# CHAPTER 1

# INTRODUCTION

The oxidation reaction directly affects food quality and is commonly associated with the changes of flavor and texture. Therefore, prevention of lipid oxidation has been of concern in the food industry. The use of synthetic antioxidants is an old practice and their safety could be questioned by the consumer. The alternative natural compounds with the efficient antioxidative activity have been paid increasing attention. Protein hydrolysates from many plant and animal sources have been reported to exhibit antioxidative activity. Some peptides in protein hydrolysate play an essential role in acting as antioxidant via not only singlet oxygen and free radical scavenging, but also metal chelation (Egorov *et al*., 1992). Protein hydrolysates from herring byproduct, capelin, yellowfin sole frame, Alaska pollack frame, round scad muscle and yellow stripe trevally meat also possessed antioxidative activity (Sathivel *et al*., 2003; Amarowicz and Shahidi, 1997; Jun *et al*., 2004; Je *et al*., 2005; Thiansilakul *et al*., 2007a; Klompong *et al*., 2007). Additionally, protein hydrolysate from squid skin gelatin showed the antioxidant activity in a linoleic acid oxidation system (Mendis *et al*., 2005).

Shrimp and shrimp products have attracted considerable attention due to their delicacy. Additionally, they are enriched in amino acids, peptides, proteins and other useful nutrients. Penaeid shrimps have become the economically important species for Thailand and are widely cultured in ponds. Black tiger shrimp (*Litopenaeus monodon*) and white shrimp (*Litopenaeus vannamei*) are commonly cultured and exported with a catch volume over 1,000 tons per year and Thailand exported 249,570 tons of shrimp and products with the value of 2.19 billions US dollars in year 2001 (Suphamongkhon, 2002). By-products from the processing of shrimp may account for up 80% of the original weight of raw material (Shahidi *et al.,* 1992). Those by-products consist of 71.4% head and 28.6% shell (Meyers, 1986) and may pose a disposal problem due to the ease of spoilage. Better utilization of wastes from seafood industry has gained an increasing interest over the past 10 years. Generally, shrimp wastes are processed as animal feed and as protein feedstuff in aquaculture diets (Sudaryono *et al.,*1995; Sudaryono *et al*., 1996; Fagbenro,1996; Nwanna *et al*., 2004). To increase the market value of the discards,

many approaches have been used. Protein and amino acids (Mandeville *et al.,* 1992), colorant (Chen and Meyers, 1982), flavourant (Pan, 1990) and chitin and chitosan (Coward-kelly *et al.,* 2006) can be recovered from shrimp head and shell. Enzymatic protein hydrolysates from shrimp waste have been also produced (Ruttanapornvareesakul *et al*., 2005). Protein hydrolysate or peptides from fish and shellfish as well as their by-products have been shown to exhibit antioxidative activity (Mendis *et al*., 2005; He *et al*., 2006). Natural antioxidant, mainly phenolic compound, from shrimp shell waste was characterized by Seymour *et al*. (1996).

Mungoong, the shrimp extract paste, is a traditional Thai fishery product and can be produced by boiling the shrimp cephalothorax to extract the soluble substances. The extract is then evaporated until the product turns to sticky paste. Some ingredients including sugar are added to improve the taste (TCPS 324, 2004). Thus, Mungoong can be a potential source of valuable nutrients, flavorants as well as neutracutical components, especially antioxidative compound. However, a low yield of Mungoong is generally obtained for Mungoong produced using the typical process. The use of some proteases might be an effective means to increase the yield. With greater hydrolysis, smaller peptides could be formed and exhibit their antioxidative activity to a greater extent. Thus, production of Mungoong with antioxidant activity can pave a way to fully use shrimp waste as the value-added product and used as a novel antioxidant for health benefit.

# Literature Review

## 1. Lipid oxidation

Oxidation of lipids is a major cause of deterioration of food and food products, especially those containing high content of unsaturated fatty acids. Lipid oxidation is mainly associated with the rejection by consumer due to the off-odor and off-flavor. The direct reaction of a lipid molecule with a molecule of oxygen, termed autoxidation, is a free-radical chain reaction and mainly involves in food deterioration. Lipid oxidation is known to proceed by a free radical chain reaction mechanism involving initiation, propagation/branching and termination stages (Monahan, 2000).

### 1.1 Initiation

The autoxidation of fat is initiated with the formation of free radicals. Initiation reactions take place either by the abstraction of hydrogen radical from an allylic methylene group of an unsaturated fatty acid or by the addition of a radical to a double bond. The rearrangement of the double bonds results in the formation of conjugated diene (-CH=CH-CH=CH-), showing a characteristic UV absorption at 232-234 nm (Nakayama *et al*., 1994).

The formation of lipid radical ($R^{\bullet}$) is usually mediated by trace metals, irradiation, light or heat (Eq. 1). Also, lipid hydroperoxide, which exists in trace quantities prior to the oxidation, breaks down to yield radicals as shown by Eqs. (2) and (3). Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids (Jadhav *et al*., 1996).

$$RH + \text{initiator} \longrightarrow R^{\bullet} + H^{\bullet} \quad (1)$$

$$ROOH \longrightarrow RO^{\bullet} + HO^{\bullet} \quad (2)$$

$$2ROOH \longrightarrow RO^{\bullet} + ROO^{\bullet} + H_2O \quad (3)$$

### 1.2 Propagation

In propagation reaction, free radicals are converted into other radicals. Propagation of free-radical oxidation processes occurs by chain reactions that consume oxygen and yield new free-radical species (peroxy radicals, $ROO^{\bullet}$) or by the formation of peroxides

(ROOH) as in Eqs. (4) and (5) (Jadhav *et al*., 1996). The product $R^{\bullet}$ and $ROO^{\bullet}$ can further propagate free-radical reactions.

$$R^{\bullet} + {}^{3}O_2 \longrightarrow ROO^{\bullet} \quad (4)$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet} \quad (5)$$

Lipid peroxy radicals ($ROO^{\bullet}$) initiate a chain reaction with other molecules, resulting in the formation of lipid hydropeoxides and lipid free radicals. This reaction, when repeated many times, produces an accumulation of hydroperoxides. The propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available (Pryor and Porter, 1990). Lipid hydroperoxides may also be formed by the reaction of an unsaturated fatty acid such as linoleic acid with oxygen in the singlet excited state or enzymatically by the action of lipoxygenase (Wilcox and Marnett, 1993). Lipid hydroperoxide, the primary products of autoxidation, are odorless and tasteless (Jadhav *et al*., 1996).

**1.3 Termination**

A free radical is any atom with unpaired electron in the outermost shell. Free radicals are electrically neutral, and salvation effects are generally very small. Owing to the bonding-deficiency and structural unstability, radicals therefore tend to react whenever possible to restore normal bonding. When there is a reduction in the amount of unsaturated lipids (or fatty acids) present, radicals bond to one another, forming a stable nonradical conpounds (Eqs. 6, 7 and 8). Thus the termination reactions lead to interruption of the repeating sequence of propagating steps of the chain reaction (Jadhav *et al*., 1996).

$$R^{\bullet} + R^{\bullet} \longrightarrow R\text{-}R \quad (6)$$

$$R^{\bullet} + ROO^{\bullet} \longrightarrow ROOR \quad (7)$$

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2 \quad (8)$$

**1.4 Factors influencing rate of lipid oxidation**

Many factors have been known to be associated with lipid oxidation (Nawar, 1996).

1. Fatty acid composition: The number, position, and geometry of double bonds affect the rate of oxidation. Relative rates of oxidation for arachidonic, linolenic, linoleic, and oleic acids are approximately 40:20:10:1, respectively. *Cis* acids oxidize more readily than their *trans* isomers, and conjugated double bonds are more reactive than non conjugated (Nawar, 1996). Autoxidation of saturated fatty acids is extremely slow; at room temperature, they remain practically unchanged when oxidative rancidity of unsaturates becomes detectable. At high temperatures, however, saturated acids can undergo oxidation at significant rates (Pryor and Porter, 1990).

2. Pro-oxidants: Transition metals, particularly those possessing two or more valency states and a suitable oxidation-reduction potential between them (e.g., cobalt, copper, iron, manganese, and nickel), are effective pro-oxidants. If present, even at concentrations as low as 0.1 ppm, they can decrease the induction period and increase the rate of oxidation (Oleary *et al*., 1992). Trace amout of heavy metals are commonly encountered in edible oils, and they originate from the soil in which the oil-bearing plant was grown, from the animal, or from metallic equipment used in processing or storage. Trace metals are also naturally occurring components of all food tissues and of all fluid foods of biological origin (eggs, milk, and fruit juices), and they are present in both free and bound forms (Nawar, 1996).

