CHAPTER I
INTRODUCTION

1.1. STATEMENT AND SIGNIFICANCE OF THE PROBLEM

A free radical is an atom or a molecule with an unpaired electron, such as, superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$). Free radicals are produced by various cellular processes, such as the electron transport system, phagocytosis, or the redox system. There are two main types of free radical, reactive oxygen species (ROS), and reactive nitrogen species (RNS). ROS and RNS can rapidly interact with intracellular proteins, lipids and DNA. Tissue inflammation results from oxidative stress and is involved in many diseases, including cardiovascular, musculoskeletal and pulmonary diseases.

There are two main antioxidant systems to control oxidative status; i.e., enzymatic antioxidant such as superoxide dismutase (SOD), catalase, glutathione reductase, and non-enzymatic antioxidants such as beta-carotenoid, bilirubin, vitamin E, albumin, uric acid and glutathione. Moreover, there are many antioxidant compounds from plants such as phenolic compounds, flavonoid, tannin, quercetin, catechins, epicatechins, and gallic acid. The mechanism of antioxidant activity of these compounds involves trapping free radical molecules, electron transference, or scavenging of free radical.

Many common Thai vegetables contain chemicals with anti-oxidant and anti-inflammatory properties. Examples are garlic (*Allium sativum* L.) which contains S-allyl cysteine (SAC) and shallot (*Allium ascalonicum* L.).

Tissue inflammation results from oxidative stress or intracellular injury, which induces chemotactic factors as chemokines, C5a, lipoxygenase products or formylated peptides. Recruitment and stimulation of inflammatory cells either from acute inflammation such as polymorphonuclear leukocytes (PMNs), platelets, mast cells, or chronic inflammation such as macrophages, lymphocytes or plasma cells are very active. The common intracellular pathways during inflammatory cell activation relates to arachidonic acid degradation, elevated cytosolic free calcium, protein

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phosphorylation, protien kinase C, tyrosine kinases, or protein phosphatases. The binding of a chemotactic factor to a specific receptor on the cell membrane results in the formation of a ligand-receptor complex. A guanine nucleotide regulatory protein (G protein) couples the ligand-receptor complex to the activation of specific enzymes associated with the leukocyte plasma membrane, including phospholipase C. The outcome of these signaling mechanisms involves the induction or enhancement of specific functional responses and following phagocytosis, degranulation, aggregation and oxidant production. This study examined the anti-oxidant and anti-inflammatory activities of shallot extract. The research focused on scavenging organic radicals and GSH depletion from protein hydroperoxide, or peroxyl radicals. The bioactive compounds were identified, which were phenolics, allyl sulfide compound (diallyl mono-, di-, tri- and tetra-sulfides), and allyl-2 propenethiosulfinate (allicin). A second goal of the study was to examine the activity of Thai shallot extracts on oxidative stress in monocytic cell line by measuring the total glutathione, total hydroperoxide levels after activation via gamma irradiation.

1.2. LITERATURE REVIEWS

1.2.1. Oxidative stress

1.2.1.1. Oxidative stress and free radical generation

1.2.1.1.1 General procedure

Oxidative stress is a well-known phenomenon present in the cells (Fig. 1.) and relates to many pathogenesis of life style-related diseases (Yoshikawa and Naito, 2002). Free radicals are usually unstable and highly reactive because of the unpaired electrons, which tend to couple with other electrons. Therefore, the reactive oxygen metabolite is more highly reactive than the original oxygen molecule. Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) are active oxygen species.

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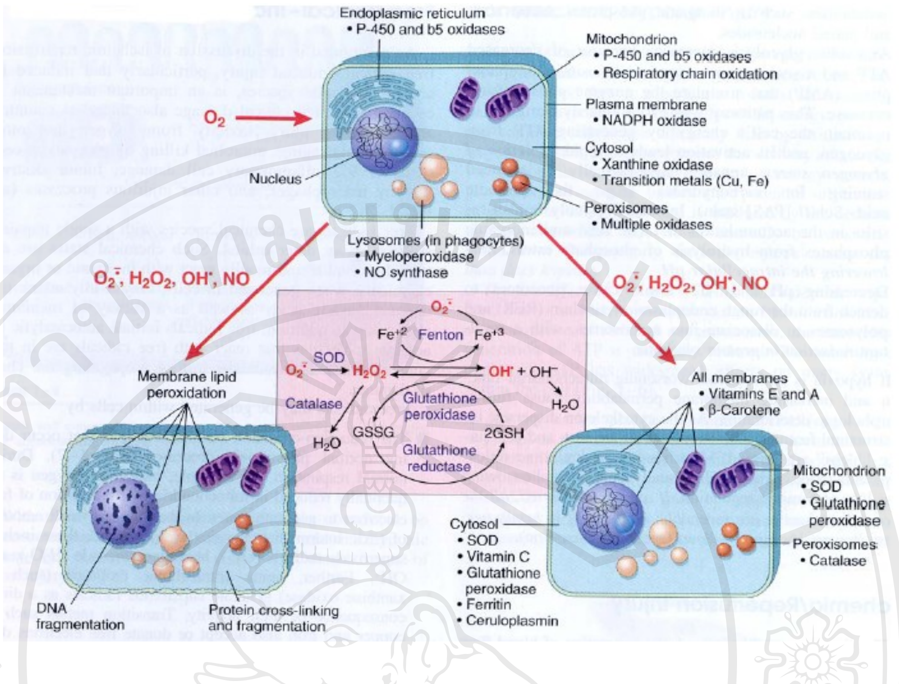

Figure 1. Relationship between free radical production and antioxidant regulation in the cells. Regulation of free radicals by antioxidant that generated is occurred in many parts of cell. Free radicals involve intracellular components such as protein, lipid, and DNA. The oxidative stress can be controlled by antioxidants such as SOD, vitamin C, GSH, peroxidase, ferritins and catalase (Mitchell and Corain, 2003).

Free radicals can be defined as any species capable of independent existence that contains one or more unpaired electrons (Halliwell and Gutteridge, 1990). Some general procedures for producing free radicals and inducing hydroperoxide groups are:

(i) Gamma radiation
(ii) Fenton reaction
(iii) Thermal decomposition of AAPH
(iv) Xanthine / Xanthine oxidase
(v) Neutrophils stimulation

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(i) **Gamma radiation:** Gamma rays are emit from a series of cobalt-60 rods and are used to produce $HO^{\bullet}$ and $O_2^{\bullet-}$ free radicals in water via a series of reactions (O'Donnell and Sangster, 1970).

$$H_2O \rightsquigarrow e^-, H_2O^{\bullet+}, H_2O^*$$

(H_2O^* = excited water molecules)

Within about 10^{-11} seconds, the electrons become solvated.

$$e^- + nH_2O \longrightarrow e^-_{aq}$$

and within seconds, the positive ions decompose

$$H_2O^{\bullet+} \longrightarrow H^+ + HO^{\bullet}$$

or $$H_2O^{\bullet+} + H_2O \longrightarrow H_3O^+ \; HO^{\bullet}$$

The hydroxyl radical is one of the most reactive chemical species known (Halliwell and Gutteridge, 1990). The excited molecules are thought to give $H^{\bullet}$ atoms (O'Donnell and Sangster, 1970).

$$H_2O^* \longrightarrow H^+ + HO^{\bullet}$$

$$H_2O^* \longrightarrow e^- + H_2O^{\bullet+}$$

The electrons and $H^{\bullet}$ atoms can react with molecular oxygen (O'Donnell and Sangster, 1970).

$$e^- + O_2 \longrightarrow O_2^{\bullet -} \quad (K = 1.9 \times 10^{10})$$

$$H^{\bullet} + O_2 \longrightarrow HO_2^{\bullet} \quad (K = 2 \times 10^{10})$$

$HO_2^{\bullet}$ is the conjugate acid of $O_2^{\bullet -}$ with a pH of 4.7, i.e., at neutral pH the molecule is predominantly in the $O_2^{\bullet-}$ form.

