

**Subcritical Water Treatment of Isada Krill
for Producing Seasonings**

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General Introduction

Krill is a small shrimp-like crustacean (euphausiid) and is found in all oceans. It plays an important role as a food for larger animals in the oceanic food chain [1]. Isada krill (*Euphausia pacifica*) also known as North Pacific krill that is found mainly around the Sanriku coast in Japan. The biomass of Isada krill is abundant, but its annual harvest is only about 70 thousand tons [2]. Most of the harvested krill was frozen for export to the industrial companies where use it in the production of animal feed or pet food, aquaculture, and only a small amount is directly consumed in Japan [2]. Isada krill was mostly caught from the ocean when the estimated life span of 1–2 year, the body length ranges from 14–19 mm [3] and weight of 0.1–0.2 g.

Isada krill contains a protein content of 40% (dry basis), a lipid content of 5% and an ash content of 4% and small amount of vitamins and minerals. Krill readily deteriorates after harvesting, owing to the action of endogenous proteases in the digestive tract, the autolysis is the most serious problem. After harvesting, krill endogenous enzymes catalyze the deterioration that results in unpleasant odor and color, rapidly [4]. High content of fluoride in exoskeleton of euphausiids species raised concerns of toxicity of using krill for a direct human diet [5]. Fluoride in krill has been reported to be as high as 1.37 and 2.40 mg/g dry mass *Euphausia crystallorophias* and *Euphausia superba*, respectively [6]. Recommended Daily Allowance (RDA) values of fluoride are only 4 and 3 mg for male and female adults, respectively. These limit its usage for human consumption.

While the harvested krill is majorly used for animal and aquaculture feed production, public awareness on nutritive value is leading to an extensive investigation on its potential for pharmaceutical industry, direct human consumption and encouraging the development of new products to increase its value. Isada krill is an excellent source of protein, lipids and astaxanthin oil with high polyunsaturated fatty acid and astaxanthin contents [5]. The protein content in raw krill is approximately 40–60% (dry basis) and its nutritional value is relatively high because of the high content of lysine, leucine and glutamic acid. Krill meal is utilized as a protein source in the feed of a number of marine organisms such as salmon, trout [7], red sea bream [8], yellow tail, and shrimp [9]. The krill lipid contains triacylglycerol (33–40%), free fatty acids (8–16%) and sterols (1.4–1.7%). Krill lipid is rich in EPA (23%), DHA (14%) and its phospholipid fatty acids, and contains the bioactive compounds of astaxanthin (115–175 ppm; dry basis).

The shrimp-like flavor is widely used as a savory flavor enhancer in food products, including seafood sauce, soup, and surimi [10]. Identification and characterization of the volatile components of shrimp have been undertaken in numerous studies. For example, Morita *et al.* [11] identified 2-acetyl-1-pyrroline and 2-acetylthiazole as active volatile compounds in boiled prawn, whereas Tachihara *et al.* [12] found that pyrazines, e.g., 2,5-dimethylpyrazine and 3,5-dimethylpyrazine, are developed through the Maillard reaction in roasted shrimp. Kim *et al.* [10] developed the cooked-shrimp flavor from the enzymatic hydrolysate of shrimp by the Maillard reaction. Kubota *et al.* [13] extracted and concentrated the shrimp-like flavor of roasted shrimp by distillation. However, few studies have focused on utilizing subcritical water extraction for producing extracts rich in shrimp-like flavor.

Subcritical or superheated water is water in the liquid state in the temperature range of 100–374 °C, at pressures of 1–22 MPa. Under subcritical conditions, the relative dielectric constant and polarity of water decrease with the increase in temperature. The reduced polarity of subcritical water gives it properties of organic solvents; thus, it can be used for extraction of organic compounds [14, 15]. Another feature of subcritical water is a high ion product, which indicates that the water acts as a stronger acid or base catalyst than water at ambient. Therefore, subcritical water has been widely used to extract and hydrolyze the natural materials for producing valuable compounds, such as proteins, essential oils, and bioactive compounds (including phenolic compounds and antioxidants). Subcritical water treatment has been applied to rice bran [16, 17], poultry waste [18], squid viscera [19-21], and Baker's yeast cells [22] to produce useful substances.

Furthermore, the production of hydrolysates from fish skin by using subcritical water improved or modified their functionalities [23]. Extraction under subcritical water conditions in a closed vessel also promoted the flavor development of tea [24].

The aim of this study was to develop a simple, efficient and preferable method for producing seasoning having shrimp-like flavor and valuable compounds (such as antioxidant, astaxanthin, and amino acids) by subcritical water treatment. Furthermore, efficient extraction of protein, lipid and astaxanthin as the component in the extract by subcritical water, and molecular mass distribution of the extract by hydrolysis reaction in subcritical water medium were also investigated.

There are five chapters in this thesis. In Chapter 1 provides a discussion on the results of subcritical water treatment of semi-dried Isada krill and the composition and flavor of the extract and residue. This chapter includes the results of production yield measurement, total

protein, total solid, total lipid, freezing point, odor intensity, size exclusion of the products and preference score of flavor at different temperatures. Chapter 2 is devoted to extend the knowledge base for utilizing subcritical water treatment for producing shrimp-flavored ingredients, raw krill was used as a starting material in this study and the effect of temperature on the extraction was studied. In addition, the treatment of ambient pressure boiling is also evaluated. Chapter 3 deals with extraction of high concentration of the liquid extract from krill. Raw Isada krill, without any addition of water, was treated under subcritical water conditions. The effect of temperature on the quality of the krill extract and residue was analyzed. Chapter 4 describes the applicability of severity factor ($\log R_0$) as a parameter for optimizing the product characteristics of both solid residue and liquid extract from subcritical water treatment of raw krill. The observation on the protein content, odor intensity, and preference score plotted against the severity factor were investigated and the optimum severity factor was determined. In Chapter 5, the properties of the liquid extract and solid residue prepared using the large vessel were compared with the materials prepared using a small vessel of batch-type reactor to prove the applicability of using the steam injection.

Chapter 1

Subcritical water treatment for producing seasoning from semi-dried Isada krill

1.1 Introduction

Semi-dried krill, which contains 30% of water obtained from minimal process of blanching and drying, can be used as ingredients in homemade food or mixed with commercial product as a supplement. Raw krill is blanched in brine water to inhibit enzyme, reduce the microorganism and develop flavor and color, and then place to dry in hot-air oven at low temperature to remove 50% of water content in krill.

The biomass of Isada krill is abundant, but its annual harvest is only about 70 thousand tons because of its restricted usage. However, much attention has been paid on use of krill for human diet and its beneficial fatty acids, isolated proteins, and amino acids [25-27] were characterized. This research is among the few that has been conducted on the feasibility of subcritical water treatment as a simple and efficient method for producing the seasoning from semi-dried krill.

In this study, subcritical water treatment of semi-dried Isada krill is investigated herein for the production of seasoning with shrimp-like flavor and analyzed the effect of treatment parameters on the total protein, total solid, total lipid, freezing point, odor intensity, size exclusion of the products and preference score. Analysis of the liquid extract and solid residues of krill treated by subcritical water and ambient pressure boiling were carried out.

1.2 Materials and Methods

1.2.1 Materials

Semi-dried Isada krill (*Euphausia pacifica*) was purchased from Kinoya Ishinomaki Suisan (Miyagi, Japan). The krill contained 0.33 g/g (w.b.) of water and 0.0308 g/g (w.b.) of lipid. The semi-dried krill samples were packed in 10-g vacuum aluminum foil bags and kept at -20°C prior to use. Diethyl ether and bovine serum albumin (BSA, 99% purity) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Folin-Ciocalteu reagent was purchased from ICN Biochemicals (OH, USA). Other reagents were of analytical grade.

1.2.2 Boiling and Subcritical Water Treatment of Isada

Eight gram of the semi-dried krill and 52 mL of distilled water were loaded into a 117-mL pressure-resistant vessel (Taiatsu Techno, Osaka, Japan) and heated to the desired temperature (100–240 °C) for 10 min using a mantle heater (200 W, Heater Engineer, Tokyo, Japan) and a TXN 700B thermo-controller (As One, Osaka, Japan). As a control treatment (boiling), the krill (24 g) was combined with 156 mL of distilled water in an open aluminum pot and boiled for 10 min. After subcritical water treatment or boiling, the vessel or aluminum pot was immediately cooled in an ice-water bath. The extract was filtered through an Advantec No. 2 filter paper (Toyo Roshi, Tokyo, Japan) under reduced pressure. All the wet residue was packed in a nylon sheet bag (25×100 mm) and was pressed at 7.86 MPa using a hydraulic press. The obtained liquid was pooled with the filtered extract. Boiling and subcritical water treatments were performed in triplicate.

1.2.3 Analysis

1.2.3.1 Moisture and Solid Contents

The residue and extract obtained by pressing were immediately subjected to analyze the moisture and solid content. The moisture content and total solid content of the krill residue was determined by the oven-drying method according to Ileleji *et al.* [28] and ISO method 6496 [29], respectively. The sample was dried in a hot-air oven at 135 °C for 2 h. The solid content of the krill extract was also determined by the same method.

1.2.3.2 Lipid Content

The lipid content in the krill extract or residue was determined by Soxhlet extraction [30]. Prior to analysis, the residue was dried in the drying oven at 105 °C for 4 h and ground. The lipid in extract (1.0 g) dehydrated with sodium sulfate (2.0 g) or residue powder (1.0 g) was extracted with diethyl ether (100 mL) in a conventional Soxhlet apparatus for 10 h. The remaining solvent was removed by drying in a hot air oven at 90 °C until constant weight was attained.

1.2.3.3 Protein Content

The protein content of the krill extract was determined by the Lowry-Folin method [31]. Briefly, the krill extract was centrifuged at 6000 rpm (4270×g) for 20 min. The appropriately diluted supernatant (0.4 mL) was mixed with Lowry reagent (2 mL) and 50% (w/v) of Folin

reagent (0.2 mL) and left at room temperature for 30 min. The absorbance of the mixture at 750 nm was measured for the determination of the protein content. BSA solutions with concentrations ranging from 100 to 500 mg/L were used to obtain a calibration curve.

1.2.3.4 Freezing Point Depression

The krill extract was filtered through an Advantec No. 2 filter paper. As an indication of the overall molality of the extract, its freezing point depression was measured using an osmometer (OM802, Vogel, Kevelaer, Germany). The extract would contain a many kinds of molecules at different concentrations.

1.2.3.5 Size Exclusion Chromatography

The extract was subjected to size-exclusion chromatography using an HPLC system (SPD-10A pump and SPD-10A UV-Vis detector, Shimadzu, Kyoto, Japan) equipped with a YMC-pack Diol-120 column (500×8.0 mm i.d., YMC, Kyoto, Japan) at ambient temperature.

Distilled water (flow rate: 1.0 mL/min) was used as the mobile phase. The krill extract was centrifuged at 6000 rpm for 20 min and filtered through a 0.45- μ m syringe filter before injecting 17 μ L of the sample into the column. The elution profile was monitored at the wavelength of 280 nm. The elution time was related to molecular mass using the calibration curve provided by the manufacturer (YMC general catalog 2007/2008, YMC, Kyoto, Japan).

1.2.3.6 Odor Concentration

An odor concentration meter (XP-329IIIR, Cosmos, Tokyo, Japan) was used to measure the concentrations of volatile compounds from the krill extracts and residues. The instrument detects odor molecules using a tin dioxide (SnO_2) thermal semiconductor sensor [32, 33]. The krill extract (1 mL) or krill residue (1 g) was placed in a 5-mL amber glass bottle. The bottle was capped and incubated at 25 °C in a water bath for 10 min. The cap was opened at the desired incubation time and the electronic probe was then immediately placed on the opening of the bottle until a constant signal was attained. The odor concentration was expressed as odor unit/mL of head space.

1.2.3.7 Flavor Preference Evaluation

The extract (1 mL) or residue (1 g), placed in an amber bottle, was presented to 10 untrained panelists for flavor preference scoring. Characteristic flavors (shrimp-like, fishy, smoky and burnt flavor) of the samples were evaluated as the overall preference score using

the 5-point hedonic scale (−2: dislike very much, −1: dislike, 0: neither like nor dislike, +1: like, and +2: like very much). The scores were presented as the mean $\pm$ SD and analysed the difference by analysis of variance (ANOVA) using SAS version 9.0 (SAS Institute, Cary, NC) for randomized complete block design (RCBD) at the $p < 0.05$ level.

1.3 Results and Discussion

1.3.1 Appearance

Semi-dried Isada krill was boiled or treated with subcritical water in the temperature range 100–240 °C for 10 min. Figure 1-1 shows the photographs of the krill extracts and residues treated at various temperatures. The krill extracts obtained by boiling and subcritical water treatment at 100–140 °C were light yellow, whereas the extract prepared at 160 °C was reddish-yellow. The extracts prepared at higher temperatures gradually became brownish-yellow, and the extract prepared at 240 °C was dark brown. The residues from subcritical water treatment at low temperatures were reddish-pink, similar to the residue of boiled krill. The residue from treatment at 160 °C was light brown, and a dark brown residue was obtained from treatment at 240 °C.

Astaxanthin is a natural carotenoid found in krill. In krill, it is normally conjugated with chromoprotein; this conjugate is degraded by cooking, leading to the development of a reddish color in cooked krill [34]. These results indicate that more astaxanthin is extracted from the krill by subcritical water treatment at higher treatment temperatures. The dark brown color obtained with higher treatment temperatures results from the products of browning reactions such as the Maillard reaction or caramelization of sugars [35].

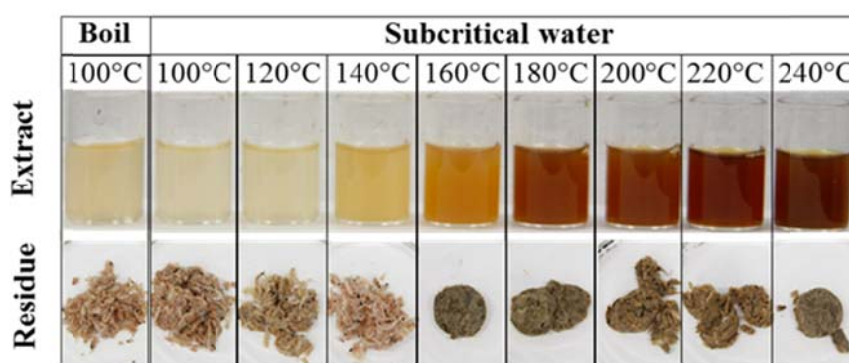


Fig. 1-1. Appearance of extracts and residues prepared by boiling and subcritical water treatment at various temperatures.

1.3.2 Yields of Krill Extract and Residue

Boiling and subcritical water treatment of semi-dried krill followed by hydraulic pressing yielded two fractions: aqueous krill extract and solid krill residue. Figure 1-2 shows the yields of the krill extract and residue obtained with different treatment temperatures. The overall yield, which was defined as a sum of the yield of extract and that of residue, obtained by boiling was approximately 0.75 kg/kg-mixture, because some water was lost by evaporation during boiling using the open pot. The yield of the residue from the boiling treatment was 0.20 kg-wet residue/kg-mixture, whereas the yield obtained from the subcritical water treatment decreased with increasing treatment temperature. Notably, the yield declined significantly at treatment temperatures higher than 140 °C. The yield of the extract increased at higher treatment temperatures. The respective increase and decrease of the yield of the extract and residue with increasing temperature indicate that subcritical water promotes the extraction and decomposition of components more efficiently at higher temperatures.

1.3.3 Properties of Krill Extracts

The total solid, lipid, and protein contents, the freezing point, and molecular mass distribution were determined for the extracts prepared by boiling and subcritical water treatment at various temperatures. The lipid content of the extract obtained by boiling using the open pot (0.0053 kg/kg-extract) was similar to that obtained by treatment at 100 °C using the closed vessel (0.0068 kg/kg-extract). The lipid content increased gradually with increasing temperature, and the highest lipid content of 0.0121 kg/kg-extract was achieved by treatment 240 °C (Fig. 1-3). This is because subcritical water has a higher capacity to dissolve (closed symbols) and subcritical water treatment at various temperatures (open symbols) at lipophilic substances because of its lower polarity [36]. In addition, the lower surface tension and viscosity of subcritical water at higher temperature may facilitate extraction under subcritical conditions [37].

