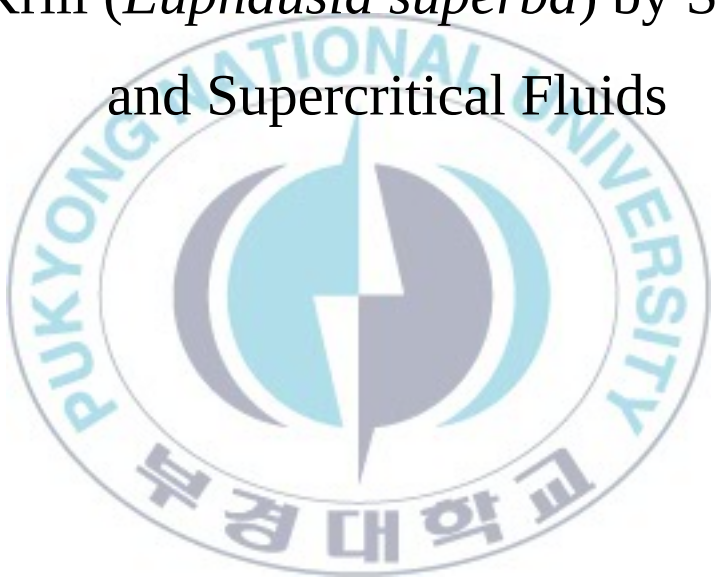
Thesis for the Degree of Doctor of Philosophy

Characterization of Bioactive Compounds Obtained from Antarctic Krill (*Euphausia superba*) by Sub- and Supercritical Fluids



by

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August 2011

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(아임계 및 초임계유체공정를 용하여 남극 크릴새우(*E. superba*) 로부터 어진생리활성 물질의 특성)

Advisor: Prof. Byung Soo Chun

by

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August 26, 2011

Dedicated

To my Parents, brothers and sisters,

My lovely wife,

And to the spirit of my mother Khadija in its heaven…

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Characterization of Bioactive Compounds Obtained from Antarctic Krill (*Euphausia superba*) by Sub- and Supercritical Fluids Processes

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Abstract

Antarctic krill (*Euphausia superba*) is a species which belongs to the order of Euphausiacea within the crustacean superorder Eucarida, playing a central role in the Southern Ocean pelagic ecosystem. Due to its high biomass (60-155 million tons) compared with the commercial fish resources; it is a new potential source for the fishing industry. Currently, krill is mostly commercially exploited for the fishing industry and aquaculture and only a small percentage is used for human consumption. However, Krill is a rich source of high-quality protein, with the advantage over other animal proteins of being low in fat and a rich source of polyunsaturated fatty acids (PUFA), mainly omega-3 fatty acids. Antioxidant levels in krill are higher than in other fish, signifying benefits against oxidative damage. Finally, the by-product generated by the processing of krill into edible products can be used for the production of value-added materials. In this thesis, the application of sub- and supercritical fluids (SCF) processes for the recovering and the characterization of the bioactive compounds from Antarctic Krill was investigated. Initially, krill oil was extracted using an environmental friendly solvent, supercritical carbon dioxide (SC-CO_2) and an organic solvent, hexane. The SC-CO_2 extraction was carried out at temperatures range of 35 to 45ºC and pressures ranging from 15 to 25 MPa. The flow rate of CO_2 (22 g min^{-1}) was constant during the extraction period of 2.5 hrs. The maximum oil yield was found at higher extraction temperature and pressure. The

extracted oil was analyzed using gas chromatography (GC) for fatty acid profile compositions. High percentage of PUFAs was found, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The quality of krill oil obtained by SC-CO_2 extraction was compared with that of hexane extracted oil. The SC-CO_2 extracted oil demonstrated higher stability than the oil obtained by hexane extraction. High performance liquid chromatography (HPLC) was used for astaxanthin analysis among the different extraction conditions. The highest yield of astaxanthin was found in krill oil extracted at 25 MPa and 45ºC. These results indicate that SC-CO_2 is effective in obtaining nonpolar lipids from krill by only one-step extraction. On the other hand, the effect of SC-CO_2 extraction on the fluorine content of krill was investigated. Almost 40 % of the fluorine content was removed from the initial concentrations during the SC-CO_2 extraction. Further works with a special SC-CO_2 extraction design are required to confirm these results.

In the second part of this study, Phospholipids was isolated and characterized from krill residues extracted by SC-CO_2 extraction. The purity of phospholipids ranged between 93 and 97% and was evaluated by HPLC–ELSD. The purified phospholipids were characterized by their acid value, peroxide value, and the oxidative stability. Thin layer chromatography (TLC) was performed to purify the individual phospholipids. The fatty acid compositions of total phospholipids, Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) were analyzed by GC. The oxidative stability of krill phospholipids was high in spite of its high content of PUFAs.

The third step of the thesis consists of the applicability of SC-CO_2 in enzyme purification system. Three major classes of digestive enzymes of krill were investigated after oil extraction using SC-CO_2, hexane and acetone. The residues of krill extracted at optimum conditions (25 MPa and 45ºC) were used to characterize the digestive enzymes. The digestive enzyme activities of protease, lipase and amylase of SC-CO_2 treated krill residues were slightly decreased comparing to organic solvent, hexane and acetone treated residues. In SC-CO_2 treated samples, all of the digestive enzymes showed slightly higher temperature stability. On the other hand the crude extracts of SC-CO_2, hexane and acetone treated krill samples showed almost same optimum pH and pH stability for each of the digestive enzymes. It was also found in SDS-PAGE that there are no significant

differences in protein patterns of the crude extracts of untreated and SC-CO_2, hexane and acetone treated krill indicating no denaturation of proteins.

The last part of the thesis deals with the use of subcritical water hydrolysis to produce valued materials from krill residues extracted by SC-CO_2 and to compare the results with those obtained from raw krill. Subcritical water hydrolysis efficiency from raw and deoiled krill was examined over the temperature range of 200 to 280ºC, ratio of material to water for hydrolysis was 1:50 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 of 0.20MPa. 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 deoiled krill hydrolysate was at 280ºC. While, the highest amino acid yield in raw krill hydrolysate was at low temperature 200ºC. 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.

Chapter 1

General introduction

1.1. Background

Antarctic krill *(Euphausia superba*), with an estimated biomass of approximately 500 million tonnes and with its ecological role as the most important trophic link between primary production and vertebrate predators, is incontestably the keystone species in the Antarctic marine ecosystem. Total standing biomass of krill (Euphausiacea) in the Nordic and Barents Seas has been estimated to be between 91-161 million tonnes with an annual production of 242 million tonnes (Dalpadado et al., 1998; Sakshaug et al., 1994), and in the Southern Ocean between 125 to 725 million tons (FAO, 2005b) up to seven times the total present marine harvest of capture fisheries (FAO, 2003). The enormous biomass together with the major key position it holds in the food web also means that krill plays an important role in biogeochemical cycles, exporting carbon directly to the deep ocean (Bathmann, 1992). Thus, Antarctic krill is the target of one of the world's largest crustacean fisheries (Nicol and Endo, 1997). Further, because of the high biomass of krill compared with the 100 million tonnes of the world-wide commercial fish resources; it is a new prospective source for the fishing industry. Currently, krill is generally commercially exploited for the aquaculture and fishing industry and has not been yet a traditional food in the human diet. Public acceptance of krill for human consumption will depend partly on its nutritive value. However, due to its biochemical composition (e.g. high protein content, omega 3 phospholipids, astaxanthin, digestive enzymes); Antarctic krill has recently received considerable increasing commercial interest (Nicol et al., 2000). As like other fishes, krill can be a unique source of oil with polyunsaturated fatty acids (PUFAs), lipid soluble bioactive compounds, amino acids and functional proteins etc.

The main focus of this thesis will be the demonstration of the utilization of krill as a new food product instead of just as fish bait. The following part will therefore give an introduction to the high nutritional value of krill and the major benefits to its use as food and in highly processed food with added value. The last part of the introduction will then

investigate the suitable processes for recovering and isolation of the bioactive compounds rich in krill as resources which can be benefited economically and also environmentally, and will further state the detailed aims of this thesis.

1.2. Krill (*Euphausiids*)

Euphausiids called Krill are a group of huge importance in marine ecosystems, comprises over 80 species, most of which are planktonic. Krill are widespread with species to be found in all the oceans of the world. Their biomass density in some area increases their importance to marine ecosystems and has led to commercially successful krill harvesting (Everson, 2000c). The subsequent species of Euphausiid crustaceans commonly arise in the southern ocean: *Euphausia superba, E. triacantha, E. vallentini, E. crystallorophias, E. frigida, Thysanoessa vicina and T. macrura.* Only two of them commonly occur in dense swarms and are of particular interest to commercial fisheries: *E. superba* and *E. crystallorophias*. Table 1.1 gives some biological information on species of krill that are commercially harvested.

The southern ocean euphausiids have a circumpolar distribution and are largely separated by their latitudinal ranges (Everson, 2000a). The appearance of krill is similar to that of shrimp belonging to the order of Decapoda of the same superorder Eucarida. Smaller size and exposed gill are points to generally distinguish the order Euphausiacea from Decapodae. A further difference is that the former spends its whole life floating as plankton, whereas the latter, in most species, floats only in the larva stage.

Experimental fishing for krill started in the early 1960s by the former USSR, with catch levels being initially low. From the first commercial fishing activities in the early 1970s, krill catches increased progressively from 19 700 tonnes in 1973-1974 to a maximum of 528 000 tonnes in 1981-1982. Catches then declined sharply until 1983-1984 to a stable level of around 100 000 tonnes (Nicol and Endo, 1997; Ichii, 2000). The Commission for the Conservation of Antarctic Marine Living Resources Scientific Committee (SC-CCAMLR) has recommended a precautionary catch limit of 4 million tonnes of krill for the Atlantic Sector (Area 48) (Hewitt et al., 2002). Harvesting of 4 million tonnes would supply nearly 400 000 tonnes of marine protein and 80 000 tonnes of marine lipids. Table 1.2 shows the annual catch of krill by the major fishing nations

during the last two decades. In 2003-2004 less than 120 000 tonnes were exploited (CCAMLR, 2005). For the season 2004-2005 about 160 000 tonnes were estimated to have been caught (SC-CCAMLR, 2005a). South Korea, Japan, Poland and Ukraine have been the main harvesting countries and it is expected that they will continue this fishery (Nicol and Endo, 1997).

1.2.1. Antarctic krill (*Euphausia superba*)

E. superba is the species commonly referred to as "Antarctic krill" and it is a widespread species, which is subject to significant commercial fishing (Everson, 2000a). The morphology of a typical krill is shown in Figure 1.2. The taxonomy of *E. superba* is as follows:

Phylum	Arthropoda	**Superorder**	Eucardia
Subphylum	Mandibulata	**Order**	Euphausiacea
Class	Crustacea	**Family**	Euphausiidae
Subclass	Malacostracea	**Genus**	Euphausia
Series	Eumalacostraca	**Species**	Euphausia superba

Antarctic krill is one of the most abundant and successful animal species on Earth. The biomass of Antarctic krill may be the largest of any multi-cellular animal species on the planet (Nicol, 2004). Antarctic Krill is also recorded as forming the largest aggregation of marine life (Macauley et al., 1984) and it has the most powerful proteolytic enzymes yet found (Anheller et al., 1989).

1.2.2. Habitat and biology of *Euphausia superba*

As like all Euphausiaceas, *E. superba* have gills clearly visible below the cephalothorax and eyes at the front of the head; the stomach is behind the eyes and they have a characteristic big muscle system from the middle to the back of the body (Everson, 2000a). *E. superba* is the best characterized of all 85 known Euphausiid species. Their length varies from 1.5 to 6.0 cm, and mature animals have a live weight of around 1.0 to 1.5 g. Antarctic krill has a high nutritional value (Grantham, 1977) and a broad range of products has been developed from it (Grantham, 1977; Suzuki and Shibata, 1990).

Distribution of Antarctic krill is circumpolar (Fig.1.2). High concentrations exist in the South Atlantic and in some regions close to the Antarctic continent in the Indian Ocean. The total surface of the distribution of Antarctic krill is approximately 36 millions square kilometers which represents for example, almost five times the area of Australia. The Antarctic krill is distributed south of 60 °S around the South Pole, with high density in the cold waters with low salinity. During daylight hours, schools of the Antarctic krill are found mainly in depths of 50 to 100 m, while in the evenings they float up to the surface (Quetin and Ross, 1991). Important life-cycle parameters of krill such as metabolic activity and maturity constitute annual patterns that specify the time for reduced metabolic and reproductive activity. As it is shown in Fig. 1.3, graduations in green color indicate different phytoplankton concentrations in the water column. Bars show oxygen uptake rates per body dry mass of krill in different seasons in the Lazarev Sea. It is clear that the oxygen uptake rates in spring/summer (green color) are significantly different from the rates measured in autumn (blue color) and winter (red color).

The large variety of food for krill includes plant and animal detritus material; transferring energy from this lower trophic level to several vertebrate predators like whales, seals, fishes, and birds (Fig.1.4). Thus, it plays an important role in the food chain system in the Antarctic ecosystem.

Table 1.1: Biological information on krill species those are commercially harvested (Nicol and Endo, 1997)

Species	Common names	Maximum weight (g)	Maximum length(mm)	Estimated life span	Depth distribution	Where commercially fished
Euphausia superba	Antarctic krill	2	65	5-7 years	Surface to 500m	Antarctic
Euphausia pacifica	North Pacific krill Isada	0.1	20	1-2 years	Surface to 300m	Japan, British Columbia
Euphausia nana	Ami-ebi	0.01	10	<1 year	Surface to 300m	Japan
Thysanoessa inernis	smaa krill	0.15	32	2 years	Surface to 300m	Japan Gulf of St Lawrence
Thysanoessa raschii	smaa krill	0.13	30	2 years	Surface to 300m	Gulf of St Lawrence
Meganyctiphanes norvegica	North Atlantic krill stor krill suil dhu	0.5	40	2+ years	Surface to 300m	Gulf of St. Lawrence, Mediterranean Scotian Shelf(proposed)
Nyctiphanes australis	Brit	0.02	17	1 year	Surface to 150m.	Tasmania, Australia (proposed)

Table 1.2: Annual catch of *E. superba* by the major fishing nations. Data up to 2006 are from the FAO databases and for 2007 are from the CCAMLR statistical bulletin. A dash means "no catch", a zero indicates a small catch < 500 t

Countries	**Annual catch (in 1000 tonnes, rounded)**																	
	1990	**91**	**92**	**93**	**94**	**95**	**96**	**97**	**98**	**99**	**2000**	**01**	**02**	**03**	**04**	**05**	**06**	**07**
Japan	69	69	78	57	61	63	59	60	67	66	81	67	51	60	34	23	33	24
South Korea	4	1	1	-	-	-	-	-	3	0	7	8	14	20	25	27	43	33
Norway	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9	40
Poland	3	10	15	7	8	13	22	14	20	20	20	14	16	9	9	4	5	7
Ukraine	-	-	55	-	13	59	10	-	-	7	-	14	32	18	12	22	15	-
USSR/Russia	326	249	103	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U.S.	-	-	-	-	-	-	-	-	-	-	-	2	12	10	9	2	-	-
Vanuatu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	29	48	-	-

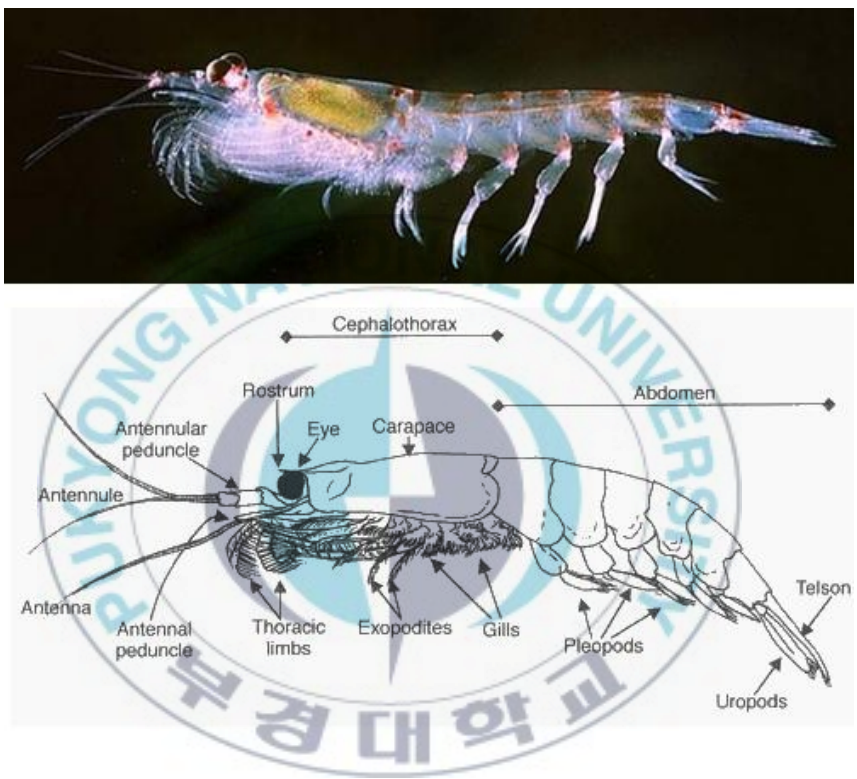

Fig. 1.1: General view of krill showing main morphological features.
(Extracted from Everson, 2000a)

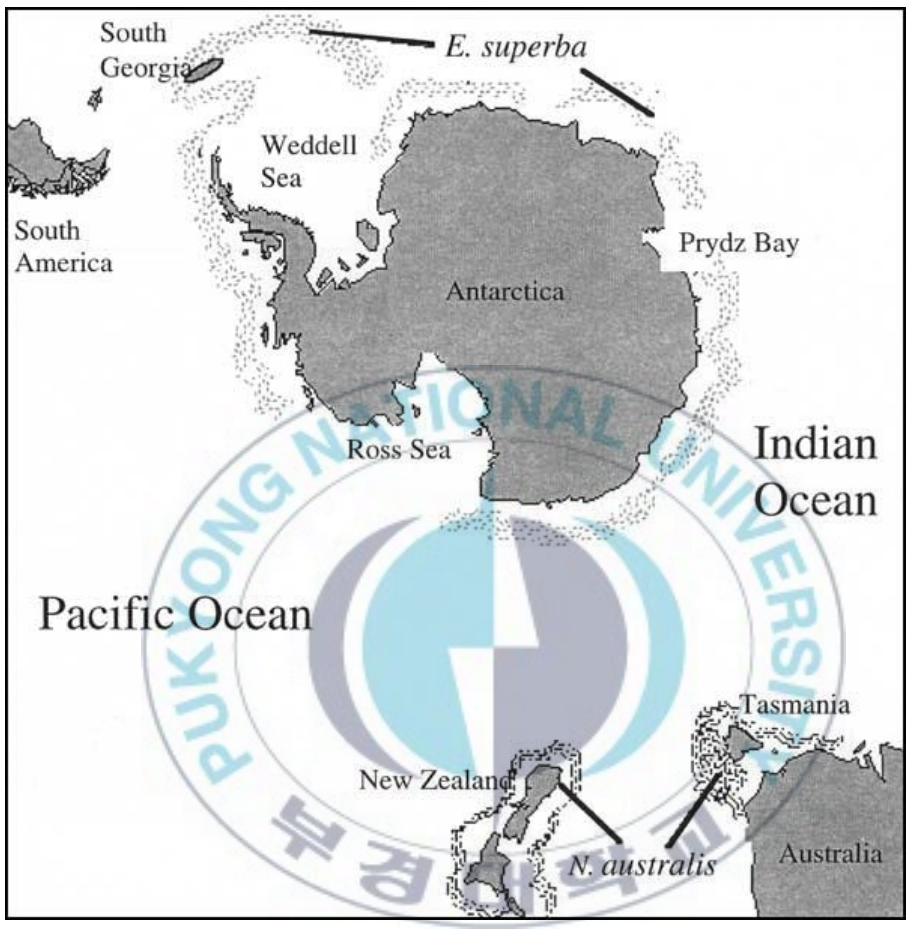

Fig. 1.2: Distribution of Antarctic krill (*E. superba*) in Southern Ocean (FAO, 1997)

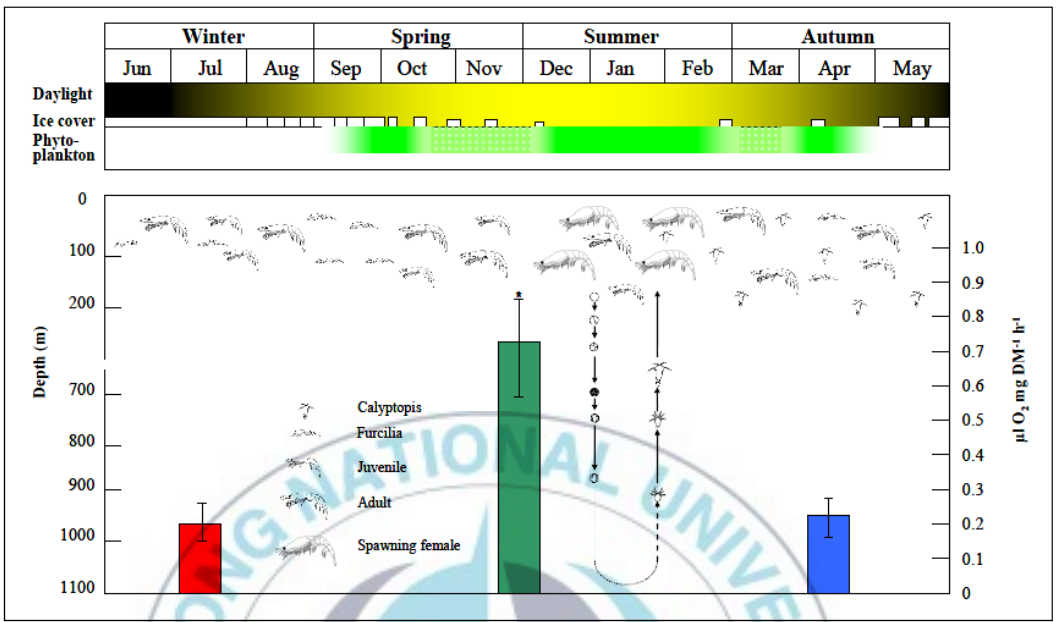

Fig. 1.3: Seasonal cycles of metabolic activity and reproduction in krill corresponding to seasonal cycles of food, ice, and light (Quetin and Ross, 1991).

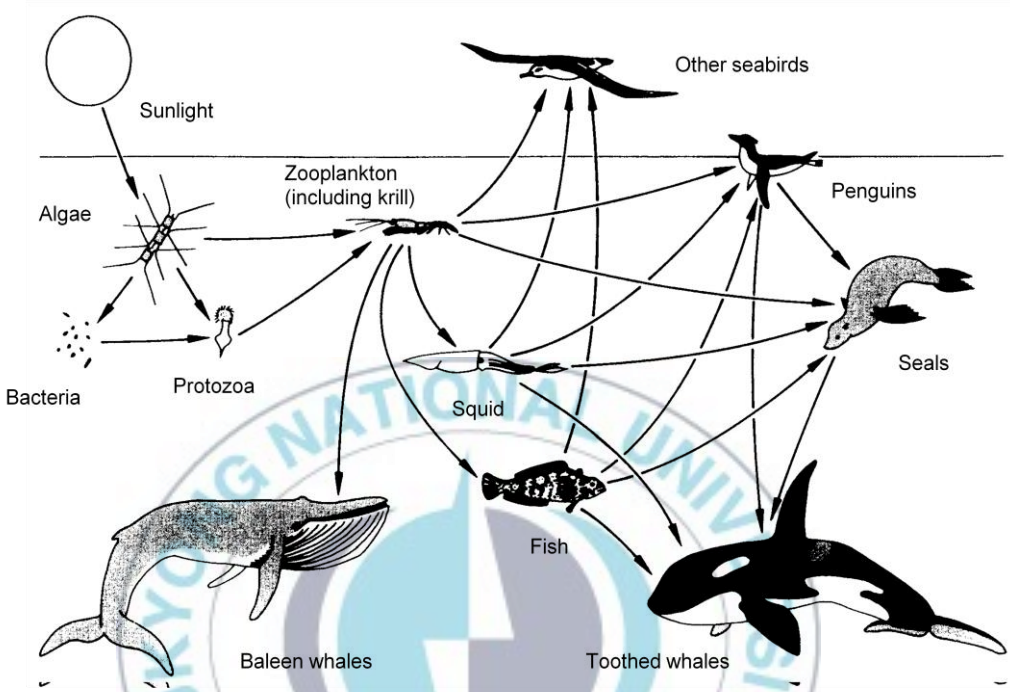

Fig. 1.4: A simplified representation of the southern ocean food web linkages that are centered on krill as presented in Everson (2000b).

1.3. Krill oils

The high quality krill oil type is obtained as a byproduct from the krill meal process, being the final product 100% natural. Krill oil has characteristics that are very desirable not only in cosmetics and neutraceuticals, but also as a pigment source for animal feed and the pet food industry. Krill oil is a source of natural carotenoids, being over 95% of the pigments present in krill oil in the form of asthaxanthin. Asthaxanthin has important metabolic functions in many organisms such as the improvement of the quality of sea bream eggs (Nakazoe et al., 1984). Asthaxanthin is not only a pigment, but also works as a photo protector and antioxidant, like beta-carotene. In addition, krill oil is a rich source of cholesterol (3-8% of the fat) which is an essential ingredient for crustacean diets (Saether et al, 1986).

Krill oils are manufactured following a solvent-extraction procedure, normally taking place on onshore plants, and alternatively following a partial onboard trawlers' processing and/or entirely manufactured onboard such trawlers. Quality will therefore depend on the type of processing the oil follows. The most market-offered oil though would come from onboard processes following the dried aqua-feed processing line concept.