Several mechanisms for metal catalysis of oxidation have been postulated (Nawar, 1996):

(1) Acceleration of hydroperoxide decomposition:

$$M^{n+} + ROOH \longrightarrow M^{(n+1)+} + OH^- + RO^\bullet$$

$$M^{n+} + ROOH \longrightarrow M^{(n-1)+} + H^+ + ROO^\bullet$$

(2) Direct reaction with the unoxidized substrate:

$$M^{n+} + RH \longrightarrow M^{(n-1)+} + H^+ + R^\bullet$$

(3) Activation of molecular oxygen to give singlet oxygen and peroxy radical:

$$M^{n+} + O_2 \longrightarrow M^{(n+1)+} + O_2^{-} \begin{cases} \xrightarrow{-e^-} {}^1O_2 \\ \xrightarrow{+H^+} HO_2^\bullet \end{cases}$$

3. Other factors.

(1) Oxygen concentration: When oxygen is abundant, the rate of oxidation is independent of oxygen concentration, but at very low oxygen concentration the rate is approximately proportional to oxygen concentration (Ahn *et al*., 1993). However, the effect of oxygen concentration on rate is also influenced by other factors, such as temperature and surface area. Jakobsen and Bertelsan (2000) reported that the fresh beef kept in modified atmosphere packaging at 20% oxygen content showed less lipid oxidation than that with 80% oxygen.

(2) Temperature: The rate of oxidation increase as the temperature is increased. Temperature also influences the relationship between rate and oxygen partial pressure (Ahn *et al*., 1993). As temperature is increased, changes in oxygen partial pressure have a smaller influence on rate because oxygen becomes less soluble in lipids and water as the temperature is raised. Silva *et al*. (2004) reported that the oxidation of potato crisp during storage was greater at 40°C, compared with that found at room temperature.

(3) Surface area: The rate of oxidation increases in direct proportion to the surface area of the lipid exposed to air (Gil *et al*., 2002). Furthermore, as surface-volume ratio is increased, a given reduction in oxygen partial pressure become less effective in decreasing the rate of oxidation. In oil-in-water emulsions, the rate of oxidation is governed by the rate at which oxygen diffuse into the oil phase (Nawar, 1996).

(4) Light: Fatty acids are particularly sensitive to photolytic autooxidation since they generally exhibit great absorption of UV radiation, but absorb less light in the visible spectrum. However, exposure to visible light can bring about oxidative changes in foods as well, primarily through photosensitized oxidation, which occurs in the presence of photosensitizers. Photosensitizers are substances containing conjugated double bond systems which thereby have the ability to absorb light in the visible region. The absorption of light leads to an electron being excited to a higher energy level, resulting in an unstable singlet-excited state of the photosensitizer (Ahn *et al*., 1993). The singlet-excited state of the photosensitizer may undergo three physical processes; internal conversion, emission of light, or intersystem crossing. Conversion of the excited singlet state via intersystem crossing yields the triplet-excited state that is the reactive intermediate in photosensitized oxidation (Sang and Jin, 2004).

(5) Moisture: In model lipid system and various fat-containing foods, the rate of oxidation dependent strongly on water activity. In dried foods with very low moisture contents ($a_w$ values of less than about 0.1), oxidation proceeds very rapidly. Increasing the $a_w$ to about 0.3 retards lipid oxidation and often produces a minimum rate. This protective effect of small amounts of water is believed to occur by reducing the catalytic activity of metal catalysts, by quenching free radicals, and/or by impeding access of oxygen to the lipid. At higher water activities ($a_w$ = 0.55-0.85), the rate of oxidation increases again, presumably as a result of increased mobilization of catalysts and oxygen (Nawar, 1996).

## 2. Antioxidant

Antioxidants are the chemical substances that reduce or prevent oxidation and have the ability to counteract the damaging effects of free radicals in tissues and thus are believed to protect against cancer, arteriosclerosis, heart disease and several other diseases (Bandyopadhyay *et al*., 2007). Antioxidant may be defined as any substance which is capable of delaying, retarding or preventing the development in food of rancidity or other flavor deterioration due to oxidation (Gordon, 2001). Antioxidant can act at different levels in an oxidative sequence. Oxidative stress can cause damage by stimulating the free radical chain reactions within a material and can be inhibited either by adding chemicals that retard the formation of free radicals or by introducing substances that compete with the existing radicals and remove them from the reaction medium (Jun *et al*., 2004).

### 2.1 Classification of food antioxidants

#### 2.1.1. Primary antioxidants

Primary antioxidants terminate the free-radical chain reaction by donating hydrogen or electron to free radicals and converting them to more stable products. They may also interact with the lipid radicals, forming lipid-antioxidant complexes (Decker, 1998). Many of the naturally occurring phenolic compounds like flavonoids, eugenol, vanillin and rosemary antioxidant also have chain-breaking properties (Rajalakshmi and Narasimhan, 1996). Protein hydrolysate from many plant and animal sources also process the primary antioxidative activity (Decker and Crum, 1993; McCarthy *et al*., 2001; Sathivel *et al*., 2003; Sakanaka *et al*., 2004,

Seymour *et al*., 1996). Primary antioxidants are effective at very low concentrations but at higher levels they become prooxidants (Rajalakshmi and Narasimhan, 1996).

### 2.1.2 Secondary antioxidants

Secondary or preventive antioxidants such as thiopropionic acid and dilauryl thiodipropionate function by decomposing the lipid peroxides into stable end products (Rajalakshmi and Narasimhan, 1996). Synergistic antioxidants can be broadly classified as oxygen scavengers and chelators. However, they may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant (Decker, 1998). Hence phenolic antioxidants can be used at lower levels if a synergist is added simultaneously to the food product. Oxygen scavengers such as ascorbic acid, ascorbyl palmitate, sulfites and erythobates react with free oxygen and remove it in a closed system (Rajalakshmi and Narasimhan, 1996). The synergistic effect of citric acid is attributed to metal chelation (Frankel, 1998). Other polyvalent acids such as tartaric, malic, gluconic, oxalic, succinic and dehydro glutaric acids, as well as sodium triphosphate and pyrophosphate also show the synergistic properties similar to those of citric acid (Yanishlieva-Maslarova, 2001). Moreover, ascorbic acid can act as a synergist with tocopherols by regenerating or restoring their antioxidant properties (Niki, 1987). Ascorbic acid and its derivatives also function as oxygen scavengers (Yanishlieva-Maslarova, 2001).

Flavonoids and related compounds and amino acids function as both primary antioxidants and synergists (Weber, *et al*., 2006). Nitrites and nitrates, which are used mainly in meat curing, probably function as antioxidants by converting heme proteins to inactive nitric oxide forms and by chelating the metal ions, especially nonheam iron, copper and cobalt that are present in meat (Cortas and Wakid, 1990). β-carotene and related carotenoids are effective quenchers of singlet oxygen and also prevent the formation of hydroperoxides. The Maillard reaction prepared from glucose-glysine, glucose-lysine and fructose-lysine exhibited the metal chelators (Yoshimura *et al*., 1997; Wijewickreme *et al*., 1997)

## 2.2 Mode of action of antioxidants in food

### 2.2.1 Radical scavenger

Radical scavenging is the main mechanism by which antioxidants act in foods (Gordon, 2001). Antioxidants can delay or inhibit lipid oxidation by inactivating or scavenging

free radicals, thus inhibiting initiation and propagation reactions. Free radical scavengers or chain-breaking antioxidants are capable of accepting a radical from oxidizing lipids species such as peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals to form stable end products (Decker, 1998). Two different mechanisms as radical scavengers have been proposed for antioxidant; either as hydrogen donors or as electron donor that form charge-transfer complexes (Namiki, 1990; Osawa, 1994).

Hydrogen donor

$$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet}$$

$$RO^{\bullet} + AH \longrightarrow ROH + A^{\bullet}$$

$$R^{\bullet} + AH \longrightarrow RH + A^{\bullet}$$

Electron donor

$$ROO^{\bullet} + A^{\bullet} \longrightarrow (AH\text{-}ROO)^{\bullet}$$

$$(AH\text{-}ROO)^{\bullet} + ROO^{\bullet} \longrightarrow \text{Stable product}$$

The free antioxidant radicals ($A^{\bullet}$) may undergo additional reactions that remove radical from the system. Termination reactions of antioxidant with other free antioxidant radicals or lipid radicals can form nonradical species.

$$ROO^{\bullet} + A^{\bullet} \longrightarrow ROOA$$

$$RO^{\bullet} + A^{\bullet} \longrightarrow ROA$$

$$A^{\bullet} + A^{\bullet} \longrightarrow AA$$

Antioxidants may act as hydrogen donors to the phenoxy radicals, which are stabilized by resonance delocalization of the unpaired electron in the aromatic ring and are further stabilized by bulky group at the ortho position as shown in Figure 1 (Shahidi and Wanasundara, 1992).

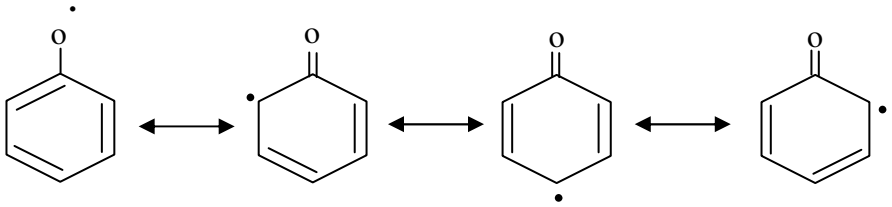

Figure 1 Delocalization of the unpaired electron in the aromatic ring of phenoxy radicals.

