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Antioxidant and biochemical properties of protein hydrolysates prepared from Silver carp (*Hypophthalmichthys molitrix*)

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Abstract

The antioxidant and biochemical properties of enzymatically hydrolyzed silver carp (*Hypophthalmichthys molitrix*) protein were studied. The molecular weight of the main peaks of the hydrolysates by both Alcalase and Flavourzyme was lower than 5000 Da. The hydrolysates treated by Alcalase for $\geqslant$1.5 h (hydrolysis time) showed that the relative proportion of <1000 Da fraction was more than 60%. For the biochemical properties, hydrolysis by both enzymes increased protein solubility to above 75% over a wide pH range; and when the hydrolysis time was prolonged (>3 h), the colour of the hydrolysates turned yellowish. The protein hydrolysates exhibited significant hydroxyl radical-scavenging activity and inhibited linoleic acid peroxidation. For Alcalase treatment, the hydroxyl radical-scavenging activity and the inhibition of linoleic acid peroxidation of hydrolysates appeared to reach a maximum level for 1.5, 2.0 h of hydrolysis, respectively; and their antioxidant activity was close to that of α-tocopherol in a linoleic acid emulsion system, and carnosine in the 2-deoxyribose oxidation system. The hydrolysate with lower molecular weight distribution possessed stronger Fe^{2+} chelation ability at a sample concentration of 5.0 mg/mL. The results suggested that the antioxidant activity of silver carp protein hydrolysates were related to its degree of hydrolysis (DH), hydrolysis time and molecular weight.
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Keywords: Silver carp; Protein hydrolysate; Antioxidative activity; Hydrolysis

1. Introduction

Silver carp is a prevalent freshwater fish in China, with an estimated annual harvest of 3,524,800 metric tons in 2005 (Ministry of Agriculture of the people's Republic of China, 2006). However, silver carp with extremely muddy flavour and many fish bones is a low market-value resource. The hydrolysis of protein with proteolytic enzymes can provide more marketable and value-added products of fish protein hydrolysate (Guerard, Guimas, & Binet, 2002; Jeon, Byun, & Kim, 2000; Shahidi, Han, & Synowiecki, 1995; Suthasinee, Sittiwat, Manop, & Apinya, 2005).

Lipid peroxidation leads to the development of undesirable off-favours and potentially toxic reaction products (Maillard, Soum, Meydani, & Berset, 1996). Many synthetic antioxidants may be used to retard lipid peroxidation in a number of fields. However, the safety and negative consumer perception of synthetic antioxidants restricts their applications in food products (Park, Jung, Nam, Shahidi, & Kim, 2001). Moreover, reactive radicals are implicated in the ethiology of age-associated chronic diseases such as cardiovascular diseases, neurodegenerative disorders, diabetes, and certain types of cancer (Ames, Shigenaga, & Hagen, 1993). Therefore, there is a growing interest to identify antioxidative properties in many natural sources including some dietary protein compounds.

Protein hydrolysates from different sources, such as milk protein (Tong, Sasaki, McClements, & Decker, 2000), maize zein (Kong & Xiong, 2006), egg-yolk (Sakanaka &

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Tachibana, 2006), porcine proteins (Saiga, Tanabe, & Nishimura, 2003), yellow stripe trevally (Klompong, Benjakul, Kantachote, & Shahidi, 2007), yellowfin sole frame (Jun, Park, Jung, & Kim, 2004), herring (Sathivel et al., 2003), mackerel (Wu, Chen, & Shiau, 2003), have been found to possess antioxidant activity. The operational conditions employed in the processing of protein isolates, the type of protease and the degree of hydrolysis affect the antioxidant activity (Peña-Ramos & Xiong, 2002). Levels and compositions of free amino acids and peptides were reported to determine the antioxidant activities of protein hydrolysates (Wu et al., 2003). Moreover, the utilization of proteins or their hydrolysates for food and/or cosmetic applications not only presents additional advantages over other antioxidants, but also they confer nutritional and functional properties (Chen, Muramoto, & Yamauchi, 1995; Moure, Domínguez, & Parajó, 2006).

However, there is little information regarding the antioxidant effect of protein hydrolysates from the silver carp (*Hypophthalmichthys molitrix*) by enzymatic treatment. The objective of this study was to determine the antioxidant activity of the protein hydrolysate by Alcalase and Flavourzyme from the low-value silver carp. Meanwhile, the solubility and colour of hydrolyastes derived from silver carp were evaluated. The understanding of antioxidative properties of silver carp protein hydrolysate may lead to utilize fish protein as a potent natural antioxidant.

2. Materials and methods

2.1. Materials

Silver carp (*Hypophthalmichthys molitrix*), 850–900 g/fish, were purchased from Chengyang supermarket in Qingdao, China. Fish after capture were filleted and the ordinary muscle (contained 18.5% protein) was collected and ground to uniformity. The ground muscle was stored in a polyethylene bag at −40 °C until used.

