

*Full Length Research Paper*

# Production of value added materials by subcritical water hydrolysis from krill residues extracted by supercritical carbon dioxide

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Accepted 25 November, 2011

The aim of this study was the determination of the best experimental conditions for the production of useful materials such as amino acids by subcritical water hydrolysis from supercritical carbon dioxide extracted krill residues and to compare the results with raw krill. Subcritical water hydrolysis efficiency from raw and de-oiled krill was examined over the temperature range of 200 to 280 °C, ratio of material to water for hydrolysis was 1:50 g/ml and for water sample contact equilibration times of 5 min to decrease the decomposition of amino acids. Nitrogen and air were used as atmosphere at pressure estimated to be between 0.101 and 6.41 MPa. The hydrolysis efficiencies of glycine, arginine, and leucine were found to be increased with increasing water temperature, consistent with higher solubility at higher temperatures. The highest yield of amino acids in de-oiled krill hydrolysate was at 280 °C. While, the highest amino acid yield in raw krill hydrolysate was at low temperature 200 °C. Also, reducing sugar content was analyzed in both samples and the results showed that the yield of reducing sugar in deoiled krill hydrolysate was higher than that of raw krill hydrolysate.

**Key words:** Subcritical water hydrolysis, krill, amino acid, value added materials.

## INTRODUCTION

Krill represents a very large biomass, little contaminated by organic pollutants and heavy metals. It has been harvested since 1975 for animal feed and aquaculture (Martin, 2007). It is the food for not only the now greatly depleted populations of whales but also many of the seals, penguins and other sea birds, as well as fish and squid (Murphy, 2001). The marine crustacean krill has not been a traditional food in the human diet. Public acceptance of krill for human consumption will depend partly on its nutritive value (Tou et al., 1997). Its expanded use in human health is possible through enzymes, various krill extracts and oils. The fat content of krill is low, but it is rich in EPA and DHA of high bioavailability given their presence in phospholipids. A number of overlapping

characteristics demonstrates the potential of krill consumption in controlling cardiovascular diseases; and in other areas of health, there are promising, although few, studies (Martin, 2007). As fisheries, krill is not directly usable in food and feed: The usual life conditions of this organism have led to the development of extremely efficient hydrolytic systems (proteolytic and lipolytic), leading to a very quick autolysis after fishing (Ellingsen and Mohr, 1987).

The protein derived from krill is considered to be of high quality based on the chemical analysis showing that the krill protein contains all nine essential amino acids in sufficient quantities to meet the FAO/WHO/UNU requirements for human adults (Chen and Jaczynski, 2007). The hydrolysis of krill into value-added products (proteins, amino acids, reducing sugar etc.) is an alternative and effective way. Current industrial hydrolysis methods include chemical (acid, alkali or catalytic) and enzymatic hydrolysis. However, the chemical hydrolysis needs violent

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reaction conditions and often causes serious pollution of the environment. Enzymatic hydrolysis is expensive, and of long production cycle (Cheng et al., 2008). Most of biomass waste is easily hydrolyzed in super- or sub-critical water, which is structurally different from normal liquid water, and possesses some marvelous properties. Pollution-free, hydrolysis in super- or sub-critical water is an environment friendly technology (Cheng and al., 2008). Sub-critical water is a promising clean medium for dissolution of biomass. The thermal protein hydrolysis is very important economically as well as ecologically. Currently, the possibility of extracting and fractionating oils receives widespread interest due to the direct applications in the food and pharmaceutical industries for the generation of high-value products (L. Danielski, Technical University of Hamburg-Harburg, Germany, personnel communication). Supercritical fluids extraction of lipids has received attention as an alternative to organic solvent extraction and has been shown to be an ideal method for extracting certain lipids (Park et al., 2008; Garcia et al., 1996). Moreover, conventional methods are usually carried out at high temperatures, which can be responsible for the destruction of valuable substances (Roh et al, 2006). In this study, lipid was extracted from krill for fatty acids and bioactive compounds by supercritical carbon dioxide (SC-CO<sub>2</sub>) which is also environmental friendly extraction technology. After SC-CO<sub>2</sub> extraction of oil, the krill residues may be used as a source of valuable materials. Therefore, the objectives of this study were to produce the useful materials by sub-critical water hydrolysis from super-critical extracted residues of krill and also to investigate the effect of oil in the krill on the sub-critical water hydrolysis by comparing the production obtained from the extracted residues with that obtained from raw krill.

