CHAPTER 1

INTRODUCTION

Lipid oxidation is of great concern to the food industry and consumers since it contributes to the development of poorer flavor, color and texture, reduces nutritive value and produces potentially toxic reaction products. Therefore, antioxidants are increasingly used to improve the stability of foods. Due to the anxiety of possible toxicity of synthetic antioxidants, natured antioxidants have been paid increasing attention among the consumers. Protein hydrolysates from many plant and animal sources have been reported to possess antioxidative activity. Whey and soy protein hydrolysates acted as natural antioxidants in cooked meat (Decker and Crum, 1993; McCarthy et al., 2001). Egg-yolk protein hydrolysates showed the strong antioxidant activity in a linoleic acid oxidation system (Sakanaka et al., 2004). Protein hydrolysates from herring byproduct, capelin, yellowfin sole frame and Alaska pollack frame also possessed antioxidative activity (Sathivel et al., 2003; Amarowicz and Shahidi, 1997; Jun et al., 2004; Je et al., 2005).

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). Histidine-containing peptides expressed their metal chelating ability as well as lipid-radical trapping potential of the imidazole ring (Uchida and Kawakishi, 1992; Murase et al., 1993; Wu et al., 2003). Generally, greater levels of free amino acids, anserine, carnosine and other peptides were obtained in the hydrolysates produced using proteases, compared with those prepared by autolysis (Wu et al., 2003). Jun et al. (2004) reported that the 13,000 Da peptide purified from yellowfin sole frame protein hydrolysate showed the pronounced antioxidant activity, while the 672 Da peptide purified from Alaska pollack frame protein hydrolysate had the remarkable activity (Je et al., 2005). The levels, sequences and compositions of amino acids and peptides may influence the antioxidant activities of protein hydrolysates (Chen et al., 1995).

Large amounts of protein-rich byproducts from the seafood industry, especially dark fleshed fish, have the limited uses due to their dark color, susceptibility to oxidation and off-flavor. As a sequence, they are discarded or processed into low market value-products such as

fish meal, fertilizer, etc. Round scads are the abundant dark fleshed fish in Thailand with an estimated catch quantity of 104,000 metric tons per year (Department of fisheries, 2002). Recently there have been relatively many studies on fish protein hydrolysates based on whole fish, fish fillet or muscle. Production of round scad protein hydrolysates with antioxidant activity can pave a way to fully use these species as the value-added product and used as a novel antioxidant to prevent the lipid oxidation in the food systems. Therefore, natural antioxidative peptides with the negligible toxicity can be the alternative additive for consumer and industries.

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. The mechanism of autoxidation can be distinguished in three distinct steps: initiation, propagation and termination (Jadhav *et al*., 1996).

1.1 Initiation

The autoxidation of fat is thought to be 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).

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

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

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

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.

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

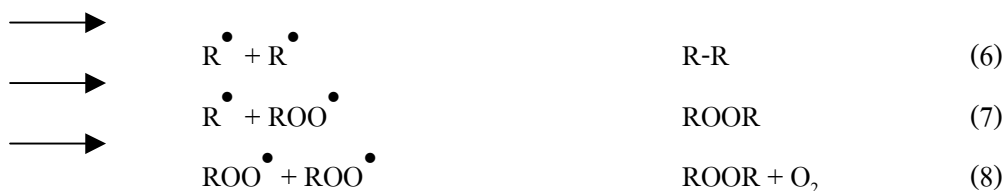$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet} \qquad (5)$$

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. 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. 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).

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

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

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

1.4 Factors influencing lipid oxidation

Many factors have been known to be associated with lipid oxidation.

1. Fatty acid: In general, free fatty acid is oxidized faster than their glyceryl esters. Relatively large amounts of free fatty acid in commercial oils can increase the rate of oxidation, especially when the pick up of trace metals from equipment or storage tanks is enhanced (Nawar, 1996; 1998). The susceptibility and rate of oxidation of fatty acids in lipids depend on the degree of their unsaturation. The autoxidation of major fatty acids of meat follows the order C18:0 < C18:1 < C18:2 < C18:3 (Shahidi, 1994a). Since seafoods contain highly unsaturated fatty acids, the oxidation of muscle from fish is higher than poultry, pork and lamb, respectively (Hsieh and Kinsella, 1989; Tichivangana and Morrissey, 1985).

2. Oxygen concentration: At very low oxygen pressure, the rate of oxidation is approximately proportional to oxygen pressure. If the supply of oxygen is unlimited, the rate of oxidation is independent of oxygen pressure. The availability of oxygen therefore plays a critical role in determining competitive oxidative pathways (Nawar, 1996; 1998). 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.

3. Temperature: Rate of reaction increases with increasing temperature. However, as the temperature increases, the increase in rate with increasing oxygen concentration becomes less evident, because oxygen becomes less soluble (Nawar, 1996; 1998). 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. Jakobsan and Bertelsan (2000) also found that the lipid oxidation of beef stored at 2°C was lower than that of beef kept at 8°C.

4. Water content: In dried food with very low moisture content (a_w <0.1), oxidation proceeds very rapidly. Increase in a_w to about 0.3 retards lipid oxidation by reducing metal catalysis, quenching free radicals, promoting nonenzymatic browning, and/or impeding oxygen accessibility. At higher a_w (0.55-0.85), the rate of oxidation increases again, presumably due to increased mobilization of the catalysts (Nawar, 1996; 1998).

