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Research article

Effects of salt extraction and heating conditions on protein characteristics and antioxidant activity of salmon (*Salmo salar)* bone extract

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Abstract

The salmon fillet industry in Thailand contributes a large amount of salmon bone (approximately 443 t/yr) as a by-product from final production. The salmon bone contains 16.65% protein and could be utilized to prepare human food. This study evaluated the effects of various salt concentrations and extraction conditions on the protein characteristics of salmon bone extract. The salmon bone extract was prepared by combining two levels (0.5%, 3.5%) of salt solution (1:6, weight per volume) at various temperatures (55–95°C) for 0.5–8 hr prior to centrifugation. The properties were determined of color, light transmission, total solids, protein content, protein pattern and radical scavenging activity. Lower lightness and light transmission were found in the extract with 3.5% salt addition compared to 0.5% salt at 55°C. At 95°C for 8 hr, extracting the salmon bone with 3.5% salt produced the highest values of 1.62% protein and 4.20% total solids, while extraction with 0.5% salt produced 0.76% protein and 1.31% total solids. Proteins with a molecular weight above 250 kDa, myosin heavy chains, collagen and tropomyosin were found in the extracts at 85°C and 95°C. The extracts with 0.5% salt addition at 55°C and 95°C had greater radical scavenging activity than extraction with 3.5% salt. Based on the protein content and the value of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, extraction with 0.5% salt at 95°C for 8 hr showed potential for use as a commercial soup product.

Introduction

Salmon meat is one of the popular seafoods in Thailand because of its nutritional and special flavor qualities (Clarke, 2015). Moreover, the salmon filleting industry in Thailand in 2010–2014 produced 5,156 t (Fisheries Foreign Affairs Division, 2014). Filleting activity produces approximately 3.1–8.6% salmon bones as a byproduct (Ramirez, 2007). However, salmon bones are underutilized, resulting in discarding problems and resultant environmental concerns (Ramirez, 2007). Salmon bone is composed of 50.06 ± 0.95% moisture, 16.65 ± 0.38% protein, 15.36 ± 0.56% lipid, 11.42 ± 0.93% ash and 6.51 ± 1.17% carbohydrate (Syahidawati and Limpisophon, 2017). Thus, from a food security viewpoint, important nutritional values (especially protein) in salmon bone could be utilized for human consumption.

Myofibrillar protein and collagen are the main proteins in salmon bone (Kristinsson and Rasco, 2000). Myofibrillar proteins, containing mainly myosin heavy chain (MHC; 250 kDa), actin (45 kDa), troponin T (41 kDa), and tropomyosin (38 kDa) are the dominant proteins in fish and meat products (Undeland et al., 2002; Wang et al., 2013b; Lu et al., 2017). Myofibrillar proteins can be extracted using either low salt concentration (below 2%, weight per volume) or high salt concentration (greater than or equal to 2%, w/v). Previous study reported that muscle proteins could be extracted using either a low or high salt concentration in the range 0.0001–5.85% NaCl (Stefansson and Hultin, 1994; Feng and Hultin, 1997).

Collagen is the main structural protein of connective tissue, composed of three α-chains intertwined as a triple-helix structure that is insoluble in the aqueous phase (Asghar and Henrickson, 1982). The triple-helix structure is approximately 300 nm in length, and the

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chain has a molecular weight of approximately 105 kDa (Papon et al., 2006). Kolodziejska et al. (2008) found that collagen from Baltic cod (*Gadus morhua*) backbone started to solubilize in water after being heated at 45°C for 15 min. Tornberg (2005) reported that the collagen of meat could break the hydrogen bond and loosen the fibrillar structure at 53–63°C, and then it could be dissolved to form gelatin on further heating.

The molecular weight has a relationship with scavenging activity value, with a high scavenging activity being caused by a low molecular weight protein or a peptide and amino acid composition or a combination of both (Jun et al., 2004; Udenigwe and Howard, 2013). Thermal extraction processes including temperature, time and salt addition, can significantly influence the protein characteristics of salmon bone (Tornberg, 2005). There is evidence in the literature that explains the effect of a combination of the salt concentration and thermal extraction on the protein characteristics of salmon bone extract. Therefore, this study aimed to evaluate the effects of various salt concentrations and extraction conditions on the characteristics of salmon bone extract.

Materials and Methods

Souree of salmon bone

Atlantic salmon (*Salmo salar*) bones were kindly provided by Thai Union Frozen Co.; Thailand. The bone was stored at -18 ± 1°C until used. Table salt (Prung Thip; Thailand) composed of 99.9% NaCl was purchased at the local market. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Aldrich (Sigma Aldrich Co., Ltd; USA).