2.2. Chemicals and reagent

Alcalase 2.4 L and Flavourzyme 500 L were obtained from Novozymes China Inc. (Jinan, Baitai), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (Ferrozine), thiobarbituric acid (TBA), α-tocopherol, linoleic acid, butylated hydroxytoluene (BHT), ascorbic acid, cytochrome C, insulin, Vitamin B_{12}, hippuryl-histydilleucine, Glutathione, 2-deoxy-D-ribose,were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). All other chemicals used in the experiments were of analytical grade.

2.3. Production of protein hydrolysates

The mince was mixed with isopropanol at a ratio of 1:1 (w/v), homogenized and defatted at 40 °C under slow agitation. The supernatant was then removed and the precipitate was air-dried at room temperature (25–28 °C).

The resulting isopropanol-defatted mince was mixed with distilled water in a ratio of 1:7 (w/v) and homogenized at a speed of 20,000 rpm for 1 min using homogenizer (Shanghai, China). The homogenate was preincubated at 50 or 60 °C for 20 min prior to enzymatic hydrolysis using Flavourzyme and Alcalase, respectively. The hydrolysis reaction was started by the addition of the enzyme (Alcalase or Flavourzyme) at a level of 0.5% (w/w). The reaction with Alcalase was conducted at pH 8.0, 60 °C and with Flavourzyme at pH 7.0, 50 °C for 0.25, 0.5, 1, 2, 3, 4, 5 and 6 h. The pH of the mixture was maintained constant during hydrolysis using 2 M NaOH. After hydrolysis, the pH of the broths was brought to 7.0, and the solutions were then heated at 90 °C for 10 min to inactivate the enzymes. Hydrolysates were centrifuged at 9000*g* for 15 min, using a centrifuge MR 23i (JOUAN, Saint-Herblain, France). The hydrolysates were freeze-dried (EYELA freeze-dryer, Shanghai, China), pulverized, placed in sealed bags, and stored at −20 °C before use.

The DH of hydrolyzed protein was determined using the pH-stat method (Alder-Nissen, 1986). DH was then calculated as follows:

$$\mathrm{DH}\ (\%) = \frac{BN_{\mathrm{b}}}{M_{\mathrm{p}}\alpha h_{\mathrm{tot}}} \times 100$$

where B is the amount of alkali consumed (ml), N_{b} is the normality of alkali, M_{p} is the mass of the substrate (protein in grammes, % N × 6.25), $1/\alpha$ is the calibration factors for pH-stat, and h_{tot} is the content of peptide bonds. For silver carp protein, $h_{\mathrm{tot}} = 7.24$ mmol/g of protein.

2.4. Determination of the molecular weight distribution

Molecular weight distributions of silver carp hydrolysates were determined by gel permeation chromatography (GPC) using a high-performance liquid chromatography (HPLC) system (Agilent 1100, USA). A TSK gel 2500 PWXL column (30 mm i.d. × 7.8 mm, Tosoh, Tokyo, Japan) was equilibrated with 50% acetonitrile (v/v) in the presence of 0.1% trifluoroacetic acid. The hydrolysates were applied to the column and eluted at a flow rate of 0.5 mL/min and monitored at 220 nm at room temperature. A molecular weight calibration curve was prepared from the average retention time of the following standards: cytochrome C (12,500 Da), insulin (5734 Da), vitamin B12 (1355 Da), hippuryl-histydilleucine (429.5 Da), and glutathione (309.5 Da) (Sigma Co., St. Louis, MO, USA).The total surface area of the chromatograms was integrated and separated into five ranges (>10,000, 5000–10,000, 1000–5000, 500–1000, <500 Da), expressed as a percentage of the total area.

2.5. Protein Solubility

Freeze-dried hydrolysates of silver carp protein were suspended (1.5%) in distilled water and pH of the mixture was adjusted to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 with 1 or 6 N

HCl and 1 or 6 N NaOH. The mixture was stirred at room temperature for 30 min and centrifuged at 7500*g* for 15 min. Protein contents in the supernatant were determined using the Biuret method (Robinson & Hodgen, 1940). Total protein content in the sample was determined after solubilization of the sample in 0.5 N NaOH.

$$\text{Solubility } (\%) = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}} \times 100$$

2.6. Colour measurement

The colour of freeze-dried hydrolysate was measured by colorimeter (Shen Guang, Model WSC-S, Shanghai, China). L^*, a^* and b^* parameters indicate lightness, redness and yellowness, respectively.

2.7. Amino acid analysis

The PICO TAG method, with modification, was used for measuring the amino acid profile of the hydrolysate (Bildlngmeyer, Cohen, Tarvin, & Frost, 1987). The dry sample (weight equivalent to 4% protein) was added with 6 N HCl (15 ml) and placed in the oven at 110 °C for 24 h. Internal standard (10 ml) was added to the mixture. After derivatisation, 100 μl PICO TAG diluent was added and mixed. Sample (100 μl) was then injected into the HPLC and analysed with a Water's PICO TAG amino acid analyzer.

