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Characteristic and antioxidant activity of retorted gelatin hydrolysates from cobia (*Rachycentron canadum*) skin

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Abstract

Alkali-pretreated cobia (*Rachycentron canadum*) skin was extracted in a retort (121 °C) for 30 min to obtain a retorted skin gelatin hydrolysate (RSGH). The molecular mass distributions and antioxidant activities of cobia RSGH and enzyme-treated RSGHs (ET-RSGHs) derived from bromelain, papain, pancreatin, and trypsin digestion were then characterized. The molecular mass distribution of the RSGH ranged mainly between 20,000 and 700 Da and those of ET-RSGHs ranged between 6500 and 700 Da. The DPPH (α,α-diphenyl-β-picrylhydrazyl) radical scavenging effects (%) of 10 mg/ml of RSGH and 10 mg/ml of the four ET-RSGHs were 55% and 51–61%, respectively. The lipid peroxidation inhibition (%) of RSGH and ET-RSGHs (10 mg/ml) were 58% and 60–71% on the fifth day in a linoleic acid model system, respectively. The 3Kd-ET-RSGHs, obtained by using a series of centrifugal ultrafiltration filters (molecular weight cut-offs of 10, 5, and 3 kDa done sequentially with decreasing pore size), exhibited dramatically improved antioxidant activity, with most of the molecular mass ranging below 700 Da. Compared to 10 mg/ml of the RSGH, 10 mg/ml of 3Kd-ET-RSGHs exhibited 45–65% more scavenging of DPPH radical and 24–38% more inhibition of lipid peroxidation. The peptides with molecular masses below 700 Da in the ET-RSGHs or 3Kd-ET-RSGHs significantly affect the antioxidant properties. These peptides are composed of a small number of amino acids or free amino acids and have the potential to be added as antioxidants in foods.

Keywords: Cobia; Retorted skin gelatin hydrolysate; Antioxidant activity; Enzymatic hydrolysis; Ultrafiltration

1. Introduction

Cobia (*Rachycentron canadum*) aquaculture is expanding throughout the world (Liao et al., 2004), most notably in Taiwan, mainland China, and Vietnam because of its rapid growth and high quality flesh (Franks, Warren, & Buchanan, 1999). More recently, the species has been cited as producing a high quality fillet suitable for sashimi or restaurant menus (Craig, Schwarz, & McLean, 2006).

Gelatins are produced on a large scale from skin and bones of mammalian origin. In recent years, fish gelatin extraction from cod (Gudmundsson & Hafsteinsson, 1997), croaker (Cheow, Norizah, Kyaw, & Howell, 2007), hake (Montero, Gómez-Guillén, & Borderías, 1999), lumpfish (Osborne, Voigt, & Hall, 1990), megrim (Sarabia, Gómez-Guillén, & Montero, 2000), pollock (Kim et al., 2001; Zhou & Regenstein, 2004), scad (Cheow et al., 2007), shark (Cho et al., 2004; Yoshimura, Terashima, Hozan, & Shirai, 2000), tilapia (Jamilah & Harvinder, 2002; Zhou, Mulvaney, & Regenstein, 2006), and tuna (Cho, Gu, & Kim, 2005) have been reported. Fish skin gelatins may provide an alternative source for gelatin production.

Nagai and Suzuki (2000) have reported that almost 30% of the fish waste produced during the filleting process consists of skin and bones with high collagen content. This can be used to produce fish gelatin, which can provide an alternative source of food grade gelatin. Cobia skin left over from fillet processing or sashimi making accounts for approximately 6% of the fish weight and this skin has been described as a good raw material for the production of gelatin (Chow, 2004).

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Some fish protein hydrolysates have been found to have noticeable antioxidant activities and it has been suggested that they might be a candidate to be a natural antioxidant (Jao & Ko, 2002; Jeon, Byun, & Kim, 1999; Kim et al., 2001; Mendis, Rajapakse, & Kim, 2005; Wu, Chen, & Shiau, 2003). It has been reported that the skin gelatin hydrolysates of Alaska pollock (Kim et al., 2001) and hoki fish (Mendis et al., 2005) contain antioxidant peptides, with those from Alaska pollock showing linoleic acid peroxidation inhibition and hoki exhibiting noticeable free-radical scavenging activities. Moreover, the skin gelatin hydrolysate from jumbo squid has also been reported to have potent free-radical scavenging activity (Mendis, Rajapakse, Byun, & Kim, 2005).

However, most previous studies of fish skin gelatin mainly focused on determination of its physicochemical characteristics. To our knowledge, antioxidant activities of cobia skin gelatin hydrolysates have not been studied. In this work, we investigated the molecular mass distributions and antioxidant activities of the cobia retorted skin gelatin hydrolysate (RSGH) and its derivatives, which we obtained by further enzyme treatments (ET-RSGHs) and ultrafiltration (3Kd-ET-RSGHs).

2. Materials and methods

2.1. Raw materials

The cobia (*Rachycentron canadum*) was cultured in marine cages located offshore of Pingtung, Taiwan. When the cobia reached 6–8 kg, they were harvested and transported to a processing factory located in Pingtung. Upon arrival, the fish were killed and gutted and then their skins were removed with automatic skinners (CF 560, First Victory Machinery Co., Kaohsiung, Taiwan). The weight of skin was about 360–480 g per fish. The skin samples (stored on ice during transportation) were taken to the laboratory, where upon arrival, they were immediately packed in plastic bags and stored at −50 °C until use (no longer than 1 week). All reagents were analytical grade.