5. Surface area: The rate of oxidation increases in direct proportion to the surface area of the lipid exposed to air. Furthermore, as surface-volume ratio is increased, a given reduction in oxygen partial pressure becomes less effective in decreasing the rate of oxidation. The rate of oxidation in oil-in-water emulsions is governed by the rate at which oxygen diffuses into the oil phase (Nawar, 1996; 1998)

6. Emulsification: In oil-in-water emulsions, or in foods where oil droplets are dispersed into an aqueous matrix, oxygen must gain access to the lipid by diffusion into the aqueous phase and passage through the oil-water interface. The rate of oxidation will depend on the interplay between a number of factors including type and concentration of emulsifier (Fomuso *et al*., 2002; Osborn and Akoh, 2004), size of oil droplets, surface area of interface, viscosity of the aqueous phase, composition, porosity of the aqueous matrix and pH (Nawar, 1996; 1998) and droplet charge of emulsifier (Mei *et al*., 1998; Hu *et al*., 2004).

7. Pro-oxidants: Enzymes such as lipoxygenase, peroxidase and microsomal enzymes can catalytically promote lipid oxidation (Slabyi and Hultin, 1982; Hsieh and Kinsella, 1989; Josephson *et al*., 1987). Transition metal ions, particularly copper and iron, have been known as the major catalysts for oxidation. At very low concentration, < 0.1 ppm, they can decrease the induction period and increase the rate of oxidation. Tichivangana and Morrissey (1985) reported that the ferrous ion at 1-10 ppm levels acts as a strong prooxidant in cooked fish muscles. Castell *et al*. (1965) found that the relative prooxidant activity of ions in fish muscle decreased in the order of $Cu^{2+} > Fe^{2+} > Co^{2+} > Cd^{2+} > Li > Ni^{2+} > Mg^{2+} > Zn^{2+} > Ca^{2+} > Ba^{2+}$. Such metal ions either in free or bound forms occur maturely in plant and animal tissues, membranes and enzymes. They are also introduced into food by contact with metallic equipment used in processing or storage. St. Angelo (1996) reported that iron bound to protein such as myoglobin, heamoglobin and ferritin may be released during postharvest storage and cooking, resulting in the initiation of lipid oxidation.

Transition metal ions in their lower valence state (M^{n+}) react very quickly with hydroperoxide. They act as one-electron donors to form an alkoxy radical and this can be considered as the branching of the propagation step as in Eq. 9. In a slow consecutive reaction, the reduced state of the metal ion may be regenerated by hydroperoxide molecule as in Eq. 10. Furthermore metals can abstract a hydrogen atom from the fatty acid themselves according to Eq. 11 (Gordon, 2001).

$$ROOH + M^{n+} \longrightarrow RO^{\bullet} + OH + M^{(n+1)+} \qquad (9)$$

$$ROOH + M^{(n+1)+} \longrightarrow ROO^{\bullet} + OH^{+} + M^{n+} \qquad (10)$$

$$RH + M^{(n+1)+} \longrightarrow R^{\bullet} + H^{+} + M^{n+} \qquad (11)$$

8. Salts: Organic or inorganic salts, commonly present in foods as natural components, ingredients or additive, can affect the hydrophobic/hydrophilic interactions among food components. Ionic species forming salts could affect the kinetics of chemical reactions occurring in foods. Calligaris and Nicoli (2005) found that potassium carbonate and potassium acetate present a strong antioxidant capacity, whereas no effect was detected for NaCl and KCl on lipid oxidation.

2. Antioxidants

Antioxidant in food is defined as any substance which is capable of delaying, retarding or preventing the development of rancidity or other flavor deterioration due to oxidation (Gordon, 2001). In general, antioxidants function by reducing the rate of initiation reaction in the free-radical chain reactions and are functional at very low concentrations, 0.01% or less (Rajalakshmi and Narasimhan, 1996).

The use of antioxidants in food products is controlled by laws and regulations of the country or by international standards. Even though many natural and synthetic compounds have antioxidant properties, only a few of them have been accepted as "generally recognized as safe" (GRAS) substances for use in food products by international bodies such as the Joint FAO/WHO Expert Committee for Food Additives (JECFA) and the European Community's Scientific Committee for Food (SCF).

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. Many of the naturally occurring phenolic compounds like flavonoids, eugenol, vanilin and rosemary antioxidant also have chain-breaking properties (Rajalakshmi and Narasimhan, 1996). Protein hydrolysate from many plant and animal sources also possess the primary antioxidative activity (Decker and Crum, 1993; McCarthy *et al.,* 2001; Sathivel *et al.,* 2003; Sakanaka *et al.,* 2004). Primary antioxidants are effective at very low concentrations but at higher levels they may 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. 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).
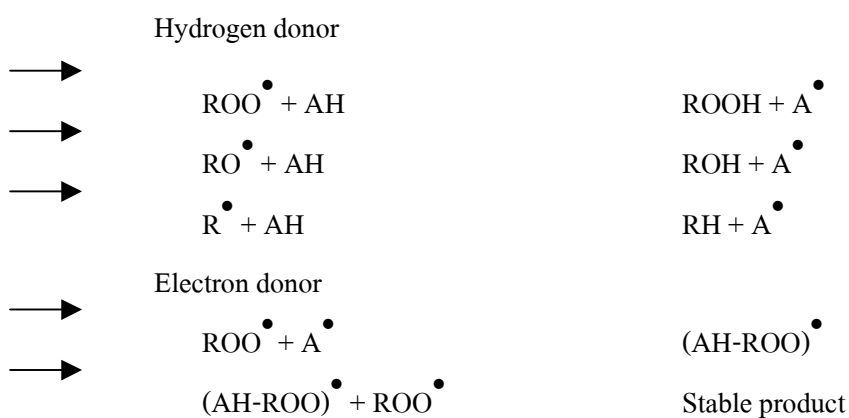
Flavonoids and related compounds and amino acids function as both primary antioxidants and synergists. Nitrites and nitrates, which are used mainly in meat curing, probably function as antioxidants by converting heme proteins to inactive nitric oxide forms and by

chelating the metal ions, especially nonheam iron, copper and cobalt that are present in meat. 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