During irradiation, H_2O_2 can be formed by reaction of two $HO^{\bullet}$ radicals (pH 7.0) within the radiolytic spur or by spontaneous dismutation of $O_2^{\bullet-}$ (Halliwell and Gutteridge, 1990).

$$HO^{\bullet} + HO^{\bullet} \longrightarrow H_2O_2$$

$$2H^+ + 2\,O_2^{\bullet -} \longrightarrow H_2O_2 + O_2 \; (K = 5 \times 10^5)$$

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The yield of the two free radical species $HO^\bullet$ and $O_2^{\bullet-}$ at neutral pH in 2.8 and 3.2 respectively per 100 eV energy absorbed (O' Donnell and Sangster, 1970). At the protein concentration used, direct ionization of the solution occurred only to a negligible extent.

(ii) Fenton reaction

The fenton reaction, sometimes also called the "metal-catalyzed Haber-Weiss reaction" occurs in solution which contain $O_2^{\bullet -}$ and iron or copper. It leads to the free radical product $HO^\bullet$ at acid (Kehrer, 2000) or complex oxidizing iron species at neutral pH (Bielski, 1988). In biological systems, $O_2^{\bullet -}$ is a frequently encountered intermediate. It can be generated by radiolysis, photosensitized oxidations, autoxidations and redox cycling, as well as by enzyme-catalyzed reactions and other metabolic processes.

$$O_2^{\bullet -} + \text{Fe (III)} \longrightarrow O_2 + \text{Fe (II)}$$

$$H_2O_2 + \text{Fe (II)} \longrightarrow HO^\bullet + HO^- + \text{Fe (III)}$$

The hydrogen peroxide in reaction is derived from the dismutation of $O_2^{\bullet -}$.

As pointed out by Bielski (1988), if the iron is chelated, the oxidation-reduction potential of the reaction is changed, e.g.

$$\text{Fe (II)} \longrightarrow \text{Fe (III)} + e^- \qquad E_0 = -0.771 \text{ v}$$

$$\text{FeEDTA}^{2-} \longrightarrow \text{FeEDTA}^- + e^- \qquad E_0 = +0.771 \text{ v}$$

(iii) Thermal decomposition of AAPH

In the aqueous phase, the water soluble azo compound 2,2'-anobis(2-amidinopropane) hydrochloride (AAPH) generates peroxyl free radicals at a constant rate which depends only on the temperature (Niki, 1990).

(iv) Xanthine/Xanthine Oxidase

Xanthine can be oxidized to uric acid by the enzyme xanthine oxidase. Fridovich (1970) found that approximately 22% of the electron flux through the

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enzyme resulted in the univalent reduction of oxygen with the $O_2^{\bullet -}$ yield representing 44% of urate concentration (Fridovich, 1970).

The superoxide radical reduces metals in the solution. These reduced metals react with H_2O_2 generated by the dismutation in the fenton reaction, which results in the production of the highly reactive $HO^{\bullet}$ radicals.

(v) Neutrophil stimulation

When neutrophils are exposed to certain stimuli they produce microbicidal oxidants in a reaction known as "respiratory burst". The key reaction in this process is the reduction of molecular oxygen to form $O_2^{\bullet -}$. This reaction is catalyzed by the enzyme NADPH oxidase (Babior, 1988).

$$2\,O_2 + NAD(P)H \longrightarrow 2\,O_2^{\bullet -} + NAD(P)^{+} + H^{+}$$

The superoxide radical enters into the fenton reaction as mentioned above. Hydrogen peroxide produced by dismutation can also react as substrate for myeloperoxidase (MPO), resulting in the formation of hypochlorous acid (HOCL) and chloramines (Winterbourn, 1985; Dahlgren, 1989). The stimulant that is used to elicit the oxidative burst is the ester phorbol myristate acetate (PMA).

1.2.1.1.2. Inflammation process and free radical generation

Inflammation is a reaction between tissue and microcirculation via generation of inflammatory mediators that move from blood into extravascular tissue (Fantone and Ward, 1999). Initiation of the inflammatory response begins as the result of direct injury or stimulation of the cellular or structural components of a tissue, including parenchymal cells, microvasculature, tissue macrophage or mast cells, mesenchymal cells and the extracellular matrix.

Acute inflammation is an immediate and early response to injury designed to deliver leukocytes to the site of injury. Leukocytes destroy microbes and begin the process of breaking down necrotic tissues. Two characteristic phenomena are 1) vascular dilatation, permitting plasma protein to leave the circulation and 2)

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emigration of leukocytes from microcirculation and accumulation at the local injury (Mitchell and Corain, 2003).

Chronic inflammation arises from various factors, such as viral infection, persistent microbial infections, and prolonged exposure to potentially toxic agents, or auto-immune diseases. Besides inflammation with mononuclear or chronic inflammatory cells, there is also tissue destruction, which is largely directed by the inflammatory cells, and repairing system, which involves new vessel proliferation and fibrosis formation (Mitchell and Cotran, 2003).

Reactive oxygen species (ROS) are a key inflammatory factor and produced from different reaction (Table 1). In the process of acute inflammation, phagocytosis and elaboration of degradation enzymes are two major benefits of having recruited leukocytes to the site of inflammation. Phagocytosis occurs in three steps; (1) recognition and attachment of the particle to the ingested leukocytes, (2) engulfment to form a phagocytic vacuole, and (3) killing and degradation of the digestive material (Mitchell and Corain, 2003). The final step in phagocytosis of microbes is killing and degradation by ROS. This stimulates an oxidative burst of oxygen consumption, glycogen catabolism (glycogenolysis), glucose oxidation, and production of reactive oxygen metabolites (Mitchell and Corain., 2003). This stimulates oxidative metabolism due to rapid activation of a leukocyte NADPH oxidase, which oxidizes NADPH. (Mitchell and Corain., 2003) (Fig. 2).

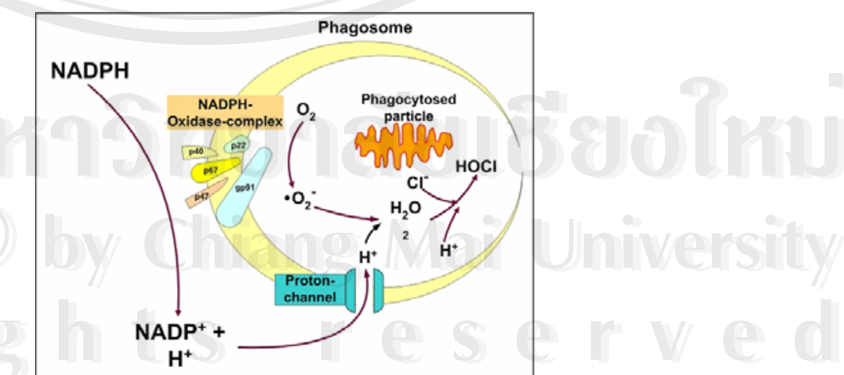

Figure 2. Free radical generation during phagocytosis. ROS are produced during phagocytosis and induce NADPH oxidase-complex on macrophage cell. (http://www.egms.de/ egms/ servlet/ Figure?id = cto000002 &figure =f12 &vol = 2004-3)

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Superoxide from NADPH oxidase undergoes spontaneous dismutation into H_2O_2. The quantity of H_2O_2 produced is generally insufficient to effectively kill most bacteria. However, lysosome of neutrophils contain myeoperoxidase (MPO), and in the presense of a halide such as Cl^-, MPO converts H_2O_2 to HOCl, which is a powerful oxidant and anti-microbial agent.

Reactive nitrogen species (RNS) (Poli, 2002; Beckman and Koppenol, 1996) are able to regulate inflammatory protein expression, and are synthesized by induction of nitric oxide synthase (iNOS). ROS and RNS interact with each other that is a result not only by induction of future new reactive species, but, via changes in the concentration of these two classes of molecules (Beckman and Koppenol, 1996). The recognition of nitric oxide (NO) production by activated macrophages as a part of the inflammatory process is important for assessing both the biological production of NO and the phenomenon of induction of NOS activity (Fig. 2 and 3). Cytokines such as interleukin 1-β (IL-1β) and tumor necrosis factor (TNF-α) are involved in the biological tissue (Boveris, 2002).