2.8. Determination of antioxidative activities

2.8.1. Inhibition of linoleic acid autoxidation

In vitro lipid peroxidation inhibition activity of peptides was determined by assessing their ability to inhibit oxidation of linoleic acid in an emulsified model system (Osawa & Namiki, 1985). Briefly, peptide sample was dissolved in 2.5 ml of 50 mM phosphate buffer (pH 7.0) and added into 2.5 ml of 50 mM linoleic acid in ethanol (95%) were mixed in a 10 ml tube, and the final volume was adjusted to 6.25 ml with distilled water. In a single experiment, sample was replaced with α-tocopherol, carnosine or butylated hydroxytoluene (BHT) for comparative purposes.

The reaction mixture was incubated in tubes with silicon rubber caps at 45 °C in dark and degree of linoleic acid oxidation was spectrophotometrically measured at 48-h intervals. Aliquot (0.1 ml) of reaction mixture was mixed with 75% ethanol (4.7 ml) followed by the addition of 30% ammonium thiocyanate (0.1 ml) and 0.02 M ferrous chloride solution (0.1 ml) in 3.5% HCl. After 3 min, the degree of colour development, which represents the linoleic acid oxidation, was measured at 500 nm with a Hitachi UV-2550 spectrophotometer (Hitachi, Tokyo, Japan).

2.8.2. Hydroxyl radical-scavenging activity

The effect of hydroxyl radicals was assayed using the 2-deoxyribose oxidation method (Sakanaka & Tachibana, 2006) with slight modifications. The reaction mixture contained 1.0 ml of 0.2 M sodium phosphate buffer (pH 7.4), 0.20 ml of 10 mM 2-deoxyribose, 0.20 ml of 10 mM $FeSO_4$–EDTA, 0.20 ml of 10 mM hydrogen peroxide, 0.2 ml of distilled water, and 0.2 ml of the sample solution in a tube. The reaction was started by the addition of hydrogen peroxide.

The reaction solution was incubated at 37 °C for 1 h, then the reaction was stopped by adding 1.0 ml of 2.8% trichloroacetic acid and 1.0 ml of 1.0% thiobarbituric acid. The mixture was boiled for 10 min, cooled in ice, and then measured at 532 nm. For comparison, carnosine was used. Hydroxyl radical-scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radicals. The results were calculated as the percentage inhibition according to the following formula:

$$\%\ \text{inhibition} = \left[\frac{(C - \text{CB}) - (S - \text{SB})}{(C - \text{CB})}\right] \times 100$$

where S, SB, C, and CB are the absorbance of the sample, the blank sample, the control, and the blank control, respectively.

2.8.3. Metal-chelating activity

The ability of peptides to chelate ferrous ions was assessed using the method of Decker and Welch (1990). One milliliter of peptide solution (5 mg/ml) was first mixed with 3.7 ml of distilled water. Then it was reacted with a solution containing 0.1 ml 2 mM $FeCl_2$ and 0.2 ml of 5 mM Ferrozine. After 10 min, the absorbance of the reaction mixture was measured at 562 nm. Chelating activity was calculated as a percentage using {1 − (absorbance of the sample at 562 nm-absorbance of the blank sample at 562 nm)/(absorbance of control at 562 nm)} × 100.

2.9. Statistical analysis

All the tests were done in triplicate and data were averaged. Standard deviation was also calculated. Duncan's multiple-range test (Steel & Torrie, 1980) was used to evaluate significant differences ($P < 0.05$) between the means for each sample.

3. Results and discussion

3.1. Enzymatic hydrolysis

The hydrolysis of silver carp protein with Alcalase or Flavourzyme proceeded at a high rate during the initial 15 min and then slowed down thereafter (Fig. 1), which indicated that maximum cleavage of peptides occurred within 15 min of hydrolysis. This result was similar to be reported for enzymatic hydrolysis of different protein substrates such as fish (Benjakul & Morrissey, 1997; Guerard et al., 2002), whey (Mutilangi, Panyam, & Kilara, 1995), wheat gluten (Kong, Zhou, & Qian, 2007). With the same protein substrate and the same amount of enzyme, Alcalase

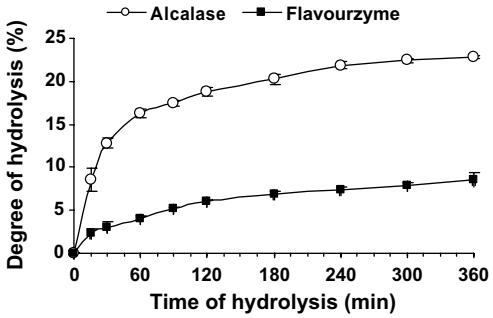

Fig. 1. Degree of hydrolysis (DH) of silver carp defatted meat during hydrolysis with Alcalase and Flavourzyme at 0.5:100 (w/w) enzyme/substrate and 1:7 (w/w) substrate/water. Bars represent standard deviations from triplicate determinations.