## MATERIALS AND METHODS

The krill (*Euphausia superba*) were collected from Dongwon F & B Co., S. Korea. The krill samples (mean body length, 5.15 cm; mean body weight, 0.65 g) were dried in a freeze-drier for about 72 h. The dried samples were crushed and sieved (700 µm) by mesh before to be used for SC-CO<sub>2</sub> extraction at the optimum conditions (25 MPa and 45°C) and for subcritical water hydrolysis. All reagents used in this work were of analytical or HPLC grade.

### Proximate composition

The moisture content, ash content and crude protein content were determined according to AOAC (1990) and lipid content was measured by conventional soxhlet apparatus using hexane as solvent for 12 h. Non protein content was estimated by subtracting the sum of weight of moisture, ash, protein and lipid from total weight.

### Supercritical CO<sub>2</sub> extraction

The set up of a laboratory scale of SCF extraction process can be operated at pressure up to 25 MPa. In order to investigate the optimum conditions of extraction, 50 g of freeze dried krill sample

were loaded into 200 ml stainless steel extraction vessel, containing cotton at the bottom of the SFE unit for extracting oil from krill. Before plugging with cap another layer of cotton was used at the top of the sample. CO<sub>2</sub> was pumped into the vessel by high pressure pump up to the desired pressure, which was regulated by a back pressure regulator. The vessel temperature was maintained by heater. Flow rates and accumulated gas volume passing through the apparatus were measured using a gas flow meter. The effects of temperature and pressure on lipid extraction from krill were studied at 35 to 45°C and 15 to 25 MPa at a constant extraction time of 2.5 h. The flow rates of CO<sub>2</sub> were kept constant at 22 g/min for all extraction conditions. After the determination of optimum conditions of pressure and temperature (25 MPa and 45°C); the extraction of oil from krill was performed. Three run (run 1, 2 and 3) were conducted depending on the extraction time; 50, 100, and 150 min, respectively. The extraction yield was determined and the krill residues remaining in the vessel were stored at -80°C until further analysis.

### Subcritical water hydrolysis

The schematic diagram of subcritical water hydrolysis unit is depicted in Figure 1. The subcritical hydrolysis was carried out in 80 ml of a batch reactor made of 276 Hastelloy with temperature control and stirring. The raw material and SC-CO<sub>2</sub> (run 1, 2 and 3) extracted residues were prepared separately with de-ionized water to get the homogeneous sample at the concentration of 0.5 g 100 ml<sup>-1</sup>; and then charged into the reactor. The reactor was filled by chosen reaction atmosphere (nitrogen, air) before to be closed and heated by an electric heater to the desired temperature (200 to 280°C). The sample was stirred by stirrer at 140 rpm. In order to avoid the massive decomposition of amino acids into organic acids; short reaction time was considered for each sample (5 min). The pressure in the reactor was estimated to be between 0.101 and 6.41 MPa based on saturated steam table for the temperature range studied. After cooling by immersing into cool water, the hydrolyzed sample which was in boiling-like status was collected from the reactor and then filtered before to be subjected to protein, amino acids and reducing sugars analysis. All experiments were performed in duplicate.

### Protein content of hydrolysates

For the protein content measurement, bovine serum albumin (BSA) was used as a standard according to Lowry et al. (1951). Protein content of hydrolysate was calculated depending on a calibration curve constructed from BSA standard (Figure 2).

### Reducing sugar content of hydrolysates

Reducing sugars content was measured by dinitrosalicylic (DNS) acid method (Miller, 1959) using D-glucose as a standard. Briefly, 3 ml of each hydrolysate were injected into test tubes and 3 ml of DNS reagent was added and well mixed. Then, the test tubes were heated for 5 min in a boiling water bath. After the color has developed and when the contents of the tubes were still warm, 1 ml of 40% potassium sodium tartrate (called Rochelle salt) solution was added to each. The test tubes were then cooled under a running tap water. A reagent blank was prepared by taking 3 ml of water and 3 ml of DNS reagent in a tube and treated in the same way. The spectrophotometric reading was taken at 575 nm using a double beam UV/VIS spectrophotometer (UVIKON 933, Kontron Instruments). Reducing sugar content of hydrolysate was measured depending on a calibration curve constructed from D-glucose standard.