Table 1. Chemical reaction of radical production in biological system. Reactions involving reactive oxygen metabolites produced by phagocytic cells. (Mitchell and Corain, 2003)

Reaction	Radicals
Reduction of molecular oxygen $O_2 + e^- \longrightarrow O_2^-$	Superoxide anion
Fenton reaction (iron-catalyzed) $H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$	Hydroxyl radical
Haber-Weiss Reaction $H_2O_2 + O_2^- \longrightarrow OH^- + OH^{\bullet}$	Hydroxyl radical
Dismutation of O_2^- $O_2^- + O_2^- + 2H^- \longrightarrow O_2 + H_2O_2$	Hydrogen peroxide
Myeloperoxidase reaction $H_2O_2 + Cl^- + H^+ \longrightarrow HOCl$	Hypochlorous acid

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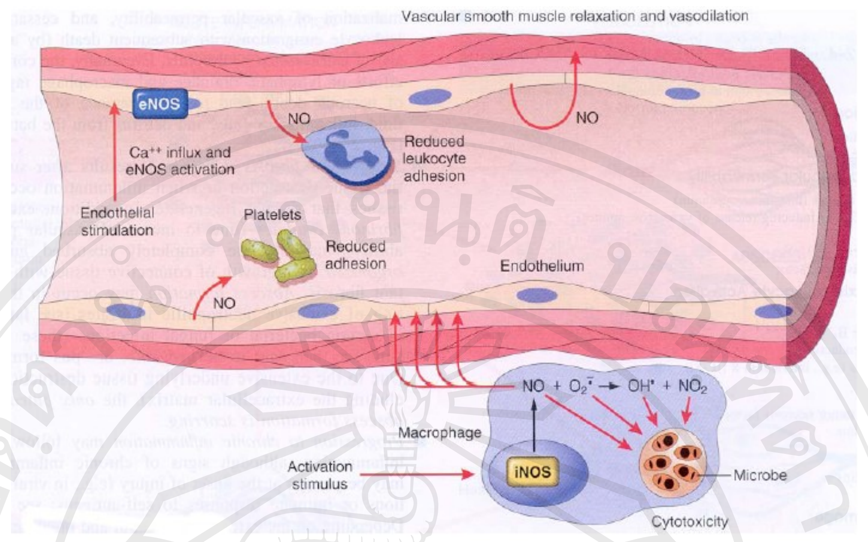

Figure 3. Production of reactive nitrogen species (RNS) in macrophage. RNS was produced from nitric oxide synthase (NOS) in vascular smooth muscle by macrophages (Mitchell and Corain, 2003).

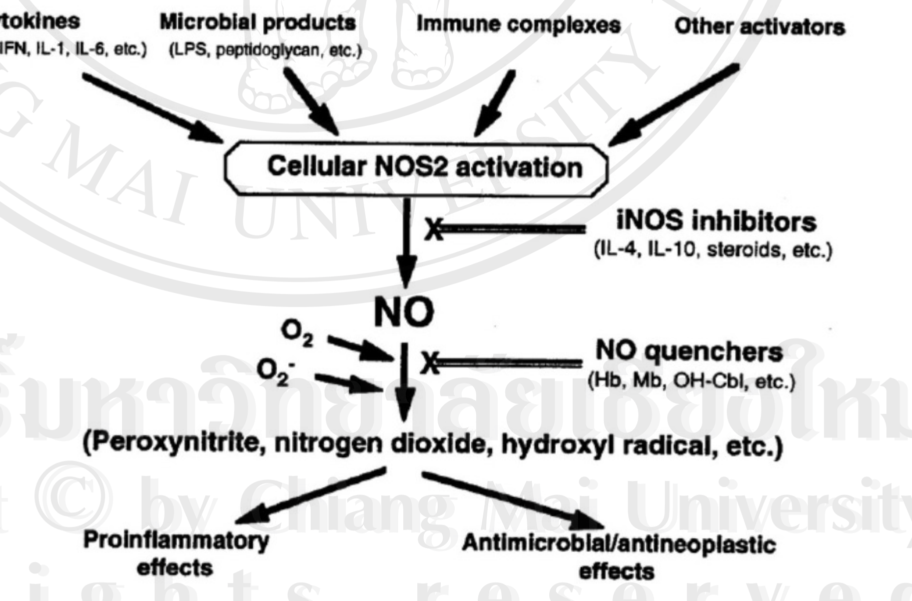

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Figure 4. The nitric oxide production pathway. NO was produced from various stimulants: cytokine, microbial production, immune complexes, or other activators that involved in regular phenomena such as inflammation and infection in human body (Weinberg, 1999).

NO is able to act directly on the cell, and induces cyclooxygenase function which stimulates pain (Fig 4.). It also destroys certain protease inhibitors, and enhances production of IL-1, TNF, or NADPH oxidase activity in myeloid cells (Weinberg, 1999). NO acts as an intracellular signal second messenger after being activated with a primary stimulant, i.e., cyclic guanosine 3′,5′- monophhosphate (cGMP) (Mille, 1999). NO not only reduces intracellular calcium level and attenuates platelet and neutrophil aggregation, but also blocks the receptor of growth factor and inhibits signaling by inositol 1, 4,5- triphosphate (IP3).

NO mediates inflammation and contributes to cell death by acting directly on transcription factors, nuclear factor κ-B (NF-κB) (Miller, 1999), apoptosis signal-regulating kinase 1 (ASK-1), c-Jun-NH_2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) (Jibiki *et al*., 2003). NO induces tissue injury at extracellular sites via diffusion through the plasma membrane.

1.2.1.1.3. Protein and lipid hydroperoxide formation

There is evidence of a relationship between reactive oxygen species (ROS) or reactive nitrogen species (RNS) and cell damage. Hydroperoxide may be formed during oxidative stress. In the oxidative stress pathway, previous evidence has shown that free radical molecules may become trapped on protein, lipid, and DNA molecules, and then transfer electrons to the target molecules.

1.2.1.1.3.1. Lipid peroxidation and mechanism (Halliwell and Guteridge, 1989)

Lipid peroxidation has been defined as “the oxidative deterioration of polyunsaturated lipids”. Polyunsaturated fatty acids (PUFAs) contain two or more carbon-carbon double bonds. The PUFAs are present in the lipid membrane as phospholipids.

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The lipid peroxidation proceeds as follows:

Initiation of lipid peroxidation: attack upon a lipid of any species at a hydrogen atom from a methylene (-CH_2-) group. An adjacent double bond weakens the energy of attachment of the hydrogen atoms. The free radicals such as the hydroxyl radical ($OH^•$), and peroxyl radical ($HOO^•$) can absorb the hydrogen atom.

$$- CH_2- + OH^\bullet \longrightarrow -C^\bullet H- + H_2O$$
$$-CH_2- + HOO^\bullet \longrightarrow -C^\bullet H- + H_2O$$

Propagation of lipid peroxidation: after a hydrogen atom is absorbed from a methylene group, a carbon radical is usually stabilized by a molecular rearrangement to form a conjugated diene.

$$R\text{-}C^\bullet H + R\text{-}C^\bullet H \longrightarrow R\text{-}CH\text{-}CH\text{-}R$$

Lipid hydroperoxide (LOOH) formation during lipid peroxidation is formed because of aerobic conditions. Oxygen is a hydrophobic molecule that concentrates in the interior of a membrane and reacts with lipid bilayer to give a peroxyl radical (LOO•) that is able to absorb H• from another lipid molecule to form a lipid hydroperoxide (LOOH) molecule.

$$R + O_2 \longrightarrow ROO^\bullet$$
$$ROO^\bullet + H^\bullet \longrightarrow LOOH$$

In physiological systems, the lipid hydroperoxide (LOOH) is also able to activate the lipid peroxidation by iron regulation.

$$LOOH + Fe^{2+}\text{-chelate} \longrightarrow Fe\ (III)\text{-chelate} + OH^- + RO^\bullet \text{ (alkoxyl radical)}$$
$$RO^\bullet + LH \longrightarrow ROH + L^\bullet$$
$$LOOH + RO^\bullet \longrightarrow LOO^\bullet + R\text{-}OH$$

1.2.1.1.3.2. Protein oxidation and mechanism (Scott and Eaton, 1997)

Proteins are a major component of biological membranes. The oxidative damage of proteins can be evaluated via two methods:

Oxidation on Thiol : protein thiol oxidation or loss of protein activity
: thiol titration
: thiol alkylation
: thiol affinity chromatography or
: ^{3}H-NEM-PAGE visualization of –SH

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Nonthiol : Carbonyl
: Oxidative deamination/ decarboxylation events
: Proteolysis
: Oxidant damage to metalloproteins
: Oxidant cross-linking of proteins
: Oxidant scission of proteins

