CHAPTER 3

RESULTS AND DISCUSSION

1. Enzymatic hydrolysis and antioxidative activities of round scad protein hydrolysates prepared using Alcalase (HA) and Flavourzyme (HF)

1.1 Effect of heating time on DH of round scad protein hydrolysates

The enzymatic hydrolysis of round scad mince was carried out at 50°C, pH 8.0 for Alcalase and 50°C, pH 7.0 for Flavourzyme. The hydrolysis was run for different times. The DH of both HA and HF increased as the heating time increased ($p<0.05$) (Figure 4). A rapid increasing in DH was obtained within the first 20 min. Thereafter, the lower increasing rate was generally observed. Possible inhibition of protein hydrolysis by the presence of products or total cleavage of all susceptible peptide bonds by the enzyme might be contemplated (Shahidi *et al*., 1995). Enzymatic hydrolysis of fish muscle proteins is characterized by an initial rapid phase, during which a large number of peptide bonds are hydrolyzed. The rate of enzymatic hydrolysis decreases and reaches a stationary phase where no apparent hydrolysis takes place (Shahidi *et al*., 1995). From this result, DH of HA and HF increased from approximately 1.86-23.17 to 11.08-59.52% and varied with type and concentration of enzyme used. At the same level of enzyme, Alcalase rendered the hydrolysates with a higher DH than did Flavourzyme. Proteinase at a concentration of 0.5% showed a greater DH of resulting hydrolysates than did 0.1% enzyme. DH has been used as an indicator for the cleavage of peptide bond and needed to be controlled during protein hydrolysis. The result was in accordance with Guerard *et al*. (2001) who reported that DH of yellowfin tuna hydrolysates increased with increasing hydrolysis time and was proportional to the amount of enzyme added.

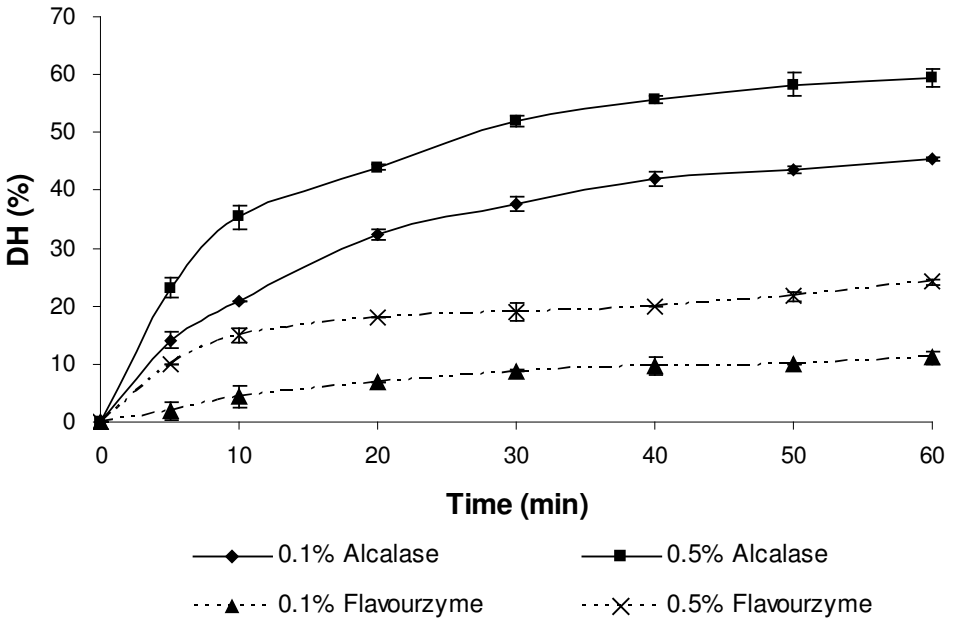

Figure 4 Changes in DH of round scad protein hydrolysate prepared using Alcalase and Flavourzyme during hydrolysis with different times. The reaction was performed at 50°C, pH 8 for Alcalase and 50°C, pH 7 for Flavourzyme. Bars represent the standard deviation from triplicate determination.

1.2 Effect of enzyme concentration on DH of round scad protein hydrolysates

Round scad mince was hydrolyzed for 1 h with different concentrations of Alcalase and Flavourzyme (0.05-5%). An increase in DH was generally observed with increasing enzyme concentration used (Figure 5). At the same amount of enzyme, round scad mince treated with Alcalase showed appreciably higher DH than that treated with Flavourzyme, indicating a higher hydrolytic activity of Alcalase towards round scad muscle proteins, compared with that of Flavourzyme. Alcalase 2.4L has been generally found to be very efficient in hydrolyzing fish protein from various species, such as Pacific whiting (Benjakul and Morrissey, 1997), shark (Onodenalore and Shahidi, 1996), dogfish (Diniz and Martin, 1996), capelin (Shahidi *et al*., 1995; Gildberg *et al*., 1989) and herring (Hoyle and Merritt, 1995). Alcalase also exhibited the greater activities towards fish protein than acid or neutral proteases such as Flavourzyme (Rebeca *et al*., 1991). When $\log_{10}$ (enzyme concentration) versus DH (%) was plotted, a linear relationship was found with the correlation coefficient (R^2) of 0.9726 for Alcalase and 0.9728 for Flavourzyme

(Figure 5). The result was in agreement with Benjakul and Morrissey (1997) and Guerard *et al.* (2001) who reported the linear relationship between $\log_{10}$ (enzyme concentration) and DH for enzymatic hydrolysis of Pacific whiting solid wastes and yellowfin tuna wastes, respectively. From this relationship, the amount of enzyme required for round scad hydrolysis to obtain a required DH (20, 40 and 60%) under the same hydrolytic condition can be calculated. Many parameters, such as substrate, enzyme-substrate ratio, temperature and time involved in enzymatic hydrolysis generally determined the DH of hydrolysate obtained (Kristinsson and Rasco, 2000a).

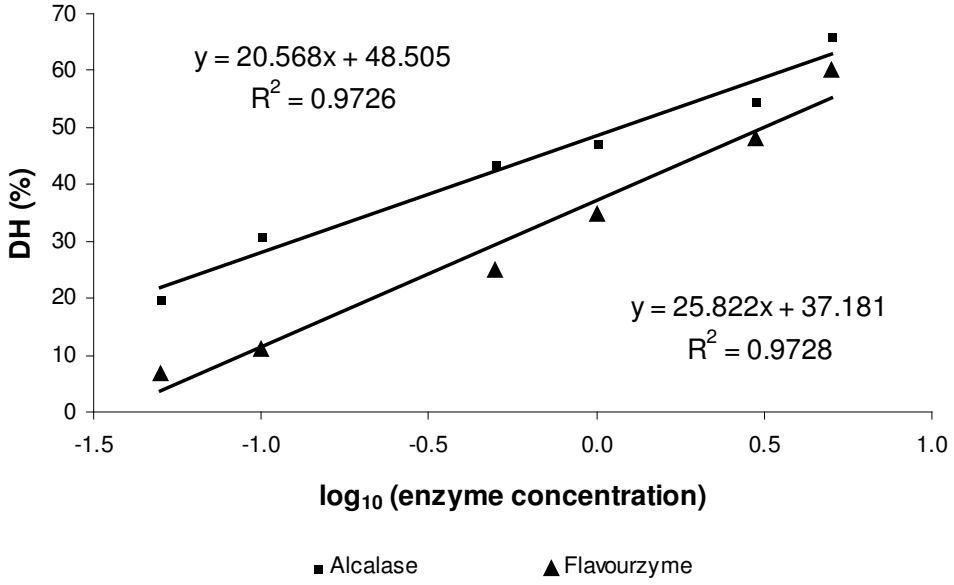

Figure 5 The relationship between $\log_{10}$ (enzyme concentration) and DH (%) in enzymatic hydrolysis of round scad by Alcalase and Flavourzyme. The reaction was performed for 1 h at 50°C, pH 8 for Alcalase and 50°C, pH 7 for Flavourzyme.

1.3 Effect of DH on antioxidative activities of round scad protein hydrolysates

1.3.1 DPPH radical scavenging activity

DPPH radical scavenging activities of HA and HF with 20, 40 and 60% DHs are shown in Figure 6 (a). Hydrolysates showed the varying DPPH scavenging activities ranging from 5.91 to 56.35%. The DPPH radical scavenging activity of HF increased with increasing DH up to 60%, whereas those of HA increased when DH increased up to 40% ($p<0.05$). At the same

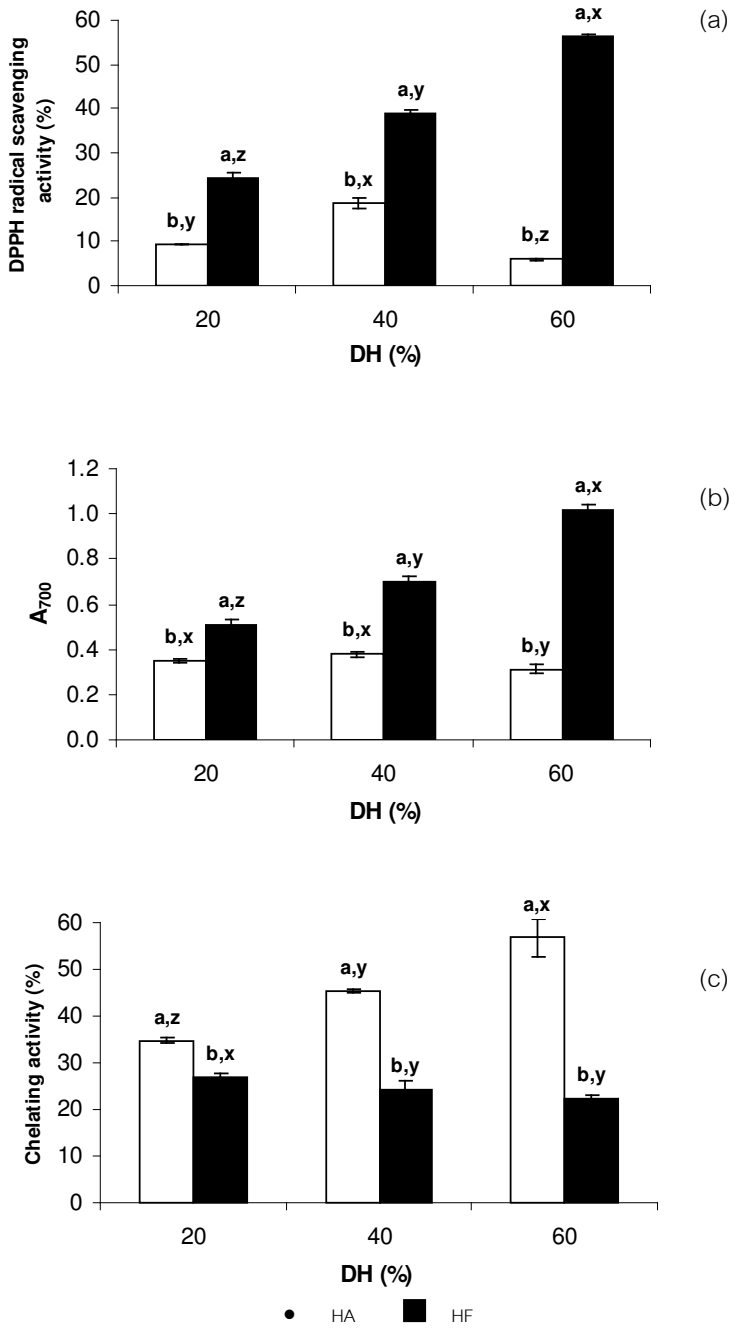

Figure 6 Antioxidative activities of round scad protein hydrolysates prepared using Alcalase (HA) and Flavourzyme (HF) with various DH (protein concentration = 35 mg/ml). a: DPPH radical scavenging activity, b: reducing power and c: Fe^{2+} chelating activity. Bars represent the standard deviation from triplicate determinations. Different letters a and b within the same DH indicate significant differences ($p<0.05$) and different letters x, y and z within the same proteinase used indicate significant differences ($p<0.05$).