1.3.1. Nutritional value

Present dietary recommendations suggest reducing fat consumption because high-fat diets have been implicated in weight gain and in increased risk of various diseases, especially cardiovascular disease. In addition to the amount of fat, the type of fat also has an important impact on health. Foods high in saturated fatty acids (SFAs) have been linked to increased risk of cardiovascular disease, whereas the omega-3 PUFAs, particularly eicosapentanoic acids (EPA) and decosahexanoic acid (DHA), have been linked to reduced risk of cardiovascular disease and have proven beneficial effects in the prevention of some other diseases (Hu et al., 2001). As like marine organisms, krill is considered to be the unique source of PUFAs. PUFAs, especially omega-3 fatty acids, have been very often subject of scientific studies in the last years. Thus, the nutritive value of krill oil was evaluated due to the consumer's desire for foods that contain low fat and SFAs but high omega 3 PUFAs.

1.3.2. Cardiovascular health benefits

Omega-3 fatty acids have proven beneficial effects in the prevention of some diseases. The risks of suffering from diseases such as diabetes, obesity, asthma, and others have been shown to decrease with increasing omega-3 consumption (Simopoulos, 2002). Omega-3 fatty acids have been shown to have beneficial effects on the cardiovascular system. Although krill oil is being advertised as a supplement with protective effects against heart disease, few published studies exist. Shagaeva et al. (1993) reported that feeding krill meat to patients with type 1 diabetes reduced their incidence of atherosclerosis. Effects of omega-3 fatty acids on coronary heart disease have been shown in animals and in humans, tissue culture studies, and clinical trials (Leaf et al. 2003; Uauy and Valenzuela 2000). Fish oil has been reported to reduce cardiovascular diseases risk through diverse mechanisms of reducing blood pressure, inflammation, arrhythmia, and atherosclerotic plaque growth, as well as by promotion of endothelial function, anti-thrombosis, and the improvement of insulin sensitivity (Din et al., 2004). Schacky and Harris (2007) have proposed using an “omega-3 index” as an indication of overall heart health. Their research has shown that measuring the combined percentage of DHA and EPA out of the total fatty acids in red blood cell membranes is as accurate as the presently used low-density lipoprotein cholesterol measurement for heart safety. Thus, patients with higher omega-3 indices are less expected to experience a sudden cardiac death.

1.3.3. Other possible health benefits

The major focus on omega-3 PUFAs has been their effects on cardiovascular diseases. However, omega-3 PUFA research has expanded into other health issues such as neurological function, retinal and brain development, cancer, arthritis, immunological conditions, diabetes, kidney disease, and skin disorders (Sidhu et al., 1970; Jiang et al., 1998; Hering et al., 2007; Smyth and McGlynn, 2005; Moyad, 2005). In the other hand, Foods rich in PUFAs are highly susceptible to lipid peroxidation, which results in oxidative products that cause deterioration of food quality and upon ingestion may cause cellular damage. However, despite its high PUFA content, krill oil is considered relatively resistant to oxidation. The stability of krill oil has been attributed to its antioxidant content (Suzuki and Shibata, 1990).

1.4. Krill protein quality

Krill is a high-protein food, having a protein content estimated in the range of 60% to 65% dry weight. Similar to other animal foods, the protein derived from krill is a complete protein, as indicated by the presence of all nine of the indispensable amino acids for adults required by FAO/WHO/UNU (Nicol et al., 2000; Young and pellet, 1991). In fact, protein quality is determined not only by the amino acid composition but also by its digestibility. Tamura (1980) reported no differences in digestibility between boiled krill and whole egg those fed to adult men for 21 days. Therefore, krill appears to be a good source of high-quality protein. The huge biomass and high-quality protein offered by krill provides an economical replacement for commercially available protein sources.

1.4.1. Health benefits of krill protein

Adequate protein intakes are necessary for synthesis of structural components of the muscle and of enzymes, hormones, hemoglobin, and other body tissues. Sidhu et al. (1970) reported that rats fed krill protein concentrate showed no difference in organ weights and hemoglobin counts than rats fed casein, indicating that krill protein is capable of supporting protein synthesis. High-protein diets have also been suggested to increase the risk of cardiovascular diseases by inducing negative effects on blood lipid profiles. However, the negative effects on blood lipids are more expected due to the high SFA intakes associated with most high-protein diets. Krill, unlike other animal sources of proteins, is low in SFAs. In addition, all food proteins have the potential to be allergenic to some people. Mills and Breiteneder (2005) reported that up to 5% to 7% of children and approximately 1% to 2% of adults experience food allergies with symptoms ranging from a mild rash to life-threatening anaphylaxis. The Food and Agricultural Organization of the United Nations includes crustaceans on its list of the eight most significant food allergens (FAO, 1995).

1.5. Value added products

1.5.1. Chitin

Chitin is the raw material used for the production of chitosan, which has been promoted as a supplement for reducing body weight, hypercholesterolemia, and hypertension. Chitin and chitosan have a wide variety of actual and potential uses ranging from loudspeaker membranes to cholesterol lowering applications (Maezaki et al., 1993; Nicol, 1991). Krill have been promoted as a possible source of chitin. The chitin composition of whole Antarctic krill was reported to be between 2.4 and 2.7% of their dry weight (Nicol and Hosie, 1993).

1.5.2 Krill enzymes

Antarctic krill contain very effective hydrolytic enzymes including proteases, carbohydrases, nucleases and phospholipases. There is growing interest in the medical applications of krill enzymes, which have been studied for use in treating ulcers and promoting wound healing due to their effective debridement of necrotic tissue (Westerhof et al., 1990). Furthermore, krill's powerful hydrolytic enzymes have an interesting potential for pharmaceutical uses, Melrose et al. (1995) indicated that krill enzymes may be used as a chemonucleolytic agent to reduce the height of vertebral disks. Thus, should a market develop for high value products such as krill enzymes, this may stimulate the krill fishery with the production of food items for human consumption and medical applications or aquaculture feed as secondary products.

1.6. Supercritical fluids (SFs)

Supercritical fluid (SF) is any substance above its critical temperature and critical pressure (Fig.1.5). It shows unique properties that are different from those of either gases or liquids under standard conditions. A supercritical fluid has both the gaseous property of being able to penetrate solids, and the liquid property of being able to dissolve materials into their components. The critical properties of the main solvents are presented in Table 1.3.

In general, lipids are extracted by organic solvents. Several methods have been

reported for extracting fish oils, with varying yields. In most cases, hazardous organic solvents are often employed in large quantities. Extraction and isolation of the lipids by conventional methods, such as hexane extraction, vacuum distillation, urea complexation or conventional crystallisation have the disadvantages of requiring high-temperature processing that results in decomposition or degradation of the thermally labile compounds or employing toxic solvents that have adverse health effects (Hultin, 1994; Sahena et al., 2010).

Currently, and due to the increasing awareness of environmental problems, the use of SF extraction for the removal of organic compounds from different liquid and solid matrices has attracted much attention. This technique has some advantages over more conventional separation techniques, largely due to the unique physical properties of SFs. SF extraction using CO_2 has been gaining ground today as an effective extraction method in the food and pharmaceutical industries, mostly due to the less health and environmental hazards involved. The following are the major areas in which SC-CO_2 can be employed: Extraction and refining of edible oils and fats, extraction of animal fats, removal of Cholesterol from oils and food, decaffeination, extraction of hops and spices (Krukonis, 1988; Saldaña et al., 1999).

1.6.1. History of supercritical fluids

The discovery of what we now call the critical point came about with Cagniard de la Tour's experiments with Papin's digester. In 1822, Baron Charles Cagniard de la Tour showed experimentally that there is a critical temperature above which a single substance can only exist as a fluid instead of either being a gas or a liquid. A liquid placed in a sealed container is in equilibrium with its vapor. When the liquid is heated and compressed the density of the vapor increases (Cagniard, 1822). Above a certain value of the temperature and pressure, which is called the critical point, the density of the vapor becomes equal to the density of the liquid and the interface between liquid and vapor disappears. In 1879, Hannay and Hogarth were the first workers who established the solvating power of supercritical fluids for solids. A few years later, Eduard Buchner (1906) became the first in a long line of researchers to measure the solubility of a model compound, naphthalene, in supercritical carbon dioxide. Also, he investigated the

solubility of certain nonvolatile organic materials in CO_2 under supercritical conditions (Buchner, 1906). In 1936 Wilson et al. (1936) devised a propane deasphalting process for refining lubricating oils. Few years later, a process was developed for the purification and separation of vegetable and fish oils. This process concentrated the polyunsaturated triglycerides in vegetable oils and vitamin-A from fish oils using propane as a selective solvent.

An important progress in SF extraction had been done by Zosel's in seventy decade who provided incentive for extensive future works (Stahl et al., 1988). He used SC-CO_2 for the decaffeination of green coffee. That decaffeination was accomplished by soaking the beans in water and then immersing them in SC-CO_2. Since 1980, there has been a fast development of SFE in foods and pharmaceuticals area e.g. the extraction of hopes (Laws et al., 1980), cholesterol from butter (Krukonis, 1988), perfumes and flavors from natural products (Coenan et al., 1983), residual solvents and monomers from polymers (Krukonis, 1985), unsaturated fatty acids from fish oils etc.

Since 1990, the joint association for the advancement of SF technology was established in the United States to develop and disseminate knowledge concerning the application of SFs for cleaning purposes (Taylor, 1996). Now a day, the application of SFs has been extended to the field of environment, energy, chemistry etc.

1.6.2. Properties of supercritical fluids

Due to its unique and advantageous properties, SF has been chosen for several engineering applications. These properties can be explained by considering the density, diffusivity and viscosity, and solvating strength which mainly depend on temperature and pressure. SF is capable to change density upon minor changes in temperature or pressure that makes it suitable for extraction. The physical stage of a substance can be described by a phase diagram of temperature and pressure as shown in Fig.1.5. In pressure-temperature phase diagram, there are three lines - the sublimation, melting and boiling process defining the region corresponding to the gas, liquid and solid states. The vapour pressure starts in triple point and ends at the critical point. The critical region arises at critical point. In critical region, there is only one phase and it possesses some of the properties of both gas and liquid (Taylor, 1996).

On the other hand, solvating strength depends on the density of the fluids. High density causes higher efficiency. Alternatively, density depends on temperature and pressure. At low pressure the solvent power of SFs decreases with rising temperature, while at high pressures solvating strength increases with increasing temperature. If the parameter 'pressure' is replaced by the parameter 'density', the solubility-temperature relationship becomes much simpler. It happens because density decreases dramatically with an increase in temperature at low pressure; whereas at higher pressure, changes in temperature have less effect on density. Therefore, density is the first consideration regarding solvating power of SFs (Brogle, 1982).

SFs demonstrate physicochemical properties intermediate between those of a liquid and a gas. At high pressure, SFs show liquid like density which affords good solvating power and mass transfer relative to liquid is rapid. Because its diffusivity is higher than liquid and its viscosity is 10-100 times lower than liquid, SF is able to diffuse through solids like a gas, and dissolve materials like a liquid (Taylor, 1996).

As it is shown in table 1.4, at high pressure SF has good density, high diffusivity and low viscosity. Thus, SF shows high solvating strength. This is the main advantages of SF in which their physical properties are similar to those of both liquids and gases. In addition, the combination of low viscosities and high diffusion coefficients found in SF is a major advantage because low viscosity leads to good infiltration of the extraction material, a small pressure drop, good mass transfer, and improved phase separation.

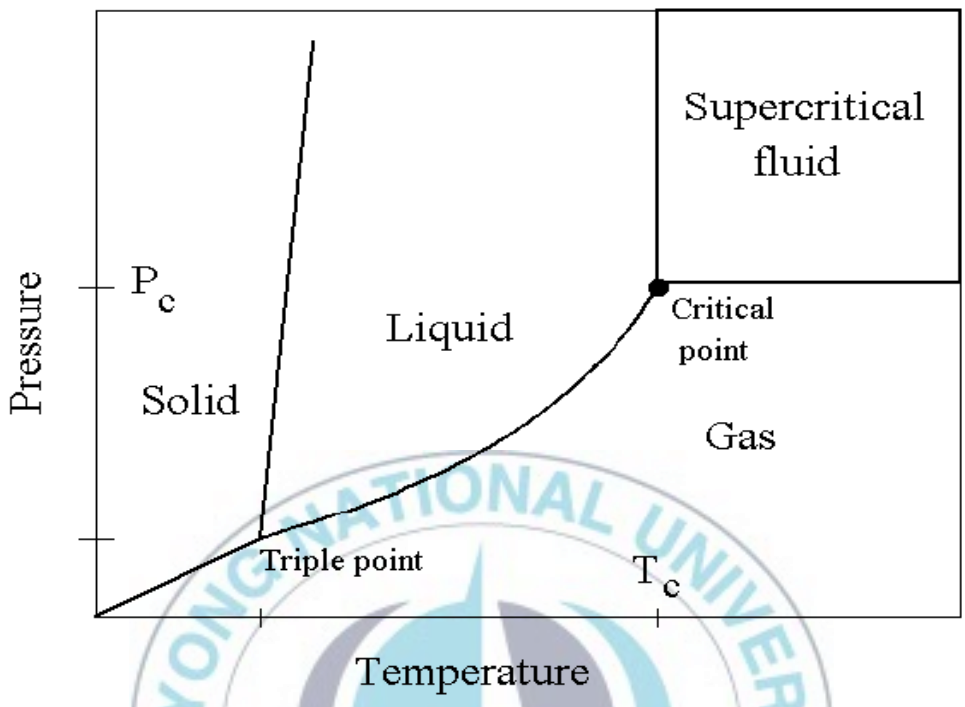

Fig. 1.5: Pressure-temperature phase diagram of a substance with critical temperature (T_C) and pressure (P_C)

Table 1.3: Critical properties of various solvents (Reid et al., 1987)

Solvent	Molecular weight (g/mol)	Critical temperature (K)	Critical pressure (MPa)	Critical density (g/cm^3)
Carbon dioxide	44.01	304.1	7.38	0.469
Water	18.02	647.3	22.12	0.348
Methane	16.04	190.4	4.60	0.162
Ethane	30.07	305.3	4.87	0.203
Propane	44.09	369.8	4.25	0.217
Ethylene	28.05	282.4	5.04	0.215
Propylene	42.08	364.9	4.60	0.232
Methanol	32.04	512.6	8.09	0.272
Ethanol	46.07	513.9	6.14	0.276
Acetone	58.08	508.1	4.70	0.278

Table 1.4: Physical properties of gases, compressed gases and liquids (Stahl et al., 1988)

	Density (g/mL)	Dynamic viscosity (g/cm.sec)	Diffusion coefficient (cm^2/sec)
Gas (ambient)	0.0006-0.002	0.0001-0.003	0.1-0.4
Supercritical fluid (T_c, P_c)	0.2-0.5	0.0001-0.0003	0.0007
Liquid (ambient)	0.6-1.6	0.002-0.03	0.000002-0.00002

1.6.3. Supercritical carbon dioxide (SC-CO_2) extraction

Carbone dioxide is the most frequently used as a SF for extraction of natural substances both from plants and animals. A SF separation process using CO_2 as a solvent offers potential advantages because it is non-flammable, non-toxic, inert to most materials, inexpensive, and can be used under mild operational conditions (Ge et al., 2002). The process consists basically of two steps: the extraction itself, and the separation between extracted components and the solvent. SC-CO_2 has excellent extractive properties such as high compressibility, liquid-like density, low viscosity, high diffusivity (Lim et al., 2002). It has also a greater ability to diffuse through the ultra fine complex matrix than conventional organic solvents and can be easily separated from the products by depressurizing process. Furthermore, low critical temperature of carbon dioxide means that the SC-CO_2 system could be operated at moderate temperature, preventing the degradation of the substance due to heat induction (Krichnavaruk et al., 2008; Lopez et al., 2004; Vasapollo et al., 2004; Machmudah et al., 2006). Therefore, SC-CO_2 is used as an alternative green solvent for various reactions, extractions, and separation processes.

Fig. 1.6 shows the CO_2 density as a function of temperature and pressure. Near to the critical point, a slight change in the operational conditions (pressure and temperature) may cause a severe variation in its density, affecting consequently the solubility of the solute in the supercritical phase (Brunner, 1994).

1.6.4. Comparison between SC-CO_2 and organic solvent extraction

Solvent extraction is a common method of lipid extraction. The advantages of SFE over other conventional processes such as extraction by solvents and separation by distillation are automation, the reduction in operational steps, safe operation due to the use of nonorganic solvents and the use of moderate temperature in the critical range favorable for heat labile foods (Raventós et al., 2002). The most important advantage of SFE is the exceptional quality of the resulting product. Table 1.5 shows the comparison between SFE and organic solvent extraction. Several investigations have been done in order to compare SFE with conventional extraction methods. SFE yields were similar to those from a hot hexane extraction (Ikushima et al., 1986). Myer et al. (1992) reported that SC-CO_2 process obtained recoveries from 97% to 100% of a Soxhlet extraction in

potato chips and puff-dried products. Similar data were obtained for seeds and seed meals (Taylor et al., 1993), and several other food products (Hopper et al., 1995). In fact, the main weaknesses are the cost of supercritical extraction equipment, incomplete lipid extractions under some conditions, and the extraction of nonfat material such as water (Dunford et al., 1997). Therefore, further development is needed since each biological system is distinctive. Conditions must be examined and improved in order to optimize yields for each kind of sample.

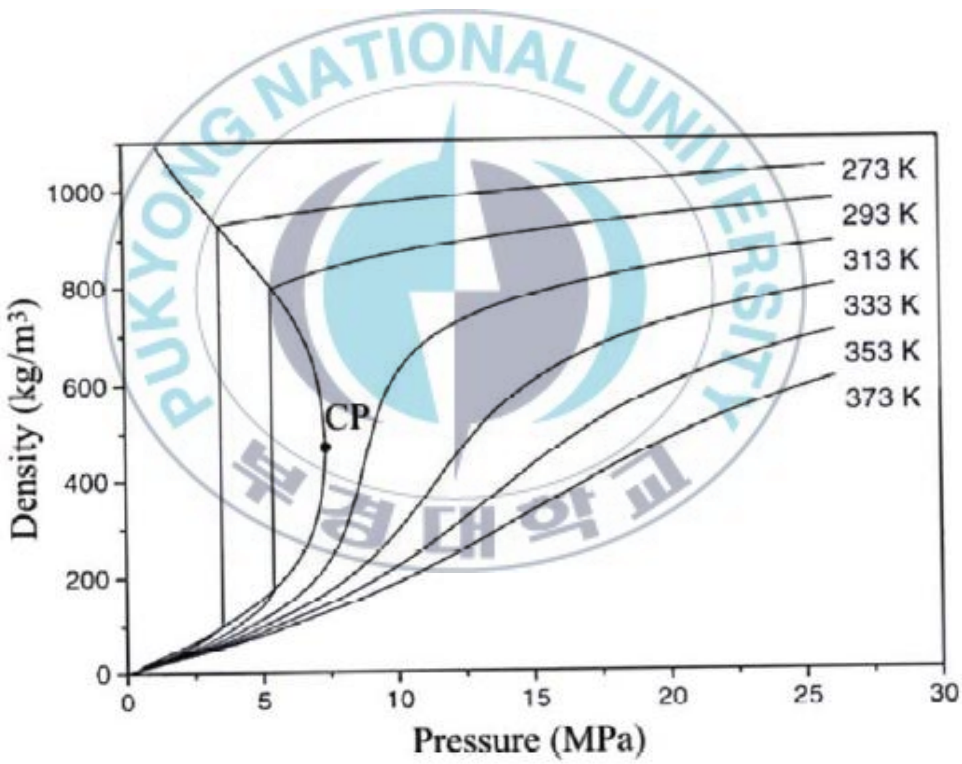

Fig. 1.6: Density of pure CO_2 at different pressures and temperatures

Table 1.5: Comparison between SC-CO_2 and organic solvent extraction (Sahena et al., 2009).

Solvent extraction	Supercritical extraction
• Solvent presence is unavoidable. The residual level of the solvent depends on the type of solvent used	• Is totally free of solvents and hence very pure
• Heavy metal content is also unavoidable and depends on the solvent, the method of solvent recycling, the source of the raw material, and the material used to construct the contact parts of the apparatus	• Totally free of heavy metals since they are not extractable even if they are present in the raw material. No heavy metals are present in CO_2 or the equipment
• Inorganic salt content cannot be avoided	• Totally free of inorganic
• Polar substances get dissolved along with the lipophilic substances from the raw material due to poor selectivity of the solvent. During solvent removal operations, these polar substances form polymers, which lead to discoloration of the extract and poor flow characteristics. All this causes the extract to look different from the basic components in the raw material and hence it is more of a ‘‘pseudo” natural extract	• No such possibility exists since CO_2 is highly selective and no chance of polar substances forming polymers exists. In addition the operating temperature is only 40–80 °C
• Both polar and non-polar colours are extracted	• Only non-polar colours can be extracted
• Solvent removal requires extra unit operations resulting in higher cost and lower recovery of useful material	• No extra unit operations needed and yield of useful material is very high

1.7. Objectives of the thesis

The specific objectives of this study were to extract oil from krill using SC-CO_2 and the use of extracted krill residues for recovering other bioactive and useful materials. Therefore, in order to obtain optimum yield of extracted oil and to recover the maximum useful materials having good quality, the following tasks as outlined below have been carried out:

- Extraction and characterization of krill oil using SC-CO_2
- Identification of bioactive and useful materials
- Isolation and characterization of krill phospholipids
- Characterization of digestive enzymes for applicability of SC-CO_2 in the field of enzyme purification systems
- Investigation possibilities of useful material production by sub-critical water hydrolysis
- Comparative study of the results obtained by SC-CO_2 extraction with those obtained by conventional organic solvent extraction

The detailed experimental procedures and the findings of the above mentioned tasks are discussed in the subsequent chapters.

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Chapter 2

Characterization of oil including astaxanthin extracted from krill (*E. superba*) using SC-CO_2 and organic solvent as a comparative method*

Abstract

This study was to investigate the efficient method for the extraction of krill oil including astaxanthin. Krill oil was extracted using SC-CO_2 and an organic solvent, hexane. The effects of different parameters on the SC-CO_2 extraction for oil, such as pressure (15 to 25 MPa), temperature (35 to 45°C), and extraction time, were investigated. The flow rate of CO_2 (22 $gmin^{-1}$) was constant entire the extraction period of 2.5 hrs. The maximum oil yield was found at higher extraction temperature and pressure. The extracted oil was analyzed by GC for fatty acids compositions. The oil obtained by SC-CO_2 extraction contained high percentage of polyunsaturated fatty acids particularly EPA and DHA. The acidity of krill oil obtained by SC-CO_2 extraction was lower than that of the oil obtained by soxhlet extraction with hexane. The peroxide value was also high in hexane extracted oil. The SC-CO_2 extracted oil showed more stability than the oil obtained by hexane extraction. These results indicate that SC-CO_2 is effective in obtaining nonpolar lipids from the krill by only one-step extraction. The amount of astaxanthin in krill oil was determined by HPLC and compared at different extraction conditions. In SC-CO_2 extraction, the maximum yield of astaxanthin was found in krill oil extracted at 25 MPa and 45ºC. On the other hand, almost 40 % of the fluorine content was removed from the initial concentrations during the SC-CO_2 extraction.

Keywords: Supercritical carbon dioxide extraction; organic solvent; krill oil; astaxanthin

* This work has been presented in 9th International Symposium on Supercritical Fluids (2009), Arcachon, France and it has been submitted to Fisheries Sciences Journal.

2.6. Introduction

Krill is considered by many scientists to be the largest biomass in the world. The vast majority is harvested for feed for fish farms; a small percentage is harvested for human consumption. Lipids of the Antarctic krill, *Euphausia superba* like in aquatic organisms are generally rich in highly unsaturated fatty acids (UFAs) and phospholipids, which have been attracted much attention for health benefits (Yamaguchi et al., 1986). There is commercial interest in obtaining PUFAs. Recently, attention has focused on n-3 PUFAs, especially EPA (C 20:5 n-3) and DHA (C 20:6 n-3), due to their association with the prevention and treatment of several diseases (Shahar et al., 1994; Von Schacky et al., 1999). Studies show that a diet rich in omega-3 fatty acids may help lower triglycerides and increase HDL cholesterol (the good cholesterol). Omega-3 fatty acids may also act as an anticoagulant to prevent blood from clotting. Several other studies also suggest that these fatty acids may help lower high blood pressure (Correa et al., 2008).

Carotenoid is a generic name used to designate the most common groups of naturally occurring pigments found in the animal and plant kingdoms. Carotenoids are considered suitable as components of various types of products due to their high antioxidant activity, e.g. cancer prevention agents, potential life extenders, inhibiting agents for heart attack and coronary artery disease (Li and Chen, 2001; Lopez et al., 2004; Sun and Temelli, 2006). In addition to Omega-3 fatty acids, Krill oil consists of three components: omega-3 fatty acids similar to those of fish oil, omega-3 fatty acids attached to phospholipids, mainly phosphatidylcholine (alternatively referred to as marine lecithin) and natural fat soluble antioxidant, astaxanthin which is one of the most effective carotenoids whose antioxidant activity is 10 times stronger than those of any other carotenoids such as zeaxanthin, lutein, canthaxanthin and β-carotene and was up to 500 times stronger than vitamin E (Shimidzu et al., 1996).

Conventional methods based on solvent extraction from natural matrices are time consuming as they involve a multiple extraction steps and require large amounts of organic solvents, which are often expensive and potentially hazardous (AOAC, 1992;SOP, 1992). The problems associated with traditional solvent extraction techniques have aroused growing interest in developing simpler, faster, more efficient methods for the extraction of carotenoids from foods and natural products (Illés et al., 1999; Careri et

al., 2001). Decomposition or degradation of thermolabile compounds cannot be avoided in a conventional extraction method, since relatively high temperatures are required for these processes. Organic solvents are also harmful to human health as well as the environment.

