Thesis for the Degree of Doctor of Philosophy

Recovery of Bioactive and Valued Materials from Squid (*Todarodes pacificus*) Viscera Using Sub- and Supercritical Fluids

by

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The Graduate School

Pukyong National University

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(아임계 및 초임계 유체를 이용한 오징어 (*Todarodes pacificus*) 내장으로부터 유용성분 회수)

Advisor: Prof. Byung Soo Chun

by

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Dedicated

To My Parents, Wife and Lovely Daughter

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Abstract

Squid is a popular food in many countries including Korea and Japan. Fish processing industries produce lot of squid viscera as a waste product. It contains lot of biomolecules that are useful to living beings. The disposal of these waste products is also a big problem for the fish processing industries. However, recovery of useful materials from squid viscera will be beneficial economically and environmentally. In this study, squid (*Todarodes pacificus*) viscera 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 the temperatures ranging from 35 to 45ºC and the pressures ranging from 15 to 25 MPa. The flow rate of CO_2 (22 g/min) was constant at the entire extraction period of 2.5 hrs. The highest oil yield was found at higher extraction temperature and pressure. The extracted oil was analyzed by gas chromatography (GC) for fatty acid compositions. The oil obtained by SC-CO_2 extraction contained high percentage of polyunsaturated fatty acids (PUFAs) especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The quality of squid viscera oil obtained by SC-CO_2 extraction was compared with that of hexane extracted oil. The SC-CO_2 extracted oil showed more stability than the oil obtained by hexane extraction. The amount of astaxanthin in squid viscera oil was determined by high performance liquid chromatography (HPLC) and compared at different extraction conditions. In SC-CO_2 extraction, the highest yield of astaxanthin was found in squid viscera oil extracted at 25 MPa and 45ºC.

Marine lecithin was isolated and characterized from squid viscera residues deoiled by SC-CO_2 extraction. The major phospholipids of squid viscera lecithin were quantified by HPLC. Phosphatidylcholine (PC- 80.54±0.68%) and phosphatidylethanolamine (PE- 13.18±0.24%) were the main phospholipids. Thin layer chromatography (TLC) was performed to purify the individual phospholipids. The fatty acid compositions of lecithin, PC and PE were analyzed by GC. A

significant amount of EPA and DHA were present in both phospholipids of PC and PE. Emulsions of lecithin in water were prepared by a homogenizer. The oxidative stability of squid viscera lecithin was high in spite of its high concentration of long chain polyunsaturated fatty acids (LC-PUFAs).

Subcritical water hydrolysis was carried out to produce valued materials from squid viscera, the byproduct of fish processing industries. The reaction temperatures for hydrolysis of freeze dried raw and SC-CO_2 extracted squid viscera were maintained from 180 to 280ºC for 5 min. The ratio of material to water for hydrolysis was 1:50. Most of the proteins from SC-CO_2 extracted squid viscera were recovered at high temperature. The protein yield in raw squid viscera hydrolyzate decreased with the rise of temperature. The reducing sugar yield was higher at high temperature in subcritical water hydrolysis of both raw and SC-CO_2 extracted squid viscera. The highest yield of amino acids in raw and SC-CO_2 extracted squid viscera hydrolyzates were 233.25±3.25 and 533.78±4.13 mg/g at 180 and 280ºC, respectively. Most amino acids attained the highest yield at the reaction temperature range of 180-220ºC and 260-280ºC for raw and SC-CO_2 extracted samples, respectively. The recovery of amino acids from SC-CO_2 extracted squid viscera was about 1.5 times higher than that of raw squid viscera.

Finally, the applicability of SC-CO_2 in enzyme purification system was checked. Three major classes of digestive enzymes of squid viscera were characterized following extraction of oil by SC-CO_2 and hexane. The highest oil extracted residues of squid viscera (25 MPa and 45ºC) were used to characterize the digestive enzymes. The activities of protease, lipase and amylase were highest in hexane extracted squid viscera samples and lowest in SC-CO_2 extracted samples. The crude extracts of SC-CO_2 and hexane extracted squid viscera samples had almost same optimum pH and pH stability for each of the digestive enzymes. The optimum temperature of protease, lipase and amylase were found to be very similar in SC-CO_2 and hexane extracted samples. However, the thermal stability for each digestive enzyme in SC-CO_2 extracted squid viscera were slightly higher than that of hexane extracted squid viscera. Studies using SDS-PAGE showed no significant differences in protein patterns of the crude extracts of freeze dried raw and SC-CO_2 and hexane extracted squid viscera, indicating that the proteins were not denatured.

Chapter 1

General introduction

1.1. Background

In 2008, the annual world capture production of fish was about 90.8 million metric tons (FAO, 2010). One-third of the catch fish is not utilized for human consumption and considered as fishery by-products (Barlow & Windsor, 1984). The processing of fish for filleting, canning, and surimi production results in an immense quantity of by-products which include trimmings, belly flaps, heads, frames, fins, skins, and viscera (Choudhury & Bublitz, 1996). Every year, thousands of tons of fish by-products of high nutrient content are dumped or discarded by fish processing plants throughout the world. Discarding these by-products creates two major problems. The first one is the underutilization of a huge amount of nutrients such as protein, minerals, oil and functional materials. Second, disposal of such huge quantities of highly polluting organic matter contributes to major environmental and economic problems. The fish processing industry is faced with the need to develop efficient by-product recovery and utilization methods to comply with the pollution control regulations (Choudhury & Bublitz, 1996). However, the processing of fish by product as resources may be benefited economically and also environmentally.

Among the fishes, the captured squid comprises a significant portion of all total fisheries. Squid is a common marine resource which is popular and in demand in the market and is an important species for commercial fisheries. As like other fishes, internal organs or viscera from squid are an abundant and underused byproduct that can be a unique source of oil with polyunsaturated fatty acids (PUFAs), lipid soluble bioactive compounds, amino acids, functional proteins and others.

1.2. Squid

Squid are marine cephalopods of the order Teuthida, which comprises around 300 species. Fisheries for cephalopods, especially squid, have attracted interest worldwide

over the last two decades. Cephalopods make up only 3% of global fisheries catches, but have the third highest commercial value, after shrimp and tuna (Suaer et al., 2002). Declining catches in many traditional fisheries have led to increased effort to develop the potential of non-traditional species, especially invertebrates such as the cephalopods. More than 2 million metric tons of squid are landed annually throughout the world. Although almost a hundred species of squid are fished commercially, two species, Japanese flying squid (*Todarodes pacificus*) and Argentine shortfin squid (*Illex argentinus*), account for over half the world's squid harvest (Robert Mazurek, 2006). In 2008, only Japanese flying squid has been caught about 402,350 metric ton (FAO, 2010). Estimates of global squid consumption by higher predators, especially sperm whales suggest that they consume a greater mass of squid than the total world catch of all marine species combined (Voss, 1973; Clarke, 1983). This is interesting because of both the commercial potential of squid fisheries and the role they might have in the provision of high quality protein for human consumption (Caddy, 1983; Roper et al., 1984).

Generally, cephalopods "live fast and die young" (O'Dor and Webber, 1986; Boyle 2002). Their life cycles are short ranging from 6 to 18 months. It is difficult to predict their recruitment due to high variability (Pauly, 1985; Moltscaniwskyj et al., 2002). They also exhibit wide fluctuations in abundance. Table 1.1 shows the life history and population characteristics of commonly landed three squid species.

1.2.1. *Todarodes pacificus*

Japanese common squid, *Todarodes pacificus*, belongs to the family of Ommastrephidae. It is a commercially important species for Japan, Korea and China (Kang et al., 2005). Like all other cephalopods, *T. pacificus* have a distinct head, bilateral symmetry, a mantle, eight arms and two longer tentacles arranged in pairs (Fig. 1.1). It is one of the most heavily exploited squid species in the world (FAO, 2003). Up to three or four intraspecific stocks of *T. pacificus* are recognized, based primarily on spawning seasons, spawning sites, growth rates, size at maturity, fecundity and migration patterns (Katugin, 2002). Its spatial distribution ranges from the Kamchatka Peninsula of Russia to Taiwan of China, and is especially abundant around Japan. The autumn spawning population, mainly distributed in the Sea of Japan, has become the major fishing target

since the 1970s. The annual catch from this population by Japan, Korea and China in the Sea of Japan varies between 210 and 320 thousands metric tons since 1995 (Wang and Chen, 2005).