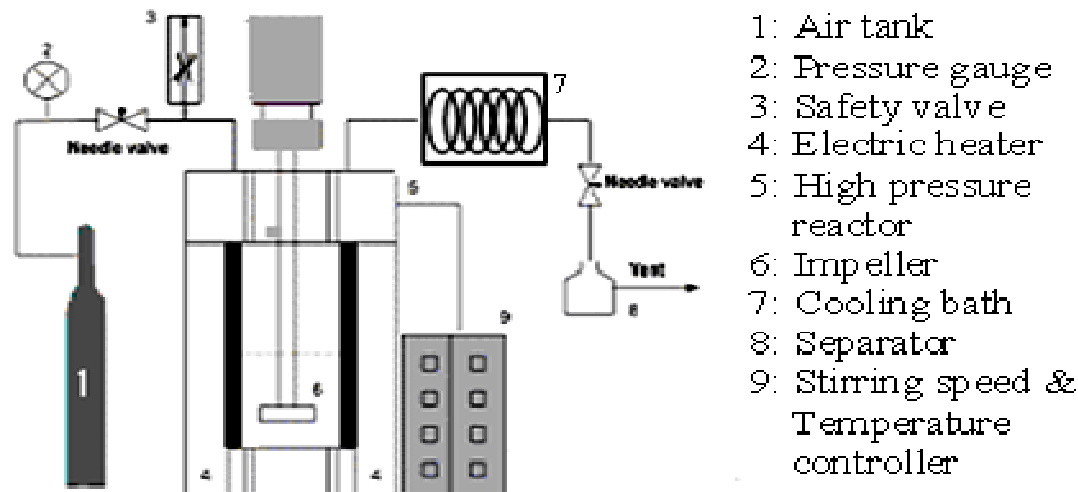


Figure 1. Schematic diagram of subcritical water hydrolysis.

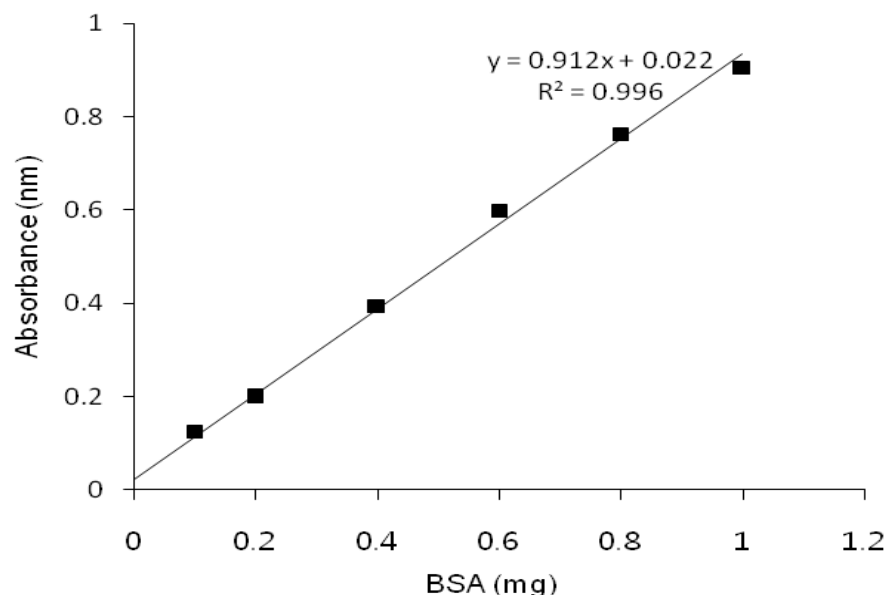


Figure 2. BSA calibration curve for the estimation of protein.

#### Amino acids analysis

The hydrolysates of freeze dried raw and different SC-CO<sub>2</sub> runs were diluted to the protein concentration of 0.25 mg/ml by 0.02 N HCl. The diluted samples were then filtered and analyzed by an amino acid auto analyzer (Hitachi L-8900, Tokyo, Japan)