The hydroxyl radical (OH•) is the most important free radical because it can destroy many cellular molecules. Although much is known about their action on lipid and DNA (Park *et al*., 2003), less is known concerning protein damage. There is evidence for significant formation of peroxides in amino acids and protein (BSA and lysozyme) (Gebicki *et al*., 2000). Proteins can trap part of the energy of the ROS in the form of peroxide groups (Simpson *et al*., 1992). Protein hydroperoxide is a highly produced, stable and reactive compound (Fig. 5)

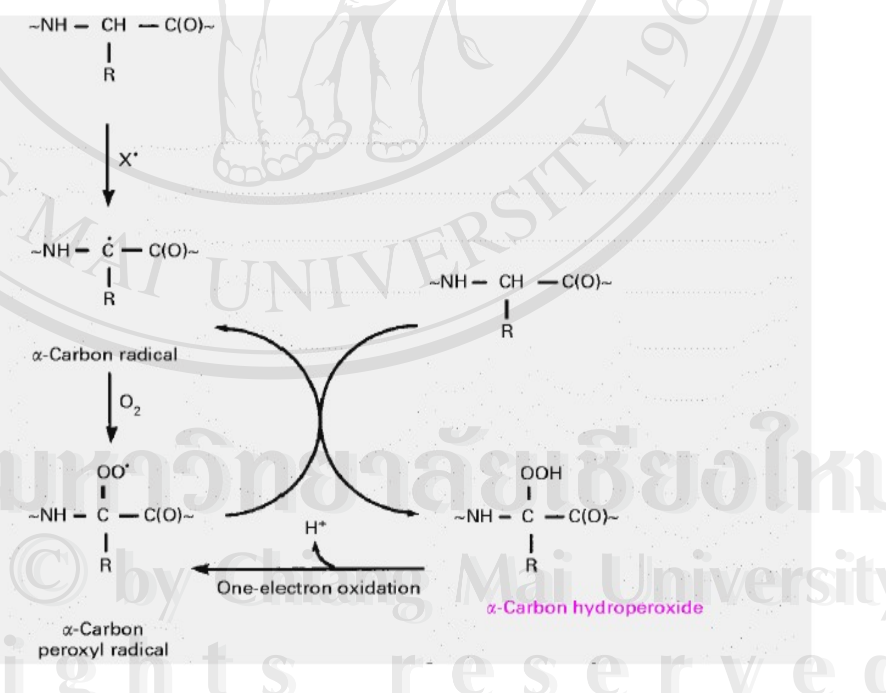

Figure 5. Protein hydroperoxide formation pathway. Free radical (X•) with free electron transfers free electron to α-carbon atom on back bone of polypeptide chain. (Dean *et al*., 1997)

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$PrH + {}^{\bullet}HO \rightarrow Pr^{\bullet} + H_2O$

$Pr^{\bullet} + O_2 \rightarrow PrOO^{\bullet}$

$PrOO^{\bullet} + e^{-} \rightarrow PrOO^{-}$

$PrOO^{-} + H^{+} \rightarrow PrOOH$

$PrOO^{\bullet} + PrH \rightarrow PrOOH + Pr^{\bullet}$

The protein radical is able to damage other molecules and propagate the free radical recycle in biological system because of radical formation in different molecules (Hawkins and Davies, 2001).

Carbon-centred radicals	($-C^{\bullet}-OH$)
Peroxl radicals	($ROO^{\bullet}$)
Alkoxyl radicals	($RO-OR^{\bullet}$)
Thiyl radicals	($RSSR^{\bullet -}$)
Nitrogen–centred radicals	($RNH^{\bullet}$)

There is previous research showing oxidation of protein via detection of protein carbonyl group, such as the oxo acid and aldehyde with the same number or fewer carbon atoms than the parent amino acid, e.g., glycine giving rise to glyoxal and glyoxylic acid, formaldehyde and formic acid; alanine giving rise to acetaldehyde and acetic acid, etc. This general scheme has been confirmed for many amino acids, including aromatic amino acids, although other reactions may also occur (Table 2).

During the oxidation of aliphatic amino acids by $HO^{\bullet}$, hydroxylated derivates, notably of the side chains, are formed. During the oxidation of aromatic residues, the formation of phenoxyl radicals from tyrosine, and their conversion into dityrosine and further products, can occur, especially if there are no reductants to repair the tyrosil radicals (e.g. thiols, vitamin E). Hydroxylation of phenylalanine, tyrosine and tryptophan is also a characteristic reaction of hydroxyl radicals, and similar reactions of histidine (giving 2-oxohistidine) are important. Histidine in reactions with free radicals can form some imidazole decay products or in some cases aspartic acid and

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some histidine derivatives. Fenton chemistry can generate both the aliphatic and aromatic products.

Early radiation studies on lysozyme, ribonuclease and other enzymes were carried out mainly in the absence of O_2 and showed that $HO^{\bullet}$ was the most effective inactivator, and characterised other more selective (but less efficiently inactivating) species such as $(SCN)_2^{-\bullet}$, $Br_2^{-\bullet}$, $Cl_2^{-\bullet}$ and $I_2^{-\bullet}$. For example, $(SCN)_2^{-\bullet}$ was found to react with an important tryptophan residue in pepsin and so inactivate the enzyme, although the damage could be reversed.

Protein receptors (such as beta-adrenoceptors) and transport proteins such as albumin, including maintenance of essential ion gradients between cells and extracellular fluids by ion channels may be affected.

Protein and lipid hydroperoxides, as well as peroxide formation in proteins, liposomes, and amino acids, can be produced by exposure to γ–irradiation or azo-compound (such as AAPH) (Gebicki *et al*., 1993). The formation of relatively stable hydroperoxide and other groups on these molecules, which are subjected to $HO^{\bullet}$ or $ROO^{\bullet}$ radicals, effectively traps some of chemical energy, extending their lifetime and radius of irradiation. It is likely that other intracellular and extracellular proteins can also be oxidized by free radicals and as a result can turn into toxic second messengers. These amino acid and protein hydroperoxides *in vitro* can be scavenged by standard antioxidants such as ascorbic acid (AA) and glutathione (GSH). Fu and Gebicki (1995) suggested that protein peroxides readily reacted with some important physiological reductants like ascorbic acid and GSH in biological systems.

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Table 2. The products of amino acid oxidation. Products from protein oxidation with radical and non-radicals are produced from various free radicals. Some of the major reactions believed to be important in the oxidation of the side chain and the backbone respectively (Dean *et al*., 1997)

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Oxidative insult	Product
Tyr+HO$^{\bullet}$ or RNIs (reactive nitrogen species)	Dopa
Tyr+HOCl	3-Chlorotyrosine
Tyr+RNIs	3-Nytrotyrosine
Tyr+HO$^{\bullet}$, or one electron oxidation of Tyr or HOCl, followed by radical- radical combination	Dytrosine
Trp+ HO$^{\bullet}$,or on electron oxidation	*N*-Formylkynurenine; kynurenine
Phe+ HO$^{\bullet}$, before or after dimerization	Dimers of hydroxylated amino acids
Phe+ HO$^{\bullet}$, one electron oxidation	*o*- and *m*-tyrosine
Trp+ HO$^{\bullet}$,or on electron oxidation	5-Hydroxytryptofan; 7-hydroxytryptofan
His+ HO$^{\bullet}$, or one electron oxidation	2-Oxohistidine
Glu+ HO$^{\bullet}$in presence of O_2	Glutamic acid hydroperoxide
Leu+ HO$^{\bullet}$ in presence of O_2	Leucine hydroperoxides and hydroxides; *a*-ketoisocapronic acid; isovaleric acid; isovaleraldehyde; isovaleraldehyde oxime; carbonyl compounds;
Val+ HO$^{\bullet}$ in presence of O_2	Valine hydroperoxides and hydroxides; carbonyl compounds;
Lys+ HO$^{\bullet}$ in presence of O_2	Lysine hydroperoxides and hydroxides; carbonyl compounds;
Pro+ HO$^{\bullet}$ in presence of O_2	Proline hydroperoxides and hydroxides; 5-hydroxy-2-aminovaleric acid; carbonyl compounds;
Arg+ HO$^{\bullet}$ in presence of O_2	5-Hydroxy-2-aminovaleric acid;
Ile+ HO$^{\bullet}$ in presence of O_2	Isoleucine hydroperoxides; isoleucine hydroxides; carbonyl compounds;
Gly: hydrogen atom abstraction from a α-carbon followed by reaction with $CO_2^{\bullet-}$ radicals;	Aminomalonic acid
Met+ HO$^{\bullet}$ or one electron oxidation	Methionine sulphoxide
Cys+ HO$^{\bullet}$ or other hydrogen atom abstracting species	Cystine; oxy acids
All amino acids exposed to photo-oxidation, oxidizing radicals or HOCl	RCHO species formed by decarboxylation or deamination