Source: Shahidi and Wanasundara (1992)

**2.2.2 Peroxide decomposer**

Some phenols, amine, dithiopropionic acid and thiopropionic acid function by decomposing the lipid peroxide into stable end products such as alcohol, ketone and aldehyde (Dziezak, 1986; Namiki, 1990). Puerta *et al*. (1999) reported that oleuropein and hydroxytyrosol were capable of scavenging hydrogen peroxide by using the guaiacol reaction.

**2.2.3 Singlet oxygen quenching**

The mechanism of converting triplet oxygen to singlet oxygen is initiated by the transfer of the photosensitizer to its electronically exited state due to the absorption of light in the visible or near-UV region. Subsequently, the photosensitizer is able to transfer its excessive energy to an oxygen molecule, giving rise to singlet oxygen (Shahidi and Wanasundara, 1992). Antioxidant can inhibit singlet oxygen by changing singlet oxygen form ($^1O_2$*) into triplet oxygen ($^3O_2$) and released heat energy. One molecule of antioxidant can interact with 1,000 molecules of singlet oxygen (Sies *et al*., 1992).

**2.2.4 Lipoxygenase inhibitor**

Lipoxygenases constitute a family of dioxygenases, that catalyze the oxygenation of free and esterified polyunsaturated fatty acids containing 1,4-pentadiene system to produce the corresponding hydroperoxy derivatives (Kuhn, 1999). They are monomeric proteins that contain a 'non-heme' iron per molecule in the active site as high-spin Fe(II) in the native state, and high-spin Fe(III) in the activated state, and they are categorized with respect to their positional specificity of arachidonic acid (AA) oxygenation (Stallings *et al*., 1990). Phenolic compounds such as flavonoid, phenolic acid and gallates exert selective inhibitory activity against

the 5-LO (leukotriene) pathway of arachidonate metabolism in activated leukocytes without affecting the cyclo-oxygenase pathway. Phenolic compounds may also bind iron ions and/or reduce them to the catalytically inactive ferrous form and both properties may be important for inhibition of lipoxygenase activity (Puerta *et al*., 1999).

**2.2.5 Synergists**

**1) Chelating agents**

Metal chelation can result in prevention of metal redox cycling, occupation of all metal coordination sites, formation of insoluble metal complexes, steric hindrance of interactions between metals, and formation of lipid intermediates (Hider *et al*., 2001; Moridani *et al*., 2003). Chelating agents play a valuable role in stabilizing foods. Chelating agents that improve the shelf-life of lipid containing food are EDTA, citric acid and phosphoric acid derivatives (Jadhav *et al*., 1996). Polyphenols were also act as chelating agents (Hider *et al*., 2001; Rice-Evans *et al*., 1996). The metal chelating ability of polyphenols is related to the presence of ortho-dihydroxy polyphenols, i.e., molecules bearing catechol or galloyl groups (Khokhar and Apenten, 2003; Moran *et al*., 1997).

**2) Reducing agents or oxygen scavengers**

Reducing agents or oxygen scavengers function by various mechanisms. They may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant or may react with free oxygen and remove it in a closed system (Giese, 1996; Rajalakshmi and Narasimhan, 1996). Ascorbic acid is a strong reducing agent, readily losing $H^+$ to become dehydroascorbic acid, which also has vitamin C activity. However, vitamin C activity is lost, when the lactone ring of dehydroascorbic acid is hydrolyzed to yield diketogluconic acid (Gordon, 2001).

**2.3 Type of antioxidant**

**2.3.1 Synthetic antioxidants**

Many synthetic compounds, which are characterized as a better antioxidant activity than natural antioxidants and are more easily available, have been used in a wide variety of food products (Pinho *et al*., 2000). Some of more popular synthetic antioxidats used are phenolic compounds such as butylated hydroxyanisol (BHA), butylated hydroxyl toluene (BHT),

tertiary butylhydroquinone (TBHQ) and esters of gallic acid, e.g. propyl gallate (PG) (Yanishlieva-Maslarova, 2001). The chemical structure of these synthetic antioxidants are shown in figure 2.

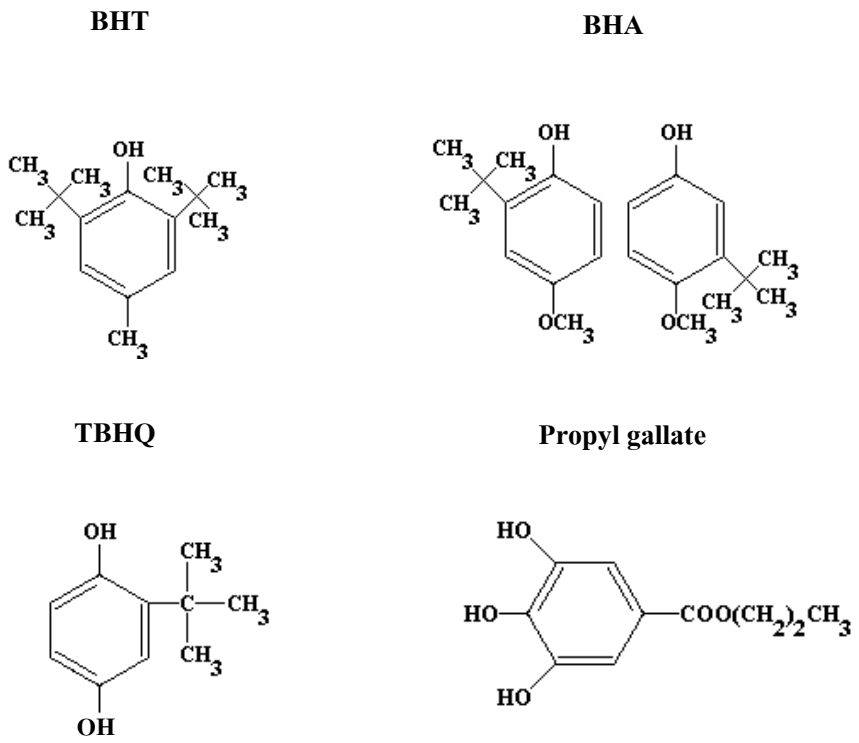


Figure 2 Chemical structure of synthetic antioxidants

Source: Yanishlieva-Maslarova (2001)

Synthetic antioxidants are widely used in food, pharmaceutical and other commercial products to prevent oxidative rancidity. The most suitable antioxidant for vegetable oils is TBHQ. BHA and BHT are fairly stable to heat and are often used for stabilization of fats in baked and fried products (Yanishlieva-Maslarova, 2001). However, it has been found that these artificial antioxidants may cause a loss of nourishment, and even produce toxic effects (Madhavi *et al*., 1996). The use of BHA and BHT at extremely high concentrations has been shown to pose potential health risk in animal models, prompting strict regulations for their use in foods (Chen *et al*., 1992; Sun and Fukuhara, 1997). In many countries, the use of these antioxidants is controlled.

The use of TBHQ in foodstuffs for antioxidant purposes was banned in the EU owing to toxicological reasons (Pinho *et al*., 2000). Therefore, natural products appear as healthier and safer than synthetic antioxidants. Since 1980, natural antioxidants have been appeared as alternative to synthetic antioxidants (Yanishlieva-Maslarova, 2001).

### 2.3.2 Natural antioxidants

Natural antioxidants are found in almost all plants, microorganisms, fungi and even in animal tissues (Pokorny, 1999). The majority of natural antioxidants are phenolic compounds and the most important groups of natural antioxidants are the tocopherols, flavonoids and phenolic acids (Yanishlieva-Maslarova, 2001). Natural antioxidants, such as tocopherol, vitamin C, and phenolic compounds from plant extracts (e.g. tea, catechins) and spices (rosemary, sage, oregano, etc.), have been shown to decrease lipid oxidation as effectively as synthetic antioxidants in cooked meat products (Fernandez-Lopez *et al*., 2003; Karpinska *et al*.,2001; Mansour and Khalil, 2000; Tang *et al*., 2001). In order to develop natural and safer antioxidants, the antioxidant or synergistic effects of amino acids, peptides, proteins and proteolytic hydrolysates from animal have been attracting considerable research attention. Carnosine, anserine and ophidine are histidine-containing dipeptides, which can chelate metals and scavenge radicals (Clifford-Hall, 2001). Peptides with N-terminal sequence could terminate metal-catalysed oxidation (Clifford-Hall, 2001). Additionally, Protein hydrolysate from fish and shellfish as well as their by-products have been shown to exhibit antioxidative activity as natural antioxidant (Je *et al*., 2005; Jun *et al*., 2004; Kim *et al*., 2001; Mendis *et al*., 2005).