showed the higher DH values for silver carp protein hydrolysis than Flavourzyme over the entire hydrolysis period. The higher ($p < 0.01$) level of DH by Alcalase treatment suggested that Alcalase has higher affinity and, therefore, is a more efficient enzyme choice than Flavourzyme for preparing silver carp protein hydrolysates. In general, alkaline proteases, including Alcalase, showed higher activities than acid or neutral proteases such as Flavourzyme (Klompong et al., 2007; Rebeca, Pena-Vera, & Diaz-Castaneda, 1991).

3.2. The molecular weight distribution of protein hydrolysates

The molecular weight distribution of protein hydrolysates obtained with Alcalase and Flavourzyme is presented in Fig. 2 by TSK gel 2500 PWXL column. The molecular weight of the main peaks of the hydrolysates by Alcalase or Flavourzyme was lower than 5000 Da. The hydrolysates treated by Alcalase for 1.5 and 4 h indicated similar molecular weight distribution, which showed that the relative proportion of <1000 Da fraction was more than 60%, and the reaction time prolonging did not produce any significant improvement after 1.5 h. From this result, the protein hydrolysates by Alcalase were likely a mixture of extremely small peptides. Chromatographic determination of Alcalase-hydrolyzed whey protein revealed numerous oligopeptides with molecular weight estimated to <1000 Da and between 1000 and 2500 Da (Peña-Ramos, Xiong, & Arteaga, 2004). Doucet, Otter, Gauthier, and Foegeding (2003) also reported that >80% of Alcalase-hydrolysated whey protein, was small peptides with of molecular weight <2000 Da after 5 h of hydrolysis.

3.3. The solubility of protein hydrolysates

The solubilities of silver carp protein hydrolysates of different hydrolysis time in the pH range of 2–12 are shown in Fig. 3. The solubility increased from 5% for silver carp protein to over 75% for all hydrolysates over a wide pH range. The solubilities of silver carp protein hydrolysates were quite low at pH 5, whereas solubilities above 80% were noticeable at other pHs tested. The hydrolysates had higher solubilities with higher DH.

The hydrolysates of yellow stripe trevally meat protein (Klompong et al., 2007) and salmon byproduct (Gbogouri, Linder, Fanni, & Parmentier, 2004) had an excellent solubility at high degrees of hydrolysis. From these results, we can deduce that the solubility increases with the protein fraction with lower molecular mass (Fig. 2) at higher degrees of hydrolysis. The smaller peptides from proteins are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water and increase solubility (Gbogouri et al., 2004).

3.4. The colour of Protein hydrolysates

The colours of the freeze-dried protein hydrolysates were also investigated by colorimeter (Table 1). L^* values of silver carp protein hydrolysates with Alcalase and Flavourzyme were markedly ($p < 0.05$) decreased after 4 h hydrolysis, and a^*, b^* values of silver carp protein hydrolysate were markedly ($p < 0.05$) increased as the hydrolysis time was prolonged.

After 6 h of hydrolysis, the increase in a^*, b^* values of nonhydrolyzed silver carp protein were, respectively, 3.34,

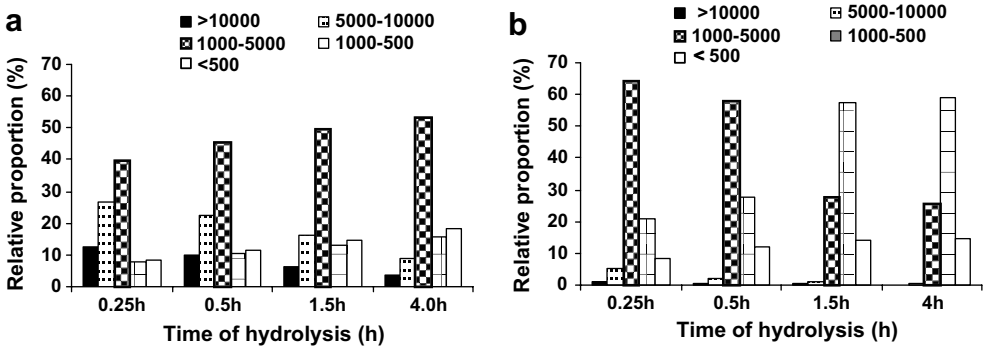

Fig. 2. Molecular weight distribution profiles of silver carp protein hydrolysates with different hydrolysis of time: (a) 0.25 h (DH = 2.3%), 0.5 h (DH = 3.1%), 1.5 h (DH = 5.1%), 4.0 h (DH = 7.7%) of hydrolysis with Flavourzyme, respectively; (b) 0.25 h (DH = 8.6%), 0.5 h (DH = 12.8%), 1.5 h (DH = 17.4%), 4.0 h (DH = 21.8%) of hydrolysis with Alcalase, respectively.