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1.2.1.2. Signaling pathway

Oxidative stress has cytotoxic effects via modulating of messengers regulating essential cell membrane functions and by stimulation of signaling pathways that involve lipids, proteins and nucleic acids (Wiseman *et al*., 1996). Superoxide is generated from the one-electron removal of the oxygen molecule, by activation of neutrophils and macrophages from the membrane NADPH oxidase system (Antonicelli *et al*., 2002; Hill *et al*., 2000). This free radical can be converted to hydrogen peroxide by intracellular superoxide dismutase (SOD) and extracellular non-enzymatic dismutation. Formation of a hydroxyl radical ($OH^{\bullet}$) from a superoxide radical and hydrogen peroxide is achieved in the presence of trace amounts of catalytic transition metal ions such as iron and copper ions. The hydroxyl radical has the ability to extract hydrogen atoms from polyenoic fatty acids. Superoxide can be changed to hydrogen peroxide and a hydroxyl radical by the Fenton reaction. The latter extraction of hydrogen from polyunsaturated fatty acid (PUFA) produces lipid hydroperoxide. Decomposition of the peroxidized fatty acid, involving a breakdown of the molecule, yields a variety of end products including malondialdehyde (MDA) and other aldehydes (alkenals, 4-hydroxyalkenals, alkadienals, etc.). These aldehydes are the most commonly detectable markers of lipid peroxidation. Overall this indicates the effect of free radicals and propagation of radicals on biological system involving lipid, protein, and DNA.

The action of free radicals within cells depends on the intracellular redox status via different mechanisms; for example; leading to the activation of protein kinases including receptor and non-receptor tyrosine kinase, protein kinase C (PKC) and mitogen activated protein (MAP) kinase cascade with phosphorylation and dephosphorylation. These protein kinases play an important role in cellular response such as activation, proliferation, and differentiation (Kass, 1997) (Fig. 6).

The reactive species also involves the inflammatory signaling pathway via AP-1 and NF-κB that is regulated with glutathione (GSH) and thioredoxin (TXR). The activated signaling induced various transcription protein as pro-inflammatory gene, anti-apoptotic protein, p53 function, pro-apoptotic protein, or antioxidant gene expression (Fig. 7).

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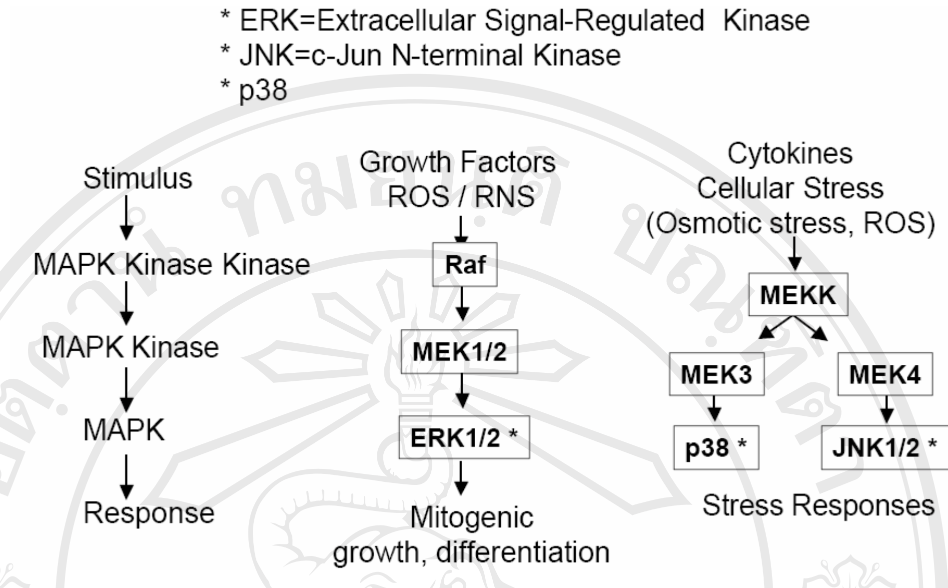

Figure 6. The mitogen-activated protein (MAP) kinase pathway. The ROS in the cells involves in many proteins that the transcription factors such as MAP kinase, ERK1/2, p38 or JNK1/2. These proteins affects the intracellular response such as growth, stress or differentiation (Cho and Choi, 2002).

The MAPKs family includes extracellular signal-regulated kinases (ERKs), which are generally activated by mitogens, c-Jun NH2 –terminal kinase (JNKs) and p38 MAPKs, both activated by cytokines and cellular stresses (Mossman and Stern, 2002). Upon activation, JNKs and ERKs phosphorylate Jun and Fos proteins, i.e., AP-1 family members. Although p38 MAPKs activate AP-1 proteins directly, they can regulate jun and fos transcription by phosphorylating enhancer binding proteins (C/EBPs) binding to their promoter elements. By selective dimerization of AP-1 family members (Jun/Jun or Fos/Jun partners) and diverse binding specificities with the promoter regions of genes, the AP-1 transcription factor regulates gene expression important in cell injury, repair, proliferation, and differentiation.

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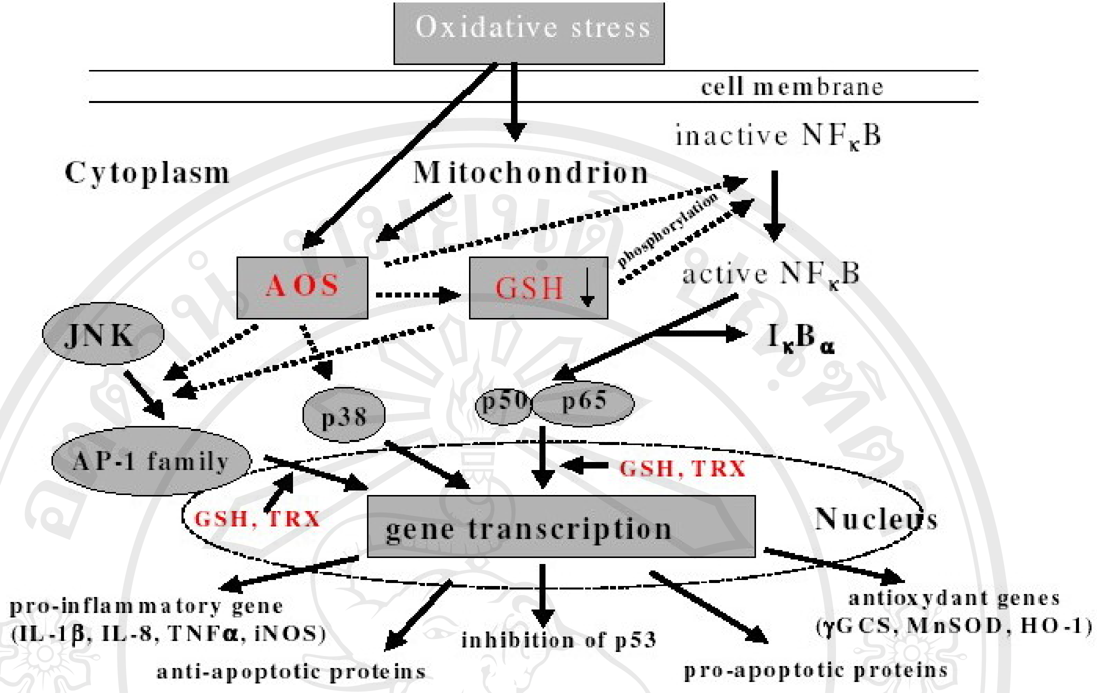

Figure 7. Oxidative stress related to inflammation and antioxidant genes. Oxidative stress affects to the antioxidant glutathione directly and depletion of GSH induced the many gene expression that relates to inflammation, apoptosis, or antioxidant protein production (Mossman and Stern, 2002).