## RESULTS AND DISCUSSION

#### Proximate compositions of freeze dried raw and SC-CO<sub>2</sub> extracted krill residues

The proximate compositions of both raw and SC-CO<sub>2</sub>

extracted residues (run3) are shown in Table I. In order to get higher efficiency of SC-CO<sub>2</sub> extraction of oil and bio-active compound; the sample was dried in a freeze dryer. The result shows that the highest yield of oil by SC-CO<sub>2</sub> extraction was approximately 12.2%, while, it was about 16.12% by conventional organic solvent extraction. SC-CO<sub>2</sub> extraction conditions used in this study (25 MPa, 45 and 150 min) is insufficient to extract all lipids from the by hexane extraction sample. In other hand, the lipid content obtained from SC-CO<sub>2</sub> extracted residues was  $4.15 \pm 0.11\%$ . The moisture content of raw and SC-CO<sub>2</sub> extracted krill was  $3.4 \pm 0.31$  and  $2.61 \pm 0.27\%$ , respectively. It was found to be decreased in SC-CO<sub>2</sub> extracted residues.

**Table 1.** Proximate compositions of freeze dried raw and SC-CO<sub>2</sub> extracted krill.

Composition (%)	Raw Krill	SC-CO <sub>2</sub> extracted residues
Moisture	3.4±0.31	2.61±0.27
Ash	4.75±0.19	5.14±0.41
Protein	62.45±0.35	73.03±0.53
Lipid	16.4±0.22	4.15±0.11
Non protein	13±0.18	15.07±0.25

**Table 2.** Protein yield from freeze dried raw and SC-CO<sub>2</sub> extracted krill residues by subcritical water hydrolysis at different temperatures.

Temperature (°C)	Freeze dried raw sample hydrolysate (mg/g)	SC-CO <sub>2</sub> extracted sample hydrolysate (mg/g)		
		Run 1	Run 2	Run 3
200	391.27±3.71	403.93±3.12	416.56±3.44	479.21±4.72
220	373.22±1.48	422.16±2.31	471.3±1.34	520.71±2.63
240	370.29±3.69	426.07±4.12	482.13±3.25	538.51±4.17
260	364.41±2.56	458.16±2.68	552.26±1.72	646.5±2.57
280	345.76±3.62	456.86±3.17	567.09±2.43	680.58±3.90

Mean value of two replicates ± S.E.

The moisture might be reduced by the effect of SC-CO<sub>2</sub>; as it is well known that SC-CO<sub>2</sub> strips the water from the medium. The protein content in freeze dried raw sample was 62.45 ± 0.35%. However, the protein content was determined to reach 73.03±0.53%. For the ash and non protein content, high values were observed in SC-CO<sub>2</sub> extracted residues comparing with those of raw sample. It is to mention that the values obtained for raw krill content are in agreement with other studies reporting proximate analysis of krill on a dry basis of 45 to 80% crude protein, 7 to 30% total lipid and 8 to 20% total ash (Grantham, 1977; Savage and Foulds, 1987; Sidhu et al., 1970).

### Protein yield in hydrolysates of raw and SC-CO<sub>2</sub> extracted samples

The protein contents in both hydrolysates at different temperatures are shown in Table 2. It was found that the hydrolysate of SC-CO<sub>2</sub> extracted sample at different experimental runs contained more protein than that of raw sample hydrolysate. This result can be explained by the effect of hydrophobic oil content in the raw materials which made them less accessible to water hydrolysis. Watchararuij et al. (2008) reported that the protein yield in subcritical water hydrolysis of soybean decreased when temperature increased from 200 to 220°C. In this study, the protein yield was found to increase with the increase in temperature in the hydrolysate of SC-CO<sub>2</sub> extracted sample. The highest protein yields in raw and SC-CO<sub>2</sub> extracted krill hydrolysates were 391.27 ± 3.71