Antioxidants can retard 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; Akoh and Min, 1998). Antioxidants can scavenge free radical either as hydrogen donors or as electron donors 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.

$$ROO^\bullet + A^\bullet \longrightarrow ROOA$$
$$RO^\bullet + A^\bullet \longrightarrow ROA$$
$$A^\bullet + A^\bullet \longrightarrow AA$$
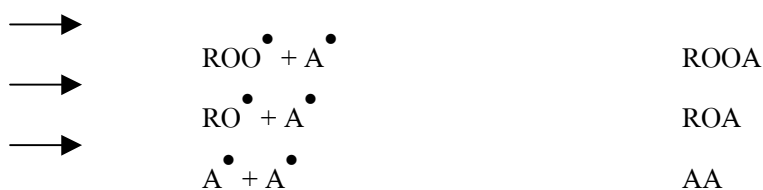
Antioxidants may act as hydrogen donors to the phenoxy radicals, which are stabilized by resonance delocalization of the unpaired electron in the aromatic ring and are further stabilized by bulky group at the ortho position as shown in Figure 1 (Shahidi and Wanasundara, 1992).
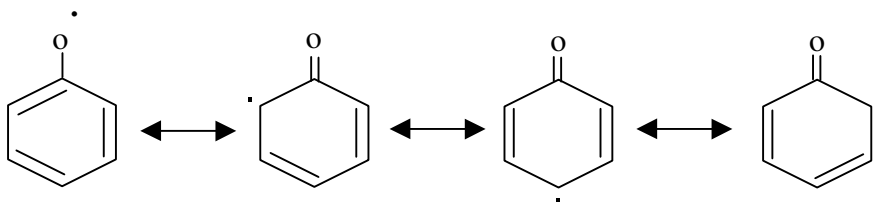

Figure 1   Delocalization of the unpaired electron in the aromatic ring of phenoxy radicals.

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).

2.2.3 Singlet oxygen quenchers

Singlet oxygen is generated from the triplet state oxygen. The mechanism of converting triplet oxygen to singlet oxygen is initiated by the transfer of the photosensitizer to its electronically excited state due to the absorption of light in the visible or near-UV region. Subsequently, the photosensitizer is able to transfer its excess energy to an oxygen molecule, giving rise to singlet oxygen (Shahidi and Wanasundara, 1992). Thus, the singlet oxygen can react with a lipid molecule to yield a hydroperoxide. Singlet oxygen reacts about 1,000-10,000 times as fast as normal oxygen with methyl linoleates (Jadhav *et al*., 1996). Lipid oxidation initiated by xanthine oxidase can be inhibited by β-carotene because of its ability to quence singlet oxygen (Rajalakshmi and Narasimhan, 1996; Namiki, 1990). The Maillard reaction derived from xylose-lysine, tryptophan-glucose and glucose-glycine model systems had a high scavenging effect on active oxygen (Tanaka *et al*., 1992; Yen and Hsieh, 1995; Yoshimura *et al*., 1997).

2.2.4 Lipoxygenase inhibitor

Lipoxygenase is a non-heme iron-containing enzyme that catalyzes the oxygenation of the 1,4-pentadiene sequence of polyunsaturated fatty acid to produce their corresponding hydroperoxide (Salas *et al.*, 1999). Free-radical intermediates occur during lipoxygenase catalysis, and these can lead to cooxidation of easily oxidized compounds, e.g. carotenoids and polyphenols (Rajalakshmi and Narasimhan, 1996).

2.2.5 Synergists

A. Chelating agents

Chelating agents are not antioxidants, however, they 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). Chelating agents form stable complexes with prooxidant metals such as iron and copper. Chelating agents bind metal ions and forms sigma bonds with a metal. It is considered as an effective secondary antioxidant because of the stabilized oxidation form of the metal ion. An unshared pair electron in their molecule structure promotes the chelating action (Dziezak, 1986; Rajalakshmi and Narasimhan, 1996; Jadhav *et al.*, 1996).

B. 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 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).

3. Fish protein hydrolysates

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).

3.1 The production of protein hydrolysates

Chemical and biological methods are the most widely used for protein hydrolysis. Chemical hydrolysis has been used in industrial practices. Biological processes using added enzymes are employed more frequently and enzyme hydrolysis holds the most promise for the future because it renders the products of high functionality and nutritive value.

3.1.1 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 $118^{\circ}C$ (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. Another drawback of acid hydrolysis is the destruction of tryptophan, which is an essential amino acid (Kristinsson and Rasco, 2000a). 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 results in less degradation of tryptophan. 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 (Kinsella, 1976). These may lead to the formation of toxic substances (Lahl and Braun, 1994; Linder *et al.*, 1995)

3.1.2 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. They are characterized further by their hydrolyzing mechanism into endopeptidases which cleave the peptide bonds within protein molecules or exopeptidases which hydrolyze the terminal peptide bonds (Adler-Nissen, 1986). 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).