2.2. Cleaning of fish skins

The frozen cobia skins, all from the same batch, were thawed to 4 °C in a cool room, cut into pieces (about 10 × 5 cm) and then washed under running tap water. Afterwards, they were immersed in 0.4 N NaOH (1:10, w/v) in a cool room (4 ± 1 °C) for 3 h and then placed in a blender (Model LB10G, Waring, Torrington, CT, USA) with variable-speed paddles. The blender was operated at the lowest speed for 1 min and skin samples were rubbed against each other with the rotating paddles. Almost all of the scales could be removed. The scaled skin samples were then soaked in a container filled with clean water that was exchanged slowly with running tap water for 3 h in a 4 °C cool room. The cleaned skins were drained with cheesecloth, which was squeezed by hand.

2.3. Preparation of cobia RSGH

The cleaned skins were further soaked in 6% phosphoric acid (1:2, w/v) for 1 h, drained and then flushed with distilled water to remove any remaining acid. Afterwards, distilled water (1:2, w/w) was added and the gelatin was extracted by retorting in an autoclave (Hirayama HA-300M, Saitama, Japan) at 121 °C for 30 min. After retorting, the extracted solution was filtered through a metal sieve (100 mesh, InterNet Inc., Minneapolis, MN, USA) to remove skin residues and the solution was then neutralized with saturated $Ca(OH)_2$ to pH 7 (MP220 pH meter, Mettler-Toledo, Greifensee, Switzerland). Finally, the neutralized solution was filtered through a Buchner funnel with Whatman No. 4 filter paper (Maidenstone, England) to remove fine residues and insoluble precipitants. The filtrated solution was concentrated at 30 °C using a vacuum evaporator (Büchi® rotary evaporator Model R-205, Flawil, Switzerland) and then freeze-dried in a lyophilizer. The freeze–dried product, RSGH, was stored at −40 °C until use.

2.4. Determination of nitrogen content and protein concentration

The nitrogen content of skins and extracts was determined by the micro-Kjeldahl method (AOAC., 1997). The protein concentrations of samples were determined by the Lowry assay (Lowry, Rosebrough, Farr, & Randall, 1951), using bovine serum albumin (BSA; Sigma–Aldrich Inc., St. Louis, MO, USA) as a reference protein. The protein content of the BSA was checked and corrected by the micro-Kjeldahl method mentioned above.

2.5. Enzymatic treatment of cobia RSGH with commercial proteinases

To further digest the RSGH, four proteases were used: bromelain (Merck, Darmstadt, Germany), papain (Sigma, St. Louis, MO, USA), pancreatin (Sigma), or trypsin (Wako, Osaka, Japan). One g of the freeze–dried RSGH was added to one of the four proteinases (hydrolysate:enzyme = 100:1, w/w) in a 0.1 M sodium phosphate buffer (pH 7.0). The hydrolysis was performed at 50 °C for 0, 0.5, 1, 2, or 3 h in a 100 rpm shaking water-bath incubator (BT-150, Yih-der Inc., Taipei, Taiwan). The enzymatic hydrolysis was ended by heating the mixtures at 100 °C for 10 min to inactivate the protease activity. The solution containing hydrolysate was centrifuged at 5000*g* for 10 min at 4 °C (05PR-22 centrifuge, Hitachi, Tokyo, Japan). Then, the supernatants were lyophilized and stored at −40 °C. The products obtained from the enzyme-treated RSGHs were RSGH-Bm (bromelain-treated), RSGH-Pp (papain-treated), RSGH-Pc (pancreatin-treated), and RSGH-Tp (trypsin-treated).

2.6. Preparation of ultrafiltrates from RSGH and ET-RSGHs through centrifugal ultrafiltration filters with 10, 5, and 3 kDa molecular weight cut-off (MWCO) membranes

Each of the lyophilized ET-RSGHs (obtained from 3 h enzymatic hydrolysis) or RSGH was subsequently dissolved in a 0.1 M sodium phosphate buffer (pH 7.0, 1%, w/v). The solution containing each hydrolysate was processed through a series of centrifugal ultrafiltration (UF) filters with MWCO of 10, 5, and 3 kDa (Millipore, Bedford, MA, USA). Each hydrolysate solution (12 ml) was first passed through a centrifugal filter with 10 kDa MWCO. Its permeate (10Kd-RSGH or 10Kd-ET-RSGH) was passed through the 5 kDa and then the 3 kDa MWCO membranes. By passing the solution sequentially through these three filters with different pore sizes, three permeates were obtained: 10Kd-hydrolysate (permeated from 10 kDa MWCO), 5Kd-hydrolysate, and 3Kd-hydrolysate. The three ultrafiltrates were lyophilized and stored at −40 °C until use.

