurs elicited by various gastrointestinal enzymes. These proteolytic activities result in the release of a mixture of amino acids and small peptides which in turn proficiently absorbed by small intestine enterocytes (Erickson & Kim, 1990). Those breakdown products of proteins within 3-20 amino acids per peptide have bioactive function after released from the parent protein source, and hence termed as 'bioactive peptides' (Pihlanto & Korhonen, 2003; Kitts & Weiler, 2003). The amino acid sequence of these peptides have significant role in determining the bioactive properties (Pihlanto & Korhonen, 2003). The amino acids such as Cys, Met, Try, Tyr, Phe and His were reported to have antioxidative properties (Elias et al., 2008).

Several *in vivo* and *in vitro* antioxidant studies on peptides hydrolyzed from animal and plant sources have been reported (Pihlanto & Korhonen, 2003). The pepsin and trypsin hydrolysates of fish protein was identified with antioxidant peptides such as Leu-Asn-Leu-Pro-Thr-Ala-Val-Tyr-Met-Val-Thr and His-Gly-Pro-Leu-Gly-Pro-Leu (Je, Qian, Lee, Byun, & Kim, 2008; Mendis, Rajapakse, & Kim, 2005). Various antioxidant peptides were purified from milk including potent superoxide scavenging peptide, Tyr-Phe-Try-Pro-Glu-Leu from pepsin digest of milk protein casein (Suetsuna, Ukeda, & Ochi, 2000). Short peptides with strong antioxidant activities from whey protein, soya protein, maize zein, canola protein hydrolysates were also reported (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Cumby, Zhong, Naczk, & Shahidi, 2008; Kong & Xiong, 2006;

Peng, Xiong, & Kong, 2009). These studies show potential role of enzymatically modified proteins as a natural source of antioxidants.

Egg is an excellent source of protein and many bioactive components (Kovacs-Nolan, Phillips, & Mine, 2005). Research on egg derived peptides shows its alternative role as a natural antioxidant source. Earlier reports on the antioxidant activity of pepsin digest of crude egg white identified Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu with strong radical scavenging activity from ovalbumin, the major egg white protein (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004). Ovalbumin pepsin hydrolysate was reported for inhibitory action on superoxide anion, hydroxyl radical and linoleic acid oxidation *in vitro* (Xu, Shangguan, Wang, & Chen, 2007). Antioxidant peptides were also characterized from ovotransferrin, the second major egg white protein (Huang, Majumder, & Wu, 2010; Shen et al. 2010). Other renowned components with antimicrobial and antiviral properties like lyzozyme and ovoinhibitor were found to have antioxidant activity (Frenkel, Chrzan, Ryan, Wiesner, & Troll, 1987; Liu et al., 2006; You, Udenigwe, Aluko, & Wu, 2010). Egg yolk protein hydrolysates were also identified with antioxidant properties (Sakanaka, Tachibana, Ishihara, & Raj, 2004). Sugino *et al.* (1997) reported the antioxidant activity of the egg yolk phospholipids. The tryptic digest of egg yolk phosvitin showed strong inhibiting property on lipid oxidation in linoleic acid system and efficient radical scavenging activity. The presence of His, Met and Tyr was suggested as responsible for the strong antioxidant activity of those phosvitin peptides (Xu, Katayama, & Mine, 2007). Alcalse hydrolysates of lecithin free egg yolk and egg shell membrane protein were studied for antioxidant property (Huang, Zhou, Ma, Cai, & Li, 2010; Park, Jung, Nam, Shahidi, & Kim, 2001). Furthermore the presence of carotenoids such as lutein and zeaxanthin improves the antioxidant capacity of the egg yolk (Handelman, Nightingale, Lichtenstein, Schaefer, & Blumberg, 1999; Karadas, Grammenidis, Surai, Acamovic, & Sparks, 2006; Nelson, Bernstein, Schmidt, Von Tress, & Askew, 2003). Latest research reports the presence of two aromatic amino acids Try and Tyr in egg yolk extracts as the major contributor to its antioxidant activity (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2011).

In spite of several studies conducted on antioxidative property of egg-derived peptides, there exists a paucity of information about the effect of cooking as well as gastrointestinal digestion on the antioxidative activity of eggs. In our study on the effects of different cooking methods on the antioxidant activity, the boiled egg white subjected to pepsin and pancreatin enzymatic hydrolysis possessed the highest antioxidant activity. Hence the boiled egg white hydrolysate was further purified and characterized for the study of antioxidant peptides.

3.2 MATERIALS AND METHODS:

3.2.1 Materials

Fresh white-shell eggs were obtained from Poultry Research Centre of the University of Alberta (Edmonton, AB, Canada). The enzymes, pepsin (porcine gastric mucosa), pancreatin (porcine pancreas) were purchased from Sigma

(Oakville, ON, Canada). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was obtained from Acros-organics (Morris Plains, NJ, USA) and AAPH (2, 2'-azobis (2-amidino-propane) dihydrochloride and fluorescein (FL) (Na salt) were obtained from Aldrich (Milwankee, WI). Ammonium acetate, ammonium carbonate, HPLC-grade acetonitrile, and trifluoroacetic acid (TFA) were obtained from Fisher Scientific Canada (Ottawa, ON, Canada).

3.2.2 Preparation of boiled egg white hydrolysate

Fresh eggs were boiled for 10 min in a saucepan with sufficient water covering the eggs, cooled the eggs by keeping under running water for 5 min and then peeled and separated the egg white. A 5 % of the boiled egg white slurry (w/v, dry weight) was prepared in the distilled water and then kept in the water bath at 80°C for 15 min with continuous shaking. The temperature of Lauda (A103) water bath (Brinkman, Missisauga, ON, Canada) was then adjusted to 37°C and transferred the egg sample to jacketed beaker and adjusted the pH to 2 with 1 N HCl. After the pH was stabilized, the proteolysis was initiated by the addition of pepsin (2% w/w of protein) at consistent temperature of 37 °C. After 3 h digestion, the hydrolysis was terminated by increasing the pH to 7. Then the temperature was increased to 40°C and pancreatin (2%, w/w of protein) was added for another 3 h digestion. And then the reaction was stopped by increasing the temperature to 95°C and kept it for 15 min. The enzyme hydrolysate was then centrifuged at 10,000 x g for 25 min, the supernatant was collected, freeze dried and stored for further analysis. The hydrolysate preparation was carried out using Titrando (Metrohm, Herisan, Switzerland) and circulating water bath to maintain consistent pH and temperature during the course of digestion.

3.2.3 Measurement of peptide concentration

Modified Lowry's protein assay (Lowry et al., 1951) was used to determine the protein concentration of the fractions from cation and anion exchange chromatography, and bovine serum albumin (BSA) was used as the standard.

3.2.4 Measurement of antioxidant activity

The antioxidant activity was determined using three different methods.

3.2.4.1 Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was performed according to Davalos, Gomez-Cordoves, & Bartolome (2004) with slight modifications, using fluorescein as a fluorescent probe. 100 µL of trolox standard solutions at final concentrations ranging from 1 to 8 µM or the egg white hydrolysate samples from the chromatographic fractions (serial dilutions) were placed in wells of a 96 well microplate, followed by addition of 50 µl of the fluorescein solution. The mixture was preincubated for 15 min at 37^{o}C. And then 50 µL of AAPH was added rapidly using a multichannel pipette. The microplate was immediately placed in a Fluoroskan Ascent microplate reader with 485-P excitation and 538-P emission filters and the fluorescence recorded every minute for 100 min. All readings were recorded

using Fluoroskan Ascent software. The area under the curve of fluorescence decay (AUC) was calculated using Graphpad prism software (trial version). Regression equations between AUC and antioxidant concentrations were calculated for all the samples. The ORAC value was calculated by dividing the slope of sample regression curve by the slope of Trolox regression curve. The following equation was used for the calculation of area under the fluorescence decay curve (AUC) using the normalized curves.

$$AUC = 1 + \sum_{i=0}^{i=100} f_i / f_0$$

f_0 is the initial fluorescence reading at time,0 min; f_i is the fluorescence reading at time *i*. The final ORAC values were expressed as µmol of Trolox equivalent/mg of peptide.