3.2 Enzymatic hydrolysis process of fish muscle 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 2.

3.2.1 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
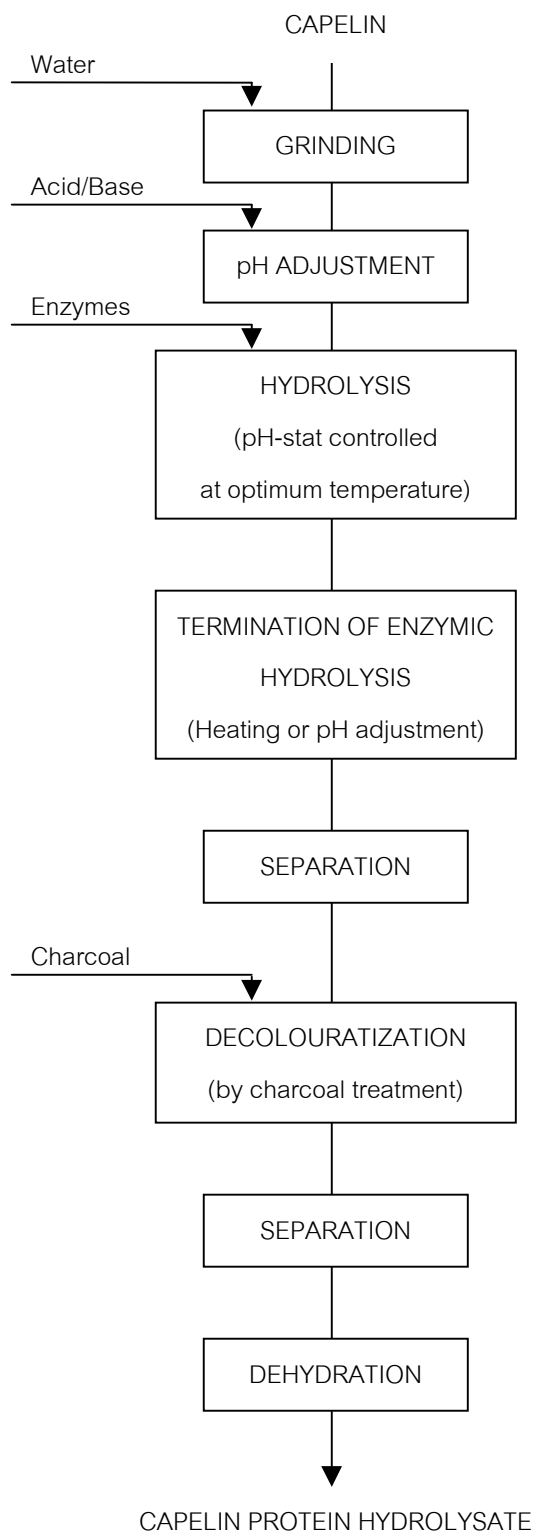
CAPELIN

Water → GRINDING

Acid/Base → pH ADJUSTMENT

Enzymes → HYDROLYSIS (pH-stat controlled at optimum temperature)

TERMINATION OF ENZYMIC HYDROLYSIS (Heating or pH adjustment)

SEPARATION

Charcoal → DECOLOURATIZATION (by charcoal treatment)

SEPARATION

DEHYDRATION

CAPELIN PROTEIN HYDROLYSATE

Figure 2   Flowchart for the production of capelin protein hydrolysate.

Source: Shahidi *et al*. (1995)

buffer is added to the minced fish (Baek and Cadwallader, 1995). However, the presence of buffer salts may affect the final properties of the hydrolysates. 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 excess fat (Ritchie and Mackie, 1982; Mackie, 1982). A fish protein hydrolysate with high lipid content turned to be darken from lipid oxidation (Hoyle and Merritt, 1994). Šližyte *et al*. (2005a) reported that the highest amount of dry cod-byproducts hydrolysate was obtained from the raw material containing the lowest amount of lipid. As established by FAO, a fish protein hydrolysate suitable for human consumption contains a lipid content not exceeding 0.5% by weight (FAO, 1971). Hoyle and Merritt (1994) used an ethanol extraction to remove fat from minced herring at the fish/ethanol ratio of 1:2 at 70°C for 30 min. Lipid content in final product was reduced to 0.9 from 4.0% of raw herring. Before placing the treated substrate in the reaction vessel, chemical reagent such as NaCl, sorbic acid or ethanol are occasionally added to the minced fish to minimize bacterial degradation (Sikorski and Naczk, 1981).

3.2.2 The enzymatic hydrolysis

A wide variety of commercial enzymes has been used successfully to hydrolyze fish and other food proteins (Table 1). 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 (Gildberg *et al.,* 1989; Rebeca *et al.,* 1991; Guerard *et al.,* 2001; Lalasidis *et al.,* 1978; Liaset *et al.,* 2002). 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).