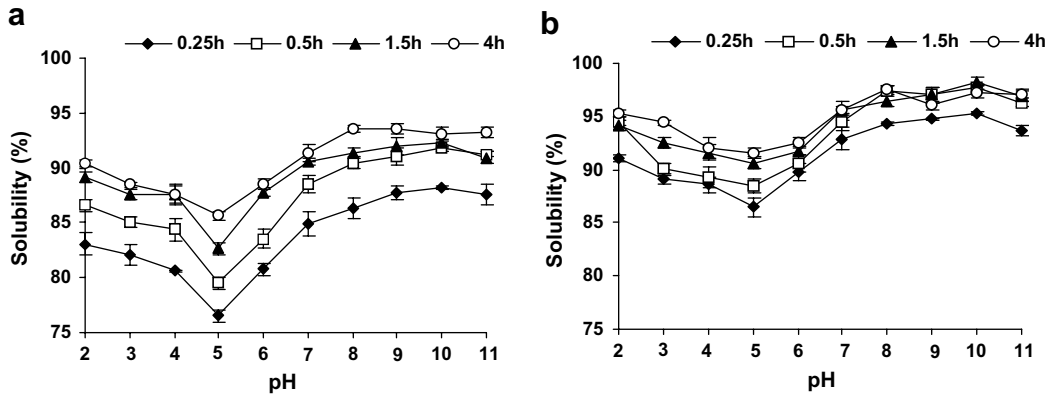

Fig. 3. Solubility of silver carp protein hydrolysates prepared using Flavourzyme (a) and Alcalase (b) with different hydrolysis of time as influenced by pHs. Bars represent standard deviations from triplicate determinations.

Table 1
L^*, a^* and b^* values of silver carp protein hydrolysates produced at different hydrolysis times

Enzyme	Color parameter	Hydrolysis time (h)							
		0	0.25	1.0	2	3	4	5	6
Alcalase	L^*	86.08b	89.28a	88.06a	86.11b	85.56b	82.44c	81.13c	78.18d
	a^*	−5.51d	−3.29c	−2.87bc	−2.77b	−2.70b	−2.57ab	−2.30a	−2.17a
	b^*	10.27d	13.72c	14.50c	14.40c	14.67c	18.34b	20.03a	20.26a
Flavourzyme	L^*	86.08a	86.30a	85.94a	85.44a	85.56a	83.43b	83.16b	82.95b
	a^*	−5.51d	−3.19c	−2.68b	−2.70b	−2.71b	−2.56b	−2.27ab	−2.06a
	b^*	10.27e	12.84d	13.48d	14.45c	14.67c	17.51b	18.46b	20.51a

Different letter in the same row indicate significant differences ($p < 0.05$).

9.99 for Alcalase; and 3.45, 10.24 for Flavourzyme. However, the decrease in L^* values of nonhydrolyzed silver carp protein were 7.90, 3.13 for Alcalase and Flavourzyme, respectively, after 6 h of hydrolysis. The colour of silver carp protein hydrolysate with Alcalase and Flavourzyme changed slowly within 3 h hydrolysis, and then was yellowish after 6 h hydrolysis.

The colour of protein hydrolysate from defatted round scad was brownish yellow in colour ($L^* = 58.00$, $a^* = 8.38$, $b^* = 28.32$) during hydrolysis (Thiansilakul, Benjakul, & Shahidi, 2007). Dark-fleshed fish, such as sardine, mackerel and round scad, contained a high amount of myoglobin (Chaijan, Benjakul, Visessanguan, & Faustman, 2004). The dark color of fish protein hydrolysate resulted from the oxidation of myoglobin and the melanin pigment of the raw material (Benjakul & Morrissey, 1997). Moreover, the formation of brown pigments might result from aldol condensation of carbonyls produced from lipid oxidation upon reaction with basic groups in proteins via Maillard reaction (Van Boekel, 1998).

Silver carp contains dark meat. Therefore, the longer hydrolysis time with higher temperature probably accelerated the pigments oxidation and Maillard reaction in the muscle. As a consequence, longer hydrolysis time with Alcalase or Flavourzyme led to the darkening and browning of silver carp protein hydrolysates.