1.2.1.1. Habitat and biology of *T. pacificus*

An oceanic and neritic species occurring within abroad temperature range from about 5 to 27ºC, usually in surface waters to 100 m depth and, to a minor extent, down to 500 m depth. During its lifespan of about 1 year a northward migration occurs first, followed by another one in southward direction, usually in close correlation with changes of the main surface currents. Fig. 1.2 shows early life cycle of an Ommastrephid squid. Large aggregations occur in small gyres and along oceanic fronts. Three independently breeding subpopulations can be distinguished in Japanese waters. The main group spawns in winter in the East China Sea, the second in autumn, west of Kyushu, and the third, minor group in spring/summer in the Sea of Japan as well as off northeastern Japan. Post spawning mortality is very high. The males of all 3 subpopulations mature before the females and transfer their spermatophores on the still immature females (in water temperatures of 13 to 18° C). With the progressing southward migration, females mature and spawn 300 to 4000 small, elliptical or semi-spherical eggs (greatest diameter 0.7 to 0.8 mm) embedded in a gelatinous capsule (egg mass). Spawning occurs usually at water temperatures between 15 and 20°C and depending on the temperature, the larvae hatch after an incubation period of 102 to 113 hours. Growth rates are directly related with temperature and inversely with size. Main food items are myctophids, anchovies (i.e., *Engraulis japonicus*), crustaceans, gastropod larvae, and chaetognaths. Cannibalism is common. Predators include rays, dolphins (*Coryphaena hippurus*), balaen whales and the northern fur seal (Roper et al., 1984).

Table 1.1. Life history and vulnerability characteristics of commonly landed squid species (O'Dor and Webber, 1986; Nesis, 2002)

Species	Intrinsic Rate of Increase	Age at 50% Sexual Maturity	Maximum Age	Reproductive Potential	Species Range	Special Behaviors	Population Variability
Ommastrephes bartramii	Unknown	Unknown	12-18 months	Unknown, but thought to be highly fecund	Pacific Ocean/shelf regions	Aggregates to spawn	Susceptible to changes in oceanographic conditions
Todarodes pacificus	Unknown	8-12 months	12-18 months	Unknown, but thought to be highly fecund	Throughout Pacific Ocean	Aggregates to spawn	Susceptible to changes in oceanographic conditions
Illex argentinus	Unknown	6-8 months	10-18 months	Unknown, but thought to be highly fecund	Shelf and oceanic waters off Eastern South America	Aggregates to spawn	Susceptible to changes in oceanographic conditions

This table has been adopted from Mazurek (2006).

Fig. 1.1. Photograph and schematic representation of *T. pacificus*.

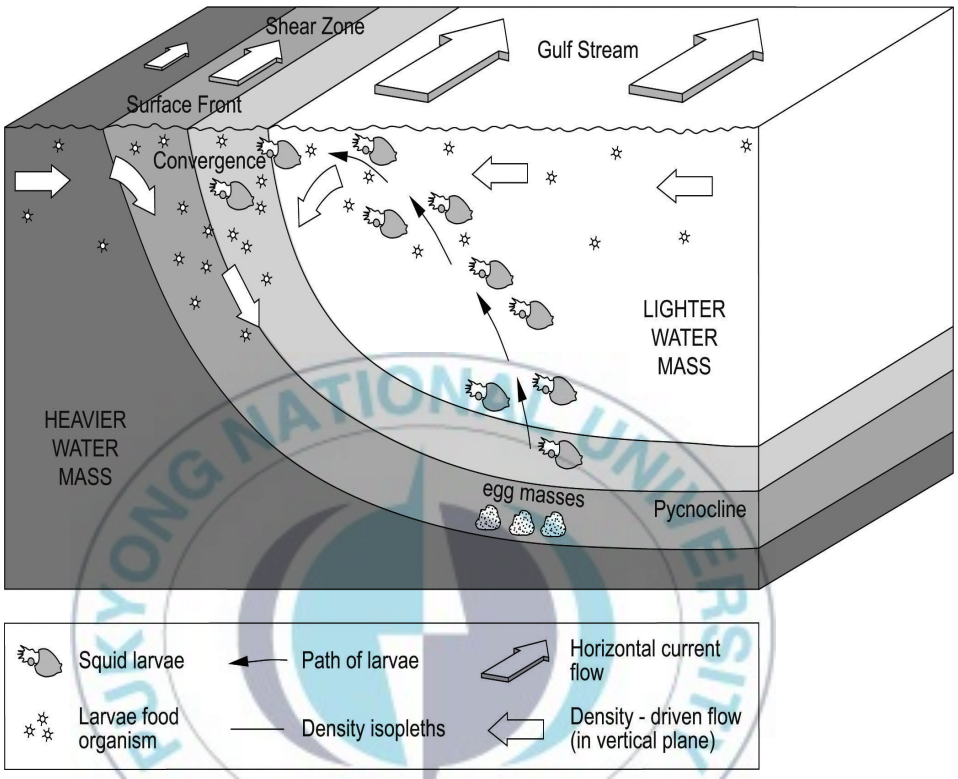

Fig. 1.2. Schematic diagram showing the early life cycle of an Ommastrephid squid (Bakun and Csirke, 1998).

1.3. Polyunsaturated fatty acids

Marine organisms are the unique source of polyunsaturated fatty acids (PUFAs). PUFAs, especially ω-3 fatty acids, have been very often subject of scientific studies in the last years. The ω-3 fatty acids have proven beneficial effects in the prevention of some diseases. The health benefits of ω-3 fatty acids are shown in following sections.

1.3.1. Cardiovascular diseases

The ω-3 fatty acids have been shown to have beneficial effects on the cardiovascular system. The risks of suffering from diseases such as diabetes, obesity, asthma, and others have been shown to decrease with increasing ω-3 consumption (Simopoulos, 2002). Effects of ω-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; Arkhipenko and Sazontova, 1995). Recent studies have shown that ω-3 fatty acids may be incorporated into cardiac cell membranes, leading to cardioprotective effects against several diseases (Masson et al., 2007). Schacky and Harris (2007) have proposed using an "ω-3 index" as an indication of overall heart health. Their research has shown that measuring the combined percentage of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) out of the total fatty acids in red blood cell membranes is as accurate as the currently used low-density lipoprotein cholesterol measurement for heart health. Patients with higher ω-3 indices are less likely to experience a sudden cardiac death.

1.3.2. Cancers

ω-3 fatty acids clearly have beneficial effects against several cancers. It is clear that some properties of PUFAs make them attractive options in the treatment of cancer (Jiang et al., 1998). The mechanisms that lead to these effects are not fully understood, but it is clear that ω-3 fatty acids can be useful in fighting various cancers. The proliferation of pancreatic cancer cells is inhibited after treatment with ω-3 fatty acids (Hering et al., 2007). It is thought that the ω-3 fatty acids may restore certain regulatory processes within the cells. With these processes restored, the cells have "restored apoptosis" (i.e. the cells are no longer chemoresistant) and can be fought by the immune system or traditional

cancer treatments (Hering et al., 2007). ω-3 fatty acids can also help prevent colorectal cancer (Nowak et al., 2007). Many studies have shown that fish oil containing PUFAs has important roles in prevention of some types of cancer, including colon (Nano et al., 2003; Moyad, 2005; Jiang et al., 1998), breast (Horia and Watkins, 2005; Mahéo et al., 2005; Barascu et al., 2006; Jiang et al., 1998), renal (Smyth and McGlynn, 2005; Moyad, 2005), prostate, pancreatic cell and liver (Jiang et al., 1998; Moyad, 2005).