Table 1   Type of enzymes and substrates used to prepare fish protein hydrolysates

Enzymes	Substrates	References
Alcalase, Neutrase, papain	Sardine (*Sardina pilchrdus*)	Qualia and Orban (1987)
Alcalase, papain	Herring (*Clupea harengus*)	Hoyle Merritt (1994)
Alcalase, Neutrase, papain	Capelin (*Mallotus villosus*)	Shahidi *et al*. (1995)
Alcalase, Neutrase	Pacific whiting (*Merluccius productus*)	Benjakul and Morrissey (1997)
Alcalase, Flavourzyme, Corolase PN-L, Corolase 7089, pyloric caeca extract	Atlantic salmon (*Salmo salar*)	Kristinsson and Rasco (2000c)
Alcalase	Yellowfin tuna (*Thunnus albacares*)	Guerard *et al*. (2001)
protease	Tuna cooking juice	Jao and Ko (2002)
protease N	Mackerel (*Scomber austriasicus*)	Wu *et al*. (2003)
Alcalase	Atlantic salmon head (*Salmo salar*)	Gbogouri *et al*. (2004)
Alcalase, α-chymotrypsin, mackerel intestine crude enzyme, Neutrase, papain, pepsin, pronase E, trypsin	Yellowfin sole (*Limanda aspera*)	Jun *et al*. (2004)
mackerel intestine crude enzyme	Alaska pollack (*Theragra chalcogramma*)	Je *et al*. (2005)
Flavourzyme, Neutrase	Cod (*Gadus morhua*)	Šližyte *et al*. (2005a)

Alcalase (Subtilisin carlsberg : EC 3.4.21.14), an alkaline enzyme produced from *Bacillus licheniformis*, has been proven repeatedly by many researchers to be one of the best enzymes used to prepare functional fish protein hydrolysates and other protein hydrolysates (Qualia and Orban, 1987; Hoyle and Merritt, 1994; Shahidi *et al.*, 1995; Benjakul and Morrissey, 1997; Kristinsson and Rasco, 2000c). Alcalase is endopeptidase which is able to hydrolyze proteins with broad specificity for peptide bonds and prefers a large uncharged residue. The optimal working conditions for Alcalase are reported to be at pH 6.5-8.5 and at a temperature of 55-70°C. Shahidi *et al*. (1995) successfully used Alcalase to optimize processing conditions to produce capelin protein hydrolysates. Alcalase treated hydrolysates exhibited superior protein recovery (70.6%) compared with Neutrase and papain. Hoyle and Merritt (1994) reported that Alcalase hydrolyzed herring meat to a higher degree and produced less bitter hydrolysates than papain. Furthermore, Benjakul and Morrissey (1997) reported that Alcalase had a higher proteolytic activity to produce protein hydrolysates from Pacific whiting solid wastes than Neutrase and optimum conditions for hydrolysate production using Alcalase were pH 9.5, 60°C, 1 h reaction time, waste : buffer ratio of 1 : 1 (w/v) and 20 AU/kg waste.

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 (Nilsang *et al.,* 2004).

The enzymatic hydrolysis of fish muscle proteins is characterized by an initial rapid phase, during 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) (Figure 3). 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).
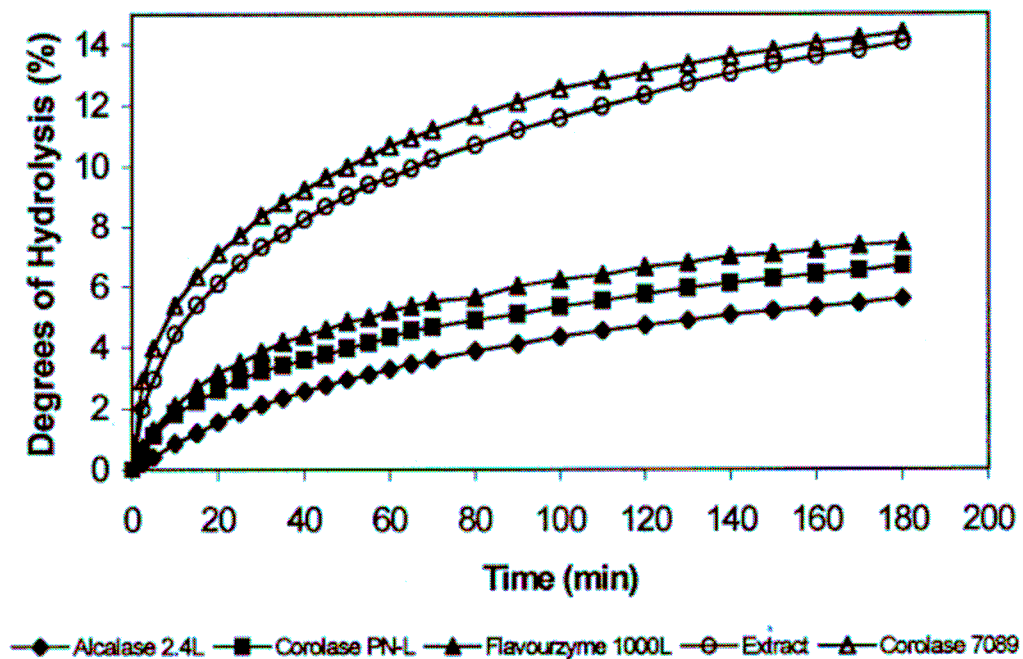

Figure 3   Enzymatic hydrolysis of salmon muscle mince with different alkaline proteases (pH 7.5, 40°C, 180 min and 7.5% substrate concentration).

Source: Kristinsson and Rasco (2000b)

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 demonstrates the four processing variables including substrate, enzyme-substrate ratio, temperature and time (Kristinsson and Rasco, 2000a). These are essential because 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).

3.2.3 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.