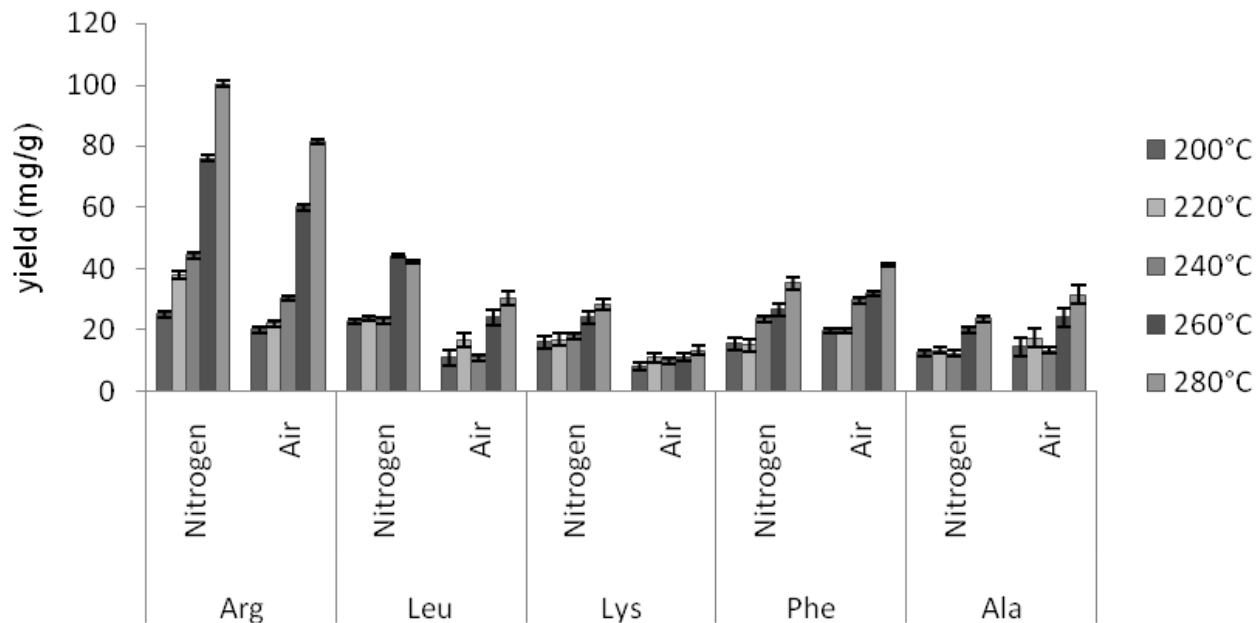
mg/g at 200°C and 680.58 ± 3.90 mg/g at 280°C, respectively. Similar results were reported by Watchararuij et al. (2008) for subcritical water hydrolysis of rice bran and soybean meal. By comparing with the crude protein of krill, this result suggested that almost all protein content could be obtained from the hydrolysate of deoiled material. In fact, because of its strong aggregation through hydrophobic interactions, protein usually has low solubility in water at ambient temperature. However, the solubility of protein in water increased at higher temperature. In addition, at high temperature the protein yield increased due to the increased rate of hydrolysis caused by the raise in water ionization constant.

### The effect of atmosphere used on amino acid yield

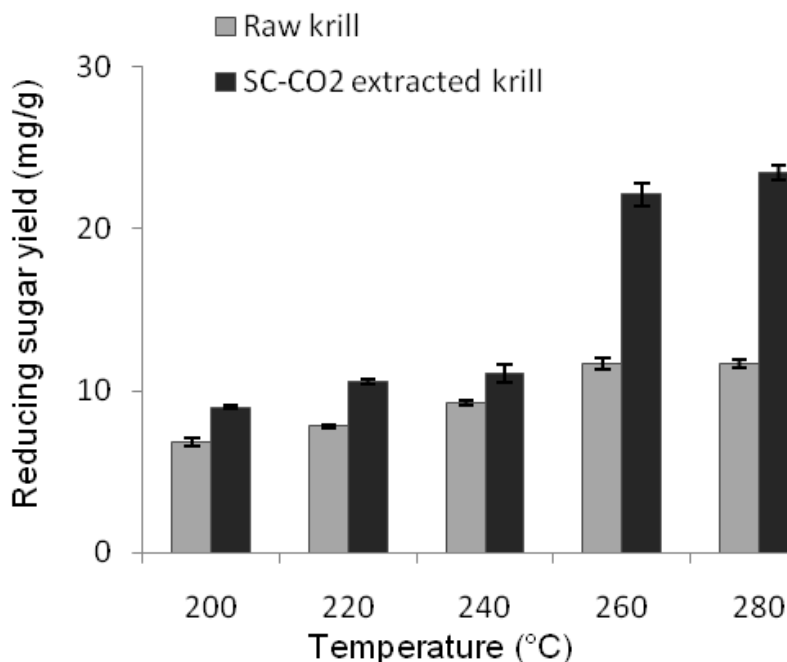
Figure 3 shows the effect of different reaction atmosphere used (nitrogen and air) on amino acid yield in hydrolysates. The results showed that no matter whatever atmosphere is used in term of maximum yield. However, it was suggested that lysine, leucine and arginine should be hydrolyzed with nitrogen as atmosphere, but for phenylalanine and alanine the yield in hydrolysate under air atmosphere was higher than that obtained under nitrogen atmosphere. Similar results for fish proteins hydrolysis was reported by Zhu et al. (2008).