1.2.1.3. Oxidative stress and inflammation in monocytic cells.

Reactive oxygen species (ROS) have been implicated in the etiology of numerous physiological and pathophysiological states, e.g., inflammation, aging, carcinogenesis, atherosclerosis, and neurodegeneration (Lee *et al*., 1999). Oxidative stress and free radical stimulation in human monocytic cells are related to inflammation processes in biological systems. Oxidative stress is produced from various sources, either internal or external. Stimulants such as infection, cytokine, radiation or organic chemical activation can cause oxidative stress. Numerous studies have shown that an imbalance in the function of human monocytic cell induces functional disorder in the human body.

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In Thailand, there are many people suffer from chronic lung diseases; asthma, chronic obstructive pulmonary disease (COPD) or bronchitis from chronic smoking, which can provoke lung cancer development. Cigarette smoke causes an inflammatory cascade resulting in the production of many inflammatory mediators in important regulatory cells within the airway, including epithelial cells, neutrophils, and alveolar macrophages. Cigarette smoke contains high concentrations of reactive oxygen species (ROS), such as superoxide and other free radicals. Exposure of the lungs to these ROS leads to an accumulation of neutrophils in alveolar walls and BAL fluid. This accumulation is caused by the induction of chemotactic cytokines such as IL-8 in the lung (Hill *et al*., 2000) and particularly alveolar macrophages. In addition to neutrophils, IL-8 is also chemotactic to eosinophils, and cigarette smoke has been shown to induce airway eosinophilia. *Ex vivo* studies have also shown that cigarette smoke can enhance TNF-alpha production from human monocytes (Ito *et al*., 2001). Thus the study of oxidative stress or free radicals on human monocytic cell is important. A leukemic cell line has been used as a model cell because of its phenotype (IgG receptor expression and inducible differentiation) and functional features (inducible cytokine expression, as in normal monocytes in blood circulation (Vlahopoulos *et al*., 1999). Some studies have demonstrated oxidative stress in the U937 cell line, for example; tobacco carcinogen and U937 human macrophages. Oxidant-stimulated U937 was related to the activation of NF-κB (Rioux, 2000), modification of low density lipoprotein (LDL) (Cathcart *et al*., 1988), stimulates the expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells (Vanhee *et al*., 1994), and changes in some cytokines (Desmarquest *et al*., 1998).

In addition, the antioxidant glutathione in U937 cells affects the progression of inflammation. Thiol antioxidant compounds (such as reduced glutathione; GSH) and antioxidant enzymes effectively provide an important intracellular and extracellular antioxidants in the lungs (Rahman and MacNee, 2000). There is evidence that oxidized glutathione (GSSG) induces apoptosis in U937 cells (Filomeni *et al*., 2003). In addition, the depletion of thiols in U937 cells also induces apoptosis from costunolide oxidation (Choi *et al*., 2002). At present, synthesized thiol antioxidants such as N-acetylcysteine (NAC) and N-acystelyn (NAL) are capable of blocking oxidative stress by increasing intracellular GSH and decreasing NF-κB activation

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(Antoncielli *et al*., 2002). NAC is able to protect U937 cell apoptosis from ricin oxidaion (Oda *et al*, 1999).

Oxidative stress in human monocytic cells is critical to the induction of inflammation in many studies. The potential significance of such reactions lies in the depletion of cellular reductants *in vivo*, and thus exerts oxidative stress. There is evidence showing that exposure to peroxyl radicals on U937 cells induceds hydroperoxide formation in protein prior to lipids (Gieseg *et al*., 2000) and produces protein hydroperoxide formation as in other cell lines, such as Sp2/0-Ag14 (Du and Gebicki, 2002), and a human monocyte-like cell line (THP-1) (Wright *et al*., 2003).

1.2.2. Antioxidant system

1.2.2.1. Antioxidant in plants

There have lots of antioxidant compounds containing in the plants (Table 3). Polyphenolic compounds such as catechin and epigallocatechin gallate (EGCG) are also present in green and black tea. Some flavonoids have anti-lipoperoxidant, anti-tumorogenic, anti-platelet, anti-ischemic, anti-allergic and anti-inflammatory activities (Cao *et al*., 1997). For example, epigallocatechin gallate (EGCG) protects U937 cells against nitric oxide production (Kelly *et al*., 2001).

Mechanisms of antioxidant action are to suppress ROS formation either by inhibition of enzymes, chelating trace elements involves in free radical production (Dixon and Steele, 1999), scavenging ROS with hydroxyl group (Fig. 8), or controlling antioxidant defense system (Hendrickson, 1994; van Acker *et al*., 1996; Ahrene, 2002).

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Table 3. The flavonoid contents in common food. The sources of antioxidant flavonoid in food, fruit, vegetables or beverages (Duthie *et al*., 2000).

Flavonoids	**Subclass**	**Common food source (total flavonoid subclass content mg. aglycone/kg food item)**
Flavonols	Quercetin Kaempferol Myricetin etc.	Fruit; apple (34.2 mg/kg), plums (12.5 mg/kg), cranberries (170 mg/kg), strawberries (39mg/kg), grapes (31.7 mg/kg) Vegetables; kale (35-32 mg/kg), onions (0.2-1096 mg/kg), braccoli (36-231 mg/kg), celery stalks (ND), tomatoes (3-191 mg/kg) Beverages; red wine (13.4 mg/kg), green tea (39 mg/kg), black tea (30.4 kg/kg), grape juice (4.2 mg/kg)
Flavones	Apigenin Luteolin	Vegetables; celery; (130 mg/kg), green olives (142.3 mg/kg), sweet peppers (11 mg/kg)
Flavan-3-ols	Catechin Epigallocatechin	Fruit; apples (84.3 mg/kg), plums (23.6 mg/kg) Beverages; green tea, black tea, red wine (110 mg/kg), grape juice (5.2 mg/kg)
Flavanones	Hesperetin Naringenin etc.	Fruit; citrus fruits; oranges (577 mg/kg), lemons (219 mg/kg) Beverages; grape juice (2 mg/kg)
Anthrocynidins	Cyanidin Delphinidin etc.	Fruit; Black grapes (92.5 mg/kg) Beverages; Red wine (2 mg/l), grape juice (2 mg/l)
Isoflavones	Daidzein Gemosteom etc.	Legumes; Soybeans (373-1403 mg/kg), chickpeas (11.5-36 mg/kg) Processed products; Soya-based, non-dairy, cream cheese (177 mg/kg), vegetarian chilli (32 mg/kg)

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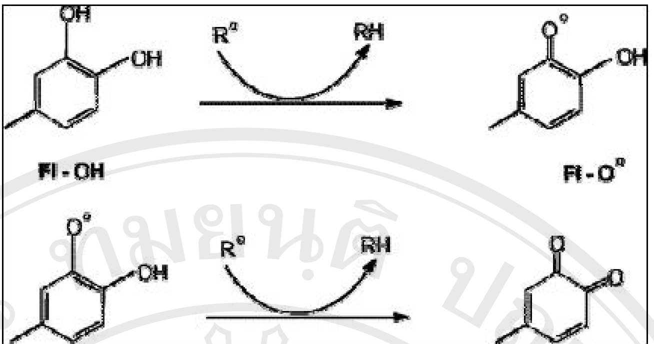

Figure 8. Scavenging activity of hydroxyl group (-OH) on free radicals. The phenolic group of ROS by flavonoids. $\dot{R}$ represents the superoxide anion. The aroxyl radical (Fl-$\dot{O}$) reacts with a second radical, acquiring a stable quinone structure (Pietta , 2000).

1) Flavonoids inhibit the enzymes responsible for superoxide anion production, for example xanthine oxidase and protein kinase C. Flavonoids have also been shown to inhibit the enzymes involved in the generation of ROS, such as cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, and NADH oxidase. Flavonoids reduce the superoxide radicals in the pH range from 7 to 10, depending on their redox properties (Jovanovic *et al*., 1994). In addition to redox properties, the reactivity of superoxide also depends on the charge. For example, the rate constant for reaction of superoxide with uncharged catechin at pH 7 (determined by pulse radiolysis) is approximately 4 times higher than the corresponding rate at pH 10, k= $1.8*10^{4}$ M^{-1} s^{-1} where catechin is doubly negatively charged. Furthermore, flavonoids efficiently chelate trace metals, which play an important role in oxygen metabolism. Free iron and copper help in formation of reactive oxygen species (ROS), as exemplified by the reduction of hydrogen peroxide with generation of the highly aggressive hydroxyl radical. However, these metal ions are essential for many physiological functions, constituents of hemoproteins and cofactors of different enzymes, e.g., iron for catalase, Cu for Cu,Zn-superoxide dismutase.