1.3.3. Human immune and inflammatory responses

The ω-3 family of PUFAs possesses anti-inflammatory activity in vitro and in vivo (Pacht et al., 2003; Simopoulos, 2002a; Hong et al., 2003). During the past years, many studies have investigated the effects of ω-3 PUFA on human immune and inflammatory responses (Kelley, 2001; Calder, 2001; Uauy and Valenzuela, 2000; Horrocks and Yeo, 1999; Curtis et al., 2004; Teitelbaum and Walker, 2001). The effects of long chain PUFA supplementation on different physiologic functions may be explained by production of various eicosanoids (Uauy and Valenzuela, 2000). Eicosanoids are a second group of chemical messengers which act within the immune system (Calder, 2001). These compounds provide a link between PUFAs, inflammation and immune functions. Eicisanoids are synthesised from PUFAs and include prostaglandins (PG), thromboxanes, leucotrienes (LT), lipoxins, hydroperoxyeicosatetraenoic acids (HPETE) and hydroxyeicosatetraenoic acids (HETE). Prostaglandins, prostacyclins, throboxanes, and leucotrienes derived from long chain PUFA play a key role in modulating inflammation, cytokine release, immune response, platelet aggregation, vascular reactivity, thrombosis, and allergic phenomenon (Uauy and Valenzuela, 2000).

1.3.4. Psychiatric effects: Alzheimer's and Schizophrenia

Alzheimer's is a devastating disease affecting the elderly. While no clinical trials have been performed on human subjects, it has been shown that withholding DHA from the diets of rats led to a deficit in higher order learning ability (Catalan et al., 2002; Hashimoto et al., 2002), similar to the effects of Alzheimer's. Furthermore, Little et al. (2007) found that aged rats given a daily dose of 10 mg of DHA experienced less age-related impairment in long-term potentiation than control rats. This effect was attributed

to changes in brain membrane fluidity caused by the DHA. ω-3 fatty acids also have other effects on the brain. Branchey and Branchey (2008) found that substance abusers that were treated daily with 3 grams of ω-3 fatty acids for 3 months became less angry or aggressive than patients given a placebo. This result could be due to modification of serotonergic neurotransmitters. Increasing the serotonin level in the brain eventually resulted in less aggressiveness in patients (Branchey and Branchey, 2008).

Studies have also shown that ω-3 fatty acids have beneficial effects on treating human schizophrenia. Peet and Stokes (2005) state that "five of six double-blind, placebo-controlled trials in schizophrenia" reported therapeutic benefits from ω-3 fatty acid supplementation. These beneficial effects are due to alterations in the fatty acid composition of brain membranes that lead to changes in "several intra-neural signal transduction systems" (Peet and Horrobin, 2002).

1.3.5. Infant brain and vision development

DHA plays an important role in the development of the brain and vision in infants. Makrides et al. (1995) showed that infants fed with DHA-supplemented formula exhibited improved visual acuity over infants fed with standard formula. However, infants that were breastfed exhibited even higher performance levels, perhaps due to some unknown ingredients in the maternal milk. Children who received formula with no supplementation had significantly lower scores, showing the ω-3 fatty acid supplementation is of utmost importance at the early stages of life (Birch et al., 2007).

ω-3 PUFAs are very essential for the brain (Garcia et al., 2004; Meza et al., 2003). The tissue of brain is particularly rich in DHA, showing a close correlation between the consumption of this acid and its deposition in the cellular membrane. DHA is particularly abundant in the membranes of retinal photoreceptors and in neural tissue, especially in the grey matter of the brain, comprising 30-50% of the lipids in these tissues (Arterburn et al., 2000). DHA take part in the brain development and retina formation of a child during pregnancy (Garcia et al., 2004) and the most rapid rate of retinal development and brain growth occurs in third trimester of gestation, and DHA accretion rates are highest during this period. Brain development and DHA accretion continue at a rapid pace throughout the first 2 years of life (Arterburn et al., 2000). Long-chain polyunsaturated

fatty acid (LC-PUFA) composition of neural membranes is a key factor for brain development (Högyes et al., 2003) and a normal adult brain contains more than 20 g of DHA (Paez et al., 2002). DHA is required for maintenance of normal brain functions in adults (Horrocks and Yeo, 1999). Maintaining concentrations of PUFA is likely to favour enhanced cognitive, learning and memory functions (Youdim et al., 2000).

1.4. Supercritical fluids (SFs)

The term 'supercritical fluid (SF)' describes a gas or liquid at conditions above its critical temperature and pressure - i.e., above the critical point. It can diffuse through solids like a gas, and dissolve materials like a liquid. SFs exhibit a liquid-like density, while their viscosity and diffusivity remain between gas-like and liquid-like values.

Generally, lipids are extracted by organic solvents. Several methods have been reported for extracting fish oils, with varying yields. Extraction and purification 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; Staby & Mollerup, 1993; Sahena et al., 2010). In recent years, the use of supercritical fluid extraction (SFE) 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. SFE using CO_2 is a promising process for the extraction and fractionation of edible oils containing labile PUFAs and lipid soluble bioactive compounds.

1.4.1. History of SFs

In 1822, Baron Charles Cagniard de la Tour (1822) 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. 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. Hannay and Hogarth (1879) were the first workers who demonstrated the solvating power of SFs for solids. A few years later, Eduard Buchner became the first in a long line of researchers to measure the solubility of a model compound, naphthalene, in supercritical carbon dioxide. He also reported that the solubilities of certain nonvolatile organic materials in CO_2 under supercritical conditions (Buchner, 1906). The orders of magnitude in SFs were higher than that of expected from vapour pressure alone.

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.

A significant development in SFE had been done by Zosel's in seventy decade who provided incentive for extensive future works (Stahl et al., 1988). He reported the decaffeination of green coffee with supercritical carbon dioxide (SC-CO_2). That decaffeination was accomplished by soaking the beans in water and then immersing them in SC-CO_2. From 1980, there has been rapid 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.

In 1990, the joint association for the advancement of SF technology was formed in the United States to develop and disseminate knowledge regarding the application of SFs for cleaning purposes (Taylor, 1996). Basically, the application of SFs has been extended to the field of environment, energy, chemistry etc. since 1990. Now a day, SFs are used in different sector including in bio-industries for specified functional materials.

1.4.2. Properties of SFs

The properties of SF 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. 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. Fig. 1.3 shows the pressure-temperature phase diagram of SFs. 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).

Solvating strength depends on the density of SFs. High density causes higher efficiency. On the other hand, density depends on temperature and pressure. At low pressure the solvent power of SFs decreases with rising temperature, whereas at high pressures it 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 exhibit physicochemical properties intermediate between those of a liquid and a gas. SFs show liquid like density at high pressure which affords good solvating power and mass transfer relative to liquid is rapid. High diffusivity and low viscosity substances can easily penetrate the material. SF is able to diffuse through solids like a gas, and dissolve materials like a liquid. Because, diffusivity of SF is higher than liquid but viscosity is 10-100 times lower than liquid (Taylor, 1996).

Since at high pressure SF has good density, high diffusivity and low viscosity it shows high solvating strength (Table 1.2). This is the main advantages of SFs in which their physical properties resemble those of both liquids and gases. The combination of low viscosities and high diffusion coefficients found in SFs 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. High diffusion coefficients will improve mass transport, and mass transfer rates in SC-CO_2 are higher than in conventional organic solvents. Critical properties of some solvents are shown in Table 1.3.

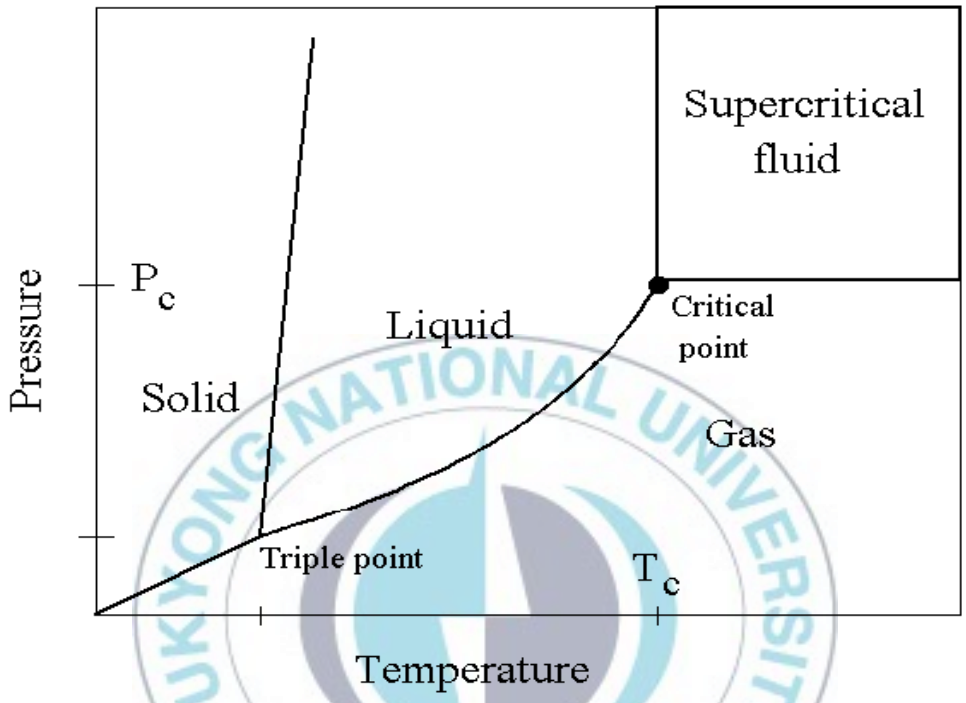

Fig. 1.3. Pressure-temperature phase diagram of a substance with critical temperature (T_C) and pressure (P_C).