In recent years, SFE has proved one of the most appealing techniques for solid sample treatment. In fact, supercritical fluids diffuse more readily into matrices than to ordinary liquids, thereby improving the extraction yields of analytes from complex matrices. The SFE technique is a desirable alternative to the solvent extraction of some classes of natural substances from foods (Sun and Temelli, 2006; Mendes et al., 2003). Supercritical fluids possess excellent extractive properties such as high compressibility, liquid-like density, low viscosity, high diffusivity (Lim et al., 2002). One advantage of SC-CO_2 relative to traditional organic solvents is that it can be used at a moderate temperature; this allows carotenoid losses through heat-induced degradation to be reduced. In addition, because it avoids the use of organic solvents, the extracted compounds can be employed as nutritional additives and in pharmacological applications (Lopez et al., 2004). The application of the extraction technique with SC-CO_2 has been widely used in many industrial applications, i.e. decaffeination of coffee, the extraction of hops and carotenoids, the synthesis of polymers, the purification and the formation of nano particles (Lim et al., 2002; Sahena et al., 2010; Kopcak and Mohamed, 2005, Machmudah et al., 2006). Yamaguchi et al., 1986 reported that when applied SC-CO_2 extraction to krill samples, the extracted oils were composed solely of nonpolar lipids without contamination by phospholipids and their deteriorated lipids. Previous studies also investigated SC-CO_2 extraction of oils rich in PUFAs introducing this technology, as a clean technology, with negligible environmental effect, (Rizvi et al., 1988; Temelli et al., 1995; Eisenbach, 1984). In the other hand, a limitation of SC-CO_2 is that it often fails in quantitative extraction of polar analytes from solid matrices, because of its poor solvating power and the insufficient interaction between SC-CO_2 and the matrix (Pawliszyn, 1993). Polar co-solvent such as ethanol is often used to enhance the solute solubility in SC-CO_2 by interacting with the solute, and thus improving the extraction efficiency. SC-CO_2 can extract majority of astaxanthin without ethanol from matrices containing high lipid; since astaxanthin molecule is lipid soluble and considered

containing no strong polar moieties (Lopez et al., 2004). In our work, we have avoided the use of the co-solvent to prevent products quality; since heat is required to separate the organic solvent and also, for the measurement of oil stability at different extraction conditions.

Thus, the purpose of this study was to obtain extraction data of krill using SC-CO_2 determined at various conditions (from 35 to 45°C and from 15 to 25 MPa). At the optimal condition, analyses of extracts were conducted. Extraction yield of extracts (fatty acids, astaxanthin) at different conditions was compared with traditional organic solvents. Also, the stability of oil obtained by SC-CO_2 extraction was also compared to the oil obtained by soxhlet extraction at hexane.

2.7. Materials and Methods

2.7.1. Materials

The krill (*Euphausia superba)* were collected from Dongwon F & B Co., S. Korea. The krill blocks were stored at -80°C for no longer than 1 year before being used experimentally. The carbon dioxide (99.99% pure) was supplied by KOSEM, Korea. All other chemicals used in different analysis were of analytical or HPLC grade.

2.7.2. Sample preparation

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 ㎛) by mesh. The dried samples called freeze dried raw krill were then stored at -80°C until using for SC-CO_2 and organic solvent extraction.

2.7.3. SC-CO_2 extraction

The extraction scale of SFE process used in this work is shown in Fig. 2.1. It consisted of a pump (ILSHIN Metering, Korea) with a maximum capacity of about 30 MPa , a water bath (DW-15 S, Dongwon Scientific system), a chiller (DW-N30L, Dongwon Scientific system, Korea), an extraction cell and a wet gas meter (WNK-1A,

Sinagawa Corp., Tokyo). Thirty five grams of freeze dried krill sample were applied in 200 mL stainless steel extraction vessel containing a thin layer of cotton at the bottom. Before plugging with cap another layer of cotton was used at the top of the sample. CO_2 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 flow rates of CO_2 were kept constant at 22 g/min for all extraction conditions. Cyclone separating vessel was used for the collection of the oil extracted by SC-CO_2. The amount of extract obtained at regular intervals of time was established by weight (g) using a balance with a precision of ±0.001 g. The extracted oil and krill residues were then stored at -80°C until further analysis and used. Krill extracted oil and residues obtained by SC-CO_2 extraction are shown in Fig. 2.2.

The effects of temperature and pressure on oil extraction from krill were performed at 35-45°C and 15-25 MPa at a constant extraction time of 2.5 hrs. Marine oil is rather involatile and thermally sensitive (Singh, 2004). Therefore, and in order to prevent the activity of bioactive compounds in both of extracts and extracted residues; the extractions were performed at low temperature.

2.7.4. Soxhlet extraction by hexane

In order to compare the extraction strength of SC-CO_2 with conventional organic solvent extraction, soxhlet extraction was selected. The extraction was carried out in a soxhlet apparatus using hexane as solvent. An amount of three gram of freeze dried raw krill sample was placed into the extraction thimble (28 x 100 mm, Advantec, Tokyo, Japan) and the extraction was run 12 hrs until the colour of the condensed solvent at the top of the apparatus was clear. The sample was then dried in the oven at 80 ± 1°C for 2 hrs after which it was cooled in desiccators before reweighing. After that, the extracted oil was stored at -20°C until further analysis.

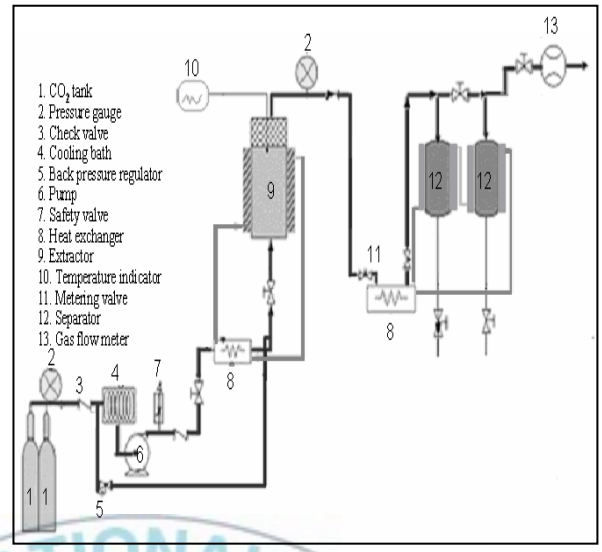

Fig. 2.1: The schematic diagram process of supercritical carbon dioxide extraction.

A) B) C)

Fig. 2.2: A) Freeze dried krill B) SC-CO_2 extracted oil C) SC-CO_2 extracted krill residues

2.7.5. Determination of extraction yield

The weight of the initial freeze-dried krill prior to the extraction with different extraction processes was recorded. Following extraction, the weight of extracted oil was also recorded. The extraction yield was determined according to the following equation:

$$\text{Oil yield} = \frac{weight\ of\ extracted\ krill\ oil\ (g)}{weight\ of\ freeze-dried\ krill\ subjected\ to\ extraction\ (g)} \times 100$$

2.7.6. Gas Chromatography analysis for fatty acid compositions

The fatty acid profiles of both krill oil obtained by SC-CO_2 and organic solvent, hexane extraction were analyzed by gas chromatography using a Hewlett Packard gas chromatograph (5890 Series II GC system). The fatty acid methyl esters were prepared according to AOCS official method Ce 2-66 (AOCS, 1998). An Agilent equipped with a flame ionization detector (FID) and DB-Wax capillary column (30 m length x 0.250 mm internal diameter, 0.25 µm of film). Carrier gas of fatty acid methyl esters was nitrogen at a flow rate 1.0 mL/min. The column temperature was raised from a constant temperature of 130ºC for 3 min, and then increased to 240ºC at a rate of 4ºC/min and hold at 240ºC for 10 min. The split ratio was fixed at 50:1. Both injector and detector temperatures were 250ºC. Fatty acid methyl esters were identified by comparison of retention time with standard fatty acid methyl esters mixture (Supleco, USA).

2.7.7. Measurement of oil stability

Fats and oils are prone to oxidation. The rapidity of oxidation depends on the degree of unsaturation, the presence of antioxidants, and prior storage conditions (AOCS, 1997). Several methods are used to determine the stability of oil. In our study, oil stability was measured by evaluating free fatty acid content and peroxide value.

2.7.7.1. Free fatty acid content of extracted oils

Free fatty acids (FFA) of krill oils were determined twice for each sample, and the average values are reported. As described by Bernardez et al. (2005), precisely 50 mg of oil was placed into pyrex tubes with the addition of 3 ml of cyclohexane. Then, 1 ml of cupric acetate-pyridine reagent was added, and tubes were vortexed for 30 sec. After

centrifugation at 2000g for 10 min, the upper layer was read at 710 nm. The measurement of FFA content of oils was based on a calibration curve obtained from oleic acid standard (Fig. 2.3).

Copper reagent was prepared according to Lowry and Tinsley (1976). Briefly, 5% (w/v) aqueous solution of cupric acetate was prepared and, after filtration, the pH of cupric acetate solution was adjusted to 6.1 using pyridine.

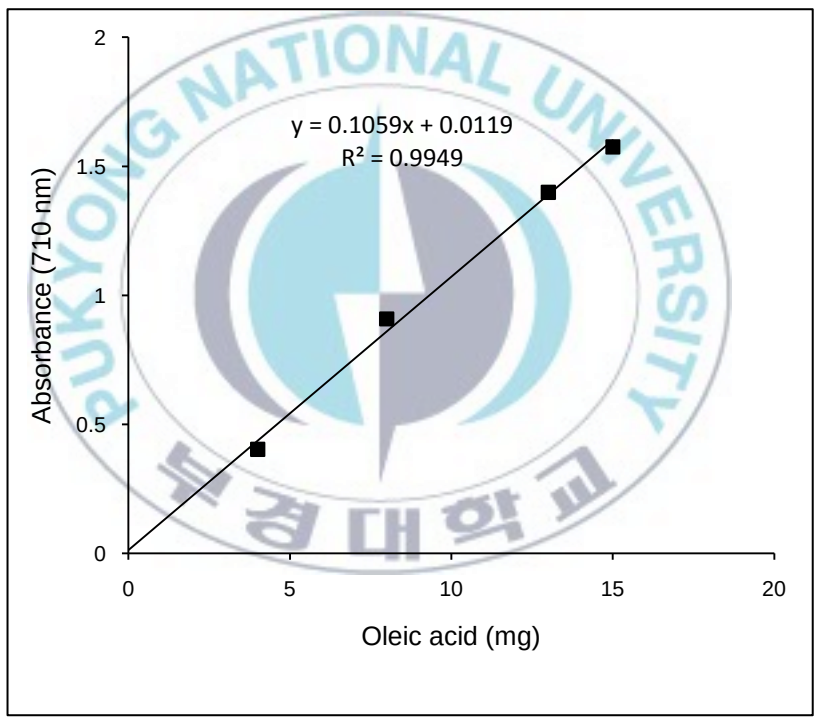

Fig. 2.3: Calibration curve of oleic acid for estimation of FFA content in extracted oils

2.7.7.2. Peroxide value

Peroxide value was determined according to the AOCS method Cd 8-53 (AOCS, 1998) with modified amount of sample taken. 1.0 gm of krill oil was dissolved in 6 mL of acetic acid-chloroform (3:2) solution. Then 0.1 mL of saturated potassium iodine (KI) solution was added to the mixtures and allowed the solution to stand with occasional shaking for 1 min. Distilled water (6 mL) was immediately added to the solution to allow the mixture to stand. The solution was titrated with 0.1N of sodium thiosulfate until the yellow iodine colour had almost disappeared. Then 0.4 mL of starch indicator solution was added with shaking to extract iodine from chloroform layer, and again titrated until the blue colour disappeared. A blank determination was performed with the same procedure. Peroxide value was expressed as milliequivalents peroxide/1000 g sample.

$$\text{Peroxide value} = \frac{(A - B) \times M \times 1000}{W}$$

Where:

A = Volume of titrant, mL of sample

B = Volume of titrant, mL of blank

M = Molarity of sodium thiosulfate solution

W = weight of sample, g

2.7.7.3. Colour

The colour of the extracted oils was measured in triplet by means of reflectance spectra in a spectrophotometer (Lovibond, USA). For measurements, samples were placed in a white cup and covered with optical glass. CIE L*a*b* colour coordinates (considering standard illuminant D65 and observer 10°) were then calculated. Colour changes were measured by the lightness (L*) and the coordinates greenness – redness (a*) and blueness – yellowness (b*).

2.7.8. Astaxanthin analysis by High Pressure Liquid Chromatography

A Waters model 600E system controller (Milford, USA) High Pressure Liquid Chromatography (HPLC) equipped with a model 484 UV/VIS detector and an Eclipse Plus C18 column (5µm, 4.6 x 250 mm, Agilent, USA) was used for astaxanthin analysis. Elution was carried out using a mobile isocratic phase prepared with 10% dichloromethane, 85% ethanol and 5% acetonitrile. Flow rate was 1 mL per minute (Krichnavaruk et al., 2008). Astaxanthin was detected at the wavelength of 470 nm. The amount of astaxanthin in the extract was measured based on the peak area of the standard astaxanthin. For astaxanthin content determination, an external standard (Sigma chemical Co., St. Louis, MO, USA) was used. The standard was dissolved in a mixture to prepare further dilutions to build the calibration curve (Fig. 2.5). The extraction efficiency was investigated by the determination of the total amount of astaxanthin in samples using soxhlet extraction with dichloromethane as solvent. The ratio between the amount of astaxanthin in the extracts and the total amount in the samples was defined as the extraction efficiency.

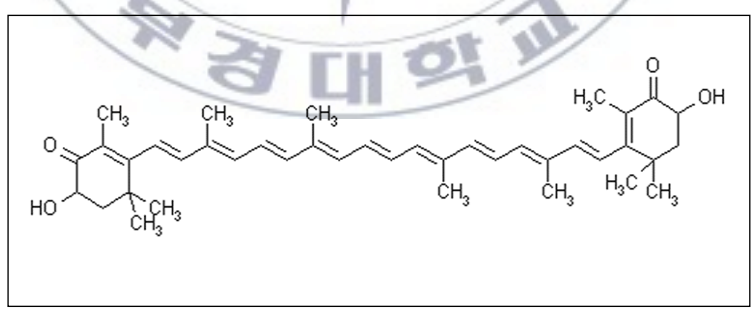

Fig. 2.4: Structure of carotenoid investigated (Astaxanthin).

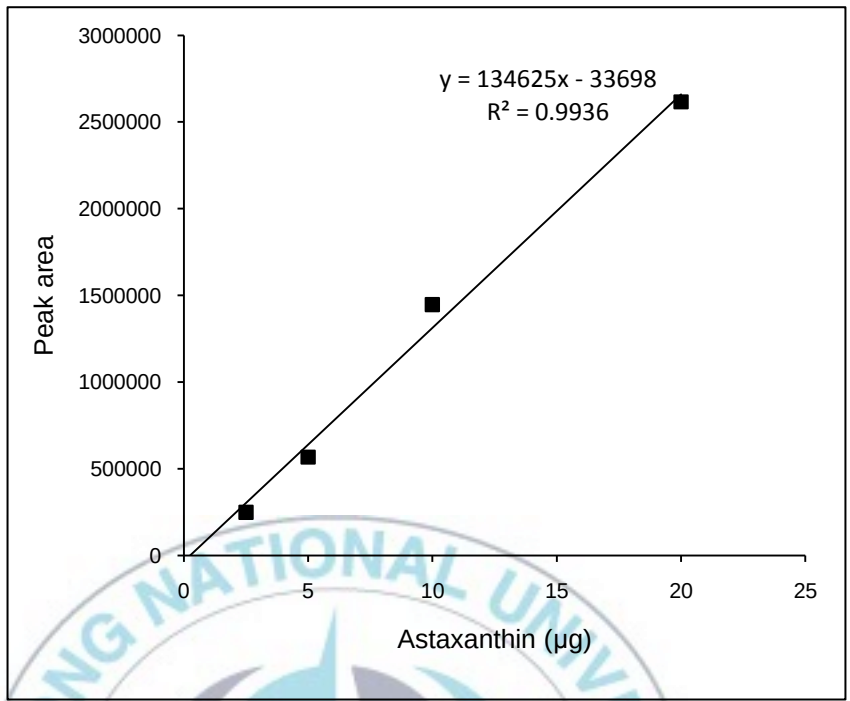

Fig. 2.5: Calibration curve of standard astaxanthin for the estimation of astaxanthin content.

2.7.9. Measurement of fluorine content of the raw and extracted residues

Since Soevik and Braekkan (1979) found high concentrations of fluoride in Antarctic krill, the problems in it using for human nutrition increased. The aim of this investigation was to study the effect of SC-CO_2 extraction on the fluorine content of krill. To achieve this objective, raw krill and extracted krill residues by SC-CO_2 and hexane extraction were prepared for fluorine content measurement. The total fluorine was determined by combusting a weighed krill samples in an oxygen bomb with a dilute base absorbing the fluorine vapors. The bomb was rinsed into a beaker with water and following the distillation, the fluorine was determined by the photometric method using lanthanum-alizarin complex.

2.7.9.1. Procedure of bomb Combustion

The bomb combustion was used as described in ASTM Standards (1996). Approximately 1 g ± 0.1 mg of sample was held in a cup in which to be combusted. The ignition wire (100 mm, nickel-chromium) was attached to the electrodes to start the combustion reaction. 5 mL of 1 *N* NaOH solution was transferred into the combustion bomb. After the sample and the wire have been properly placed in the bomb, it was charged with oxygen gas from a commercial cylinder to the pressure of about 25 to 30 atm. The oxygen was slowly admitted into the bomb so as not to remove or displace any powdered material from the sample holder. Then, the bomb was placed in a cooling bath with water flowing before the ignition wire to be attached from the firing circuits to ignite the sample. In order to allow the cooling and the absorption of soluble vapors, the bomb was remained in the cooling water for 15 min. The bomb was removed and the pressure was released at a uniform rate. The bomb interior, electrodes and the cub were thoroughly rinsed into a 100-mL beaker with several small washings of water keeping the volume below 50 mL. The collected solution, approximately 50 mL will be used for fluorine analysis.

2.7.9.2. The photometric method

As described by Greenhalgh and Riley (1961), the photometric method is based on the colourimetric reaction between fluoride and lanthanum-alizarin reagent. Briefly, the distillation was conducted using heating flask containing 400 mL distilled water and 200 ml sulfuric acid. The collected solution of samples (10 mL) was added to the boiling liquid mixture at 180°C. Following the collection of 100 mL of the distillate, the lanthanum-alizarin reagent was added and well mixed to form a stable complex with fluoride. After 60 minutes, the optical density was read at 622 nm. The measurement of fluorine content of samples was based on a calibration curve obtained from identically-prepared standard for the range of 0 to 0.04 mg (Fig. 2.6).

2.7.10. Statistical analysis

All extractions and analysis of samples from each extraction were carried out in duplicate in randomized order and means were reported. Data were evaluated by Duncan's multiple range test using SAS 9.1 (SAS institute Inc., Cary, NC, USA) to evaluate differences in mean values. The least significant difference at the 95% confident level was calculated for each parameter.

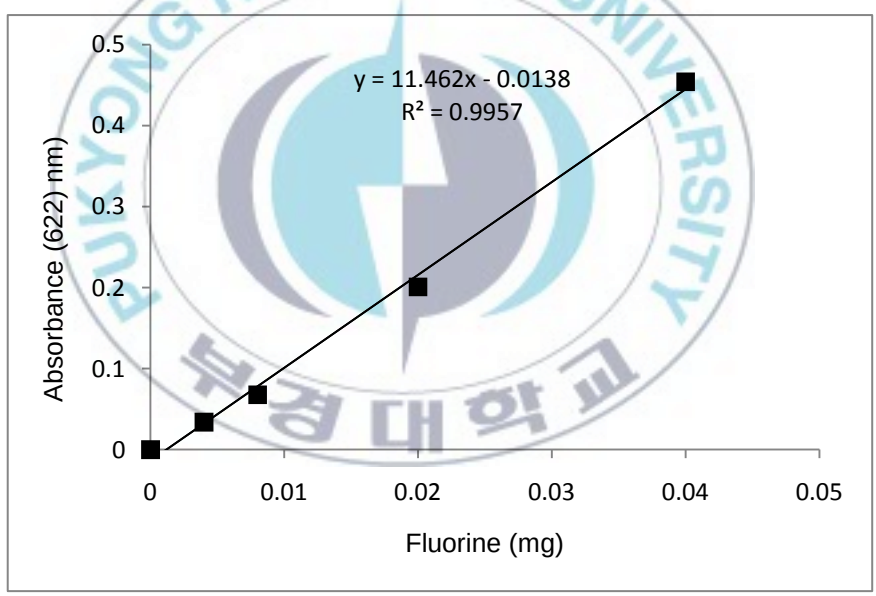

Fig. 2.6: Calibration curve of standard fluorine for the estimation of fluorine content.

2.8. Results and Discussion

2.8.1. SC-CO_2 extraction

The extracted oil from krill was fluid and bright red due to the presence of astaxanthin in it (Yamaguchi et al., 1986). Fig. 2.7A-C presents the SC-CO_2 extraction curves of krill oils at different temperatures (35, 40 and 45°C) and pressure (15, 20 and 25 MPa). The extraction yields ranged from 4.1 to 12.2% depending on the experimental conditions. The highest yield obtained was 4.27± 0.08 g/35 g of krill at temperature, 45°C and pressure, 25 MPa. Ooi et al. (1996) mentioned that SC-CO_2 pressure affects both yield and solubility of palm oil. They showed that at higher pressures, the yield increased with temperature from 323.2 to 338.2 K and they attributed this to the solubility of palm oil increasing with increasing temperature, even though increasing temperature causes the density of SC-CO_2 to decrease. In this work, an increase in temperature lead to an increase in the total yield of SC-CO_2 extracted krill oil at a given mass flow rate and pressure. The applied pressure and temperature variation greatly affected the oil solvating power of SC-CO_2 and hence the amount of yield. The yield of extracted oil was increased with the increasing of CO_2 mass, depending on the pressure and temperature. The extracted oil per solvent (CO_2) mass used was increased constantly over the entire extraction time, until nearly all oils was extracted. The change in the slope of the extraction curve (45°C and 25 MPa) shows that SC-CO_2 extracted almost all extractable oil. The lower yields at low pressure and low temperature are attributable to the fact that krill residues contained still oil. At constant temperature, the density of the SC-CO_2 was increased with the pressure and hence the solvating power; that is, the amount of oil extracted was increased. The effect of pressure can be attributed to the increase in solvent power and by the rise of intermolecular physical interactions (Morita and Kajimoto, 1990; Bai et al., 1997; Bulgarevicg et al., 2002). Similar trends have been reported by De Azevedo et al., 2008 and Park et al., 2008 in the extraction of oil from green coffee and boiled anchovy respectively.

Compared to other experimental conditions the amount of oil extracted was highest at 45°C. Regardless of the decreasing of density of the solvent, the oil extraction yield was increased with the temperature which probably attributed to the increase of the oil

components vapour pressure. Yamaguchi et al., 1986 has reported that up to 45℃ the amounts of extracted oils were almost constant in spite of temperature and pressure examined. Thus, the effect of the increase of solute vapour pressure may have dominated over solvent's density.

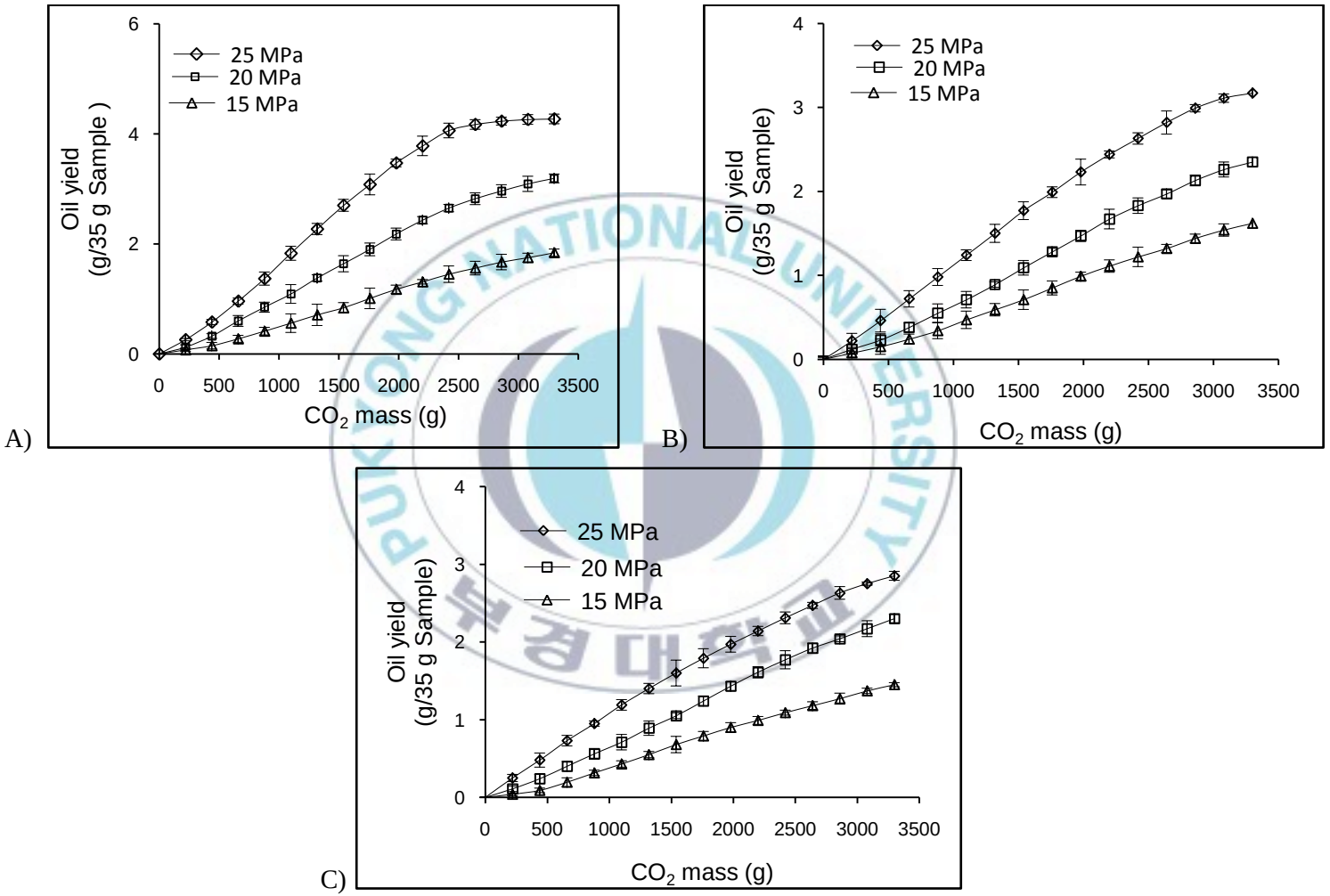

Fig. 2.7A-C: SC-CO_2 of oil from krill at different temperatures. A) 45°C, B) 40°C and C) 35°C

2.8.2. Comparison of oil yield obtained by SC-CO_2 and hexane extraction

The comparison of the yield of the oil obtained by SC-CO_2 and by Soxhlet extraction with hexane is reported in Fig. 2.8. The highest yield obtained in SC-CO_2 extraction was 12.2% from the run conducted at 25 MPa and 45ºC. The oil yield obtained by hexane extraction was 16.12% (w/w in freeze dried sample). By considering that the extraction of oil using hexane as organic solvent was nearly complete, the highest yield by SC-CO_2 extraction was almost 75.6%. Sanchez-Vicente et al., 2009 reported that the maximum yield of peach seed oil obtained by SC-CO_2 was 70%. However, the findings of this work concur with that reported by Yamaguchi et al., 1986. These differences in maximum yield may be occurred due to variation of processing unit used, experimental conditions, sample size, percentage lipid in sample, moisture contain, etc.