Preparation of salmon bone extract

The salmon bones were thawed and cut horizontally into 2–3 cm strips. The salmon bones were washed twice using a cold water (4°C or colder) process to remove some lipids and the fishy odor according to Syahidawati and Limpisophon (2017). After washing, the salmon bones were composed of 50.06 ± 0.95% moisture, 16.65 ± 0.38% protein, 15.36 ± 0.56% lipid, 11.42 ± 0.93% ash, and 6.51 ± 1.17% carbohydrate as reported by Syahidawati and Limpisophon (2017). Low (0.5%, w/v) or high (3.5%, w/v) salt solutions combined with temperatures (55°C, 85°C or 95°C) and times (0.5 hr, 2 hr, 5 hr or 8 hr) were used to study the characteristics of the salmon bone extract. The extraction process took place at 55°C, 85°C or 95°C in a water bath (Memmert, GmbH; Germany) for 0.5 hr, 2 hr, 5 hr or 8 hr. To avoid evaporation during extraction, the process was performed in a container covered with a lid. The ratio of bone to solution was 1 to 6 (w/v). The extract was centrifuged at 3,500×*g* for 15 min at 4°C. The supernatant remaining after centrifugation and filtration was defined as the salmon bone extract and its chemical and physical properties were quantified.

Determination of color and light transmission

Salmon bone extract (45 mL) was poured into a 50 mL transmission cell to determine color and light transmission. The appearance of color (CIE L*a*b* system) and light transmission of the salmon bone extract were analyzed using an Ultrascan Pro Hunter Lab colorimeter with illuminant D65 (Hunter Laboratory Co., Ltd.; USA).

Determination of total solids

Determination of total solids was carried out following the method developed by AOAC (2000). The aluminum dish (W_1) was dried to constant weight in an oven. Five mL of the salmon bone extract was placed in the dish and the dish containing the sample was dried in an oven at 105°C until constant weight (W_2). The total solids were determined according to the Equation 1:

$$\text{Total solids (\% w/v)} = \frac{(W_2 - W_1)}{V} \times 100\% \quad (1)$$

where W_1 is the weight in grams of the empty aluminum dish, W_2 is the weight in grams of the aluminum dish containing the sample after drying and V is the volume in milliliters of the sample.

Determination of protein content

The protein content was determined according to Lowry et al. (1951). Sample measurement was conducted at 750 nm in an ultraviolet-visible spectrophotometer (Thermo Fisher Scientific Co. Ltd.; USA). Distilled water was used as a blank and bovine serum albumin was used as a protein standard. The protein concentration was determined as a percentage (%, w/v).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

Protein pattern in the salmon bone extract was analyzed by using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lowry's method was used to determine the protein concentration. All extracts were prepared to obtain the final protein concentration at 1 mg/mL in an SDS-PAGE sample. SDS-PAGE was performed based on the procedure of Laemmli (1970) using 4% stacking gel and 10–15% separating gel both with and without 0.01% β-mercaptoethanol as a reducing agent, staining used Coomassie Brilliant Blue G-250 and broad molecular weight standards were used in the range 3.5–225 kDa.

2,2-Diphenyl-1-picrylhydrazyl radical-scavenging activity

DPPH in ethanol was used to determine the electron donation capability in the extracts. The assay was carried out following the method of Girgih et al. (2013). The protein concentration in the sample was adjusted to 1 mg/mL using salt solution. An amount of 0.85 mL of each sample was mixed with 0.85 mL of 0.1 mM DPPH solution. The mixture was incubated in the dark at room temperature for 15 min prior to centrifugation at 5,590×*g* for 10 min. After centrifugation, the supernatant was kept in the dark for 30 min. The absorbance was read at 517 nm. The salt solution with DPPH reagent was used as a control and the blank was the salt solution. The bleached purple color of the DPPH reagent was evaluated a positive antioxidant activity. The scavenging activity was determined according to Equation 2:

$$\text{Relative scavenging activity (\%)} = \frac{(X - Y)}{X} \times 100\% \quad (2)$$

where, X is the absorbance at 517 nm of the control and Y is the absorbance at 517 nm of the sample.

Statistical analysis

Data were subjected to analysis of variance using a full factorial design with the confidence interval at 95% level ($p < 0.05$) to explore differences resulting from the effects of temperature and time on the

salmon bone extract characteristics. The experiments were carried out using two replications. A comparison of means within the same salt concentration was appraised using Duncan's multiple range tests. A comparison of two levels of salt concentration at the same extraction condition was approased using Student's t test. All statistical analyses were performed using the SPSS statistical software (version 17; SPSS Inc.; USA).

Results and Discussion

Color and light transmission

The lightness (L*) value of the salmon bone extracts with 0.5% and 3.5% salt were in the range 83.27–96.46 and 78.03–91.46, respectively, while the light transmission values were in the ranges 64.48–91.10 and 53.28–79.46, respectively, as shown in Table 1. Among the different salt concentrations, extraction at either 85 or 95°C from 0.5 hr to 8 hr did not significantly change the lightness and light transmission values. On the other hand, the lightness and light transmission values from heating at 55°C with 0.5% salt were higher ($p < 0.05$) than those with 3.5% salt, which was supported by the appearance of those with 0.5% salt being slightly clearer than those with 3.5% salt. The redness (a*) values of salmon bone extracts with 0.5% and 3.5% salt were in the ranges -0.21 to 1.54 and 0.26 to 1.90, respectively, and the yellowness (b*) values with 0.5% and 3.5% salt were in the ranges 3.37–14.37 and 8.45–15.18, respectively. The redness and yellowness values for heating at 55°C of salmon bone samples with 0.5% salt were slightly lower than those with 3.5% salt ($p < 0.05$).