Figure 1-3 also shows the protein content of the extracts obtained by boiling and subcritical water treatment at various temperatures. The protein contents of the extracts prepared at 100 °C using the open pot and closed vessel were similar. The protein contents of the extracts obtained from subcritical water treatment increased with increasing treatment temperature. The highest protein content of 0.047–0.049 kg/kg-extract was obtained at 180–200 °C; the protein content of the extract decreased at higher temperatures. These results are consistent with those of previous studies. For example, Wiboonsirikul *et al.* [17] reported that the

protein content of rice bran extract obtained by subcritical water treatment increased as the treatment temperature was increased from 50 to 200 °C, with a marked decrease at the treatment temperature 250 °C. The increase in the protein content of the extracts is indicative of the hydrolysis of proteinaceous substances in the krill to soluble peptides and amino acids [37]. The decrease of in protein content of the extract at 220 and 240 °C indicated that the proteinaceous components were further decomposed or converted to other products. For example, amino acids may react with carbohydrates to form brown products via Maillard reaction.

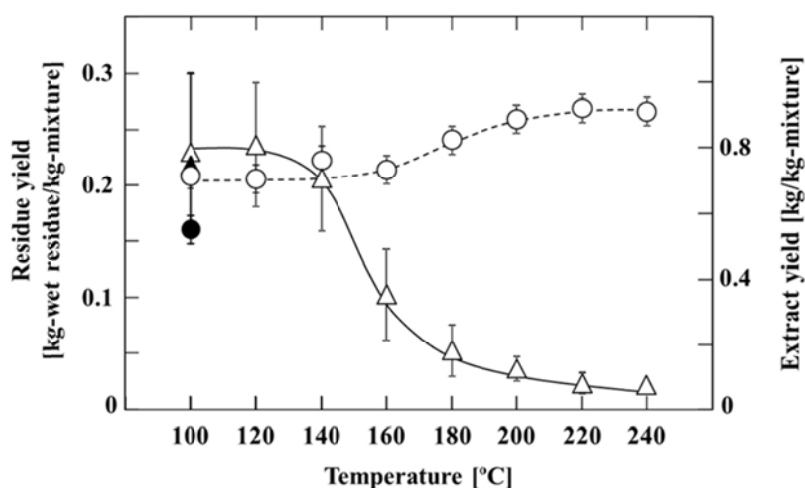


Fig. 1-2. Yields of residue (▲, △) and extract (●, ○) of *Isada* krill obtained by boiling (closed symbols) and subcritical water treatment at various temperatures (open symbols).

The total solid content of the krill extract increased with increasing treatment temperature and reached a constant value of 0.073 kg/kg-extract (Fig. 1-3). The freezing points of the subcritical water extracts decreased with increasing solid content. The total solid was almost constant between 200 and 240 °C, but the freezing point of the extract decreased at those temperatures. These facts indicate that molecule in the extract would be converted to small ones at high temperature. The freezing point of the extract obtained by boiling was significantly lower than those of the extracts derived from subcritical treatment at various temperatures (except at 240 °C), because the solid content of the extract obtained by boiling was higher than those of the extracts derived from subcritical water treatment owing to evaporation of water during boiling.

1.3.4 Molecular Mass Distributions

Size Exclusion Chromatography was used to qualitatively determine the molecular mass distribution of the extracts (Fig. 1-4). Boiling and subcritical water treatment at 100–140 °C

led to similar chromatograms, i.e., a group of peaks was present at retention times of 6–13 min which corresponded to molecular mass of 10^6 – 10^8 Da and another large peak was observed at 19.3 min (10^4 Da). For subcritical water treatment at ≥ 160 °C, the intensity of the group of higher molecular mass (10^6 – 10^8 Da) became significantly higher because of the increased solid content of the extracts (Fig. 1-3). In addition, several peaks of molecular mass of 10^4 – 10^6 were observed; some small peaks of molecular mass of 10 kDa were also detected after 20 min. These results indicate that a greater number of lower-molecular-mass (molecular mass < 10 kDa) compounds were produced at higher temperatures, although the compounds were not conclusively identified.

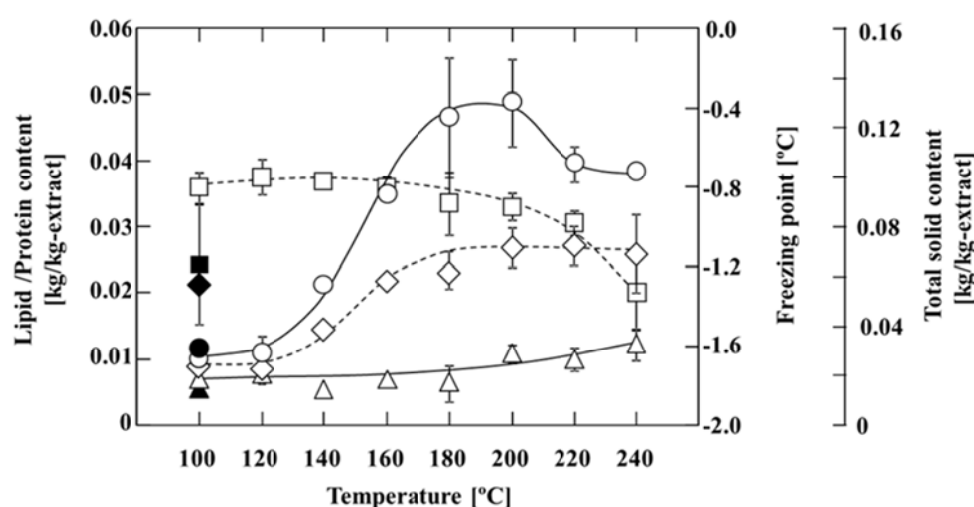


Fig. 1-3. Lipid content (▲, △), protein content (●, ○), freezing point (■, □), and total solid content (◆, ◇) of Isada krill extract prepared by boiling (closed symbols) and subcritical water treatment at various temperatures (open symbols).

1.3.5 Properties of Krill Residues

Moisture content analysis of the pressed residues was performed *via* the hot-air oven method. The moisture contents of the residues obtained at 100 °C using the open pot and closed vessel were almost the same (Fig. 1-5). The moisture content of the residues obtained by subcritical water treatment at 100–140 °C was *ca.* 0.21–0.28 kg-H₂O/kg-dried residue; the value gradually decreased with increasing treatment temperature. This is ascribed to the destruction of the cellular structures of krill by heat and pressure, resulting in the loss of water-binding capability. The lipid contents of the dried krill residues were determined after hot-air oven drying (Fig. 1-5), because the lipid content of the residue obtained using the open pot was almost the same as that of the residue prepared at 100 °C using the closed

vessel. The lipid remained in the dried residues obtained by using subcritical water at 100–240 °C (0.053–0.161 kg/kg-dried residue). Subcritical water treatments at high temperature, the most of compounds were extracted into the extract solution, but the lipid was not completely. The remaining lipid in the residues on dry basis presented higher content at higher temperature.

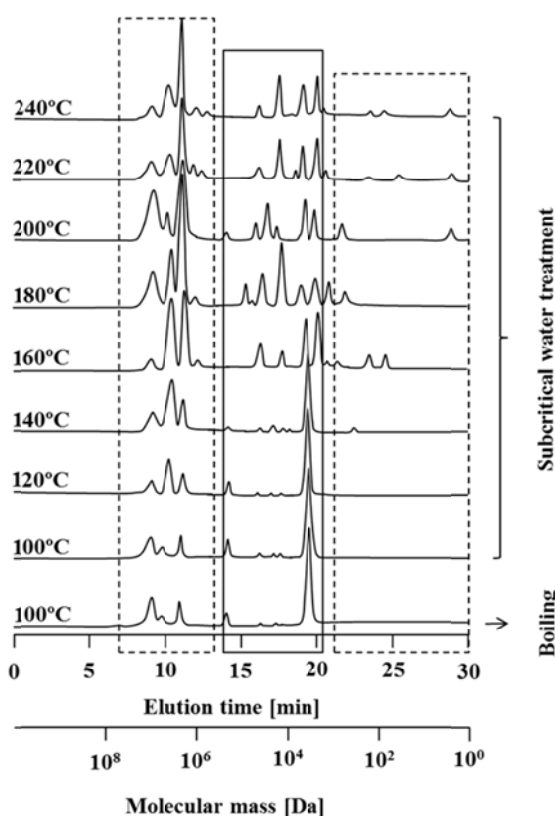


Fig. 1-4. Size-exclusion chromatograms of krill extracts obtained by boiling and subcritical water treatment at various temperatures.

1.3.6 Odor Concentration and Flavor Preference

The flavor of the extract and residue is an important characteristic for their use as seasoning. The suitability of the extract and residue as seasoning was analyzed on the basis of odor concentration measurement and flavor preference evaluation. The odor intensity increased gradually with increasing treatment temperature for both the extract and residue (Fig. 1-6A) with a more pronounced increase in the case of the extract. This might indicate that the volatile compounds are easily dissolved in water.

Figure 1-6B shows the flavor preference score of the extracts and residues prepared at different temperatures. The extracts and residues were presented to 10 Japanese panelists who

scored and described the characteristics of the flavor of each sample. Boiling using the open pot and treatment at 100 °C using the closed vessel imparted a fishy odor to the samples accompanied by a low preference score. Giogios *et al.* [38] evaluated the volatile compounds of the shrimp solution extracted *via* simultaneous steam distillation, and showed that the extract contained sulfur-compounds and amines that impart a fishy odor. The highest preference scores, which reflected the property of shrimp-like flavor, were given for the extracts and residues obtained at 160 and 180 °C. The Maillard reaction is inextricably linked to the desirable flavor and color characteristics of thermally processed food [39].

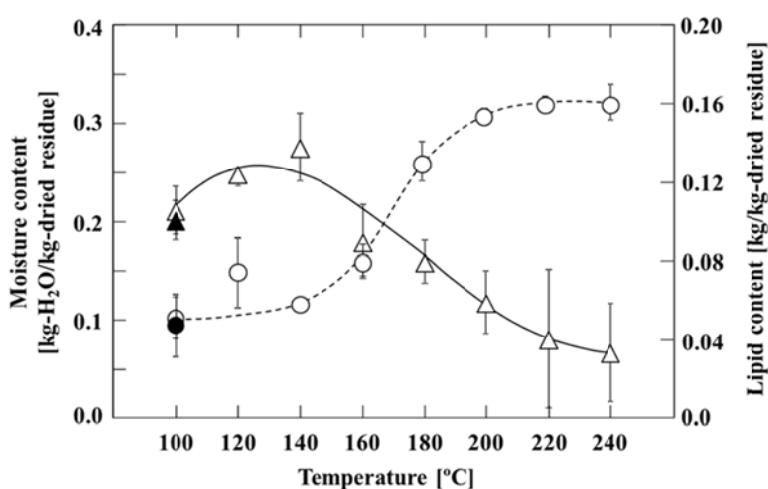


Fig.1-5. Moisture content (▲, △) and lipid content (●, ○) of Isada krill residues obtained by boiling (closed symbols) and subcritical water treatment at various temperatures (open symbols).

The Maillard reaction during the thermal treatment results in the formation of many volatile compounds such as aliphatic compounds, alcohols, ketones, and pyrazine [40]. The low preference scores for the severe treatments at 200–240 °C may be ascribed to the detection of smoky and burnt flavors. It is possible that the subcritical water treatment at elevated temperature produced an off-flavor because of protein hydrolysis. Therefore, the extract obtained by subcritical water treatment at 160 and 180 °C is deemed suitable for use as food seasoning.

1.4 Conclusions

The protein content, freezing point, and odor preference score of krill extracts and residues prepared by boiling and subcritical water treatment at various temperatures differed on the

basis of the conditions employed. There were tendencies that high protein content and flavor preference score were achieved by subcritical water treatment at 160 and 180 °C. Therefore, we concluded that the subcritical water treatment at 160 and 180 °C for 10 min produces a krill extract and residue that can be used as seasoning.

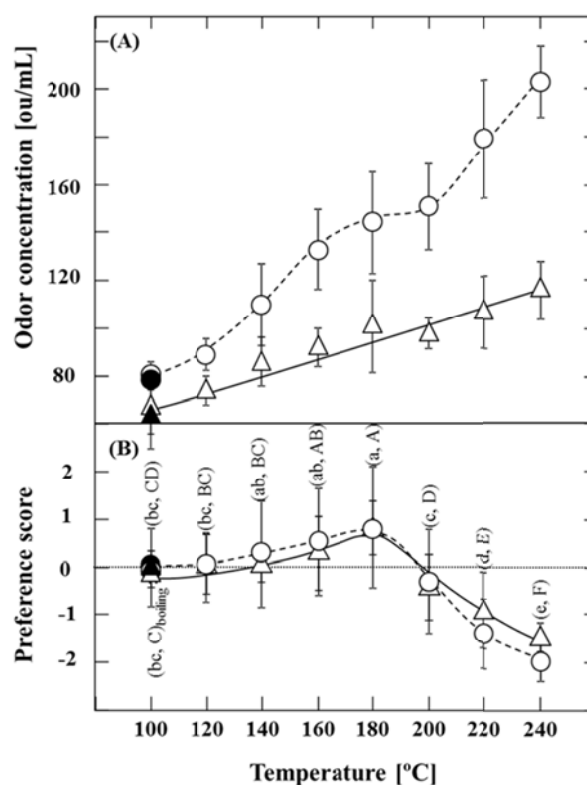


Fig. 1-6. (A) Odor concentration and (B) flavor preference score of krill extracts (●,○) and residues (▲, △) prepared by boiling (closed symbols) and subcritical water treatment at various temperatures (open symbols).

A, B, C,... and a, b, c,... the significant difference ($p < 0.05$) of the residue and extract, respectively.

Chapter 2

Compositions, flavor and antiradical properties of products from subcritical water treatment of raw Isada krill

2.1 Introduction

As described that treatment of semi-dried krill with subcritical water can produce extracts and solid residues that provide a pleasant flavor and may prospectively be used as seasoning ingredients in the previous chapter, availability of the shrimp-like flavor in the treatment products depends on a few parameters including treatment temperature and time and a kind of starting material, which may be either prospective or unpleasant. The objective of the studies in this chapter was to extend the knowledge base for utilizing subcritical water treatment for producing shrimp-flavored ingredients, raw krill is used as a starting material and the antiradical activity of the products is evaluated.

2.2 Materials and Methods

2.2.1 Materials

Isada krill frozen and packed in a 1-kg plastic shrink wrap was purchased from Hamaichi (Wakayama, Japan) and stored in a freezer at -30°C until use. The average mass and length of the krill were 0.07 ± 0.01 g and 18.00 ± 0.65 mm, respectively. Diethyl ether, Folin-Ciocalteu reagent and bovine serum albumin (BSA) were the same as those described in Section 1.2.1. Trisodium citrate dihydrate, copper(II) sulfate pentahydrate, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfuric acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and potassium persulfate, and uracil were purchased from Wako Pure Chemical Industries (Osaka, Japan). Myoglobin, tri -tyrosine, di-L-phenylalanine, and L-phenylalanyl-L-alanine dihydrate were purchased from Sigma-Aldrich (Tokyo, Japan). Glucose oxidase was purchased from Nacalai Tesque (Kyoto, Japan).

2.2.2 Subcritical water and boiling treatment

For subcritical water treatment, the pack of frozen krill was thawed in the pack using tap water and 30 g of the thawed krill was mixed with 30 g of distilled water. The mixture was poured into a 117 mL batch-type vessel (Taiatsu Techno, Osaka, Japan). The closed vessel was then heated with a temperature-controlled ribbon heater (200 W, As One, Osaka, Japan)

to a desired temperature in a range of 100–240 °C. After reaching the desired temperature, the system was maintained at that temperature for 10 min, after which the vessel was immediately cooled by immersion in ice water.

For the ambient pressure boiling treatment, 90 g of the thawed krill was mixed with 90 g of distilled water and boiled at atmospheric pressure in an open aluminum pot for 10 min.

The solid residue and liquid extract were separated by filtering and pressing the same method as described in Section 1.2.2.

2.2.3 Analysis

Total solid, protein and crude oil contents, freezing point depression, odor intensity and flavor preference in the krill extract and residue evaluated using the same method as described in Section 1.2.3.

2.2.3.1 Antiradical activity analyses

The DPPH radical scavenging activity of the krill extract was determined as described by Faithong *et al.* [41] with slight modifications. The extract was centrifuged at 6000 rpm for 5 min and diluted with distilled water. Trolox was dissolved and serially diluted with 95% ethanol to concentrations in the range of 5–50 µmol/L. Subsequently, 1.5 mL of the diluted extract, 95% ethanol (control) or Trolox solution were combined with 1.5 mL of 0.15 mmol/L DPPH in 95% ethanol. The mixture was vortex-mixed and kept in the dark at ambient temperature for 30 min. The absorbance of the mixture was measured at 517 nm using a UV 1600 spectrophotometer (Shimadzu, Kyoto, Japan). The DPPH radical scavenging activity was calculated using Eq. (2-1):

$$\text{Scavenging activity(\%)} = 100 \times (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \quad (2-1)$$

where A_{control} and A_{sample} are the absorbance of the mixture of DPPH and 95% ethanol and the sample, respectively.