3.2.4.2 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ABTS decolorization assay

$ABTS^{\bullet+}$ decolorization assay was based on the method of Strljbe, Haenen, Berg, & Bast (1997) with slight modifications. ABTS radical cation was generated by mixing 7 mM ABTS with 2.45 mM potassium persulfate, and diluted 13 fold with assay buffer (3mM phosphate buffer at pH 7.5 containing 150 mM NaCl) immediately before use. For each run, 20 µL of the egg white hydrolysate samples from the chromatographic fractions (serial dilutions) and 80 µL of phosphate buffer were placed in wells of a 96-well microplate, followed by addition of 100 µL of the ABTS radical solution. Absorbance was monitored at 734 nm after 5 min incubation at 37 ^{o}C. A Trolox regression equation between absorbance and Trolox concentrations was calculated and used to calculate the Trolox equivalent antioxidant capacity (TEAC) value for all the samples. TEAC value is expressed as µmol of Trolox equivalent/mg of peptide.

3.2.4.3 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) assay

Scavenging of DPPH radical assay was performed according to Bersuder, Hole, & Smith (1998). 20 µL of the egg white hydrolysate samples from the chromatographic fractions (serial dilutions) and 80 µL of water were placed in wells of 96-well microplate, followed by addition of 100 µL of 0.2 mM DPPH in 95% ethanolic solution. Absorbance was monitored at 517 nm after 45 min of incubation at 37 ^{o}C. A Trolox regression equation between absorbance and the standard (Trolox) concentrations was calculated and the DPPH radical scavenging activity was estimated for all the peptides. The results were expressed as µmol of Trolox equivalent/mg of peptide.

3.2.5 Purification of antioxidant peptides from hydrolysate

The boiled egg white hydrolysate was dissolved in 10 mm ammonium acetate (pH 4) buffer and then filtered the sample by using 3000 Da ultra filtration membrane. Fractionation of hydrolysate was performed using a HiPrep 16/10 SP FF cation exchange column (16 x 100 nm, GE Healthcare Sweden) coupled with an AKTA explorer 10XT system. The column was equilibrated with 10 mM ammonium acetate (pH 4) and eluted with 0.5 M ammonium carbonate buffer at a flow rate of 5 mL/min. The injection volume was 4 mL and the elution was detected at 280 nm. The most potent fraction collected in the unadsorbed fraction was further applied to HiPrep Q FF 16/10 anion exchange column (16 x 100 nm, GE Healthcare Sweden). The column was equilibrated with 10 mM ammonium acetate (pH 8.5) and eluted with 10 mM ammonium carbonate and 1 M NaCl buffer at a flow rate of 5 mL/min. The fractions exhibiting the most potent antioxidant activity was further purified by reverse-phase-high-performance-liquid chromatography (RP-HPLC) on a Xbridge C18 column (10 mm x 150 mm, 0.5µm, Waters Inc, Milford, MA, USA) coupled with a guard column (40 x 10 mm, Waters Inc, Milford, MA, USA) attached to Waters 600 HPLC system, under the control of the software of Empower Version 2 for the instrument control and data acquisition. Sample was injected automatically at 500 µL by Waters 2707 autosampler, and was eluted using a linear gradient starting from 100% solvent A (HPLC-grade water containing 0.1% TFA) to 40 % solvent B (HPLC-grade acetonitrile with 0.1% TFA) over 40 min at a flow rate of 5 mL/min, followed by washing the column at 100% solvent B for 10 min before next run. The elution was monitored at a wavelength of 220 nm using Waters 2998 photodiode array. Fractions were collected at 2 min intervals from 3 min to 50 min (19 fractions), concentrated using vacuum-rotary evaporator at 35°C, and the antioxidant assays (ORAC, DPPH and ABTS) of each were determined.

3.2.6 Liquid chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Identification of the peptides in the most antioxidant active fractions from the RP-HPLC separation was carried out by liquid chromatography tandem mass spectrometry (LC-MS/MS). The analysis was carried out by Waters ACQUITY UPLC system connected online to Waters (Micromass) Q-TOF Premier (Milford, MA, USA). Peptides were separated by Waters Atlantis dC18 (75 µm x 150 mm, 3 µm) UPLC column (Milford, MA, USA). The separation was carried out using solvent A, 0.1% formic acid in optima LC/MS grade water and solvent B, 0.1% formic acid in optima grade acetonitrile. Samples in Solvent A (5 µL) was injected to the 5 µm trapping column for 2 min at a flow rate of 10 L/min using 99% solvent A, followed by a gradient from 99% A to 90% A over 5 min, to 70% A over 30 min, to 60% A over 3 min and 5% A over 1 min at a constant flow rate of 0.350 L/min, increased the flow rate to 0.500 µL/min and held at 5% A for 2 min, with subsequent increased to 98% A over 1 min, held for another 27 min, and then decreased the flow rate to 0.350 L/min over 1 min. Further ionization was performed by electrospray ionization technique (ESI) by NanoLockspray ionization source in a positive ion mode (capillary voltage at 3.80 kV and the

source temperature at 100^{o}C). Quadrupole Time-of-Flight (Q-TOF) analyzer operated in a positive ion MS/MS mode was used for peptide mass detection. A MS/MS full-scan was performed for each sample with an acquisition m/z range of 0-1000 Da. Instrumental control and data analysis were executed using MassLynx software (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K.). Peaks Viewer 4.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used in combination with manual *de novo* sequencing to process the MS/MS data and to perform peptide sequencing. The peptide sequences were identified from the respective monoisotopic mass.

3.2.7 Statistical analysis

The results were analyzed by one-way analysis of variance (ANOVA) using statistical analysis system software (SAS, version 9.0, SAS Institute, Cary, NC). The estimated the significant differences using Duncan's multiple range test at $p < 0.05$ (Duncan, 1955).

3.3 RESULTS AND DISCUSSION:

3.3.1 Fractionation of antioxidant peptides from boiled egg white hydrolysate

Cation exchange chromatography of boiled egg white hydrolysate gave 3 major peaks (A, B and C) and a minor peak (D) as shown (Fig. 3.1). The antioxidant activity was determined for all the fractions using ORAC, ABTS and DPPH assays as shown in Table 3.1. The fraction A showed the most potent antioxidant activity was then subjected to anion exchange chromatography. Five fractions were collected and the antioxidant activity was estimated (Table.3.2). The most potent fraction B was then subjected to further purification using an Xbridge C18 RP-HPLC column. 19 fractions were collected and the antioxidant activity of each fraction was shown in Table.3.3; Fractions 1, 5, 8 and 14 exhibited main antioxidant activity were used for further analysis by LC MS/MS. Fraction 12 showed the highest peptide concentration was also subjected for characterization.

3.3.2. Identification of peptide sequences

MS spectrums of each fraction and one representative peptide MS/MS interpretation from each fraction were shown in Fig 3.4. Peptides having intensity above the cutoff of 40% were sequenced using Peaks Viewer 4.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) in combination with manual *de novo* sequencing to process the MS/MS results (Table 3.4). A total of 63 peptides derived from boiled egg white were identified: 10 peptides from F1, 11 from F5, 13 from F8, 16 from F12, and 13 from F14 with amino acid residues ranging from 3 to 10 (Table 3.4).

Ovalbumin, contributing to 54-58% (w/w) of the total egg white protein, contains 386 amino acids sequences with a molecular weight of 45 kilo Dalton (kDa), (Huntington & Stein, 2001; Li-Chan et al., 1995; Lopez-Exposito et al., 2008). 18 peptides identified from the pepsin and pancreatin hydrolysate of boiled egg white were derived from ovalbumin. Several studies revealed the presence of

antihypertensive peptides like RADHPFL (Matoba, Usui, Fujita, & Yoshikawa, 1999), YAEERYPIL (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004), and IVF present in the egg white from enzymatic hydrolysates (Miguel, Recio, Gómez-Ruiz, Ramos, & López-Fandiño, 2004). YAEERYPIL was also characterized as a potent radical scavenging peptide (Davalos et al., 2004).

Ovotransferrin, the second major protein (12-14 %, w/w) in egg white, consists of 686 amino acid residues with a molecular mass of 78 kDa. This is a disulfide bond rich single chain glycoprotein that has been reported to have involvement in the redox linked signals and response to free radicals and specifically attacks superoxide radicals (Williams, Elleman, Kingston, Wilkins, & Kuhn, 1982; Li-Chan et al., 1995; Ibrahim, 1997). A total of 19 antioxidative peptides identified from boiled egg white hydrolysate were derived from ovotransferrin. LGFEYY (residues 339-344) characterized from the study was also reported from our previous study as a potent antioxidant peptide (Shen et al., 2010). Antioxidant peptides were also released from lyzozyme (5 peptides), ovostatin (6), ovomucoid (1), ovomucin α (7) and β (4) subunits and flavoprotein (3). The lyzozyme has a role in protecting against the oxidative damage in the body (Liu et al., 2006). Ovomucin was reported for its immunomodulatory property (Tsuge, Shimoyamada, & Watanabe, 1997); it is interesting to note a total of 11 peptides were characterized from ovomucin in the study. Ovostatin was reported as an antimicrobial protein (Molla, Matsumura, Yamamoto, Okamura, & Maeda; 1987); our study showed its derived peptides also possess antioxidant activity.