Low lightness and light transmission from the extracts at 85°C and 95°C indicated that the salmon bone extracts had high extracted components. Zhang et al. (2013) reported that crucian carp cooked at high temperature resulted in a soup with a higher turbidity (low light transmission) than soup cooked at a low temperature for the same time. Crucian carp soup cooked at 75°C and 85°C resulted in a milk-like color but soup cooked at 55°C was clear, like water. These results indicated that the appearance of the soup was correlated with the amount of extracted components in crucian carp. In the current study, the lightness and light transmission values of the salmon bone extract at 55°C with 3.5% salt were slightly lower than with 0.5% salt, indicating a higher amount of components in the extract. Extraction of salt-solubilized myofibrillar protein from chicken, pork and beef meats with 3.5% salt (high concentration) also had higher yields than those with lower added salt (Keever, 2011).

Total solids and protein content

The values of total solids at 55°C to 95°C for 0.5 hr to 8 hr were in the range 0.80–1.31% when extracted with 0.5% salt, as shown in Table 2. Extraction with 3.5% salt resulted in total solids from 3.51% to 4.20%. The total solids content of samples significantly increased with increased salt concentration, temperature and time. Extraction at 95°C for 8 hr (the highest temperature and the longest time) produced the highest total solids for the extract with 0.5% salt of 1.31 ± 0.07%, while with 3.5% salt it was as 4.20 ± 0.01%. These results indicated that a high temperature and extraction time increased the total solid component of the salmon bone extract and was in agreement with Wang et al. (2016) who also found that increasing temperature and time extraction yielded total solids. They used high pressure to prepare the extract of chicken bone residue at 120°C from 0 hr to 2 hr, which resulted in total solids in the range 0.90–5.20%.

The current experiment covered the container of the salmon bone extract during the heating process so that the volume of extract did not change during extraction. It was also confirmed that the total solids amount was strongly influenced by the protein value of the salmon bone extract as extraction with 0.5% salt, a ratio of salmon bone to water of 1:6 (w/v) and heating from 55°C to 95°C for 0.5 hr to 8 hr yielded a protein content from 0.30% to 0.76% (Table 2). Interestingly, extraction with 3.5% salt at 95°C for 2–8 hr resulted in a protein content that was twice as high than for extraction with 0.5% salt at the same temperature and for the same time. Extraction with 3.5% salt at 95°C for 8 hr yielded 1.62% protein content in salmon bone extract which was close to the protein content of chicken bone extract (Wang et al., 2016), which used a hot-pressure process at 120°C for 40 min with a ratio of chicken bone to water of (1:1.5, weight per weight) and produced a protein content of 1.67%. The statistical analysis confirmed that increasing the salt concentration, temperature and times significantly increased the protein yield from the salmon bone extract. The protein value of the salmon bone extract was close to that in sheep and pig bone soup by Zhang et al. (2014), who reported that the protein contents of sheep and pig bone soups cooked at 95°C for 2 hr at a ratio of bone to water of 1:10 (w/v) were 1.1 ± 0.1% and 1.9 ± 0.1%, respectively. In addition, extraction with 0.5% salt at 95°C for 8 hr produced 0.76% protein which was similar to the protein level (0.6%) in commercial fish soup (Campbell's kitchen, 2015). This supported the potential of using salmon bone extract with 0.5% salt at 95°C for 8 hr as fish soup.

Table 1 Color and light transmission of salmon bone extracts with 0.5 and 3.5% salts at various temperatures and times