DHs tested, DPPH radical scavenging activity of HF was greater than that of HA ($p<0.05$). DPPH is a stable free radical that shows the maximal absorbance at 517 nm in ethanol. When DPPH encounters the proton-donating substance, the radical would be scavenged by changing color from purple to yellow and the absorbance is reduced (Shimada *et al*., 1992). The result suggested that round scad protein hydrolysates possibly contained the amino acids or peptides, which functioned as electron donors and could react with free radicals to form more stable products. Type of proteinase used for hydrolysis had a major impact on radical scavenging activity of obtained product since the compositions of amino acids and peptides were mainly governed by enzyme used (Chiang *et al*., 1999). Wu *et al*. (2003) reported that changes in levels of free amino acids, anserine and carnosine, of mackerel hydrolysates derived from different enzymes during hydrolysis had an effect on the DPPH radical scavenging activity.

1.3.2 Reducing power

The reducing power of HF increased with increasing DH up to 60% ($p<0.05$), as shown by an increase in A_{700} (Figure 6 (b)). Nevertheless, no differences were observed for HA with increasing DH up to 40% ($p>0.05$). Marked decreases in reducing power were found in HA with DH of 60%. HF showed the higher reducing power than HA at all DHs tested which varied from 0.51 to 1.02 and from 0.31 to 0.38, respectively. For the reducing power assay, the presence of ruductants (antioxidants) in tested samples would result in reducing Fe^{3+}/ferricyanide complex to the ferrous form. The Fe^{2+} can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm. From the result, it was suggested that protein hydrolysates from round scad mince could function as electron donor to the free radicals and the efficacy was governed by DH and the enzyme used. The reducing power of bioactive compounds had been reported to be associated with their antioxidant activity (Meir *et al*., 1995; Juntachote and Berghofer, 2005).

1.3.3 Metal chelating activity

Both HA and HF showed chelation toward Fe^{2+} ranging from 22.26 to 56.80% (Figure 6 (c)). The Fe^{2+} chelating ability of HA increased with increasing DH up to 60% ($p<0.05$), while that of HF decreased as DH increased to 40% and no difference was observed when DH increased up to 60% ($p>0.05$). HA had a better Fe^{2+} chelating ability than HF at the same DH tested. The chelating of ferrous ions was used to determine the ability of hydrolysates in metal chelating activity. Ferrozine can quantitatively form complexs with Fe^{2+} ion. In the presence of

chelating agents, the complex formation is disrupted, resulting in the decrease in color formation. The results indicated that round scad protein hydrolysates had a pronounced capacity of iron binding. Transition metals such as Fe, Cu and Co in foods affect both the speed of autooxidation and hydroperoxide breakdown to volatile compounds (Nawar, 1996). Transition metal ions react very quickly with hydrogen peroxides. They act as one-electron donor to form an alkoxy radical and this can be considered as the branching of the propagation step of oxidation (Gordon, 2001). Therefore, protein hydrolysate from round scad mince could chelate the prooxidative iron, resulting in lowered oxidation.

From the results, antioxidative activities of both HA and HF were governed by DH, which was most likely associated with the size and amino acid sequence of the resulting peptides. Changes in size, level and composition of free amino acid and small peptides affect the antioxidative activity of protein hydrolysate (Wu *et al*., 2003). During hydrolysis, the wide variety of smaller peptides and free amino acids were generated, depending on enzyme specificity and hydrolysis conditions used. Saiga *et al*. (2003) revealed that the hydrolysate produced by actinase E exhibited the larger metal chelating activity than that of papain produced hydrolysate. Although the amino acid compositions of both hydrolysates were similar, the chelating activities differed. The difference might be caused by the differences in the structure and length of the peptides in the hydrolysates. Alcalase is endopeptidase, which is able to hydrolyze proteins with broad specificity for peptide bonds and prefers a large uncharged residue. Flavourzyme is the endo- and exopeptidase enzyme mixture, which can produce both amino acids and peptides (Hrč ková *et al*. 2002). From the result, Flvourzyme brought about the higher antioxidative activity of hydrolysate obtained than Alcalase. Peña-Ramos and Xiong (2002) reported that chymotrypsin- and Flavourzyme-hydrolyzed preheated soy protein isolate had the greatest inhibitory effect on lipid oxidation, compared with those produced from other enzymes such as Alcalase, Protamex, pepsin and papain. In addition, enzymatic extract from brown seaweed, *Ecklonia cava*, hydrolyzed with Flvourzyme afforded the higher antioxidative activity than did the extract prepared using Alcalase (Heo *et al*., 2005). From the result, amino acids and peptides in HA and HF could act both primary and secondary antioxidants. Since Flavourzyme rendered hydrolysates with a better antioxidant activity than Alcalase, it was used for further studies.

2. Effect of defatting on enzymatic hydrolysis and antioxidative activities of round scad protein hydrolysates

2.1 Fat and moisture contents of round scad mince and defatted mince

Different solvents were used to remove fat from round scad mince. The round scad mince contained 74.89% moisture content, whereas ethanol-defatted mince and isopropanol-defatted mince comprised 73.43 and 72.66% moisture content, respectively. Ethanol and isopropanol have been known to compete with protein in water binding. As a result, water was more removed from protein molecules in the presence of solvent (Sikorski and Naczk, 1981; Hoyle and Merritt, 1994). After defatting using ethanol or isopropanol, the fat content (1.75%) was decreased to 1.31 and 1.09%, respectively. Owing to the different polarity index of isopropanol (3.9), ethanol (5.2) and water (9), the lowest fat content was consequently observed in the round scad mince defatted with isopropanol ($p<0.05$). The result was in agreement with Sikorski and Naczk (1981) who found that isopropanol could remove the fat in fish muscle effectively prior to hydrolysis.

2.2 Enzymatic hydrolysis of round scad mince and defatted mince using Flavourzyme

Hydrolysis of round scad mince and defatted mince using Flavourzyme was carried out for 1 h at 50°C and pH 7. When $\log_{10}$ (enzyme concentration) versus DH (%) was plotted, a linear relationship was obtained with the correlation coefficient (R^2) of 0.9744, 0.9716 and 0.9932 for HF derived from raw round scad mince, ethanol-defatted mince and isopropanol-defatted mince, respectively (Figure 7). At the same level of enzyme, HF derived from round scad mince exhibited the higher DH (%) than HF derived from ethanol-defatted mince and isopropanol-defatted mince, respectively. Hoyle and Merritt (1994) reported that the high temperatures used during fat extraction might inactivate the endogenous enzymes and also denature fish proteins. As a result, poor wetting abilities of denatured substrates reduced dispersibility of the added enzymes. This contributed to a lower DH in hydrolysates prepared from defatted mince and a higher amount of enzyme was needed to reach the DH required.

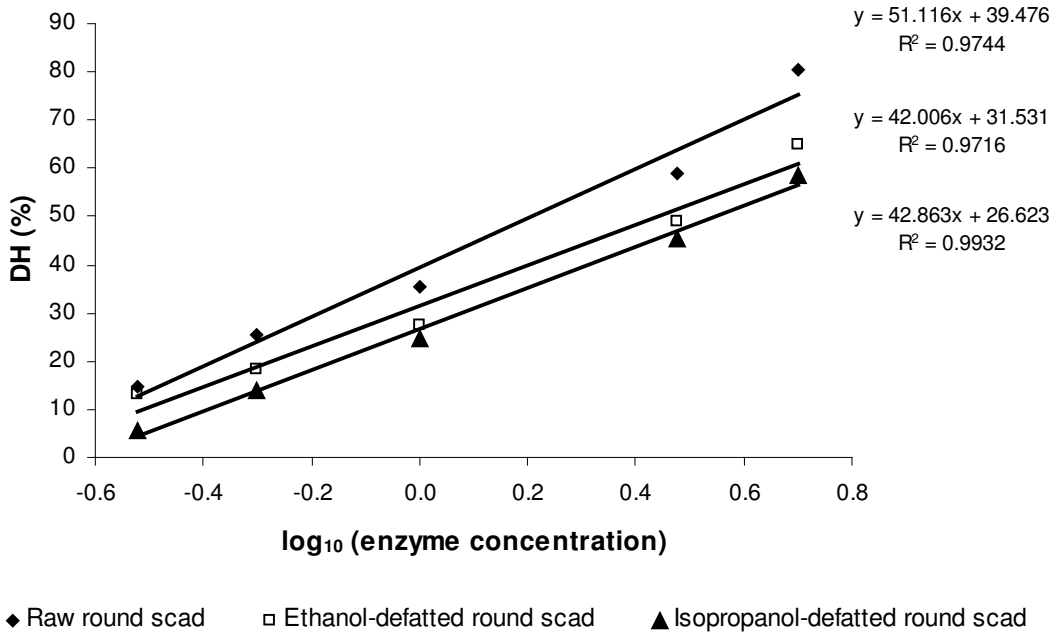

Figure 7 The relationship between $\log_{10}$ (enzyme concentration) and DH (%) in enzymatic hydrolysis of round scad mince and defatted mince using Flavourzyme. The reaction was performed for 1 h at 50°C and pH 7.

2.3 Yield of round scad protein hydrolysates from mince and defatted mince using Flavourzyme

Protein hydrolysates with DH of 20, 40 and 60% were prepared from round scad mince, ethanol-defatted mince and isopropanol-defatted mince and varying yields were obtained (Table 5). Hydrolysate derived from round scad mince with DH of 60% showed the highest yield (65.86%) while that derived from isopropanol defatted mince rendered the lowest yield at all DHs ($p<0.05$). For all protein substrates used, the yield increased with increasing DHs ($p<0.05$). This indicated that enzyme concentration had a marked effect on the yield of the final product. Hoyle and Merritt (1994) reported that raw herring showed the higher recovery of soluble nitrogen than ethanol extracted herring or herring presscake when Alcalase was used. During the defatting process, the removal of water from protein molecules most likely caused the aggregation of proteins via hydrophobic interaction. The larger aggregate formed was less susceptible to

hydrolysis by Flavourzyme. The denatured proteins of defatted mince are highly resistant to enzymatic breakdown (Cheftel *et al*., 1985). As a consequence, the lowered amount of proteins solubilized during subsequent enzymatic hydrolysis was attained. In addition, the abilities of enzyme to disperse and hydrolyze the proteins can be reduced, leading to the decreased yield (Šližyte *et al*., 2005a).

Table 5 Yields of round scad protein hydrolysate derived from mince and defatted mince using Flavourzyme with different DHs

Substrates	DH (%)	Yield (%)*
Mince	20	$43.07\pm3.96^{c**}$
	40	51.40 ± 0.84^{b}
	60	65.86 ± 5.74^{a}
Ethanol-defatted mince	20	19.40 ± 0.29^{f}
	40	31.57 ± 1.26^{e}
	60	42.73 ± 0.36^{c}
Isopropanol-defatted mince	20	12.53 ± 0.36^{g}
	40	22.69 ± 1.44^{f}
	60	37.46 ± 0.26^{d}

*Means ± SD from three experiments.