2.7. Molecular mass distributions of cobia RSGH and ET-RSGHs

Molecular weight distributions of the RSGH and ET-RSGHs were determined by gel filtration chromatography on a Superdex Peptide HR 10/300 column (column size 10 × 300 mm, Amersham Pharmacia Biotech, Uppsala, Sweden), using a Hitachi 2130 HPLC system and a UV/Vis detector, (Tokyo, Japan). The mobile phase used was a 0.02 M sodium phosphate buffer (pH 7.2) containing 0.25 M NaCl. The flow rate was 0.5 ml/min. Absorbance was monitored at 214 nm. The column was calibrated using trypsin inhibitor (Wako), aprotinin (Amersham Pharmacia Biotech), cytochrome C (Sigma), pepstatin A (Serva, Heidelberg, Germany), Gly–Gly–Gly (Amersham Pharmacia Biotech), and glycine (Sigma), according to the manufacturer's instructions. The molecular masses were 20,000, 12,500, 6511, 686, 189, and 75 Da, respectively. This yielded a near linear correlation between the retention times and the log of the calibration molecules' masses in the range of 75–20,000 Da. For quantitative determinations, peak areas in the sample chromatograms were calibrated by using Hitachi EZChrom Elite 3.1 chromatography data system software operating with Windows® XP.

2.8. DPPH radical scavenging activity

The scavenging effects of the cobia RSGH and ET-RSGHs on the α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical were measured as previously described in Shimada, Fujikawa, Yahara, and Nakamura (1992) with some modifications. Briefly, a volume of 1.5 ml of RSGH (2–30 mg/ml), ET-RSGH (10 mg/ml), or 3Kd-ET-RSGH (10 mg/ml) in 95% ethanol was added to 1.5 ml of 0.1 mM DPPH in 95% ethanol. The mixture was Vortexed (Vortex Maxi Mix® II, Barnstead, Dubuque, IO, USA) for 10 s and left for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm (Hitachi U-2800 Spectrophotometer). A lower absorbance indicated higher DPPH scavenging activity. Ten ppm butylated hydroxyanisole (BHA) (Sigma), 100 ppm α-tocopherol (Sigma), and 1000 ppm ascorbic acid (Sigma) were used as positive controls. The scavenging effect can be expressed in the following formula as: DPPH radical scavenging activity = [(blank absorbance − sample absorbance)/blank absorbance] × 100%; where the DPPH blank is the value of 1.5 ml of ethanol mixed with the 1.5 ml of ethanol containing 0.1 mM DPPH. All experiments were carried out in triplicate.

2.9. Inhibition of lipid peroxidation in the linoleic acid model system

The lipid peroxidation inhibition by cobia skin gelatin hydrolysates was determined according to the ferric thiocyanate method (Mitsuda, Yasumoto, & Iwami, 1966) with some modifications. Briefly, a 2.5 ml aliquot of 2 mg/ml linoleic acid solution (in 95% methanol) was mixed with a 0.5 ml aliquot of RSGH (2–10 mg/ml) or other hydrolysates (10 mg/ml in 0.2 M sodium phosphate buffer, pH 7.0) and 2 ml of 0.2 M sodium phosphate buffer (pH 7.0). After incubation at 37 °C in darkness for 1–5 d, a 0.1 ml aliquot of the above-mentioned mixture was mixed with 4.7 ml ethanol (75%), 0.1 ml ammonium thiocyanate (30%) (Wako), and 0.1 ml ferrous chloride (20 mmol/l) (Merck). After stirring the mixture for 3 min, the peroxide value was determined spectrophotometrically at 500 nm. The percentage of lipid peroxidation inhibition can be expressed by the following formula: inhibition percent (%) = $[1 - (A_1/A_0)] \times 100$; where A_0 is the absorbance of the blank (phosphate buffer without hydrolysates), and A_1 the absorbance in the presence of the hydrolysate samples. Ten ppm BHA (Sigma) was used as the positive control.

2.10. Statistical analyses

The SAS statistical software program (SAS Institute Inc., Cary, NC, USA) was used for analyses of variance. Significance was determined at $P \leqslant 0.05$. Duncan's multiple ranges test was used to determine significant differences among means. All data reported is based on the means of three replicates.

3. Results and discussion

3.1. Recovery yield of cobia RSGH

To convert insoluble native collagen to gelatin, a treatment is required to break noncovalent bonds and disorganize the protein structure, producing adequate swelling and cleavage of intra- and intermolecular bonds, leading to subsequent collagen solubilization (Stainsby, 1987). After

alkali pretreatment, acid soaking, and draining, the skin samples were added with distilled water and then extracted by retorting in an autoclave. The yield of RSGH obtained from cobia skin was 46.9% (data not shown). Zhou and Regenstein (2004) reported that the H^+ concentration of the solvent for fish gelatin extraction has a significant effect on yield of gelatin. Cheow et al. (2007) reported that the acid treatment causes swelling of skin gelatin and removal of non-collagen protein and hot water extraction cause thermohydrolysis and subsequent solubilization of swollen gelatin. In fact, the retorting treatment resulted in sufficient denaturation of soluble collagen and the strong thermohydrolysis provides an effective method for the production of skin gelatin hydrolysates.

3.2. Determination of molecular mass distributions of cobia RSGH and ET-RSGH

HPLC gel filtration chromatography was used to evaluate the molecular mass distributions of the RSGH, ET-RSGHs, or 3Kd-ET-RSGHs. The calibration curve using reference proteins is shown in Fig. 1A. There was a linear relation between the retention time and the log of the molecular mass of the reference proteins in the range of 20,000–75 Da ($r^2 = 0.993$). About 47.9% and 31.5% of the total hydrolysates were in the 20,000–6511 and 6511–686 Da fractions (Fig. 1B), respectively. After hydrolysis by the four enzymes for 0.5 h, the major molecular masses of the four ET-RSGHs (RSGH-Bm, RSGH-Pp, RSGH-Pc, and RSGH-Tp) were mainly in the 6511–686 Da fraction (Fig. 2A). Although the hydrolysis time was extended to 3 h, there was no obvious change in the main molecular mass distributions of the four hydrolysates.