The percentage DPPH radical scavenging activity of the sample was compared with that of Trolox in order to express the DPPH radical scavenging activity as µmol Trolox equivalent (TE)/g-extract.

$$\text{TE} = C_{\text{Trolox}} / W_{\text{extract}} \quad (2-2)$$

where C_{Trolox} is the Trolox equivalent concentration of the extract ($\mu\text{mol/mL}$) and W_{extract} is weight of the extract (g).

The ABTS assay was conducted as described by Arnao *et al.* [42] with slight modifications. A stock solution of ABTS radical was prepared by mixing 7.4 mmol/L ABTS solution and 2.6 mmol/L potassium persulfate solution (1:1 by volume); the mixture was allowed to react for 12 h at ambient temperature in the dark. The solution was then diluted by mixing 1 mL of the solution with 50 mL of methanol. The sample (150 μL) was mixed with 2850 μL of freshly prepared ABTS solution. The mixture was kept at room temperature for 2 h and the absorbance of the mixture was spectrophotometrically determined at 734 nm. A standard curve for Trolox was prepared in the 100–500 $\mu\text{mol/mL}$ concentration range.

The ABTS radical scavenging activity was calculated and expressed as μmol Trolox equivalent (TE)/g-extract based on Eqs. (2-1) and (2-2), respectively.

2.2.3.2 Statistical analysis

Statistical analysis of experimental data was performed by analysis of variance (ANOVA) using SPSS 17 (IBM, NY, USA) program and indicated as means $\pm$ standard deviation. The least significant difference test (LSD) for complete randomized design (CRD) was performed to determine the significant difference among the means of treatments at $p = 0.05$. For sensory evaluation, the randomized complete block design (RCBD) was conducted for preference score at the same level.

2.3 Results and Discussion

2.3.1 Yield of krill extract and residue

Two fractions (the liquid krill extract and solid krill residue) were obtained from ambient pressure boiling and subcritical water treatments of raw krill followed by hydraulic pressing. The visual appearance of the extract and residue obtained by ambient pressure boiling and subcritical water treatments at various temperatures is shown in Fig. 2-1. Light yellow extracts were obtained from the ambient pressure boiling and subcritical water treatments at 100–140 $^{\circ}\text{C}$, whereas the residues were pink and retained the krill appearance. Dark brown extracts were obtained with the use of higher temperatures (160–240 $^{\circ}\text{C}$) and the residues were gray and lost the krill appearance completely.

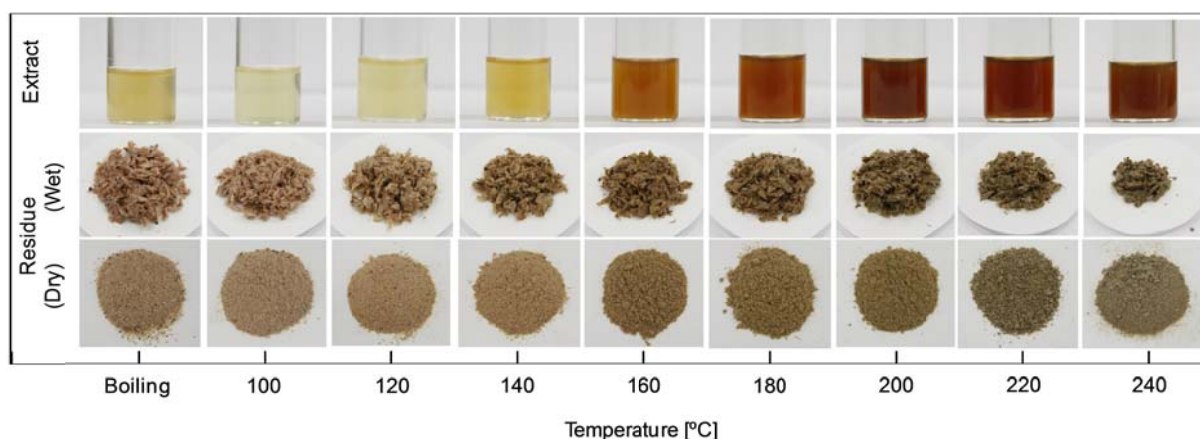


Fig. 2-1. Krill extracts and residues obtained by ambient pressure boiling at 100 °C and subcritical water treatments at various temperatures for 10 min. Dry residues were obtained by hot-air drying (50 °C for 4 h) followed by grinding.

The yields of the extract and residue obtained by ambient pressure boiling were 0.55 kg-extract/kg-mixture and 0.16 kg-wet residue/kg-mixture, respectively. Ambient pressure boiling in an open container permitted evaporation of water and thus resulted in considerable loss of the extract. For the subcritical water treatment, the yield of the residue decreased whereas the yield of the extract increased with increasing treatment temperature. Drastic changes in the yield of the extract and residue from subcritical water treatment were observed at 140–200 °C (Fig. 2-2). The corresponding, respective decline and rise in the yield of the residue and extract demonstrated that krill extraction with subcritical water was more efficacious at higher temperatures.

2.3.2 Protein, lipid, and solid contents

Protein was the major component in the krill extract obtained by subcritical water treatment. A gradual increase in the protein content from 0.018 to 0.038 kg/kg-extract (45.7–65.0% of total solid) was observed as the treatment temperature increased from 100 to 200 °C; however, no further increase in the protein content of the extract was achieved with the use of higher temperatures (Fig. 2-3A). This observation is consistent with that of our previous report [43]. The increase in the protein content of the subcritical water extracts is an indication of hydrolysis of proteinaceous components in the krill to soluble peptides and amino acids [37].

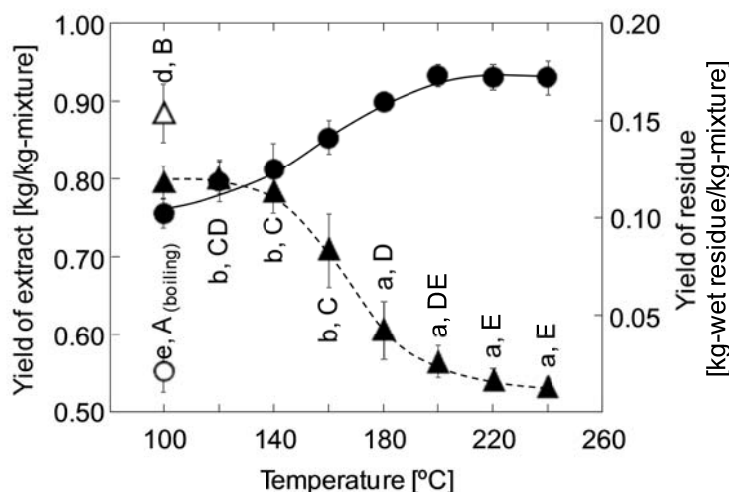


Fig. 2-2. Yields of krill extract (●, ○) and residue (▲, △) obtained by ambient pressure boiling (open symbols) and subcritical water treatments at various temperatures (closed symbols). Capital and small letters indicate significant differences ($p < 0.05$) for the residue and extract, respectively.

The lipid content of the krill extract and residue is defined as the weight of lipid produced from 1 kg of raw krill (Fig. 2-3A). The lipid content of the extract prepared by subcritical water treatment at 100 °C did not differ from that obtained by ambient pressure boiling; however, the lipid content of the subcritical water extract gradually increased from 0.0039 to 0.0225 kg/kg-raw krill with an increase in the temperature from 120 to 240 °C. The dissolution of lipophilic substances is enhanced by the lowered polarity, surface tension, and viscosity of subcritical water [36], which facilitates extraction of nonpolar components [16] at high temperature, thereby accounting for the observed increase in the lipid content of the subcritical water extract with increasing temperature. However, residual lipid was detected in the residues (28.7–67.6% of total lipid), indicating that complete lipid extraction could not be achieved with subcritical water at the evaluated temperatures.

The solid content of the subcritical water extracts increased from 0.04 to 0.06 kg/kg-extract with increasing treatment temperature (Fig. 2-3B). Due to evaporation of water during treatment in the open container at 100 °C, the freezing point depression of this extract was notably larger than that of the extract obtained using the closed vessel. The freezing point depression of the extracts obtained at 100–180 °C were almost the same; however, ΔT_f of the extract obtained at 240 °C was higher than that of the extract obtained at 220 °C despite the similarity of the solid content of these extracts. Thus, it can be inferred that the components of the krill were hydrolyzed to soluble components at higher temperatures.

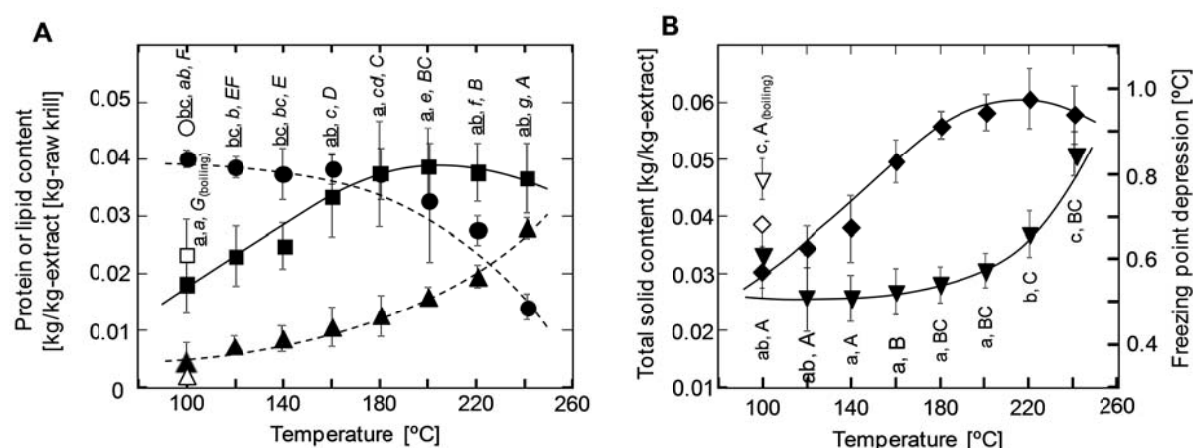


Fig. 2-3. Lipid content of extract (▲, △) and residue (●, ○) and protein content of the extracts (□, ■) (A) and total solid content (◆, ◇) and freezing point depression (▼, ▽) of krill extract (B) obtained by ambient pressure boiling (open symbols) and subcritical water treatment at various temperatures (closed symbols).

Underlined small letters, upright capital and small letters, and slant capital and small letters indicate significant differences ($p < 0.05$) for the protein content, freezing point depression, solid content, and lipid content in the residue and extract, respectively.

2.3.3 Molecular mass distribution

The molecular mass distribution of the extracts was qualitatively determined using SEC (Fig. 2-4A). Three groups of peaks, i.e., high ($>10^5$ Da), moderate (10^3 – 10^5 Da), and low ($<10^3$ Da) molecular-mass peaks, were observed in the typical chromatogram. Only minor differences were observed in the moderate-molecular mass profiles of the extracts obtained using ambient pressure boiling and subcritical water at 100 °C.

At temperatures in the range of 100–140 °C, the extracts obtained using subcritical water treatment all had similar patterns and signal heights. For the samples subjected to subcritical water treatment at 160–180 °C, the intensity of the peaks corresponding to high- and moderate-molecular mass species was greatly enhanced and low-molecular-mass species were also detected with subcritical water treatment at these temperatures. Certain peaks corresponding to high-molecular-mass species disappeared with subcritical water treatment at 240 °C and the intensity of the low-molecular-mass peaks also declined to some extent at this temperature. These results are consistent with those of previous reports [17, 19]. Wiboonsirikul *et al.* [17] reported that subcritical water treatment resulted in an increase in

the content of low and high molecular-mass components in protein extracts from defatted rice bran with an increase in the temperature from 50 to 200 °C, but caused a decrease at 250 °C. Uddin *et al.* [19] also found that the protein yield from subcritical water hydrolysis of raw squid viscera decreased when the temperature was increased from 200 to 280 °C. The decrease in the content of the high- and low-molecular-mass substances at 240 °C may be ascribed to the decomposition of these molecules by subcritical water. The decomposition of protein and amino acids under hydrothermal conditions generates small and volatile components, i.e., organic acids and volatile compounds [37].

Figure 2-4B shows the molecular mass of solute in the extracts; the molecular mass was estimated from the freezing point depression using Eq. (2-1). The highest molecular mass of the solutes in the extract was obtained by subcritical water treatment at 180 °C. The gradual decrease in the estimated molecular mass with increasing temperature agreed well with the previously described decline in the peak height of the high-molecular mass components in the chromatograms at the corresponding temperatures. Because protein was the main component found in the hydrolysates, it is possible that subcritical water treatment at high temperature promoted the formation of lower-molecular mass substances due to protein hydrolysis. For instance, Mekonnen *et al.* [44] reported that the subcritical hydrolysis of proteins from waste proteinaceous biomass produced a narrower molecular weight distribution and smaller molecules at higher degrees of hydrolysis.

2.3.4 Odor intensity and flavor preference

Flavor plays an important role in food product acceptance. The odor intensity and flavor preference of the extracts and residues were determined to evaluate the effect of the subcritical treatment temperature on these parameters as indicators of product acceptance. Figure 2-5 shows the odor intensities detected by a semi-conductor sensor. The odor intensities of the extracts and residues from both treatments increased with increasing treatment temperature. Generally, during thermal treatment of a material containing protein and carbohydrate, many compounds such as aromatic compounds, alcohols, and ketones are developed through the Maillard reaction [45]. Because these products are volatile and water-soluble, the odor intensity of the extract is higher than that of the residue.

The preference test by panelists is important for selection of desirable products for use as food ingredients. Kubota *et al.* [13] found that pyrazines (i.e., 5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 2-ethyl-6-methylpyrazine) were important constituents of the cooked-shrimp flavor in the extract of cooked shrimp. The desirable flavor of cooked shrimp was also

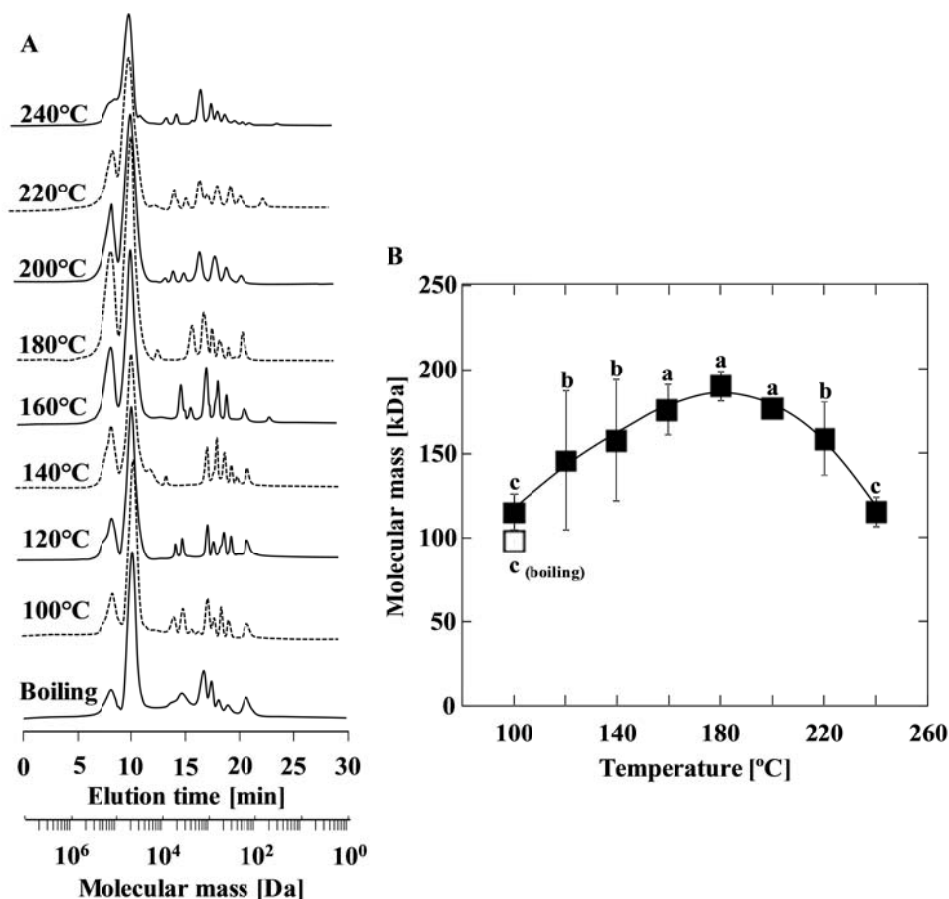


Fig. 2-4. Size exclusion chromatograms (A) and treatment-temperature dependence of the molecular mass estimated from the freezing point depression and solid mass (B) of krill extracts obtained by ambient pressure boiling (open symbol) and subcritical water treatment at various temperatures (closed symbols). The letters indicate significant differences ($p < 0.05$).

generated by subcritical water treatment of Isada krill. The highest scores of overall flavor were assigned to the extract and residue obtained by subcritical water treatment at 140 and 160 °C, respectively. The detection of a fishy odor for the extracts from ambient pressure boiling and subcritical water treatments at 100 °C may have led to the low scores of these samples. The low scores of the extracts and residues from treatment at more severe temperatures (180–240 °C) was due to the detection of burnt odor. Cooking at elevated temperature led to the development of overheating products of the Maillard reaction, i.e. alkylpyridine and furans [45], which resulted in the detection of an off-flavor. Therefore, the extract and residue obtained by subcritical water treatment at 140 and 160 °C, respectively, are considered to be prospective candidates for use as seasonings with shrimp-like flavor.