It was recently reported that peptide containing Pro (P), Asp (D), Tyr (Y), Trp (W) or His (H) tends to show greater antioxidant activity (Park et al., 2001; Ren et al., 2010). Pyrrolidine ring present in the proline has remarkably low ionization potential and forms charge transfer complex with $^1O_2$ and proline forms stable radicals with $^{\bullet}OH$ under hydrogen abstration (Matysik et al., 2002); Thus proline acts as a scavenger of $^1O_2$ and $^{\bullet}OH$, in addition to its reaction to H_2O_2 induced stress (Young, Martin, Feriozi, Brewer, & Kayser, 1973; Wondrak, Jacobson, & Jacobson, 2005; Krishnan, Dickman, & Becker, 2008); 27 peptides identified in the study contain proline (Table 3.4). The presence of indole group in Trp (W) and phenol group in Tyr serves as potent hydrogen donors and helps in converting the reactive oxygen species to more stable and less active indoyl and phenoxyl radicals (Park et al., 2001; Hernández-Ledesma et al., 2005). The presence of Trp was found in peptides from ovotransferrin (RIQWCAVGKD, SAGWN), ovalbumin (WTSSN) and ovostatin (GWIESPS). Tyr, another amino acid with antioxidant property was present in peptides from ovalbumin (2), ovotransferrin (3), ovmucin (2) and lyzozyme (2). Recent quantitative structure and activity relationships of antioxidant peptides indicated that a peptide with a hydrophobic amino acid at N-terminus, a basic amino acid residue at C-terminus, and a hydrophilic amino acid residue next to C terminus shows greater antioxidant activity (Li et al., 2011). Hydrophobic amino acid residues such as Val or Leu at the N terminus were also reported to increase the antioxidant activity (Park et al., 2001; Li, Li, He, & Qian, 2011). The ovalbumin derived peptides LQPSSVD and VLQPSSVD, and flavoprotein-derived peptide VAQ and VPN, contain Val or

Leu at their N termini, suggestive of increased antioxidative property (Park et al., 2001). Among the total 10 peptides identified from F1, 6 peptides (VPGAT, LHPI, LVELI, VKYNV, VLLPDEV, and LVLLPDEV) possess Val or Leu as the N terminus. The imidazole ring in His contributes to the antioxidant activity as a proton donor and a metal chelator (Chen et al., 1995; Park et al., 2001; Li et al., 2011). It was reported previously that the removal of the histidine from the C terminus could decrease the antioxidant activity of the peptides (Chen, Muramoto, Yamauchi, & Nokihara, 1996). Tsuge *et al.* (1991) reported 3 peptides (AHK, VHH, and VHHANQN) from egg proteins containing His and Val residues with strong antioxidant property. In this study, histidine containing peptides, AAHAV, LAEVPTH and VAAH from ovotransferrin, and AVHAAH from ovalbumin, were identified. Amino acid residues such as Ile, Phe, Ala and Lys at the N terminus also increase antioxidant activity (Guo, Kouzuma, & Yonekura, 2009). 17 peptides identified in the study contain one of these amino acid residues, which might contribute to the antioxidant activity of the peptides.

3.4. CONCLUSIONS:

Boiled egg white protein hydrolysate was fractionated using ion exchange chromatography and reverse-phase high performance liquid chromatography, five fractions showing potent antioxidant activities were subjected to LC-MS/MS characterization. A total of 63 peptides were identified, mainly from ovalbumin, ovotransferrin, ovomucin, lysozyme, and ovostatin. Our previous study has shown the presence of antioxidative aromatic amino acids in egg yolk; results from the present study implied that gastrointestinal digestion of egg white proteins could further enhance the antioxidant activity of egg by releasing a number of antioxidant peptides from egg proteins. Further research on the antioxidant activity of each peptide *in vivo* will help to understand the most potent peptide from the boiled egg white hydrolysates.

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Figure 3.1: Cation exchange chromatogram of boiled egg white hydrolysate using HiPreP 16/10 SP FF cation exchange column as described in materials and methods.

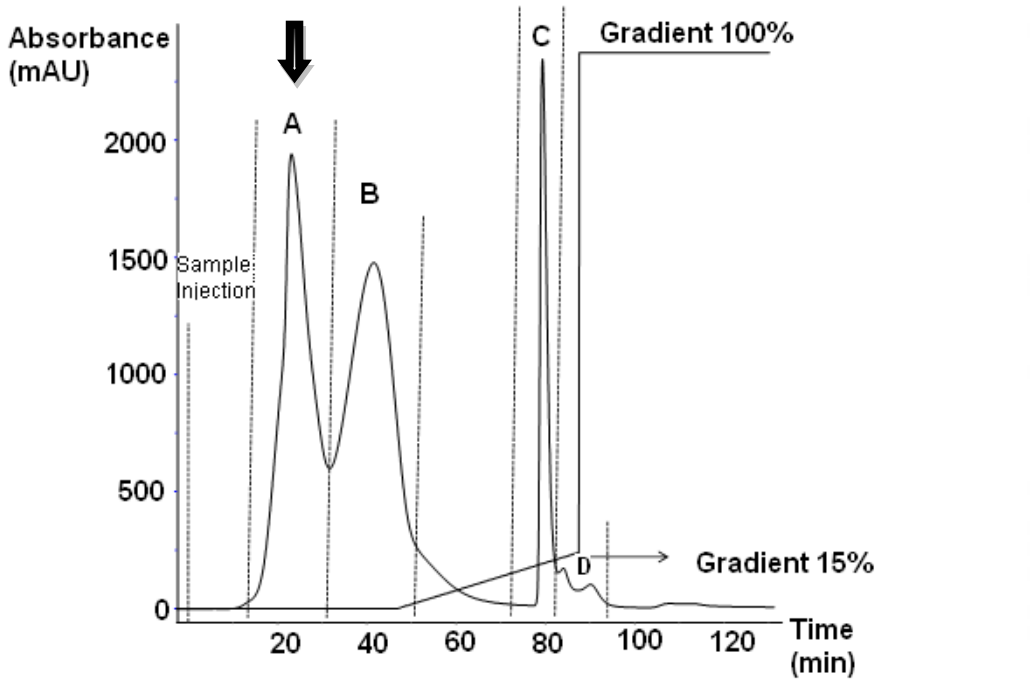


Table 3.1: Antioxidant activity of fractions from cation exchange chromatography using DPPH, ABTS and ORAC assays.

	DPPH (µmol TE/mg of protein)	ABTS (µmol TE/mg of protein)	ORAC (µmol TE/mg of protein)
Fraction A	1.04 ± 0.04^{a}	1.09 ± 0.24^{a}	2.38 ± 0.04^{a}
Fraction B	0.93 ± 0.02^{b}	0.94 ± 0.12^{b}	1.94 ± 0.34^{b}
Fraction C	0.52 ± 0.15^{c}	$0.55 \pm .03^{c}$	1.22 ± 0.01^{c}
Fraction D	0.01 ± 0.04^{d}	$0.15 \pm .03^{d}$	0.23 ± 0.07^{d}

The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Duncan's multiple range test; different letters (a, b, c, d) denotes significant difference with the treatment groups ($P < 0.05$).

Figure 3.2: Anion exchange chromatogram of Fraction A, which exhibited the most potent antioxidant activity using HiPrep Q FF 16/10 anion exchange column as described in 3.2.5.