2.8.3. Fatty acid compositions

The comparison of the fatty acid profile of the oil obtained by SC-CO_2 in different conditions and by Soxhlet extraction with hexane is reported in Table 2.1. Total of 23 fatty acids were identified in the different extracts analyzed. It is observed that under certain extraction conditions there were slight changes in the fatty acid compositions of the extracted oil. The run at 20 MPa and 40ºC showed the highest percentage (95%) of the total fatty acid. Among saturated fatty acids, palmitic acid (C16:0) was present in the highest concentration ranging from 18.57 to 22.75% of total identified fatty acids. Oleic acid (C18:1) was also found in significant amounts ranging from 18.16 to 20.90%, among the UFAs. EPA (C20:5) in extracts analyzed was present in higher amounts comparing to other PUFAs. The percentage of EPA (C20:5) and DHA (C22:6) in total identified fatty acids were ranged from 9.41 to 11.27 and 3.11 to 4.91, respectively. The composition of PUFAs obtained in krill oil was indistinguishable with that reported by Yamaguchi et al., 1986. Also, it has been shown that marine fish oils such as cod liver oil and anchovy oil contained about 14-31% of EPA and DHA (AOCS, 1997).

On the other hand, results showed that the oil extracted by SC-CO_2 present a higher value of total fatty acids particularly PUFAs, than the oil extracted with hexane. This difference can be explained bearing in mind that the soxhlet extraction with hexane

occurs at a higher temperature than SC-CO_2. Thus, may lead to thermal degradation of fatty acids, mainly UFAs (Rubio-Rodriguez et al., 2008).

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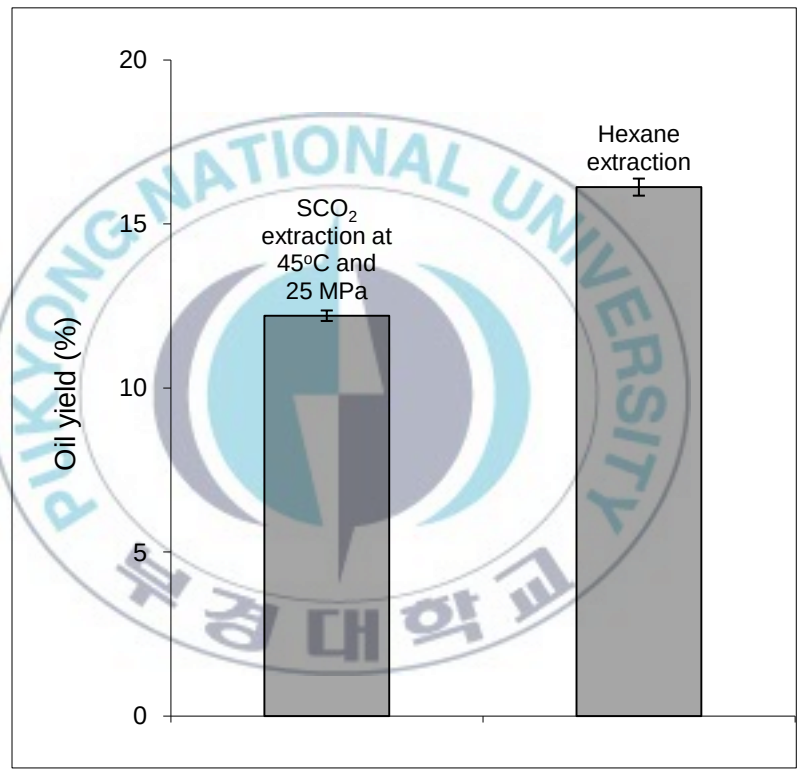


Fig. 2.8: Krill oil yield extracted by SC-CO_2 and hexane extraction.

Table 2.1: Fatty acid profile of krill oil extracted with SC-CO_2 and with hexane as determined by the AOAC method (Fatty acids showed only that were found more than 1% of total fatty acids)

Fatty Acids[a] (%)	**SC-CO_2**									**Hexane**
	25 MPa			20 MPa			15 MPa			
	45ºC	40ºC	35ºC	45ºC	40ºC	35ºC	45ºC	40ºC	35ºC	
C14:0	14.80	15.04	12.86	14.95	15.29	14.89	13.06	15.33	15.42	14.20
C16:0	21.50	22.07	19.78	21.96	22.75	22.13	18.57	21.78	22.24	23.20
C16:1	9.28	9.11	8.23	9.91	10.12	9.33	8.26	9.45	9.98	7.99
C17:1	1.03	1.57	0.92	1.76	2.14	1.06	0.87	1.16	1.97	1.67
C18:0	1.49	1.39	1.22	1.96	2.03	1.95	1.39	1.76	1.66	1.39
C18:1	19.63	19.44	19.18	20.90	20.68	19.84	18.56	19.69	20.64	18.16
C18:2	1.93	1.87	1.14	1.99	1.87	1.95	1.74	1.97	1.88	1.91
C20:0	1.66	1.78	1.57	1.61	1.89	1.67	1.44	1.48	1.66	1.33
C20:1	0.63	0.51	0.48	1.02	0.87	0.59	0.43	0.61	0.74	0.58
C20:2	2.97	2.86	2.56	3.08	2.89	2.48	2.41	2.62	2.81	2.66
C20:5 (EPA)	11.03	10.87	10.13	11.27	10.93	10.27	10.34	10.27	10.76	9.41
C22:6 (DHA)	4.91	3.68	3.11	4.69	3.71	3.59	4.63	3.45	3.52	3.74

[a]Data are the mean value of three replicates. Standard error of the fatty acid constituents were on the order of about ± 2%.

2.8.4. Oil stability

Fats and oils are prone to oxidation. The degree of oxidation depends strongly on the level of unsaturation, the presence of antioxidants and prior storage condition (Kamal-Eldin and Yanishlieva, 2002). High level of PUFAs is found in marine fish oil. Peroxide value and FFA analyses give an idea of how good or bad oil is at a particular time. FFAs are responsible for the acidity of oil. Changes of FFA content are mainly correlated to hydrolytic reactions in the oil. Peroxide value and FFA content of the oil extracts are shown in Table 2.2. Comparing the FFA and peroxide value in several oil extracts, it has been observed that the amount of FFA and peroxide value were significantly high in hexane extracted oil than SC-CO_2 extracted. It was also found that among oils obtained by SC-CO_2 in different condition, oil extracted at higher extraction temperature contained high amount of FFA and peroxide value. This result arranged with the high FFA content and peroxide value in hexane extracted oil due to higher temperature. Rubio-Rodriguez et al., 2008 has reported that higher temperature and storage time caused an important increase of the FFA content in the hake by-products oil. Also, peroxide value is used as a measurement of rancidity of oils which occurs by auto oxidation. Low exposure of oxygen in SC-CO_2 extraction process caused minimal oxidation, especially if CO_2 used is free of O_2 (in this study CO_2 used is 99.99% pure). Thus, the oil obtained by SC-CO_2 extraction showed more stability compared to the oil extracted by hexane.

2.8.5. Colour

The changes in the lightness (L*), redness (a* value) and yellowness (b* value) over different krill oils extracted are shown in Table 2.2. Lightness does not seem to be affected by extraction conditions, slight difference of L* value was recorded between SC-CO_2 extracted oil and oil extracted by hexane. The most significant changes are observed in a* values, which are related with the tonality of colour, changing from redness (a*) to greenness in hexane extracted oil. The characteristic red colour of krill oil is denoted by a high value of b* that shows a slight increase at 45°C and 20 MPa related to other SC-CO_2 conditions.

2.8.6. Extraction yield of astaxanthin

Previous literature stated that the solubility of organic compounds in SC-CO_2 depended basically on the balance between fluid density and solute vapour pressure, which are controlled by fluid temperature (Krichnavaruk et al., 2008). In the other word, SC-CO_2 is suitable for the extraction of thermally labile compounds due to its low critical temperature. However, previous papers indicate that some substances like astaxanthin could be unstable under high pressure of SC-CO_2 at a temperature, for example 80°C that never provoke the decomposition of astaxanthin under atmospheric pressure (Miki et al., 1983). Thus attention should be paid to this fact during the extraction of natural products using SC-CO_2. In this study, the SC-CO_2 extraction of astaxanthin yield obtained at different pressures and temperatures were compared with those obtained with hexane. As shown in Fig. 2.10 the astaxanthin yield increased with pressure at temperatures of 35 and 45ºC. The highest yield of astaxanthin was 8.62 ± 0.31 mg/100g at 25 MPa and 45ºC. But, at the temperature 40ºC the maximum amount was found at the pressure 20 MPa. This result can be explained by the increase in the fluid density and the decrease in diffusion coefficient. Careri et al., 2001 has reported that raising the pressure increases the solvent density which has double effect-an increase in the solvating power of the SCF, which enhances the extraction process and reduced the interaction between the solvent and the matrix resulting from the decrease in diffusion coefficient, as result, the extraction yield decrease. At 35 and 45ºC the increase in solvating power of the fluid was dominant than the decrease in diffusion coefficient between the fluid and matrix. However, at 20 MPa and 40ºC, the leading effect was the decrease in diffusion coefficient.

On the other hand, at the pressure 15 and 25 MPa the astaxanthin yield was the maximum with the temperature 45ºC. Rising the temperature decreases the solvent density, however can increase the solute vapour pressure, which enhances the extraction yield. The same effect of temperature was reported by Lopez et al. (2004) for the extraction of astaxanthin from crustaceans.

For hexane extraction, the amount of astaxanthin obtained was 10.32 ± 0.13 mg/100g. The highest extraction efficiency of astaxanthin obtained by SC-CO_2 was almost 86% of the total amount of astaxanthin estimated by dichloromethane extraction (Fig.2.9). This result agreed with that reported by Thana et al. 2008 (83.05%) from *Haematococcus pluvialis*.

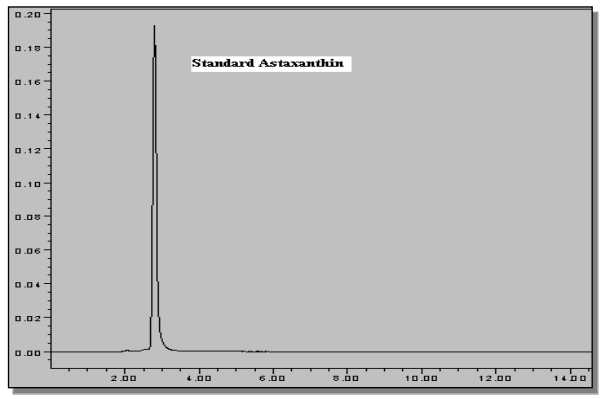
Table 2.2: Free fatty acids, peroxide value and colour of krill oil obtained by SC-CO_2 and hexane extraction

		SC-CO_2 extraction									**Hexane extraction**
		15 MPa			20 MPa			25 MPa			
		35ºC	40ºC	45ºC	35ºC	40ºC	45ºC	35ºC	40ºC	45ºC	
Free fatty acids[a] (g/100 g oil)		2.21 ± 0.08	2.61 ± 0.15	3.02 ± 0.06	2.75 ± 0.12	3.13 ± 0.09	3.21 ± 0.14	3.11 ± 0.13	3.27 ± 0.07	3.94 ± 0.15	6.47 ± 0.17
Peroxide value[a] (milliequivalent/kg)		3.14 ± 0.08	4.03 ± 0.13	4.63 ± 0.14	4.12 ± 0.07	4.64 ± 0.12	5.25 ± 0.15	5.02 ± 0.13	5.41 ± 0.17	6.11 ± 0.21	8.12 ± 0.28
Colour[b]	**L***	24.38	24.41	24.25	24.04	25.51	24.21	24.13	24.79	24.38	23.54
	a*	+9.21	+9.35	+9.42	+9.32	+10.02	+9.17	+9.26	+9.33	+9.12	+0.34
	b*	+3.75	+4.18	+4.21	+4.15	+4.11	+4.27	+4.25	+4.29	+4.35	+1.42

[a]Mean value of three replicates ± Standard Error (S.E.). *Lightness (L*), redness (a*) and yellowness (b*)*

[b]Values in each column are not significantly different ($p < 0.05$).

A)



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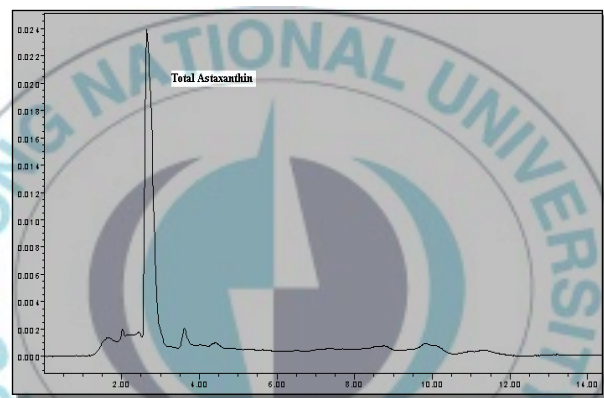

Fig. 2.9: HPLC picks of astaxanthin, standard (A) and extracted from krill (B)

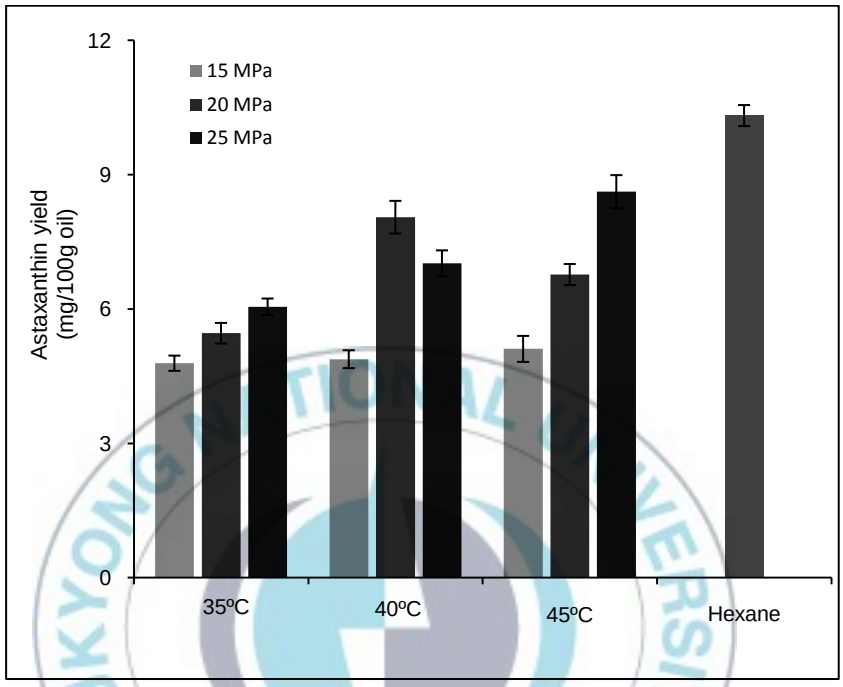

Fig. 2.10: Astaxanthin yield from krill at different SC-CO_2 extraction conditions and hexane extraction. Data are the mean value ± S.E. ($n \leq 3$)

2.8.7. Correlation of astaxanthin solubility using Chrastil model

In order to correlate the solubility of astaxanthin in the SC-CO_2, Chrastil (1982) model was used. The model related the solubility directly to the density of the gas solvent, avoiding the complexity of the equation of state. It is easy to use and it usefulness has been proved in SFE studies. The correlations based on experimental density are very useful to determine the solubility of solids and liquids in compressed fluids, as they are both simple and do not require physicochemical properties of the solute. Fig. 2.11 shows the experimental solubility data. It evidently showed the isotherms and the effects of temperature and solvent density. Wahyu et al., 2008 has reported that solubility of lipids in SC-CO_2 might be correlated directly to the pressure and temperature by the equation of state. The calculation of mole fractions of astaxanthin in solvent were calculated by the equation (1) and the density relationship in Chrastil model is based on the equation (2) which is derived from Antoine:

$$\alpha_{asta} = \frac{n_{asta}}{n_{asta} + n_{CO_2}} \quad (1) \qquad y_{asta} = \rho_{CO_2}^{k} \exp\left(\frac{a}{T} + b\right) \quad (2)$$

Where: α_{asta} is the mole fraction of astaxanthin, n_{asta} is moles of astaxanthin and n_{CO_2} is moles of carbon dioxide. y_{asta} is the solubility of astaxanthin (mol/mol), ρ_{CO_2} is the SC-CO_2 density, T is experimental temperature (K) and a, b and k are empirical fitting parameters (Chrastil constants). The solubility data of fucoxanthin extracted by SC-CO_2 from brown seaweeds was fitted well with Chrastil model at most experimental conditions (Roh et al., 2008). In this work, at a given temperature, nearly a linear relation between solubility of astaxanthin and SC-CO_2 density was obtained. Thus, the solubility data of astaxanthin show it correlation with Chrastil model. Chrastil parameters obtained are shown in Table 2.3.

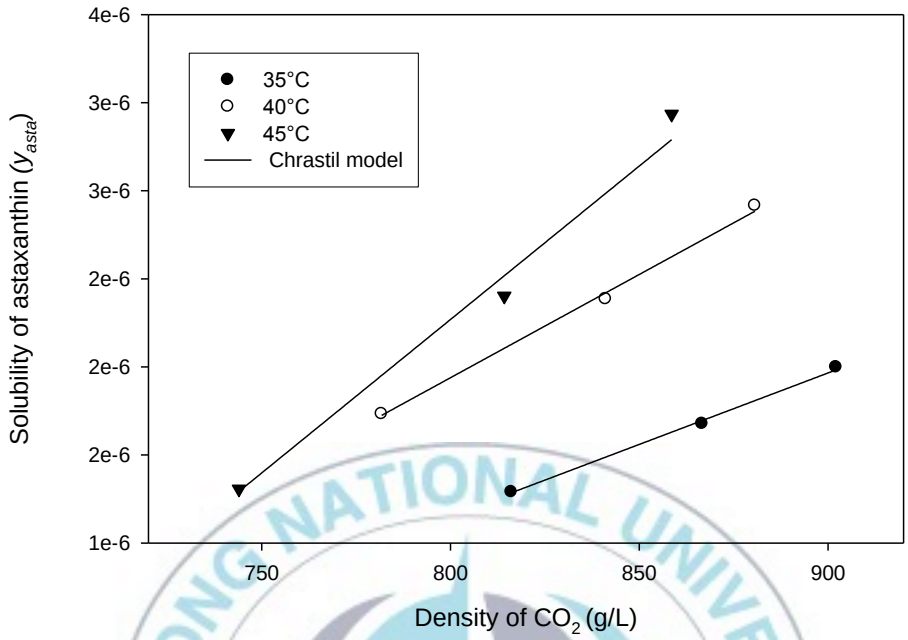

Fig. 2.11: Correlation of astaxanthin solubility experimental data using Chrastil model

Table 2.3**:** Chrastil equation Parameters of astaxanthin mole fraction

Parameters of Chrastil equation	**Temperature (°C)**		
	35	40	45
k	2.74	2.85	2.92
a	1.02	1.04	1.09
b	-21.64	-21.38	-21.73

2.8.8. Fluorine content in krill residues extracted by SC-CO_2

The majority of foods are low in fluorine, but krill is an exception. Fluorine is essential for the mineralization of bone and teeth and in the prevention of dental caries. In addition, the use of high-dose fluorine is being investigated for the avoidance of osteoporosis (Palmer and Wolfe, 2005). The fluorine content of krill is concentrated in the shell, where it may attain concentrations of 350 mg/100g dry weight (Virtue et al., 1995). After catching, the fluorine in krill is capable of migrating from the shell into the muscle tissues. Adelung et al. (1987) has reported the careful removal of the shell before consuming krill and immediate removal of the shell upon harvest to prevent migration of fluorine into the muscle can minimize potential toxicity because over 99% of the fluorine content of krill is associated with the exoskeleton. In this study, the fluorine contents measured in different krill samples are shown in the Fig.2.12. It has found that the amount of fluorine in the different parts of raw krill agreed with that reported in literature. The residues extracted by hexane demonstrated a slight reduce of fluorine amounts. However, the fluorine content in the residues extracted by SC-CO_2 showed that almost 40 % of the fluorine content was removed during the SC-CO_2 extraction. Little information is available in the literature concerning SFE of metal ions. On the other hand, if metal ions are bound to organic ligands, their solubility in SC-CO_2 may be significantly increased. In fact, the amounts of fluorine removed from the initial concentrations after SC-CO_2 extraction can be explained by the role of CO_2 as carrier of fluorine under supercritical conditions. Thus, further experimental works are required to confirm that the amounts removed were carried out by CO_2. Therefore, a special SC-CO_2 extraction design with special adsorption column is needed in order to find out the removed amounts.

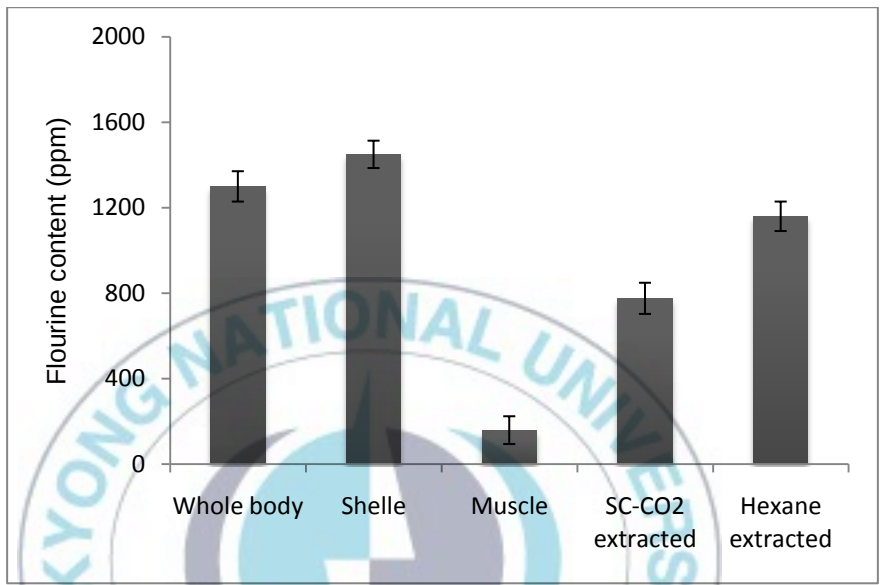

Fig. 2.12: Fluorine content in raw and extracted krill by SC-CO_2 and hexane extraction

2.9. Conclusion

In this study, the extraction of krill oil using SC-CO_2 and soxhlet extraction with hexane was investigated. The optimal extraction conditions chosen, regarding extraction rate, were 25MPa, 45°C and around 22 g CO_2/min. Under those conditions, the extraction yield obtained after 2.5 h was 75.6% comparing with the oil obtained by organic solvent extraction. The lipid compositions of oil showed a high amount of omega-3 PUFA, especially EPA and DHA. The main fatty acids were C14:0, C16:0, C16:1, C18:1 and C20:5 in oils of both obtained by SC-CO_2 extraction and organic solvent extraction. The result showed that the highest amount of astaxanthin was extracted at 25 MPa and 45ºC which represent 86% as extraction efficiency. The solubility of astaxanthin was examined and correlated with Chrastil model satisfactorily. In addition, SC-CO_2 extracted oil showed more stability and better quality than organic solvent extracted oil. Thus, krill oil obtained by SC-CO_2 extraction would be a good source of PUFAs and astaxanthin. On the other hand, the fluorine content in the SC-CO_2 extracted residues demonstrated that almost 40 % of the fluorine content was removed from the initial concentrations during the SC-CO_2 extraction. Further work is still needed to confirm and to improve the investigation of the effect of SC-CO_2 extraction on the fluorine content.

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Chapter 3

Purification and characterization of phospholipids from Krill (*E. superba*) residues deoiled by SC-CO_2*

Abstract

Purification of phospholipids from the Antarctic krill (*Euphausia superba*) using two step extraction processes has been investigated. Using SC-CO_2 extraction with optimal extractions conditions of 45°C, 25 MPa, and CO_2 flow rate of 22 g/min; most of the neutral lipids were extracted. PC, PE and PI were then extracted in a second step conducted with modified existing method using ethanol, hexane and acetone as solvents. The purified phospholipids were characterized by their acid value, peroxide value, and the oxidative stability. The purity of phospholipids ranged between 93 and 97% and was evaluated by HPLC–ELSD.