Table 1.2. Physical properties of gases, compressed gases and liquids

	Density (g/mL)	Dynamic viscosity (g/cm-see)	Diffusion coefficient (cm^2/sec)
Gas (ambient)	0.0006-0.002	0.0001-0.003	0.1-0.4
Supercritical fluid (critical temperature and pressure)	0.2-0.5	0.0001-0.0003	0.0007
Liquid (ambient)	0.6-1.6	0.002-0.03	0.000002-0.00002

(Stahl et al., 1988)

Table 1.3. Critical properties of various solvents

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

(Reid et al., 1987)

1.4.3. Supercritical carbon dioxide

CO_2 is widely used as a SF for extraction of natural substances both from plants and animals. A SF separation process using carbon dioxide as the 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). SC-CO_2 also possesses excellent extractive properties such as high compressibility, liquid-like density, low viscosity, high diffusivity (Lim et al., 2002). It has 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).

Fig. 1.4 shows the CO_2 density as a function of temperature and pressure. Close to the critical point, a slight change in the operational conditions (pressure and temperature) may cause a drastic variation in its density, affecting consequently the solubility of the solute in the supercritical phase (Brunner, 1994).

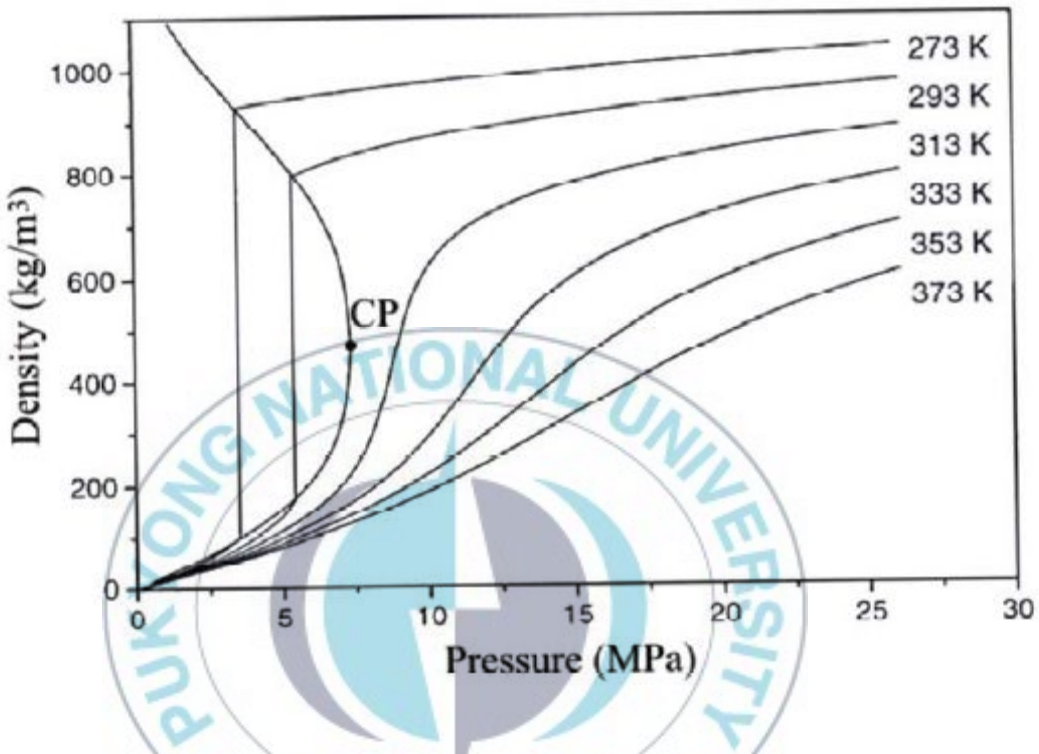

Fig. 1.4. Density of pure CO_2 at different pressures and temperatures.

1.5. Outline of the thesis

Till now, it is a big problem to dispose fish viscera produced by fish processing industries. As far our knowledge, limited work has been conducted for producing useful material from fish byproduct. No detail works have been carried out in which squid viscera were properly used for recovery of bioactive and other useful materials. In this study, we have used SC-CO_2 to extract oil from squid viscera and the extracted viscera residues have been used for recovery of other bioactive and useful materials. Therefore, to recover the maximum useful materials having good quality, the following tasks have been carried out:

- Extraction and characterization of squid viscera oil by SC-CO_2
- Identification of bioactive materials
- Isolation and characterization of squid viscera lecithin
- Useful material production by sub-critical water hydrolysis
- Characterization of digestive enzymes for applicability of SFs in the field of functional protein processing systems
- Comparative study of the results obtained by SC-CO_2 extraction with conventional organic solvent extraction

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

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Chapter 2

Supercritical carbon dioxide extraction of squid viscera oil *

2.1. Introduction

Squid is very popular food in many countries especially Korea and Japan. Squid viscera as like as fish viscera are non-edible parts produced in large quantities in Korea by the fish processing industry as a waste-product. These wastes contain a lot of protein, lipid and many kinds of biological active matter. Marine lipids especially PUFAs and lipid soluble bioactive compounds have been attracted much attention for health benefits. There is commercial interest in obtaining PUFAs, in particular EPA and DHA. These ω-3 fatty acids have an important role in the prevention of human diseases, such as in lowering blood cholesterol and thus preventing heart diseases. Recently, it has been known that ω-3 fatty acids are essential for normal growth and development and may play an important role in the prevention and treatment of coronary artery disease, hypertension, arthritis, others inflammatory and autoimmune disorders, and cancer (Correa et al., 2008). We have already discussed the health benefits of ω-3 fatty acids in details in chapter 1.

In addition to ω-3 fatty acids, the oil from marine organisms also contains some natural fat soluble antioxidants including carotenoids. Carotenoid is a generic name used to designate the most common groups of naturally occurring pigments found in the animal and plant kingdoms. These lipid-soluble pigments comprise well over 700 compounds that account for beautiful red, orange, and yellow colors. Due to high antioxidant activity, carotenoids are considered suitable as components of various types of products, e.g. cancer prevention agents, potential life extenders, inhibiting agents for heart attack and coronary artery disease (Lopez et al., 2004; Li and Chen, 2001; Sun and Temelli, 2006). There are several types of carotenoids which can be obtained from

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different natural sources. Astaxanthin is one of the most effective carotenoids whose antioxidant activity is approximately 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). It is the main carotenoid pigment found in aquatic animals especially in many well known sea foods such as salmon, trout, red seabream, shrimp, lobster, and fish eggs (Miki, 1991; Torissen et al., 1989).