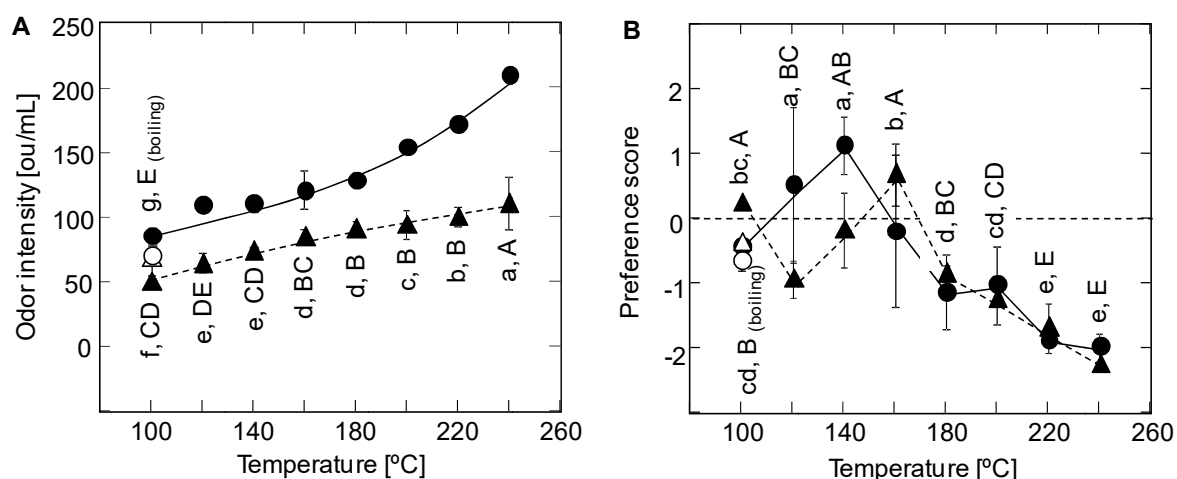


Fig. 2-5. Odor intensity (A) and preference score of overall flavor (B) from krill extract (●, ○) and residue (▲, △) obtained by ambient pressure boiling (open symbols) and subcritical water treatment at various temperatures (closed symbols).

Capital and small letters indicate significant differences ($p < 0.05$) for the residue and extract, respectively.

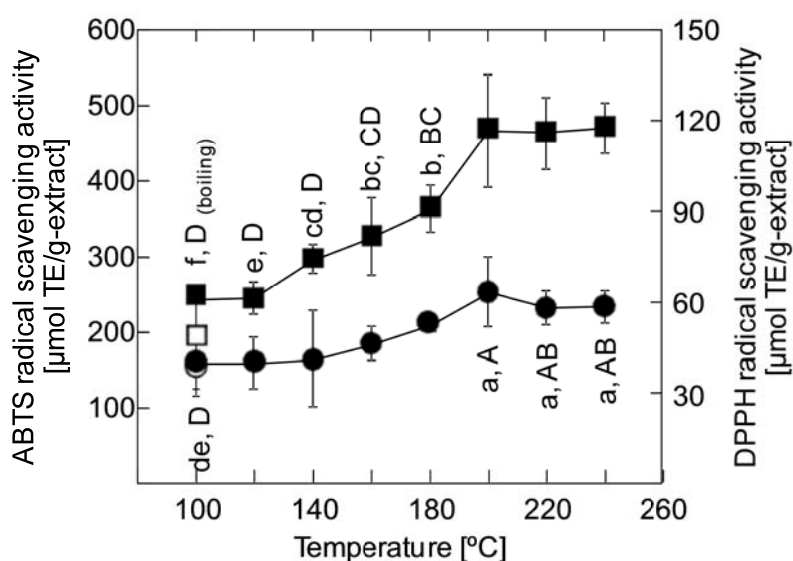


Fig. 2-6. DPPH (●, ○) and ABTS (■, □) radical scavenging activities of krill extracts obtained by ambient pressure boiling (open symbols) and subcritical water treatment at various temperatures (closed symbols).

Capital and small letters indicate significant differences ($p < 0.05$) for the DPPH and ABTS, respectively.

2.3.5 Antiradical activities

Figure 2-6 shows the antioxidant activities of the extracts obtained from the ambient pressure boiling and subcritical water treatments. The DPPH and ABTS radical scavenging activities of the extract obtained by subcritical water treatment ranged from 38.35–62.92 and 193.32–469.35 $\mu\text{mol TE/g-extract}$, respectively. The extracts prepared at higher temperatures exhibited higher DPPH and ABTS radical scavenging activities. It has been reported that the hydrolysates of shrimp, krill, and marine materials possess antioxidant activity [41]. The antiradical activity of the extract depended on the degree of protein hydrolysis and the amount of protein hydrolysate [46]. In addition, Maillard reaction products may also act as antioxidants [47]. Plaza *et al.* [35] reported that increasing the subcritical water extraction temperature from 100 to 200 °C increased the antioxidant capacity of brown algae (*U. pinnatifida*) extract.

2.4 Conclusions

Subcritical water treatment of raw Isada krill produced promising products for use as shrimp-flavored seasoning ingredients with functionalities. Its indications, i.e., protein, solid and lipid contents, and antiradical activity of these products, increased with the use of higher treatment temperatures. Subcritical water treatment at high temperatures (≥ 180 °C) promoted the hydrolysis of high-molecular-mass substances. However, the most desirable shrimp-like flavor was obtained from the 1:1 (w/w) mixture of raw krill and water by subcritical water treatment at 140 and 160°C. Eventually, the knowledge presented in this study extend a method for using the krill as a starting material for seasoning with shrimp-like flavor that would increase the value of the krill as a food resource for human diet.

Chapter 3

Direct treatment of Isada krill under subcritical water conditions to produce seasoning with shrimp-like flavor

3.1 Introduction

Previous chapters, we have applied the treatment to semi-dried or raw krill by mixing the krill with water at a mass ratio of 1:1, to produce extracts and residues having pleasant, shrimp-like flavors. However, mixing of the krill with water resulted in the dilution of the extract, which decreased the flavor intensity. Astaxanthin has a role in determining the color of crustacean products [48]. Its stability during drying and storing processes has been studied [49]; however, its stability during a hydrothermal processing, as in subcritical water treatment, has not been reported.

In this study, we described the direct treatment of raw krill, without the addition of water, under subcritical conditions, to produce extracts and residues, which is possible because the water content of raw krill is around 80% of its fresh mass. The effects of treatment temperature on the properties of the extracts and residues were examined to determine the optimal treatment conditions. Sensory evaluations of the extracts and residues were also carried out.

3.2 Materials and Method

3.2.1 Materials

Raw krill and all of chemical reagents were the same as those described in Section 2.2.1.

3.2.2 Treatment under subcritical water conditions

The plastic wrapped frozen krill pack was thawed by immersion in water, and 60 g of the thawed krill was put into a 117 mL pressure-resistant batch-type vessel (Taiatsu Techno, Osaka, Japan). The closed vessel was then heated with a 200 W mantle heater (Heater Engineer, Tokyo, Japan) equipped with a TXN 700B thermo-controller (As One, Osaka, Japan) to temperatures of 100–240 °C, at intervals of 20 °C. Extractions at these eight temperature points were performed in triplicate. After reaching the desired temperature, it

was maintained for 10 min, and then the vessel was immediately immersed in ice water to cool it down to room temperature.

The treated krill was separated into liquid and solid residues by filtration under reduced pressure, followed by pressing as described in Section 1.2.2.

3.2.3 Properties of the extracts

The Properties of the extracts including total solid, protein and lipid contents and odor intensity and sensory evaluated using the methods described in detail in 1.2.3 and the pH of the extract was measured using a pH meter (D-51; Horiba, Kyoto, Japan).

3.2.3.1 Molecular mass distribution

Molecular mass distribution was determined by size exclusion chromatography, using an HPLC system consisting of an LC-10AD pump (Shimadzu) and an YMC-Pack Diol-120 column (500 mm length, 8.0 mm internal diameter; YMC, Kyoto, Japan). Filtered extract (20 μ L) was loaded onto the column and eluted with distilled water at a flow rate of 1.0 mL/min. Elution was monitored with an UV–Vis light detector (SPD-10A; Shimadzu, Kyoto, Japan) at 280 nm. The mass distribution in the extract was determined by comparison with molecular mass standards comprising glucose oxidase (153 kDa), BSA (66 kDa), myoglobin (16.9 kDa), Tyr-Tyr-Tyr (507 Da), Phe-Phe (312 Da), Phe-Ala (236 Da), and uracil (112 Da), dissolved in distilled water. The elution volume of the extract was converted to the molecular mass using the calibration curve of the standards. The curve was expressed by the following equation:

$$\log M = 7.31 - 0.334V_e \quad (3-1)$$

where M and V_e are molecular mass (Da) and elution volume (mL), respectively.

The freezing-point depression, ΔT_f ($^{\circ}$ C), was also measured, using an osmometer (OM802; Vogel, Kevelaer, Germany) to estimate the average molecular masses of solutes (M_{solute}) in the extracts, using the following equation:

$$M_{\text{solute}} = w_{\text{solute}} K_f / \Delta T_f \quad (3-2)$$

where w_{solute} is the fraction of total solid content (g/kg) and K_f is the molal freezing-point

depression constant of water (1.86 (°Ckg)/mol).

To quantify peak areas, the printed chromatogram was carefully cut along a peak shape, and the paper was divided into regions of high, medium, and low molecular mass by division at 10^4 and 10^2 Da. The divided chromatograms were weighed to calculate the relative peak areas of the regions of high, medium and low molecular mass to the total area.

3.2.3.2 Astaxanthin analysis

Astaxanthin in the liquid extracts or solid residues was extracted and saponified to convert it into its free form. Krill residues were first dried in a hot air oven at 70 °C for 2 h and ground using a mortar and pestle to produce a powder. Extraction and saponification were performed using a modified method of Yuan and Chen [50]. Residual powder (0.5 g) or extract solution (5 mL) was mixed with 10 mL of an extraction solvent, which was a mixture of dichloromethane and methanol (1:3, by volume) in a 50-mL screw-capped vial. The headspace was flushed with nitrogen and the mixture was stirred in the dark for 3 h. The vial was then centrifuged at $9600\times g$ for 5 min at 4 °C. The precipitated residue or the upper layer of the extract was separated from the solution containing astaxanthin. The volumes of astaxanthin-containing solutions obtained from residues and extracts were adjusted to 10 and 5 mL, respectively, with the extraction solvent. Astaxanthin in the solution was de-esterified by mixing with 0.1 mol/L sodium hydroxide in methanol at the ratio 5:1. Headspace was flushed and kept at 4 °C in the dark for 12 h. The solvent was then removed by evaporation under a stream of nitrogen. Dry, free astaxanthin was re-dissolved in chloroform for HPLC analysis. Standard solutions of astaxanthin containing 5–25 µg/mL were prepared from a stock solution of 25 µg/mL.

The astaxanthin content was determined using HPLC (Shimadzu, Kyoto, Japan), with a Luna[®] 3µm Silica (2) 100 Å column (150 mm×4.6 mm; Phenomenex, Torrance, CA, USA) at ambient temperature. The mobile phase was a mixture of hexane and acetone (82:18, by volume), and the flow rate was 1.2 mL/min. The sample injection volume was 20 µL. Astaxanthin loss during the subcritical water treatment was calculated in percentage using the following equation:

$$\text{Astaxanthin loss} = [A_{\text{raw}} - (A_{\text{ext}} + A_{\text{res}})] / A_{\text{raw}} \times 100 \quad (3-3)$$

where A_{raw} , A_{ext} , and A_{res} are the absorbances that correspond to astaxanthin concentrations

in raw krill, liquid extract and solid residue, respectively.

3.3 Results and Discussion

3.3.1 Appearance of extracts

The appearance of extract solutions and solid residues obtained by treatment of krill under subcritical water conditions varied depending on the treatment temperature (Fig. 3-1). The brown color of extracts was more intense at higher treatment temperatures. Extracts obtained at 100 and 120 °C were light yellow, whereas extracts prepared at 140 and 160 °C were reddish-yellow. Extracts prepared at higher temperatures gradually became brownish-yellow, and the extract prepared at 240 °C was dark brown. The residues treated at low temperatures were reddish-pink. The residues from treatment at 140–180 °C were light brown, and residue color became lighter with treatments at temperatures above 200 °C.

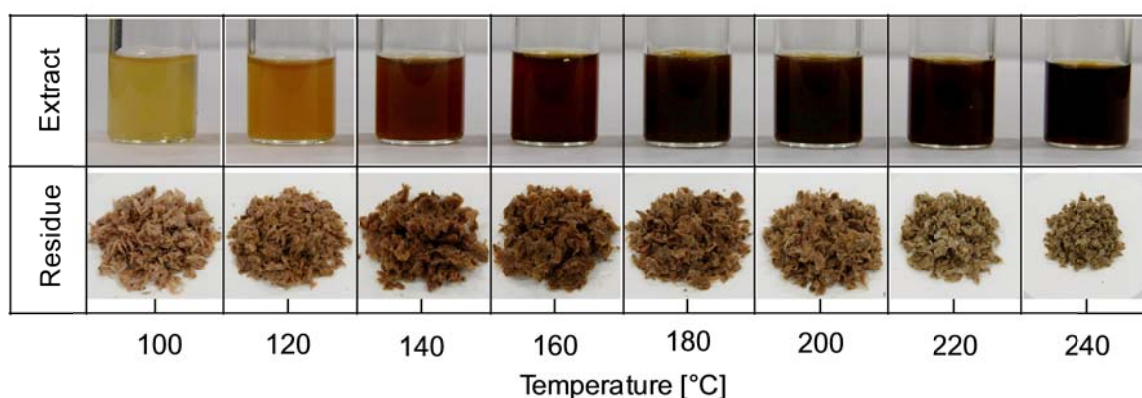


Fig. 3-1. Photographs of the liquid extracts and solid residues obtained after subcritical water treatment of raw krill at various temperatures.

3.3.2 Yields of extract solutions and solid residues

Raw krill was treated at various temperatures and separated to give extract solutions and solid residues. Yields of extracts and residues varied depending on treatment temperature (Fig. 3-2). Inset of the figure shows the temperature profiles during the treatment of the krill using the batch-type vessel. The yields of extracts and residues showed little variation with treatment from 100 to 140 °C, but extract yields greatly increased, and residue yields greatly decreased, with the treatments below 140 to 180 °C. The yields of residues on net mass basis at temperatures below 140 °C were around 0.3–0.4 kg per kg of raw krill, declining significantly at treatment temperatures above 140 °C. The yields of extracts increased with

increasing treatment temperature. At temperatures above 180 °C, the yields of extracts were ≥ 90 % of the raw krill. The respective increases and decreases in the yields of extracts and residues with increasing temperature indicate that subcritical treatment at higher temperature decomposed the krill more efficiently by increasing the ability of the krill water content to act as a solvent.