3.5. Inhibition of linoleic acid autoxidation

The silver carp protein hydrolysates were determined by inhibition of lipid peroxidation in a linoleic acid model system. As shown Fig. 4a, all hydrolysates could act as significant retarders ($p < 0.01$) of lipid peroxidation. The hydrolysates inhibiting lipid oxidation exhibited a nonlinear pattern. For Alcalase-hydrolyzed protein, the effect of inhibiting lipid oxidation increased initially and peaked on 1.5 h of hydrolysis, followed by a slight decline during the 6 h of hydrolysis. For Flavourzyme-hydrolyzed protein, the shape of these progress curves was similar to Alcalase-hydrolyzed protein, and the effect of inhibiting lipid oxidation peaked on 4 h of hydrolysis. This activity of 1.5 h hydrolysate with Alcalase was higher than natural antioxidant α-tocopherol, carosine and was close to highly active synthetic antioxidant, BHT (Fig. 4b).

Generally, the lack of a direct relationship between antioxidant activity and DH suggested that the specific composition (e.g., type of peptides, ratio of different freed amino acids) was an important factor as well (Kong & Xiong, 2006; Pihlanto, 2006). Many researches reported that low molecular weight peptides showed higher antioxidant activity (Rajapakse, Mendis, Byun, & Kim, 2005). The hydrolysate of jumbo squid skin gelatin was fractionated using ultrafiltration technique, and the highest antioxidant

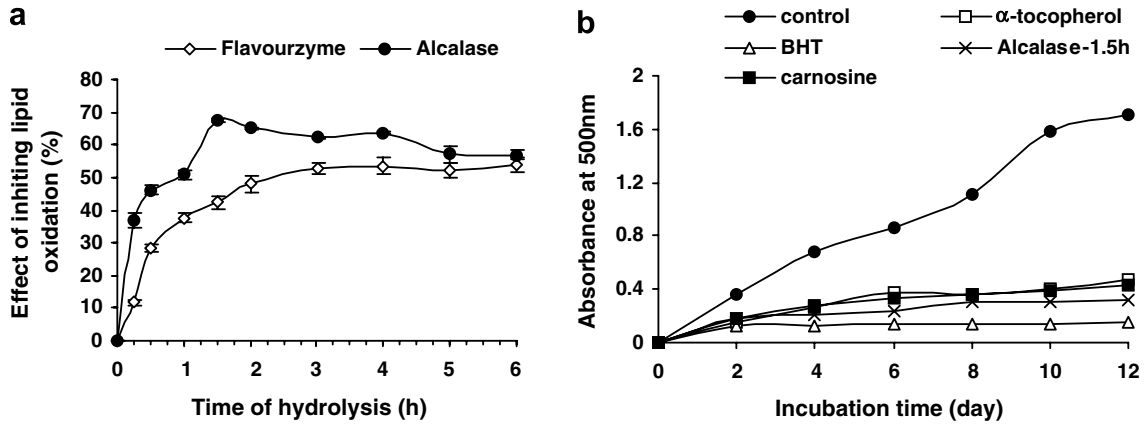

Fig. 4. Effect of silver carp protein hydrolysates on oxidation of linoleic acid: (a) Inhibition of lipid peroxidation by silver carp protein hydrolysates at different hydrolysis time was determined as described in the text after 8 days; (b) Comparison of antioxidant activities between Alcalase-hydrolyzed protein (1.5 h of hydrolysis) and α-tocopherol, caronsine and BHT. Bars represent standard deviations from triplicate determinations.

activity was observed in the lowermost molecular weight peptide fraction (<3000 Da) (Mendis, Rajapakse, Byun, & Kim, 2005). Hernández-Ledesma, Dávalos, Bartolomé, and Amigo (2005) also investigated the 3000 Da fraction was mainly responsible for the antioxidant activity (oxygen radical absorbance capacity -fluorescein values) found in the whole hydrolysates from whey proteins α-lactalbumin and β-lactoglobulin. Wu et al. (2003) found that the antioxidant activity of hydrolysate derived from mackerel protein reached a maximum after 10 h of hydrolysis and then a slight decline during the 25 h of hydrolysis. The levels of FAAs and peptides also reached a maximum at 10 h of hydrolysis, and a peptide from fish protein hydrolysates with a 1400 Da MW possessed stronger antioxidant activity than a 900 or 200 Da peptide. In addition, Kong and Xiong (2006) reported that if the hydrolysis of zein protein with Alcalase became too extensive (time of hydrolysis >4 h), the hydrolysate could reduce the peptide's ability to act as a physical barrier to prevent oxidants from reaching the lipid fraction in the liposome.

From this result, the hydrolysate by Alcalase treatment showed the higher effect of inhibiting lipid oxidation than Flavourzyme. The ability of hydrolysates to chemically inhibit lipid oxidation was influenced by DH. This might be due to the possibility that hydrolysates may contain both antioxidative and pro-oxidative components making them a less efficient antioxidant system against lipid oxidation (Pihlanto, 2006). Moreover, the hydrolysates which contained the non-protein composition (e.g. the brown pigments during the hydrolysis) might influence the antioxidative activity against lipid oxidation.