2) Flavonoid (Fl-OH) is thermodynamically able to reduce highly oxidizing free radicals with redox potentials in the range of 0.13-1.0 V, such as superoxide radicals

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by hydrogen atom donation, due to their lower redox potentials ($0.23 < E_7 < 0.75$ V), as compared to other anti-oxidizing species like trolox (E_7=0.48V) and 4-methoxyphenol (E_7=0.73V) (Jovanovic *et al*., 1994),

1.2.2.2. Antioxidant glutathione

Glutathione is an intracellular antioxidant involved in normal cell growth, development, and detoxification. The chemical structure of GSH is composed of three amino acids, i.e., glycine, cysteine, and glutamate. GSH or γ–glutamylcysteine-glycine, is found in several prokaryotes and in most eukaryotic cells in concentration ranging from 10^{-6} to 10^{-3} M (Fig. 9).

Several mechanisms exist for the transport of amino acids across cell membranes. Many are symport or antiport mechanisms that couple amino acid transport to sodium transport. The γ-glutamyl cycle is an example for a group transfer mechanism of amino acid transport. Although this mechanism requires more energy input, it is rapid and has a high capacity. The cycle function is primarily in the kidney, and particularly in renal epithelial cells.

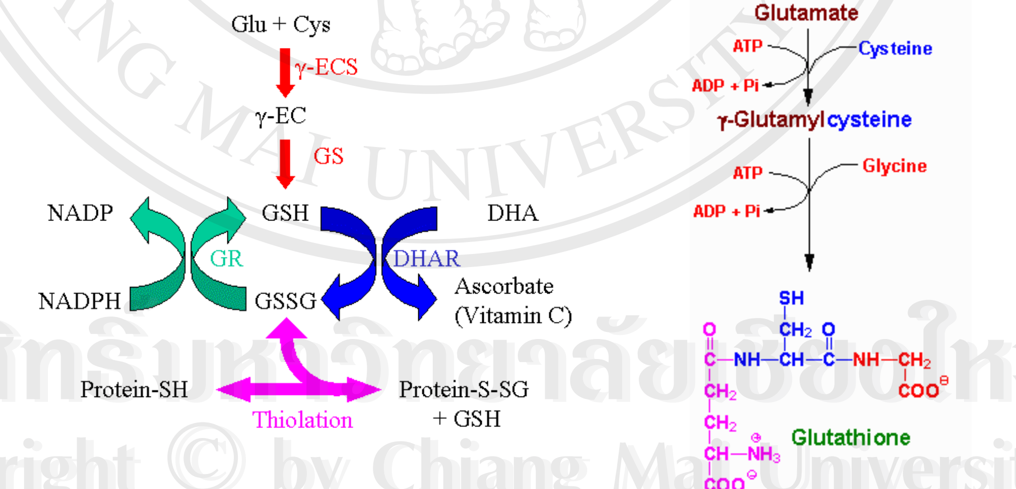

Figure 9. The chemical structure of GSH and its synthesis pathway. The pathway involves co-action of NADPH from pentose phosphate pathway and DHA (http://www.dur. ac.uk/d.p. dixon /GSH.gif).

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The enzyme γ-glutamyl transpeptidase is located in the cell membrane and shuttles GSH to the cell surface to interact with an amino acid. Reaction with an amino acid liberates cysteinylglycine and generates a γ-glutamyl-amino acid which is transported into the cell and hydrolyzed to release the amino acid. Glutamate is released as 5-oxoproline and the cysteinylglycine is cleaved to its component amino acids. Regeneration of GSH requires an ATP-dependent conversion of 5-oxoproline to glutamate and then the 2 additional moles of ATP that are required during the normal generation of GSH.

GSH function

Role of GSH is extremely important particularly in the highly oxidizing environment of the erythrocytes with the sulfhydryl group. The resulting oxidized form of GSH consists of two molecules disulfide bonded together (abbreviated as GSSG). The enzyme glutathione reductase utilizes NADPH as a cofactor to reduce GSSG back to two moles of GSH. Hence, the pentose phosphate pathway is an extremely important pathway within erythrocytes for the continuing production of the NADPH needed by glutathione reductase (GPX) (Fig. 10).

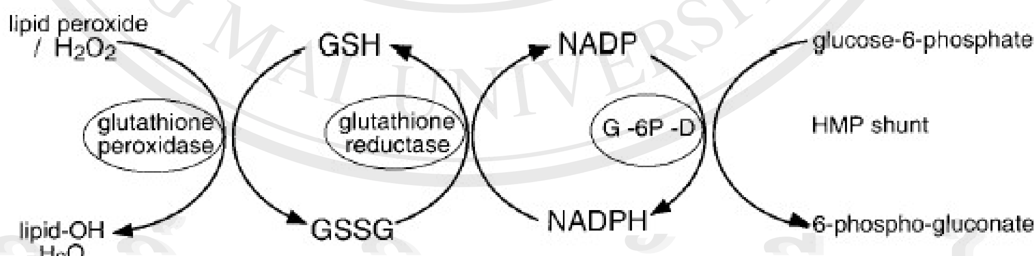

Figure 10. Detoxification of radicals by glutathione. Detoxification of H_2O_2 or lipid peroxide by GSH, and the recovery system to maintain GSH via NADPH (http://ethesis.helsinki.fi/julkaisut/laa/ kliin/vk/ pietarinen-runtti/review.html).

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Glutathione acts to detoxify H_2O_2 by glutathione peroxidase (GPX), and also scavenges peroxides other than H_2O_2 that can catalyze GSH-dependent reduction of fatty acid hydroperoxide, cholesterol hydroperoxide, and t-butyl hydroperoxide.

Glutathione biosynthesis and degradation

GSH is synthesized in two steps. First, glutamylcysteine synthetase catalyzes dipeptide formation, and this is converted to GSH by glutathione synthetase. Cells can make the necessary cysteine from methionine. The second step is activated by a regulatory enzyme, glutamylcystenyl synthetase, which can be inhibited by buthionine sulphoximine (BSO) (Fig. 11). The enzyme is synthesized GSH from γ−glutamylcysteine and glycine by using ATP.

The degradation of the glutamyl moiety of GSH (GSH or GSH S-conjugates) is catalyzed by γ−glutamyl transpeptidse, a membrane-bound enzyme whose active site is on the external surface of cells. GSH is normally transported out of cells to undergo transpeptidation.

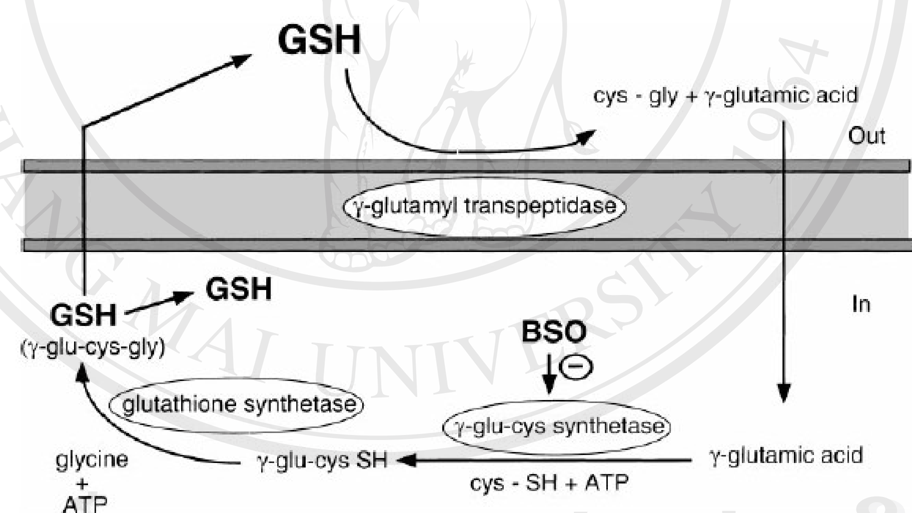

Figure 11. GSH synthesis pathway. GSH is synthesized by three amino acids, glutamate, glycine and cysteine by γ-glutamylcystenyl synthetase and glutathione synthetase. The step of γ-glutamylcysteinyl synthetase is inhibited by BSO (Chen *et al*., 2002).