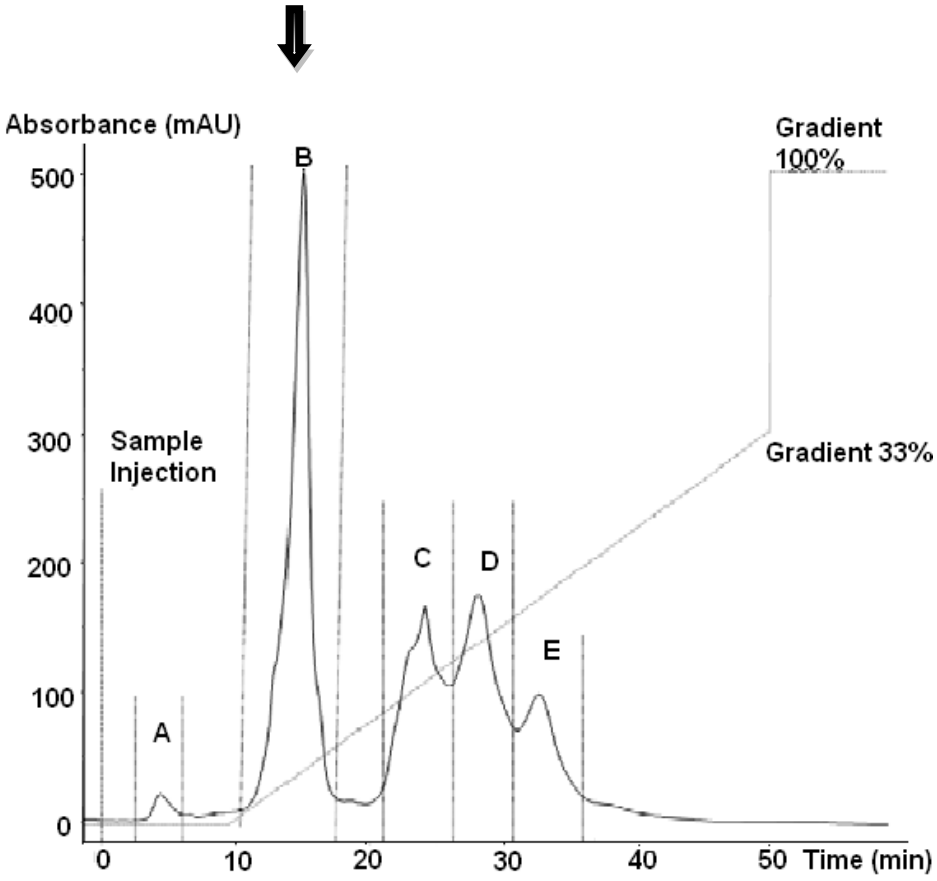


Table 3.2: Antioxidant activity of fractions from anion exchange chromatography using ORAC, DPPH and ABTS assays.

	DPPH (µmol TE/mg of protein)	ABTS (µmol TE/mg of protein)	ORAC (µmol TE/mg of protein)
Fraction A	1.39 ± 0.92^{b}	2.19 ± 0.06^{b}	2.45 ± 0.86^{b}
Fraction B	2.84 ± 0.38^{a}	2.88 ± 0.08^{a}	3.06 ± 0.10^{a}
Fraction C	1.25 ± 0.05^{d}	1.65 ± 0.05^{c}	2.16 ± 0.05^{c}
Fraction D	1.03 ± 0.04^{c}	0.92 ± 0.01^{d}	1.10 ± 0.05^{d}
Fraction E	1.38 ± 0.35^{b}	0.80 ± 0.01^{d}	0.90 ± 0.08^{d}

The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Duncan's multiple range test; different letters (a, b, c, d) denotes significant difference with the treatment groups ($P < 0.05$).

Figure 3.3: RP-HPLC chromatogram of fraction B in Figure 3.3 by Xbridge C18 column (10 mm x 150 mm, 0.5 M) under linear gradient condition of 100% solvent A (0.1%TFA in water) to 40% solvent B (0.1% TFA in acetonitrile) over 40 min at a flow rate of 5 mL/min.

Total of 19 fractions were collected at 2 min interval and antioxidant activity were determined.

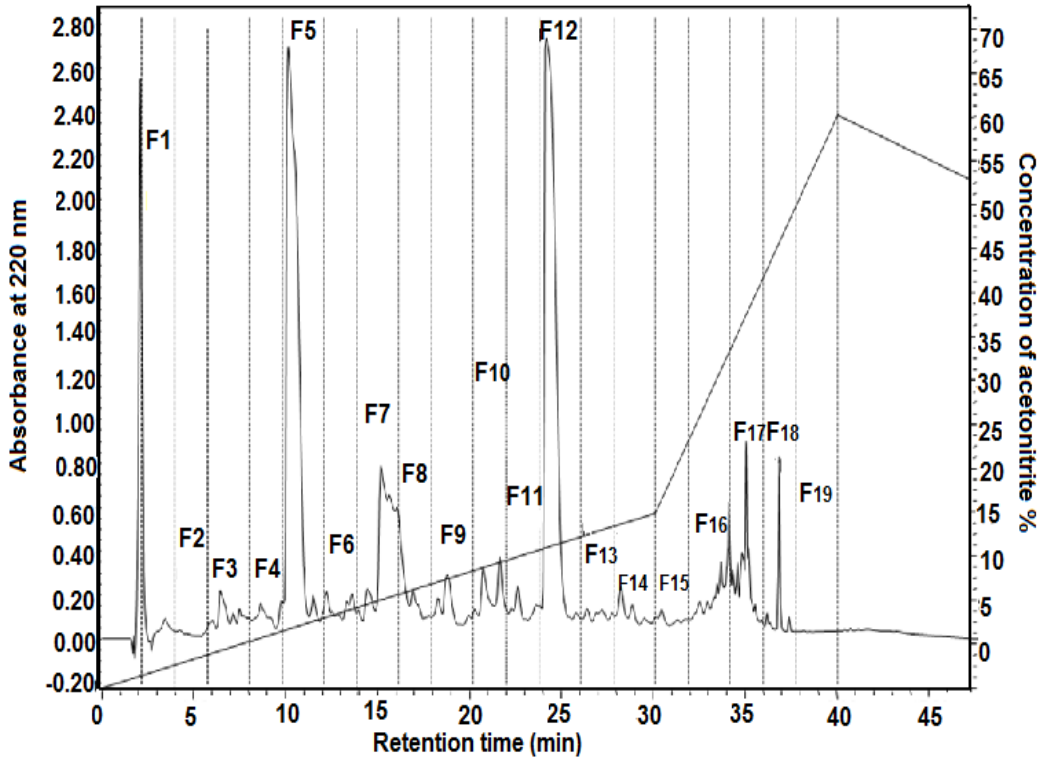
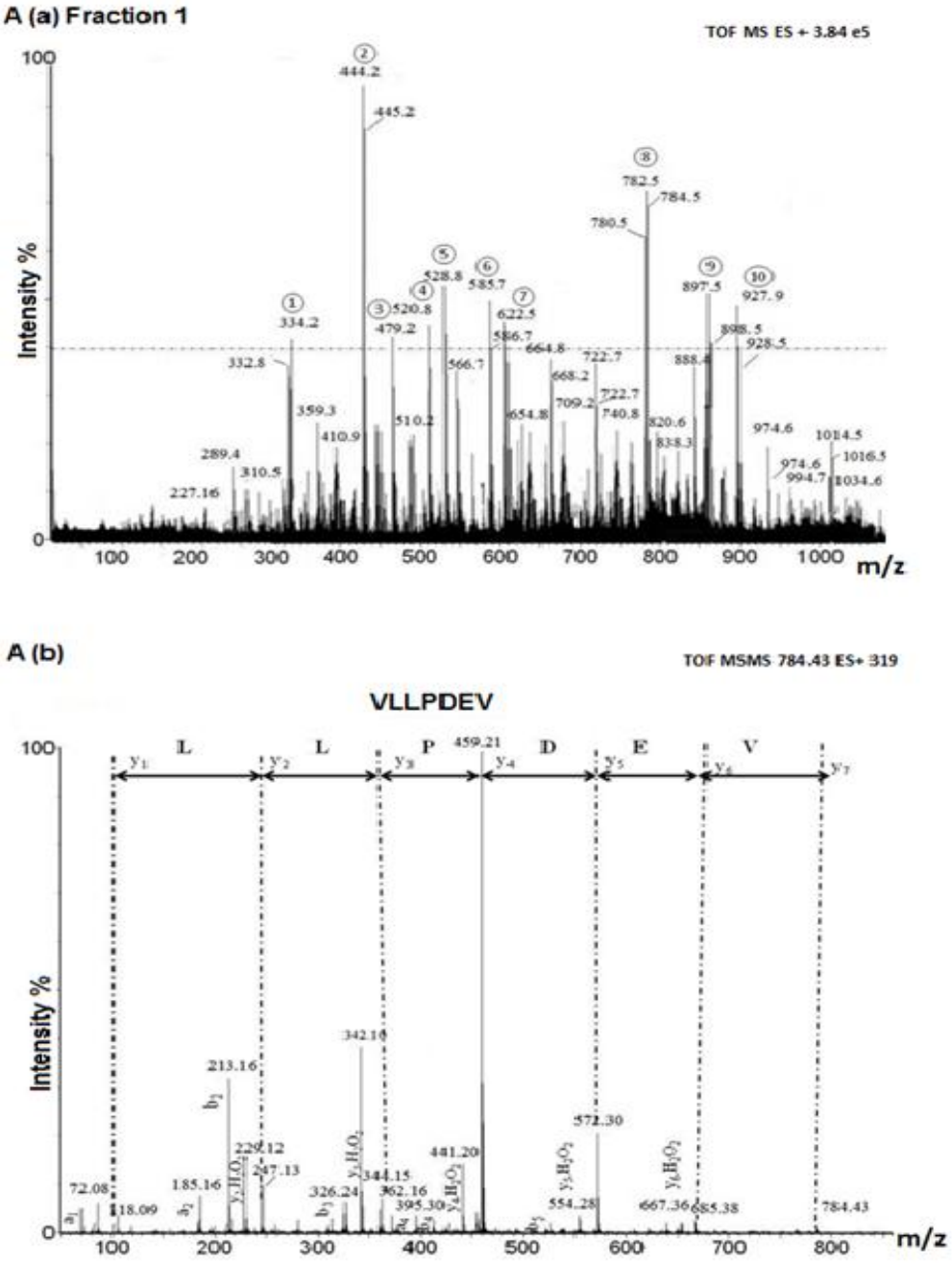