Keywords: Supercritical carbon dioxide, Krill, Phospholipids purification and characterization

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3.7. Introduction

As with fish, the Antarctic krill is a rich source of the long-chain omega-3 fatty acids EPA and DHA. However unlike fish oil, the EPA and DHA of krill oil are in the form of phospholipids giving it new properties and making it potentially more potent (Martin, 2007). Phospholipids which is a natural and integral part of cell function and is more readily absorbed increasing bioavailability; is a general term that includes all lipids containing phosphorus. Usually, the analysis of phospholipids is based either on the determination of their total fatty acids by GC or the determination of the phospholipids classes (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine, sphingomyelin, lysophosphatidylcholine, etc.) with HPLC (Peterson and Cummings, 2005).

Commercially, phospholipids are obtained from soybeans, egg yolk, or brain tissue (Budavari, 1989). Until now, the soybean is the most frequent and studied source of lecithin. However, lecithin from soybean is rich in mainly saturated fatty acids with some lower unsaturated fatty acids. It does not contain some important PUFA including EPA and DHA. Egg yolk has also been used widely as a source of lecithin.

Several methods were compared for recovery and purification of mixtures of lipids and, more specifically, for phospholipids; however, for more difficult isolation, the results and recoveries vary, depending on the type of phase used and the nature of the sample matrix and composition (Caboni et al., 1996). Some methods that were originally used for phospholipids separation from meat (Boselli et al., 2008) gave low recoveries when applied to other matrixes. In recent years, SFE which is used as an alternative for lipid extraction to organic solvent extraction; has received much attention, because it allows a reduction in extraction time, requires little sample manipulation, and involves a much lower solvent consumption, leading to extracts of increased purity (Sihvonen et al., 1999; Hong et al., 2010). Some works refer to the application of SC-CO_2 extraction of marine materials to obtain PUFA. Yamaguchi et al., 1986 reported on the extraction of lipids from Antarctic krill. According to their results, only non-polar components such as cholesterol, carotenoid, triacylglycerols and their derivatives were extracted. Phospholipids did not appear in the extracted fractions. SC-CO_2 does not provide a means to dissolve phospholipids, but it can be recovered by the addition of a polar entrainer to

$SC\text{-}CO_2$ (Yukihisa et al., 2004). The choice of a suitable co-solvent must be based on some considerations such as thermodynamic and food safety (Montanari, 1996). Some researchers have already studied the role of ethanol as a co-solvent; Prosise (1985) reported that ethanol was an excellent solvent for isolating phospholipids for food use. But all other neutral lipids with phospholipids are also extracted by ethanol. Therefore, further steps are needed to purify the phospholipids.

In this study, the objective was to optimize and improve existing method to isolate an enriched phospholipids fraction from freeze dry krill and characterize the purified phospholipids. To achieve this objective, firstly SFE conditions were optimized to extract the majority of neutral lipids. Then, in a second processing step, the residual extracted krill, containing phospholipids, was extracted with ethanol solvent.

3.8. Materials and methods

3.8.1. Materials and chemicals

The freeze stored krill residues extracted by $SC\text{-}CO_2$ extraction at the optimum conditions (25 MPa and 45ºC) were used for phospholipids isolation. Standard of PC, PE, trolox, oleic and linoleic acid were purchased from Sigma-Aldrich, St. Luis, Mo., USA. All reagents used in this study were of analytical or HPLC grade.

3.8.2. Isolation of phospholipids

The multiple-step procedure for extracting phospholipids from krill is outlined in Fig.3.1. The sample (45 g) was extracted according to the procedure described by Palacios and Wang (2005), with modification. For the initial extraction, 200 mL of ethanol (95%) was added to 45 g of krill extracted residues (at 45°C and 25 MPa) in a 300-mL centrifuge bottle and stirred by a magnetic stirrer about 12 hr. The mixture was then centrifuged at 1900 rpm for 10 min, and the supernatant containing ethanol, some polar lipids, and some neutral lipids was transferred to a separator funnel with double volume of hexane. After one hour (1hr), the hexane phase was removed, and the ethanol phase was mixed with an additional double volume of hexane and left for 2 hr for phase separation. The ethanol was removed from the ethanol extract by rotary vacuum

evaporator at 40ºC, the remaining lipid material was dissolved in 10 mL of hexane and transferred to a 150-mL centrifuge bottle where 60 mL (about fifth volume) of chilled acetone (4°C) was added and carefully stirred to precipitate the phospholipids. The centrifuge bottle was then placed in an ice-water bath for 15 min and centrifuged at 1500 rpm. After discarding supernatant, the collected precipitate called purified phospholipids was stored at -40ºC until further analysis. In order to investigate the effect of the presence of neutral lipids on the ethanol extraction yield, raw krill samples were used in the same purification method. Three replicates of the samples were carried out.

3.2.3. Quantification of purity of isolated phospholipids

The phospholipids content of lecithin was measured according to Stewart (1980) by a colorimetric method based on the formation of a complex between phospholipids and ammonium ferrothiocyanate. Briefly, 0.35 mg of dry lecithin was dissolved in 2 mL of chloroform. Then, 1 mL of a solution prepared from ferric chloride (27 g/L) and ammonium ferrothiocyante (30 g/L) was added. After vortexing, the mixture was centrifuged at 1000 rpm for 15 min. The lower phase was collected and the absorbance was recorded at 488 nm. A calibration curve was made by standard PC and the result was expressed as gram equivalents of PC per gram of lecithin.

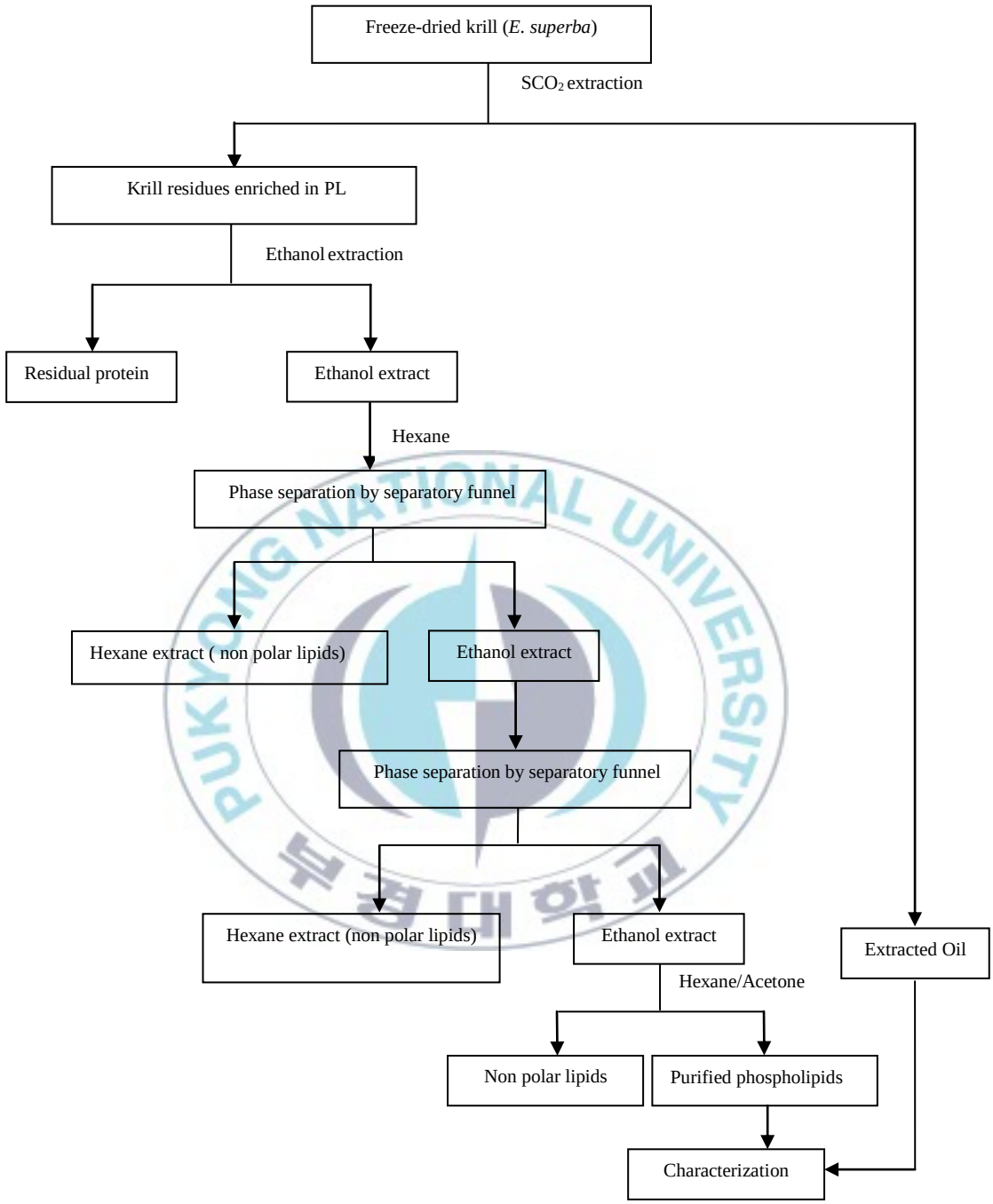


Fig. 3.1: Summary of krill phospholipids purification steps.

3.2.4. Major phospholipids quantification by HPLC–ELSD

Phospholipids composition was determined using a Jasco (LC-Net II/ADC) HPLC system (Japan) coupled with an evaporative light scattering detector (ELSD), model 400 (USA), model 126 solvent delivery modules. Appropriate dilutions of sample were injected (20 ul injection loop) on to a diol normal-phase silica column (250 mm × 4.6 mm i.d., with integral guard column; Advanced Separations Technologies, Waters). The analysis was carried out according to the method of Letter (1992). Extracted lecithin was dissolved in chloroform and injected (20 μL) into injector. The mobile phase was isopropyl alcohol, hexane and water. The drift tube temperature of ELSD was set to 60ºC. The pressure of nitrogen gas as a nebulizer was 50 PSI. The quantification of phospholipids was performed based on the peak area of standard phospholipids, PC, PE and PI. The millennium software was used to analyze the data obtained by HPLC.

3.2.5. Characterization of purified phospholipids

Hexane-insoluble matter, humidity and acid value of the purified phospholipids were determined using the AOCS (2001) official methods (Ja 3-87, Ja 2b-87, Ja 6-55). Analyses were performed by triplicate.

3.2.5.1. Free fatty acids

Free fatty acids of purified phospholipids from krill were analyzed as described by Bernardez et al. (2005). We have already discussed this method in Chapter 2 for the determination of free fatty acids in krill oil. Briefly, 50 mg (Approximately) of sample was placed into pyrex tubes with the addition of 3 ml of cyclohexane and then 1 ml of cupric acetate-pyridine reagent was added. Tubes were vortexed for 30 sec. After centrifugation at 2000 rpm for 10 min, the upper layer was read at 710 nm. The FFA content was measured on a calibration curve constructed from oleic acid standards. Copper reagent was prepared according to Lowry and Tinsley (1976). A 5% (w/v) aqueous solution of cupric acetate was prepared and filtered. Then the pH of cupric acetate solution was adjusted to 6.1 using pyridine.

3.2.5.2.Peroxide value

Peroxide value was determined according to AOCS (2001) method Cd 8-53 by modified amount. 1.0 g of krill purified phospholipids was dissolved in 6 mL of acetic acid-chloroform (3:2) solution. Then 0.1 mL of saturated KI solution was added to the mixtures and allowed the solution to stand with occasional shaking for 1 min. Distilled water (6 mL) was immediately added to the solution. The solution was titrated with 0.1 N of sodium thiosulfate until the yellow iodine colour had almost disappeared. Then 0.4 mL of starch indicator solution was added and again titrated until the blue colour disappeared. A blank determination was conducted with the same procedure. Peroxide value was expressed as milliequivalents peroxide/1000 g sample.

3.2.5.3.Thin layer chromatography of the purified phospholipids fractions

The phospholipids were separated by thin layer chromatography (TLC) for determination of fatty acid compositions of individuals. TLC was carried out using silica gel 60 plates (pre-coated with a 0.20 mm layer of silica gel 60) were obtained from ALUGRAM (Germany). The sample was applied (0.3 mg) on the plate and developed with a solvent system of dichloromethane: methanol: glacial acetic acid (60: 30: 10) (Fricke et al., 1984). Spots were visualized by exposure to iodine vapor or by charring with 50% sulphuric acid at 130°C for 30 min. Standard mixtures of phospholipids were run in parallel with the sample for identification of spots. Spots were then scraped off in screw cap tube separately and then extracted from the silica using the solvent system of chloroform:ethanol:water (2:2:1, v/v/v). The chloroform phase were collected by phase separation and evaporated by vacuum rotary evaporator. The purity of the remaining residues of each polar lipid was again checked by TLC. PC, PE and PI from the spots were extracted as described above. This purified PC, PE and PI were used for fatty acid compositions analysis.

3.2.5.4.Analysis of fatty acids composition by Gas chromatography

Fatty acids analysis of total phospholipids, and purified PC, PE and PI were carried out by GC. Methyl esters of fatty acids from total lipid extracts were prepared according to AOCS (1997). The gas chromatographic apparatus for the analysis of fatty acid

composition was a HP 5790II equipped with a flame ionization detector (FID) and a capillary column DB-wax. Nitrogen was used as a carrier gas (1.0 $mLmin^{-1}$) of fatty acid methyl esters. The oven temperature was programmed starting at a constant temperature of 130ºC for 3 min, and then increased to 240ºC at a rate of $4ºCmin^{-1}$ and hold at 240ºC for 10 min. Injector and detector temperatures were 250ºC. Fatty acid methyl esters were identified by comparison of retention time with standard fatty acid methyl esters mixture (Supleco, USA).

3.2.5.5. Oxidative stability

The oxidative stability was measured by the oxidation of emulsion of purified phospholipids in water (deionized and degassed water) at 37ºC. Four emulsions of phospholipids in water (w/w) (linoleic acid 5%, phospholipids 5%, water 90%; phospholipids 5%, water 95%; torolox 1%, phospholipids 4%, water 95%; linoleic acid 10%, water 90% (control)) were prepared. The mixtures were properly homogenized by a homogenizer. Oxidative stabilities were checked by thiocaynate and thiobarbituric acid method (TBA). Linoliec acid and standard trolox were used to compare oxidative stability of purified phospholipids.

3.2.5.5.1. Thiocyanate method

This method was conducted according to Mitsuda et al. (1966). The peroxide formed by lipid peroxidation reacts with ferrous chloride and form ferric ions. Ferric ions then unite with ammonium thiocyanate and produce ferric thiocyanate. Briefly, 0.1 mL of emulsion solution was added to 4.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of red color was measured at 500 nm (UVIKON 933, Kontron Instruments). Every 24 hr interval during incubation, the absorbance was recorded.

3.2.5.5.2. TBA method

The TBA method measures free radicals present after peroxide oxidation. The TBA value was measured according to Ottolenghi (1959). Briefly, 2 ml of 20% trichloroacetic acid

and 2 ml of 0.67% 2-thiobarbituric acid were added to 1 ml of emulsion solution. The mixture was placed in a boiling water bath (100ºC) for 10 min. After cooling the mixture was centrifuged at 3000 rpm for 20 min. Absorbance of supernatant was measured at 532 nm by a uv/visible spectrophotometer (UVIKON 933, Kontron Instruments).

3.3. Statistical analysis

All experiments were performed in triplicate and each set of result were averaged. The standard deviations were used as the basis for the error bars shown in the figures. The least significant difference at the 95% confident ($P < 0.05$) level was calculated by Duncan test using Statistical Analysis System (SAS Ver. 9.1, SAS Institute, USA).

3.4. Results and Discussion

3.3.1. Organic solvent extraction for comparison and solvent selection

In order to investigate the effect of neutral lipids on the efficiency of the extraction by ethanol, forty five gram of raw krill were treated using the same purification method; and the purified phospholipids composition was compared with that obtained from SC-CO_2 extracted residues. Also, with the aim of comparing the extraction efficiency of organic solvents in order to select the solvent with highest yield of phospholipids content and safety for human body; different organic solvents were used for the extraction of phospholipids from both raw materials and SC-CO_2 extracted residues. The results of the comparison are reported in Table 3.1. The highest yield was from SC-CO_2 extracted residues, and the most effective solvent was ethanol with 42.7% extraction efficiency.

3.3.2. Quantification of major phospholipids composition

Major phospholipids compositions are shown in Table 3.2. The mains phospholipids of krill were PC and PE. PC and PE content of krill were 80.4±0.64% and 14.9±0.46% of total phospholipids, respectively. PI was also present but with very low percentage (0.7±0.72%) of total phospholipids (Fig.3.2). Kusumoto et al. (2004) reported that phospholipids from krill (*Euphausia pacifica)* contained (36.2%-53.8%) of PC and

(3.4%- 17.5%) of PE. Gordeev et al. (2004) and Fricke et al. (1984) also reported that PC content of phospholipids from krill (*Euphausia superba)* was 3 to 5 times higher than PE content, which was similar to this study. The phospholipids content of krill from different catches may vary considerably, and the variations are probably due to both differences in nutritional status and maturity of the roe of krill. And also, to different isolation and quantification process used.

3.3.3. Phospholipids characterization

The isolated phospholipids were almost pure (97%). It was found that the deoiled krill residues contained 8.06 % of total phospholipids. Analysis of the purified krill phospholipids shows a composition in agreement with the range reported in the literature for krill phospholipids (Fricke et al., 1984). Phospholipids content variation in krill may due on different factors, such as sample age, fishing area, season, extraction process and time, extraction efficiency by different solvents etc. The results from krill phospholipids characterization are summarized on Table 3.3. Hexane-insoluble matter of phospholipids was low (<1%) indicating almost the purity of phospholipids.

3.4.3.1. Free fatty acids, acid value and peroxide value

The acid value, peroxide value and free fatty acids of purified phospholipids from SC-CO_2 extracted krill residues are given in Table 3.3. Acid value and free fatty acids were used to determine the acidity of phospholipids. On the other hand, peroxide value is used as a measurement of rancidity of lipids which occurs by auto oxidation. Generally, peroxide and acid values of commercially available soy lecithin (mainly phospholipids) are found up to 12 milliequivalent/1000 g and 30 mg KOH/ g of lecithin, respectively. The phospholipids isolated from krill contained free fatty acids which increased its acid value. Changes of free fatty acids content are mainly related to hydrolytic reactions in the lipid. Free fatty acids content of the purified phospholipids was 2.34 g/100 g of phospholipids. It was found that the amount of free fatty acids and peroxide value agreed with the results of Ackman et al. (1970) and Sargent and Falk-Petersen (1981), who reported values in the range of 2-8% of the dry weight. But contrast the result (0.6%) reported by Saether et al. (1986) who mentioned about the special precautions taken in his

study to avoid postmortem lipolysis. Also, higher temperature and storage time caused a significant increment of the free fatty acids in the hake byproducts oil (Rodriguez et al., 2008). Previous studies reported that the oxidation rate of Antarctic krill (*Euphausia superba*) lipid is very slow and no peroxides are accumulated even after a long-term storage (Yanagimoto et al., 1979).

3.4.3.2. Fatty acid compositions of total phospholipids, PC, PE and PI

In order to analyze fatty acid compositions of major phospholipids, the phospholipids were separated by preparative TLC (Fig.3.3). The fatty acid compositions of isolated phospholipids, PC, PE and PI are shown in Table 3.4. The predominant fatty acid in total phospholipids and in subfraction PC was oleic acid (18:1) (29.16% and 33.9%, respectively). Arachidonic acid (20: 4 n-6**)** was abundant in PC subfraction (24.35%) and in total phospholipids in different percentages (18.07%). Palmitic acid (16:0) was predominant in PI subfraction (36.02%), but also was highly present in all subfractions. Stearic acid (18:0) varied from 1.33% to 10.28% in all subfractions. PC can be an important source of essential PUFA; the present study showed that PC contained almost 40% of PUFA. Several works have confirmed that in marine crustaceans, PE is a highly unsaturated PL (Chapelle, 1977). Because of the high levels of PUFA that contain, particularly EPA and DHA, PE has been considered as a reserve for the adaptation of membrane fluidity to changes in temperature (Chapelle, 1986). In agreement with these previous studies, these results showed that PE contained 26.30% of EPA and 10.79% of DHA. The amount of the unsaturated fatty acids in total phospholipids was very high (82.41%) while the percentage of the saturated fatty acids was found 11.59%. The unsaturated / saturated ratio varied from 0.6 to 7.11 for the total phospholipids and for all subfractions. The PUFA were the most abundant of the unsaturated fatty acids of total phospholipids, PC, and of the PE subfraction. The results obtained are in harmony with those of other authors (Gordeev et al., 2004).

Table 3.1: Organic solvent extraction from raw krill and SC-CO_2 extracted residues for comparison and solvent selection

Solvents	Total phospholipids purified[a] (%)	
	Raw krill	SC-CO_2 treated krill
Petroleum Ether	26.5±0.52	29±0.41
Acetone	18.6±0.35	20±0.24
2-Propanol	33.5±0.43	36±0.35
Ethanol	37.4±0.51	42.7±0.16

[a]The results showed mean value ± standard deviation.

Table 3.2: Major phospholipids composition obtained from SC-CO_2 extracted krill

Phospholipids distribution[a] (%)	
PC	80.4±0.64
PE	14.9±0.46
PI	0.7±0.72

[a]The results showed mean value ± standard deviation.

Table 3.3: Characterization of the purified phospholipids.

Parameters	Value
Hexane-insoluble matter[a] (%)	0.91±0.73
Moisture[a] (%)	2.13±0.65
Acid value[a] (mg KOH/g)	23.07±0.24
Peroxide value[a] (milliequivalent/1000g)	4.66±0.28
Free fatty acids[a] (g/100 g)	2.34±0.34

[a]The results showed mean value ± standard deviation.

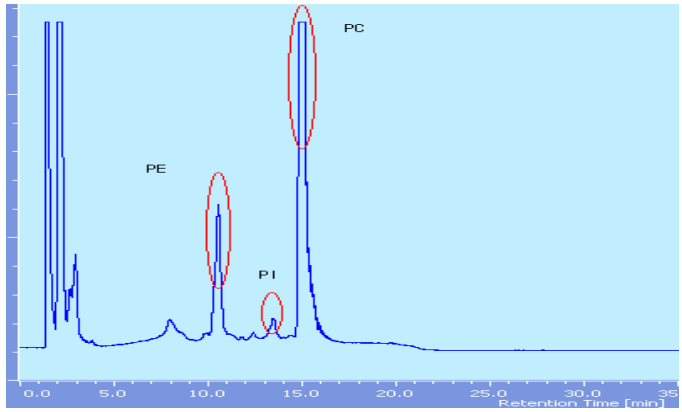


Fig. 3.2: HPLC peak of phospholipids purified from SC-CO_2 extracted residues

Fig. 3.3: Thin-layer chromatograms of phospholipids purified from SC-CO_2 extracted residues (1) and from raw krill (2): PA, PC, PI, and PE

Table 3.4: Fatty acid composition of the phospholipids fraction

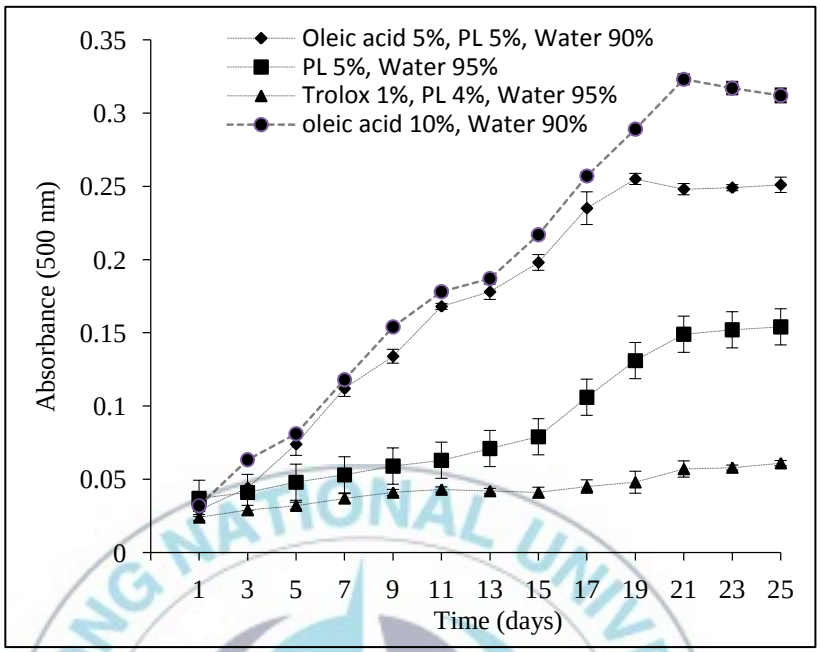
Fatty acids [a] (%)	Total phospholipids	PC	PE	PI
14 : 0	1.12	2.78	4.32	10.15
15 : 0	1.50	1.21	1.55	5.63
16 : 0	4.69	18.05	25.66	36.02
16 : 1	0.21	0.70	1.16	19.33
18 : 0	10.28	6.20	1.33	8.72
18 : 1	29.16	33.09	16.77	20.15
18 : 2 n-6	3.50	11.54	2.17	ND
20 : 4 n-6	18.07	24.35	9.50	ND
20 : 5 n-3	20.57	2.08	26.30	ND
22 : 6 n-3	10.90	ND	10.79	ND
Total (%)	100.0	100.0	100.0	100.0
Saturated	11.59	28.24	33.31	60.52
Unsaturated	82.41	71.76	66.69	39.48

[a]Results were average of two determinants. Standard error of the fatty acid constituents were on the order of about ±2%. For total phospholipids, fatty acids showed which was present more than 1% of total fatty acids. ND- Not detected

3.4.4. Oxidative stability

Instead of determining the absolute state of oxidation of incubated sample, oxidation trend was evaluated. The oxidative stabilities of phospholipids are shown in Fig.3.3. It has been reported that the oxidation rate of Antarctic krill (Euphausia superba) lipid is very slow (Yanagimoto et al., 1979). In this study, phospholipids with linoleic acid showed increase in absorbance values from first day. The increase in absorbance value was an indicator of auto oxidation by formation of peroxides during incubation. Phospholipids showed low absorbance value indicating low level of lipid peroxidation until 17 days. And it showed increased oxidation after 19 days. On the other hand, phospholipids with trolox showed higher oxidative stability than that of the others emulsion. Trolox, which is an antioxidant, inhibited the peroxide formation by lipid peroxidation in a certain period. The control showed increase in absorbance values from day 1 and reached on day 19 and dropped on day 23. In addition, the phospholipids showed slightly high absorbance comparing to phospholipids with linoleic acid. It might be happened due to the presence of peroxide from the oxidation of some neutral lipid (impurities) exist in the emulsion of phospholipids. In TBA method, the absorbance measured on day 15 was slightly similar between phospholipids and phospholipids with trolox emulsion. However, it was high on phospholipids with linoleic acid emulsion indicating low oxidative stability (Fig.3.4). On the other hand, a significant increase in absorbance was found on day 25 for phospholipids emulsion. It has also been shown that the major constituents of the PUFA of phospholipids in krill are EPA and DHA which were the most susceptible to oxidation; in soybean, linoleate, and in egg yolk, arachidonate. Therefore, it can be supposed that the oxidative deterioration of phospholipids proceeds most rapidly in krill, less rapidly in egg yolk and least rapidly in soybean (Lee et al., 1981). However, phospholipids showed high oxidative stability which can be explained by the presence of the natural antioxidant, astaxanthin in phospholipids; since Krill is a major source of astaxanthin, which has strong antioxidant activity (Shinichi et al., 2003). Gogolewski et al. (2000) also reported that long chain PUFA esterified with polar lipids had synergistic effect with antioxidant. Previous studies reported the high oxidative stabilities of phospholipids from animal and plant sources by applying different methods (Palacios and Wang, 2005; Belhaj et al., 2010).