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1.2.3. Allium Genus (Fenwich and Hanley, 1985)

1.2.3.1. Garlic

Garlic (*Allium sativum* L.) has historically been one of the most common vegetables used as both a spice and medicinal herb. Its usage varies in Thailand. Garlic is a traditional vegetable that has been grown in various areas in Thailand and is used in Thai food, especially in spicy soup, or stir-fried vegetable. The strong odor from the garlic bulb is characteristic as its spicy taste. Garlic belongs to the Alliaceae family. The bulb grows under the ground, and is white in color (Fig. 12).

Figure 12.Garlic bulbs

Chemical composition of garlic

Garlic or aged garlic extract (AGE) contains bioactive organosulfur compounds, mainly S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC) (Imai *et al*., 1994). This compounds have high potential antioxidant activity. Diallyl sulfide (DAS), triallyl sulfide, diallyl disulfides (DADS) and diallyl polysulfides are lipid-soluble compounds found in AGE.

S-allylcysteine $CH_2-CH-CH_2-S-CH_2-CH(NH_2)-COOH$

Diallyl sulfide $CH_2=CH-CH_2-S\ (O^-)^+-CH=CH-S-S-CH_2-CH=CH_2$

Diallyl disulfide $CH_2=CH-CH_2-S-S-CH_2-CH=CH_2$

Diallyl trisulfide $CH_2=CH-CH_2-S-S-S-CH_2-CH=CH_2$

Allicin (thiosulfonate) $CH_2=CH-CH_2-S\ (O^-)^+-S-CH_2-CH-CH_2$

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Pharmacological activities of garlic

Onions, garlic and shallots have anti-inflammatory and anti-oxidative properties. Studies have shown that garlic exhibits anti-bacterial, anti-cataract, anti-cytotoxic, anti-fungal, anti-hypertensive, hypolipidemic, anti-mutagenic, anti-tumor,

anti-carcinogenic and cancer chemopreventive activities. In addition, it can stimulate cell proliferation and activity of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD).

Both lipid- and water-soluble compounds provide antioxidant effects, (Ying *et al*., 2002; Amagase, 1997; Imai *et al*., 1994; Wei and Lau., 1998; Geng *et al*., 1997). Some evidences suggest that a synthesized diallyl disulfide (DADS) can induce GSH synthesis, anti-proliferation, anti-inflammation, and anti-cancer progression in cell lines (Hong *et al*., 2000, Sundaram *et al*., 1996).

In plants, the sulfide is a precursor of GSH (Fig. 13). Diallyl sulfide, diallyl disulfide and diallyl tri-sulfide can modulate GSH-related antioxidant systems in rats (Wu *et al*., 2001). These compounds could reduce carcinogens, serve as an intracellular antioxidant by protecting cell membrane and intracellular component from damaging by free radicals, and also assists in the regulation of DNA synthesis in normal liver and mammary cells (Liu *et al*., 2000).

Onion (*Allium cepa* L.) has been shown to contain various phenolic compounds having high antioxidant capacity and which promote GSH synthesis (Myhrstad *et al*., 2002).

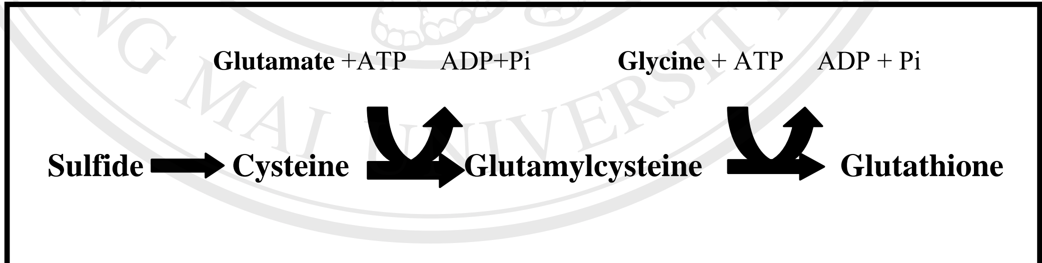

Figure 13. Glutathione synthesis pathway from sulfide in plant. Glutathione is synthesized from sulfide by the glutathione synthetase. (De Kok *et al*., 2005). Sulfide glutamate and glycine are importance for glutathione systhesis.

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Aged garlic extract (AGE) has been shown to inhibit lipid peroxidation on LDL membrane (Lau, 2001) and DNA damaging, while promoting antioxidant enzyme activity and GSH synthesis. Moreover, diallyl tri- and di-sulfides inhibits cell proliferation and controlled NF-κB nuclear binding in adenocarcinoma cell lines (Sundaram and Milner, 1996).

1.2.3.2. Shallot

Shallot (*Allium ascalonicum* L.) is classified into the Allium family and useful in many Thai foods. The pink color of the small shallot bulb has a stronger odor than the garlic bulb (Fig. 14).

Figure 14. Shallot bulbs

Chemical composition of shallot

Previous studies reported that vegetables in Allium family (onion and garlic) contain high amount of flavonol compounds (Terrance *et al*., 1992), especially in edible portion (less than 0.03 to more than 1 g/kg). Shallot uniformly contains a high concentration of total flavonols (especially 4'-glucoside and quercetin aglycone, over 800 mg/kg) and some isorhamnetin or kaempferol monoglycosides. Flavonoids can be found in the alcohol extract (Lucienne *et al*., 1967). Fattorusso and co-workers (2002) report isolation of phytochemicals in the polar extracts from bulbs of shallot. Interestingly, Eric and coworkers (1992) claimed that thiosulfur compounds differed among members of the onion family and there are different thiosulfinates in each Allium species.

Pharmacological activities of shallot

Presently, there are some evidence shown that the shallot activities in immune system. Amin and Kapadins (2005) showed that the shallot extract from drying and autoclaved preparation inhibited the fungi growth better than garlic extracts. Whereas the fresh shallot juice inhibits microbial activity less than fresh garlic juice. There are also some reports indicated that shallot bulb can relieve catching a cold, and induce hair growth. But there are no scientific evidence supporting.

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From previous evidence, there is no evidence of shallot activity against oxidative stress either on protein and lipid oxidation, and the activity in monocytic cells. Thus, in this study the investigations of antioxidant activity involved in protein hydroperoxide formation, including protection of GSH from protein hydroperoxide were performed using three shallot extracts: from crude, and from extraction with water or hexane that contained phenolics or organosulfide (diallyl di-, tri-, tetra-sulfide), and comparing with garlic extracts. The goal of the research was to study the effects of shallot on inhibition or scavenging of protein hydroperoxide from oxidative stress induced via γ-irradiation, and lipid hydroperoxide from auto-oxidation *in vitro*. The regulatory function of shallot extracts on the oxidative stress via hydroperoxide formation and GSH synthesis in human U937 monocytic cells was also evaluated and compared with garlic extracts, a standard antioxidant, and standard diallyl disulfide (DADS).

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1.3. OBJECTIVES

1.3.1. To evaluate the content and capacity of antioxidant in Thai shallot extracts compared with garlic extracts and standard antioxidants.

1.3.2. To investigate the potential effect of Thai shallot extracts on inhibition or scavenging the protein, amino acid, and lipid hydroperoxide during oxidation process.

1.3.3. To investigate the potential effects of Thai shallot extracts on prevention of GSH depletion from protein hydroperoxide or AAPH-oxidation in human erythrocytes.

1.3.4. To differentiate between shallot and garlic extracts in regard to bioactive compounds, total phenolics, and organosulfide between shallot extracts and garlic extracts.

1.3.5. To demonstrate the capacity of Thai shallot extracts involved in GSH synthesis in a human monocytic cell line whether they can protect the cells from oxidative stress by hydroxyl radicals.

1.3.6. To demonstrate the capacity of Thai shallot extracts on oxidative stress and inflammation from H_2O_2 or LPS in monocytic or macrophage cell lines.

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