Interestingly, the molecular mass distribution of the RSGH-Bm was similar to that of the RSGH-Pp, while the molecular mass distribution of the RSGH-Pc was similar to that of the RSGH-Tp (Fig. 2B). Fig. 2 shows that bromelain or papain essentially finished digesting RSGH within 0.5 h. However, it took pancreatin or trypsin at least 2 h to get to completion. The hydrolysis reactions of the four enzymes were run under the same condition, 50 °C and pH 7.0, and these conditions might not be the most favorable hydrolysis condition for all of these enzymes. The similarity in patterns may be related to bromelain and papain being thiol proteases, while pancreatin and trypsin are serine proteases (Fersht, 1984).

After 0.5 h hydrolysis, the fractions of RSGH-Br, RSGH-Pp, RSGH-Pc, and RSGH-Tp with molecular weights smaller than 6511 Da were 86.1%, 83.1%, 72.5%, and 74.7% of the total hydrolysates, respectively. However, regardless of which enzymatic treatment was used for 3 h, fractions smaller than 6511 Da accounted for about 85% of the total hydrolysates. Apparently enzymatic hydrolysis was essentially finished in 3 h using any of the four enzymes. In fact, when RSGH was further treated with bromelain for 24 h, the main molecular mass of the RSGH-Bm was still distributed within the 6511–686 Da fraction (data not shown).

3.3. Antioxidant activities of cobia RSGH and ET-RSGHs evaluated by DPPH radical scavenging activity

DPPH radicals can be scavenged by proton-donating substances such as natural or artificial antioxidants. Fig. 3A shows that the RSGH is able to quench DPPH radicals and that there is a good correlation ($r = 0.982$) between its concentrations (2–20 mg/ml) and DPPH radical scavenging effects (8.6–85.8%). Moreover, RSGH was able to scavenge almost 90% at concentrations between 22 and 30 mg/ml. The DPPH scavenging effect (%) of 10 mg/ml RSGH was 54.9% and close to that of 10 ppm BHA (Fig. 3B). Enzymatic and chemical modifications have been extensively employed to improve the functional properties of proteins. This study, investigating DPPH radical scavenging activities of the cobia ET-RSGHs, found the maximal DPPH scavenging effects of 10 mg/ml RSGH-Bm, RSGH-Pp, RSGH-Pc, and RSGH-Tp to be 50.9 (2 h), 57.6 (2 h), 60.7 (3 h), and 59.6% (3 h), respectively (Fig. 3C).

Both the RSGH and ET-RSGHs may contain certain peptides that are electron donors and can react with free

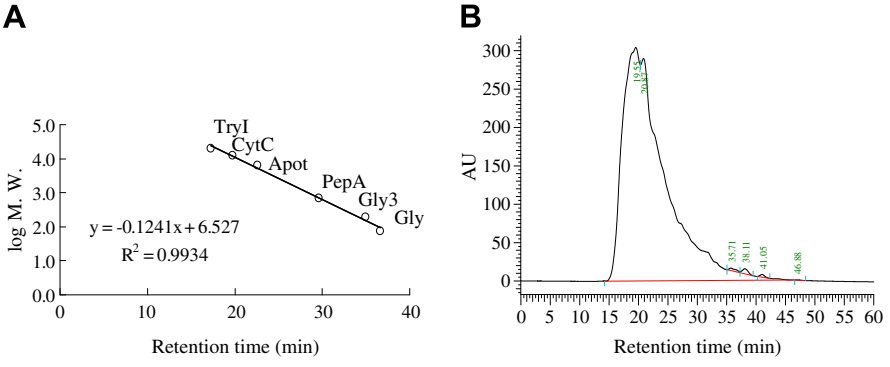

Fig. 1. (A) Relationship between the molecular weight (MW) of standard markers and their retention time using Superdex peptide 10/300 GL column for analysis. (Column size: 10 × 300 mm; flow rate: 0.5 ml/min; elution buffer: 0.02 M phosphate buffer containing 0.25 M NaCl, pH 7.2.) Standards: (1) trypsin inhibitor (molecular mass: 20,000 Da), (2) cytochrome *c* (12,500 Da) (3) aprotinin (6500 Da), (4) pepstatin A (686 Da), (5) Gly-Gly-Gly (189 Da), (6) Glycine (75 Da). (B) HPLC profiles of cobia RSGH.