Temperature (°C)	Time (hr)	L*		a*		b*		Light transmission (%)	
		0.5% Salt	3.5% Salt	0.5% Salt	3.5% Salt	0.5% Salt	3.5% Salt	0.5% Salt	3.5% Salt
55	0.5	96.46 ± 0.06^{a**}	81.90 ± 0.80^{d}	-0.21 ± 0.00^{c**}	1.49 ± 0.12^{b}	3.37 ± 0.22^{c**}	12.42 ± 0.44^{bc}	91.10 ± 0.15^{a**}	60.12 ± 1.47^{e}
	2	89.57 ± 0.06^{b**}	83.80 ± 0.15^{cd}	0.39 ± 0.01^{b**}	1.19 ± 0.07^{bc}	9.08 ± 0.16^{b**}	12.07 ± 0.04^{bc}	75.37 ± 0.14^{bc**}	63.67 ± 0.29^{cde}
	5	88.97 ± 0.24^{b**}	81.88 ± 1.29^{d}	0.48 ± 0.03^{b**}	1.29 ± 0.09^{bc}	9.44 ± 0.15^{b**}	12.61 ± 0.45^{bc}	74.10 ± 0.52^{bc**}	60.09 ± 2.38^{e}
	8	90.37 ± 1.49^{b**}	78.03 ± 1.51^{e}	0.30 ± 0.06^{b**}	1.90 ± 0.11^{a}	8.99 ± 1.27^{b**}	15.18 ± 0.88^{a}	77.12 ± 3.24^{b**}	53.28 ± 2.57^{f}
85	0.5	88.69 ± 1.93^{b}	84.07 ± 0.50^{cd}	0.58 ± 0.31^{b}	0.97 ± 0.17^{cd}	10.67 ± 0.85^{b}	11.74 ± 0.16^{bc}	73.54 ± 4.06^{bc}	64.20 ± 0.95^{cde}
	2	88.88 ± 2.47^{b}	85.69 ± 0.04^{c}	0.69 ± 0. 42^{b}	0.82 ± 0.01^{de}	11.26 ± 2.1^{b}	11.10 ± 0.64^{c}	74.04 ± 5.14^{bc}	67.37 ± 0.06^{c}
	5	83.27 ± 4.92^{c}	85.36 ± 0.37^{c}	1.54 ± 0.45^{a}	0.86 ± 0.16^{d}	14.37 ± 1.90^{a}	11.79 ± 0.33^{bc}	64.48 ± 0.92^{d}	66.71 ± 0.73^{cd}
	8	86.82 ± 3.10^{bc}	82.87 ± 1.49^{d}	0.40 ± 0.20^{b}	1.23 ± 0.18^{bc}	9.17 ± 1.52^{b}	12.93 ± 0.17^{b}	68.16 ± 4.09^{cd}	61.94 ± 2.81^{de}
95	0.5	88.96 ± 2.22^{b}	91.11 ± 1.16^{a}	0.48 ± 0.12^{b}	0.34 ± 0.06^{f}	9.88 ± 1.65^{b}	8.45 ± 0.58^{d}	74.13 ± 4.70^{bc}	78.75 ± 2.57^{a}
	2	87.81 ± 0.66^{bc}	91.46 ± 1.51^{a}	0.66 ± 0.16^{b}	0.40 ± 0.24^{f}	8.38 ± 2.79^{b}	8.66 ± 1.32^{d}	71.68 ± 1.36^{bc}	79.46 ± 3.27^{a}
	5	88.90 ± 0.24^{b}	88.18 ± 1.48^{b}	0.50 ± 0.08^{b}	0.54 ± 0.20^{f}	9.47 ± 0.30^{b}	11.83 ± 1.21^{bc}	73.94 ± 0.50^{bc}	72.47 ± 3.09^{b}
	8	89.23 ± 2.18^{b}	90.01 ± 0.06^{ab}	0.38 ± 0.23^{b}	0.26 ± 0.02^{f}	10.00 ± 1.03^{b}	11.07 ± 0.45^{c}	74.71 ± 4.65^{bc}	76.32 ± 0.12^{ab}

L* = measure of lightness; a* = chronic scale from greenness (- a) to redness (+ a); b* = chronic scale from blueness (- b) to yellowness (+ b).
Values are expressed as mean ± SD.
$^{a, b, c}$ = mean values in the same column within the same color parameter are significantly ($p < 0.05$) different from two replications.
** = mean values are significantly ($p < 0.05$) different between 0.5% salt and 3.5% salt under same extraction conditions.

Table 2 Total solids and protein content of salmon bone extracts with 0.5% and 3.5% salt at various temperatures and times

Temperature (°C)	Time (hr)	Total solids (% weight per volume)		Protein content (% weight per volume)	
		0.5 % salt	3.5 % salt	0.5 % salt	3.5 % salt
55	0.5	0.87 ± 0.03^{f}	3.52 ± 0.08^{f}	0.30 ± 0.04^{e}	0.30 ± 0.01^{gh}
	2	0.88 ± 0.02^{f}	3.51 ± 0.02^{f}	0.31 ± 0.01^{e}	0.37 ± 0.03^{fg}
	5	0.95 ± 0.02^{cde}	3.54 ± 0.04^{ef}	0.41 ± 0.02^{d}	0.46 ± 0.00^{ef}
	8	0.96 ± 0.02^{cde}	3.59 ± 0.04^{e}	0.40 ± 0.01^{d}	0.48 ± 0.04^{e}
85	0.5	0.80 ± 0.00^{g}	3.55 ± 0.03^{ef}	0.32 ± 0.00^{e}	0.26 ± 0.03^{h}
	2	0.85 ± 0.03^{fg}	3.65 ± 0.02^{d}	0.38 ± 0.01^{d}	0.40 ± 0.02^{ef}
	5	0.92 ± 0.04^{ef}	3.63 ± 0.01^{d}	$0.40 \pm 0.02^{d**}$	0.63 ± 0.07^{d}
	8	1.00 ± 0.01^{cd}	3.77 ± 0.02^{c}	$0.49 \pm 0.02^{c**}$	0.74 ± 0.00^{c}
95	0.5	0.94 ± 0.01^{de}	3.55 ± 0.01^{ef}	$0.40 \pm 0.00^{d**}$	0.64 ± 0.01^{d}
	2	1.02 ± 0.01^{c}	3.75 ± 0.01^{c}	$0.47 \pm 0.01^{c**}$	0.89 ± 0.07^{b}
	5	1.18 ± 0.01^{b}	4.06 ± 0.01^{b}	$0.58 \pm 0.01^{b**}$	1.56 ± 0.07^{a}
	8	1.31 ± 0.07^{a}	4.20 ± 0.01^{a}	$0.76 \pm 0.05^{a**}$	1.62 ± 0.06^{a}