3.3.3 Total solid and protein contents and pH of extracts

Total solid and protein contents, and pH of extracts varied according to treatment temperature (Fig. 3-3). The total solid content in the extracts increased gradually from 0.06 to 0.13 kg per kg of extract with increasing treatment temperature up to 220 °C, leveling off to 0.12 kg per kg at 240 °C. Protein content also increased from 0.03 to 0.11 kg per kg of extract at treatment temperatures up to 200 °C, and slightly decreased, to 0.10 kg per kg at higher temperatures. The pH of the extracts showed similar variation and might result from the formation of amines, which is a basic product obtained by cooking materials containing protein at high temperature [51]. These observations suggest that proteinaceous substances would be the main components in the extracts obtained at higher temperatures [51]. However, the decrease of protein content at 220 and 240 °C suggested that the degradation of proteins and the generation of small components, such as organic acids, at these temperatures occurs [17, 37].

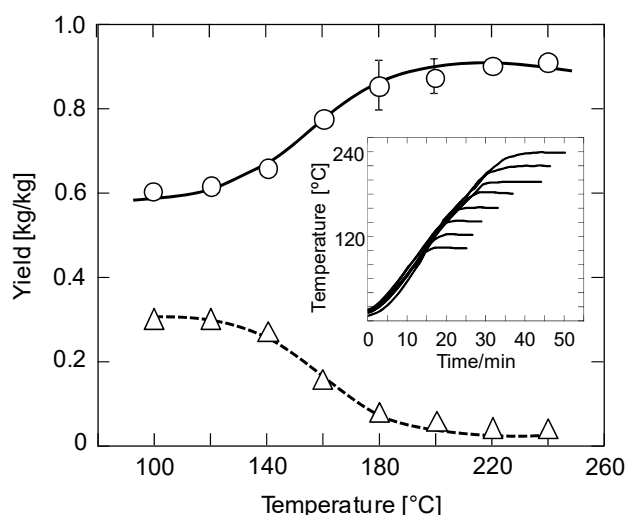


Fig. 3-2. Yields of the liquid extracts (○) and solid residues (△) obtained after subcritical water treatment of raw krill at various temperatures. Inset: temperature profiles during the treatments.

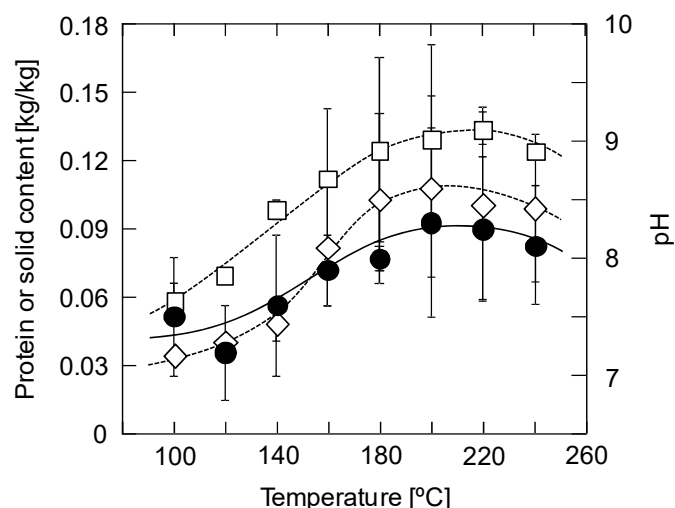


Fig. 3-3. Total solid (□) and protein (◇) contents, and pH (●) of the liquid extracts obtained after subcritical water treatment of raw krill at various temperatures.

3.3.4 Molecular mass distribution

Molecular mass distribution profiles of liquid extracts varied depending on treatment temperature. The molecular masses ranged from 10^1 to 10^6 Da (Fig. 3-4A). Chromatograms of extracts obtained at 100–140 °C showed similar patterns, although the signal intensity of the extract at 140 °C was greater than those of the extracts at 100 and 120 °C. Similarly, the extracts obtained at 220 and 240 °C showed similar distribution profiles.

The chromatograms were divided into regions of high, medium and low molecular mass, and peak areas of the regions were measured (Fig. 3-4B). The peak areas of the regions of high and medium molecular mass increased with increasing treatment temperature up to 180 °C, and decreased at higher temperatures. The total solid content of the extracts showed little variation from 180 to 240 °C (Fig. 3-3). Therefore, the decline could be ascribed to hydrolysis of high-molecular-mass substances such as protein. The peak area of the low-molecular-mass region sharply decreased at temperatures above 140 °C, suggesting degradation of low-molecular-mass substances. Small-molecular-mass substances were observed in the extracts obtained from treatments at 100–160 °C, but their levels were much reduced with treatments at higher temperatures. A small peak at $<10^1$ Da was observed at 220 and 240 °C, and might be ascribed to low-molecular-mass substances, such as organic acids, amines, aldehyde, and imines, formed by the thermal decomposition of amino acids [52]. Yoshida *et al.* [37] reported that pyroglutamic and acetic acids were generated by the hydrolysis of fish meat protein after treatment for 5 min under subcritical water conditions at

513 K (240 °C) and 533 K (260 °C), respectively. These results indicated that a greater number of lower-molecular-mass compounds were produced during subcritical water treatment at higher temperatures.

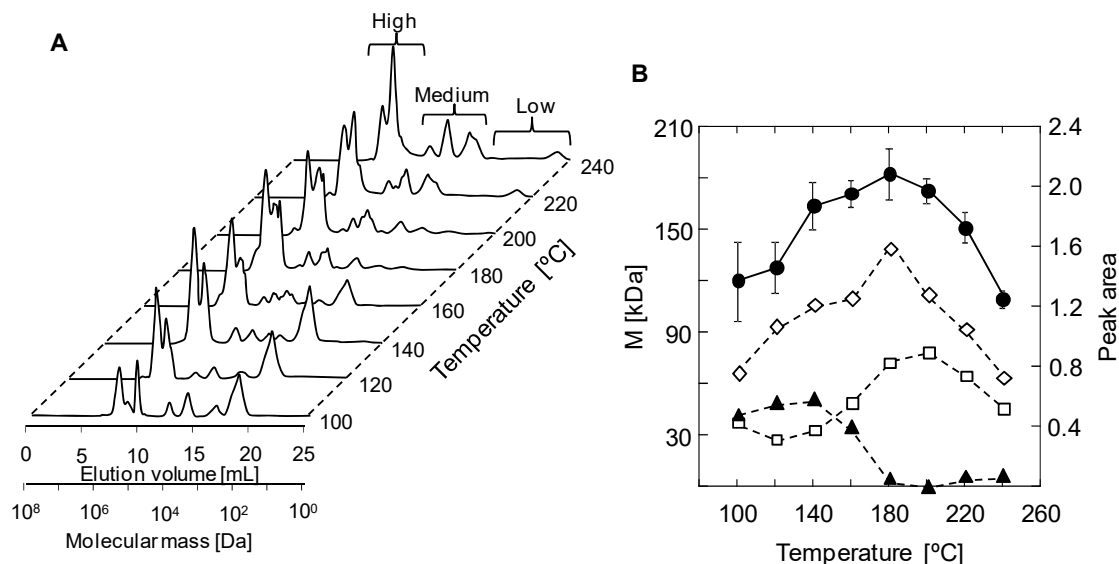


Fig. 3-4. Size exclusion chromatograms (A) of liquid extracts obtained from subcritical water treatment of raw krill at 100–240 °C, and the mean molecular mass (M) of the liquid extract (B), estimated by freezing-point depression (●), and the relative peak areas of regions of high (◇), medium (□), and low (▲) molecular mass of the chromatograms.

3.3.5 Lipid content

Lipid contents of extracts and residues varied depending on treatment temperature (Fig. 3-5). Lipids moved more easily from the krill to the liquid extract at higher treatment temperatures. With increasing treatment temperature, the lipid contents of liquid extracts increased from 0.006 to 0.031 kg per kg of raw krill (from 12 to 58 % of total lipid content), whereas those of solid residues decreased from 0.045 to 0.022 kg per kg (from 88 to 42 % of total lipid). Because subcritical water has a low relative dielectric constant (like an organic solvent), which decreases with increasing temperature, more lipids should be extracted and solubilized in the liquid phase at high temperatures [36]. For instance, the use of subcritical water to pretreat the oil seed of *Datura stramonium* increased the yield of extractable lipids by 50 % [53]. In addition, collapse of krill shell material could become increasingly important at elevated temperatures, facilitating lipid extraction. Chitosan and protein, which are the shell components of the crustacean organism [54], could be decomposed by subcritical water hydrolysis [55].

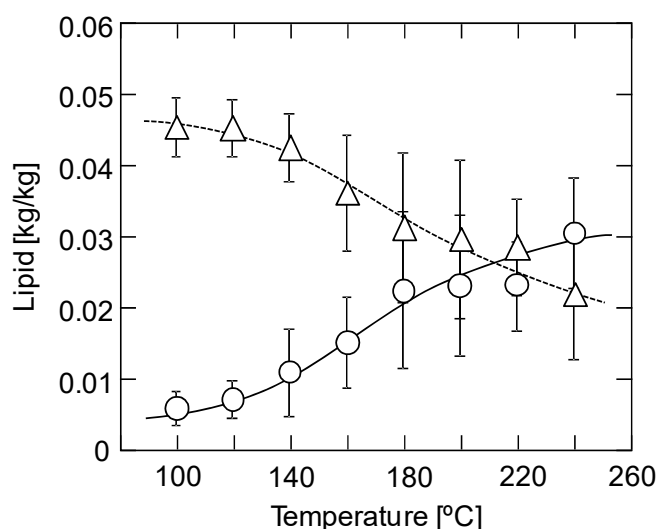


Fig. 3-5. Lipid content of the liquid extracts (○) and solid residues (△) obtained after subcritical water treatment of raw krill at various temperatures.

3.3.6 Astaxanthin content and stability

Astaxanthin is the main carotenoid that has a role as a pigment in the krill and it exhibits excellent antioxidant capacity. The overall astaxanthin contents in krill extracts and residues obtained by subcritical water treatment, as well as astaxanthin loss during treatment (determined by comparison with total astaxanthin composition of raw krill) vary depending on treatment temperature (Fig. 3-6). The overall astaxanthin content in the extracts prepared at 100–140 °C increased with increasing treatment temperature, and the highest yield was achieved at 140 °C, with a corresponding reduction in residual astaxanthin content in the residues. This increase in the extraction of astaxanthin from the krill might be due to the reduced viscosity and polarity of subcritical water at higher temperatures, increasing the ability to solubilize the hydrophobic astaxanthin [55]. In addition, heating of the krill also helped releasing astaxanthin from protein complex [48]. Nevertheless, it was found that the overall astaxanthin content under subcritical conditions at 100–140 °C remained constant even though some astaxanthin was extracted into the liquid solution.

Astaxanthin loss was determined as the difference between the astaxanthin content of raw krill and the sum of the contents of the extracts and residues. In the treatment temperature range of 160–240 °C, the levels of astaxanthin in both the extracts and residues decreased with increasing temperature, resulting in a sharp increase in astaxanthin loss, presumably due to degradation. Almost all the astaxanthin was lost at 220–240 °C (Fig. 3-6). Astaxanthin has previously been shown to be thermally labile, with Becerra *et al.* [57] reporting that 14.2 %

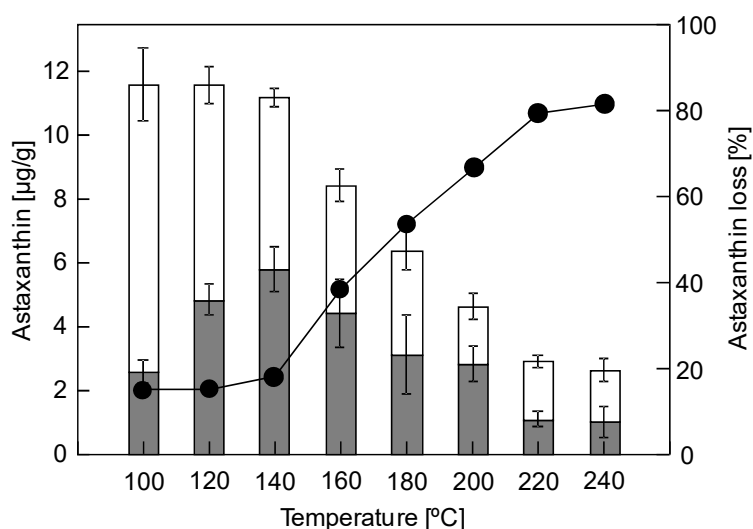


Fig. 3-6. Astaxanthin content in the liquid extracts (gray bars) and solid residues (white bars), and astaxanthin loss (●), after subcritical water treatment of raw krill at various temperatures.

of astaxanthin was degraded by boiling shrimp in brine (5 % NaCl, by mass per volume) for 15 min.

3.3.7 Odor intensity and sensory evaluation

Treatment temperature had a remarkable influence on the odor intensities of krill extracts and residues (Fig. 3-7). The odor intensity became stronger as the treatment temperature increased for both the extracts and residues. Degradation of proteins and other substances, which are precursors of odor compounds [58], could increase at higher treatment temperatures.

Shrimp-like flavor intensity and preference scores of the krill extracts and residues at various treatment temperatures were measured (Fig. 3-8). The preference score expressed an indication of the overall quality of the extract or residue as a seasoning. Shrimp-like flavor intensity was evaluated as the magnitude of cooked shrimp aroma. Both the extract solutions and solid residues exhibited a shrimp-like flavor, but its subjective intensity, measured by panellists, did not correlate with the objective odor intensity, measured electronically (Fig. 3-7). The strongest shrimp-like flavor was detected in the extracts and residues prepared at 140–160 °C. These results are similar to those for the treatment of raw krill mixed with water [10]. The temperature dependence of preference scores was similar to that of the shrimp-like-

flavor intensity (Fig. 3-8). The extract and residue obtained at 140 °C had the highest values of both preference score and shrimp-like flavor intensity. Burnt odor, which is ascribed to very dark roast and ash-like substances, was recognized in extracts and residues prepared at temperatures of 180–240 °C, and the preference scores and shrimp-like flavor intensities for these treatment temperatures were low, especially for the extract and residue obtained at 240

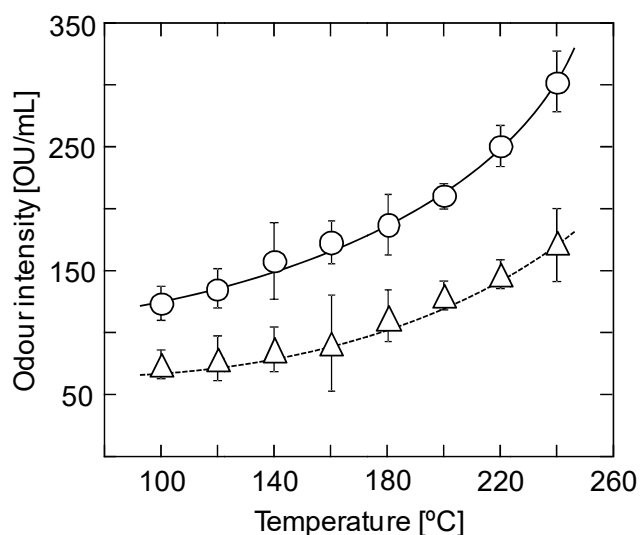


Fig. 3-7. Odor intensity of the liquid extracts (○) and solid residues (△) obtained from subcritical water treatment of raw krill at various temperatures.

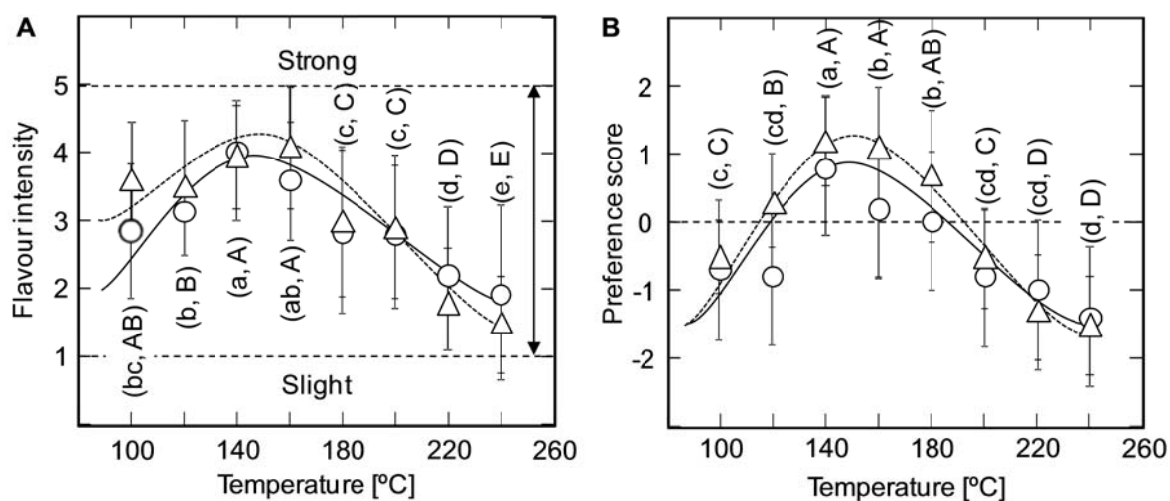


Fig. 3-8. Shrimp-like flavor intensity (A) and preference score (B) of the liquid extracts (○) and solid residues (△) obtained from subcritical water treatment of raw krill at various temperatures. Different upper and lower letters indicate significant differences ($p < 0.05$) for the residue and extract, respectively.