### Reducing sugar yields

The reducing sugar content in freeze dried raw and SC-



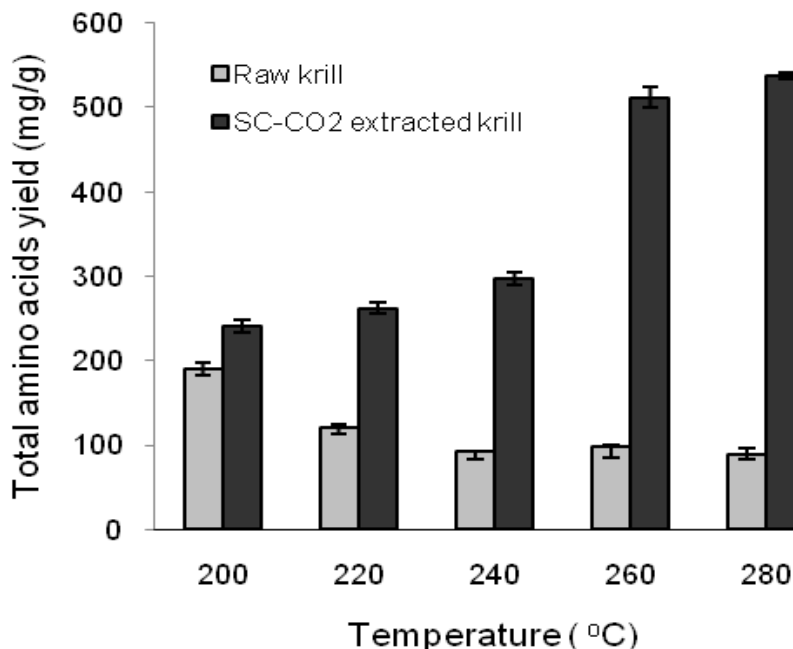
**Figure 3.** The yield of some amino acids by subcritical water hydrolysis of SC-CO<sub>2</sub> extracted krill under nitrogen and air atmosphere.



**Figure 4.** Reducing sugar yield by subcritical water hydrolysis of raw and SC-CO<sub>2</sub> extracted krill at different temperatures.

CO<sub>2</sub> extracted sample hydrolysates are shown in Figure 4. Reducing sugars are produced from carbohydrate which reacts with hydronium and hydroxide ions. It was found that the amount of reducing sugar in both raw and SC-CO<sub>2</sub> extracted sample rose with increasing temperature.

Thus, within the temperature range of 200 to 280°C, the decomposition of reducing sugar did not occur due to the short reaction time. Previous work has reported similar results from rice bran and soybean meal (Watchararujij et al., 2008). The reducing sugar yield in hydrolysate of SC-



**Figure 5.** Total amino acid yield by subcritical water hydrolysis of freeze dried raw and SC-CO<sub>2</sub> extracted krill at different temperatures.

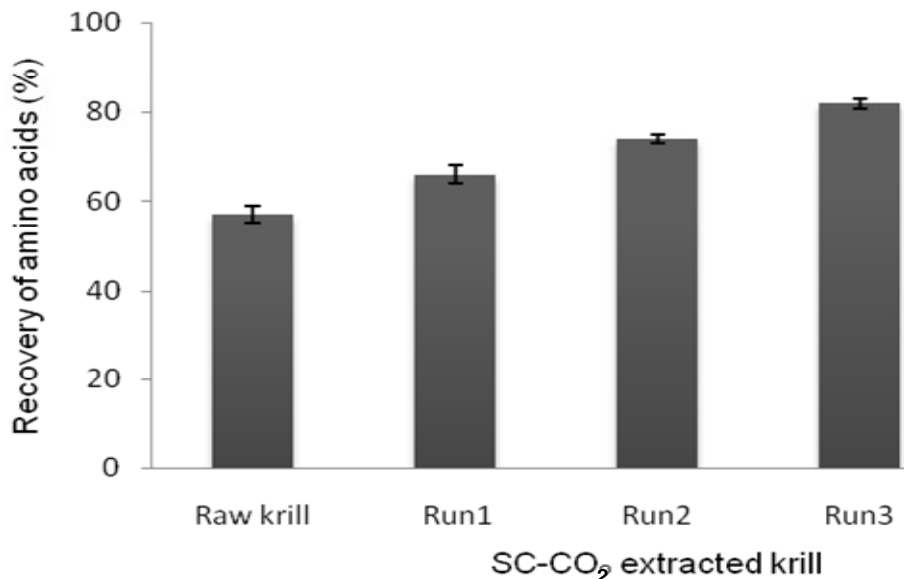
CO<sub>2</sub> extracted sample was found to be higher than that of raw sample. This result was approved with the high content of non-protein substances in SC-CO<sub>2</sub> extracted krill (Table 1).