Table 3.3: The antioxidant activity of HPLC fractions determined by DPPH, ABTS and ORAC assay

	DPPH(µmol TE/mg of peptide)	ABTS(µmol TE/mg of peptide)	ORAC(µmol TE/mg of peptide)
Fraction 1	**2.13 ± 0.09**	**2.99 ± 0.06**	**4.92 ± 0.32**
Fraction 2	0.12 ± 0.03	0.14 ± 0.02	0.13 ± 0.10
Fraction 3	0.10 ± 0.14	0.09 ± 0.03	0.11 ± 0.05
Fraction 4	0.73 ± 0.06	0.82 ± 0.06	0.81 ± 0.07
Fraction 5	**1.80 ± 0.10**	**2.13 ± 0.04**	**3.37 ± 0.11**
Fraction 6	0.12 ± 0.02	0.23 ± 0.12	0.10 ± 0.03
Fraction 7	0.99 ± 0.06	1.36 ± 0.05	1.53 ± 0.04
Fraction 8	**2.04 ± 0.04**	**2.76 ± 0.05**	**3.44 ± 0.02**
Fraction 9	0.10 ± 0.03	0.09 ± 0.07	0.09 ± 0.04
Fraction 10	0.45 ± 0.10	0.90 ± 0.05	0.55 ± 0.21
Fraction 11	1.04 ± 0.04	1.99 ± 0.06	2.08 ± 0.14
Fraction 12	**0.74 ± 0.12**	**0.98 ± 0.10**	**1.58 ± 0.08**
Fraction 13	0.10 ± 0.18	0.09 ± 0.09	0.09 ± 0.06
Fraction 14	**1.70 ± 0.11**	**2.93 ± 0.12**	**2.80 ± 0.03**
Fraction 15	0.12 ± 0.05	0.28 ± 0.01	0.24 ± 0.20
Fraction 16	0.09 ± 0.04	0.09 ± 0.13	0.08 ± 0.22
Fraction 17	0.10 ± 0.14	0.07 ± 0.04	0.06 ± 0.15
Fraction 18	0.12 ± 0.03	0.09 ± 0.09	0.09 ± 0.19
Fraction 19	0.39 ± 0.07	1.54 ± 0.16	1.47 ± 0.22

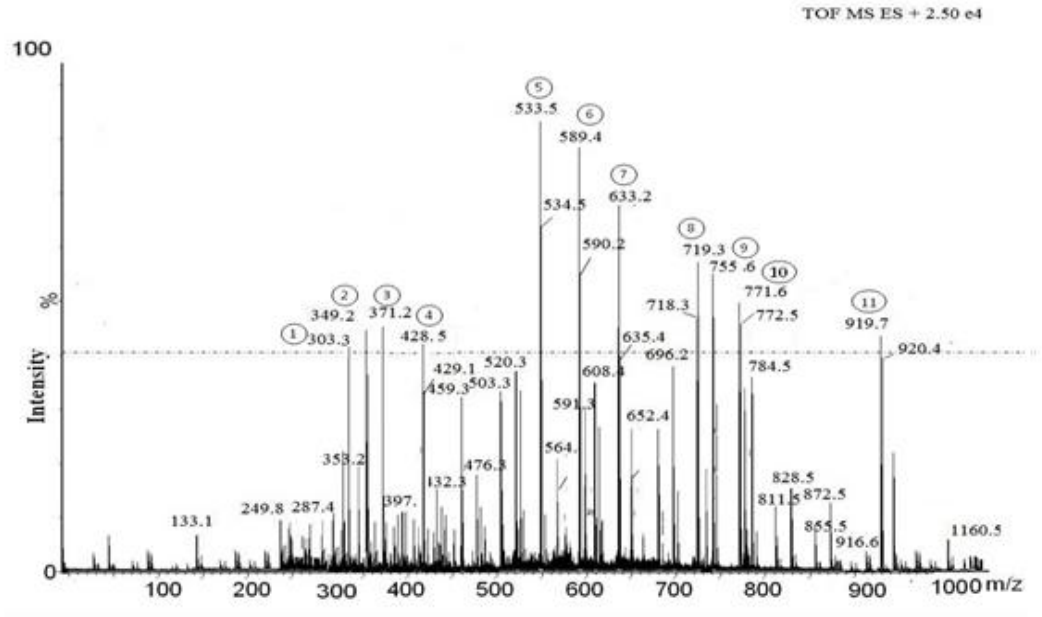
Data presented as means ± standard deviations (n = 3; each with duplicate measurements)

Figure 3.4: LC-MS spectra of fractions from RP-HPLC. The dashed line represents the cutoff ion intensity (40 %) of selected parent ions in the peptide sequencing. One candidate peptide was shown as *de novo* sequencing by using their MS/MS spectra by monoisotopic mass of the amino acids.

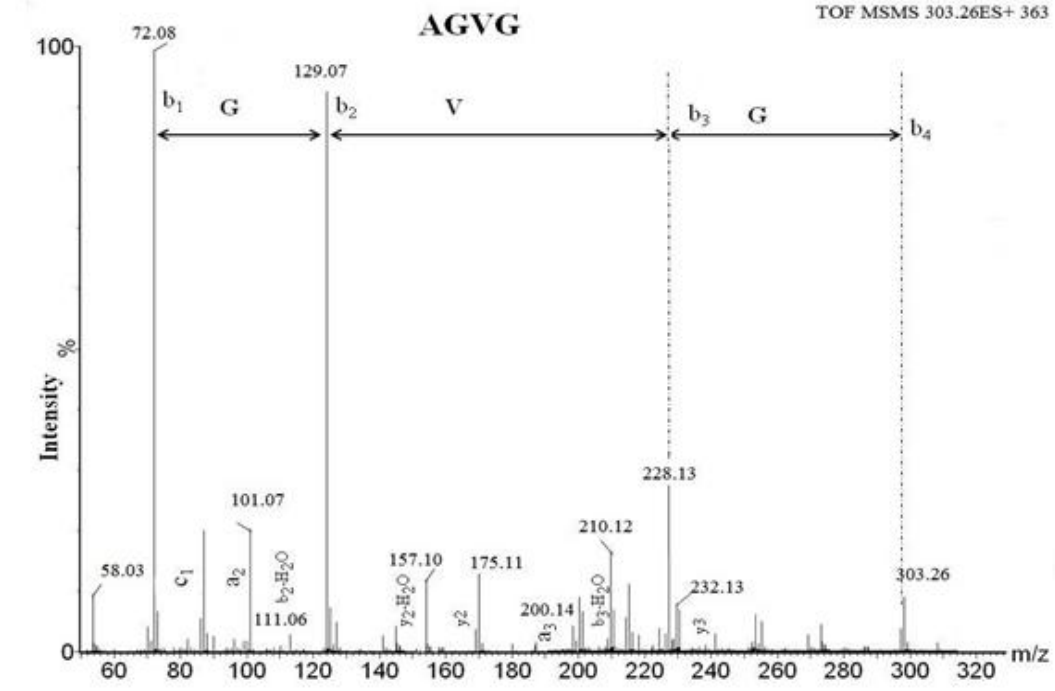


A (a) Fraction 1(1-10 parent ions); A (b) Interpretation of LC-MS/MS spectrum of the ion *m*/*z* 784.45, derived from ovalbumin peptide VLLPDEV.