**Different superscripts in the same column indicate the significant difference ($p<0.05$).

2.4 DPPH radical scavenging activity

DPPH radical scavenging activities of hydrolysates derived from round scad mince and defatted mince with various DHs are depicted in Figure 8 (a). Markedly increased DPPH radical scavenging activity was observed in all hydrolysates when DH (%) increased. With the more pronounced hydrolysis, the peptides with higher antioxidative activity were produced. Similar DPPH radical scavenging activities were observed among the hydrolysates derived from round scad mince and defatted mince when DHs were 20 and 40%. At 60% DH, the marked differences in DPPH radical scavenging activity were noticeable ($p<0.05$). Hydrolysate derived

from round scad mince defatted with isopropanol with DH of 60% exhibited the highest DPPH radical scavenging activity, compared with the hydrolysate derived from ethanol-defatted mince

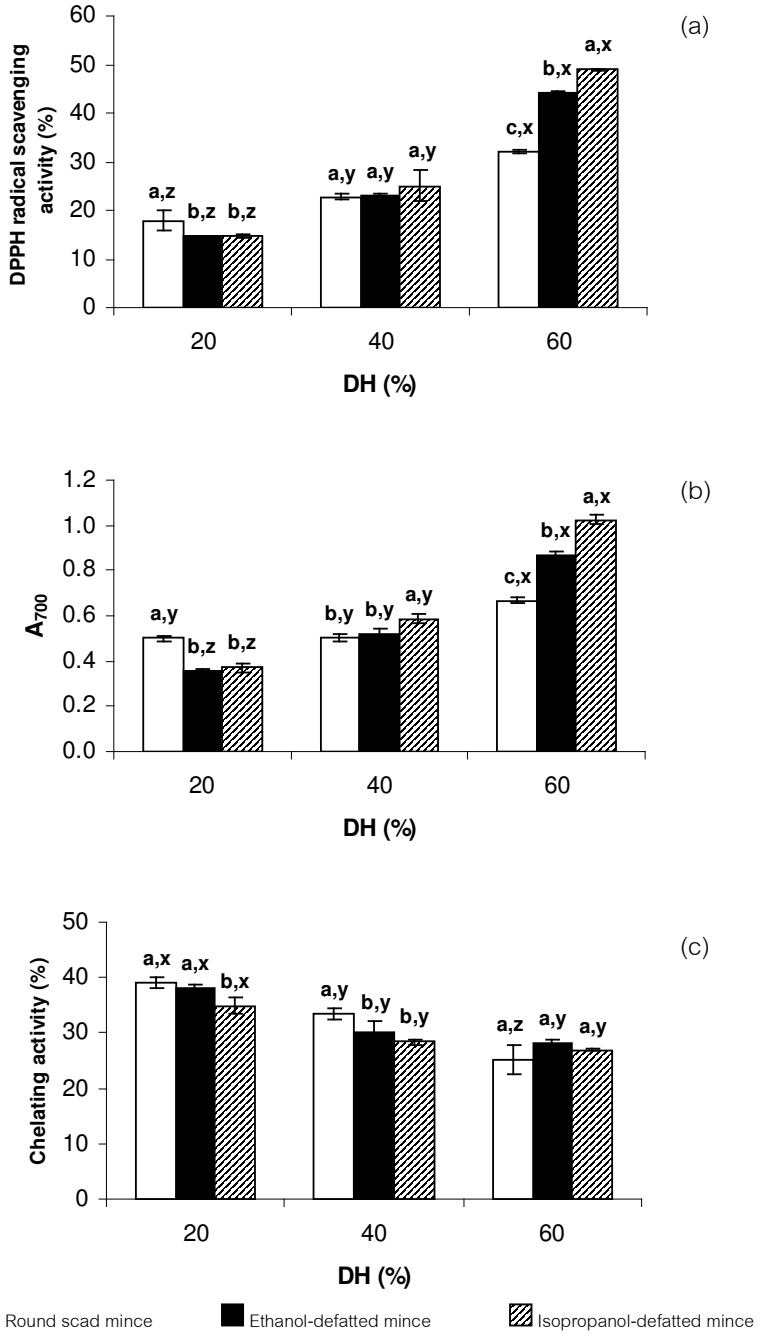

Figure 8 Antioxidative activities of HF prepared from round scad mince, ethanol-defatted mince and isopropanol-defatted mince with various DH (protein concentration = 25 mg/ml). a: DPPH radical scavenging activity, b: reducing power and c: Fe^{2+} chelating activity. Bars represent the standard deviation from triplicate determinations. Different letters a,

b and c within the same DH indicate significant differences ($p<0.05$) and different letters x, y and z within the same substrate indicate significant differences ($p<0.05$).

and from the original mince ($p<0.05$). In general, the reduction of $DPPH^{\bullet}$ by antioxidant ($DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$) or by radical species ($DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH\text{-}R$) results in a loss of absorbance at 515 nm (Fukumoto and Mazza, 2000). Free radical scavenger, particularly of the peroxyl radical, which is the major propagator of the oxidation chain of fat, is able to terminate the chain reaction (Frankel, 1991; Yen *et al*., 2002). Nevertheless, the different scavenging activities of hydrolysates derived from different substrates might be caused by the differences in amino acids or peptides generated. Even the same number of peptides was cleaved, the resulting peptides might be different in term of amino acid type and sequences. Thus, this might contribute to the varying radical scavenging activity. Rajapakse *et al*. (2005) reported that purified peptides derived from fermented mussel sauce which contained two aromatic amino acids and two histidine residues exhibited the high radical scavenging properties. Generally, aromatic amino acids are considered as effective radical scavengers, because they can donate protons easily to electron deficient radicals. At the same time, their antioxidative stability can remain via resonance structures. Strong antioxidative properties of carnosine (β-alanyl-L-histidine), a natural dipeptide present in human body, have been suggested due to the combined effects of free radical scavenging and metal ion chelation via histidine residue (Kohen *et al*., 1988). Not only the presence of some favorable amino acids, but also their correct positioning in the peptide sequence is trivial to its activity. The proper positioning of Glu, Leu and His has been reported to improve radical scavenging activities of antioxidative peptides (Chen *et al*., 1996; Suetsuna *et al*., 2000).

2.5 Reducing power

Reducing power of hydrolysate derived from round scad mince and defatted mince at various DHs is shown in Figure 8 (b). Increase in reducing power was observed in the hydrolysate derived from ethanol- and isopropanol-defatted mince when DH (%) increased ($p <0.05$). Nevertheless, no difference was found in the hydrolysate derived from round scad mince when DH increased up to 40% ($p>0.05$). At 60% DH, the difference in reducing power varying from 0.67 to 1.03 was found among the samples. Hydrolysate derived from round scad mince showed the lowest reducing power, whereas that derived from isopropanol defatted mince exhibited the highest reducing power ($p<0.05$). Similar result was found when comparing with

DPPH radical scavenging activity (Figure 8 (a)). For the measurement of reducing power, the Fe^{3+} - Fe^{2+} transformation was investigated (Zhu *et al*., 2006). Moure *et al*. (2006) reported that the ferric reducing antioxidant power measures the reducing capability of the ferric ion and has been correlated to the radical scavenging capacity. The result revealed that all hydrolysates are electron donors and can react with free radicals and convert them to more stable products, thus terminating the radical chain reactions. Thus, hydrolysates from round scad mince and defatted mince possessed the primary antioxidant, but the antioxidative activity was affected by defatting process.

2.6 Metal chelating activity

The chelating activity of all hydrolysates on Fe^{2+} slightly decreased when DH increased up to 60% (Figure 8 (c)). At the same level of DH, all hydrolysates derived from different substrates showed the differences in chelating activity. At DH of 20 and 40%, hydrolysate derived from mince without defatting gave the highest chelating activity ($p<0.05$). No differences were observed between hydrolysates derived from round scad mince defatted by ethanol and isopropanol ($p>0.05$). However, no differences in metal chelating activity were found among all samples derived from different substrates when DH was 60% ($p>0.05$). Fe^{2+} ion is the most powerful prooxidant among various species of metal ions (O'Brien, 1969; Halliwell and Gutteridge, 1984; Yamauchi *et al*., 1988). It can catalyze the generation of reactive oxygen species, hydroxyl radical ($OH^{\bullet}$), by which the lipid peroxidation chain reaction is accelerated (Stohs and Bagchi, 1995). Peptides obtained from other protein sources such as porcine myofibrillar proteins and fermented mussel sauce have been reported to act as metal chelator (Saiga *et al*., 2003; Rajapakse *et al*., 2005). Rajapakse *et al*. (2005) reported that Fe^{2+} chelation activity of the mussel sauce was estimated to be 75% relative to EDTA at the same concentration. Histidine is frequently observed in sequences of peptide having the ion chelating activity. 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 chains (Suetsuna *et al*., 2000).

From the result, it was suggested that the deffatting of round scad mince prior to hydrolysis affected the antioxidant properties of obtained hydrolysates. Fat extraction might remove membrane lipids and induce the denaturation of proteins. During extraction, proteins were vulnerable to solvent as well as heat treatment (Mutilangi *et al*., 1996), leading to the aggregation

of protein and exposure of hydrophobic domain (Sikorski and Naczk, 1981). Peptides derived from many protein sources with increased hydrophobicity have been reported to relate with antioxidative properties (Chen *et al*., 1995). Rajapakse *et al*. (2005) presumed that hydrophobic amino acids such as Leu, Val and Ala present in fermented mussel sauce peptides have favored the radical scavenging properties. Moreover, these amino acids were reported to be effective in inhibiting oxidation of fatty acids tested in a linoleic acid model system (Marcuse, 1962). As a result, different peptides with varying properties might be produced from different substrates. Hydrolysate derived from isopropanol-defatted mince with 60% DH showed the high antioxidative activity. Therefore, it was produced and used for fractionation studies.

3. Fractionation of antioxidative peptides from round scad protein hydrolysate

Fractionation was carried out to separate or concentrate the antioxidative compounds from round scad protein hydrolysate. Size exclusion and the extraction with different solvents possessing varying polarity were carried out.

3.1 Fractionation of round scad protein hydrolysate by gel filtration chromatography and solvent extraction

HF derived from isopropanol-defatted mince with 60% DH (HFIP 60) was separated by Sephadex G-75 gel filtration chromatography and two fractions with the MW of 206 kDa (F1) and 9 kDa (F2) were obtained (Figure 9). The absorbance at 220 nm indicates peptide bond, whereas the absorbance at 280 nm represents proteins, peptides or amino acids with aromatic ring (Amarowicz and Shahidi, 1997). Generally, the absorbance at 220 nm of both fractions was greater than that at 280 nm. Relatively high ratio of absorbance at 280 nm/220 nm was noticeable for F1, suggesting the higher content of peptides containing aromatic ring. High absorbance at 280 nm might indicate the presence of tyrosine and tryptophan in the peptides or the existence of other aromatic compounds (Amarowicz and Shahidi, 1997). From the result, the peak of F2 was larger than that of F1, indicating a higher proportion of F2 in HFIP 60. 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. Recently, antioxidative peptides from hydrolysate of yellowfin sole frame

protein (Jun *et al*., 2004), Alaska pollack frame protein (Je *et al*., 2005), fermented mussel sauce (Rajapakse *et al*., 2005) and hoki frame protein (Kim *et al*., 2006) were identified.

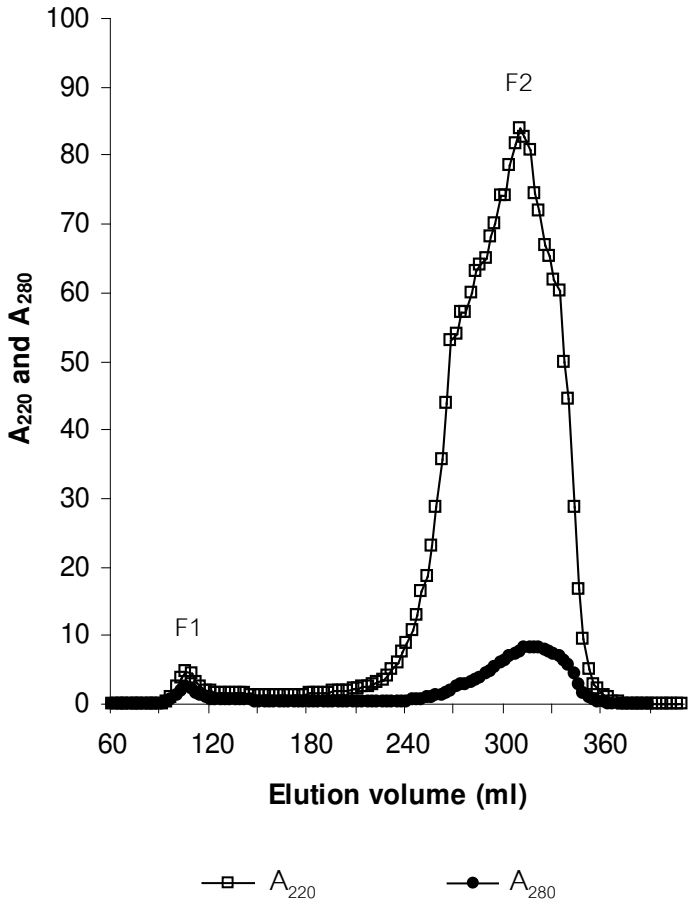

Figure 9 Separation of peptides from round scad protein hydrolysate derived from isopropanol-defatted mince with 60% DH by Sephadex G-75.