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°C for 5 to 30 min, depending on the type of enzyme. For example, papain is very heat tolerant, and has been reported to need at least 90°C for 30 min to be fully inactivated (Hoyle and Merritt, 1994). Alcalase and Neutrase activities were stopped by heating at 90°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.

3.2.4 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). Because lipid in the final hydrolysate is a major concern for fish protein hydrolysate, it is important to remove it. 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 (Onodenalre and Shahidi,

1996) and filtering the slurry by passing it 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. 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).

3.3 Compositions and functional properties of fish protein hydrolysates

Enzymatic hydrolysis of fish proteins is one of the methods for effective protein recovery from fishery industry and can be applied to improve and upgrade the functional and nutritional properties of proteins. The substrates and proteases employed and the degree to which the protein is hydrolyzed affect the compositions and properties of the resulting hydrolysates (Mullally *et al.,* 1995).

3.3.1 Chemical composition and nutritive value

Normally, fish muscle contains the nutritive and easily digestible proteins with an excellent amino acid composition (Yáñez *et al.,* 1976). Fish protein hydrolysates consist of high protein content ranging from 62 to 90 % (Liceaga-Gesualdo and Li-Chan, 1999; Šližyte *et al.,* 2005b), which depends on the substrates and the preparation. The proximate composition of spray-dried fish protein hydrolysates from herring as influenced by method of fat extraction before hydrolysis and type of enzyme used is shown in Table 2. Fat content was, however, significantly different among samples. The lowest value was found in ethanol-extracted herring hydrolysate. Lipid residues retained in dried fish protein hydrolysates after hydrolysis must be lower than 0.5% to reduce development of rancidity during storage (Spinelli *et al*., 1972). Ash contents of hydrolysate from herring were higher when Alcalase was used, compared with those found in hydrolysate prepared using papain (Hoyle and Merritt, 1994). Quaglia and Orban (1987) also reported a similar finding, in which the greater amount of alkali was required to maintain and control the pH conditions during hydrolysis when Alcalase was used in comparison with papain.

Šližyte *et al*. (2005b) reported that the composition of freeze-dried protein hydrolysates from cod by-products contained 3.9-6.5% moisture, 75.0-91.6% protein, 1.4-11.5% lipid and 9.7-13.9% ash. The dried hydrolysate had a low water activity (0.07-0.21), which is low enough to prevent microbial growth in the powders (Fennema, 1996). Raw material containing the highest amount of lipids gave highest amount of oil fractions and the lowest percentage of solubilized proteins. In addition, the heat denatured proteins seem to be highly resistant to enzymatic breakdown and the presence of lipids in sludge fraction was noticeable (Šližyte *et al*., 2005a).

Table 2   Proximate composition, nitrogen solubility index and yields of protein hydrolysates from raw herring, herring presscake and ethanol-extracted herring

(%)	Raw herring	Herring presscake	Ethanol-extracted herring	Raw herring	Herring presscake	Ethanol-extracted herring
		Alcalase			Papain	
H_2O	4.7[a]	3.9[b]	3.3[c]	4.8[a]	3.2[c]	3.9[b]
Protein	87.9[a]	82.3[b]	83.7[b]	85.3[b]	83.4[b]	85.7[b]
Fat	4.0[a]	3.7[a]	1.8[b]	4.7[c]	3.6[a]	0.9[d]
Ash	12.5[a]	13.3[b]	12.0[a]	9.6[c]	9.9[c]	7.5[d]
NSI	89.7[a]	92.9[a]	96.9[b]	93.1[a]	95.2[ab]	98.7[b]
Yield	5.5[a]	4.8[a]	3.6[b]	4.8[a]	3.4[b]	2.9[b]

Source: Hoyle and Merritt (1994)

The nutritive value of a protein depends primarily on its capacity to satisfy the needs for nitrogen and the essential amino acids. Fish protein hydrolysates have similar amino profiles to that of original material except for the sensitive amino acids such as methionine and tryptophan that are affected to a relatively large extent during enzymatic hydrolysis (Table 3) (Shahidi *et al.,* 1995). Extensively hydrolyzed proteins also have reduced immunological reactivities and can be used in formulas for hyperallergic infants (Mahmoud, 1994). Furthermore, peptides, which are easily absorbed, may be an optimal nitrogen source in sports nutrition. Peptides with a high biological value are attractive as a general protein supplement to a wide variety of diets.

Table 3　Amino acid composition of capelin proteins and capelin protein hydrolysate

Amino acids	Composition (%)	
	Capelin protein	Protein hydrolysate
Alanine	5.57±0.04	6.00±0.01
Arginine	5.99±0.10	5.70±0.02
Aspartic acid + asparagine	8.88±0.15	9.89±0.53
Cysteine	1.33±0.01	1.34±0.00
Glutamic acid + glutamine	13.2±0.03	13.4±0.03
Glycine	5.32±0.04	5.14±0.01
Histidine	2.43±0.00	2.09±0.02
Hydroxylysine	0.09±0.00	0.17±0.02
Hydroxyproline	0.42±0.02	0.46±0.07
Isoleucine	4.72±0.08	4.25±0.04
Leucine	8.15±0.05	7.60±0.00
Lysine	8.47±0.09	8.49±0.06
Methionine	3.09±0.02	2.05±0.01
Phenylalanine	3.80±0.01	3.19±0.00
Proline	3.70±0.15	3.67±0.03
Serine	4.18±0.05	4.24±0.10
Taurine	0.73±0.00	1.66±0.01
Threonine	4.82±0.05	4.56±0.03
Tryptophan	1.07±0.01	0.43±0.01
Tyrosine	3.34±0.01	2.47±0.06
Valine	5.71±0.12	5.77±0.01