## 3. Utilization of shrimp wastes

The utilization of shrimp waste including shell and head has been reported. To increase the market value of shrimp wastes, many approaches have been used. The use of shrimp head as a protein feedstuff for replacing fish meal in aquaculture diets have been studied and protein content of the products is in the range of 40-43 % (Sudaryono *et al*., 1995; Sudaryono *et al*., 1996; Nwanna *et al*., 2004). Generally, the shrimp waste hydrolysate has a high content of essential amino acids, indicating a high nutritional value used for food and animal feed (Gildberg and Stenberg, 2001). Fabenro *et al*. (1997) produced shrimp head silage by fermented heads of

river prawn (*Macrobrachium vollenhovenii*) with *Lactobacillus plantarum* at 30°C for 7 days and found that protein content of the silage was 40-50% and amino acid composition of product consist of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Lopez-Cervantes *et al*. (2006) reported that free amino acid contents in the fermented shrimp waste ranged from 9.3 to 56.9 mg/g dry mass. The sample contained arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, asparagine, serine, glutamine, glycine, alanine and proline. Tyrosine was the most abundant amino acid (56.9%). Ruttanapornvareesakul *et al*. (2006) studied the effect shrimp head protein hydrolysates (SHPH) on the state of water and the denaturation of fish myofibrils during dehydration. SHPH was added to lizardfish myofibrils at concentration ranging from 2.5% to 10%. The amount of monolayer and multilayer water in myofibrils contained SHPH were higher than those without SHPH (control). The Ca-ATPase inactivation rate of myofibrils containing SHPH decreased during dehydration.

Shrimp waste is also an important source of pigment. Sachindra *et al*. (2006) extracted shrimp waste carotenoids using different organic solvents and solvent mixtures. A 50:50 mixture of isopropyl alcohol and hexane gave the highest (43.9lg/g waste) carotenoid extraction yield, compared to acetone, methanol, ethanol, isopropyl alcohol, ethyl acetate, ethyl methyl ketone, petroleum ether, and hexane individually and to a mixture of acetone and hexane. Furthermore, chitin and chitosan are currently produced from several crustacean wastes. Chitin is a structural component in crustacean exoskeletons, which are 15-20% chitin by dry weight (Coward-Kelly *et al*., 2006). Chitosan is made by alkaline *N*-deacetylation of chitin. The optimum condition for the deacetylation reaction of shrimp wastes was observed at a temperature of 130°C for 90 min, and the resulting chitosan had the molecular weight of 150 kDa, and a deacetylation degree of 90% (Weska *et al*., 2007). The effectiveness of chitosan in most applications depends on a complex mixture of electro-visco-elastic parameters. Chen and Hwa (1996) reported the change of thermal and physico-chemical properties of chitosan with different molecular weights but with the same degree of deacetylation. Trung *et al*. (2006) reported that chitosan with 75% degrees of deacetylation (DD) had a 1.5 times higher water absorption than chitosan with 87% and 96% DD, probably due to its 20% lower level of crystallinity. Membranes cast from this chitosan also exhibited 1.5 times more water absorption and 2 times higher permeability.

However, chitosan with 87% and 96% DD had 1.5–2 times higher absorption of fat and the orange II dye. This is attributed to the higher content of positively charged amine groups in the polymer. Cast into membrane, chitosan of higher degree of deacetylation showed a higher tensile strength and a higher elongation at break, probably due to the higher level of crystallinity (Trung *et al*., 2006).

## 4. Protein concentrate

Fish protein concentrate (FPC) can be prepared from any type of fish or fishery waste. It is prepared from fish by extracting out the oil, screening or settling out the bones and drying. The resultant product (FPC) is higher in protein (85% to 95 %) and lower in ash content (Windsor, 2001).

### 4.1 Type of protein concentrate

Protein concentrate can be classified into 3 different types as follows:

1. Type A: A virtually odorless and tasteless powder which contains at least 67.5% protein and not more than 0.75% lipid (Shahidi, 1994). Type A fish protein concentrate is the form most commonly associated with use for human consumption (Peterson and Johnson, 1978).

2. Type B: Product with a lipid content of up to 10% with a distinct fishy flavor (Shahidi, 1994).

3. Type C: Normal fish meal produced under satisfactorily hygienic conditions and their characteristically strong fishy odor and flavor (Windsor, 2001).

### 4.2 The production of fish protein concentrate

Fish protein concentrate can be produced using the following procedure (Windsor, 2001):

1) Fresh whole fish are rinsed with fresh water, weighed and fed to a mincer by conveyor.

2) First extraction: Minced fish are fed to extractor 1 which dehydrates the fish; it is an unheated vessel in which the mince is agitated for about 50 minutes together with the liquid recovered from extractor 2, which contains some isopropanol**.**

3) Centrifuging: The contents of extractor 1 are fed to a continuous centrifuge, where the slurry separates into a solid known as wet cake, and a liquid. The wet cake is conveyed to extractor 2, and the liquid to a still for recovery of solvent and fat.

4) Second extraction: Extractor 2 is jacketed, and the temperature is about 75°C. Here the liquid recovered from extractor 3 is added to the wet cake from extractor 1 and the mixture is agitated for 90 minutes. At the beginning of this stage, the cake is almost completely dehydrated, but has a fat content of about 5 percent, which is reduced to about 1 percent during the extraction.

5) Centrifuging: The contents of extractor 2 are centrifuged; the wet cake is conveyed to extractor 3, and the liquid is returned to extractor 1 for the next batch of raw material.

6) Third extraction: The extractor 3 is jacketed, and the temperature is again about 75°C. Fresh isopropanol is added to the wet cake and agitated for about 70 minutes. During this stage the fat content is reduced to about 0-3 percent.

7) Centrifuging: The contents of extractor 3 are centrifuged and the wet cake is washed with pure isopropanol for about 50 minutes. The liquid is returned to extractor 2 for the next batch.

8) Solvent removal: The wet cake is heated in a rotating vacuum dryer to evaporate the solvent; the vapors are drawn off, condensed and used again.

9) Grinding and packing: the dried material is conveyed to a hammer mill, where it is ground to a fine powder and sieved.

Fish species and drying process have influence on the quality of protein concentrate. Murueta *et al*. (2007) produced protein concentrates from 9 species of by-catch fish using three drying processes (freeze-drying, heating at 65°C and 110°C). The soluble protein content from each process and species was evaluated. Significant differences were observed among processes. The composition of protein, observed by electrophoresis, was affected by temperature. Raw and freeze-dried protein from *S. scituliceps* showed high proteolytic activity and underwent auto-hydrolysis. The highest proteolytic activity was at 65°C and its optimum pH was 7.5. *In vitro* digestibilities of the protein concentrates were higher when obtained by low-temperature methods. Type of enzyme also affects the taste and flavor of products from protein concentrate. Nilsang *et al*. (2005) produced protein hydrolysate from fish soluble concentrate

(FSC) by using Flavourzyme™ and Kojizyme™ and reported that Kojizyme™ enhanced the formation of some bitter-taste amino acids such as tryptophan during hydrolysis process whereas Flavourzyme™ did not.

## 5. Protein hydrolysate

Protein hydrolysates can be defined as proteins that are chemically or enzymatically broken down to peptides of varying sizes (Adler-Nissen, 1986). The chain length of peptides formed during the hydrolysis process is one of the parameters determining both the functional and the organoleptic properties of the hydrolysate. Normally, fish protein hydrolysate includes 85-90% protein, 2-4% lipid and 6-7% ash (Mackie, 1982). However, the use of fish by-product based hydrolysates into food and animal feed applications has been restricted by their bitterness (Daukšas *et al.,* 2004).

### 5.1 The production of protein hydrolysate

Hydrolysis is conventionally conducted by chemical means involving the use of an acidic, basic, or other catalyst. However, to gain more selective hydrolysis, enzymes are often used (Johnson, 1974).

#### 5.1.1 The chemical methods

Chemical hydrolysis of proteins is achieved by cleaving peptide bonds with either acid or alkaline (Hale, 1972). It is relatively inexpensive and quite simple to conduct. However, this method tends to be a difficult process to control and almost invariably leads to products with variable chemical composition and functional properties (Blendford, 1994). Protein hydrolysis with strong chemicals and solvents is commonly performed at extreme temperatures and pH, giving products with reduced nutritional qualities, poor functionality and restricted to use as flavor enhancers (Webster *et al.,* 1982; Leffler, 1986).

**A. Acid hydrolysis**

Acid hydrolysis of proteins is used more commonly than hydrolysis under alkaline conditions. Although the process is harsh and hard to control, it is still the preferred method for hydrolysis of vegetable proteins. Total hydrolysis of fish protein substrate can be achieved with 6 N hydrochloric acid for 18 h at 118°C (Peterson, 1978). However, the

hydrolysate contains a large amount of salt (NaCl) formed during the neutralization, making the product unpalatable and causing the interferes with functionality in food systems (Fountoulakis and Lahm, 1998). Additionally, acid hydrolysis is disadvantageous since tryptophan, asparagine, glutamine and small quantities of other amino acids are destroyed and the extended hydrolysis is necessary to release all of the amino acids (Johnson, 1974).The acid hydrolysis is also widely utilized to convert underutilized and secondary raw material from fish into fertilizer due to the low production cost and the extensive hydrolysis.