Values are expressed as mean ± SD.
$^{a, b, c}$ = mean values in the same column within the same parameter are significantly ($p < 0.05$) different from two replications.
** = mean values are significantly ($p < 0.05$) different between 0.5% salt and 3.5% salt under same extraction conditions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

Extracting salmon bone using 0.5% (low) salt and 3.5% (high) salt at different temperatures produced different protein patterns. Extraction at low (55°C) temperature with low salt as shown in Fig. 1A yielded a protein with a molecular weight around 102–150 kDa and tropomyosin (38 kDa; Lu et al., 2017) and a low molecular weight product (12 kDa). Increasing the heating time could degrade tropomyosin and the molecular weight around 102–150 kDa into lower molecular weight peptides or amino acid (less than 12 kDa). Heating for 8 hr completely removed the bands with a molecular weight in the range 102–150 kDa and tropomyosin. Extraction at low temperature (55°C) and high salt (3.5%) as shown in Fig. 1B yielded a molecular weight around 76–102 kDa and a low amount of tropomyosin. Extracting salmon bone with high (3.5%) salt at low temperature (55°C) yielded a protein with a molecular weight of 76–102 kDa but it seemed that there were no proteins with a molecular weight of 102–150 kDa. Low molecular weight (12 kDa) products were also present in the extract at 55°C with high salt as there were with low salt.

Extracting the salmon bone at 85°C with low (Fig. 2A) and high (Fig. 2B) salt levels revealed that they both resulted in protein with a molecular weight higher than 250 kDa, myosin heavy chain (MHC) (250 kDa), collagen (102–150 kDa), protein with a molecular weight around 76–102 kDa, tropomyosin (38 kDa) and protein with a low molecular weight of 12 kDa (Undeland et al., 2002; Wang et al., 2013b; Lu et al., 2017; Darmanto et al., 2014). Protein at a molecular weight between 76 kDa and 102 kDa disappeared when β-mercaptoethanol was added to the sample as a reducing agent, indicating that this protein band could be degraded by the destruction of a disulfide bond. This protein band might be α-actinin (95 kDa) of the myofibrillar protein isolated from salmon mince as reported by Lund and Nielsen (2001). Heating the sample with low salt at 85°C for 5 hr and 8 hr might degrade the molecular weight of around 76–102 kDa into a lower molecular weight around 12 kDa. However, those conditions (high salt) could degrade protein with a molecular weight higher than 250 kDa, MHC and a molecular weight around 76–102 kDa into lower molecular weight peptides or amino acids.

Extracting the salmon bone at 95°C with low (Fig. 3A) and high (Fig. 3B) salts showed that both low and high salts resulted in a protein with a molecular weight higher than 250 kDa, MHC, collagen and tropomyosin. Extracting the salmon bone at 95°C and for time longer than 5 hr resulted in no high molecular weight proteins above

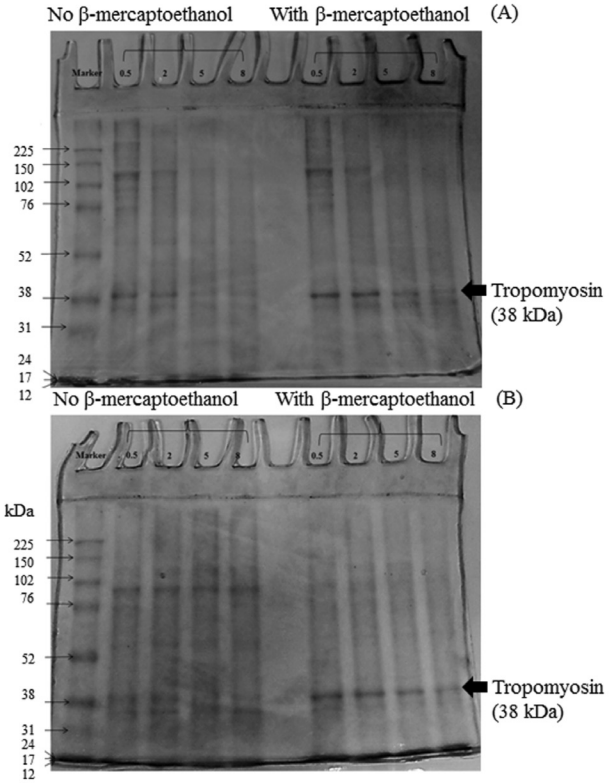

Fig. 1 Protein pattern of salmon bone extract at 55°C during heating for 0.5–8 hr in 10% gel with: (A) 0.5% salt; (B) 3.5% salt

250 kDa and MHC. Protein with a low molecular weight (12 kDa) appeared in the extract with low salt but these proteins disappeared in the extract with high salt. The addition of high salt combined with high temperature extraction resulted in a faster degradation rate of MHC and collagen after heating for 5 hr compared to the addition of low salt. On the other hand, heating at 95°C for a long time (up to 8 hr) degraded MHC and collagen even though the extraction used low salt. This experiment confirmed that a low ionic strength (0.5% salt= 0.085 M NaCl) combined with high temperature could extract myofibrillar proteins including proteins with a molecular weight higher than 250 kDa, MHC, proteins with a molecular weight of 102–150 kDa,