Source:　Shahidi *et al*. (1995)

3.3.2 Functional properties

Enzymatic hydrolysis of fish proteins generates a mixture of free amino acids and varying size of peptides. This process increases the number of polar groups with the coincidental increase in solubility of the hydrolysate. Therefore, functional characteristics of the proteins can be modified. Hoyle and Merritt (1994) found that protein hydrolysate from herring prepared with the different enzymes included a high-molecular-weight fraction (>30,000 Da), two intermediate fractions (400-1000 Da) and a low-molecular-weight fraction (<200 Da), indicating the presence of high molecular weight protein fractions, short peptides and free amino acids, respectively. Pour-EI (1981) defined protein functionality as "any property of food or food ingredients except its nutritional ones that affects its utilization". 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).

A. Solubility

Solubility is probably the most important of protein and protein hydrolysate functional properties. Many of the other functional properties, such as emulsification and foaming, are affected by solubility (Wilding *et al.,* 1984). Hydrophobic and ionic interactions are the major factors that influence the solubility of proteins. Solubility of protein and protein hydrolysates is generally measured by employing the nitrogen solubility index (NSI) (Morr, 1985; Hoyle and Merritt, 1994; Gbogouri *et al*., 2004).

Intact fish myofibrillar proteins have the lack of solubility in water over a wide range of pH (Venugopal and Shahidi, 1994a; Spinelli *et al.,* 1972). 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; Gauthier *et al.,* 1993). Sardine hydrolysates made with Alcalase at higher degrees of hydrolysis showed a decrease in high molecular weight fractions and the increased solubility (Quaglia and Orban, 1987). Although a high DH generally leads to high solubility, the negative effects on the rest of the functional properties may be obtained (Gbogouri *et al*., 2004). To maintain or improve

functionality, appropriate degrees of hydrolysis are required (Mullally *et al.,* 1994). Hoyle and Merritt (1994) found that enzymatically hydrolyzed herring hydrolysate with prior ethanol extraction had the highest solubility, compared with nonextracted enzymatically hydrolyzed hydrolysate. Solubility or protein-water interaction is affected by presence of other components in solution. This finding suggested that the lower lipid content of ethanol-extracted hydrolysates resulted in less competitive water binding of proteins, compared with hydrolysates with higher lipid contents. 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. Thus proteins and hydrolysates display low solubility at their isoelectric point (Gbogouri *et al.,* 2004).

B. Water holding capacity

The functional properties of proteins in a food system depend in part on the water-protein interaction. 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).

Kristinsson and Rasco (2000b) pointed out that fish protein hydrolysates have good water holding capacity and are useful for certain food formulations. Addition of 1.5% of 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). At even lower addition levels, there was a large reduction in the amount of drip loss, indicating that capelin protein hydrolysate has strong water binding capacity. 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).

C. Emulsifying properties

Proteins are often used as surfactants in emulsion-type processed foods (Nielsen, 1997). Proteins have interfacial properties, which are important for their application as emulsifiers in sausages or as protein concentrates in dressings. 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). Proteins adsorb to the surface of the freshly formed oil droplets during homogenization and form a protective membrane that prevents droplets from coalescence (Demetriades *et al.,* 1997). 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; Šližyte *et al*., 2005b).

The emulsifying properties of hydrolyzed proteins can also be improved by controlling the extent of hydrolysis. There is a relationship between %DH and emulsifying properties of fish protein hydrolysates. According to Adler-Nissen and Olsen, emulsifying capacity could be significantly increased by gentle hydrolysis to a DH of approximately 5% (Adler-Nissen and Olsen, 1979). Mahmoud (1994) showed that the emulsifying activity of the hydrolysates decreased linearly with increasing DH (25-67%). Quaglia and Orban (1990) and Kristinsson and Rasco (2000b) also reported that the emulsifying capacity of fish waste protein decreased with increasing DH. Extensive hydrolysis generally results in a drastic loss of emulsifying properties. Although small peptides diffuse rapidly and absorb at the interface, they are less efficient in stabilizing emulsions because they cannot unfold and reorient at the surface like a protein (Turgeon *et al.,* 1991). 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). Gbogouri *et al*. (2004) confirmed that better emulsion stabilities are obtained at low DH. 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; Lee *et al.,* 1987). Lee *et al*. (1987) suggested that peptides should have a minimum chain length of >20 residues to function as good emulsifiers. Solubility seems to play an important role in

emulsification because rapid migration to and adsorption at the interface are critical (Chobert *et al.,* 1988).

There are many different factors that may account for the difference observed between hydrolysates in both the ability to form an emulsion and emulsion stability. Peptide molecular characteristics and peptide chain length show the major impact (Kristinsson and Rasco, 2000a). Environmental conditions such as pH, ionic strength, temperature, etc. also have an effect on the emulsification properties (Gauthier *et al.,* 1993).

D. Foaming properties

The chemistry underlying foaming properties of protein and protein hydrolysates have many things in common with emulsifying properties. Both rely on the surface properties of protein. Food foams consist of air droplets dispersed in and enveloped by a liquid containing a soluble surfactant lowering the surface and interfacial tension of the liquid (Kinsella, 1976). The amphiphilic nature of proteins makes a foaming formation possible. A protein may have an excellent foamability but it may not necessarily produce a stable foam (Wilde and Clark, 1996).