Currently, the most common way for extraction is liquid solvent extraction using toluene, hexane, petroleum ether, chloroform, acetone etc. Decomposition or degradation of thermolabile compounds cannot be avoided in a conventional separation method, since relatively high temperatures are required for these processes. Organic solvents are also harmful to human health as well as the environment. Supercritical fluid extraction (SFE) is an efficient alternative for the extraction of natural substances from foods (Sun and Temelli, 2006; Mendes et al., 2003). It has been widely used in many industrial applications, i.e. extraction of fish oil for PUFAs, the decaffeination of coffee, the extraction of hops and carotenoids, the synthesis of polymers, the purification and the formation of nano particles (Sahena et al., 2010; Rubio-Rodriguez et al., 2008; Letisse et al., 2006; Lim et al., 2002; Esquivel et al., 1997; Temelli and Leblanc, 1995; Kopcak and Mohamed, 2005, Machmudah et al., 2006). Several authors also investigated SC-CO_2 extraction of oils rich in PUFAs and this technology, having a negligible environmental impact, could represent a potential tool to change the relative concentration of the various lipid moieties (Eisenbach, 1984; Rizvi et al., 1988; Temelli et al., 1995; Perretti et al., 2003a; Perretti et al., 2003b). 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. Lim et al. (2002) reported that SC-CO_2 with ethanol as a co-solvent enhanced the yield of astaxathin by 9-24% at different extraction conditions. Astaxanthin molecule is lipid soluble and considered containing no strong polar moieties (Lopez et al., 2004; Krichnavaruk et al., 2008). Therefore, SC-CO_2 can extract majority of astaxanthin without co-solvent from materials containing high lipid. Moreover, heat is required to separate ethanol that can oxidize and reduce the quality of the products. In this study, the use of ethanol as a co-solvent was avoided also for measurement of oil stability at different extraction conditions.

The objective of this study was to extract oil from squid viscera by SC-CO_2 with lipid soluble bioactive compounds and the fatty acids compositions of squid viscera oil at different extraction conditions were also analyzed. The stability of oil obtained by SC-CO_2 extraction was also compared to the oil obtained by soxhlet extraction at hexane.

2.2. Materials and Methods

2.2.1. Materials

Squid viscera were collected from F & F Co., Busan, Korea. The visceral waste was washed thoroughly with cold water and brought to the laboratory in iced condition. The pure carbon dioxide (99%) was supplied by KOSEM, Korea. All reagents used in this study were of analytical or HPLC grade.

2.2.2. Sample preparation

The squid viscera samples were dried in a freeze-drier for about 72 hrs. The dried samples were crushed by a mechanical blender and sieved (700 μm) by mesh. It was difficult to make more small size sample because squid viscera contained lot of oil that made sticky material by more crushing. These samples called freeze dried raw squid viscera were then stored at -60°C until using for SC-CO_2 and organic solvent extraction.

2.2.3. SC-CO_2 extraction

The set up of a laboratory scale of SFE process is shown in Fig. 2.1. This apparatus can be operated at pressure up to 25 MPa. Twenty five gram of freeze dried raw squid viscera sample were loaded into the stainless steel extraction vessel which was 200 mL in volume. A thin layer of cotton was placed at the bottom of the extraction vessel. Before plugging with cap another layer of cotton was used at the top of the sample. CO_2 was pumped at constant pressure into the extraction vessel by high pressure pump up to the desired pressure. A back pressure regulator was used to control the pressure of CO_2. The extraction temperature was maintained by connecting the extraction vessel with water bath. Flow rates and accumulated gas volume passing through the apparatus were measured using a gas flow meter. The oil extracted by SC-CO_2 was collected by a

cyclone separating vessel. The amount of extract obtained at regular intervals of time was established by weight using a balance with a precision of ±0.001 g. The extracted oil and viscera residues were then stored at -80°C until further used and analysis.

The effect of temperature and pressure on lipid extraction from squid viscera were studied at 35-45°C and 15-25 MPa at a constant extraction time of 2.5 hrs. The extractions were performed at low temperature because fish oil is rather involatile and thermally sensitive (Singh, 2004). Another cause for selecting low temperature was to retain the high activity of bioactive materials both in extracted oil and residues. The flow rates of CO_2 were kept constant at 22 g/min for all extraction conditions.

Squid viscera oil and residues obtained by SC-CO_2 extraction are shown in Fig. 2.2.

2.2.4. Soxhlet extraction by hexane

The extraction was carried out in a soxhlet apparatus using hexane as solvent. Three gram of freeze dried raw squid viscera sample was placed into the extraction thimble and the extraction was run 12 hrs until the colour of the condensed solvent at the top of the apparatus was clear.

2.2.5. GC analysis for fatty acid compositions

The fatty acid compositions of squid viscera oil obtained by SC-CO_2 and organic solvent, hexane extraction were determined by gas chromatography using a Hewlett Packard gas chromatograph (5890 Series II GC system). The fatty acid methyl esters were prepared firstly according to AOCS official method Ce 2-66 (AOCS, 1998) and then separated using an Agilent DB-Wax capillary column (30 m length x 0.250 mm internal diameter, 0.25 µm of film). Nitrogen at a flow rate 1.0 mL/min was used as a carrier gas of fatty acid methyl esters. The split ratio was fixed at 50:1. The oven temperature was programmed starting at 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. 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).

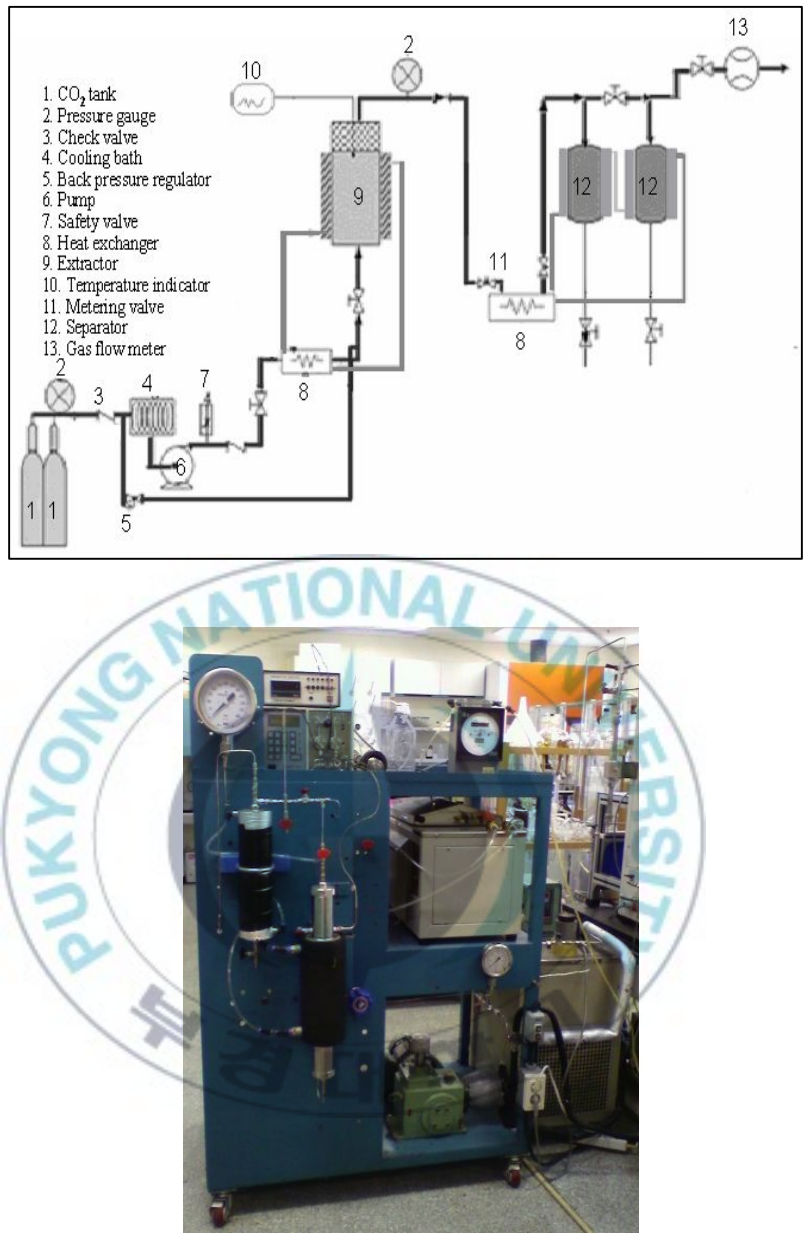


Fig. 2.1. Photograph and schematic diagram of SC-CO_2 extraction process.

A) B) C)

Fig. 2.2. A) Freeze dried squid viscera B) SC-CO_2 extracted oil C) SC-CO_2 extracted viscera residues.

2.2.6. Measurement of oil stability

Several parameters may use to determine the deterioration of oil. In this study, oil deterioration was monitored by evaluating free fatty acid content and peroxide value.