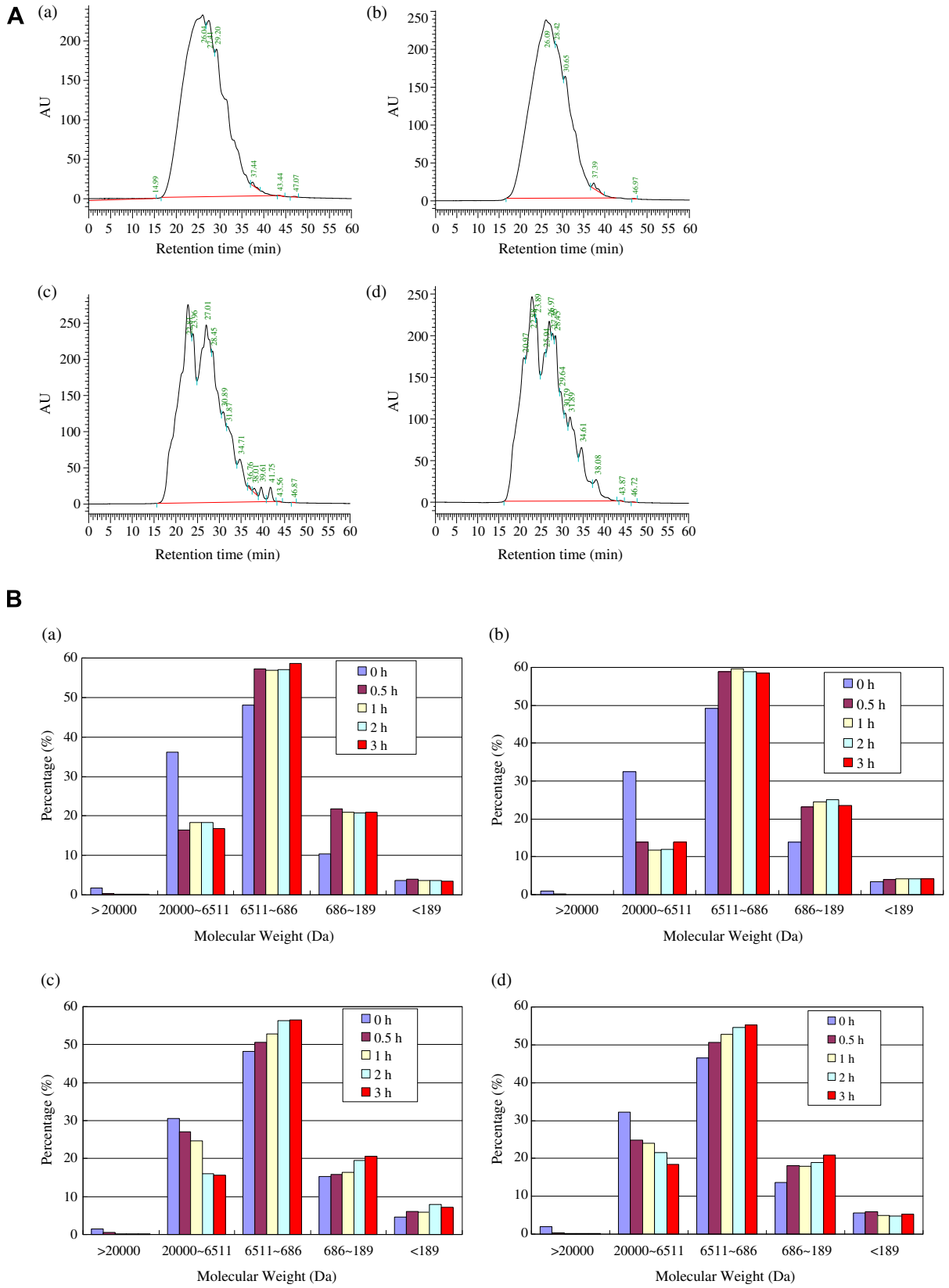

Fig. 2. (A) HPLC profiles of cobia RSGH treated with a: bromelain, b: papain, c: pancreatin, and d: trypsin (100:1, w/w) at 50 °C for 0.50 h. HPLC gel filtration chromatography was used to evaluate the molecular mass distributions of the hydrolysates. The calibration curve was calibrated using the reference proteins from Fig. 1. For quantitative determination, peak areas in HPLC chromatograms were integrated. (B) Molecular size distributions with HPLC of RSGH treated with a: bromelain, b: papain, c: pancreatin, and d: trypsin (100:1, w/w) at 50 °C for different hydrolysis times (0–3 h).