°C. Consequently, treatment of the krill at 140 °C was the most promising for producing a seasoning with the desired shrimp-like flavor.

Fluoride mass fraction in krill which has been reported to be as high as 1.37 and 2.40 mg/g dry mass *Euphausia crystallorophias* and *Euphausia superba*, respectively [59], raised concerns of utilizing of krill for direct human consumption because the Recommended Daily Allowance (RDA) values of fluoride are only 4 and 3 mg for male and female adults, respectively. However, using the extract and residue as seasoning ingredient should be possible because the amount that needs to be added to food products is usually low.

3.4 Conclusions

Raw Isada krill, without any addition of water, was treated under subcritical water conditions in the temperature range of 100–240 °C. The yields of the liquid extract and solid residue increased and decreased, respectively, as the treatment temperature increased. The total contents of solid material and protein in the extracts were higher at higher treatment temperatures. Treatment at high temperatures (220–240 °C) caused degradation of high-molecular-mass substances. Degradation of astaxanthin in both the extracts and residues was significant at treatment temperatures above 160 °C. The highest preference score and shrimp-like flavor intensity were recorded for both the extract and residue prepared at 140 °C. Although suboptimal for overall yields, treatment at 140 °C would seem to be the most promising method for avoiding degradation of krill components, and for producing a shrimp-like flavor seasoning from the krill.

Chapter 4

Using severity factor as a parameter to optimize krill treatment under subcritical water conditions

4.1 Introduction

We demonstrated that both the solid residue and liquid extract obtained from subcritical water treatment of Isada krill can potentially be used as seasoning ingredients and that the extract is also a good source of antioxidants. To evaluate the combined effect of treatment temperature and time, the severity factor ($\log R_0$) has been established to help compare the different treatment conditions. Severity factor was proposed by Overend and Chornet [60], and has been widely used to evaluate the hydrolysis efficiency of lignocellulosic materials such as wood chips [61] and rice husks [62], xylan-rich materials such as wheat bran, corn cobs, spent brewery grain, and *Eucalyptus* wood [63], and other polysaccharides such as coconut meal (mannan) [64] and fruit peel (pectic substances) [65]. However, the application of severity factor to the hydrolysis of proteinaceous material has not been reported. In this context, the objective of this study was to investigate the applicability of the severity factor as a parameter for optimizing the product characteristics of both the solid residue and liquid extract from subcritical water treatment of raw krill.

4.2 Materials and Methods

4.2.1 Subcritical water treatment

Subcritical water treatment was performed using a small batch-type vessel. The cylindrical vessel was made of SUS-316 stainless steel (Taiatsu Techno, Osaka, Japan) with a total volume of 10 mL. The plastic wrapped frozen krill pack was thawed with tap water and 6 g of the thawed sample was loaded into the vessel and tightly sealed. Vessels were placed in oil bath (Yamato Scientific, Tokyo, Japan) preheated to 120, 140, 150, 160, and 180 °C. The temperature inside each vessel was recorded every minute using a thermocouple (type K), reaching the bath temperature after 21, 26, 27, 28.5, and 31 min, respectively (Table 4-1). This constant temperature was then maintained for 2, 4, 6, 8, and 10 min, respectively. After that, the vessels were removed from the oil bath and immediately immersed in ice-water to rapidly cool the contents. Figure 4-1 shows temperature profiles in the vessel during subcritical water treatment.

Table 4-1. Severity factors ($\log R_0$) of subcritical water treatment at various holding times (0–10 min) after reaching a desired temperature (T).

	T [°C]	t [min]*	$\log R_0$ at isothermal reaction time					
			0	2	4	6	8	10
Small vessel (10 mL)	120	21	1.54	1.61	1.69	1.76	1.82	1.88
	140	26	2.25	2.32	2.38	2.43	2.48	2.53
	150	27	2.52	2.59	2.64	2.70	2.75	2.79
	160	28.5	2.90	2.99	3.01	3.06	3.10	3.14
	180	31	3.51	3.57	3.62	3.67	3.71	3.75
Large vessel (117 mL)	120	18	-	-	-	-	-	1.78
	140	19	-	-	-	-	2.37	-
	160	22	-	-	2.59	-	2.79	2.84
	180	26	2.94	3.07	3.24	-	-	-

* t : time required to reach the desired temperature (isothermal reaction time at 0 min).

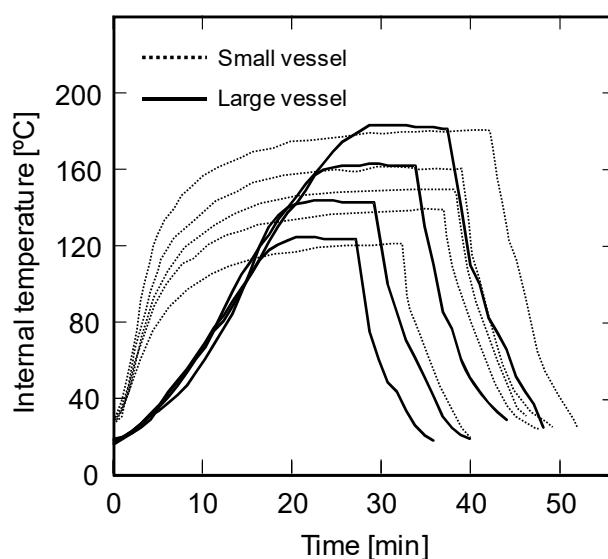


Fig. 4-1. Temperature changes in small (dotted lines) and large (solid lines) vessels during subcritical water treatment.

To evaluate the applicability of the severity factor, another eight subcritical water treatment conditions were performed in a larger batch-type vessel (117 mL, Taiatsu Techno, Osaka, Japan) using 70 g of krill sample. Treatment temperatures (120–180 °C) and isothermal treatment times (0–10 min) were also varied to obtain different severity factor values (Fig. 4-1).

The extract and residue of the treated samples were collected following the method described in our previous report [43]. The subcritical water treatments for both small and larger vessels were performed in triplicate.

4.2.2 Severity factor

To indicate the intensity of the subcritical water treatment conditions, the combined effect of temperature and time was simplified as a single parameter, severity factor ($\log R_0$), which is calculated using Eq. (4-1):

$$\log R_0 = \log \left(\int_0^t \exp \left[\frac{T(t) - 100}{14.75} \right] dt \right) \quad (4-1)$$

where T is the treatment temperature ($^{\circ}\text{C}$) in the vessel at time t (min), 100 is the base temperature ($^{\circ}\text{C}$) and the value of 14.75 is an empirical parameter related to activation energy from assumed pseudo first-order kinetics [60]. Table 4-1 shows $\log R_0$ for each treatment condition in both small and larger vessels.

4.3 Results and Discussion

4.3.1 Product Appearance

Subcritical water treatments were carried out using different temperatures and time periods. A total of 30 samples were obtained using the small vessel. To compare the results of these experiments, the severity factor ($\log R_0$) values were in the range 1.54–3.75, calculated using Eq. (4-1) (Table 4-1).

Examples of the treated samples under subcritical water conditions are shown in Fig. 4-2. Subcritical water treatment at different $\log R_0$ values affected the color and appearance of the krill extract and residue. At low $\log R_0$ values (1.54–1.88), the color of the liquid extract gradually changed from yellowish-orange to reddish-brown. The color of the solid residues started to change from reddish-pink to reddish-brown when the treatment severity increased from 2.25 to 3.51. Since treated krill has the color of astaxanthin, an important and abundant carotenoid in crustaceans, the reddish-pink color would be ascribed to astaxanthin. However, the browning color in the residue and extract may have come from melanoidins, brown nitrogen-containing compounds formed *via* the Maillard reaction [66]. At $\log R_0 > 3.51$, the residues started to change back to light-brown, while the extract became darker. This was possibly due to the higher solubility of brown products in subcritical water at higher temperatures during treatment, resulting in their leaching into the extract.

Extract					
Residue					
$\log R_0$	1.54	2.43	2.79	3.14	3.75
	120 °C 0 min	140 °C 6 min	150 °C 10 min	160 °C 10 min	180 °C 10 min

Fig. 4-2. Examples of liquid krill extracts and solid residues obtained from the treatment of krill under subcritical water conditions at different treatment severity factors ($\log R_0$).

4.3.2 Yields of extract and solid residue

Figure 4-3 shows that yields of both the extract and solid residue fractions obtained from subcritical water treatment varied with the severity factor values. Separation of the extract from residue by filtering and pressing resulted in approximately 10% loss of the total yield. A slight increase in the extract yields, from 0.54 to 0.60 kg/kg-raw krill, and decrease in the residue yields, from 0.39 to 0.33 kg/kg-raw krill, were observed as $\log R_0$ increased in the range 1.54–2.25. However, at $\log R_0 > 2.25$, the extract and residue yields drastically increased to 0.79 and decreased to 0.18 kg/kg-raw krill, respectively, perhaps caused by hydrolysis and hydrothermal decomposition of the cell structure resulting in leaching of water and decomposition of organic components into smaller molecular substances that could be dissolved in the liquid extract.

4.3.3 Compositions of krill extract

The main solid components in the extract might come from the hydrolysis products of proteins, chitin (exoskeleton), and lipids, which are peptides and amino acids, saccharides, and fatty acids, respectively. Solid and protein contents were analyzed and plotted in Fig. 4-4 to represent the amount of components solubilized in the extracts. The solid content gradually increased from 0.078 to 0.177 kg/kg-extract when the $\log R_0$ increased from 1.54 to 3.75. This increase in solids can be ascribed to the solubilization of molecules resulting from the hydrolysis and degradation of macromolecules (proteins, polysaccharides, and fats) under subcritical water treatment conditions, as shown by previous studies. The hydrolysis of polysaccharides into oligo-, di-, and mono-saccharides in coconut meal [67], and hydrolysis of triglycerides into glycerol and fatty acids in vegetable oils, are examples [68].

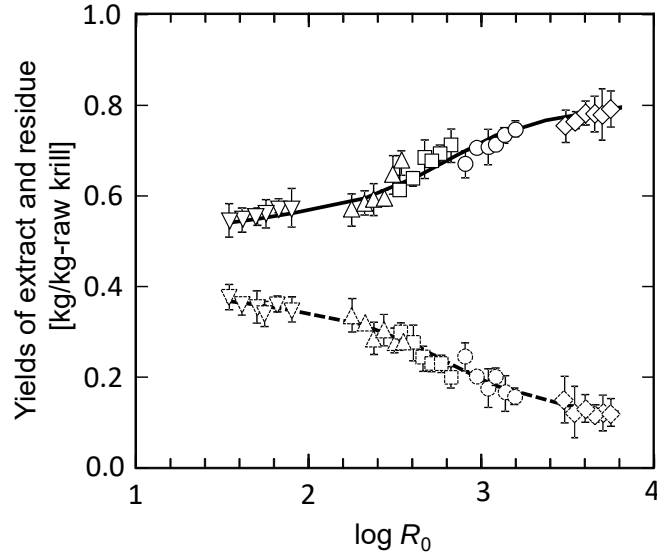


Fig. 4-3. Yields of liquid extract (solid line, open symbols) and solid residue (dotted line, open symbols) obtained from treatment of krill under subcritical water conditions at different treatment severity factors ($\log R_0$). The symbols ∇ , $\triangle$, $\square$, $\circ$, and $\diamond$, indicate treatment temperatures of 120, 140, 150, 160, and 180 °C, respectively.

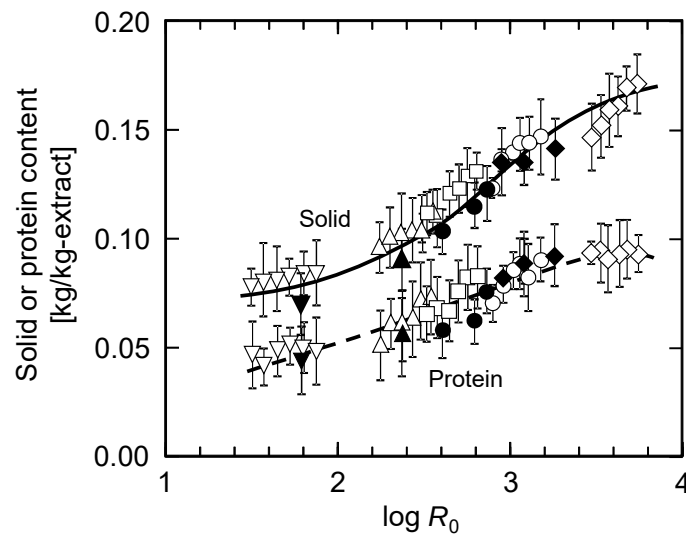


Fig. 4-4. Solid and protein contents of liquid extracts obtained from treatment of krill under subcritical water conditions at different treatment severity factors ($\log R_0$) using small (open symbols) and large (closed symbols) vessels. Other symbols are as described in Fig. 4-2.

Apart from water, protein was the main component of krill (62.2% dry basis). Protein was a major component of the liquid extract, whereas carbohydrate and lipid contents were low, *i.e.* less than 0.026 and 0.021 kg/kg-extract, respectively (data not shown). The protein

content gradually increased from 0.042 to 0.094 kg/kg-extract, which was about 85.0% of the total protein content found in raw krill at $\log R_0$ of 1.54–3.51. The increase in protein content in the extracts was expected because the hydrolysis of proteins into soluble proteins, peptides, and amino acids in subcritical water occurs readily and has often been reported [17, 37, 69]. A decrease in protein content at higher severity values ($\log R_0 > 3.51$) was also noticed. This could result from peptides and amino acids being further converted to other components under subcritical water conditions at high temperatures, particularly organic acids and amines [17, 70]. In addition, amino acids could also react with reducing sugars *via* the Maillard reaction to form other compounds [66].

4.3.4 Molecular mass distribution

Determination of the molecular mass distribution of the krill extract as a function of $\log R_0$ provided further information of the structure and composition of the extract. The HPLC chromatogram was obtained by UV absorbance detection at 280 nm, which represented proteins, amino acids, hydroxymethylfurfural (HMF), and aromatic acids in the extract [71], as shown in Fig. 4-5A. The chromatogram was divided into three regions, high (molecular mass $> 10^5$ Da), medium ($10^3 < \text{molecular mass} < 10^5$ Da), and low (molecular mass $< 10^3$ Da) molecular mass, indicating the presence of protein or long-chain peptides, short-chain peptides, and amino acids or HMF, respectively. The multimodal peaks of high molecular masses might have represented mainly protein and long-chain peptides, which gradually increased at higher $\log R_0$. Distinctive peaks of medium-sized molecular substances were observed at $\log R_0 > 2.43$ and a decline in low molecular mass peaks was observed at $\log R_0 > 2.79$. The overall increase in peak area was in agreement with the changes in solid and protein contents discussed previously.

The average molecular mass of components in the extract, estimated from the solid weight and molality (molal concentration) following the method described in our previous report [72], began increasing when $\log R_0$ was increased from 2.43 to 3.51 (Fig. 4-5B). The increase in average molecular mass was attributed to the hydrolysis of protein molecules to soluble peptides of various chain lengths due to the higher ion product concentrations, *i.e.* hydronium and hydroxide ions, in subcritical water. Further hydrolysis may lead to extensive degradation of peptides to shorter peptides and amino acids, as shown in others works [17].