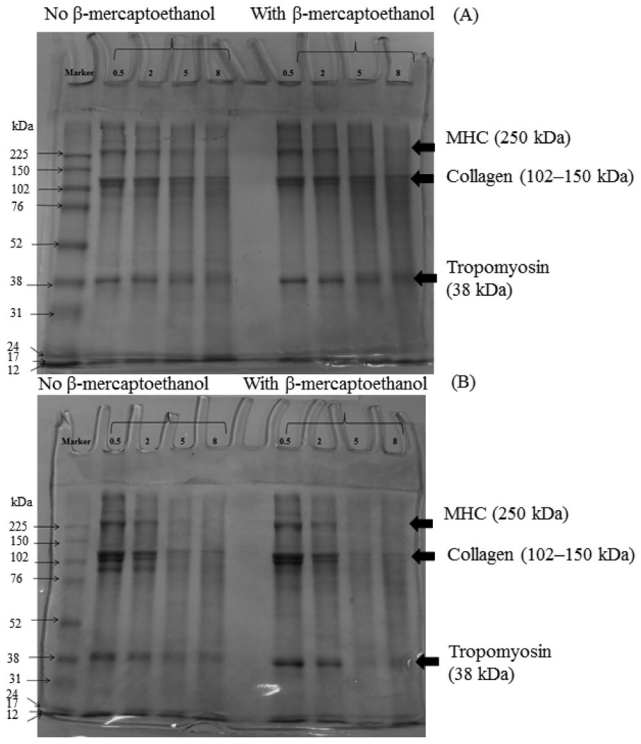

Fig. 2 Protein pattern of salmon bone extract at 85°C during heating for 0.5–8 hr in 10% gel with: (A) 0.5% salt; (B) 3.5% salt

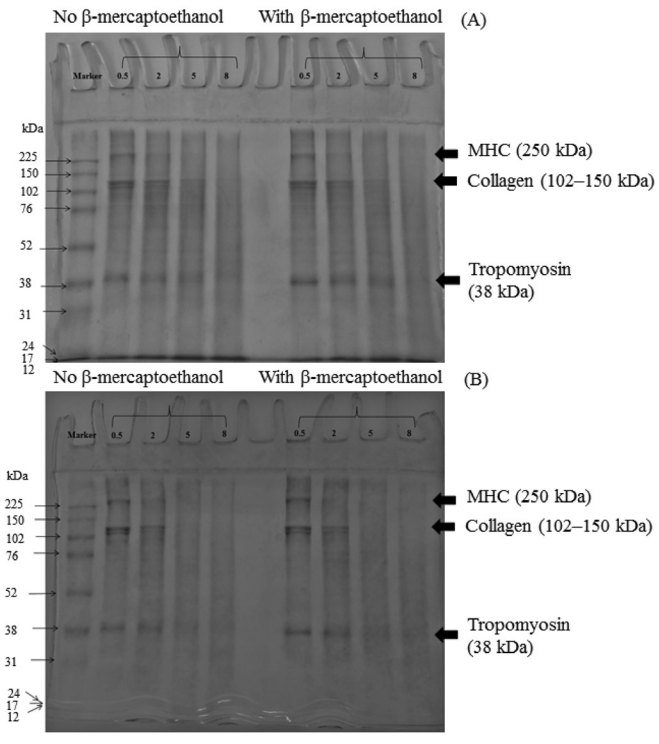

Fig. 3 Protein pattern of salmon bone extract at 95°C during heating for 0.5–8 hr in 10% gel with: (A) 0.5% salt; (B) 3.5% salt

tropomyosin and proteins with a low molecular weight (12 kDa). Zhang et al. (2013) also found that crucian carp (*Carassius auratus*) muscle protein could be extracted using a low (0.5%) salt solution at 55–100°C for 2 hr.

Collagen, which makes up the majority of protein in bone structure, contains a triple helix structure connected by a hydrogen bond (Asghar and Henrickson, 1982). Type I collagen has been reported as the major collagen in fish skin and bone and consists of two identical α_1(I) chains and one α_2 chain (Nagai and Suzuki, 2000). Based on other studies, type I collagen does not have a disulfide bond since it contains less cysteine (approximately 0.2%) and methionine (approximately 1.24–1.33%) which has an important role in the formation of the disulfide bond (Foegeding et al., 1996; Owusu-Apenten, 2002; Kittiphattanabawon et al., 2005). The collagen pattern can be defined as a protein band which did not change under either the presence or the absence of the reducing agent in SDS-PAGE. The extraction of the salmon bone at 85°C (Fig. 2) and 95°C (Fig. 3) with low and high salts confirmed that collagen (102–150 kDa) could be extracted since those protein bands did not change the form under either the presence or the absence of β-mercaptoethanol as a reducing agent. Collagen in the salmon bone started to be extracted at high temperature (85°C and 95°C); however, it degraded during increased heating time. When the sample was treated at a temperature higher than 60°C, the hydrogen bond was destroyed into a subunit which resulted in the disappearance of the band (Wang et al., 2016). The combination of high temperature and heating time could degrade high molecular weight protein, especially MHC and collagen, into lower molecular weight protein. The salt concentration in this experiment did not influence collagen extraction. This result was supported by Montero et al. (1994) who did not observe a difference in collagen solubility of plaice (*Pleuronectes platessa*) skin when the skin was extracted using acetic acid with and without 0.4 M NaCl. Collagen extraction was more influenced by high temperature.