**B. Alkaline hydrolysis**

The use of alkali reactants, primarily sodium hydroxide, to hydrolyze protein often results in poor functionality and more importantly can adversely affect the nutritive value of the hydrolysate (Peterson, 1978). Alkaline hydrolysis of fish proteins primarily uses fish protein concentrate as the starting substrate. During alkaline hydrolysis of fish protein, the rapid cleavage of proteins to water-soluble polypeptides takes place, followed by further degradation at a slower rate. Alkaline hydrolysis is almost exclusively used for determination of tryptophan which is stable under basic conditions (Fountoulakis and Lahm, 1998). Several deleterious reactions can occur during hydrolysis. These are initiated by hydrogen abstraction from the alpha carbon of an amino acid and include racemization of L-amino acids, which produces D-amino acids, which are not absorbed by humans. Also, the formations of lysinoalanine, ornithinoalanine, lanthionine and β-amino alanine can occur via β-elimination reactions (Friedman, 1999). These may lead to the formation of toxic substances (Lahl and Braun, 1994; Linder *et al*., 1995).

### 5.1.2 The enzymatic methods

Biological processes are preferred methods for improving functionality and avoiding destruction of products. This can be done via proteolytic enzymes already present in the fish viscera and muscle or by adding enzymes from other sources. Proteases are characterized based on their hydrolyzing mechanism into endopeptidases which cleave the peptide bonds within protein molecules or exopeptidases which hydrolyze the terminal peptide bonds (Adler-Nissen, 1986). The choice of an enzyme for a particular use must be based on the specificity, pH optimum, heat stability, effect of activators and inhibitors (Johnson, 1974). Added enzymes are used to obtain a more selective hydrolysis since proteases are specific for peptide bonds adjacent to certain amino acid residues (Peterson, 1978). When hydrolyzed, most food proteins liberate

bitter-testing peptides, which affect their acceptability in certain applications (Dauksas *et al*., 2004). The bitterness of peptides is associated with their mean hydrophobicity. Peptides that have above 5.85 kJ/mol are bitter, whereas those with less than 5.43 kJ/mol are not (Damodoran, 1996). The intensity of bitterness depends on the amino acid composition and sequence as well as the type of protease used. Hydrolysate of hydrophilic proteins, such as gelatin, are less bitter than the hydrolysates of hydrophobic proteins, such as caseins and soy protein (Damodoran, 1996).

### 5.2 Enzymatic hydrolysis process of fish proteins

The production of fish protein hydrolysate is influenced by the composition of raw material, type of enzyme used and hydrolysis conditions. The scheme for the production of fish protein hydrolysate using enzymes is given in Figure 3.

#### 5.2.1 The substrate and preparation

Lean fish species or their derived material is preferred as the protein substrate for enzymatic hydrolysis to avoid extensive lipid oxidation. However, the abundant underutilized pelagic fish can be used. The production is started by evisceration of the whole fish. Thereafter a fish is ground, mixed with water and homogenized until a viscous homologous mixture is obtained. In some instances, a buffer solution such as phosphate buffer and boric acid-NaOH buffer is added to the minced fish (Baek and Cadwallader, 1995). Process for fatty and lean species is different. Fatty species such as mackerel, herring, menhaden, sprat and anchovy would require additional treatments such as centrifugation or solvent extraction to remove the excess fat (Ritchie and Mackie, 1982; Mackie, 1982).

#### 5.2.2 The enzymatic hydrolysis

Proteolytic enzymes from microorganisms such as Alcalase, Neutrase, Protease N and Protamex are most suitable to prepare fish protein hydrolysates because of their high productivity (Benjakul and Morrisssey, 1997; Gildberg *et al.,* 1989; Guerard *et al.,* 2001; Rebeca *et al.,* 1991; Liaset *et al.,* 2002; Wu *et al*., 2003). Enzymes from plants and animals such as papain, bromelain, ficin and pepsin are still used for hydrolysis (Aspmo *et al.,* 2005; Liaset *et al.,* 2000). The selection of enzymes is usually based on a combination of efficacy and economics (Lahl, 1994).

*Flavourzyme* is a fungal protease/peptidase complex produced by submerged fermentation of a selected strain of *Aspergillus oryzae* which has not been genetically modified

and is used for the hydrolysis of proteins under neutral or slightly acidic conditions. The optimal working conditions for Flavourzyme are reported to be at pHs 5.0-7.0 with an optimal temperature around 50°C (Šližyte *et al.,* 2004b). Flavourzyme is the endo- and exopeptidase enzyme mixture which can minimize the bitterness in the hydrolyzed products (Imm and Lee, 1999; Liaset *et al.,* 2000). Nilsang *et al*. (2004) reported that the spray-dried fish protein hydrolysate produced from fish soluble concentrate using Flavourzyme contained high protein content (66%) and had the bitterness less than that of 1 ppm caffeine solution.

The enzymatic hydrolysis of fish muscle proteins is characterized by an initial rapid phase, in which a large number of peptide bonds are hydrolyzed. Thereafter, the rate of enzymatic hydrolysis decreases and reaches a stationary phase where no apparent hydrolysis takes place (Shahidi *et al*., 1995). The shape of the hydrolysis curve has been associated with enzyme inactivation, product inhibition by hydrolysis, products formed at high degrees of hydrolysis, the soluble peptides that act as effective substrate competitors to the unhydrolyzed fish protein (Rebeca *et al.,* 1991) and possibly autodigestion of the enzyme (Mullally *et al.,* 1995).

Degree of hydrolysis (DH), which indicates the percentage of peptide bonds cleaved (Adler-Nissen, 1976), is one of the basic parameters that describes the properties of protein hydrolysates and needs to be controlled during protein hydrolysis. DH is governed by the four processing variables including substrate, enzyme-substrate ratio, temperature and time (Kristinsson and Rasco, 2000a). Several properties of protein hydrolysates are closely related to DH. Hydrolysis of peptide bonds causes several changes such as reduced molecular weight, an increase of amino and carboxyl groups, which increase solubility and destruction of tertiary structure (Nielsen, 1997).

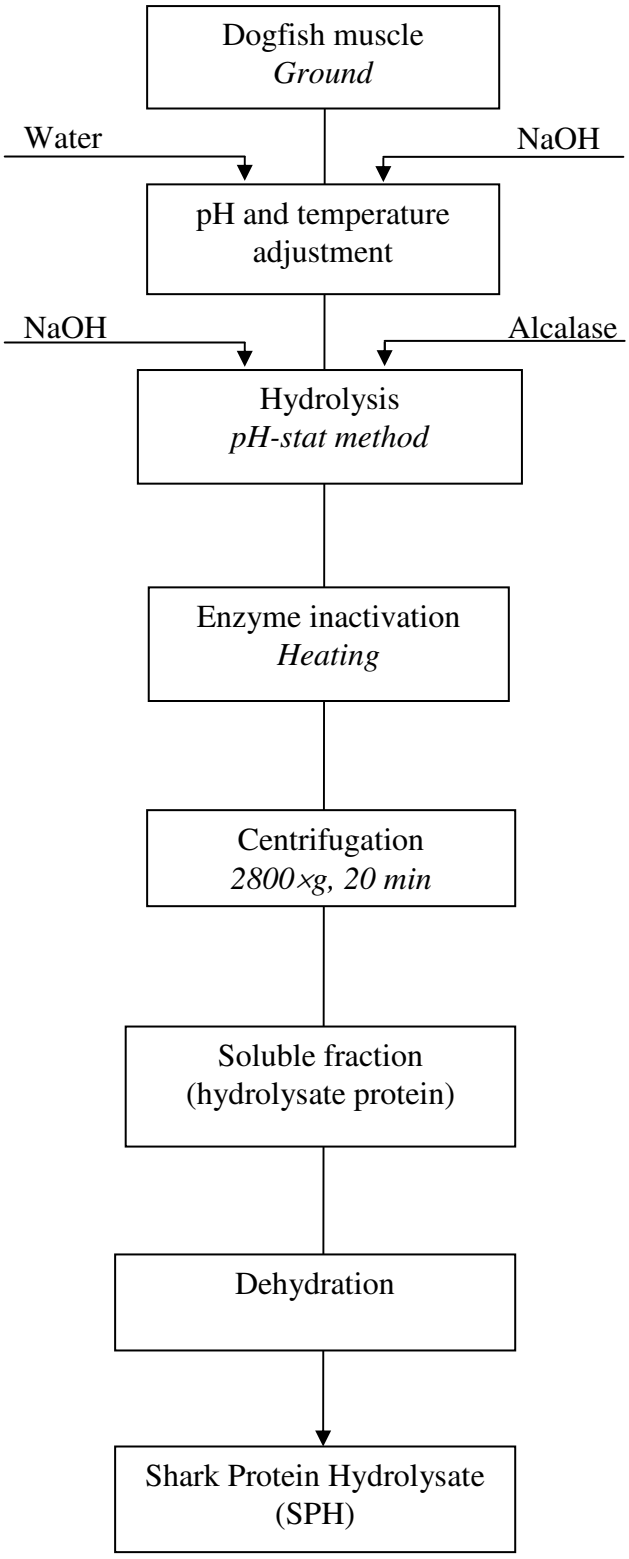


Figure 3 Schematic diagram of the process used in the production of protein hydrolysate from spiny dogfish (*Squalus acanthias*) shark.