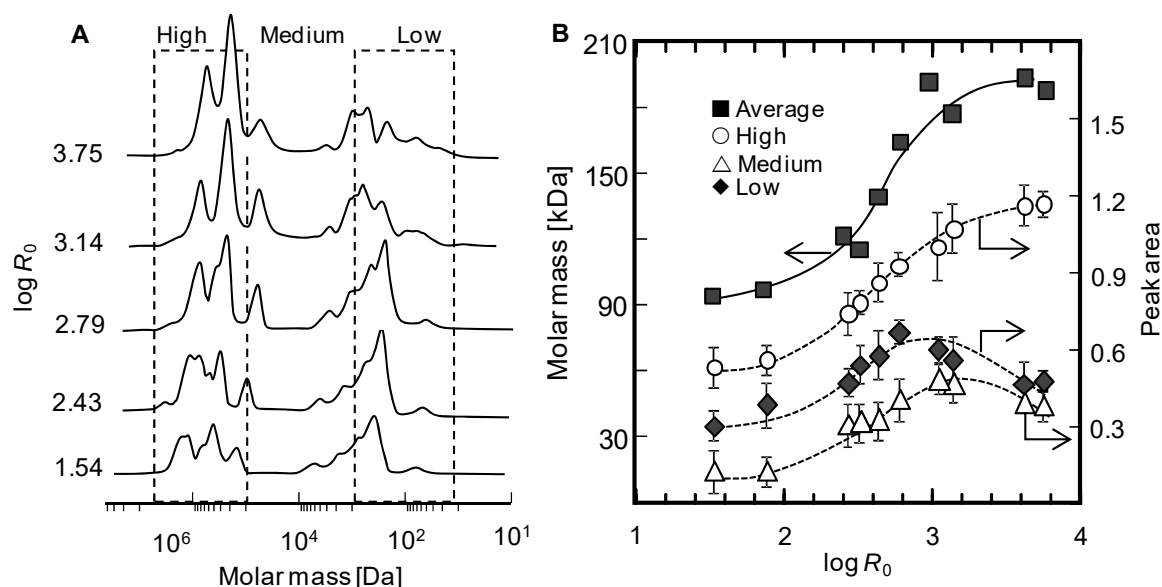


Fig. 4-5. (A) HP-SEC chromatograms and (B) average molar mass (■) and peak areas of high (○), medium (△), and low (◆) molecular mass components from the chromatograms of liquid extract obtained from treatment of krill under subcritical water conditions at different treatment severity factors ($\log R_0$).

The decrease in low molecular mass components, comprising amino acids and other degradation products, at $\log R_0 > 3.51$ might have been caused by the formation of high molecular mass melanoidins *via* polymerization reactions at higher temperatures and longer heating times, as seen in other studies [67].

4.3.5 Odor intensity

Figure 4-6 shows the odor intensities of the liquid extract and solid residue detected by an electronic sensor at ambient temperature. As the sensor used in this experiment directly measured the concentration of several, unspecified volatile compounds, the results indicated that an increase in severity factor resulted in a larger amount of volatile components in the extract and solid residue. These volatile compounds could have arisen from several reactions, such as the degradation of amino acids to ammonia and organic acids [70], formation of flavor compounds *via* Maillard reaction products (aldehydes, ketones, dicarbonyls, acryl amides, and heterocyclic amines) [67], degradation of sugar into acetic and formic acids [73], and several other volatiles characteristic to cooked shrimp. Treatment carried out at $\log R_0 > 3.51$ led to a sharp increase in the odor intensity due to the formation of burnt products.

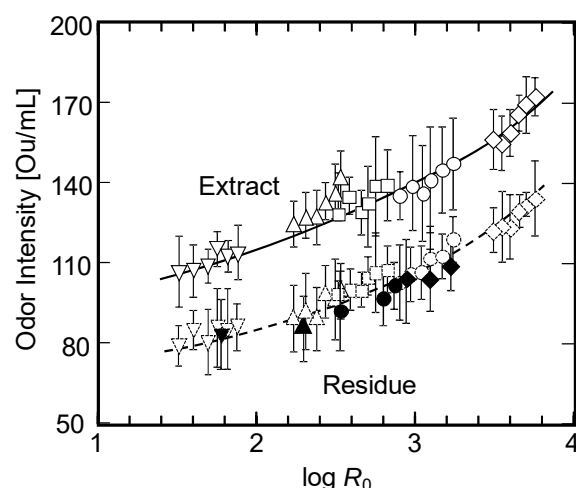


Fig. 4-6. Odor intensity of liquid extract (solid line) obtained using a small vessel and the odor intensity of the residue obtained using small (dotted line, open symbols) and large vessels (closed symbols) from the treatment of krill under subcritical water conditions at different treatment severity factors ($\log R_0$). Other symbols are as described in Fig. 4-2.

4.3.6 Sensory evaluation

Sensory evaluation was assessed to indicate the preference and shrimp-like flavor intensity of the subcritical water treated products. The overall preference scores and intensity scores for shrimp-like flavor were clearly dependent on the treatment conditions (Fig. 4-7). Stronger shrimp-like flavor was obtained from the krill extracts and residues treated at $\log R_0$ between 2.25 and 2.8. A similar trend in the preference and flavor intensity scores indicated that the shrimp-like flavor strongly influenced sample preference. The samples prepared at $\log R_0 > 3.00$ resulted in lower shrimp-like flavor intensity and preference scores. Panelists detected a smoky odor in the extract and residue obtained from subcritical water treatment under these conditions, resulting in low sensory evaluation scores.

4.3.7 Applicability of severity factor

To prove the applicability of the severity factor used in this study, another eight samples were also obtained using the larger vessel at different $\log R_0$ values (1.78–3.24) (Table 4-1). The temperature profiles inside the large vessel were different from those in the small vessels due to differences in heat transfer of the two systems. The observed data for protein content, odor intensity, and preference score plotted against the severity factor were in line with data from the smaller vessel (Figs. 4-4, 4-6, and 4-7B), indicating that $\log R_0$ was an effective

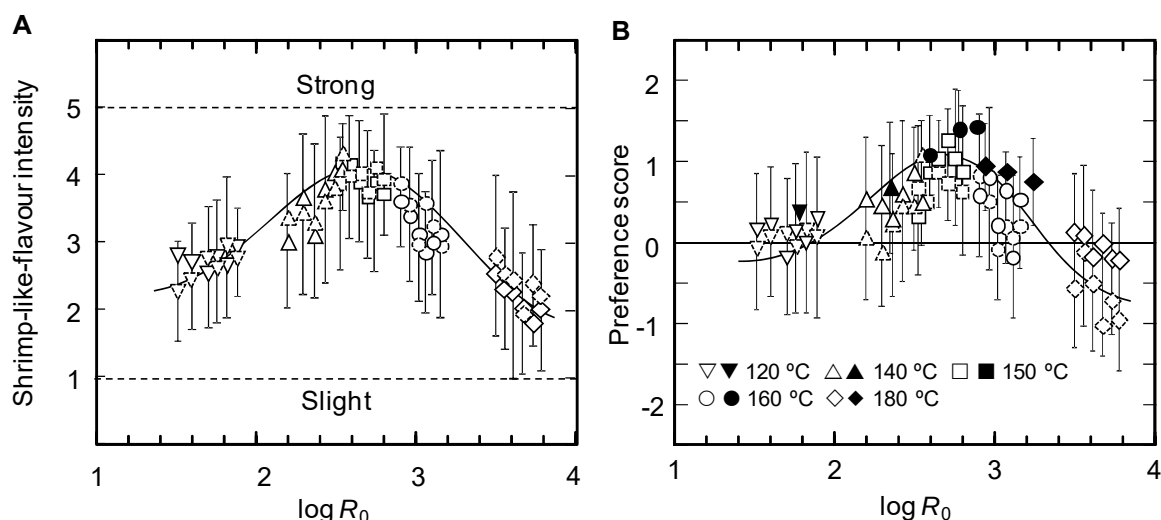


Fig. 4-7. (A) Score of shrimp-like flavor intensity of liquid extract (solid line, open symbols) and solid residue (dotted line, open symbols) obtained using a small vessel and (B) overall preference of liquid extract (solid line, open symbols) and solid residue (dotted line, open symbols,) using small vessel and overall preference of liquid extract using large vessels (closed symbols) after treatment of krill under subcritical water conditions at different treatment severity factors ($\log R_0$). Other symbols are as described in Fig. 4-2.

process parameter for determining the intensity of subcritical water treatment conditions and, therefore, should remain useful when scaling up the process.

4.4 Conclusions

In this study we showed that various properties of subcritical water-treated products from krill could be correlated with the severity factor ($\log R_0$), a combined parameter of treatment temperature and time. Solid content, protein content, and odor intensity increased with $\log R_0$. Sensory evaluation scores for shrimp-like flavor intensity and overall preference were highest at $\log R_0$ values of 2.75–3.01. The applicability of this parameter was also proven using two different sizes of bath-type vessel.

Chapter 5

Preparation of liquid and solid seasonings with shrimp-like flavor from Isada krill under subcritical water conditions by steam injection

5.1 Introduction

In the previous chapters, we used a small pressure-resistant vessel with an inner volume of 117 mL, and the vessel was heated using either an external mantle heater or by immersion in an oil bath. However, such heating methods would be inadequate or inconvenient for treating the krill in large quantities. In this study, heating by direct injection of steam into a large vessel was used to treat a 12.5 kg batch of raw krill, and the properties of the liquid extract and solid residue prepared using the large vessel were compared with the material prepared using the 117-mL small vessel.

5.2 Materials and Methods

5.2.1 Materials

Isada krill used for large-scale treatment was caught near Ishinomaki (Miyagi, Japan), and was supplied by Kinoya Ishinomaki Suisan (Miyagi). The krill used for small-scale treatment was caught from the Sanriku Coast and was obtained from Hamaichi (Wakayama, Japan). The average weight and length of the latter krill were 35.9 ± 10.5 mg and 17.9 ± 2.7 mm ($n = 20$), respectively.

Folin-Ciocalteu reagent, BSA, DPPH, and Trolox were the same describe in Section 1.2.1. Raffinose and Dextran T-10, which were used to prepare the calibration curve for size exclusion chromatography, were purchased from Nacalai Tesque (Kyoto, Japan) and GE Healthcare Japan (Tokyo, Japan), respectively. Powdered instant coffee, which was used to refresh the sense of smell, was a product of Key Coffee (Tokyo).

5.2.2 Treatment of Isada krill under subcritical water conditions

A large-scale treatment using the Isada krill caught near Ishinomaki was conducted by Communication Link (Sendai, Japan). The frozen krill (12.5 kg) was thawed at room temperature, and was then pressure-filled by N₂ gas into a large pressure-resistant vessel. The pressure in the vessel was reduced to atmospheric pressure, and high-pressure steam was

injected into the vessel to increase the temperature to the desired level (140 °C or 160 °C). After maintaining the temperature at 140 and 160 °C for 17 min and 4 min, respectively, the liquid extract was withdrawn into a cooler with a water jacket by introducing steam into the tracing line. The extract was cooled down to room temperature (*ca.* 30 °C) and then weighed. The temperature inside the vessel was recorded every minute. The extract was filled into polyethylene bags and stored in a freezer. The solid residue in the vessel was gathered up by using a scraper and was kept frozen in bags. The treatments using the large vessel at 140 and 160 °C were designated as L140 and L160, respectively, and the extract and residue were abbreviated E and R, respectively. The crude extract and residue were further refined. The crude extract was filtered through a No. 2 Advantec filter paper (Tokyo, Japan) under reduced pressure, and the crude residue was put into a Teflon mesh bag (MS-150 Moku, 144 µm mesh opening, 25×100 mm, Semitec, Tokyo, Japan) and was further pressed by a hydraulic machine at 7.86 MPa to obtain the compressed residue and the filtrate. The filtrate was combined with the liquid extract, and this mixture was designated solely as the extract (E). The residue remaining in the bag was called the residue (R).

The small-scale treatment was carried out in the same manner as previously reported [72]. In brief, the thawed krill (20 g; Hamaichi) was put into a 117-mL batch-type vessel (Taiatsu Techno, Osaka, Japan) together with distilled water (20 g). The vessel was tightly sealed and then heated to 140 °C or 160 °C by immersion in an oil-bath (MH-5E, Rikagaku Sangyo, Chiba, Japan), which was regulated at 230 °C. The temperature in the vessel was monitored using a TXN-700B thermometer (As One, Osaka, Japan). After the temperature reached 140 °C or 160 °C, the vessel was transferred to another EO-200 oil-bath (As One), which was regulated at 153 °C (or 173 °C) and kept for 17 min (or 4 min). During this period, the temperature was maintained at nearly 140 °C or 160 °C. After that, the vessel was immediately cooled by immersion in ice water. The treatments using the small vessel at 140 and 160 °C were designated as S140 and S160, respectively. Based on preliminary experiments, the treatment conditions were determined to give almost the same temperature profiles as for L1 and L2, as shown later.

As a control experiment, the ambient-pressure boiling treatment was carried out. The thawed krill (30 g; Hamaichi) and water (30 g) were put in an open aluminum pot and boiled at atmospheric pressure for 10 min. This treatment was designated as C.

The mixture from the small vessel or pot was also separated into the solid residue and liquid extract by the same method as for the crude extract and residue from the large vessel.

5.2.3 Properties of solid residue

The wet solid residue (*ca.* 2 g), which had been weighed precisely, was put in a Petri dish and dried at 135 °C for 2 h in a DNF-400 oven (Yamato Scientific, Tokyo). The moisture content of the solid residue was calculated from the difference in the weight between the wet and bone dry residues. The moisture content was expressed on the basis of the dry material in units of kg-water/kg-dry residue.

5.2.3 Color

The color of the solid residue, when put in a glass cup (16 mm I.D.×28 mm) to a height of *ca.* 10 mm, was characterized using an SA4000 color-difference meter (Nippon Denshoku Industries, Tokyo, Japan) with a D65 light source at a view angle of 10°. The color distance, ΔE^* , between the krill residues treated under subcritical and cooking (control) conditions, which are labeled r and c, respectively, was calculated by the following equation:

$$\Delta E^* = \sqrt{(L_r^* - L_c^*)^2 + (a_r^* - a_c^*)^2 + (b_r^* - b_c^*)^2} \quad (5-1)$$

where L^* , a^* , and b^* indicate brightness and intensities in redness and yellowness, respectively.

5.2.4 Molecular mass distribution

The extract was centrifuged at 1×10^4 rpm for 5 min. The supernatant (15 μ L) was applied to a high-performance liquid chromatography comprising an LC-20AD pump, an RID-10A refractive index detector (Shimadzu, Kyoto, Japan), and a YMC-Pack Diol-60 column (500×8.0 mm I.D., YMC, Kyoto, Japan). Distilled water was used as the mobile phase (flow rate: 1.0 mL/min). The mass distribution in the extract was determined using external molecular mass standards comprising glycerol (92 Da), glucose (180 Da), raffinose (595 Da), and Dextran T-10 (1.0×10^4 Da) dissolved in distilled water.

5.2.5 Properties of liquid extract

The solid content of the liquid extract (0.4 mL) was measured using a PAL-1 digital hand-held refractometer (Atago, Tokyo, Japan), and was expressed in units of Brix. The salt concentration of the extract (0.4 mL) was measured using a PAL-ES1 digital salt-meter (Atago).

The freezing point depression, DPPH radical scavenging activity, sensory evaluation and odor intensity were determined using the method describe the detail in Section 1.2.3.

5.3 Results and Discussion

5.3.1 Yields of extract and residue

Table 5-1 summarizes the mass balance of the crude extract and residue obtained from the large vessel. Because the injected steam was condensed, the total mass of the crude extract and residue was greater than the initial mass of the krill (12.5 kg). More steam condensed during treatment at 160 °C than at 140 °C.

Table 5-1. Mass balance during the treatment using the large vessel at 140 and 160 °C.

Treatment temperature [°C]	Crude extract [kg]	Crude residue [kg]	Condensed water* [kg]
140	8.88	5.37	1.75
160	17.18	2.25	6.93

*The amount of condensed water was calculated by subtracting the weight of raw krill (12.5 kg) from the sum of crude extract and residue.

5.3.2 Appearance of extract and residue

Figure 5-1 shows the temperature profiles during the large- and small-scale treatments. Because the treatment was performed non-isothermally, the temperature history during treatment affected the properties of the extract and residue. We demonstrated that severity factor R_0 , which was defined by Eq. (5-2) [60], could assess the combined effects of temperature and time during subcritical water treatments of coconut meal [64] and passion fruit peel [65].