3.6. Hydroxyl radical-scavenging activity

The scavenging effect against hydroxyl radicals was investigated by using the 2-deoxyribose oxidation method. Fig. 5a shows the hydroxyl radical-scavenging effects of Silver carp protein hydrolysates. The protein hydrolysates prepared by both Alcalase and Flavourzyme exhibited significant hydroxyl radical-scavenging activity. The radical-

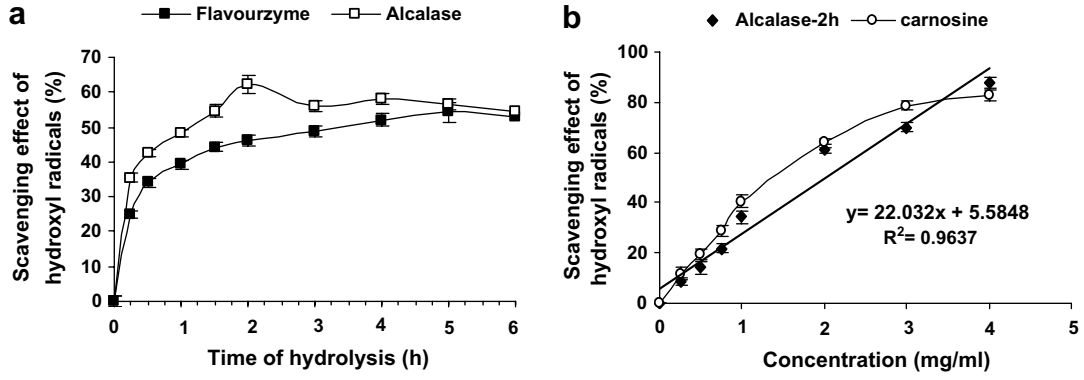

Fig. 5. Hydroxyl radical-scavenging activity of silver carp protein hydrolysates: (a) hydroxyl radical-scavenging activity at different hydrolysis time; (b) Comparison of antioxidant activities between Alcalase-hydrolyzed protein (2.0 h of hydrolysis) and caronsine at different concentrations. Regression equation was obtained from linear regression of the concentrations of Alcalase-2 h and hydroxyl radical-scavenging effects. Bars represent standard deviations from triplicate determinations.

scavenging activity of protein hydrolysate by Alcalase treatment increased initially and peaked on 2 h-hydrolysis, followed by a slight decline during the 6 h of hydrolysis. However, the protein hydrolysates by Flavourzyme treatment showed the lower radical-scavenging activity, which could be related to their lower DH with this enzyme in comparison with Alcalase. Moreover, the scavenging effect of silver carp protein hydrolysate (Alcalase-2 h) on hydroxyl radicals was concentration-dependent, and the antioxidant activity was close to that of carnosine (Fig. 5b).

3.7. Metal chelating activity

Metal chelating activity of silver carp protein hydrolysate were determined at a sample concentration of 5.0 mg/mL. The chelating activity of hydrolysates increased with longer hydrolysis time (Fig. 6a). Alcalase-hydrolyzed silver carp protein showed a higher chelating activity ($p < 0.05$) than Flavourzyme-hydrolyzed silver carp protein at any of the hydrolysis times. For Alcalase treatment, the 6 h hydrolysate of hydrolysis exhibited 92.97% metal-chelating activity.

When relative chelating activity and DH were plotted, a linear relationship was observed (Fig. 6b). Therefore, increased metal-chelating activity could be increased through hydrolysis with certain enzymes. Similar results were reported by Klompong et al. (2007).

Transitional metal ions, such as Fe^{2+} and Cu^{2+}, can catalyze the generation of reactive oxygen species which oxidize unsaturated lipids (Stohs & Bagchi, 1995). The

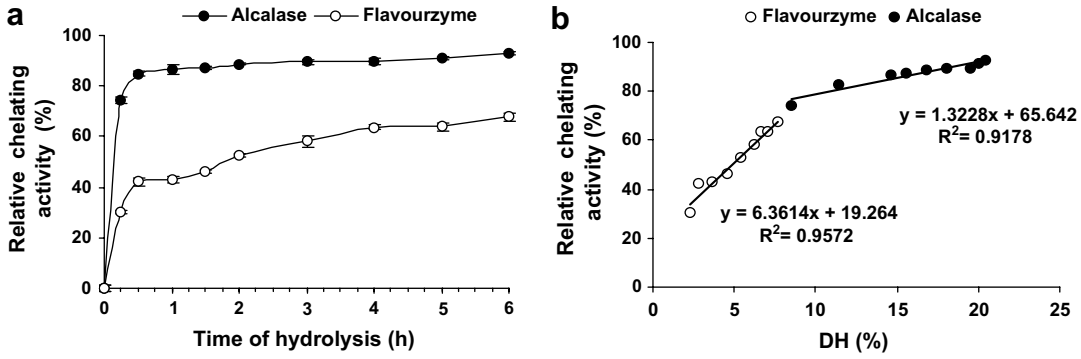

Fig. 6. The relative chelating activity of silver carp protein hydrolysate at a sample concentration of 5.0 mg/mL: (a) relative chelating activity of protein hydrolysate at different hydrolysis time; (b) relationship between DH and relative chelating activity. Bars represent standard deviations from triplicate determinations.