Foaming ability of protein hydrolysate is governed by the size of peptides. 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). According to Philips *et al*. (1994), the proteins with low molecular weight are contributory to rapid foam formation, but they may not be ideal in forming the protein-protein interactions that give rise to stable foams. 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).

E. 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.

F. 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 (Daukš as *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).

4. Antioxidative activity of fish protein hydrolysates

Hydrolysis of protein 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 (Amarowicz and Shahidi, 1997; Sathivel *et al.,* 2003; Jun *et al.,* 2004; Je *et al.,* 2005).

4.1 Mode of action of fish protein hydrolysates

4.1.1 Radical scavenging activity and reducing power

Fish protein hydrolysates have been found to possess the radical scavenging activity. Sathivel *et al*. (2003) evaluated the antioxidant activity of herring byproduct hydrolysates in linoleic acid system. The hydrolysates had the antioxidative activity about 0.5 times less than that of α-tocopherol, BHA and BHT at a level of 8 ppm. The similar results were also found in mackerel hydrolysates (Wu *et al.,* 2003) and capelin hydrolysates (Amarowicz and Shahidi, 1997). Chan and Decker (1994) postulated that peptides containing basic amino acids are electron acceptors that take electrons from radicals formed during the oxidation of unsaturated fatty acids. Therefore, fish protein hydrolysates may function as a free radical chain stopper. 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). 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)

The reducing power of mackerel hydrolysates was studied by Wu *et al*. (2003). 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).

4.1.2 Metal chelation

Although hydrolysates from other protein sources such as soybean, egg yolk, porcine myofibrillar and potato are known to act as an antioxidant via metal ion chelation (Chen *et al.,* 1998; Lu and Baker, 1986; Saiga, 2003; Wang and Xiong, 2005), there are a few reports on fish protein hydrolysate. Mendis *et al*. (2005) investigated the ferrous ion chelation ability of peptides derived from jumbo squid skin gelatin hydrolysate. The peptides 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). 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.

4.1.3 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. The synergistic effects with other antioxidants were previously demonstrated with the hydrolysates of a vegetable protein, yeast protein, Alaska pollack skin gelatin and bovine serum albumin (Bishov and Henick, 1975; Kim *et al.,* 2001; 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.

4.2 Amino acids and peptides with antioxidative activity

The levels and composition of free amino acids and peptides in hydrolysate have been found to be associated with antioxidant activities. Increased 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 higher concentrations (Amarowicz and Shahidi, 1997).

Several amino acids are generally accepted to be antioxidative and exhibit higher antioxidative activities when incorporated into peptides (Saito *et al.,* 2003). Tryptophan and histidine showed high antioxidant activity whereas glycine and alanine exhibited only weak activity. 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 of the NH_3R group (Taylor and Richardson, 1980). Six antioxidative peptides were isolated from the proteolytic digest of a soybean protein (Chen *et al.,* 1995). The peptides were composed of 5-16 amino acid residues, including hydrophobic amino acids, valine or leucine at the N-terminal positions and proline, histidine or tyrosine in the sequences (Chen *et al.,* 1996). Among 22 synthetic peptides, histidine-containing peptides can act as a metal ion chelator, an active-oxygen quencher and a hydroxyradical scavenger (Chen *et al.,* 1998). From the study on antioxidative properties of tripeptides isolated from a soybean protein hydrolysate, tyrosine containing tripeptides exhibited higher activities than histidine containing tripeptides in the peroxidation of linoleic acid and showed a strong synergistic effect with phenolic antioxidants. However, this tripeptide had only marginal reducing activity and a moderate peroxynitrite scavenging activity whereas cysteine containing tripeptides showed the strong peroxynitrite scavenging activity. When either the N-terminus or C-terminus of Pro-His-His was changed to other amino acid residues, their antioxidative activity did not significantly alter. Additionally, tripeptides containing tryptophan or tyrosine residues at the C-terminus had strong radical scavenging activities, but very weak peroxynitrite scavenging activity (Saito *et al*., 2003). Overall antioxidative activities of peptides are attributed to the cooperative effects of these properties.

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 molecular weights of peptides which

possessed the high antioxidative activity are different depending upon fish protein sources (Table 4). 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). 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. The sequence of the purified peptide was Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr and molecular weight was 13 kDa (Jun *et al.,* 2004). The similar result was found in purified peptide from Alaska pollack frame protein hydrolysate with amino acid sequence as Leu-Pro-His-Ser-Gly-Tyr (MW 672 Da) (Je *et al.,* 2005).

Table 4   The molecular weight of peptides with the antioxidative activity from fish protein hydrolysates

Substrates	MW (Da)	References
cod frame	5,000-10,000	Jeon *et al.* (1999)
tuna cooking juice	390-1,400	Jao and Ko (2002)
mackerel	1,400	Wu *et al.* (2003)
yellowfin sole frame	13,000	Jun *et al.* (2004)
Alaska pollack frame	672	Je *et al.* (2005)
hoki frame protein	1,801	Kim *et al.* (2006)

OBJECTIVES

1. To study the antioxidative activities of round scad protein hydrolysates prepared using Alcalase or Flavourzyme with the varying DHs

2. To study the effects of defatting prior to hydrolysis and fractionation on antioxidative activity of resulting hydrolysates or their fractions

3. To study the chemical composition, functional properties and antioxidative stability of round scad protein hydrolysate