2.2.6.1. Free fatty acid content of squid viscera oil

Free fatty acids of extracted oil from squid viscera were analyzed as described by Bernardez et al. (2005). Accurately, 50 mg of oil 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 2000g for 10 min, the upper layer was read at 710 nm. The FFA content of oil was measured on a calibration curve constructed 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 filtered. Then the pH of cupric acetate solution was adjusted to 6.1 using pyridine.

2.2.6.2. Peroxide value

Peroxide value was determined by the AOCS method Cd 8-53 (AOCS, 1998) with modified amount of sample taken. 1.0 g of squid viscera oil 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 0.1N 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.

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

Where, S = Volume of titrant, mL of sample; B = Volume of titrant, mL of blank; N = Normality of sodium thiosulfate solution and M = Mass of sample, g.

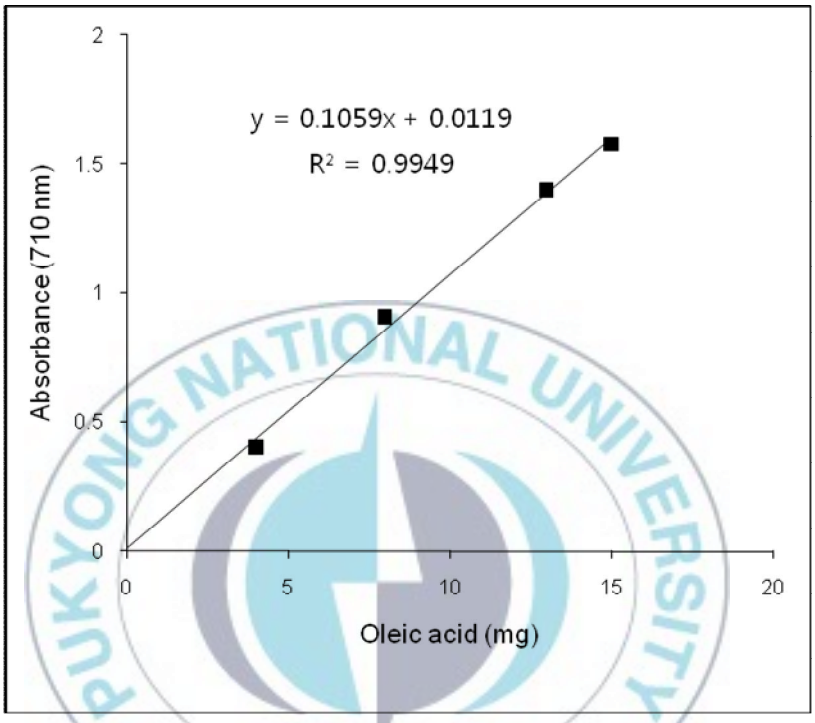


Fig. 2.3. Calibration curve of oleic acid for estimation of free fatty acids in squid viscera oil.

2.2.7. HPLC analysis for the measurement of astaxanthin

HPLC analysis was carried out using a Waters HPLC equipped with a model 600E system controller, a model 484 UV/VIS detector and a Eclipse Plus C18 column (5 μm, 4.6 x 250 mm, Agilent, USA). Astaxanthin was analyzed according to the method described by Krichnavaruk et al. (2008). A mobile phase consisting of acetonitrile, dichloromethane and ethanol at the volume ratio of 5:10:85 was eluted 1 mL/min as isocratic method. 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. Astaxanthin was purchased from Sigma, St. Louis, MO, USA. A calibration curve was constructed by the peak area with corresponding astaxanthin amount (Fig. 2.4).

2.2.8. Statistical analysis

Data were analyzed by Duncan's multiple range test using SAS 9.1 (SAS institute Inc., Cary, NC, USA) and Microsoft office excel 2007 (Microsoft Corporation, USA). The least significant difference ($p < 0.05$) at the 95% confident level was calculated for each parameter.

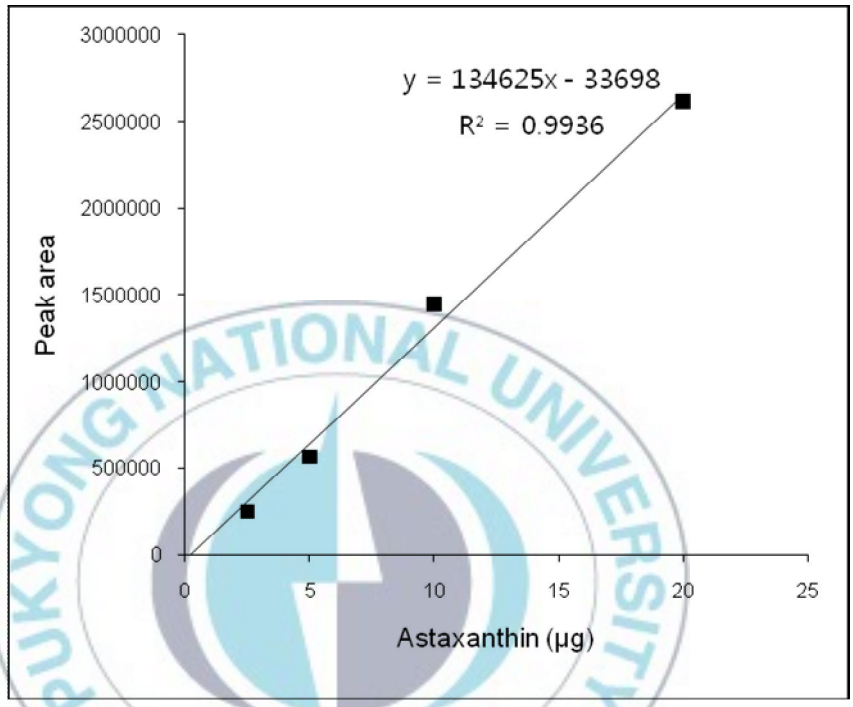


Fig. 2.4. Calibration curve of standard astaxanthin for estimation of astaxanthin in squid viscera oil.

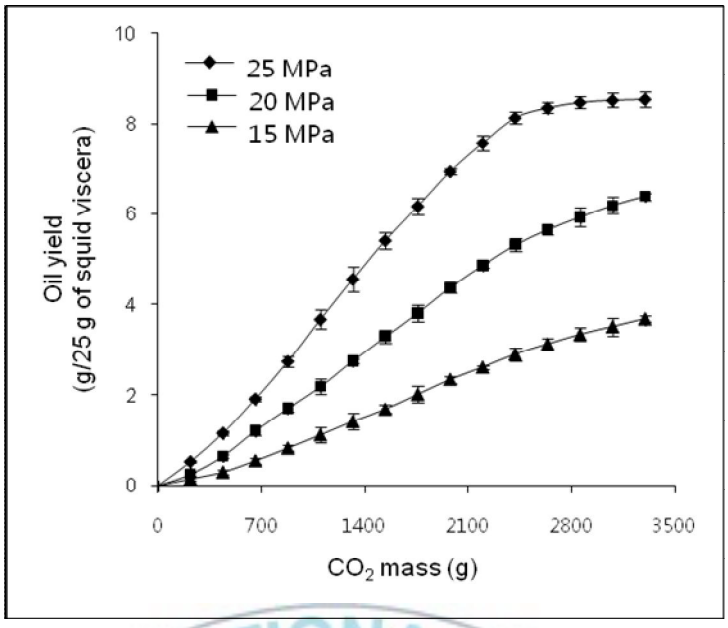
2.3. Results and Discussion

2.3.1. SC-CO_2 extraction

Squid viscera oil was characterized by a reddish brown colour and a characteristics fish odour. The extraction curves of squid viscera oil by SC-CO_2 at different temperatures (35, 40 and 45°C) and pressure (15, 20 and 25 MPa) are shown in Fig. 2.5A-C. The highest oil yield obtained by SC-CO_2 extraction was 8.54±0.17 g/25 g of squid viscera at temperature, 45°C and pressure, 25 MPa. The applied pressure and temperature variation greatly affected the oil solvating power of SC-CO_2 and hence the amount of yield. Depending on the pressure and temperature, the amount of extracted oil was increased with the increasing of CO_2 mass. The amount of extracted oil per solvent (CO_2) mass used was increased constantly over the entire extraction period, until almost all the oil was extracted. The change in the slope of the extraction curve (45°C and 25 MPa) indicated that SC-CO_2 extracted almost all squid viscera oil. After SC-CO_2 extraction at low pressure and low temperature, squid viscera residues contained still oil. At constant temperature, the amount of oil extracted from squid viscera was increased with the pressure. Due to the increase in pressure, the density of the SC-CO_2 was increased and hence the solvating power. The effect of pressure can be attributed to the increase in solvent power and by the strengthening of intermolecular physical interactions (Morita and Kajimoto, 1990; Bai et al., 1997; Bulgarevicg et al., 2002). Similar results were found in the extraction of oil from green coffee (De Azevedo et al., 2008) and boiled anchovy (Park et al., 2008).