B (a) Fraction 5

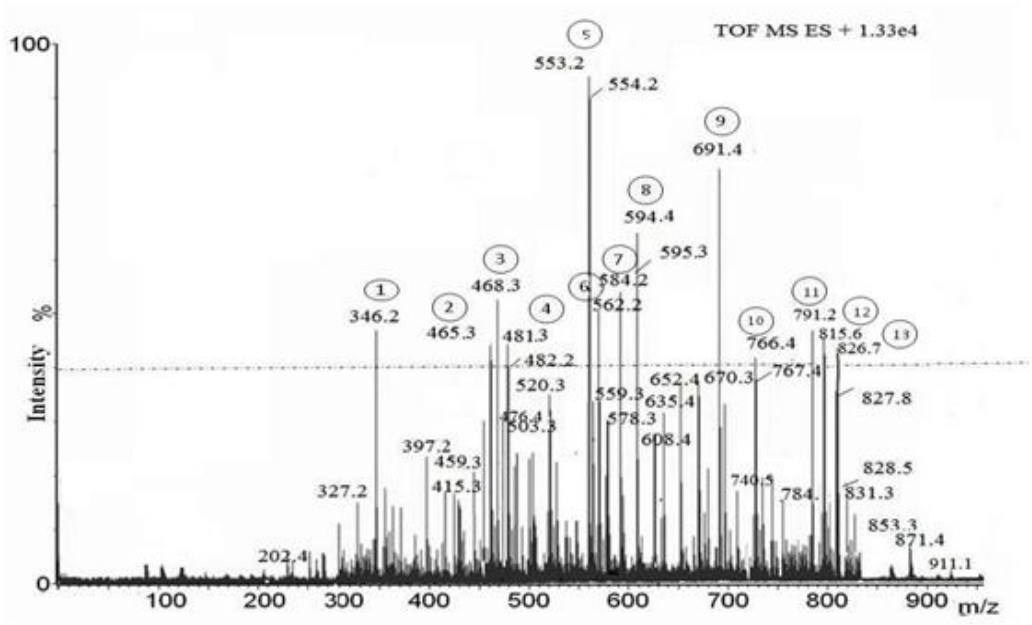


B (b)

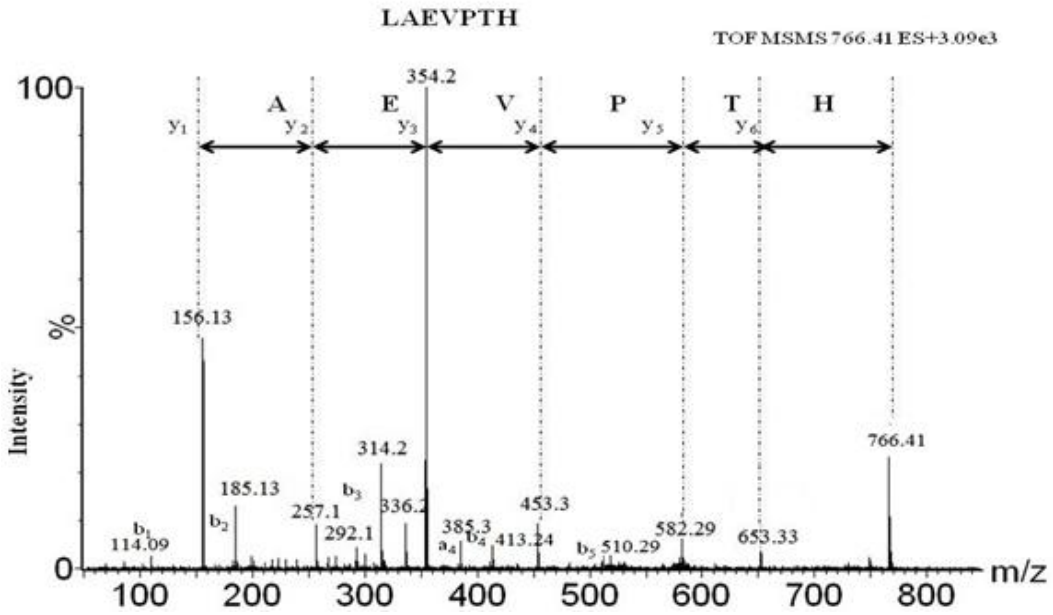


B (a) Fraction 5 (1-11 parent ions); B (b) Interpretation of LC-MS/MS spectrum of the ion *m/z* 303.36, derived from lyzozyme peptide AGVG.

C (a) Fraction 8

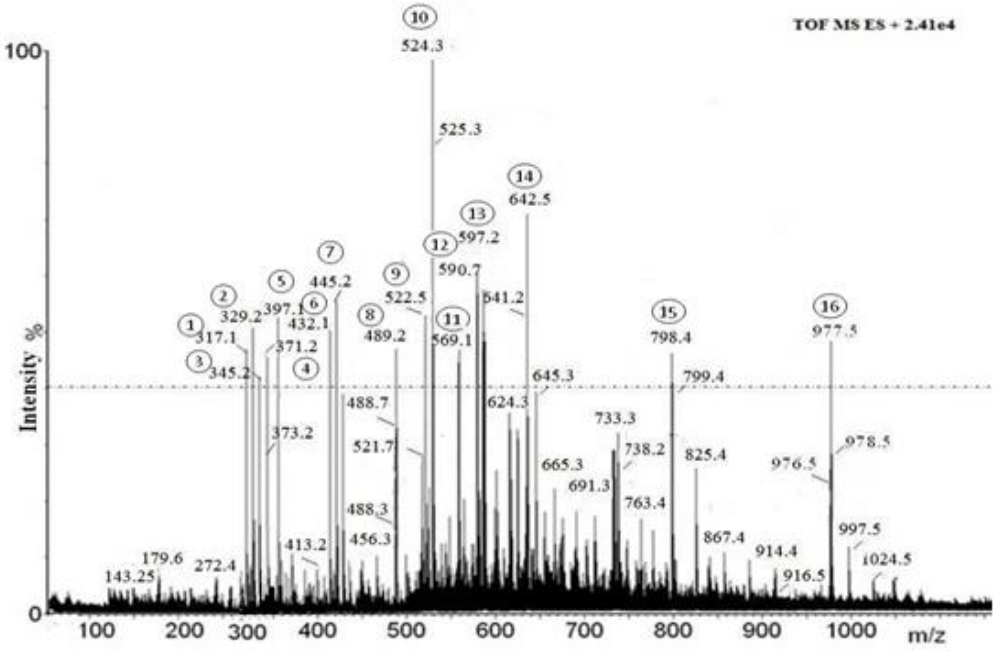


C (b)

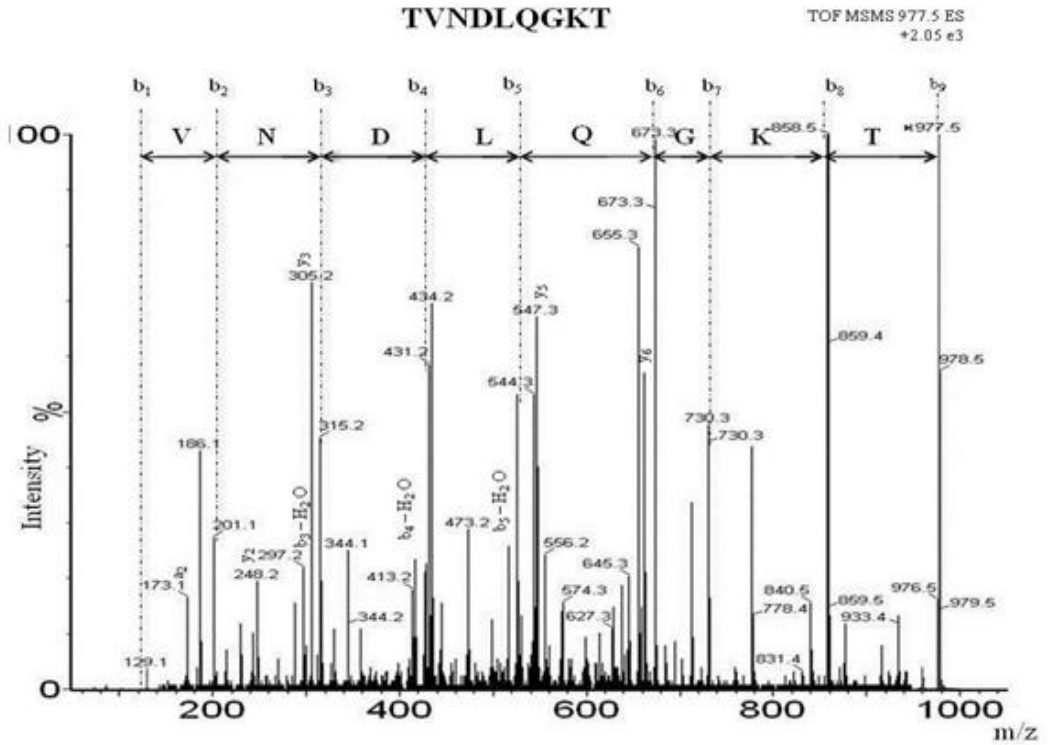


C (a) Fraction 8 (1-13 parent ions); C(b)Interpretation of LC-MS/MS spectrum of the ion *m/z* 766.41, derived from ovotransferrin peptide LAEVPTH.