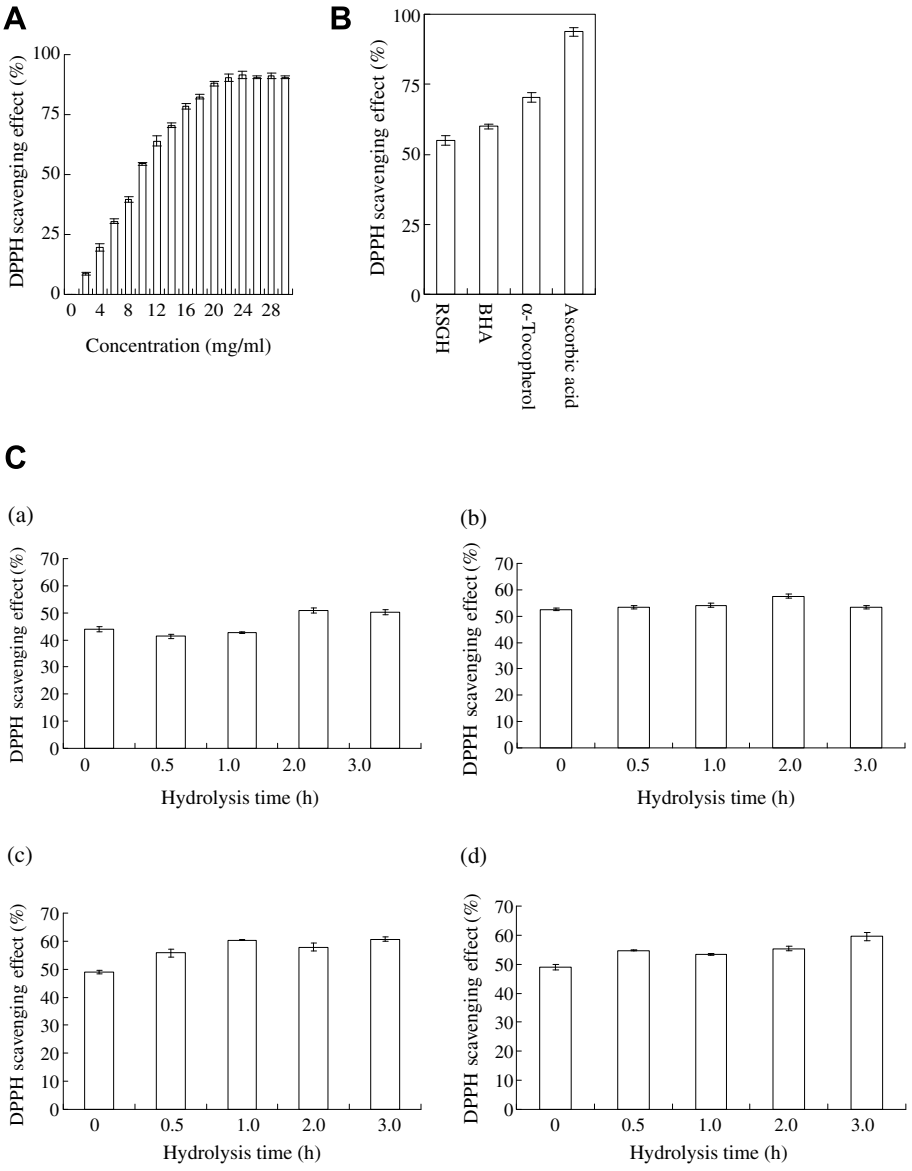

Fig. 3. (A) DPPH scavenging effects of cobia RSGH at various concentrations. (B) DPPH radical scavenging effects of cobia RSGH and other antioxidants. The concentration of RSGH was 10 mg/ml, while those of BHA, α-tocopherol, and ascorbic acid were 10, 100, and 1000 ppm, respectively. (C) DPPH radical scavenging activities of RSGH treated with a: bromelain, b: papain, c: pancreatin, and d: trypsin at 50 °C for various hydrolysis times (0–3 h). The concentration of all hydrolysates used in all experiments was 10 mg/ml.

radicals to terminate the radical chain reaction. However, the ET-RSGHs derived from the RSGH with further enzymatic digestion did not display much more activity than the RSGH. It is assumed that the retorting treatment (121 °C for 30 min) provides a sufficient hydrolysis effect to degrade cobia skin gelatin and produce most of the antioxidant peptides with smaller molecular sizes, particularly the fractions smaller than 6511 Da.

3.4. Inhibition of lipid peroxidation by cobia RSGH and ET-RSGHs in linoleic acid model system

Some fish proteins have been shown to have antioxidative activities against the peroxidation of lipids or fatty acids (Jeon et al., 1999; Kim et al., 2001; Mendis et al., 2005; Wu et al., 2003). Cobia RSGH slowed down lipid peroxidation in a linoleic acid model system (Fig. 4A). The 8 and 10 mg/ml of RSGH had a greater inhibition effect on lipid peroxidation, similar to that of 10 mg/l of BHA. Their inhibition percentages were 55.1% and 58.2% on the fifth day, respectively. The lipid peroxidation inhibition of the ET-RSGHs prepared with the four enzymes after 0, 0.5, 1, 2, and 3 h hydrolysis was also investigated (Fig. 4B). During this experiment, all ET-RSGHs exhibited noticeable inhibition on the first two days. On the fifth day, the inhibition percentages of all ET-RSGHs (10 mg/ml) remained with in the 59.7–70.8% range (Fig. 4B). Compared with the original 10 mg/ml RSGH (Fig 4A),