Different solvents with varying polarity index including hexane (0), dichloromethane (3.1) and ethyl acetate (4.4), were used for fractionation of HFIP 60. Three peptide fractions and a residual fraction (R) were obtained after fractionation, consecutively starting with hexane (E1), dichloromethane (E2) and ethyl acetate (E3), respectively. The yield of different fractions is given in Table 6. Among all fractions, F2 and R showed the highest yield (88.81 and 90.62%, respectively) and the very low yields were found in the others, ranging from 0.32 to 7.14%. Very low yields of three fractions obtained from solvent extraction were most likely due to the low amount of non-polar peptides or amino acids in HFIP 60.

Table 6 Yields of different fractions from round scad protein hydrolysate derived from isopropanol-defatted mince with 60% DH separated by Sephadex G-75 gel filtration and solvent extraction

Separation methods	Fractions	Yield (%)*
Gel filtration chromatography	F1 (206 kDa fraction)	$0.32\pm0.03^{b**}$
	F2 (9 kDa fraction)	88.81 ± 2.00^{a}
Solvent extraction	E1 (hexane fraction)	0.91 ± 0.07^{c}
	E2 (dichloromethane fraction)	7.14 ± 1.00^{b}
	E3 (ethyl acetate fraction)	1.33 ± 0.06^{c}
	R (residual fraction)	90.62 ± 2.00^{a}

*Means ± SD from three experiments.

**Different superscripts in the same column within the same separation method indicate the significant differences ($p<0.05$).

3.2 DPPH radical scavenging activity

Different fractions separated by gel filtration chromatography and solvent extraction were analyzed for DPPH radical scavenging activity in comparison with HFIP 60 (Figure 10 (a)). The concentrations of protein in HFIP 60 and its fractions were adjusted to 1.5 mg/ml prior to analysis, except F1 (0.07 mg/ml) which was excessively diluted by elution procedure. Different fractions exhibited varying DPPH radical scavenging activity as shown in Figure 10 (a). The highest DPPH radical scavenging activity was found in E2 and E3 ($p<0.05$). The lower activities were observed for HFIP 60, R, F2, E1 and F1 in the descending order. DPPH radical scavenging activity is generally used to determine hydrogen donating ability of protein hydrolysates (Brand-Williams *et al*., 1995; Wu *et al*., 2003). The result revealed that HFIP 60 consisted of different antioxidative compounds with different polarity and size. The high activity of E2 and E3 pointed out that solvent extraction was able to specifically isolate some amino acids or peptides with antioxidative activity, leading to the much increase in antioxidant activity. The lower activity in the residual fraction suggested that more polar compounds exhibited the lower

antioxidative activity, especially via the radical chain breaking. In addition, the lower radical scavenging activity of HFIP 60 in comparison with E2 and E3 might be associated with the

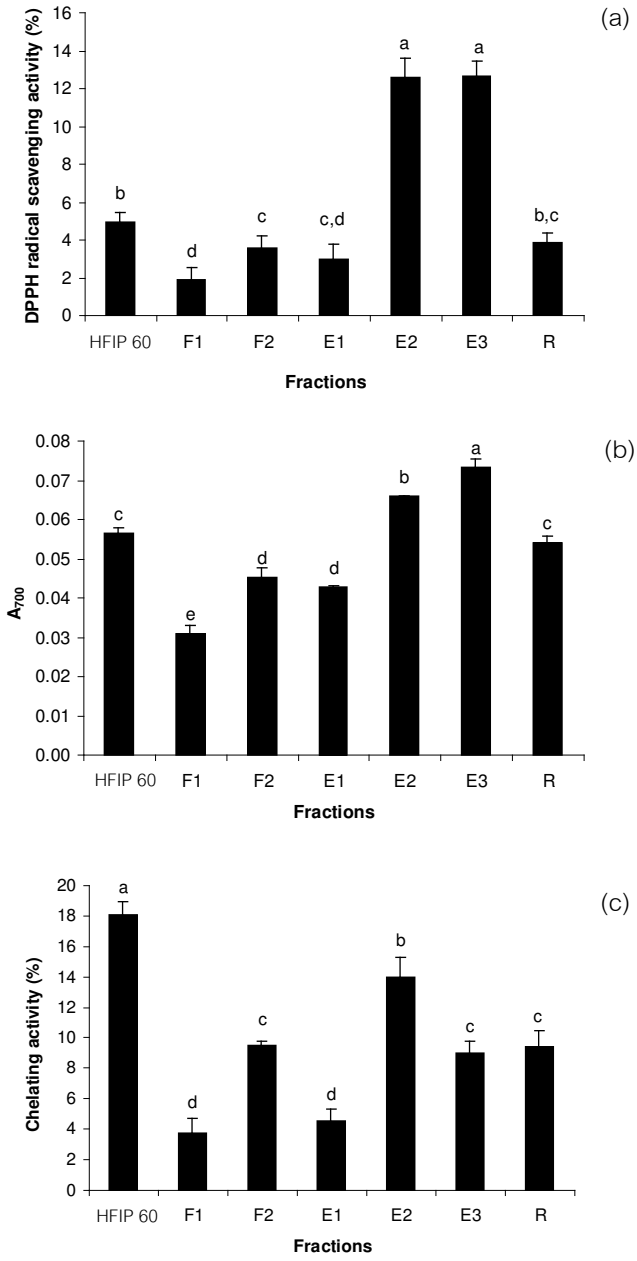

Figure 10 Antioxidative activities of fractions from round scad protein hydrolysate separated by Sephadex G-75 gel filtration and solvent extraction. a: DPPH radical scavenging activity, b: reducing power and c: Fe^{2+} chelating activity. Bars represent the standard deviation from triplicate determinations. Different letters indicate significant differences ($p<0.05$). HFIP 60: HF derived from isopropanol-defatted muscle with 60%

DH; F1: 206 kDa fraction; F2: 9 kDa fraction; E1: hexane fraction; E2: dichloromethane fraction; E3: ethyl acetate fraction; R: residual fraction.

presence of prooxidative compounds such as haemoglobin and myoglobin. According to Pratt and Hudson (1990), some amino acids such as cysteine may act as prooxidant while histidine has been shown to revert from being an antioxidant to a prooxidant at higher concentrations. From the result, the low DPPH radical scavenging activities were observed in F1 (MW = 206 kDa) and F2 (MW = 9 kDa). Therefore, size exclusion was less effective in fractionation or concentrate the peptides showing the antioxidative activity. Kim *et al*. (2006) 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). Thus, the compositions of peptides, which were governed by substrate, hydrolysis condition and purifying technique, had a pronounced effect on antioxidative activity of obtained hydrolysate or its fractions.

3.3 Reducing power

Different reducing power was noticeable among HFIP 60 and its fractions (Figure 10 (b)). The highest reducing power was observed in E3, which also exhibited the highest radical scavenging ability ($p<0.05$), followed by E2, HFIP 60, R, F2, E1 and F1, respectively. Generally, reducing power of HFIP 60 and its fractions correlated well with DPPH radical scavenging activity. This result confirmed the primary antioxidant property of the hydrolysate and its fractions. From the result, it was inferred that solvent extraction was the appropriate method for isolation the potent fractions with antioxidative activity from HFIP 60. Nevertheless, Wu *et al*. (2003) reported that the peptides derived from mackerel hydrolysate with molecular weight of approximately 1,400 Da possessed a stronger DPPH radical scavenging effect and reducing power than that of 900 and 200 Da peptides.

3.4 Metal chelating activity

The ability of HFIP 60 and different fractions in metal chelating is depicted in Figure 10 (c). HFIP 60 exhibited the highest Fe^{2+} chelating activity, followed by E2. This indicated their capacity of iron binding. Thus, it was most likely that their action as antioxidant might be related to their metal chelating activity. No differences in Fe^{2+} chelating activity were

noted among F2, E3 and R ($p>0.05$), while the lowest activity was found in E1 and F1 ($p<0.05$). Synergistic effects of antioxidants might be attributed to the formation of complexes with prooxidant metal ions and regeneration of exhausted antioxidants. Some molecules act as chelators of metal ions and contribute as indirect antioxidants (Chen *et al*., 1998; Rajapakse *et al*., 2005). From the result, all fractions derived from HFIP 60 showed the lower metal ion chelation activity, compared to HFIP 60. The fractionation might remove the compounds with metal chelating property. Generally, histidine-containing peptides have been reported to act as metal ion chelators (Chen *et al*., 1998). In addition, amino acid residues involving Gly, Asp, and Lys in porcine myofibrillar proteins interacted with metal ions through their charged properties and inactivated prooxidant activity of metal ions (Saiga *et al*., 2003). Therefore, it can be speculated that these amino acid residues might be lowered after fractionation process. Chen *et al*. (1998) demonstrated that antioxidant activity of the peptides depended on their amino acid composition and sequence.

From the result, stepwise fractionations of protein hydrolysate using different solvents possessing varying polarity provide an effective means to concentrate antioxidative peptides, especially those with primary antioxidative activity. Thus, dichloromethane or ethyl acetate effectively fractionated the antioxidative peptides of HFIP 60. Furthermore, the peptide with molecular weight of approximately 9 kDa exhibited a stronger antioxidative activity than that with 206 kDa. These results indicated that the antioxidative activity of proteins or peptides depended on their molecular weight. Thus, peptides from round scad protein hydrolysate with different amino acid compositions, sequences and sizes exhibited different antioxidative activity. Owing to their high antioxidative activities, E2, E3 and HFIP 60 were chosen for further study.

3.5 Characterization of antioxidative peptide

Thin layer chromatography (TLC) was used for separation of antioxidative peptides or amino acids in E2, which had the highest DPPH radical scavenging activity and the considerable chelating activity (Figure 10). E2 was applied and developed with chloroform : methanol : water (7:3:0.5, v/v/v). After drying and spraying with ninhydrin solution (Figure 11 (a)), seven bands with R_f of 0.83 (band A), 0.58 (band B), 0.49 (band C), 0.44 (band D), 0.31 (band E), 0.13 (band F) and 0.04 (band G) were observed. Ninhydrin technique is particularly effective for visualizing amino acid spots which displays a purple coloration (Amarowicz and

Shahidi, 1997). After dipping with DPPH solution, three major bands were observed at R_f of 0.60, 0.45 and 0.09 (Figure 11 (b)). This test can be used for the primary characterization of the scavenging potential of compounds (Yamaguchi *et al*., 1998). From the result, E2 showed DPPH radical scavenging activity as evidenced by the faint yellowish spots of diphenylpicryl hydrazine against a violet background on the TLC plate (Choi *et al*., 2002; Conforti *et al*., 2002). The similar R_f of bands between TLC plates sprayed with ninhydrin solution and with DPPH solution indicated that bands B, C, D, F and G had the antioxidative activity. However, bands A and E had the negligible antioxidative activity. Therefore, DPPH radical scavenging activity of E2 was attributed to some particular amino acids or peptides.