### Amino acid yields

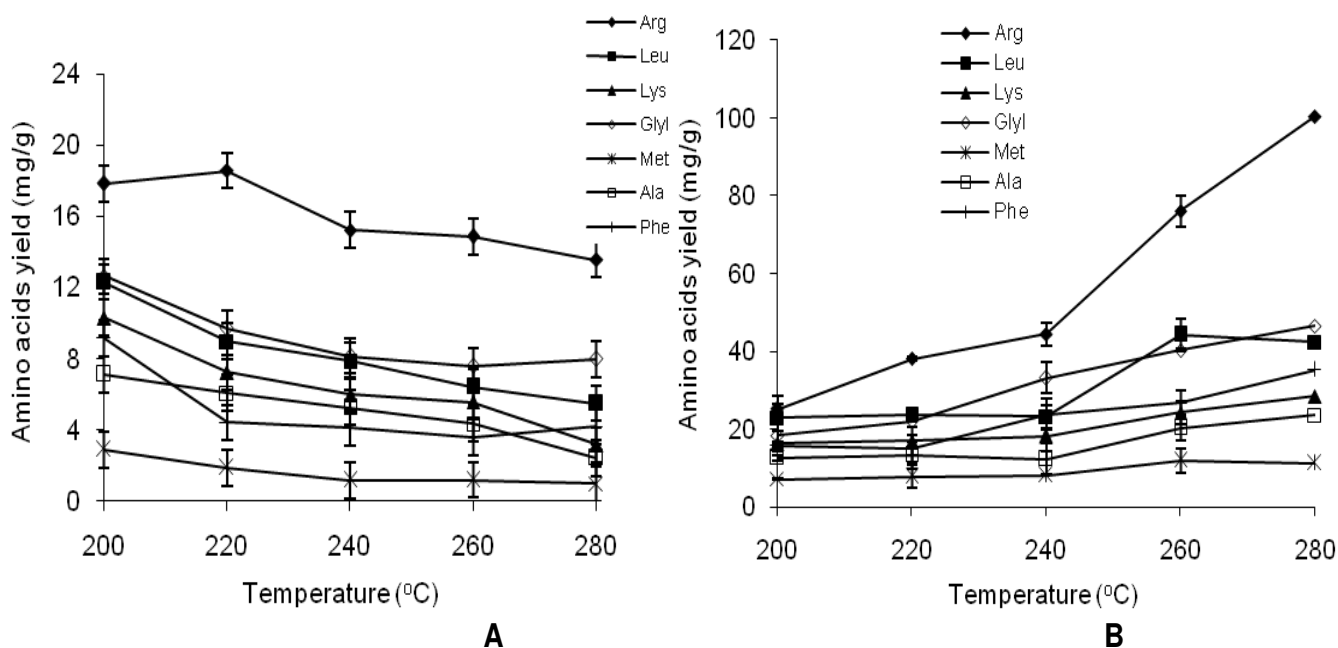
The krill protein is considered to be of high quality, the chemical analysis showed that the protein recovered from krill contains all 9 essential amino acids in adequate quantities to meet the FAO/WHO/UNU requirements for human adults (Chen et al., under review). Amino acids play an important physiological role in all life forms. Amino acids are relatively tasteless. Nonetheless, they contribute to the flavor of food. Amino acids and protein hydrolyzates are as a result, useful additives in food industry (Rogalinski et al., 2005). In this study, in order to decrease the decomposition of amino acids; short reaction time was applied for subcritical water hydrolysis (Kang et al., 2001). In the other hand, low ratio of sample to water was used considering higher efficiency of hydrolysis by subcritical water for amino acids yielding. At similar ratio of material to water, highest amino acids yield by subcritical water hydrolysis was also obtained in other work (Lamoolphak et al., 2008). Figure 5 shows the total amino acid yield in raw and SC-CO<sub>2</sub> extracted sample hydrolysates. The total amino acids yield of SC-CO<sub>2</sub> extracted sample hydrolysates was higher than that of raw sample hydrolysates. This result agreed with the high protein yield of SC-CO<sub>2</sub> extracted sample hydrolysates