A)



B)

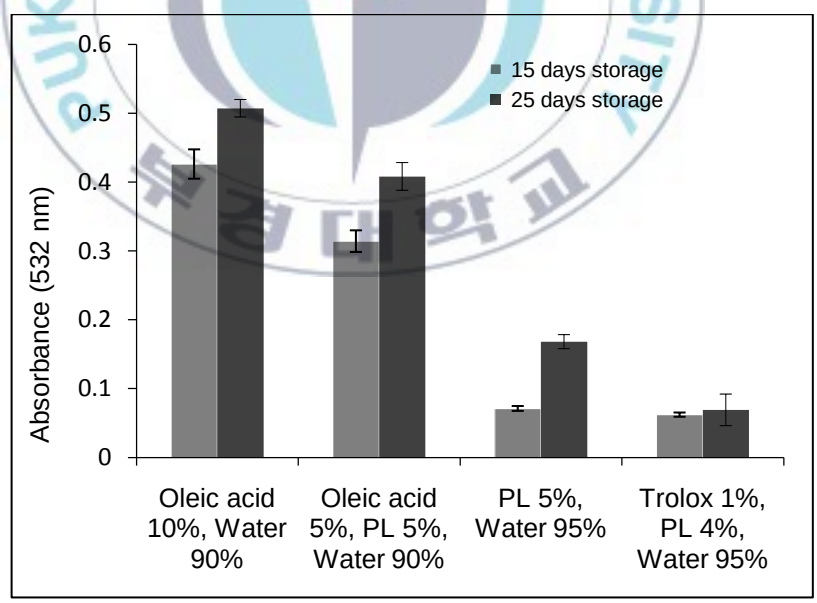


Fig. 3.4A-B: Oxidative stability of purified krill phospholipids A) Thiocyanate method and B) Thiobarbituric acid method. Results were the mean value of three replicates ± S.E.

3.5. Conclusions

In this study, the suitable conditions for the purification of phospholipids from krill were examined. With a two-step extraction process, it is possible to isolate phospholipids -rich extract from krill. By SFE with CO_2 most of the neutral lipids were extracted from the Antarctic krill. The functional lipids in the spent solids from this first stage SFE were enriched with the removal of the neutral lipids. PC, PE and PI were then extracted in a second step using ethanol, hexane and acetone as solvents. Under optimum conditions, the final yield estimated was about 42.7% (w/w) of krill lipid. EPA formed the most abundant molecular species in PE, but not in PC and PI. While, arachidonic acid (20: 4 n-6**)** was mainly present in PC and PE but was missing in phospholipids. Also, the purified phospholipids showed a potent antioxidant activity. Therefore, further work could be done on the isolation and characterization of the individual compounds in the extracts, which are responsible for antioxidant activity. These results show a great potential for utilization of SFE to obtain a functional, value-added ingredient from marine product, with great economic significance.

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Chapter 4

Comparative study of digestive enzymes of krill (*E. superba*) after SC-CO_2 and organic solvent extraction*

Abstract

In recent years, recovery and characterization of enzymes from fish and aquatic invertebrates has been achieved and some interesting and novel applications related to marine enzymes in food processing have emerged. In this study, Enzyme activities of krill were characterized before and after lipid extraction by SC-CO_2 and organic solvent, hexane and acetone. Krill SC-CO_2 extraction was performed under the conditions of temperature range from 35-45°C and pressure, 15-25 MPa for 2.5 h with a constant flow rate of 22 g/min. Extraction yield of lipids was optimum at 45°C and 25 MPa . The digestive enzyme activities of protease, lipase and amylase of SC-CO_2 treated krill residues were slightly decreased comparing to organic solvent, hexane and acetone treated residues. In SC-CO_2 treated samples, all of the digestive enzymes showed slightly higher temperature stability. In the other hand the crude extracts of SC-CO_2, hexane and acetone treated krill samples showed almost same optimum pH and pH stability for each of the digestive enzymes. It was also found in SDS-PAGE that there are no significant differences in protein patterns of the crude extracts of untreated and SC-CO_2, hexane and acetone treated krill indicating no denaturation of proteins.

Keywords: Digestive enzymes, krill, Supercritical carbon dioxide, hexane, acetone, enzyme activity

* This work has been presented in 13th European Meeting on Supercritical Fluids (2010), Graz, Austria.

5.6. Introduction

Enzymatic methods have become increasingly an important part of the processes used by the modern food and feed industry in order to produce a large and diversified range of products for both human and animal consumption (Fereidoon and Janak, 2001). In fact, enzyme technology has evolved to be an integral part of the food industry. The early advances were focused on methods to be used in order to control endogenous enzyme activities. Presently on enzymes were developed as processing aids and as valuable tools for ingredient production (Wasserman, 1990). Enzymes have also many other industrial applications. Proteases are enzymes that cleave peptide bonds with different specificities. They represent one of the three largest groups of industrial enzymes and find application in detergents, pharmaceutical industry, leather industry and bioremediation processes (Anwar and Saleemuddin, 1998). Due to their biotechnological potential, lipases are attracting an enormous attention. They constitute the most important group of biocatalysts for biotechnological applications. Lipolytic enzymes are involved in the breakdown and thus in the mobilization of lipids within the cells of individual organisms as well as in the transfer of lipids from one organism to another (Beisson et al., 2000). Various important chemicals are currently manufactured from fats and oils by chemical processes, could be produced by lipases with greater rapidity and better specificity under mild conditions (Vulfson, 1994). Amylases constitute a class of industrial enzymes, which alone form approximately 25% of the enzyme market covering several industrial processes such as sugar, textile, paper, brewing, distilling industries and pharmaceuticals (Oudjeriouat et al., 2003).

Most enzymes from fish and aquatic invertebrates are also present in terrestrial organisms (Haard, 1998). However, variations have been observed among these two groups due to differences in molecular weight, amino acid composition, pH and temperature optimum, stability, inhibition characteristics and kinetic properties (De-Vecchi and Coppes, 1996). Many research works have been carried out during the last few years for investigating the new possibilities offered by enzymes originating from fish and aquatic invertebrates. At present, the utilization of krill is limited to meat extracts, dried products, feed for fishing and culture fisheries, and a material for carotenoid

pigments. Krill also offers the advantage of being high in the omega-3 polyunsaturated fatty acids (Tou et al., 2007).

Lipids present in the sample prevent extraction and purification of enzymes. For purification of enzymes, lipid free sample is required for higher efficiency. Currently, a large number of methods are available to extract lipids from biological materials. Most of them use organic solvents, usually in mixtures containing chloroform and methanol, as in the procedures (Bligh and Dyer, 1959). However, the removal of lipids with organic solvents conducts to the protein denaturation and loss of functional properties (Pariser et al., 1978). And they are also harmful for human health as well as environment. The SFE method is very advantageous and environmentally friendly over other conventional either solvent or enzyme extraction methods for recovering natural lipids. The use of SFE technology that offers suitable extraction and fractionation appears to be promising for the food and pharmaceutical industries (Sahena et al., 2009). SC-CO_2 has been used for extraction of lipid from different marine organisms (Yamaguchi et al., 1986). We have already discussed the SC-CO_2 extraction of lipid from krill in previous chapter. However, after extraction the leftover materials are rich sources of various enzymes that may have some unique properties of interest for both basic research and industrial applications.

The present study was designed to investigate the digestive enzyme activities and characterize the crude enzymes obtained from krill after lipid extraction by SC-CO_2 and to compare the results with those obtained by organic solvent using hexane and acetone.

5.7. Materials And Methods

All experiments were carried out at least two replications and the results were calculated as a mean value.

5.7.1. Materials

For this study, four different samples of krill were used: Freeze dried raw krill, SC-CO_2 extracted residues at optimum conditions (25 MPa and 45ºC), hexane and acetone extracted residues. All other chemicals used in different analysis were of analytical or HPLC grade.

5.7.2. Digestive enzyme assay

5.7.2.1. Preparation of extracts

The four samples of krill were prepared for crude enzyme extract and also for electrophoresis. One gram for each sample was homogenized in 6 mL cold distilled water by mechanical stirring at 4°C for 2 hrs. The homogenates were then centrifuged at 9000 rpm for 15 min at 4°C, and the supernatants were removed and stored at -20°C for further analysis.

5.7.2.2. Protease assay

Protease activity was measured using the Casein Folin-Ciocalteau method (Oda and Murao, 1974) with some modification. The assay was conducted by using as substrate; one percent (1%) casein solution in 0.0125 M sodium borate-NaOH buffer (pH 10.5). Enzyme reaction mixtures consisted of 2.5 mL of substrate and 0.5 mL of crude enzyme and incubated for 10 min at 37ºC. The reaction was stopped by addition of 2.5 mL of trichloroacetic acid solution (8% w/v) and holding for 20 min. The sample was then centrifuged for 10 min at 3000 rpm. The supernatant (2 mL) was mixed with 5 mL of 0.55 M Na_2CO_3 and 1 mL of 1 N Folin-Ciocalteu reagent. After 20 min of incubation at 37°C, the absorbance of the supernatant was measured at 660 nm (UVIKON 933, Kontron Instruments). Tyrosine was used as standard to construct a calibration curve and one unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol of tyrosine per min from casein.

5.7.2.3. Lipase assay

Lipase activity was measured using the method of Vorderwülbecke et al. (1992) as modified by Hatzinikolaou et al. (1999). The substrate emulsions consisted of 0.2 mL solution A (40 mg of *p*-nitrophenyl-laurate was dissolved in 12.0 mL of isopropanol) into 3.0 mL solution B (0.4 g Triton X-100 and 0.1 g gum arabic were dissolved in 90 mL of 0.1 M potassium phosphate buffer, pH 7.0) under extreme vortexing. After the emulsions were holding steady for 1 hr at room temperature, 0.1 mL of crude extract was added to 3.2 mL of the substrate emulsion and incubated for 20 min at 35°C in a shaking water

bath. The reaction was finished by boiling for 5 min. Following centrifugation (6000 rpm, 10 min) the absorbance of the clear supernatant was recorded at 410 nm. As control, 0.1 mL of inactivate enzyme extract prepared by heating at 100°C for 5 min was treated in the same way. The *p*-nitrophenol was used as standard for the construction of the calibration curve, and one unit of enzyme activity was defined as the amount of enzyme required to liberate of 1 μmol *p*-nitrophenol from *p*-nitrophenyl-laurate per minute under the same assay conditions.

5.7.2.4. Amylase assay

Amylase activity was assayed using the starch hydrolysis method by measuring the content of reducing sugars released. The crude extract (0.5 mL) and 0.5 mL of 1.0% (w/v) potato starch (Sigma) were mixed in 100 mM acetate buffer (pH 6.5). The reaction mixture was incubated at 37°C for 10 min. The amount of liberated reducing sugar was determined according to the dinitrosalicylic (DNS) acid method described by Miller, 1959. D-Glucose was used as standard to construct a calibration curve and one unit of amylase activity was defined as the amount of enzyme that released 1 μmol of reducing end groups per minute.

5.7.3. Effect of pH and pH stability of protease, lipase and amylase

Different buffers of large range of pH values were used in order to determine the effect of pH on the crude enzyme activities. The buffers used were 0.1 M of citric acid-sodium citrate (pH 4.0-5.5), 0.1 M of potassium phosphate (pH 6.0-8.0) and 0.1 M of glycine-NaOH (pH 8.5-12). For the pH stability, the crude enzyme extracts were pre-incubated for 5 hrs at 0°C in buffers with the same ionic concentrations at different pH values ranging from pH 3.0 to pH 12.0. Then, the enzyme activities were measured instantaneously after this treatment using the standard methods as mentioned above.

5.7.4. Effect of temperature and temperature stability of protease, lipase and amylase

In order to investigate the optimal temperature for enzyme activities, two specific buffers were prepared as follow; potassium phosphate (0.1M, pH 7.5) buffer for amylase

and 0.1 M glycine-NaOH (pH 8.5) buffer for both protease and lipase. The temperature was ranged between 20°C and 80°C. The same range of temperature was used for the pre-incubation of the crude enzymes to test their temperature stability. After 1 hr of incubation under the different temperatures, the residual enzyme activities were determined under the same assay conditions.

5.7.5. Electrophoresis

Electrophoresis of crude extracts were carried out according to the method of Laemmli (1970) using sodium dodecyl sulfate-polyacrylamaide gel electrophoresis (SDS-PAGE) with 5% (w/v) stacking gel and 12% (w/v) separating gel. Electrophoresis was performed using a Mini-Protein III cell module (Bio-Rad Laboratories, CA, USA) at a constant voltage (100 V) for 2 hrs. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 for 2 hrs and destained in 50% methanol (v/v) and 10% (v/v) acetic acid solution. Molecular weight markers (Sigma) as standard protein were used.

5.8. Results And Discussion

5.8.1. Digestive enzyme activities

The activities of protease, amylase and lipase of crude enzyme extracts of krill are represented in the Figure 4.1A-C. The activity of lipase was the highest among the three classes of digestive enzymes. The highest activities of protease, lipase and amylase were found in hexane treated krill sample comparing to acetone and SC-CO_2 treated samples. The digestive enzyme activities of krill samples might be lost due to the treatment by SC-CO_2. Prior research has been showed that the loss of enzyme activity after SC-CO_2 treatment attributed to the interactions between CO_2 and the enzyme. This means CO_2 may form a covalent complex with free amino groups on the surface of the enzyme (Kamat et al., 1995; Habulin and Knez, 2001). Giessauf et al., 1999 has also suggested that the most important factor that may cause enzyme activity loss is possibly the depressurization step; in a long-term enzyme application its activity decreases with increasing number of depressurizations.

5.8.2. Optimum pH of protease, lipase and amylase

Fig. 4.2A-C shows the optimum pH of protease, lipase and amylase. The highest proteolytic activity in all treated krill extracts were found at pH 8.5. Previous studies have also reported that the high protease activity was at pH ranging from 8.0 to 10.0 in several fish species (Eshel et al., 1993; Hidalgo et al., 1999; Prasertsan et al., 2001). At acidic pHs, only minor protease activities were found in extracts. The observed results indicate that the crude extracts of krill having both the acidic and alkaline proteases. In agreement with our results, Natalia et al., 2004 also found similar results for carnivorous ornamental fish.

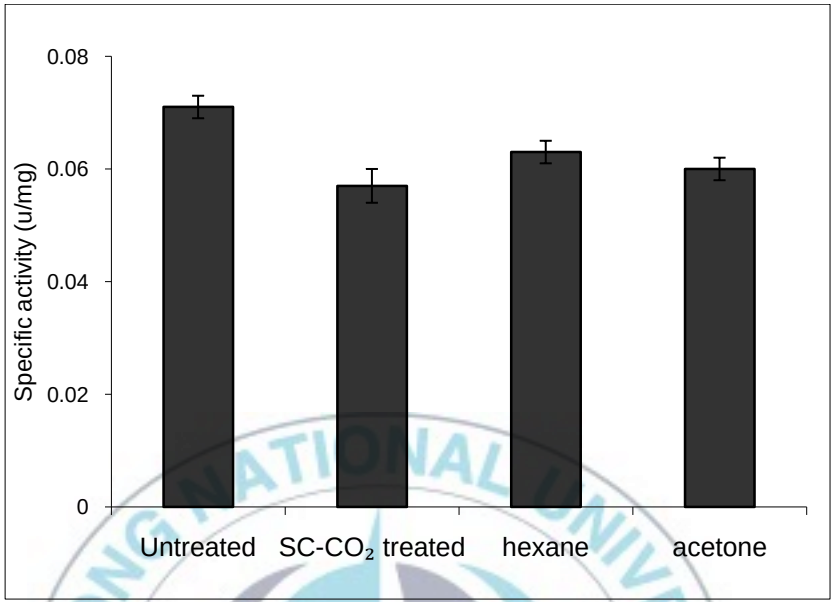
According to some authors (Mukundan et al., 1985; Raso and Hultin, 1988; Gjellesvik et al., 1992; Kumar et al., 2005), the optimum pH of 7 to 9 for lipase activities were found from fish and other sources. In our study, the optimum lipolytic activity was found at pH 8 for crude enzyme of almost all krill extracts. For the amylase activity in the crude extracts of krill, it was optimum at pH 7.5. The data on amylase activity obtained from the gut of seabream, tuber and turbot support our result (Munilla-Mordn and

Saborido-Rey, 1996; Noman et al., 2006). The minor lipase and amylase activities found at acidic pH indicate the presence of acidic lipase and amylase in the crude extracts. This results show that there is no significant difference for the pH effect on digestive enzyme activities in the crude extracts of SC-CO_2, hexane and acetone extracted krill.

5.8.3. pH stability

In Fig. 4.3A-C, pH stability of protease, lipase and amylase in the crude extracts of SC-CO_2 as well as hexane and acetone extracted krill are shown. In the pH range of 8.5-10.5, the protease activities lose less than 10% of its original value and then lose more and more with increasing pH. In agreement with this result Prasertsan et al., 2001 also found that more than 90% activity was retained from crude enzyme extracts of tuna viscera in the pH range of 9 to 11. Yamamoto, 1975 reported that the instability of acidic proteases recorded at the alkaline pH region contrasts with the activities of gastric proteases obtained from many of the lower vertebrates' fish species. For the crude extract lipase, it was observed as stable within a pH range of 7.5 to 9.5 with almost 90% activity. This pH stability recorded is in agreement with results reported by Kumar et al. (2005) and Aryee et al. (2007) who observed that lipases were stable within a pH range of 8 to 10.5 and 7 to 10, respectively. The stability of amylase determined in krill extract was in pH range of 6.5 to 8.5 where almost 90% activity was observed. Noman et al. (2006) reported almost the same pH range of 6 to 8 for α-amylase stability. On the other hand, the differences in the stable pH range for the crude extract of SC-CO_2, hexane and acetone extracted krill samples were insignificant.

A)



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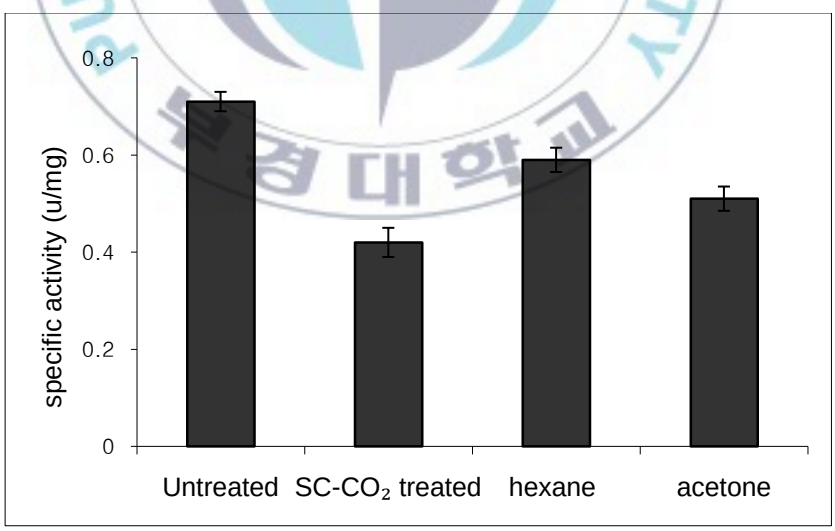


Fig. 4.1A-C: Digestive enzyme activities of crude extracts of SC-CO_2, hexane and acetone extracted krill. A) Protease, B) Lipase and C) Amylase

C)

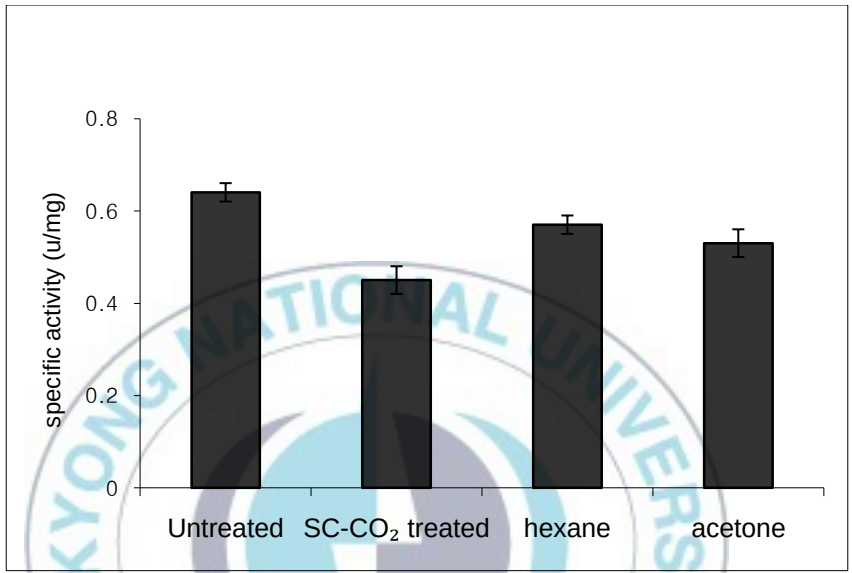
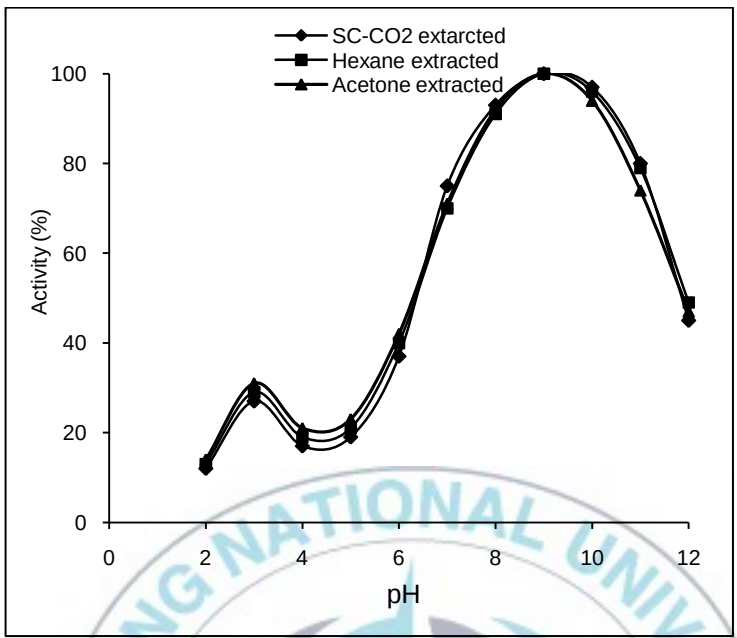


Fig. 4.1A-C: Digestive enzyme activities of crude extracts of SC-CO_2, hexane and acetone extracted krill. A) Protease, B) Lipase and C) Amylase

A)



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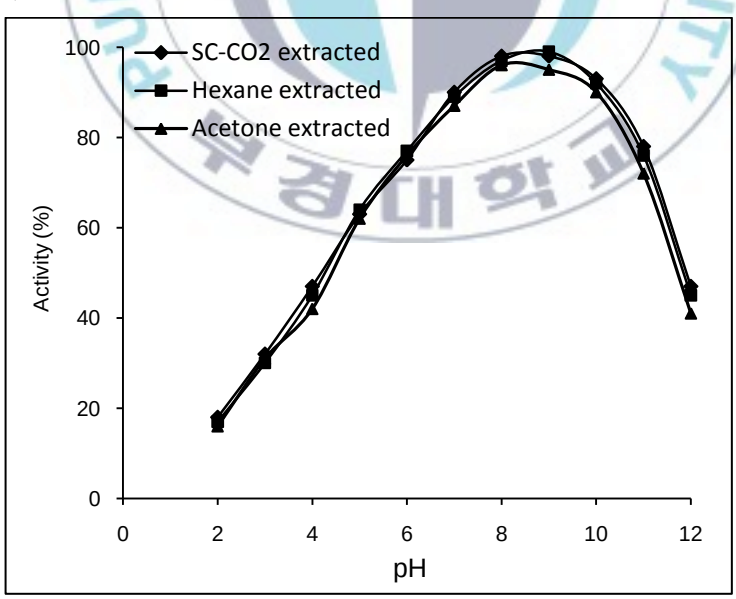