Table 2
Amino acid composition of silver carp protein and its hydrolysate (g/100 g protein)

Amino acid[a]	0 h[b]	Alcalase[c]			Flavourzyme[d]		
		0.25 h	1.5 h	4.0 h	0.25 h	1.5 h	4.0 h
Aspartic acid	9.98	9.86	9.03	9.94	10.35	9.87	8.85
Threonine	4.43	4.35	3.56	4.04	4.54	3.92	3.93
Serine	4.43	3.98	4.02	4.08	4.30	4.07	4.09
Glutamic acid	15.89	15.65	16.1	14.75	18.69	17.70	17.43
Glycine	3.51	4.46	4.07	4.63	4.71	4.87	4.45
Alanine	7.08	6.82	7.13	6.02	7.92	7.67	8.49
Valine	9.74	7.03	7.41	7.53	5.67	6.06	5.97
Methionine	0.31	0.94	0.98	0.86	0.47	0.53	0.72
Isoleucine	4.54	4.19	4.20	4.35	3.57	3.76	3.76
Leucine	8.42	8.18	8.41	8.61	8.69	8.29	8.81
Tryosine	3.14	3.04	2.82	2.96	2.13	2.46	2.40
Phenylalanine	4.03	3.89	4.35	4.75	3.21	3.25	3.43
Histidine	8.97	9.09	9.44	8.30	9.32	9.50	9.51
Lysine	2.31	2.26	2.38	2.34	2.35	2.15	2.34
Arginine	6.31	6.09	6.14	5.89	5.32	6.03	5.40
Cystine	1.86	2.70	2.38	2.95	3.92	5.06	5.23
Proline	5.09	7.37	7.60	8.01	4.85	4.76	5.20
Total	100	100	100	100	100	100	100
Total hydrophobic amino acids	42.69	42.85	44.15	44.77	39.09	39.19	40.83

[a] The aspartic and glutamic acid contents include, respectively, asparagines and glutamine.
[b] Silver protein carp.
[c] The hydrolysates obtained from 0.25 h, 1.5 h, 4 h of Alcalase hydrolysis.
[d] The hydrolysates obtained from 0.25 h, 1.5 h, 4 h of Flavourzyme hydrolysis.

chelating activity of peptides in hydrolysates could decrease lipid oxidation. Presumably, peptide cleavages led to an enhanced Fe^{2+} binding due to an increased concentration of carboxylic groups (COO^-) and amino groups in branches of the acidic and basic amino acids, thus removing the pro-oxidative free metal ion from the hydroxyl radical system. The direct relationship between soluble protein/peptide concentration and the increase in the chelation capability supported this premise (Saiga et al., 2003). From the results, the hydrolysates which contained lower molecular weight fraction (Fig. 2) showed higher metal-chelating activities. Moreover, the lower molecular weight distribution of hydrolysates played an important role in the metal-chelating activity.

3.8. Amino acid composition

Hydrolysis with Alcalase and Flavourzyme did not appreciably change the percentage of most amino acids of the hydrolysates (Table 2). However, both sulfur amino acid (Met and Cys) had 1- to 3-fold increases in hydrolyzed samples. Slight increase in proline was also noted in Alcalase-hydrolyzed silver carp protein. Several amino acids, such as Tyr, Met, His, Lys, and Trp, were generally accepted as antioxidants (Chen, Muramoto, Yamauchi, & Nokihara, 1996). In addition, the total content of hydrophobic amino acids of silver carp protein hydrolysates with Alcalase was higher than with Flavourzyme. For protein hydrolysates and peptides, an increase in hydrophobicity would increase their solubility in lipid and therefore enhances their antioxidative activity (Rajapakse et al., 2005; Zhu, Zhou, & Qian, 2006). The ability of whey protein isolate fractions to delay lipid oxidation was found to be related to the prevalence of histidine and hydrophobic amino acids (Pihlanto, 2006).This information seem to be related to the antioxidative activity of hydrolysates.

4. Conclusions

The antioxidant activity of silver carp protein hydrolysates were related to its degree of hydrolysis, hydrolysis time and molecular weight. The protein hydrolysates prepared by Alcalase or Flavourzyme exhibited significant hydroxyl radical-scavenging activity and the inhibition of lipid peroxidation. However, longer time hydrolysis with Alcalase and Flavourzyme led to the darkening and browning of hydrolysate. The effects of hydrolysates on food taste and quality, and identification of the specific peptides in silver carp protein hydrolysate responsible for its overall antioxidative capability need to be studied.

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