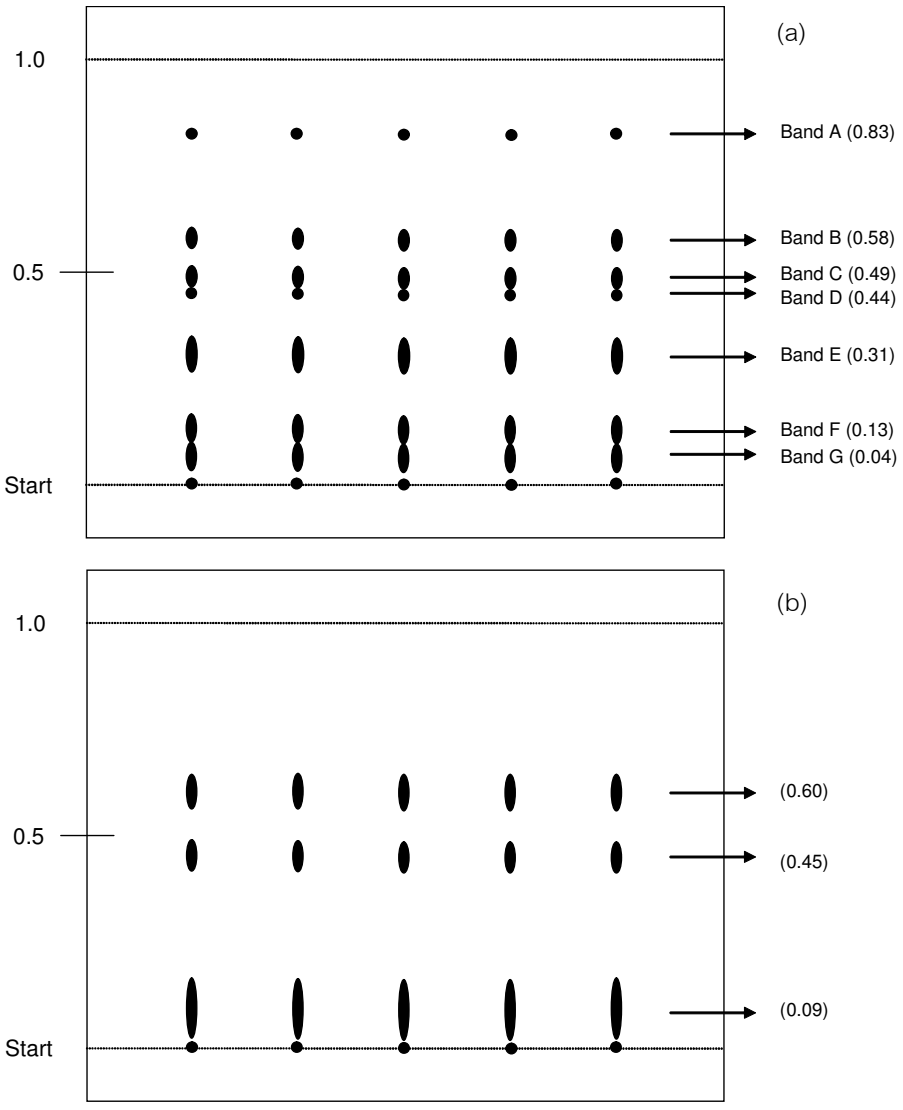

Figure 11 Thin layer chromatography of E2 (dichloromethane fraction) of round scad protein hydrolysate after separation; plates were sprayed with ninhydrin solution (a) and DPPH solution (b).

3.6 Antioxidative activity of HFIP 60 and peptide fractions in different systems

3.6.1 Antioxidative activity in a linoleic oxidation system

Linoleic acid, an unsaturated fatty acid, is usually used as a model compound in lipid oxidation and antioxidation related assays of emulsion system, in which carbon-centered, peroxyl radicals, and hydroperoxides are involved in the oxidation process (Burton and Ingold, 1986; Zhu *et al.*, 2006). Lipid peroxidation is thought to proceed via radical-mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids (Rajapakse *et al*., 2005). Inhibition of oxidation of linoleic acid by HFIP 60, E2 and E3 was monitored upon incubation at 40°C for 9 days as shown in Figure 12. During incubation, the system containing HFIP 60, E2 or E3 at all concentrations lowered the oxidation as evidenced by lower A_{500}, compared with the control system ($p<0.05$). The control showed a continuous increase in A_{500} throughout 9 days of incubation. An increase in A_{500} indicated the formation of peroxide value in linoleic acid model system (Yen and Chen, 1995). The hydrolysate and its fractions exhibited antioxidant activity in a concentration dependent manner. HFIP 60, E2 and E3 at 500 ppm completely inhibited the oxidation of linoleic acid. No changes in A_{500} were observed when BHT at 25 and 100 ppm was used. No significant differences in A_{500} were noticeable between the system containing BHT and that with HFIP 60 or its fractions at 1,000 ppm ($p>0.05$). Slight differences in antioxidative activity were found between HFIP 60 and solvent-extracted fractions, both E2 and E3. Peptides with of 5–16 amino acid residues separated from soybean protein hydrolysate could inhibit autoxidation of linoleic acid (Chen *et al*., 1995). Peptides from hydrolysate of yellowfin sole frame (Jun *et al*., 2004) and Alaska pollack frame (Je *et al*., 2005) were also reported to show inhibitory activity in linoleic acid model system. This activity was attributed to the ability of peptide to interfere propagation cycle of lipid peroxidation, thereby inhibiting radical mediated linoleic acid oxidation. HFIP 60 and its fractions might contain both hydrophilic and hydrophobic amino acids in their sequences, which contributed to peroxidation inhibition by increasing the solubility of peptides in lipid, thereby facilitating better interaction

with radical species. Also, they were soluble in an aqueous phase. Therefore, they can be used as natural antioxidant in emulsion system.

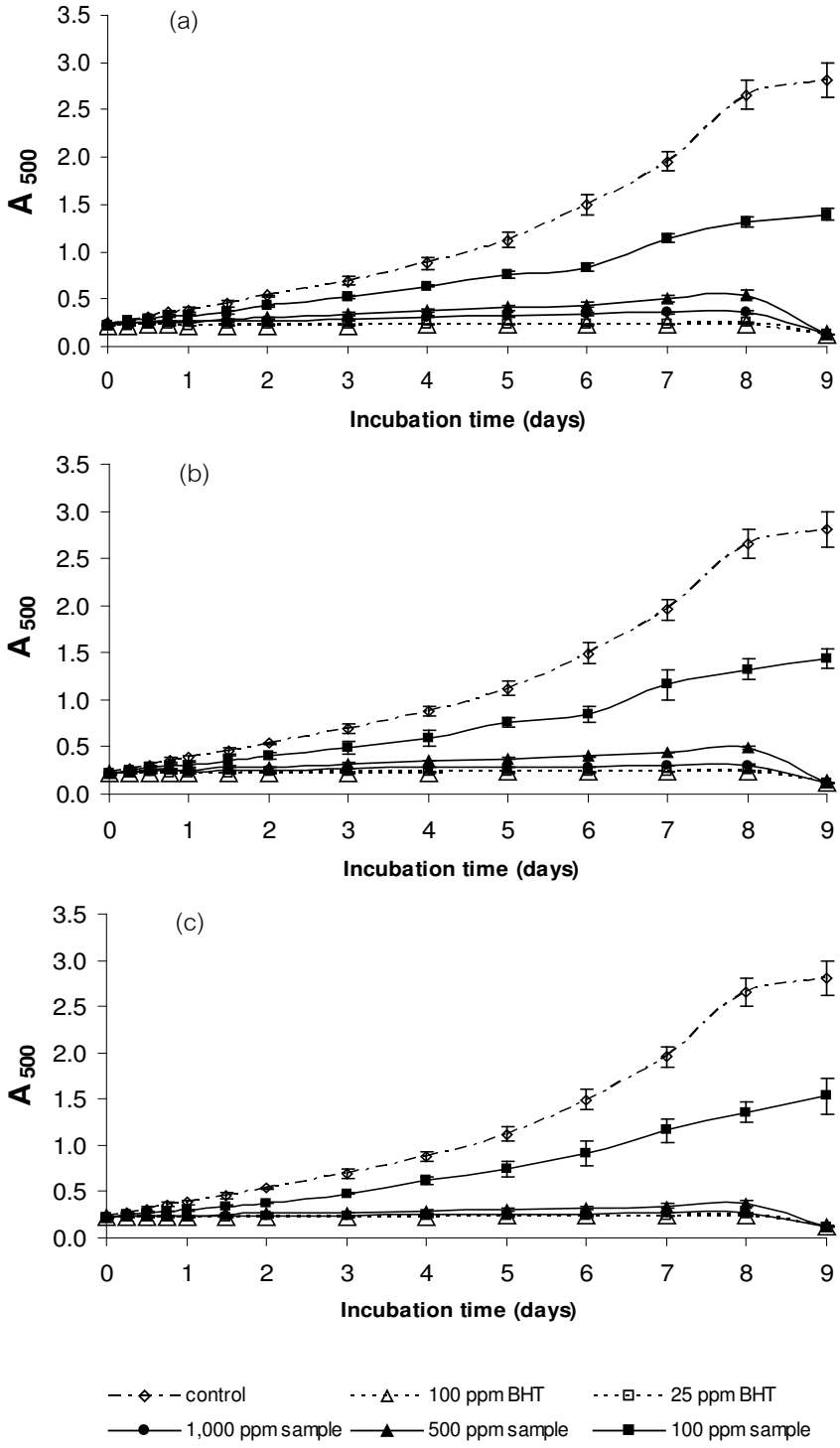

Figure 12 Changes in A_{500} of linoleic acid system in the presence of HFIP 60 (a), E2: dichloromethane fraction (b) and E3: ethyl acetate fraction (c) at different levels in

comparison with the control and the systems containing BHT at 25 ppm and 100 ppm. Bars represent the standard deviation from triplicate determinations.

3.6.2 Antioxidative activity in a lecithin liposome system

The antioxidant activities of HFIP 60 and its fractions were also estimated in a lecithin liposome system by monitoring conjugated dienes (Figure 13) and TBARS values (Figure 14). Generally, the increase in liposome oxidation was observed when incubation time increased ($p<0.05$). However, no changes in conjugated dienes and TBARS values of liposome system containing 100 ppm BHT were observed throughout the incubation period of 36 h ($p>0.05$). The formation of conjugated dienes was not noticeable in the liposome system within the first 6 h. Thereafter, the marked increases in conjugated dienes were observed, especially during 6-24 h. The rate of increase varied with the samples and concentrations used. Poirier *et al*. (2001) reported an increase in lag time of conjugated dienes formation, reflecting resistance to oxidation. Addition of HFIP 60 and E2 at 1,000 ppm resulted in a longer lag time in comparison with other samples used except for BHT at 25 and 100 ppm. Generally, TBARS decreased as the amount of hydrolysates or their fractions increased. Both HFIP 60 and solvent-extracted fractions at 1,000 ppm showed a pronounced antioxidant activity throughout the incubation period as evidenced by lesser formation of TBARS ($p<0.05$). From the result, HFIP 60 and E2 at 1,000 ppm had the ability to retard TBARS formation, and showed similar effect to BHT at 25 ppm. Negligible formation of TBARS was observed when 100 ppm of BHT was used. The ability of hydrolysate or peptide fraction in a liposome system has previously been demonstrated (Lee and Hendricks, 1997; Peña-Ramos and Xiong, 2002; Kansci *et al*., 2004). In general, liposomes may be appropriate lipid models to evaluate antioxidants for both food and lipoprotein particles containing phospholipids (Frankel *et al*., 1997). The antioxidative activity of HFIP 60 and its fractions can be explained by the interaction of the polar portion of protein hydrolysates with the polar environment of lecithin liposomes, thus affording better protection against oxidation. In addition, the antioxidative activity of HFIP 60 and its fractions was related to their chelating abilities on Cu^{2+}, which has been known to catalyze lipid peroxidation in the reaction mixtures. The decrease in both conjugated diene and TBARS was mostly found after 36 h of incubation. This might be caused by the transformation of conjugated dienes during advancement of

oxidation. Additionally, secondary products with low molecular weight were possibly lost, leading to a lower amount of such products (Stahnke, 1994; Stahnke, 1995).