Source: Diniz and Martin (1997)

### 5.2.3 Termination of enzymatic reaction

When a desired %DH is attained, it is necessary to terminate the enzymatic reaction. Otherwise the enzymes would remain active in the substrate and further hydrolyze the protein and peptides. Deactivation of enzymes is achieved either by chemical or thermal means (Kristinsson and Rasco, 2000a).

Chemical inactivation would either lower or raise the pH of the slurry to a point where the enzyme is inactivated. Some enzymes are more sensitive to pH changes rather than temperature changes. Alcalase is a relatively thermostable enzyme, but it is very sensitive to acid pH. Complete inactivation of Alcalase therefore is obtained by lowering the pH to 4.0 (Adler-Nissen, 1986; Shahidi, 1995). After protein hydrolysates from capelin using commercially available Alcalase, Neutrase and papain were produced, the hydrolytic reaction was stopped by lowering the pH to 3-4 in order to deactivate the enzyme (Shahidi *et al.*, 1995).

Thermal inactivation is the easy method for termination of enzyme. Usually the slurry of hydrolysate and enzymes are transferred to a heat bath, where the enzymes are deactivated by exposing them to temperatures ranging from 75 to 100$^{\circ}$C for 5 to 30 min, depending on the type of enzyme (Guadix *et al*., 2006). For example, papain is very heat tolerant, and has been reported to need at least 90$^{\circ}$C for 30 min to be fully inactivated (Hoyle and Merritt, 1994). Alcalase and Neutrase activities were stopped by heating at 90$^{\circ}$C for 5 min (Benjakul and Morrissey, 1997). However, terminating the reaction by thermal means is undesirable (Haque, 1993). Heat denaturation of protein leads to the exposure of hydrophobic residues and subsequently protein aggregation (Guadix *et al*., 2006).

### 5.2.4 Separation of protein hydrolysate

Hydrolysis of protein molecules brings about the peptides and free amino acids which are obtained in the soluble fraction (Mohr, 1980). Commonly, the slurry is desludged by centrifugation, which usually results in four fractions: oil layer on the top, emulsion, fish protein hydrolysate and sludge on the bottom of the centrifugation vessels (Šližyte *et al.,* 2005b). Lipid in the final hydrolysate is a major concern for fish protein hydrolysate. Lipid residues in product must be lower than 0.5% to prevent alteration of the lipid fraction during storage (Spinelli *et al.,* 1972). Other separation methods for fish protein hydrolysates have been reported such as suction filtration of the sludge (Onodenalore and Shahidi, 1996) and filtering the slurry through a 2-mm

mesh screen (Viera *et al.,* 1995). The insoluble fraction or the sludge precipitated during centrifugation may be used as animal feed.

### 5.2.5 Drying

In a commercial operation, the final soluble fraction is generally dried to convert the hydrolysate to a powder form, which can be incorporated into food formulations. Spray drying of the soluble fraction is one of the most energy consuming and expensive steps in the production of protein hydrolysates. Generally, protein hydrolysates are neutralized and freeze dried. By neutralization, the final product can have a fairly high salt content, which should be desalted before freeze-drying. Alternatively, ultrafiltration membranes have been introduced into the production of protein hydrolysates, mainly to control the molecular size of the product. Generally, the product has to be very pure and free of lipids (Kristinsson and Rasco, 2000a).

## 5.3 Functional properties of protein hydrolysate

The functionality of proteins can be changed by subjecting them to physical and chemical treatments such as pH, ionic strength, heat, mechanical shear, etc., as well as enzymatic treatments (Nielsen, 1997). The functionality of protein hydrolysate is tied to the nature and the composition of peptides generated during hydrolysis. The understanding of the relationship between the physicochemical characteristics and the functions of protein hydrolysates can lead to improvements in the quality and stability of hydrolysate-based nutritional formulas (Mahmoud, 1994). Enzymatic hydrolysis of fish proteins generates a mixture of free amino acids and varying size of peptides. The functional properties of fish protein hydrolysates are important, particularly if they are used as ingredients in food products. The main functional properties of fish protein hydrolysates include solubility, water holding, emulsifying, foaming and sensory properties (Kristinsson and Rasco, 2000a).

### 5.3.1 Solubility

Solubility is an important prerequisite for a protein to perform as a functional ingredient in foods (Nielsen, 1997). Many of the other functional properties, such as emulsification and foaming, are affected by solubility (Wilding *et al.,* 1984). The solubility of food proteins differs greatly depending on both the source of the protein and the processing treatments applied during isolation (Nielsen, 1997). Hydrophobic and ionic interactions are the major factors that influence the solubility of proteins. Nitrogen soluble index (NSI) has been used

to determine protein solubility, mainly caused by the dispersion of protein in the solvent (Cheftel *et al*., 1985). The high nitrogen solubility of protein hydrolysate indicates potential applications in formulated food systems by providing attractive appearance and smooth mouthfeel to the product (Peterson, 1981).

Generally, fish myofibrillar proteins have the lack of solubility in water over a wide range of pH (Venugopal and Shahidi, 1994). Enzymatic hydrolysis is very important in increasing the solubility of these proteins. Enzymatic breakdown of the protein involves a major structural change in that the protein is gradually cleaved into smaller peptide units. The enhanced solubility of the hydrolysates is due to the newly exposed ionizable amino and carboxyl groups of the amino acids that increase the hydrophilicity (Mahmoud, 1994). The solubility of protein hydrolysates increases with an increase in DH. This is mainly due to a reduction in the molecular weight and an increase in the number of polar groups (Nielsen, 1997). Salmon byproducts hydrolysates made with Alcalase at higher degrees of hydrolysis showed a decrease in high molecular weight fractions and the increased solubility (Gbgouri *et al*., 2004). Not only DH but solubility of protein is also a function of both pH and ionic strength. The solubility of salmon hydrolysate was high at pHs 6 to 7 and was low at pHs 3 to 4. The pH influences the charge on the weakly acidic and basic side-chain groups. Generally, proteins and hydrolysates display low solubility at their isoelectric point (Gbogouri *et al.,* 2004).

**5.3.2 Water holding capacity**

Water holding capacity refers to the ability of the protein to absorb water and retain it against gravitational force within a protein matrix, such as protein gels or beef and fish muscle (Damodaran, 1996). Fish protein hydrolysates are highly hygroscopic. The presence of polar groups such as COOH and $NH_2$ that increase during enzymatic hydrolysis has a substantial effect on the amount of adsorbed water and moisture sorption isotherm for these materials. Proper packaging and low relative humidity of air during processing has been taken into consideration (Kristinsson and Rasco, 2000a). The recommended maximum water content of fish protein hydrolysate during the storage is 0.075 g/g at RH less than 15% (Buinov *et al.,* 1977).

During storage and processing, the change of the water holding capacity of meat depend on the amount of the non-tightly bound water immobilized within the microstructure of tissue, and the amount of immobilized water depends, to a great extent on the spatial molecular

arrangement of the myofibrillar filament proteins such as myosin and actin (Pomeranz, 1991). Shrimp head protein hydrolysate presence of peptides and rich of hydrophilic amino acids such as Glx, Asx, Arg and Lys could stabilize water molecules in lizard fish myofibrils during dehydration (Ruttanapornvareesakul *et al*., 2005). The relationship between amounts of certain amino acids and water holding capacity of protein hydrolysate from cod by-products was observed: decreasing amounts of glycine/arginine, alanine, hydroxyproline and sum of hydrophobic amino acids increased the water holding capacity of the frozen comminuted fish muscle (Šližyte *et al.,* 2005b). Addition of 1.5% fish protein hydrolysate made from salmon reduced water loss after freezing to 1%, compared with 3% of the control. Addition of 3% hydrolysate resulted in an increase of approximately 4% in the cooking yield of comminuted meat (Shahidi *et al*., 1995).

### 5.3.3 Emulsifying properties

Protein are often used as surfactants in emulsion-type processed foods (Nielsen, 1997). Partial hydrolysis of proteins generally increases the number of polar groups and hydrophilicity, decreases the molecular weight, alters the globular structure of proteins and exposes previously buried hydrophobic regions. These changes will affect their emulsifying properties (Nielsen, 1997). Hydrolysates are also water-soluble and surface active and promote oil-in-water emulsions, due to their hydrophilic and hydrophobic functional groups (Wilding *et al.,* 1984). Emulsifying capacity and emulsifying stability are two parameters generally used to measure the ability of protein hydrolysates to form and stabilize emulsions (Sathivel *et al*., 2003; Gbogouri *et al*., 2004).