The amount of oil extracted was highest at 45°C as compared to other conditions. Despite of the decreasing of solvent's density, the oil extraction yield was increased with the temperature which can be attributed to the increase of the oil components vapour pressure. The effect of the increase of solute vapour pressure seems to have dominated over solvent's density. Similar findings were observed in the oil extraction yield from boiled anchovy (Park et al., 2008).

A)



B)

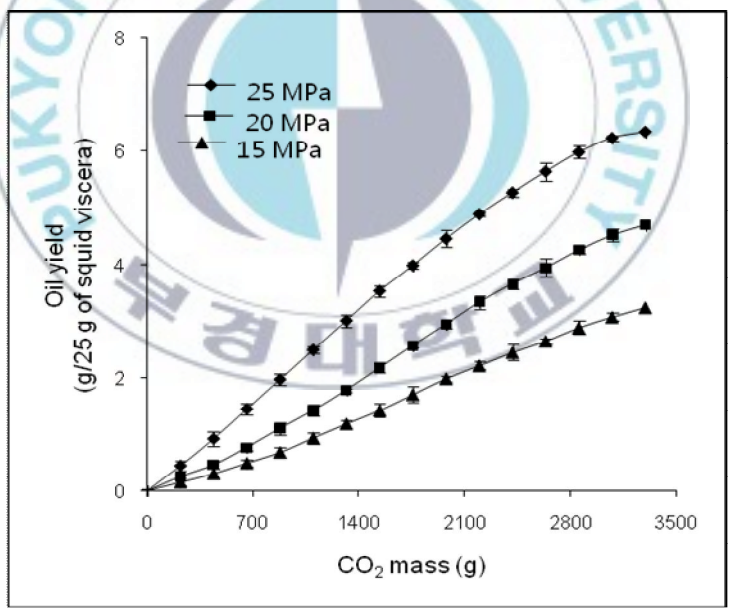


Fig. 2.5A-C. SC-CO_2 extraction of oil from squid viscera at different conditions. A) 45°C, B) 40°C and C) 35°C. Data are the mean value of three replicates ± Standard error (S.E.).

C)

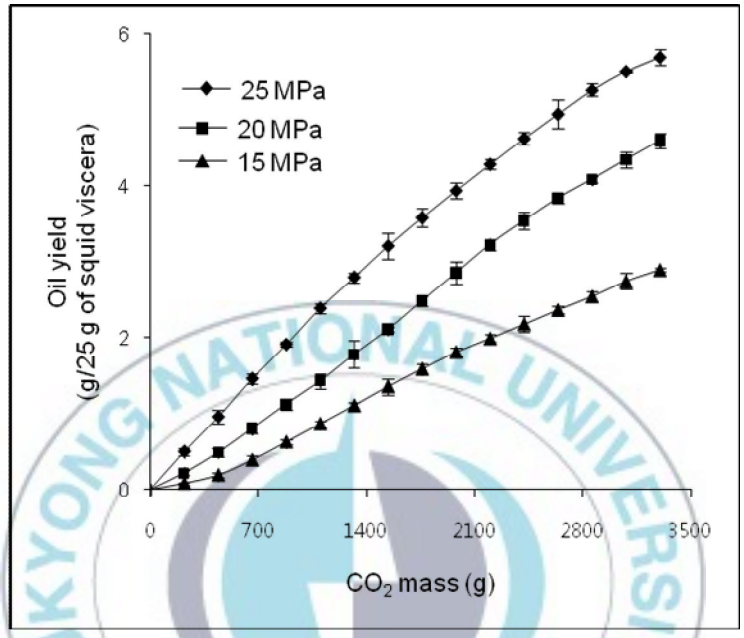


Fig. 2.5A-C. SC-CO_2 extraction of oil from squid viscera at different conditions. A) 45°C, B) 40°C and C) 35°C. Data are the mean value of three replicates ± Standard error (S.E.).

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

The oil yield obtained by soxhlet extraction at hexane from squid viscera was 39.24±1.03% (w/w in freeze dried raw sample) shown in Fig. 2.6. On the other hand, the highest yield in SC-CO_2 extraction was 34.16±0.69% for the experiment conducted at 25 MPa and 45ºC (Fig. 2.5A). By considering that the extraction of oil using hexane was complete, the highest yield by SC-CO_2 extraction was almost 87%. The maximum oil yield obtained by SC-CO_2 was 70% from peach seed (Sanchez-Vicente et al., 2009). The differences in maximum yield may be occurred due to variation of processing unit, operating conditions, sample size, percentage of lipid in sample etc.

2.3.3. Fatty acid compositions

The fatty acid compositions of the oil obtained by SC-CO_2 and hexane extraction are shown in Table 2.1. Total of 22 fatty acids were identified in the different extracts analyzed. Under certain extraction conditions there were remarkable changes in the fatty acid compositions of the squid viscera oil. The highest percentage (about 87%) of the total fatty acid was identified at 20 MPa and 40ºC. Within saturated fatty acids, palmitic acid (C16:0) was present in the highest concentration ranging from 13.20 to 24.30% of total identified fatty acids. Among monounsaturated fatty acids, oleic acid (C18:1) was also found in substantial amounts ranging from 9.10 to 11.60% of total identified fatty acids. DHA (C22:6) in squid viscera oil 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 5.89 to 11.14 and 11.01 to 19.77, respectively. The composition of total PUFAs obtained in squid viscera oil was identical with marine fish oils such as cod liver oil and anchovy oil which contained about 14-31% of EPA and DHA (AOCS, 1997).

It was also observed that the oil extracted by SC-CO_2 showed a higher percentage of PUFAs than the oil extracted with hexane. This may be happened due to applying higher temperature in soxhlet extraction as compared to SC-CO_2 extraction. High temperature may lead to thermal degradation of fatty acids, especially unsaturated fatty acids. Similar results were reported in fatty acid profile of hake byproducts (Rubio-Rodriguez et al., 2008).

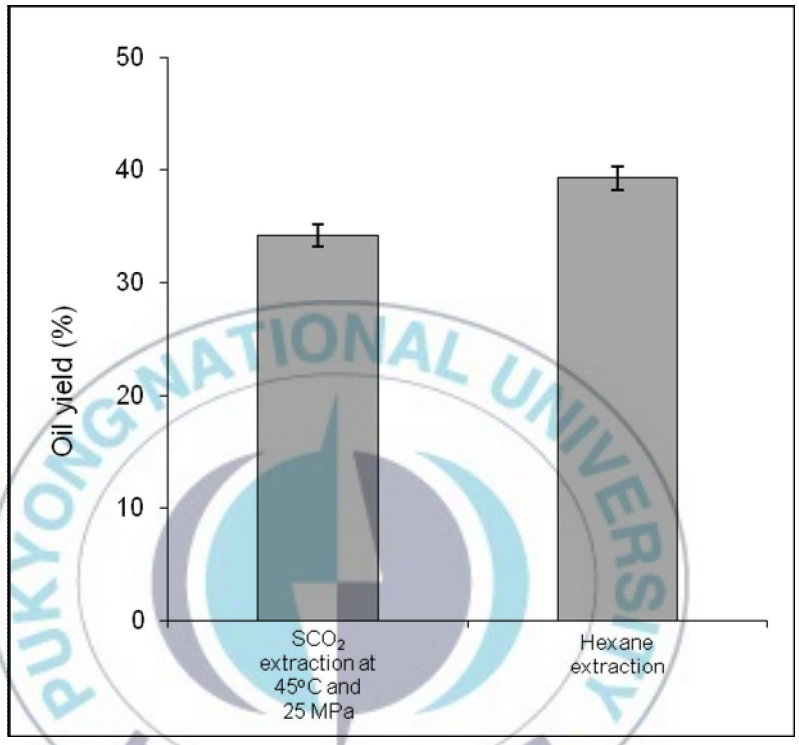


Fig. 2.6. The percentage of oil yield from squid viscera at SC-CO_2 and hexane extraction.