Fig. 4.2A-C: Optimum pH of digestive enzymes in crude extracts of SC-CO_2, hexane and acetone extracted krill. A) Protease, B) Lipase and C) Amylase

C)

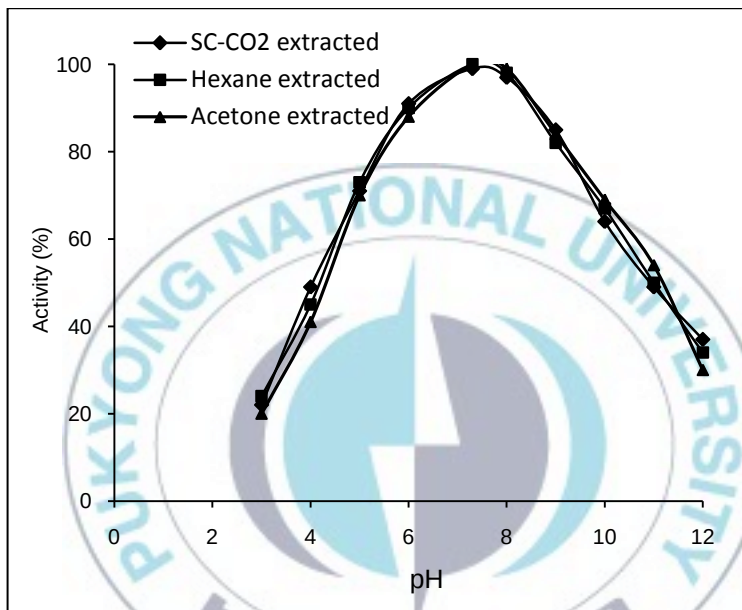
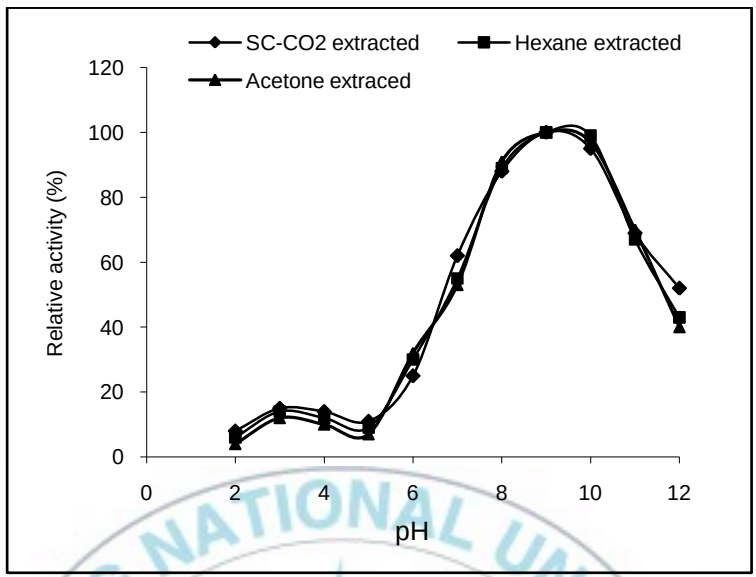


Fig. 4.2A-C: Optimum pH of digestive enzymes in crude extracts of SC-CO_2, hexane and acetone extracted krill. A) Protease, B) Lipase and C) Amylase

A)



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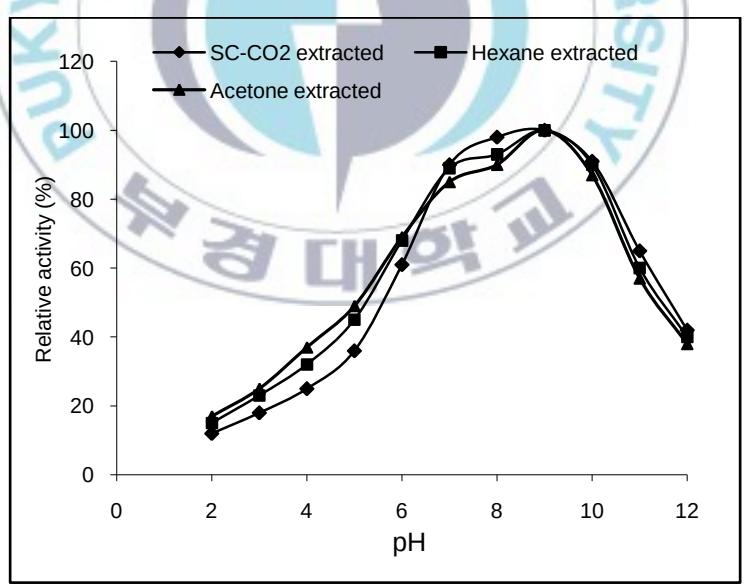


Fig. 4.3A-C: pH stability of digestive enzymes in crude extracts of SC-CO_2, hexane and acetone extracted krill. A) Protease, B) Lipase and C) Amylase

C)

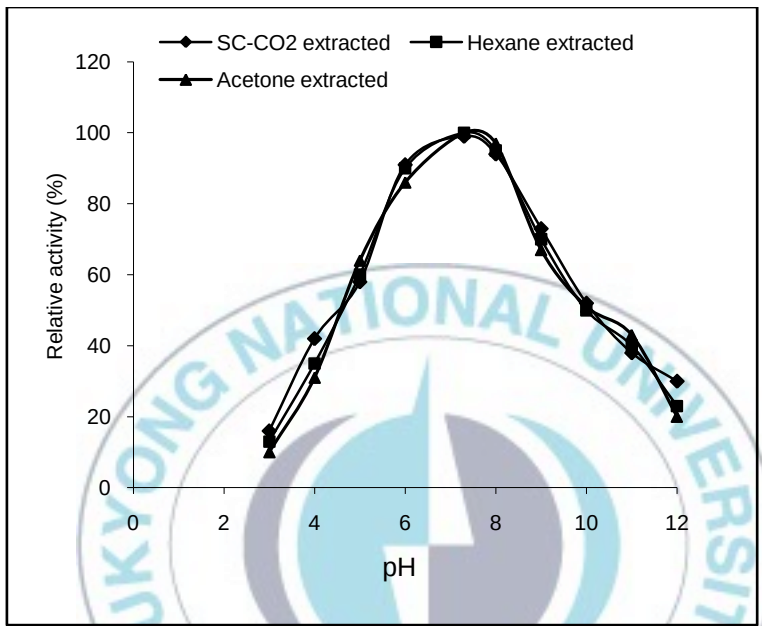


Fig. 4.3A-C: pH stability of digestive enzymes in crude extracts of SC-CO_2, hexane and acetone extracted krill. A) Protease, B) Lipase and C) Amylase

5.8.4. Optimum temperature of protease, lipase and amylase

In Fig. 4.4A-C, the optimum temperatures of protease, lipase and amylase enzymes are shown. The highest protease, lipase and amylase activity in all crude extracts of SC-CO_2 hexane and acetone extracted samples maintained above 50% of activity over the temperature range of 20 to 60°C with optimum activities at 60, 50 and 37°C, respectively. Similar optimum temperature for protease and amylase in the crude extract of tambaqui waste and tuber were reported by Esposito et al. (2009) and Noman et al., (2006), respectively. However, different optimum temperature for lipase was recorded from grey mullet as 55°C which is slightly higher than the tuna viscera lipase. These differences in the optimum of temperature may be explained by a number of factors including the different substrates used for determination and the varying mechanical properties of the homologous lipases which has been shown to have an effect on the temperature sensitive activity of enzymes (Aryee et al., 2007).

5.8.5. Temperature stability

The temperature stability of digestive enzymes investigated in the crude extracts of SC-CO_2, hexane and acetone extracted samples are given in Fig. 4.5A-C. The protease activities all crude extracts remained above 85% of activity up to 60°C. The protease activity of fish waste has been reported to maintain about 86% of its activity during 30 min incubation at the temperature 60°C (Esposito et al. 2009). Above the temperature 60°C, the activity of protease enzymes declined sharply. Though, the SC-CO_2 extracted krill maintained about 80% of its protease activity at 65°C while both hexane and acetone extracted samples retained 67% of its activities. For lipase activity, it was found that the crude extracts of krill retained more than 70% of activity up to 50°C. But, at higher temperatures the activity was quickly decreased. Aryee et al., 2007 has observed more than 70% of lipase activity in the grey mullet at temperatures up to 50°C. It is to mention that the crude extract of SC-CO_2 extracted krill demonstrated slightly higher temperature stability of lipase enzymes at temperatures higher than 50°C. More than 80% of amylase activity was retained up to 40°C, as also found by Noman et al. (2006) in tuber. In the term of comparison, At 45°C, the SC-CO_2 extracted krill extract showed higher amylase activity than that of hexane and acetone extracted krill extracts. Thus, the

digestive enzymes investigated in this study exhibit higher temperature stability in SC-CO_2 extracted krill extract comparing to hexane and acetone extracted krill extracts. This may have occurred because of very slight changes in the properties of the active site by temperature and pressurization during SC-CO_2 extraction.

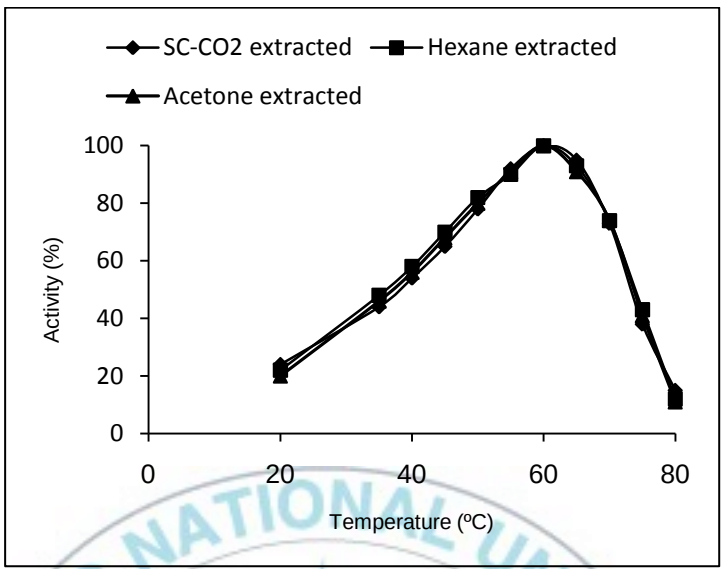
5.8.6. Electrophoresis

In Fig. 4.6, the gel electrophoresis of marker protein as control and the crude extracts of freeze dried raw, SC-CO_2 hexane and acetone extracted krill are shown. It is shown that the proteins in freeze dried raw krill were very similar in subunit composition to SC-CO_2 and organic solvent treated samples and the gel banding patterns observed were almost identical. Thus, it can be concluded that protein denaturation was not establish in SC-CO_2 extracted krill sample since there was no any change in the intensity of protein bands. This is in agreement with results reported by Stahl et al. (1984) who found very slight protein denaturation in SC-CO_2 extracted seed residues.

5.9. Conclusions

This work provided a comparative study of the digestive enzyme activities in the crude extracts of SC-CO_2, hexane and acetone extracted krill residues. In SC-CO_2 extracted samples the activity of protease, lipase and amylase were slightly lower than those observed in hexane and acetone extracted samples. However, all of the digestive enzymes showed slightly higher temperature stability in SC-CO_2 extracted samples. In the other hand, the electrophoretic patterns showed that no protein denaturation was found in SC-CO_2 extracted krill. The use of carbon dioxide for lipid extraction is environment friendly. Results obtained in the present work point to the possibility to use krill after lipid extraction by SC-CO_2 as alternative of organic solvent, for isolation and purification of different digestive enzymes. In addition, the unique pressure dependence of SCF physical properties presents opportunities for rational control of enzyme activity, specificity, and stability. Thermostable biocatalysts are highly attractive for economic purposes. Thus, further study using SC-CO_2 at different extraction conditions may also help to obtain high quality and high thermally stable functional proteins.

A)



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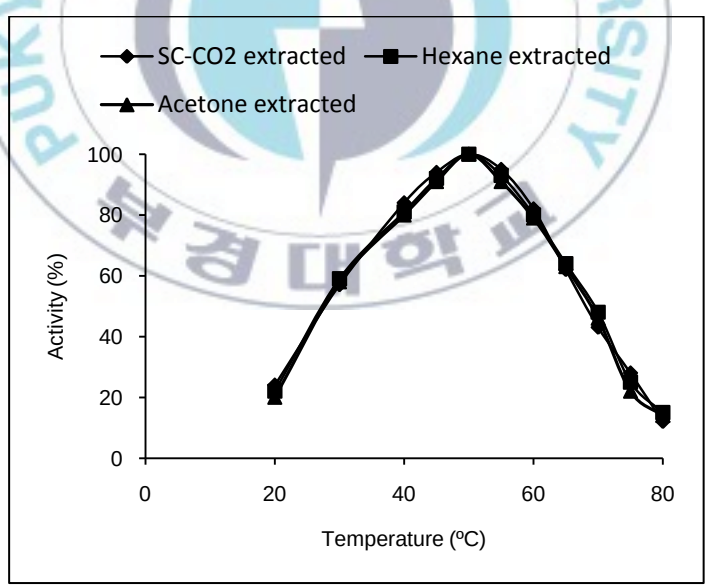


Fig. 4.4A-C: Optimum temperature of digestive enzymes in crude extracts of SC-CO_2, hexane and acetone extracted krill. A) Protease, B) Lipase and C) Amylase

C)

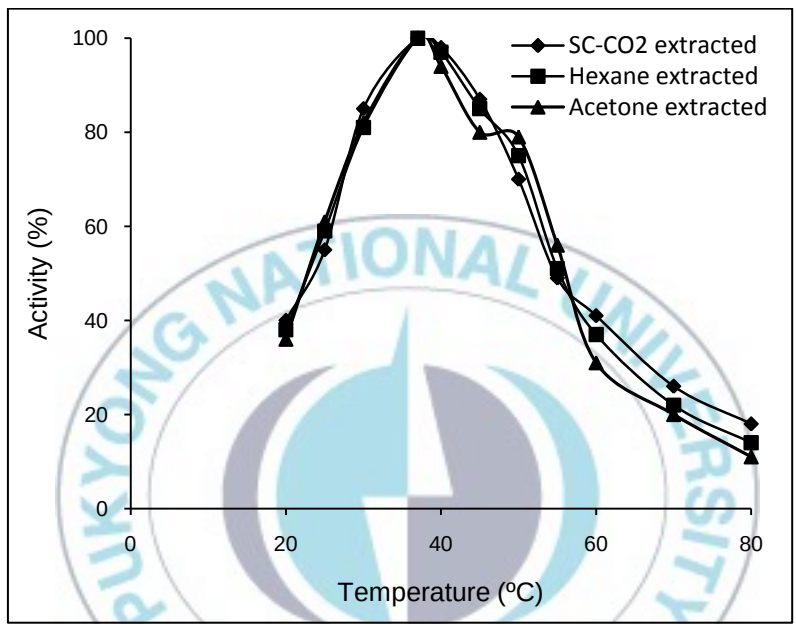
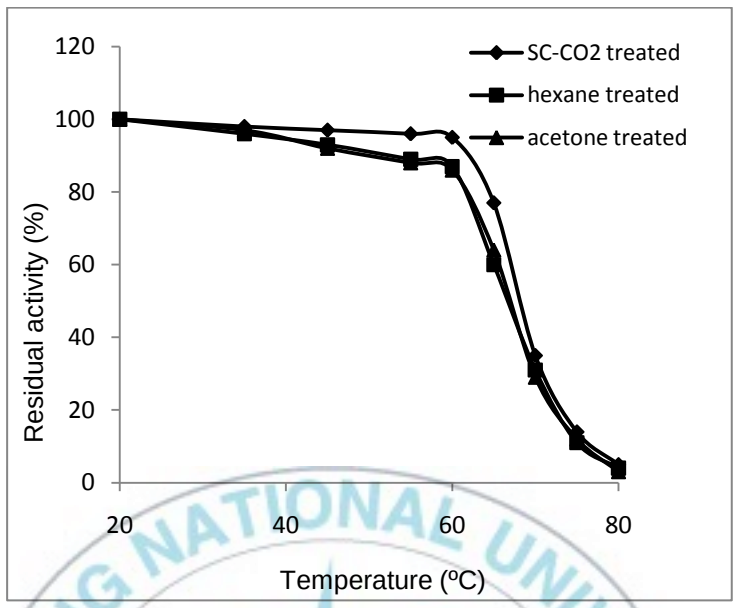


Fig. 4.4A-C: Optimum temperature of digestive enzymes in crude extracts of SC-CO_2, hexane and acetone extracted krill. A) Protease, B) Lipase and C) Amylase

A)



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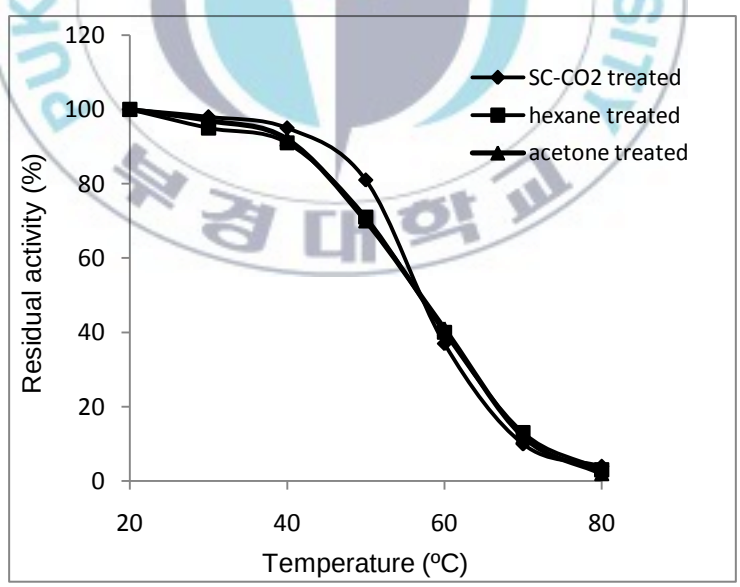

Fig. 4.5A-C: Temperature stability of digestive enzymes in crude extracts of SC-CO_2, hexane and acetone extracted krill. A) Protease, B) Lipase and C) Amylase

C)

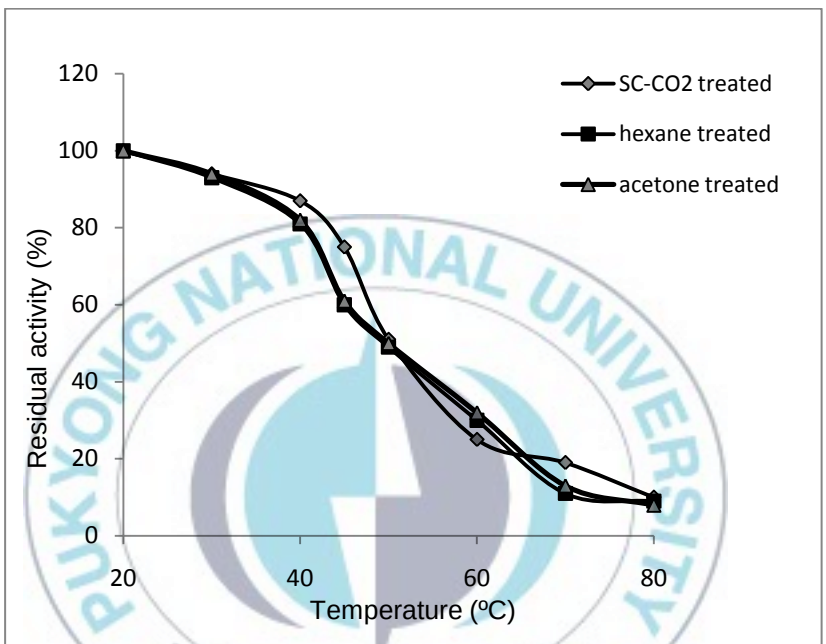

Fig. 4.5A-C: Temperature stability of digestive enzymes in crude extracts of SC-CO_2, hexane and acetone extracted krill. A) Protease, B) Lipase and C) Amylase

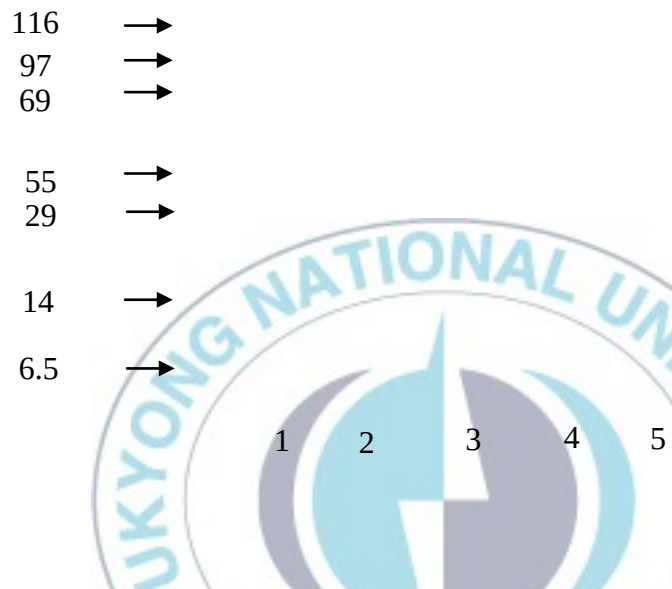

Fig. 4.6: SDS-PAGE electrophoresis of crude protein of untreated and SC-CO_2, hexane and acetone treated krill extracts. Lane 1: Molecular Weight Marker (MWM en kDa). Lane 2: SC-CO_2 treated krill extract. Lane 3: hexane treated krill extract. Lane 4: acetone treated krill extract. Lane 5: untreated krill extract.

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Chapter 5

Production of value added materials by subcritical water hydrolysis from krill residues extracted by SC-CO_2*

Abstract

The aim of this work was the determination of the best experimental conditions for the production of useful materials, such as amino acids by subcritical water hydrolysis from SC-CO_2 extracted krill residues and to compare the results with raw krill. Subcritical water hydrolysis efficiency from raw and deoiled krill was examined over the temperature range of 200 to 280ºC, ratio of material to water for hydrolysis was 1:50 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 of 0.20MPa. 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 deoiled krill hydrolysate was at 280ºC. While, the highest amino acid yield in raw krill hydrolysate was at low temperature 200ºC. 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.

Keywords: Subcritical water hydrolysis; krill; Amino acid; Value added materials

* This work has been submitted to African Journal of Biotechnology

4.6. 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 reaction conditions and often causes serious pollution of the environment. Enzymatic hydrolysis is expensive, and of long production cycle (Cheng and 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 merited in importance, and in economical as well as ecological aspects. 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 (Danielski, 2007). In this study, lipid was extracted from krill for fatty acids and bioactive compounds by supercritical carbon dioxide (SC-CO_2) which is also environmental friendly extraction technology. 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, 2006). After SC-CO_2 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 supercritical extracted residues of krill and also to compare the production with raw krill.

4.7. Materials and Methods

4.7.1. Materials

The freeze dried raw krill was used for SC-CO_2 extraction at the optimum conditions (25 MPa and 45ºC) and for subcritical water hydrolysis. All reagents used in this work were of analytical or HPLC grade.

4.7.2. 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 hrs. Non protein content was estimated by subtracting the sum of weight of moisture, ash, protein and lipid from total weight.

4.7.3. Supercritical CO_2 extraction

The set up of a laboratory scale of SCF extraction process as it is described in chapter 2 can be operated at pressure up to 25 MPa. The extraction of oil from krill was performed at optimum conditions of pressure and temperature (25 MPa and 45°C). Three

run (run1, run2, and run3) were conducted depending on the extraction time (50 min, 100 min, and 150 min) respectively.

4.7.4. Subcritical water hydrolysis

The experimental flow chart of subcritical water hydrolysis unit is depicted in Fig. 5.1. 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_2 (run1, run2, and run3) extracted residues were prepared separately with deionized water to get the homogeneous sample at the concentration of 0.5g/100 ml; and then charged into the reactor. The reactor was filled by chosen reaction atmosphere (nitrogen, air) at 0.20 MPa before to be closed and heated by an electric heater to the desired temperature (200 to 280ºC). 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). 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.

4.7.5. Protein content measurement 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 (Fig. 5.2).

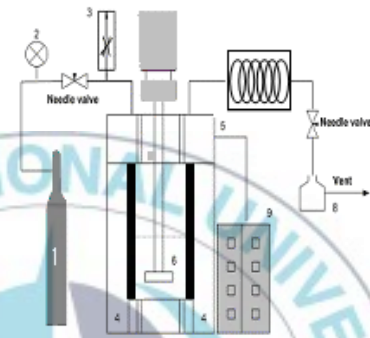
4.7.6. Reducing sugar content measurement of hydrolysates

Reducing sugars content was measured by dinitrosalicylic (DNS) acid method (Miller, 1959) using D-glucose as a standard. Briefly, 3 ml of each hydrolysates were injected into test tubes and 3 ml of DNS reagent was added and well mixed. Then, the test tubes were heated for 5 minutes 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 of the tubes. 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 (Fig. 5.3).

4.7.7. Amino acids Analysis

The hydrolysates of freeze dried raw and different SC-CO_2 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) placed in central laboratory of Pukyong National University.