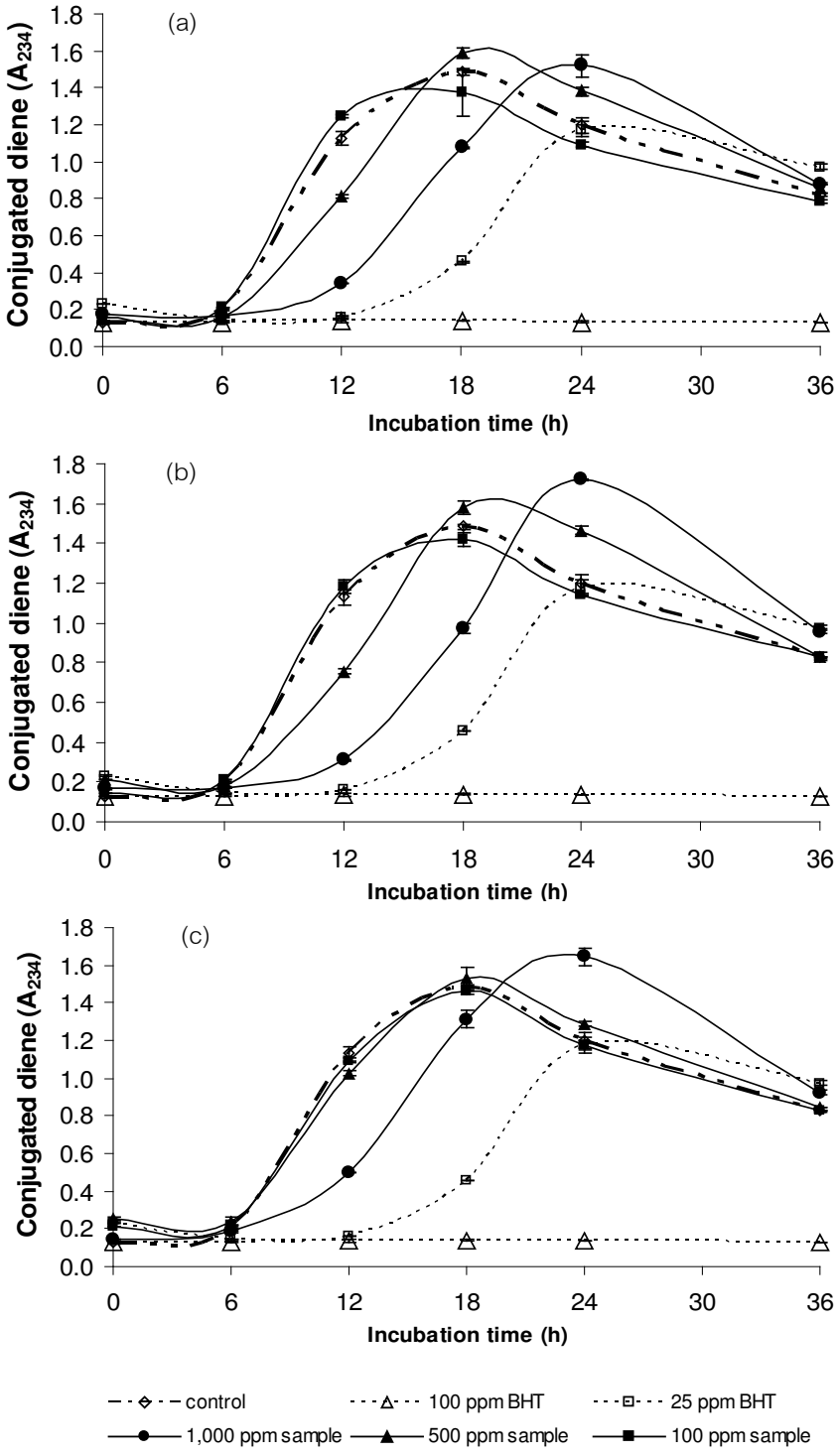

Figure 13 Changes in conjugated diene (A_{234}) of lecithin liposome system in the presence of HFIP 60 (a), E2: dichloromethane fraction (b) and E3: ethyl acetate fraction (c) at different levels in comparison with the control and the systems containing BHT at 25 ppm and 100 ppm. Bars represent the standard deviation from triplicate determinations.

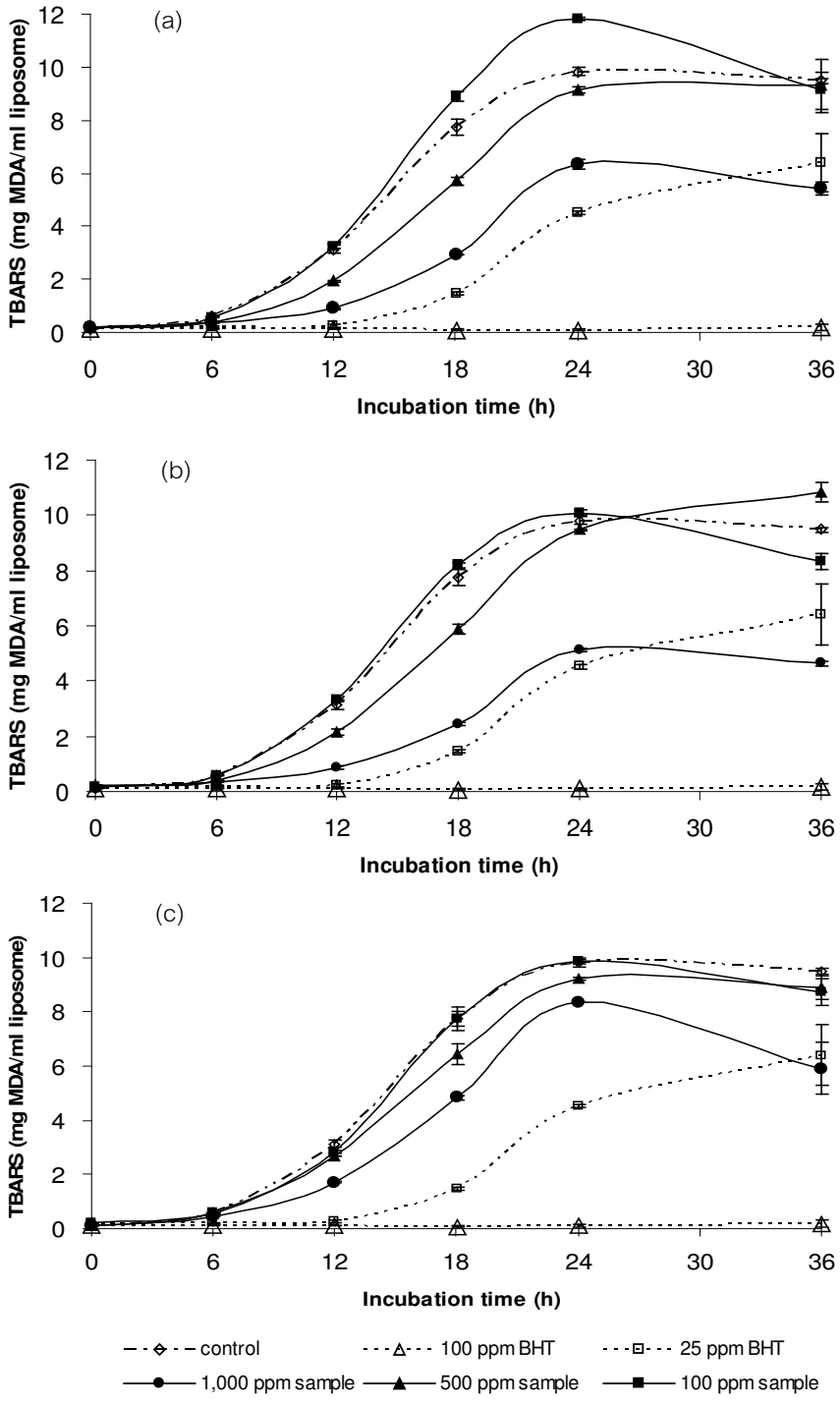

Figure 14 Changes in TBARS (mg MDA/ml liposome) of lecithin liposome system in the presence of HFIP 60 (a), E2: dichloromethane fraction (b) and E3: ethyl acetate fraction (c) at different levels in comparison with the control and the systems containing BHT at 25 ppm and 100 ppm. Bars represent the standard deviation from triplicate determinations.

Some amino acids and proteins have been reported as water-soluble antioxidants, which are able to prevent lipid oxidation in foods. However, some amino acids could function as antioxidant in oils (Cervato *et al*., 1999; Lu and Baker, 1986). Methionine and cysteine had antioxidative effect in soybean oil (Riisom *et al*., 1980), while the peptides containing two histidine or two tyrosine residues showed the antioxidative activity against the peroxidation of linoleic acid (Saito *et al.*, 2003). All amino acids have been shown to have antioxidant activity in some particular systems, which probably reflect the antioxidant nature of the NH_3R group (Taylor and Richardson, 1980). From the result, it was suggested that round scad protein hydrolysate and its fractions most likely functioned as both primary and secondary antioxidants in lipid oxidation model systems. Chan and Decker (1994) reported that anserine and carnosine, histidine-containing dipeptides, inhibited lipid oxidation through a combination of free radical scavenging and metal chelation. However, Aruoma *et al*. (1989) reported that carnosine and anserine were cabable of scavenging the hydroxyl radical, but not superoxide radical or hydrogen peroxide.

4. Compositions and some properties of round scad protein hydrolysate

Fish protein hydrolysates, obtained by controlled enzymatic hydrolysis, are among the best protein hydrolysates in term of nutritive value, balanced amino acid composition and high digestibility (Kristinsson and Rasco, 2000a). Protein hydrolysates are mainly used as animal feed because of their bitter flavor and fishy odor. Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides. Fish protein hydrolysates have been shown to be potential for nutritional or pharmaceutical applications (Amarowicz and Shahidi, 1997; Ravallec-Plé *et al*., 2001; Bordenave *et al*., 2002; Wu *et al*., 2003; Wergedahl *et al*., 2004)

4.1 Proximate analysis

Proximate composition of freeze-dried round scad protein hydrolysate is shown in Table 7. The round scad protein hydrolysate contained high protein content (68.97%). Therefore, round scad protein hydrolysate could be an essential source of proteins. The high protein content was a result of the solubilization of protein during hydrolysis, the removal of insoluble undigested non-protein substances and partial removal of lipid after hydrolysis (Benjakul and Morrissey, 1997). The percentage of solubilized protein was found to depend on the amount of lipids in the raw material. Raw material containing the highest amount of lipids gave the lowest percentage of solubilized protein (Šližyte *et al*., 2005a). Since the defatting of round scad mince with isopropanol was carried out prior to hydrolysis, the removal of fat layer after hydrolysis resulted in a low lipid content in round scad protein hydrolysate (0.15%). During the hydrolysis process, the muscle cell membranes tend to round up and form insoluble vesicles, leading to the removal of membrane structured lipids (Shahidi *et al*., 1995). Reduced lipid content was reported in the protein hydrolysate from salmon (Gbogouri *et al*., 2004), capelin (Shahidi *et al*., 1995) and herring (Sathivel *et al*., 2003; Liceaga-Gesualdo and Li-Chan, 1999). From the result, protein hydrolysate contained a high ash content (24.56%), most likely caused by the use of McIlvaine buffer required for pH adjustment for enzymatic hydrolysis.

Table 7 Chemical compositions of round scad protein hydrolysate

Compositions	Content (% wet wt. basis)*
Moisture	8.75±0.40
Protein	68.97±3.57
Lipid	0.15±0.03
Ash	24.56±2.56

*Means ± SD from three experiments.