2,2-Diphenyl-1-picrylhydrazyl radical-scavenging activity

Fig. 4A shows that salmon bone extract with the addition of low salt could provide a scavenging activity in the range 17–34%. However, Fig. 4B shows that the extract with high salt had a lower scavenging activity (13–25%) than those with low salt addition. Salmon bone extraction at 55°C and 95°C with low salt addition provided higher DPPH radical-scavenging activity value (approximately 35%) than some enzymatic crude protein hydrolysates such as a sandfish protein hydrolysate (17.21%, Alcalase at 70°C for 3 hr), a blue muscle protein hydrolysate (5–13%, neutrase at 60°C for 3 hr) and a chickpea protein hydrolysate (27.9%, trypsin at 37°C for 2 hr), compared at the same protein concentration (1 mg/mL; Jang et al., 2016; Wang et al., 2013a; Singh, 2011).

Extraction with high salt at the same temperature and time had a significantly lower scavenging activity than extraction with a low salt. Extraction with low salt at 55°C and 95°C had a significant higher scavenging activity compared to at 85°C. Extraction with low salt concentration at 85°C had the lowest antioxidant activity. This might be related to the size of the molecular weight protein obtained from the sample. Extraction at 85°C resulted in a higher band intensity of high molecular weight proteins (higher than 250 kDa and MHC) than at other temperatures at a low salt concentration. The high molecular weight protein had lower scavenging activity than low molecular weight proteins, in agreement with the study by Raghavan et al. (2008) of low molecular weight peptides (3.5–20 kDa) in Tilapia protein hydrolyzates which had more ability ($p < 0.05$) to scavenge reactive oxygen species than high molecular weight peptides (above 30 kDa).

Extraction using high salt resulted in lower scavenging activity than extraction with low salt. Fig. 4B shows that the scavenging activity of the extract with 3.5% salt slightly decreased with increased temperature and time. Increasing salt and temperature could promote lipid oxidation during extraction and increased the amount of lipid peroxyl radicals (LOO*) (Kanner, 1994; Andersen et al., 2007). Those radicals might interact and scavenge peptide and protein from the salmon bone extract that acted as antioxidants. Even though in this experiment, the lipid was reduced by centrifuging and filtering the sample, free radicals still remained in the extract. Extraction with 3.5% salt could result in a low molecular weight as with extraction using 0.5% salt. During extraction, a higher molecular weight was degraded into a low molecular weight but those of low molecular weight were prone to react with lipid peroxyl radicals. Furthermore, lipid oxidation occurred during the extraction process which decreased the ability of proteins with a low molecular weight to react with free radicals, hence the DPPH radical-scavenging activity was low.

The highest scavenging activity occurred when heating the salmon bone extract at 5 hr. The relationship of molecular weight to scavenging activity for 5 hr is illustrated in Fig. 5 and Fig. 6. Extraction with low salt at 55°C and 95°C produced higher scavenging activity compared to high salt addition. The reduced amount of high molecular weight protein and the high amount of low molecular weight protein around 3.5 kDa had a higher scavenging activity. This was supported by Kim et al. (2001) who reported the high antioxidant activity of peptides from the gelatin hydrolysate in Alaska pollack skin in the range 1.5–4.5 kDa. However, extraction with high salt also had a similar protein pattern but the lipid oxidation inhibited the protein playing a role in free radical scavenging. Therefore, the extract with high salt could not provide higher scavenging activity.

The antioxidant activity of proteins and peptides is caused by the complex interactions between their ability to inactivate a reactive oxygen species, scavenge free radicals, chelate prooxidative transition metals, reduce hydroperoxides, enzymatically eliminate specific oxidants and alter the physical properties of food systems in a way that separates reactive species (Elias et al., 2008). Udenigwe and Howard (2013) observed that mixtures of small peptides (below 3 and below 10 kDa), obtained from papain hydrolyzates of beef brisket sarcoplasmic proteins, exhibited antioxidant activities expressed as the DPPH radical-scavenging activity, chelation of metal ions and reducing power. Saiga and Nishimura (2013) reported the mechanism of peptides acted as an antioxidant from porcine myofibrillar proteins by a protease treatment in an Fe (II)-induced aqueous lipid peroxidation system. DPPH radical-scavenging activity indicates the