D (a) Fraction 12

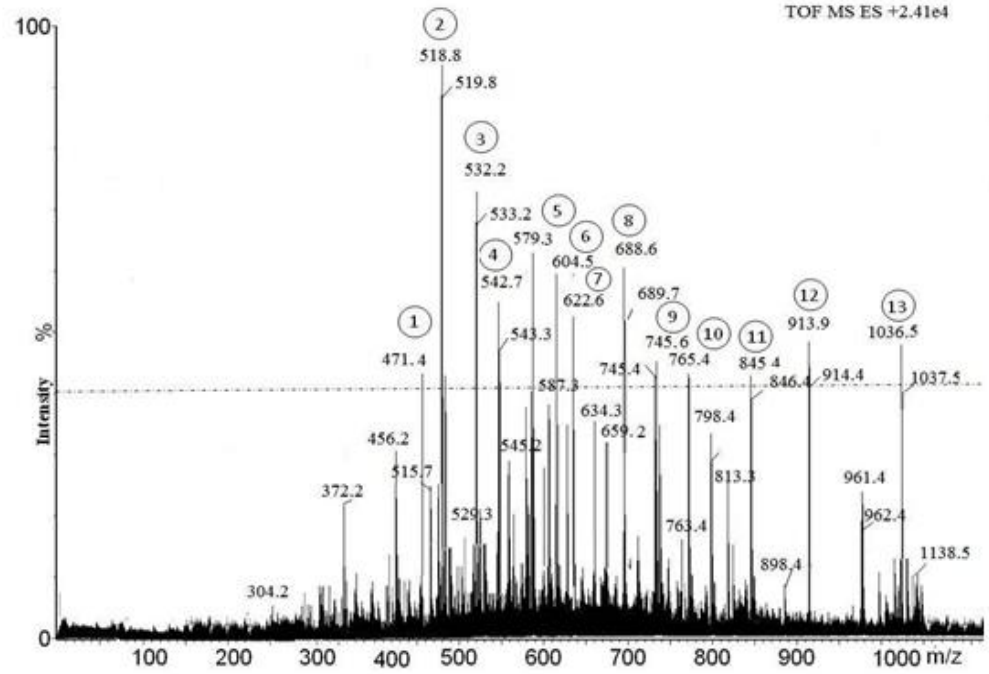


D (b)

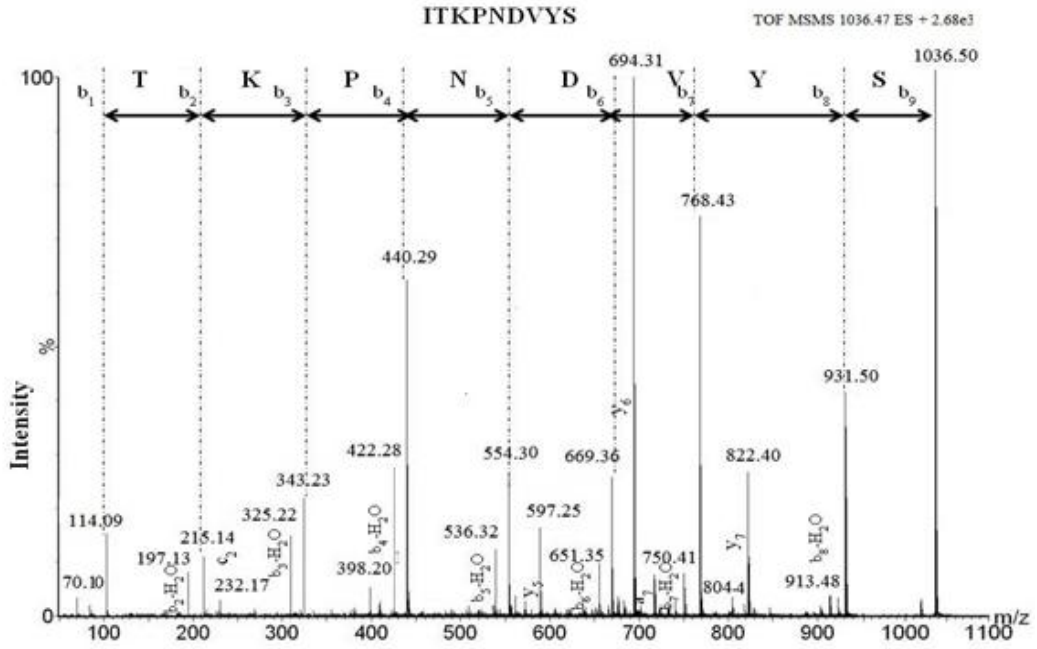


D (a) Fraction 5 (1-16 parent ions); D(b)Interpretation of LC-MS/MS spectrum of the ion *m/z* 977.5, derived from ovotransferrin peptide TVNDLQGKT.

E (a) Fraction 14



E (b)



E (a) Fraction 5 (1-13 parent ions); E (b) Interpretation of LC-MS/MS spectrum of the ion *m/z* 1036.49, derived from ovalbumin peptide ITKPNDVYS.

Table 3.4: Sequence of peptides identified by LC- MS/MS in the potent antioxidant fractions.

Molecular ion (m/z) selected for MS/MS Charge)	Sequence	Source Fragment (f)
Fraction 1		
1) 334.2 (1)	SGGI	Ovotransferrin f (524 -527)
2) 444.22 (1)	VPGAT	Ovotransferrin f (180-184)
3) 479.44 (1)	LHPI	Ovostatin f (608-611)
4) 520.75 (2)	YAEERYPIL	Ovalbumin f (107-115)
5) 528.8 (2)	RIQWCAVGKD	Ovotransferrin f (363 -372)
6) 585.66 (1)	LVELI	Ovomucin α unit f (1457-1461)
7) 622.46 (1)	VKYNV	Ovomucin β unit f (933-937)
8) 784.45 (1)	VLLPDEV	Ovalbumin f (244 -250)
9) 897.5 (1)	LVLLPDEV	Ovalbumin f (243-250)
10) 927.89(1)	RNAPYSGY	Ovotransferrin f (203 -210)
Fraction 5		
1) 303.26 (1)	AGVG	Lysozyme f (1 5 -178)
2) 349.18 (1)	ACR	Ovomucin β unit f (345-347)
3) 371.2 (1)	AGHS	Ovostatin f (1099-1102)
4) 428.48 (1)	PGKK	Ovotransferrin f (307-310)
5) 533.52 (1)	SAGWN	Ovotransferrin f(241-245)
6) 589.41 (1)	ASNGIQ	Ovomucin β unit f (97- 102)
7) 633.21 (1)	QTAADQ	Ovalbumin f (135-140)
8) 719.3 (1)	KVEQGAS	Ovomucoid f (136-142)
9) 755.58 (1)	YCGVRAS	Lysozyme f (54-60)
10) 771.64 (1)	RAAAARGV	Flavoprotein f (3-10)
11) 919.72 (1)	IESGSVEQA	Ovotransferrin f (162-170)
Fraction 8		
1) 346.18 (2)	LGAKDST	Ovalbumin f (44-50)
2) 465.25 (1)	CQGGT	Lysozyme f (24-28)
3) 468.29 (1)	AAHAV	Ovotransferrin f (267-271)
4) 481.26 (1)	FDVT	Ovostatin f (221-224)
5) 553.24(1)	ASGTMS	Ovalbumin f (236-241)
6) 565.2 (1)	TGEIK	Ovostain f (496-500)
7) 584.2 (1)	VCGLVP	Ovotransferrin f (423-428)
8) 594.36 (1)	WTSSN	Ovalbumin f (268-272)
9) 691.36 (1)	LGAKDST	Ovalbumin f (44-50)
10) 766.4 (1)	LAEVPTH	Ovotransferrin f (605-611)
11) 791.2 (1)	LGFEYY	Ovotransferrin f (339-344)
12) 815.56 (1)	QESKPVQ	Ovalbumin f (204-210)
13) 826.65 (1)	DVFSSSAN	Ovalbumin f (305-312)