Several studies have shown that the emulsifying properties of protein hydrolysates are affected by the DH. Emulsifying capacity could be significantly increased by gentle hydrolysis to a DH of approximately 5% (Adler-Nissen and Olsen, 1979). This is in accordance with Kong *et al*. (2007) who reported that the higher emulsifying capacity value (0.64) obtained for the protein hydrolysate of DH 5%. The trend was similar to that reported in emulsifying properties of salmon muscle protein hydrolysates by Kristinsson and Rasco (2000b). DH of protein hydrolysates also has a significant effect on the stability of emulsions. The emulsion stability decreases with wider amplitude as a function of proteolysis (Quaglia and Orban, 1990). Better emulsion stabilities are obtained at low DH (Gbogouri *et al*., 2004). In the

emulsion, large peptides with greater hydrophobicity can adsorb to the oil surface and induce the formation of small oil droplets, which are of higher stability than large oil droplets induced by small peptides. Generally, the molecular weight of the hydrolysates has a major influence on the emulsifying properties. An optimum molecular size or chain length of peptides provides the good emulsifying properties (Adler-Nissen and Olsen, 1979). Generally, the pH of protein solutions during emulsification affects their emulsifying properties via charge effects. The emulsifying capacity of protein hydrolysates is usually low at isoelectric pH, which is relatively low for peptides. However, addition of salt improves the emulsifying properties at the isoelectric pH (Turgeon *et al*., 1992).

**5.3.4 Foaming properties**

The digestion of protein produces a range of peptides which possess altered hydrophobicity, charge balance and conformation compared to the native molecule. Their reduced molecular weight will make them more flexible, forming a stable interfacial layer and increasing the rate of diffusion to the interface, improving the foamability properties (Wilde and Clark, 1996).

Fish protein hydrolysate from herring with its reduction in molecular weight presented an improved foamability (Liceaga-Gesualdo and Li-Chan, 1999). The digestion of the protein produces a range of peptides which possess the altered hydrophobicity, charge balance and conformation, compared to the native molecule. Protein hydrolysate with reduced molecular weight is flexible in forming a stable interfacial layer and increasing the rate of diffusion to the interface, leading to the improved foamability properties. However, protein hydrolysate with smaller peptides had the poor foam stability (Liceaga-Gesualdo and Li-Chan, 1999). The advantage of using hydrolyzed proteins as foaming agents is their insensitivity to change in pH. The pH of the dispersing medium markedly affects foaming, particularly foam stability. Foaming properties were highest when the pH is close to the isoelectric point of the protein (Kinsella, 1981). Sanchez and Patino (2005) revealed that an increase in protein concentration resulted in a higher rate of diffusion. Thiansilakul *et al*. (2007b) reported that foam expansion at 0 min after whipping indicated the foam abilities of protein hydrolysates, which increased from 23.33% to 70% when the concentrations of round scad protein hydrolysate increased from 0.1% to 3%.

### 5.3.5 Fat absorption

The ability of fish protein hydrolysates to absorb and hold oil is another important functional property. It influences not only the taste of the product but is also an important functional characteristic, especially for the meat industry (Kristinsson and Rasco, 2000a). The mechanism of fat absorption is attributed mostly to physical entrapment of the oil. The higher bulk density of the protein, the higher fat absorption is obtained (Kinsella, 1976). Fat binding capacity of proteins also correlates with surface hydrophobicity (Kristinsson and Rasco, 2000a), DH (Kristinsson and Rasco, 2000b) and enzyme/substrate specificity (Haque, 1993).

Fat absorption of salmon by-products hydrolysates produced using Alcalase was studied by Gbogouri *et al*. (2004). The 11.5% DH fish hydrolysate showed the best ability to bind corn oil than hydrolysates of higher DH and sodium caseinate due to the larger peptide sizes. The nonhydrolyzed salmon protein has a fat absorption capacity significantly higher than its hydrolysates and sodium caseinate. Šližyte *et al*. (2005b) reported that cod hydrolysate powder obtained without adding commercial enzymes showed the highest fat absorption ability due to the presence of large peptides in the powders. In addition, hydrolysate powders containing higher amounts of lipids had higher fat absorption ability while a positive relationship between fat absorption and amount of phospholipids was observed in the hydrolysate samples.

### 5.3.6 Sensory properties

Although enzymatic hydrolysis of proteins develops desirable functional properties, it results in the formation of short chain peptides, thus causing the development of bitter taste in the product. The bitterness strongly restricts the practical uses of these hydrolysates as a food ingredient. The mechanism of bitterness is not very clear that the presence of bile in the raw material may also influence the development of bitterness in fish protein hydrolysates (Daukšas *et al.,* 2004). It is widely accepted that hydrophobic amino acids of peptides are a major factor. Peptides with a molecular weight ranging from 1,000 to 6,000 Da and with hydrophobic characteristics have shown most likely to be bitter (Gonzalez-Tello *et al*., 1994). Hydrolysis of protein results in exposing buried hydrophobic peptides, which are readily able to interact with the taste buds, resulting in detection of bitter taste. An extensive hydrolysis to free amino acids is able to decrease the bitterness of these peptides because hydrophobic peptides are far more bitter, compared with a mixture of free amino acids (Kristinsson and Rasco, 2000a). However, free

amino acids are undesirable from a functional standpoint. Strict control of hydrolysis and termination at low %DH values therefore is desirable to prevent the development of a bitter taste and to maintain the functional properties (Kristinsson and Rasco, 2000a).

Hoyle and Merritt (1994) reported that Alcalase-hydrolyzed herring with a higher DH was less bitter than papain-hydrolyzed herring. Lower fishy odor in ethanol-extracted herring hydrolysate was related to the low levels of lipid in the product. Umami, bitter and fishy were the dominant tastes of threadfin bream hydrolysates produced by Alcalase. The bitter amino acids, including methionine, valine, isoleucine, phenylalanine, leucine and tyrosine constituted 317 mg/g of the total amino acids. The umaminess could be due to the presence of high contents of glutamic acid and inosine 5-monophosphate (Normah *et al*., 2004).

## 6. Antioxidative activity of protein hydrolysates

Protein hydrolysates contains free amino acids and peptides, which have been found to exhibit antioxidative activity. Fish protein hydrolysates have also been recognized to act as natural antioxidants against lipid oxidation in food model system (Je *et al.,* 2005; Jun *et al.,* 2004; Sathivel *et al.,* 2003).

### 6.1 Mode of action of fish protein hydrolysates

#### 6.1.1 Radical scavenging activity and reducing power

Protein hydrolysates have been found to possess the radical scavenging activity. Chan *et al*. (1994) reported that carnosine-related dipeptides possessed the antioxidant abilities in the phosphatidylcholine liposome model, and that the formation of the $^{\bullet}OH$ free radicals could be detained by carnosine. Protein hydrolysates possibly contained substances which scavenge the DPPH radical by donation of hydrogen atom to form a stable DPPH-H molecule (Matthaus, 2002). In addition to quenching the DPPH radical, the purified peptide from hoki frame protein hydrolysate had a scavenging effect toward hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$) and superoxide radical ($O^{2-}$) (Kim *et al.,* 2006). Je *et al*. (2007) reported that antioxidative peptide from tuna backbone protein could inhibit lipid peroxidation in linoleic emulsion system because of an important hydrophobic property of the peptide sequence to scavenge on lipid derived radicals. Due to this property, antioxidative peptide from tuna backbone protein could interact with lipid molecules and could scavenge by donating protons to lipid derived radical. Round scad

protein hydrolysate showed DPPH radical-scavenging activity with the values of 59.9% (Thiansilakul *et al*., 2007b).

Reducing power of both mackerel hydrolysates prepared by autolysis and with Protease N increased gradually with the increasing hydrolysis time. The reducing power ability might be associated with carnosine and anserine (Wu *et al*., 2003). Moreover**,** the reducing power of protein hydrolysate was found to depend on the DH and enzyme used. Klompong *et al*. (2007) found that at high DH (25%), yellow stripe trevally protein hydrolysate hydrolyzed with Flavourzyme showed a higher reducing power than the other hydrolyzed with Alcalase. Thiansilakul *et al*. (2007a) found that reducing power of round scad protein hydrolysate prepared using Flavourzyme increased with increasing DH up to 60%, whereas those of round scad protein hydrolysate prepared using Alcalase increased when DH increased up to 40%.