The R_0 values for the large-scale treatments, L140 and L160, were calculated to be 284 and 304 min, respectively. The R_0 values for the small-scale treatments S140 and S160, were 293 and 291 min, respectively, and were almost the same as those for the large-scale treatments because the profiles observed during the small-scale treatments well replicated the profiles during the large-scale treatments. The R_0 value during the control experiment was evaluated

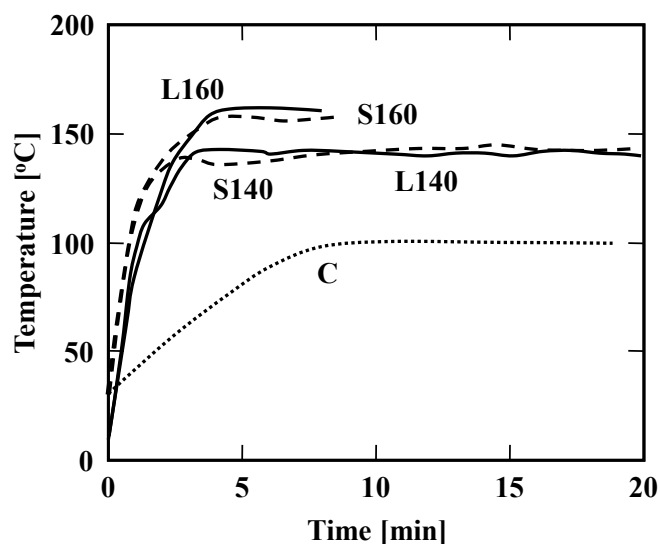


Fig. 5-1. Temperature profiles during the treatment of *Isada* krill using a large (solid curves) and small (broken curves) vessels at 140 °C and 160 °C. Labels L and S indicate the large and small vessels, respectively. Values 140 and 160 represent the treatment temperatures. The dotted curve labeled C shows the temperature profile during the boiling treatment (control).

to be 13, from its temperature history.

Figure 5-2 illustrates the appearance of the liquid extracts and solid residues obtained by the large- and small-scale treatments as well as that of the extract and residue in the control experiment. The extracts obtained by subcritical treatments were dark brownish, while the extract of the control experiment (C-E) was light brownish. The dark brownish color of the extracts L140-E, L160-E, S140-E, and S160-E, seem to be ascribed to the Maillard reaction during the treatments at high temperatures.

Figure 5-3 shows the absorption spectra of the extracts. The absorbance of C-E was much lower than those of the extracts obtained by the subcritical treatments. Among the extracts obtained by the subcritical treatment, S140-E exhibited a slightly lower absorbance than others in the visible-light range. The low absorbance of S140-E coincided with its light appearance. Because the R_0 values for the S140-E and S160-E are almost the same, some factors other than the R_0 value would greatly affect the appearance.

The solid residues obtained by the subcritical treatment, L140-R, L160-R, S140-R, and S160-R, were also dark brownish, while the residue of the control experiment, C-R, exhibited a light pinkish-brown color. The color of the residues, L140-R, L160-R, S140-R, and S160-R, was characterized by the color distance, ΔE^* , from that of the residue, C-R (Table 5-2). The ΔE^* of S140-R, whose extract exhibited a lighter brown color than the other extracts, was



Fig. 5-2. Appearance of the liquid extracts and solid residues obtained using the large and small vessels at 140 and 160 °C. Labels L and S and values 140 and 160 are the same as in Fig. 5-1. E and R indicate the extract and residue, respectively. C-E and C-R indicate the extract and residue, respectively, obtained from the boiling treatment (control).

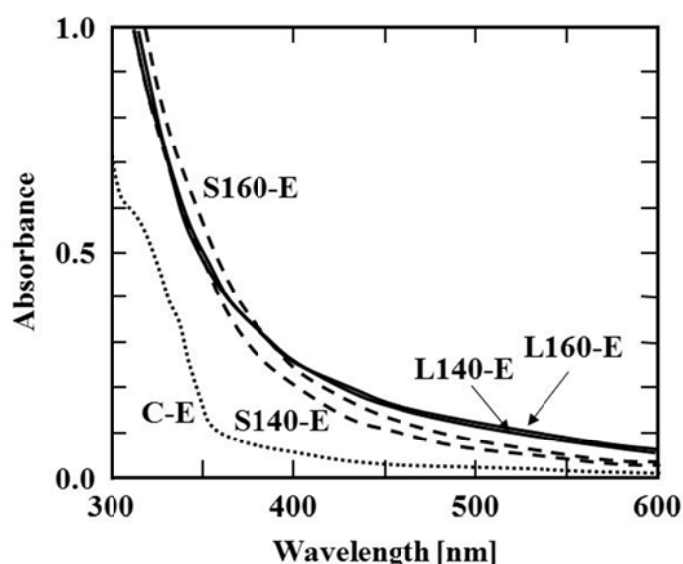


Fig. 5-3. Absorption spectra of the extracts from the large- and small-scale treatments at 140 and 160 °C, which are labeled as L140-E, L160-E, S140-E and S160-E, respectively. C-E is the same as in Fig. 5-2.

also a little smaller than those of other residues.

5.3.3 Properties of extract

The characteristics of all the extracts are summarized in Table 5-3. Yields L140-E and L160-E were calculated for the extracts refined from the crude extract and residue. The wet-basis

Table 5-2. Yield, moisture content, color difference ΔE^* and odor intensity of the solid residues.

Residue [†]	Yield [kg/kg-mixture]	Moisture content [kg/kg-d.m]	ΔE^*	Odor intensity
L140-R	0.145	1.98	9.68	467
L160-R	0.138	1.98	8.60	248
S140-R	0.071	1.58	5.93	249
S160-R	0.083	1.97	8.51	300
C-R	0.065	1.13	—	14

[†]L140-R, L160-R, S140-R and S160-R indicate the residues obtained using the large and small vessels at 140 and 160 °C, respectively. C-R is the residue obtained from the control boiling treatment

moisture content of raw Isada krill was about 0.8. The yields of the L140-E and L160-E were lower than those of the S140-E and S160-E because the steam injected into the vessel condensed to dilute the extracts. Because less steam condensed during the treatment at 140 °C than at 160 °C, the L140-E had a higher solid content, salt concentration, protein content than the L160-E. The high solid content of the L140-E resulted in larger ΔT_f than the L160-E. The L160-E had stronger radical scavenging activity than the L140-E despite the lower solid content. Although the R_0 value for the treatment at 160 °C was almost the same as that for the treatment at 140 °C, compounds with higher activity were produced at the higher temperature.

Because the small vessel was a closed system, the solid content, ΔT_f , salt concentration, and protein content of the S140-E were almost the same as those of the S160-E. The radical scavenging activity of the S160-E was higher than that of the S140-E due to the higher treatment temperature.

Because water evaporated during heating in the pan, the yield of C-E was much lower than those from the subcritical treatment. The mass of water lost during heating was added to the C-E to compensate for the loss. The compensated extract was designated as the C-E_c. The solid and protein contents of the C-E_c were much lower than those obtained by subcritical treatment. This indicated that some of solid materials in the krill were solubilized during the

subcritical treatment. The radical scavenging activity of the C-E_c was much lower than those of the extracts from the subcritical treatment. This also indicated that the subcritical treatment of the krill was more severe than boiling for producing compounds with antioxidative ability.

Figure 5-4 illustrates the size exclusion chromatograms for the extracts. The extracts obtained from the subcritical treatment showed larger peaks in the high-molecular-mass range of 10^3 to 10^4 than the C-E_c. This coincided with the higher protein content of the extracts from the subcritical treatment. In addition, this indicates that there would be polymerized compounds formed during the treatment by the Maillard reaction. There seemed to be no significant difference in the peak area among the extracts.

Table 5-3. Yield, solid content, degree of freezing point depression ΔT_f , salt concentration, protein content, DPPH radical scavenging activity, and odor intensity of the liquid extracts.

Extract [†]	Yield [kg/kg- mixture]	Solid content [Brix%]	ΔT_f [°C]	Salt concen- tration [%]	Protein content [%]	DPPH	
						scavenging activity [mmol/TE/ L]	Odor intensit y
L140-E	0.653	8.6	1.018	0.90	62.80	1.24	553
L160-E	0.746	7.4	0.875	0.76	50.70	1.51	387
S140-E	0.786	6.3	0.680	0.70	41.30	1.34	300
S160-E	0.782	6.8	0.708	0.71	46.90	2.18	453
C-E	0.443	9.2	1.234	0.72	54.60	1.43	193
C-E _c	—	5.2	0.698	—	30.9	0.81	—

[†] L140-E, L160-E, S140-E and S160-E indicate the extracts obtained using the large and small vessels at 140 and 160 °C, respectively. C-E is the extract obtained from the control boiling treatment, and C-E_c is the extract compensated by the evaporated water during the boiling treatment.

5.3.4 Properties of residue

The properties of the solid residues are summarized in Table 5-2. It should be noted that the definition of yield for the residues treated using the large vessel, L140-R and L160-R, are different from that for other residues. The yields for the S140-R, S160-R, and C-R were *ca.* 0.07, indicating that 90% or more of the krill was recovered as the liquid extract. The color

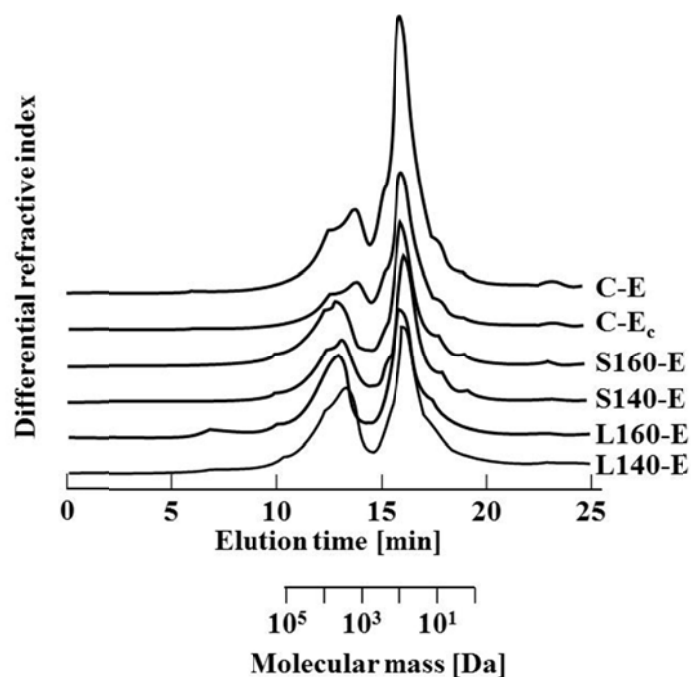


Fig. 5-4. Size exclusion chromatograms of the extracts. The labels are the same as in Fig. 5-3.

distance of the S140-R, which was a lighter shade of brown, was smaller than the others. Odor intensity was much stronger for the extracts from the subcritical treatment than the C-R. The odor characteristics of the residues were examined in detail by sensory evaluation, as shown later.

5.3.5 Sensory evaluation

As shown in Table 5-4, the residues obtained by the subcritical treatment of the Isada krill had a higher preference score than the control residue, C-R. The liquid extracts obtained at 140 °C, L140-E and S140-E, also exhibited a higher preference score than the control extract, C-E.

The smell characteristics evaluated were shrimp-like, fishy, rotten, burnt and roasted flavors for all the extracts and the residues (Figs. 5-5A and B). For both the extract and residue, the subcritical treatment weakened fishy and rotten flavors and strengthened shrimp-like, roasted, and burnt flavors compared with the boiling treatment (control). These changes in flavor characteristics are responsible for the improvement of the preference scores of the extracts and residues.

Table 5-4. Preference scores of the extract and residues ($n=12$).

Vessel	Treatment temperature [°C]	Extract	Residue
Large	140	0.75 ± 1.01	0.75 ± 0.72
	160	0.25 ± 0.72	1.17 ± 0.80
Small	140	0.67 ± 1.03	1.00 ± 1.00
	160	0.42 ± 1.26	1.08 ± 0.95
Boiling (control)		0.25 ± 1.23	0.00 ± 0.82

Values: mean $\pm$ S.D.

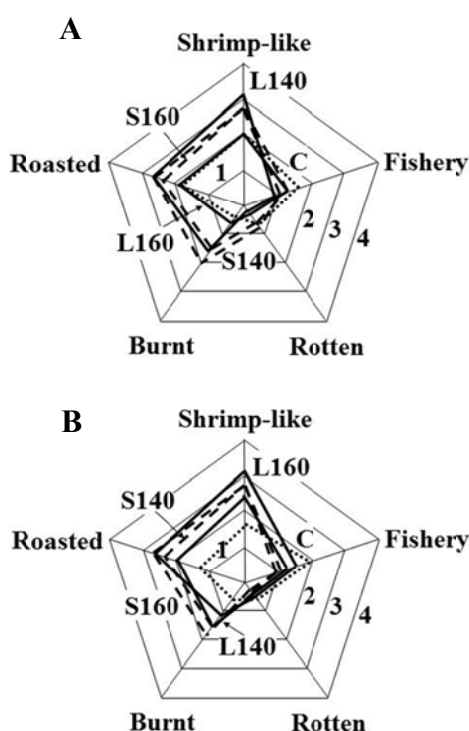


Fig. 5-5. Five-axis cobweb charts for (A) extracts and (B) residues. The labels are the same as in Fig. 5-1.

5.4 Conclusions

Based on the log R_0 value the krill extract and residue were prepared using a steam injection reactor for scale up. The results for the extracts and residues prepared by the large-scale treatment were almost the same as those for the extracts and residues prepared using a 117-mL vessel operated at similar severity factors. Success in the scale up would promise practical production of the seasonings from Isada krill.

Concluding Remarks

Chapter 1

Subcritical water treatment of the semi-dried Isada krill produced an extract solution and a solid residue, both of which possessed good score from the panelists when the shrimp-like flavor could not be achieved by the ambient pressure boiling treatment. Krill extract obtained from an ambient pressure boiling and subcritical water treatments of semi-dried krill presented protein as a major component. The increase in the protein content of the extract is indicative of the hydrolysis of proteinaceous substances in the krill to soluble peptides and amino acids. The decrease in protein content of the extract at high temperature indicated that the proteinaceous substances were further decomposed or converted to other products. For example, amino acids may react with carbohydrates to form brown products *via* Maillard reaction which is inextricably linked to shrimp-like flavor of krill extract and residue.

Chapter 2

Proteinaceous components existed and that they were hydrolyzed in the subcritical water to produce peptides and amino acids, which were soluble in the extract. The extractability of lipid and astaxanthin from the krill increased at higher temperatures might be due to the modified properties of subcritical water, increasing the ability to solubilize the hydrophobic substances. However, more than 50% of astaxanthin degradation was observed at treatment temperatures above 160 °C. The antiradical activity of the extract depended on the degree of protein hydrolysis and the amount of protein hydrolysate, and Maillard reaction products could also act as antioxidants. The observation of valuable substances demonstrated that products of Isada krill obtained by subcritical water treatment can prospectively be used as seasoning which would be a good source of functional compounds.

Chapter 3

Raw Isada krill, without any addition of water, was treated under subcritical water conditions in the temperature range of 100–240 °C for 10 min. Subcritical water treatment resulted in an increase in the content of low and high molecular-mass components with an increase in the temperature from 100 to 180 °C, but caused a decrease at 200 °C. The decrease in the content of the high-molecular-mass substances at high temperature could be ascribed to the decomposition of these molecules by subcritical water. The decomposition of protein and amino acids under hydrothermal conditions generated small and volatile

components, i.e., organic acids and volatile compounds. The direct treatment of the krill exhibited an increase of the extract concentration, which increased the flavor intensity.

Chapter 4

Various properties of the products from krill by its subcritical water treatment could be related with the severity factor ($\log R_0$), which is a combination parameter of treatment temperature and time. Solid content, protein content, and odor intensity increased with $\log R_0$. A total of 30 samples were obtained using the small reactor. The temperature profiles in 2 working vessels (10 and 117 mL) during subcritical water treatments which were varied to 38 different severity factor values. To compare the results among these experiments, the $\log R_0$ values were calculated, which were in a range of 1.54–3.75. Its applicability was examined for 8 samples obtained at different $\log R_0$ values using the larger vessel. The solid and protein contents, and preference scores for the samples obtained using the small and large vessels laid on the same curves, indicating that the $\log R_0$ was an effective process parameter for determining the subcritical water treatment conditions and that it should be useful for up-scaling the process.

Chapter 5

Based on the $\log R_0$ value the krill extract and residue were prepared using a steam injection reactor for scale up. The heating by direct injection of steam into a large vessel was used to treat a 12.5 kg batch of raw krill. To prove the applicability of using the steam injection, the properties of the liquid extract and solid residue prepared using the large vessel were compared with the materials prepared using a small vessel of batch-type reactor. The results for the extracts and residues prepared by the large-scale treatment were almost the same as those for the extracts and residues prepared using a 117-mL vessel operated at similar severity factors.

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List of Publications

1. Koomyart I, Nagamizu H, Khuwijitjaru P, Kobayashi T, Shiga H, Yoshii H, Adachi S. (2015). Subcritical water treatment for producing seasoning from semi-dried Isada krill. *J. Food Process Eng.*, **37**, 567–574.
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