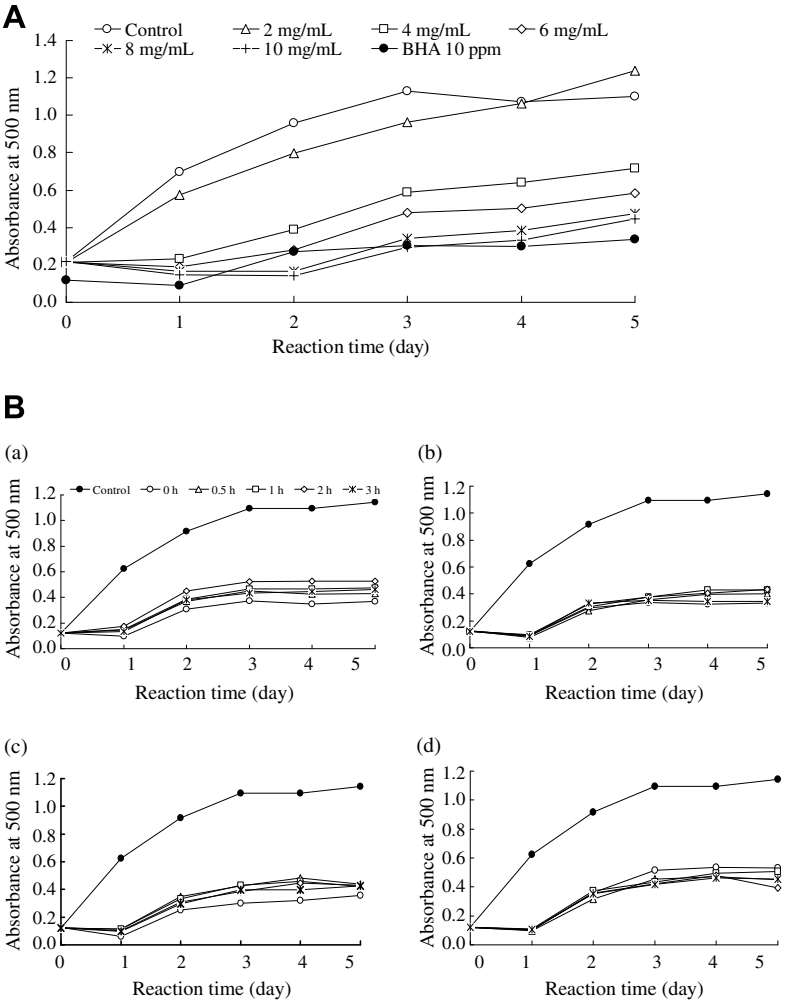

Fig. 4. (A) Inhibition of lipid peroxidation measured in a linoleic acid model system at 37 °C for 5 d in the presence of RSGH (0–10 mg/ml). The concentration of BHA, as a positive control, used in the experiments was 10 ppm. (B) Inhibition of lipid peroxidation measured in a linoleic acid model system at 37 °C for 5 days in the presence of a: RSGH-Bm, b: RSGH-Pp, c: RSGH-Pc, and d: RSGH-Tp for different hydrolysis times (0–3 h). The concentration of all hydrolysates used in the experiments was 10 mg/ml.

however, some of the ET-RSGHs did not display a significant increase in lipid peroxidation inhibition on the fifth day (Fig 4B).

It is well-known that lipid peroxidation occurring in food products deteriorates food quality, resulting in rancidity, unacceptable taste, and shorter shelf life. The RSGH and ET-RSGHs were found in this study to retard lipid deterioration and may be used as natural antioxidants for food products.

3.5. Effect of permeates from cobia RSGH and ET-RSGHs fractionated by UF on DPPH radical scavenging activity and lipid peroxidation inhibition

As mentioned above, the ET-RSGHs did not show a significant improvement in their DPPH radical scavenging activity, although the reaction time of enzymatic hydrolysis was extended. A further addition of proteinases to the RSGH would probably not significantly increase the number of small peptides with antioxidant properties. However, smaller peptides in cobia RSGH possibly play a key role in its antioxidant potency.

One method of controlling molecular size of the desired hydrolysates is the use of UF with appropriate MWCO membranes. In this study three ultrafiltrates were obtained: 10Kd-RSGH (permeate from 10 kDA MWCO), 5Kd-RSGH, and 3Kd-RSGH. HPLC analysis demonstrated that the main molecular mass distribution of the original RSGH, and its 10Kd-RSGH, 5Kd-RSGH and 3Kd-RSGH ultrafiltrates were 20,000–6511, 20,000–686, 6511–189, and below 686 Da, respectively (Table 1). It was observed that the scavenging effects of RSGH, 10Kd-RSGH, 5Kd-RSGH, and 3Kd-RSGH were 54.9%, 56.4%, 59.7%, and 66.2%, respectively. The 3Kd-RSGH possessed the highest DPPH radical scavenging activity of the four hydrolysates and its scavenging effect was approximately 20% higher than that of the original RSGH. Furthermore, we also tested the DPPH scavenging activities of the three UF fractions from the pancreatin-treated RSGH (3 h hydrolysis). The 3Kd-RSGH-Pc ultrafiltrate also had a much better

Table 1
Molecular mass distributions (%) of cobia RSGH, UF RSGHs[a], and 3Kd-ET-RSGHs[b]

Hydrolysate	Molecular mass distribution[c] (%)[d]				
	>20,000	20,000–6511	6511–686	686–189	<189
RSGH	9.8	47.9	31.5	7.9	2.9
10Kd-RSGH	0.1	30.0	44.2	14.9	10.8
5Kd-RSGH	0	2.6	32.4	43.2	21.8
3Kd-RSGH	0	0	21.0	48.5	30.5
3Kd-RSGH-Bm	0	0	14.1	57.3	28.6
3Kd-RSGH-Pp	0	0	12.6	58.9	28.5
3Kd-RSGH-Pc	0	0	17.0	55.7	27.3
3Kd-RSGH-Tp	0	0	19.3	52.3	28.3

[a] UF RSGHs (10Kd-RSGH, 5Kd-RSGH, and 3Kd-RSGH) were fractionated from the RSGH with a series of centrifugal UF filters with MWCO of 10, 5, and 3 kDa.
[b] 3Kd-ET-RSGHs (3Kd-RSGH-Bm, 3Kd-RSGH-Pp, 3Kd-RSGH-Pc, and 3Kd-RSGH-Tp) were fractionated from the ET-RSGHs with a series of centrifugal UF filters (MWCO 10, 5 and 3 kDa).
[c] The molecular mass (Dalton) distributions of the ultrafiltrates were evaluated using the reference proteins from Fig. 1.
[d] For quantitative determination, peak areas with a specific molecular mass range in HPLC chromatograms were integrated.