1: Air tank
2: Pressure gauge
3: Safety valve
4: Electric heater
5: High pressure reactor
6: Impeller
7: Cooling bath
8: Separator
9: Stirring speed &

Fig. 5.1: Flow chart and photograph of subcritical water hydrolysis experimental apparatus

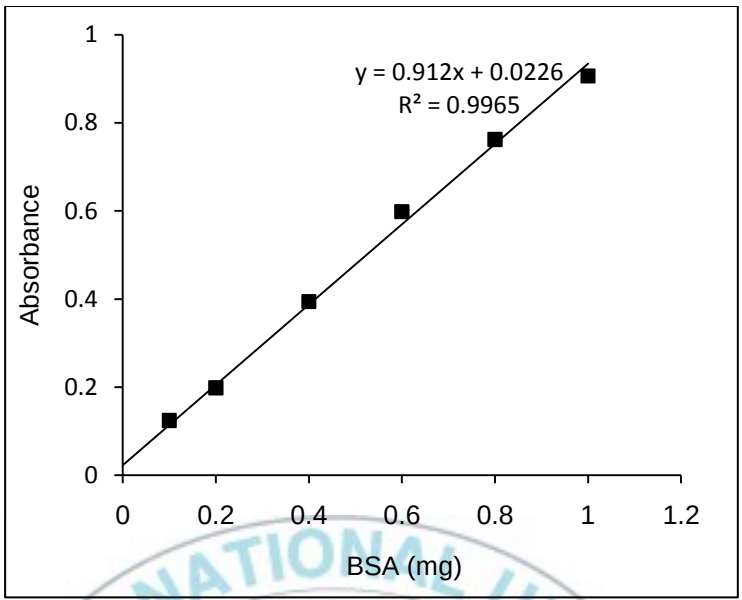


Fig. 5.2: BSA calibration curve for the estimation of protein

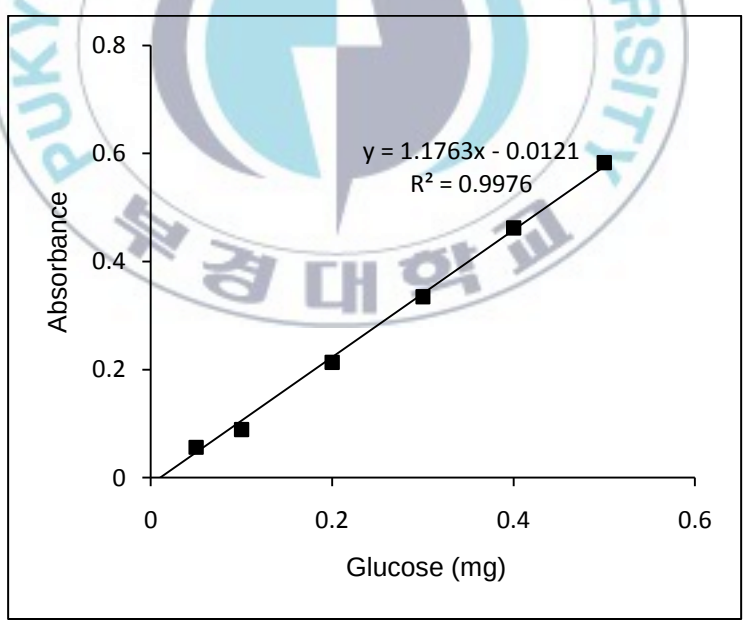


Fig. 5.3: D-glucose calibration curve for the estimation of reducing sugar

4.8. Results and Discussion

4.8.1. Proximate compositions of raw and SC-CO_2 extracted krill residues

The proximate compositions of both raw and SC-CO_2 extracted residues are shown in Table 5.1. In order to get higher efficiency of SC-CO_2 extraction of oil and bioactive compound; the sample was dried in a freeze dryer. In Chapter 2, we have discussed the SC-CO_2 extraction of oil from krill under different conditions. The result showed that the highest yield of oil by SC-CO_2 extraction was approximately 12.2%, while, it was about 16.12% by conventional organic solvent extraction. Thus, SC-CO_2 cannot extract all lipids from the sample. In this work, the lipid content obtained by hexane extraction from SC-CO_2 extracted residues was 4.15±0.11%.The moisture content of raw and SC-CO_2 extracted krill was 3.4±0.31 and 2.61±0.27%, respectively. It was found to be decreased in SC-CO_2 extracted residues. This reduce can be explained by the combination between moisture and CO_2. In the other hand, the protein content in freeze dried raw sample was 62.45±0.35%. In the SC-CO_2 extracted sample, the protein content increased to 73.03±0.53. For the ash and non protein content, high values were observed in SC-CO_2 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– 80% crude protein, 7–30% total lipid, and 8–20% total ash (Grantham, 1977; Savage and Foulds, 1987; Sidhu, et al., 1970).

4.8.2. Protein yield in hydrolysates of raw and SC-CO_2 extracted residues

The protein contents in both hydrolysates at different temperatures are shown in Table 5.2. It was found that the hydrolysate of SC-CO_2 extracted sample 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 hydrolysation. Watchararuji et al. (2008) reported that the protein yield in subcritical water hydrolysis of soybean decreased when temperature increased from 200 to 220ºC. In our study, the protein yield was found to increase with the increase in temperature in the hydrolysate of SC-CO_2 extracted sample. The highest protein yields in raw and SC-CO_2

extracted krill hydrolysates were 391.27±3.71 mg/g at 200ºC and 680.58±3.90 mg/g at 280ºC, respectively. Similar results were reported by Watchararuji 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.

4.8.3. The effect of atmosphere used on amino acid yield

Fig. 5.4 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 were reported by Zhu et al., 2008.

4.8.4. Reducing sugar yields

The reducing sugar content in freeze dried raw and SC-CO_2 extracted sample hydrolysates are shown in Fig. 5.5. 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_2 extracted sample rose with increasing temperature. Thus, within the temperature range of 200 to 280ºC, 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 (Watchararuji et al., 2008). The reducing sugar yield in hydrolysate of SC-CO_2 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_2 extracted krill (Table 5.1).

Table 5.1: Proximate compositions of freeze dried raw and SC-CO_2 extracted krill

Composition[a] (%)	**Raw Krill**	**SC-CO_2 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

[a]Mean value of two replicates (at least) ± S. E.

Table 5.2: Protein yield from freeze dried raw and SC-CO_2 extracted krill residues by subcritical water hydrolysis at different temperatures

Temperature (°C)	**Freeze dried raw sapmple hydrolysate[a] (mg/g)**	**SC-CO_2 extracted sample hydrolysate[a] (mg/g)**		
		Run1	**Run2**	**Run3**
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

[a]Mean value of two replicates ± S.E.

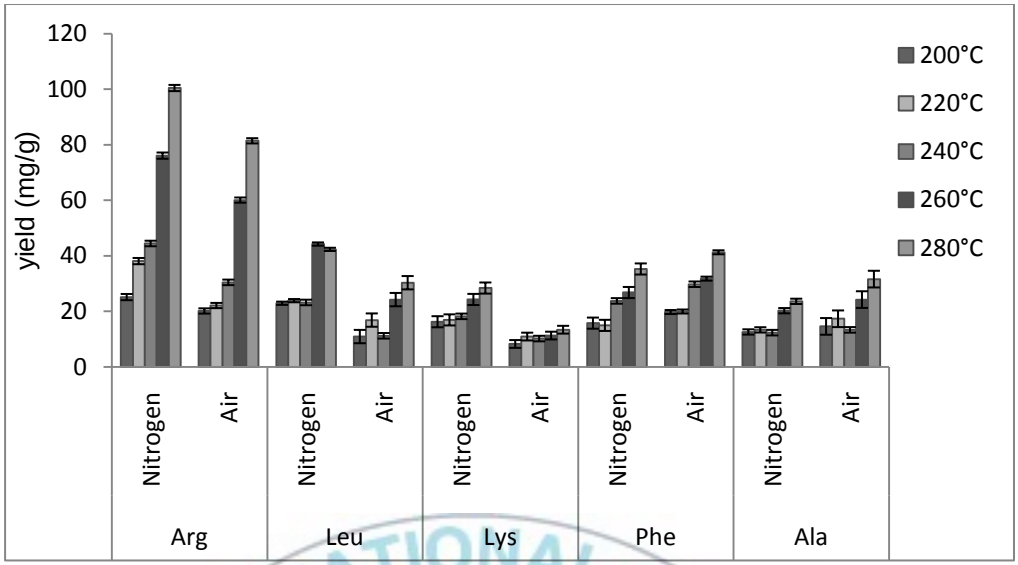


Fig. 5.4: The yield of some amino acids by subcritical water hydrolysis of SC-CO_2 extracted krill under nitrogen and air atmosphere. Data were the mean value of two replicates ± S.E.

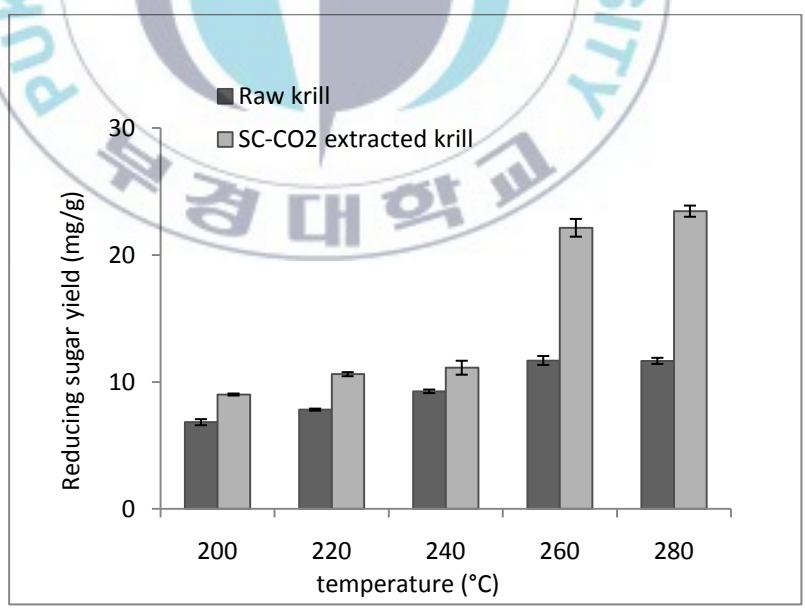


Fig. 5.5: Reducing sugar yield by subcritical water hydrolysis of raw and SC-CO_2 extracted krill at different temperatures. Data were the mean value of two replicates ± S.E.

4.8.5. 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 nine 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 hydrolysates 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). Fig. 5.6 shows the total amino acid yield in raw and SC-CO_2 extracted sample hydrolysates. The total amino acid yield of SC-CO_2 extracted sample hydrolysates was higher than that of raw sample hydrolysates. This result agreed with the high protein yield of SC-CO_2 extracted sample hydrolysates comparing to raw sample hydrolysates. For SC-CO_2 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_2 extracted sample hydrolysate was 537.78±4.13 mg/g sample at 280ºC.

For raw krill hydrolysates, the total amino acid yield was found to be decreased with the increasing temperature contrary to the results obtained from the SC-CO_2 extracted sample. The highest amino acid yield in raw sample hydrolysates was 190.65±2.74 mg/g raw sample at 180ºC. At high temperature, the total amino acid yield in raw sample hydrolysate was low. However, the amino acid yield was higher in SC-CO_2 extracted sample. Thus, this contrast can be explained by the presence of oil in the raw material which may possibly have interfered 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_2 extracted sample. It was found that the efficiency of subcritical water hydrolysis for amino acid yield was highest in SC-CO_2 run3 (150 min of extraction) extracted sample (82%) comparing with the run2 (100 min) and run1 (50 min), respectively (Fig 5.7). 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 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.

The Fig 5.8A and B showed the yield of the main amino acids recovered from raw and SC-CO_2 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 250-300ºC (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 200-290ºC. In this study, the highest yield of amino acids from SC-CO_2 extracted sample was obtained within the temperature of 260-280ºC. For raw sample hydrolysates, the highest yield was obtained within 200-220ºC.

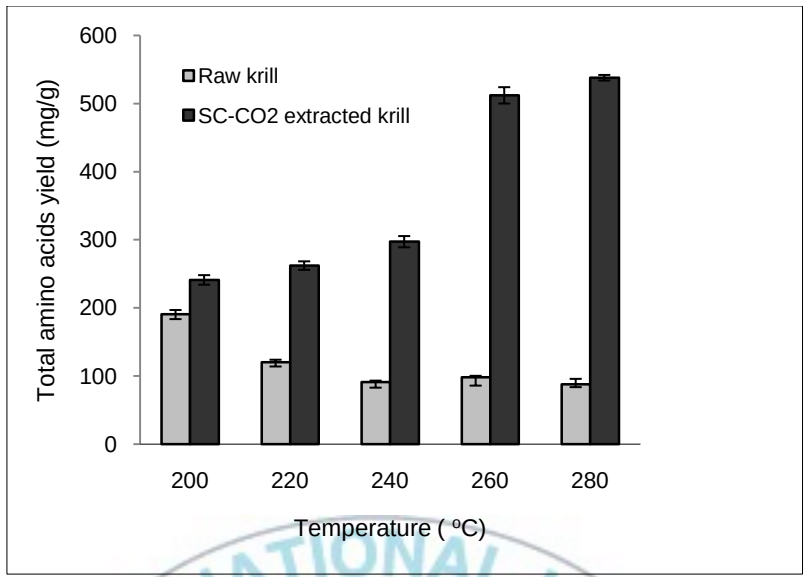


Fig. 5.6: Total amino acid yield by subcritical water hydrolysis of freeze dried raw and SC-CO_2 extracted krill at different temperatures. Data are the mean value of two replicates ± S.E.

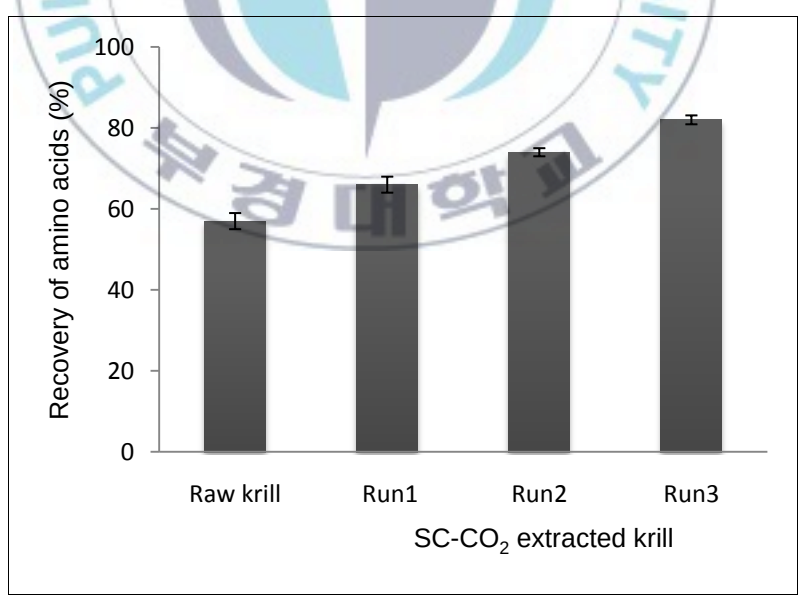
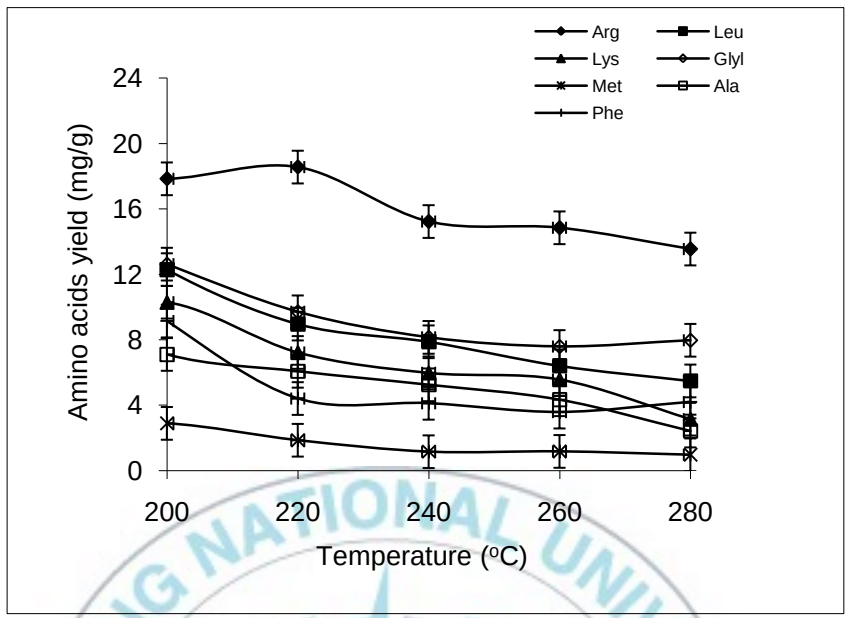


Fig 5.7: Recovery of amino acids from raw and SC-CO_2 extracted krill by subcritical water hydrolysis

A)



B)

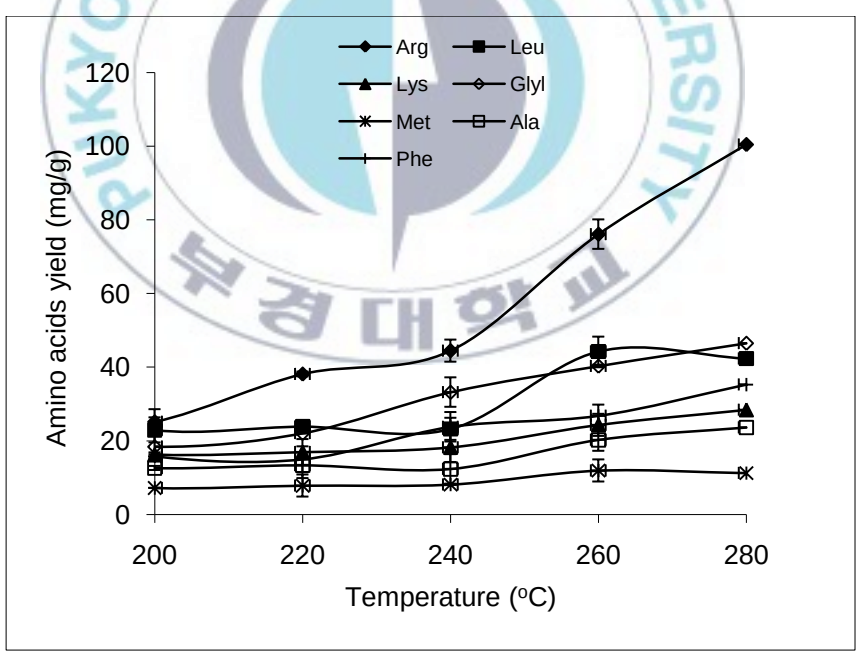


Fig 5.8A-B: The amino acids yield in hydrolysates at different temperatures. A) Raw krill, B) SC-CO_2 extracted residues. Data were the mean value of two replicates ± S.E.

4.9. 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_2 extracted krill were obtained in the hydrolysates at high temperature. Result showed 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_2 extracted sample the highest yield was found at high temperature. The recovery of the amino acids from SC-CO_2 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_2 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_2 extracted krill, respectively. In the short reaction time, subcritical water hydrolysis was more effective for amino acid recovery from SC-CO_2 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.

5.5 . References

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Summary

From overall studies we conclude that:

- Krill oil obtained by SC-CO_2 extraction would be a good source of PUFAs especially EPA and DHA and also a natural antioxidant, astaxanthin.

- The oil obtained by SC-CO_2 extraction showed more stability than organic solvent, hexane extracted oil.

- Fluorine content in the krill sample was reduced after SC-CO_2 extraction.

- The phospholipids purified from SC-CO_2 extracted krill residues showed high oxidative stability.

- The main composition of krill phospholipids were PC and PE with higher amounts of PUFAs mainly EPA and DHA.

- Despite the fact that the activities of principal digestive enzymes of SC-CO_2 extracted krill residues were reduced slightly compared to hexane and acetone extracted residues, it proved the applicability of SFs in enzyme processing.

- The majority of the proteins from SC-CO_2 extracted krill residues were obtained as amino acids by subcritical water hydrolysis.

To conclude, the quality oil including the bioactive compounds would be extracted by SC-CO_2 which is an environmental friendly solvent. Moreover, Phospholipids, proteins as amino acids and enzymes might be obtained from SC-CO_2 extracted krill for using in the food industry as well as in the pharmaceutical and cosmetic industry.

아임계-초임계유체공정를 이용하여남극 크릴새우(*Euphausia superba*) 로부터 얻어진생리활성 물질의 특성

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요 약

남극 크릴새우 (*Euphausia superba*)는 난바다곤쟁이목 난바다곤쟁이과에 속하는 종으로 남극해 생태계의 중심역할을 한다. 다른 수산자원들에 비하여 높은 생체량(60-155 만 톤)을 가지며 수산업에 새로운 가능성이 대두되고 있는 자원이다. 현재, 남극 크릴새우는 낚시, 양식업 그리고 식용 등 대부분 상업적으로 사용되고 있다. 그러나 불포화지방산(PUFAs), 주로 오메가-3 지방산이 풍부한 고품질의 저지방 동물성 단백질이 풍부한 자원이며, 항산화능이 다른 어류에 비하여 특이적으로 높다. 또한, 가공 후 부산물은 부가가치상품으로 사용이 가능하다. 본 연구에서는 아임계 및 초임계 유체(SCF) 공정을 통하여 남극 크릴새우로부터 생리활성물질을 추출하고 그 특성을 조사하였다.

먼저, 친환경적 공정인 초임계 이산화탄소 (SC-CO_2) 추출법과 hexane 을 이용한 유기용매 추출법을 통하여 남극 크릴새우 오일을 추출하였다. SC-CO_2 추출은 온도 범위 35 - 45°C, 압력 15 -25 MPa 범위에서 수행하였으며 CO_2 유량은 22g/min 로서 총 추출 시간인 2 시간 30 분 동안 지속적으로 흐르게 하였다. 오일의 최대 추출 수율은 온도와 압력이 높을수록 높았다. 오일의 지방산 분석을 위해 gas chromatography (GC)를 이용하였다. 그 결과, 고도불포화지방산(PUFAs)의 함량이 높았으며, 그 중 특히 eicosapentaenoic acid (EPA) 와 docosahexaenoic acid (DHA) 의 함량이 높았다. SC-CO_2 를 이용하여 추출한 남극 크릴새우 오일와 hexane 으로 추출한 오일의 품질을 비교해 본 결과 SC-CO_2 추출 오일이 hexane 추출 오일보다 높은 안정성을 보였다. 또한 high performance liquid chromatography (HPLC) 를 이용하여 각기 다른 추출조건에 따른 astaxanthin 함량을 비교 분석한 결과 25MPa, 45℃ 추출조건에서 astaxanthin 의 수율이 가장 높음을 확인하였다. 이는 SC-CO_2 가 남극 크릴새우로부터 지질을 효과적으로 얻을

수 있음을 나타낸다. 그리고 남극 크릴새우의 fluorine 함량에 대한 SC-CO_2 추출 효과를 조사하였다. 그 결과, SC-CO_2 추출하는 동안 fluorine 함량은 초기농도의 40%정도 줄어들었다. 이러한 결과를 확인하기 위하여 새로운 SC-CO_2 추출과정 설계가 추가적으로 필요하다.

제 2 장에서는 남극 크릴새우의 SC-CO_2 추출 후 부산물로부터 인지질을 분리, 특성을 파악하였다. 인지질 정제는 순도 93-97%범위로 HPLC -ELSD 로 정량 분석하였으며 정제된 인지질은 산, 과산화수소 값 및 산화안정성을 조사하였다, Thin layer chromatography (TLC)를 수행하여 각각의 인지질을 정제하였으며, 총 인지질, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI)의 조성은 GC 로 분석하였다. 그 결과, 남극 크릴새우 인지질의 산화안정성은 불포화지방산 함량이 높음에도 불구하고 높게 나타났다.

제 3 장에서는 효소정제시스템에서 SC-CO_2 의 적용가능성을 조사하였다. 남극 크릴새우로부터 SC-CO_2, hexane, acetone 으로 오일을 추출한 후, 주요 3 가지 소화효소에 대하여 조사하였다. 먼저 최적조건 (25 MPa, 45°C) 에서 추출된 남극 크릴새우의 추출 부산물 내의 소화효소 특성을 조사한 결과, 남극 크릴새우의 SC-CO_2 추출 부산물은 hexane, acetone 의 유기용매 추출 부산물과 비교하여 프로테아제, 리파아제, 아밀라아제의 활성을 가장 감소시켰다. 또한 SC-CO_2 추출 부산물의 모든 소화효소들이 높은 열안정성을 보였지만 SC-CO_2 와 hexane, acetone 의 추출 부산물 모두 각각의 소화효소들에 대하여 거의 동일한 최적 pH, pH 안정성을 보였다. SDS-PAGE 를 이용한 결과, SC-CO_2, hexane, acetone 을 처리하거나 처리하지 않은 남극 크릴새우의 조추출물에서 단백질 패턴의 큰 차이를 보이지 않았으며, 이는 단백질 변성이 일어나지 않았음을 나타낸다.

제 4 장에서는 남극 크릴새우의 SC-CO_2 추출 부산물로부터 고부가가치 소재 생산을 위한 아임계 수 가수분해공정을 수행하였으며, 이를 미가공 크릴새우와 비교 분석하였다. 남극 크릴새우의 탈지시료와 원시료의 아임계 수 가수분해능을 알아보기 위하여 200°C 에서 280ºC 의 조건하에서 시료와 물의 비율을 1:50 으로 하고 아미노산 분해를 줄이기 위하여 반응시간은 5 분 동안 수행하였다. 또한 0.25MPa 의 질소와 공기가 사용되었다. 글라이신, 아르기닌, 류신의 가수분해능은 높은 온도에서 높은 용해도를 가지듯이 수온이 증가됨에 따라 증가되었다. 오일이 제거된 남극 크릴새우 가수분해물의 아미노산 수율은 280 °C 에서 가장 높았다. 반면, 처리하지 않은 남극 크릴새우의 가수분해물의 아미노산

수율은 200 °C 에서 가장 높았다. 또한, 환원당 수율도 오일이 제거된 남극 크릴새우 가수분해물에서 더 높게 나타났다.

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