4.2 Amino acid compositions

The amino acid compositions of freeze-dried round scad protein hydrolysate are presented in Table 8. Protein hydrolysate was rich in arginine, lysine, histidine and leucine, which accounted for 13.95, 13.94, 11.15 and 10.05% of total amino acids, respectively. From the result, protein hydrolysate had essential amino acid/non-essential amino acid ratio of 0.92. Fish and

shellfish have been reported to contain the high essential amino acid/non-essential amino acid ratio (Iwasaki and Harada, 1985). Therefore, the obtained protein hydrolysate could possibly be a dietary protein supplement to poorly balanced dietary proteins. Fish byproducts have a high percentage of essential amino acids and can be used to produce nutritious products (Shahidi, 1994). Shahidi *et al.* (1995) reported that capelin hydrolysate had the similar amino acid profiles to that of raw material. Nevertheless the sensitive amino acids such as methionine and tryptophan were present in smaller amounts after hydrolysis and decolourisation of hydrolysate by charcoal.

Table 8 Amino acid compositions of round scad protein hydrolysate

Amino acids	Content (%)
Asp + Asn	2.04
Ser	8.16
Glu + Gln	3.47
Gly	1.49
His	11.15
Arg	13.95
Thr	5.09
Ala	5.31
Pro	0.51
Cys-S-S-Cys	0.69
Tyr	5.20
Val	6.77
Met	4.51
Lys	13.94
Ile	3.15
Leu	10.05
Phe	4.52

Recently, protein hydrolysate from round scad has been reported to exhibit the antioxidative activity (Thiansilakul *et al.,* 2006). Amino acids in round scad protein hydrolysate possibly involved in antioxidative activity. Amino acids have been known to exhibit antioxidant

activity; tryptophan and histidine showed high antioxidative activity in comparison with methionine, cysteine, glycine and alanine (Riisom *et al.,* 1980). Antioxidative activity of histidine or histidine-containing peptide may be attributed to the chelating and lipid radical-trapping ability of the imidazole ring, whereas tyrosine residue in the peptide may act as a potent hydrogen donor (Uchida and Kawakishi, 1992; Je *et al.,* 2005). Generally, aromatic amino acids are considered as effective radical scavengers, because they can donate protons easily to electron deficient radicals. At the same time, their antioxidative stability can remain via resonance structures (Rajapakse *et al.,* 2005). From the result, round scad protein hydrolysate had a high nutritional value based on its amino acid profile.

4.3 Mineral contents

Freeze-dried round scad protein hydrolysate consisted of different minerals at different levels as shown in Table 9. Na, K, Ca and Mg were found at high contents while Ni was not detectable. The high content of Na (15.98%) reflected the use of McIlvaine buffer for hydrolysate preparation. The apparent metal ions could act as the pro-oxidants in the hydrolysate. Transition metal ions, particularly Cu and Fe have been known as the major catalysts for oxidation (Thanonkaew *et al*., 2006). Sathivel *et al*. (2003) reported that K, Mg, P, Na, S and Ca were abundant in herring and herring by-product hydrolysates and varied with substrate used. Fish protein hydrolysates usually contain a moderate NaCl content due to salting for conservation or pH adjustments during the pH shift process, however it was limited for application (Picot *et al*., 2006).

Table 9 Mineral contents of round scad protein hydrolysate

Minerals	mg/kg
Fe	4.28
Cu	2.26
Mn	0.95
Cd	0.10
Ni	ND[a]
Mg	245.36
P	12.78
K	0.42[b]

Ca	714.57
S	0.69
Na	15.98[b]

[a]ND is not detectable or below detection limit

[b]K and Na are expressed as % (w/w)

4.4 Color

During hydrolysis, round scad mince turned brownish. As a result, freeze-dried round scad protein hydrolysate was brownish yellow in color (L* = 58.00, a* = 8.38, b* = 28.32). Additionally, Flavourzyme with dark color also contributed to brownish color of resulting hydrolysate. From the study of Sathivel *et al*. (2003), it was reported that the color of whole herring and herring byproduct hydrolysates prepared using Alcalase varied with substrates. Herring gonad hydrolysate was the darkest (L* = 74.6) and most yellowish (b* = 18), whereas whole herring hydrolysate was the lightest (L* = 89.4) and least yellowish (b* = 8.0). Furthermore, the dark color of fish protein hydrolysate was probably from the oxidation of myoglobin and the melanin pigment of raw material (Benjakul and Morrissey, 1997). Therefore, the varying color of fish protein hydrolysate depended on the composition of the raw material and the hydrolysis condition.

4.5 Functional properties

4.5.1 Solubility

Nitrogen Solubility Index (NSI) of freeze-dried round scad protein hydrolysate was 99%. NSI has been used to determine protein solubility, mainly caused by the dispersion of protein in the solvent (Cheftel *et al*., 1985). Solubility is one of the most important physicochemical and functional properties of protein hydrolysates (Kinsella, 1976; Mahmoud *et al*., 1992). Good solubility of proteins is required in many functional applications, especially for emulsions, foams and gels. Soluble proteins provide a homogeneous dispersibility of the molecules in colloidal systems and enhance the interfacial properties (Zayas, 1997). From the result, it was suggested that the high solubility of round scad protein hydrolysate was possibly due to the size reduction and the formation of smaller, more hydrophilic and more solvated polypeptide units (Cheftel *et al*., 1985; Adler-Nissen, 1986; Chobert *et al*., 1988; Shahidi, 1994a). In addition, insoluble protein fractions were removed by centrifugation before the round scad

protein hydrolysate was freeze-dried. 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).

4.5.2 Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of round scad protein hydrolysates at various concentrations (0.1, 0.5, 1.0 and 3.0%) are shown in Table 10. EAI of round scad protein hydrolysate decreased with increasing concentrations ($p<0.05$). ESI of protein hydrolysate was lowered when hydrolysate concentration increased up to 0.5% ($p<0.05$). However, no differences in ESI were observed as the hydrolysate concentrations were above 0.5% ($p>0.05$). The EAI estimates the ability of the protein to aid in the formation and stabilization of newly created emulsion by giving units of area of the interface that is stabilized per unit weight of protein; this is determined by the turbidity of the emulsion at a wavelength of 500 nm (Pearce and Kinsella, 1978). Emulsification of protein hydrolysates is attributed to the adsorption of protein molecules on the surface of freshly formed oil droplets during homogenization and the formation of protective membrane that inhibits droplets coalescence (Dickinson and Lorient, 1994). Protein hydrolysates are surface-active materials and promote oil-in-water emulsion because of their hydrophilic and hydrophobic groups and their charge (Gbogouri *et al*., 2004; Rahali *et al*., 2000). The dependence of emulsifying activity on concentration of protein was explained based on adsorption kinetics (Kinsella, 1976). At low protein concentrations, protein adsorption at the oil–water interface is diffusion controlled. At high protein concentration, activation energy barrier does not allow protein migration to take place in a diffusion dependent manner, resulting in the accumulation of proteins in the aqueous phase. Thus, proteins or peptides were most likely localized in the aqueous phase and those proteins or peptides at low amount were migrated to interface. This resulted in the decrease in EAI of round scad protein hydrolysate at higher concentration. ESI also diminished with increasing hydrolysate concentration, presumably because of the increase in protein-protein interaction. This resulted in the lower protein concentration at the interface (Lawal, 2004). Thus,

the thinner film stabilizing the oil droplet was most likely formed. As a result, the lower emulsion stability was obtained.

The emulsifying properties of hydrolyzed protein are improved by controlling the extent of hydrolysis. Extensive hydrolysis results in a drastic loss of emulsifying properties. Although hydrolyzed proteins and small peptides diffuse rapidly and adsorb at the interface, they are less efficient in stabilizing emulsions because they could not unfold and reorient at the interface like a protein (Turgeon *et al*., 1991). There is a relationship between %DH and emulsifying properties for fish protein hydrolysates. Enzymatic hydrolysis had a negative influence on the capacity to form and stabilize emulsions as degree of hydrolysis increased for sardine protein hydrolysates (Quaglia and Orban, 1990). Šližyte *et al.* (2005b) reported that the reduction of the emulsifying properties of cod hydrolysates treated with Flavourzyme can also be explained by higher amount of free amino acids in the samples.

Table 10 Emulsifying properties of round scad protein hydrolysates at various concentrations

Hydrolysate concentrations (%)	Emulsifying activity index (m^2/g)*	Emulsion stability index (min)*
0.1	37.28±1.88^{a**}	35.89±6.65^{a}
0.5	23.67±0.06^{b}	11.96±0.22^{b}
1.0	13.38±0.02^{c}	12.09±0.28^{b}
3.0	5.31±0.02^{d}	12.32±0.04^{b}

*Means±SD from triplicate determinations.

**Different superscripts in the same column indicate significant differences ($p<0.05$).

4.5.3 Foaming properties

Foam expansions of round scad protein hydrolysates at various concentrations (0.1, 0.5, 1 and 3%) are depicted in Figure 15. Foam expansion at 0 min after whipping indicated the foam abilities of round scad protein hydrolysates, which increased from 23.33 to 70 % when hydrolysate concentrations increased from 0.1 to 3% ($p<0.05$). Sánchez and Patino (2005) revealed that increase in protein concentration resulted in the higher rate of diffusion. Foam expansion after whipping was monitored for 60 min to indicate the foam stability of round scad

protein hydrolysates. Within the first 10 min, round scad protein hydrolysate with the concentration of 3% showed the highest foam stability ($p<0.05$). The slight differences in foam expansion were observed among hydrolysates with different concentrations. Lawal (2004) pointed out that increase in foam stability with increasing concentration was a result of formation of stiffer foams. Foam stiffness develops from small bubble size and high viscosity. Therefore, foam stability could be improved by a greater protein concentration owing to the increased viscosity and the facilitated formation of a multilayer cohesive protein film at the interface. Foaming properties are physicochemical characteristics of proteins to form and stabilize foams. The stability of foams is a consequence of the well-ordered orientation of the molecules at the interface, where the polar head was placed in the aqueous phase and the hydrophobic chain faced the apolar component (Sánchez-Vioque *et al*., 2001). It is well known that peptides are more soluble than proteins (Adler-Nissen, 1986; Chobert *et al*., 1996) and can diffuse more easily to the interface (Qi *et al*., 1997). In general, peptides generate poorly stable foams since their short chains hinder the formation of a stable film around the gas bubbles (Adler-Nissen, 1979). A good cohesiveness of films is only reached with high-molecular weight peptides or partially hydrolyzed proteins (Bombara *et al*., 1994). Another aspect that facilitates the anchorage of peptides at the interface is the presence of an amphiphilic structure which is associated with the amino acid sequence (Attwood and Florence, 1983; Caessens *et al*., 1999).

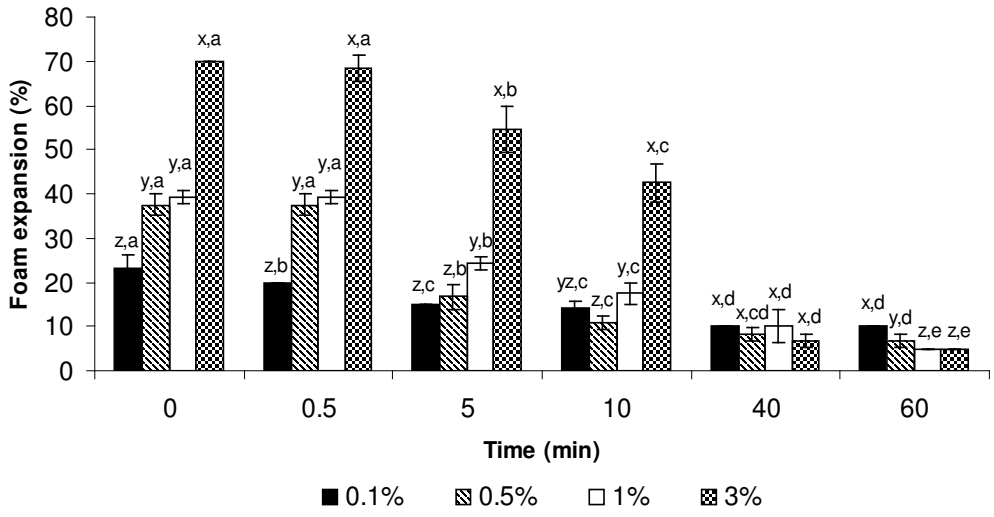

Figure 15 Foaming properties of round scad protein hydrolysates at various concentrations. Bars represent the standard deviation from triplicate determinations. Different letters a, b, c,

d and e within the same concentration indicate significant differences ($p<0.05$) and different letters x, y and z within the same time indicate significant differences ($p<0.05$).