Fraction 12

1)	317.14 (1)	VAQ	Flavoprotein f (64-66)
2)	329.2 (1)	VPN	Flavoprotein f (258-260)
3)	345.2 (1)	GAVV	Ovomucin α unit f (882-1885)
4)	371.22 (1)	PAGT	Ovomucin α unit f (350-353)
5)	397.1 (1)	VAAH	Ovotransferrin f (267-269)
6)	432.07 (1)	LKDG	Ovotransferrin f (207-210)
7)	445.19 (1)	PTDI	Ovomucin α unit f (663-665)
8)	488.74 (2)	TVNDLQGKTS	Ovotransferrin f (124-132)
9)	522.5 (2)	YNAGV	Lysozyme f (173-177)
10)	524.26 (2)	TVNDLQGK	Ovotransferrin f (124-131)
11)	569.14 (1)	VVVDP	Ovotransferrin f (613-617)
12)	590.73 (1)	AGLAPY	Ovotransferrin f (86-91)
13)	597.2 (1)	TKSDF	Ovotransferrin f (297-301)
14)	642.5 (1)	LVEPEG	Ovostatin f (886-888)
15)	798.4 (1)	QITKPND	Ovalbumin f (90-96)
16)	977.47 (1)	TVNDLQGKT	Ovotransferrin f(124-132)

Fraction 14

1)	471.38 (1)	KPGAV	Ovomucin α unit f (1880-1884)
2)	518.75(2)	ITKPNDVYS	Ovalbumin f (91-99)
3)	532.16 (1)	KGGISA	Lysozyme f (167-172)
4)	542.7 (1)	ATALAP	Ovomucin α unit f (1362-1367)
5)	579.27 (1)	PFASGT	Ovalbumin f (234-239)
6)	604.52 (1)	AVHAAH	Ovalbumin f (317-322)
7)	622.66 (1)	YAPGDT	Ovomucin β unit f (336-341)
8)	688.6 (1)	GWIESPS	Ovostain f (423-428)
9)	745.59 (1)	LQPSSVD	Ovalbumin f (162-168)
10)	765.38 (1)	ETTQGMS	Ovomucin α unit f (966-972)
11)	845.4 (1)	VLQPSSVD	Ovalbumin f (161-168)
12)	913.9 (1)	QITKPNDV	Ovalbumin f (90-97)
13)	1036.49 (1)	ITKPNDVYS	Ovalbumin f (91-99)

CHAPTER- 4 FINAL REMARKS

4.1. IMPORTANCE OF DIETARY ANTIOXIDANTS

Antioxidants play an important role in providing protection against free radicals, the harmful by products generated during normal physiological process and environmental pollution (Ames, Shigenaga, & Hagen, 1993). There exists mounting scientific evidences which shows the importance of free radicals in the pathogenesis of degenerative diseases such as cancer, cardiovascular disease, immune-system decline, brain dysfunction, and cataracts. Over the decades, there is an increase in consumer attention to the health and nutritional aspects in order to maintain the antioxidant status of the body. The role of antioxidants in the body and the antioxidants derived from various food sources have been studied intensively. Dietary intake of antioxidants may help to maintain the antioxidant status in the body (Fang, Yang, & Wu, 2002). Recent studies shows that functional foods such as fruits, vegetables, milk and eggs acts as natural source of exogenous antioxidants (Shahidi, 2000). Chemical diversity of the antioxidants makes it difficult to quantify the individual components in most of the food commodities. Research conducted on tomato derivatives and coffee shows although there was a significant reduction of natural antioxidants during thermal treatments, the total antioxidant properties were either maintained or even enhanced due to the development of novel products (Nicoli, Anese, Parpinel, Franceschi, & Lerici, 1997). Although the intake of antioxidants as supplements have been increased in Canada (Singh & Levine, 2006; Wilson, Bray, Temple & Struble, 2010); randomized clinical trials revealed that intake of vitamin supplements with claims 'rich in antioxidants' resulted in increase of about 5-6 % all cause mortality (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007; Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2008). So it is highly recommended to eat healthy food rather than depending on the supplements to meet adequate amount of antioxidants in the body. Therefore, the dietary intake of the natural food enriched with bioavailable antioxidants plays an important role as a potential source and helps to avoid the use of synthetic antioxidants.

4.2. EGGS AS A NATURAL SOURCE OF ANTIOXIDANTS: A SUMMARY OF PRESENT RESEARCH

Egg is an excellent source of macro and micro nutrients and beyond that it helps in providing beneficial properties. Recent studies have shown that many of the egg derived peptides have antioxidant activity; some of them were identified from the major egg white proteins; ovalbumin and ovotransferrin and from egg yolk derived peptides (Yamamoto, Sogo, Iwao, & Miyamoto, 1990; Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004; Shen, Chahal, Majumder, You, & Wu, 2010). In the present study with fresh samples, we noticed that egg yolk have higher antioxidant activity than egg white and the whole egg. Our study also found that cooking has decreased antioxidant activity of the egg white and yolk samples. Cooking followed by mimic gastrointestinal digestion enzymatic digestion increased the antioxidant activity of egg samples. The results suggested

though there was either denaturation and or inactivation of antioxidant components on thermal treatments. New antioxidants were further released upon gastrointestine simulated enzymatic digestion leading to increased antioxidant activity. Among the 21 different treatments, the boiled egg white treated with pepsin and pancreatin showed the maximum antioxidant activity. In the further study using boiled egg white pepsin and pancreatin hydrolysate, we identified 63 peptide sequences from ovalbumin, ovotransferrin, ovomucin, lyzozyme and ovostatin. YAEERYPIL (residues 107-115), also identified from crude egg white pepsin hydrolysate, was reported to show both angiotensin I-converting enzyme (ACE) inhibitory activity and potent radical scavenging property (Davalos et al., 2004). Our study showed that gastrointestinal digestion of egg white is capable of generating of a number of antioxidant peptides, thus could improve the antioxidant status. Bioactive peptides, with 2-10 amino acid residues can exhibit more potency than longer peptides and could augment various functions and they can easily been absorbed through the gastrointestinal tracts (Yoshikawa et al., 2000; Kitts & Weiler, 2003; Korhonen & Pihlanto, 2003). The characterization of the peptides from the boiled egg white revealed the peptides ranging from 3 to 10 amino acid residues. That shows the capability of those peptides to execute the functions as bioactive peptides in the body. The presence of the hydrophobic amino acids, Val and Leu at the N terminal of the amino acid sequence and the other amino acids like Try, Pro, Asp, or His with greater antioxidant activity was noticed in the identified peptide sequences (Ren et al., 2010). In conclusion, the effect of various cooking methods and enzyme treatment on the antioxidant activity of the egg white, yolk and whole egg were studied and found that simulated gastrointestinal digestion of boiled egg white improved the antioxidant activity due to the release of bioactive peptides.

4.3. INFERENCES OF THE PRESENT STUDY

Recent changes in the perspective concerning the relation between food and health have increased the consumption of functional foods and nutraceuticals with various health benefits. Over the decades, there is an increase in the study related to role of antioxidants in preventing various degenerative diseases, aging and cancer. Most of the food sources such as fruits, vegetables, milk, egg and soya function as good sources of antioxidants. Effects of cooking and digestion on the antioxidant activities of food commodities have not been fully understood. The protein fragments with specific biofunction derived from the intact parent protein after breakdown with proteases in the gastrointestinal tract are often referred to bioactive peptides (Kitts & Weiler, 2003). The present study showed that antioxidant activitiy of boiled egg white enhanced after simulated gastrointestinal digestion, and identified 63 antioxidant peptides. This study helps to understand the beneficial use of egg as dietary source of antioxidant and thereby helps to promote awareness about the benefits of egg consumption among the health conscious consumers.

4.4. RECOMMENDATIONS FOR FUTURE RESEARCH

- To fully characterize the antioxidants in eggs, antioxidants in egg yolk should be identified in the future.
- A continued research is essential to understand the mechanisms of antioxidative property of the boiled egg white derived peptides and to identify their possible roles in the elimination, suppression or inhibition of reactive oxygen species. Subsequent analysis of the protective role using cell cultures and animals models in order to effectively differentiate the most rational and effective use of the egg derived antioxidants is highly recommended.
- The synthetic peptides with similar amino acid residues of identified egg white peptides merit more studies to confirm their antioxidant functions.
- Further research of these peptides *in vivo* will provide the scientific evidence for the use of eggs as antioxidants in the functional foods and nutraceuticals.

4.5. LITERATURE CITED:

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