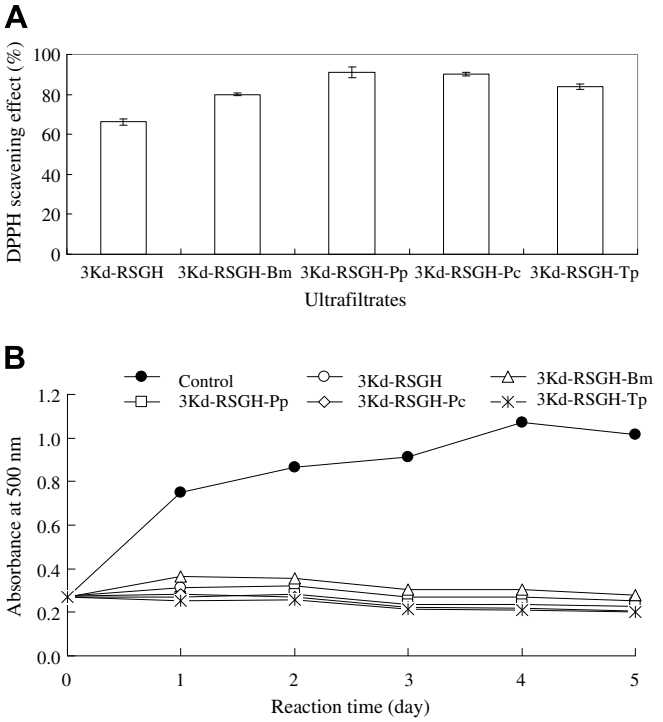

Fig. 5. (A) DPPH scavenging effect (%) of cobia 3Kd-RSGH and 3Kd-ET-RSGHs (10 mg/ml). (B) Inhibition of lipid peroxidation was measured in a linoleic acid model system at 37 °C for 5 d in the presence of the 3Kd-RSGH and 3Kd-ET-RSGHs (3Kd-RSGH-Bm, 3Kd-RSGH-Pp, 3Kd-RSGH-Pc, and 3Kd-RSGH-Tp). The concentration of ultrafiltrates used in all tests was 10 mg/ml.

DPPH scavenging effect than the other fractions (data not shown). Based on these results, the characteristics and antioxidant activities of all four 3Kd-ET-RSGHs were investigated. The major molecular mass profiles of the four 3Kd-ET-RSGHs mainly distributed within the ranges of 686–189 and 189–0 Da accounting for 52–59 and 27–29% of the total ultrafiltrates, respectively (Table 1). However, the constituent peptides with ranges of 686–189 and 189–0 Da in the ET-RSGHs only accounted for 20–23% and 4–7% of total hydrolysates, respectively (Fig. 3B). The UF treatment was an effective approach to collecting smaller hydrolyzed peptides (in 3Kd-ultrafiltrates) with molecular mass ranging below 686 Da from the four ET-RSGHs. Moreover, the DPPH scavenging effects of 3Kd-RSGH-Bm (80%), 3Kd-RSGH-Pp (91%), 3Kd-RSGH-Pc (90%), and 3Kd-RSGH-Tp (84%) ultrafiltrates showed 45%, 65%, 64%, and 53% higher DPPH scavenging activity than that of the original RSGH, respectively (Fig. 5A).

In Section 3.4, it was mentioned that 10 mg/ml RSGH had inhibited 58.2% of linoleic acid peroxidation on the fifth day (Fig. 4A) and that ET-RSGHs (10 mg/ml) had inhibited that peroxidation from 59.7% to 70.8% on the fifth day (Fig. 4B). Obviously, the inhibition of lipid peroxidation was affected by the molecular mass distributions of the hydrolysates. The 3Kd-RSGH (10 mg/ml) inhibited 73.1% and the 3Kd-ET-RSGHs (10 mg/ml) inhibited 72.2–80.1% on the fifth day in the model system (Fig. 5B). The 3Kd-RSGH and 3Kd-ET-RSGHs (10 mg/ml) inhibited 26% and 24–38% more peroxidation than the 10 mg/ml RSGH, respectively.

Because the major molecular mass profile of the 3Kd-ET-RSGHs mainly distributed below 700 Da (Table 1), it is assumed that the 3Kd-ET-RGSHs are small oligopeptides containing a small number of amino acids or free amino acids. Such small peptides in the cobia skin hydrolysates play a key role in their antioxidant activity. In addition to the cobia skin gelatin hydrolysates, other peptides in skin gelatin hydrolysates from hoki fish (His-Gly-Pro-Leu-Gly-Pro-Leu) (Mendis et al., 2005) and Alaska pollock (Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly and Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly) (Kim et al., 2001) have demonstrated noticeable free-radical scavenging activity (the first peptide) and linoleic acid peroxidation inhibition (the latter two peptides), respectively.

4. Conclusion

This study found that the cobia RSGH produced by a retorting treatment (121 °C for 30 min) and its derivatives (ET-RSGHs and 3Kd-ET-RSGHs) exhibited strong DPPH free-radical scavenging activity and lipid peroxidation inhibition. The peptides with molecular masses below 700 Da in the ET-RSGHs or 3Kd-ET-RSGHs play important roles in their antioxidant properties. These can be potential antioxidants for use in foods and dietary supplements.

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