To improve the functional properties of proteins, enzymatic modification has been extensively employed. Recently, Flavourzyme has been used to produce protein hydrolysates with acceptable functional properties (Kristinsson and Rasco, 2000b). The peptides produced by enzymatic hydrolysis have smaller molecular sizes and less secondary structures than those of the original protein (Jeon *et al*., 1999). The functional properties of hydrolyzed proteins are governed mainly by their molecular weight (Adler-Nissen, 1986) and hydrophobicity (Turgeon *et al*., 1992). Generally, fish protein hydrolysates may be expected to have increased solubility and significant changes in foaming, water holding, fat adsorption and emulsifying properties, compared to those of native proteins or common food protein ingredients (Sathivel *et al*., 2003; Gbogouri *et al*., 2004; Šližyte *et al*., 2005b). The physiochemical properties of protein hydrolysates are therefore intrinsically related to a number of parameters such as 1) the purity of the protein substrate; 2) the pretreatment of the protein substrate; 3) the specificity of the enzyme used for proteolysis; 4) the physicochemical conditions (pH, temperature, ionic strength, activator) used during hydrolysis; 5) the DH; 6) the technique used for enzyme inactivation (heat treatment, acidification, or membrane filtration); and 7) the use of posthydrolysis treatments (adsorbents for free amino acids, membrane separation) (Gauthier and Pouliot, 2003).

5. Study on the stability of round scad protein hydrolysate during storage

5.1 Antioxidative activities

Antioxidative activity of freeze-dried round scad protein hydrolysate during storage at 25°C and 4°C is depicted in Figure 16. Slight decreases in DPPH radical scavenging activity of round scad protein hydrolysate under both storage conditions were observed within the first week of storage ($p<0.05$) and no marked changes were observed thereafter (Figure 16 (a)). However, no changes in reducing power and metal chelating activity of round scad protein hydrolysate under both storage conditions were found within first 2 weeks of storage ($p>0.05$).

Subsequently, the slight decreases were noticeable ($p<0.05$) (Figure 16 (a) and 15 (b)). This might be due to the destruction of antioxidative compounds as the storage time increased, leading to some losses in antioxidative activity. From the result, round scad protein hydrolysate was more stable when stored at 4°C in comparison with 25°C.

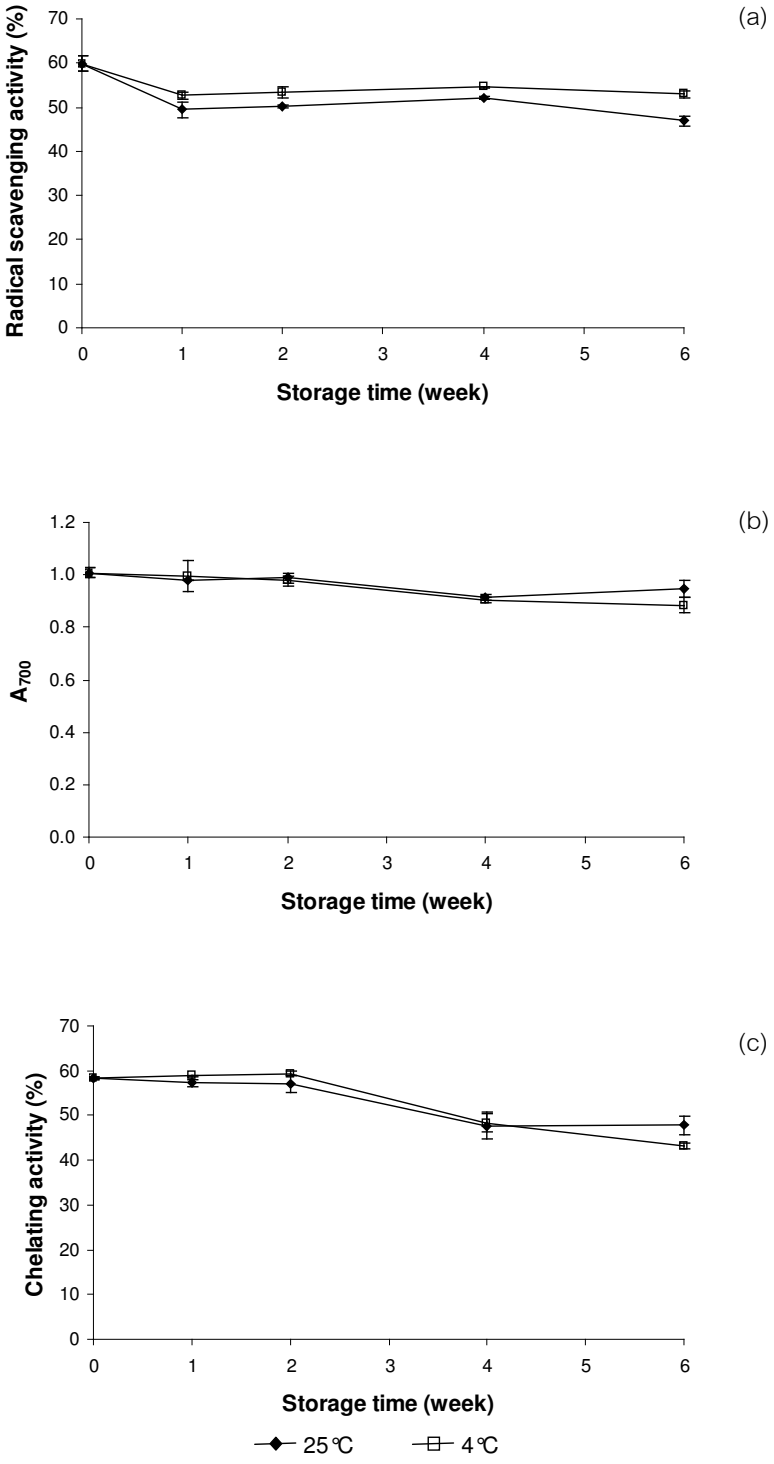

Figure 16 Changes in DPPH radical-scavenging activity (a), reducing power (b) and chelating activity (c) of round scad protein hydrolysate (protein concentration = 10 mg/ml) during storage at 25°C and 4°C for 6 weeks. Bars represent the standard deviation from triplicate determinations.

5.2 Solubility

The solubility of freeze-dried round scad protein hydrolysate was expressed as the percentage of soluble nitrogen compounds with respect to total nitrogen in each sample. Changes in solubility of round scad protein hydrolysate during storage at 25°C and 4°C are shown in Figure 17. During 6 weeks of storage, solubility of round scad protein hydrolysate stored at both temperatures slightly decreased ($p<0.05$). No substantial differences in solubility were observed between hydrolysate kept at 4°C and 25°C throughout the storage of 6 weeks. The decrease in solubility might be due to the aggregation of those peptides with the concomitant formation of a larger aggregate with the lowered solubility.

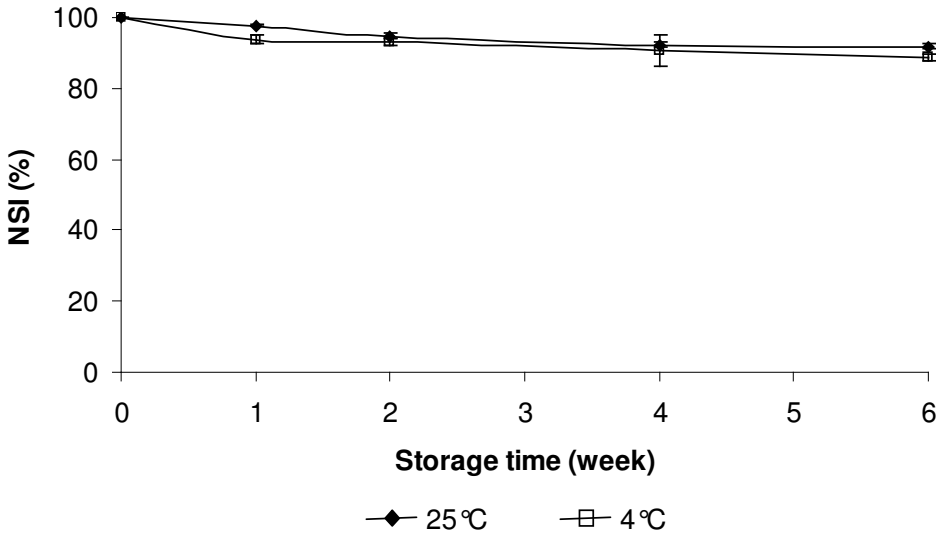

Figure 17 Changes in solubility (nitrogen solubility index: NSI) of round scad protein hydrolysate during storage at 25°C and 4°C for 6 weeks. Bars represent the standard deviation from triplicate determinations.

5.3 Color

Freeze-dried round scad protein hydrolysate stored at 25°C and 4°C was dissolved to obtain the concentration of 10 mg/ml and the color was measured (Table 11). No changes in L*, a* and b*-values of the solution of protein hydrolysate stored at 4°C were observed up to 6 weeks of storage ($p>0.05$). Conversely, changes in a* and b*-value of the solution of freeze-dried protein hydrolysate stored at 25°C were noticeable at week 6 ($p<0.05$). The increase in b*-value indicated the increase in yellowness of samples during storage at 25°C. Hoyle and Merritt (1994) reported that herring hydrolysates had the decreases in lightness scores and increases in b values, which indicated the darkening during storage. The formation of brown pigments might result from aldol condensation of carbonyls produced from lipid oxidation upon reaction with basic groups in proteins. From the result, it pointed out that the color of freeze-dried round scad protein hydrolysate was stable for up to 6 weeks when storage at 4°C and the changes in color occurred when storage temperature increased.

Table 11 Changes in L* (lightness), a* (redness/greenness) and b* (yellowness/blueness)-values[#] of the solution prepared from round scad protein hydrolysate stored at 25°C and 4°C for different times

Storage time (week)		0	1	2	4	6
25°C	L*	12.50±0.79$^{ax##}$	14.53±1.66^{ax}	13.55±1.54^{ax}	14.01±3.16^{ax}	13.44±2.30^{ax}
	a*	-1.00±0.15^{ax}	-1.15±0.05^{ay}	-1.23±0.01^{ax}	-1.19±0.17^{ay}	-1.56±0.23^{by}
	b*	0.45±0.13^{bx}	0.89±0.06^{bx}	0.39±0.06^{bx}	0.49±0.72^{bx}	1.59±0.15^{ax}
4°C	L*	12.50±0.79^{ax}	13.47±0.61^{ay}	12.72±0.81^{ay}	11.83±1.38^{ay}	12.29±0.43^{ay}
	a*	-1.00± 0.15^{ax}	-0.99±0.09^{ax}	-1.24±0.22^{ax}	-1.02±0.14^{ax}	-1.35±0.27^{ax}
	b*	0.45±0.13^{ax}	0.37±0.17^{ay}	0.36±0.26^{ax}	0.08±0.85^{ax}	0.76±0.07^{ay}

[#]Means±SD from triplicate determination.

[##]Different superscripts in the same row, a and b, indicate significant differences ($p<0.05$). Different superscripts in the same column, x and y, within the same color parameter. indicate significant differences ($p<0.05$).