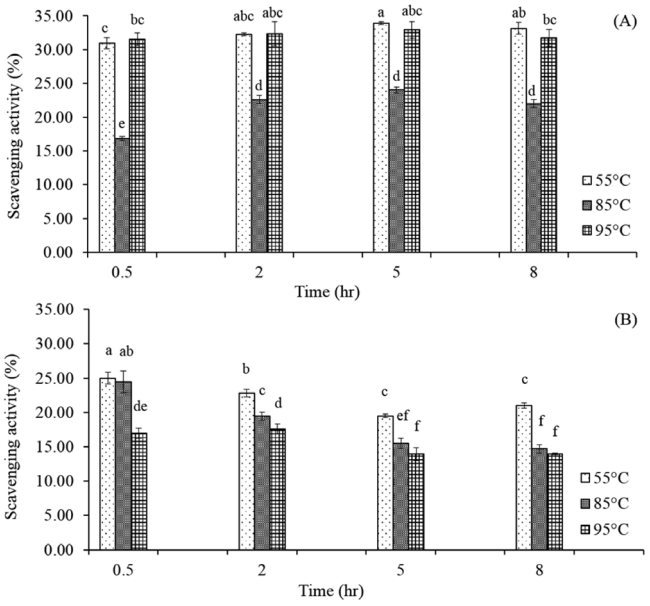

Fig. 4 Radical scavenging activity of salmon bone extracts at various temperatures and times with: (A) 0.5% salt; (B) 3.5% salt, where values are expressed as mean ± SD, a, b, c indicate mean values in the same salt addition followed by different superscripts are significantly ($p < 0.05$) different from two replications

hydrogen-donating abilities of antioxidants (Guerard and Suyama-Maritnez, 2003). Rajapakse et al. (2005) reported that mussel-derived radical scavenging peptide contained two aromatic amino acids and two histidine residues, which were assumed to contribute to higher radical scavenging properties. Aromatic amino acids (Tyr, His, Trp and Phe) are regarded as effective radical scavengers since aromatic amino acids can donate protons easily to electron deficient radicals while maintaining their stability via resonance structures and those properties improve the radical-scavenging properties of the amino acid residues (Sarmadi and Ismail, 2010). Hydrophobic amino acids including Pro, Leu, Trp, Ile, Tyr, and His are predominant amino acids in the peptides of salmon protein which could act as antioxidant as indicated in the report of Borawska et al. (2016). Therefore, smaller peptides and free amino acids from the degradation of myofibrillar proteins in salmon bone extract should play a radical scavenging activity role.

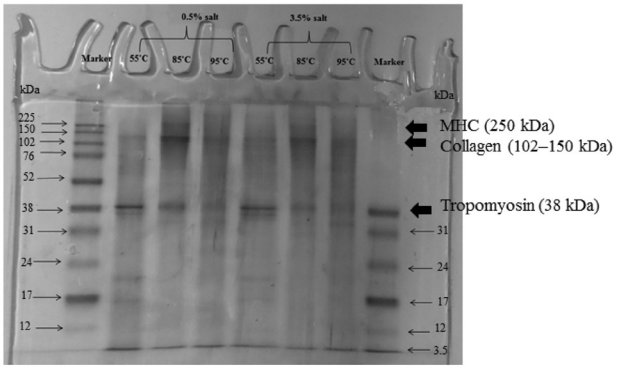

Fig. 5 Protein pattern of salmon bone extract with 0.5 and 3.5% salt concentrations at 55°C, 85°C and 95°C during heating for 5 hr in 15% gel with β-mercaptoethanol

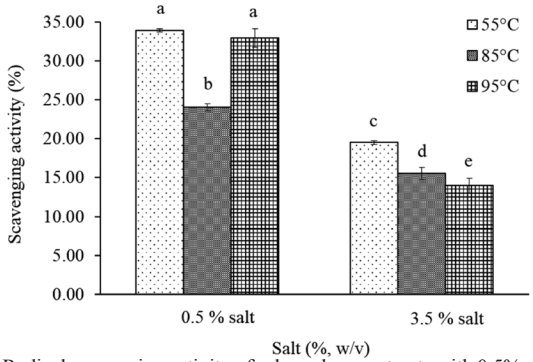

Fig. 6 Radical scavenging activity of salmon bone extracts with 0.5% and 3.5% salts at 55°C, 85°C and 95°C for 5 hr, where values are expressed as mean ± SD, a, b, c indicate that mean values with different superscripts are significantly ($p < 0.05$) different from two replications and w/v = weight per volume

Extracting the salmon bone with 3.5% (high) salt at a higher temperature for a long time gave a significantly higher protein content and total solids. Extraction using low and high salts resulted in a different protein pattern with various molecular weight proteins. High extraction temperatures (85°C and 95°C) with low and high salts yielded collagen (102–150 kDa). A molecular weight higher than 250 kDa and MHC was obtained in the extract at 85°C with either low or high salt addition. Extracting the salmon bone at 95°C could degrade protein when extracted for a longer time. Extracting salmon bone with a low salt percentage at 55°C and 95°C had a higher level of DPPH radical-scavenging activity compared to salmon bone extraction at 85°C. The salmon bone extract at 85°C had dominant proteins with molecular weights above 102 kDa while the extracts at 55°C and 95°C contained higher amounts of low molecular weight proteins. Extracting the salmon bone with 0.5% (low) salt at 95°C for 8 hr yielded a protein content of 0.76 ± 0.05%, which was close to the protein content in commercial fish soup indicating that salmon bone has potential for use as a commercial soup product.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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