**6.1.2 Metal chelation**

Interaction between lipid hydroperoxides and transition metal ions lead to excessive formation of free radicals that promote lipid oxidation (Decker, 1998). Moreover, in the presence of transition metal ions such as iron or copper, highly reactive hydroxyl radicals are generated through Fenton reactions (Mendis *et al*., 2005). Some molecules derived from protein hydrolysate act as chelators of metal ions and contribute as indirect antioxidants (Chen *et al*., 1998; Rajapakse *et al*., 2005). A higher degree of cleavage of peptide bonds rendered hydrolysates with higher metal-chelating activities. Klompong *et al*. (2007) reported that metal-chelating activity of both protein hydrolysates hydrolyzed with Alcalase and Flavourzyme increased with increasing DH and at the same DH, the hydrolysate derived from Flavourzyme showed a higher chelating activity than did other. According to Thiansilakul *et al.* (2007b), protein hydrolysate from round scad hydrolyzed with Flavourzyme had a pronounced capacity for iron binding. However, Mendis *et al*. (2005) found that peptides derived from jumbo squid skin gelatin hydrolysate did not exhibit any substantial metal ion chelation activity, compared to known ion chelators such as, EDTA and ascorbate. Generally, histidine-containing peptides have been reported to act as metal ion chelators (Chen *et al.,* 1998). In addition, it is thought that acidic and/or basic amino acids play an important role in the chelation of metal ions by carboxyl and amino groups in their side chain (Suetsuna *et al*., 2000). Therefore, it can be speculated that the

absence of metal ion chelation in squid gelatin peptide could be due to relatively low percentage of histidine residues (Mendis *et al*., 2005).

### 6.1.3 Synergistic effect with other antioxidants

The synergistic effects with the nonpeptidic antioxidant, α-tocopherol, were studied in peptide fractions of protein hydrolysate from yellowfin sole frame and Alaska pollack frame (Jun *et al.,* 2004; Je *et al.,* 2005). Most peptide fractions exhibited synergistic effect with α-tocopherol on antioxidative activity in linoleic acid autoxidation system. Jumbo squid skin gelatin peptides had strong synergistic effects with α-tocopherol at 1:1 ratio (Mendis *et al*., 2005). Kim *et al.* (2001) reported that peptides from gelatin hydrolysate of Alaska pollack skin hydrolyzed with Pronase E possessed high antioxidative activity and showed higher synergistic effect with α-tocopherol than those hydrolyzed with Alcalase and Collagenase. The synergistic effects with other antioxidants had been previously demonstrated with the hydrolysates of a vegetable protein, yeast protein and bovine serum albumin (Bishov and Henick, 1975; Hatate *et al.,* 1990). Chen *et al*. (1996) reported that the hydrolysates of soybean protein showed a strong synergistic effect with nonpeptidic antioxidants although some hydrolysates had very low antioxidative activity.

## 6.2 Amino acids and peptides with antioxidative activity

The levels and composition of free amino acids and peptides in hydrolysate are associated with antioxidant activities. Increasing levels of carnosine (β-alanylhistidine) and anserine (β-alanyl-1-methylhistidine) of the mackerel hydrolysates during hydrolysis time caused the increased antioxidative activity. These histidine-containing dipeptides may correlate with their abilities to inhibit the peroxidation (Wu *et al.,* 2003). However histidine has been shown to revert from being an antioxidant to a prooxidant at high concentrations (Amarowicz and Shahidi, 1997).

The amino acid residues at the N- termini of dipeptides have been demonstrated to be antioxidative in an oil system. It is probable that the amino acid residues play a role in increasing the interaction between peptides and fatty acids (Jun *et al*., 2004). Tryptophan and histidine showed high antioxidant activity whereas glycine and alanine showed only weak activity, and methionine and cysteine had an antioxidative effect in soybean oil (Riisom *et al.,* 1980). However, all amino acids exhibited antioxidant activity in some systems, which probably reflect the antioxidant nature the $NH_3R$ group (Taylor and Richardson, 1980). Antioxidant

peptides derived from different sources exhibit varying potencies to scavenge free radicals. Phenolic hydroxyl groups present in aromatic amino acid contribute substantially for scavenging of radicals acting as potent electron donors (Suetsuna *et al*., 2000). In addition, some other amino acid such as histidine, proline, alanine and leucine have been reported to contribute for scavenging of free radicals (Kim *et al*., 2001). The ability to quench the DPPH radical was found in protein hydrolysates derived from mackerel by autolysis and with Protease N (Wu *et al.,* 2003) and protein hydrolysates from tuna cooking juice derived by Protease (Jao and Ko, 2002).

Not only amino acid but molecular weight of peptide also related with their antioxidative activity (Jun *et al*., 2004). Separations of peptides from protein hydrolysates have been done for analyzing the relationships between molecular weight distribution and antioxidative activity (Amarowicz and Shahidi, 1997; Jun *et al*., 2004; Je *et al*., 2005). The chromatography has been used to separate a potent antioxidative peptide from protein hydrolysates such as using ion exchange, gel filtration and high performance liquid chromatography (Wu *et al*., 2003; Mendis *et al.*, 2005; Rajapakse *et al.*, 2005; Je *et al.*, 2007). Cod frame protein hydrolysates were prepared with crude proteinase extracted from tuna pyloric caeca and furthermore fractionated by ultrafiltration membranes into four sizes. The 5-10 kDa hydrolysate possessed the most effective antioxidative activity and showed approximately two fold higher activity than the original hydrolysate. The activity was also as high as that of α-tocopherol (Jeon *et al.,* 1999). The similar result was found in the report of Kim *et al*. (2006) who investigated the free radical scavenging activity of four fractions derived from hoki frame protein hydrolysates using ultrafiltration membrane. Fraction III with molecular weight distribution of 1-3 kDa exhibited the most potent DPPH radical scavenging effect in comparison with Fraction I (5-10 kDa), II (3-5 kDa) and IV (<1 kDa). Yellowfin sole frame protein hydrolyzed with mackerel intestine crude enzyme and pepsin were also fractionated into five major types. Fraction-I (10-30 kDa) exhibited the highest activity in the linoleic acid autoxidation system (Jun *et al.,* 2004). Peptide isolated from triptic hydrlysate of squid skin gelatin exhibited antioxidant properties (Mendis *et al*., 2005). Fermented marine blue mussel derived peptides were purified using ion exchange, gelfiltration and high performance liquid chromatographic techniques to identify a potent radical scavenging activity. The hepta-peptide was found to be highly effective for radical scavenging. In addition, the peptide exhibited a strong lipid peroxidation inhibition at 54 μM concentration and it was higher than α-

tocopherol (Rajapakse et al., 2005). The muscle of prawn was hydrolyzed by various proteases and antioxidant peptides from prawn muscle were also isolated. Three antioxidant peptides have been isolated from the active peptidic fraction by ion-exchange chromatography, gel filtration and ODS high-performance liquid chromatography (Suetsuna. 2000). The purified peptide from different protein sources had different amino acid sequence (Table 1).

Table 1 Amino acid sequence of antioxidative peptide from various protein sources

| Protein sources | Amino acid sequence | References |
|---|---|---|
| prawn | Ile-Lye-Lys, Phe-Lys-Lys, Phe-Ile-Lys | Suetsuna (2000) |
| Alaska pollack skin gelatin | Gly-Pro-Hyp | Kim *et al.* (2001) |
| yellowfin sole frame | Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr | Jun *et al*. (2004) |
| blue mussel | His-Phe-Gly-Asp-Pro-Pro-His | Rajapakse *et al.* (2005) |
| Alaska pollack frame | Leu-Pro-His-Ser-Gly-Tyr | Je *et al.* (2005) |
| squid skin gelatin | Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu, Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg | Mendis *et al*. (2005) |
| hoki skin gelatin | His-Gly-Pro-Leu-Gly-Pro-Leu | Mendis *et al.* (2005) |
| hoki frame | Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn | Kim *et al.* (2006) |
| tuna backbone | Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Glu-Glu-Leu-Ser | Je *et al.* (2007) |

Protein hydrolysate not only exhibited antioxidant acitivity but also exerted ACE (angiotensin I-converting enzyme) inhibition effects (Ono *et al*., 2003). Recently, many ACE inhibitory peptides have been isolated from various food proteins such as tuna muscle, sardine and bovine blood plasma (Hyun and Shin, 2000; Kohama *et al*., 1998; Ukeda *et al*., 1992). The peptides fractionated from Alaska pollack frame protein hydrolysate exhibited Angiotensin I converting enzyme inhibitory activity with $IC_{50}$ value of 14.7 μM, and the sequence of the peptide

was Phe-gly-Ala-Ser-Thr-Arg-Gly-Ala (Je *et al*., 2004). Jung *et al*. (2006) was purified ACE inhibitory peptide from yellowfin sole frame protein hydrolysate using consecutive chromatographic techniques. The ACE inhibitory peptide had molecular mass of 1.3 kDa consisting of 11 amino acids, Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-lu-Leu and $IC_{50}$ value was 28.7 µg/ml. He *et al*. (2006b) found that oligopeptide from shrimp protein hydrolysate exhibited Angiotensin I converting enzyme inhibitory activity with $IC_{50}$ value of 0.22 mg/ml.

## Objectives

1. To study the composition and properties of Mungoong from the cephalothorax of white shrimp.
2. To investigate the antioxidative activity of soluble fraction from Mungoong in various model systems and to monitor antioxidative activity of Mungoong during storage.
3. To study the effect of Flavourzyme as the processing aid on the yield, compositions and antioxidative activity of Mungoong.