comparing to raw sample hydrolysates. For SC-CO<sub>2</sub> extracted sample, it was found that the amino acid yield increased with the increase in temperature. Cheng et al. (2008) also reported the same increase of amino acids yield with the increase in temperature to a certain degree. The highest yield of amino acids in SC-CO<sub>2</sub> extracted sample hydrolysate was 537.78±4.13 mg/g sample at 280 °C.

For raw krill hydrolysates, the total amino acid yield was increasing temperature contrary to the results obtained from the SC-CO<sub>2</sub> extracted sample. The highest amino acid yield in raw sample hydrolysates was 190.65 ± 2.74 mg/g at 180 °C. At high temperature, the total amino acid yield in raw sample hydrolysate was low. However, the amino acid yield was higher in SC-CO<sub>2</sub> extracted sample. Thus, this contrast can be explained by the presence of oil in the raw material which may possibly have interfered with the breakdown of peptide bond by subcritical water hydrolysis at high temperature. Moreover, by considering the short reaction time and the high temperature; oil may form a complex with protein that make the protein hydrolysis reaction more difficult. This was also in conformity with the results of amino acids recovery from different experimental runs of SC-CO<sub>2</sub> extracted sample. It was found that the efficiency of subcritical water hydrolysis for amino acid yield was highest in SC-CO<sub>2</sub> run3 (150 min of extraction) extracted sample (82%) comparing with the run2 (100 min) and run1 (50 min), respectively (Figure 6). In addition, high temperature causes the decomposition of amino acids into organic acids or other products (Sato et al., 2004). Kang and Chun (2004) reported that the



**Figure 6.** Recovery of amino acids from raw and SC-CO<sub>2</sub> extracted krill by subcritical water hydrolysis.



**Figure 7.** The amino acids yield in hydrolysates at different temperatures. A) Raw krill; B) SC-CO<sub>2</sub> extracted residues.

significant decrease in the amino acid production from a hydrothermal process of fish wastes was due to the decomposition of amino acids into organic acids or volatile materials.

Figure 7a and b shows the yield of the main amino acids recovered from raw and SC-CO<sub>2</sub> extracted krill by subcritical water hydrolysis at different temperature reactions. Previous studies have been carried out in which

depending on the raw protein and corresponding contact time, the thermal degradation of amino acids occur at temperature above 250 to 300°C (Yoshida et al., 1999; Daimon et al., 2001; Quitain et al., 2001). Cheng et al. (2008) also reported that most of amino acids give maximum yield at the reaction temperature range of 200 to 290°C. In this study, the highest yield of amino acids from SC-CO<sub>2</sub> extracted sample was obtained within the

temperature of 260 to 280°C. For raw sample hydrolysates, the highest yield was obtained within 200 to 220°C.

## Conclusions

The result presented in this study demonstrated that the production of valued materials, especially amino acids, from krill using subcritical water hydrolysis was successful. Most proteins from SC-CO<sub>2</sub> extracted krill were obtained in the hydrolysates at high temperature. Result shows different relationship between amino acids yield and reaction temperature. The highest amino acid yield from raw sample was at low temperature while, for SC-CO<sub>2</sub> extracted sample the highest yield was found at high temperature. The recovery of the amino acids from SC-CO<sub>2</sub> extracted sample hydrolysate was higher than that of raw sample hydrolysate. The appropriate conditions for protein and amino acids production from raw and SC-CO<sub>2</sub> extracted sample by Subcritical water hydrolysis were 1:50 material to water weight ratio at 5 min and hydrolysis temperature of 200°C for raw krill and 280°C for SC-CO<sub>2</sub> extracted krill, respectively. In the short reaction time, subcritical water hydrolysis was more effective for amino acid recovery from SC-CO<sub>2</sub> extracted sample than raw sample. Thus, subcritical water hydrolysis may be a useful method for production of valued materials from krill, which can be as source of food additives.

## ACKNOWLEDGEMENT

This research was supported by Technology Development Program for Fisheries, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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