Table 2.1. Major fatty acid compositions of squid viscera oil obtained by SC-CO_2 and hexane extraction

Fatty acids (%)	SC-CO_2									Hexane
	25 MPa			20 MPa			15 MPa			
	45ºC	40ºC	35ºC	45ºC	40ºC	35ºC	45ºC	40ºC	35ºC	
C14:0	3.72	4.60	2.81	3.74	4.84	4.01	2.68	4.70	4.99	5.13
C16:0	16.94	18.00	14.52	17.84	20.97	19.00	13.20	19.43	21.94	24.30
C16:1	3.61	4.12	2.86	4.16	4.35	3.84	2.75	4.09	4.50	4.14
C18:0	3.10	3.07	2.76	3.49	3.79	3.43	2.75	3.40	3.79	3.02
C18:1	9.81	9.77	9.78	11.60	11.40	10.53	9.10	10.31	11.39	10.13
C18:2	1.18	1.17	1.01	1.53	1.37	1.24	1.08	1.25	1.37	1.14
C20:0	2.53	2.73	2.27	2.45	2.92	2.55	2.28	2.40	2.54	3.07
C20:1	3.74	3.27	3.24	4.34	4.17	3.60	3.11	3.64	3.89	3.84
C20:3	1.94	1.77	1.39	2.32	1.90	1.69	1.63	1.72	1.82	1.14
C20:5 (EPA)	10.38	9.96	9.27	11.14	9.96	9.00	9.16	8.86	9.66	5.89
C22:6 (DHA)	19.77	16.65	13.21	19.15	16.88	14.60	17.03	13.30	14.55	11.01

Data are the mean value of two replicates. S.E. of the fatty acid constituents were on the order of about ± 3%. Fatty acids showed only that were found more than 1% of total fatty acids.

2.3.4. Oil stability

High level of PUFAs is present in marine fish oil. The quality of oil is deteriorated at a varying velocity depending strongly on production and storage conditions (Kamal-Eldin and Yanishlieva, 2002). FFAs are responsible for the acidity of oil. Changes of FFA content are mainly related to hydrolytic reactions in the oil. FFA content and peroxide value of the oil extracted by SC-CO_2 and hexane are given in Table 2.2. It was found that the amount of FFA and peroxide value were significantly high in hexane extracted oil than SC-CO_2 extracted. It was also observed that oil obtained at higher extraction temperature contained high amount of FFA and peroxide value. This result agreed with the high FFA content and peroxide value in hexane extracted oil due to higher temperature. Higher temperature and storage time also caused a significant increment of the FFA in the hake byproducts oil (Rubio-Rodriguez et al., 2008). On the other hand, peroxide value of an oil or fat is used as a measurement of rancidity which occurs by auto oxidation. Low exposure of oxygen in SC-CO_2 extraction caused minimal oxidation. However, the oil extracted by hexane showed lower stability compared to the oil obtained by SC-CO_2 extraction.

2.3.5. Extraction yield of astaxanthin

The SC-CO_2 extraction of astaxanthin obtained at different pressures and temperatures are shown in Fig. 2.7. The highest amount of astaxanthin yield was 6.15±0.37 mg/g of squid viscera at 25 MPa and 45ºC. It was found that the astaxanthin yield increased with pressure at temperatures of 35 and 45ºC. On the other hand, at 40ºC the maximum value was observed at 20 MPa. This trend of astaxanthin solubility can be explained by the increase in the SC-CO_2 density and the decrease in diffusion coefficient. Rising the pressure increases the fluid density which has double effect, an increase in the solvating power of the supercritical fluid, which enhances the extraction process and a reduced interaction between the fluid and the matrix resulting from the decrease in diffusion coefficient, which makes the yield of extraction process decrease (Careri et al., 2001). At 35 and 45ºC the increase in solvating power of the SC-CO_2 was dominant than the decrease in diffusion coefficient between the SC-CO_2 and matrix. On the other hand, at 40ºC and 20 MPa, the dominant effect was the decrease in diffusion coefficient.

Solute solubility also depends on a complex balance between the decrease in the SC-CO_2 density and the increase in solute vapour pressure. At 15 and 25 MPa the yield of astaxanthin obtained was the maximum while the extraction temperature was 45ºC. Rising the temperature decreases the fluid density, but can increase the solute vapour pressure, which enhances the yield of extraction process. Lopez et al. (2004) also reported the same effect of temperature for astaxanthin extraction from crustaceans. At pressures of 15 and 25 MPa the increase in vapour pressure was dominant over fluid density. On the other hand, a decrease in the yield observed at 20 MPa when the operating temperature was 45ºC. The decrease of astaxanthin yield was due to dominancy of the decrease in the density of SC-CO_2 than solute vapour pressure.

In hexane extraction, the highest amount of astaxanthin was 7.88±0.24 mg/g of squid viscera. The highest amount of astaxanthin yield obtained by SC-CO_2 extraction was almost 78% of the total amount of astaxanthin estimated by soxhlet extraction. Thana et al. (2008) reported approximately 83.05% recovery of astaxanthin yield by SC-CO_2 extraction from *Haematococcus pluvialis*.

Table 2.2. Free fatty acids and peroxide value of squid viscera oil obtained by SC-CO_2 and hexane extraction

SC-CO_2 extraction		Free fatty acids (g/100 g oil)	Peroxide value (milliequivalent/1000 g)
Pressure (MPa)	Temperature (°C)		
15	35	2.24±0.07	3.77±0.09
	40	2.77±0.13	4.17±0.11
	45	3.19±0.17	4.88±0.12
20	35	2.96±0.10	4.26±0.08
	40	3.29±0.07	4.89±0.10
	45	3.45±0.21	5.43±0.18
25	35	3.32±0.16	5.08±0.11
	40	3.56±0.06	5.66±0.16
	45	4.34±0.14	6.52±0.22
Hexane extraction		6.57±0.16	8.29±0.26

Mean value of three replicates ± S.E.

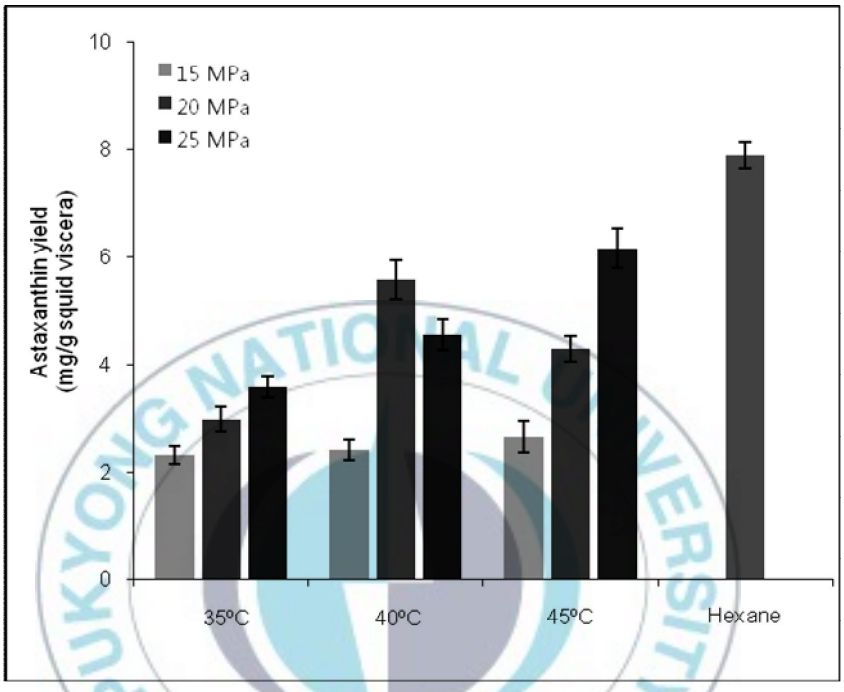


Fig. 2.7. Astaxanthin yield from squid viscera at different extraction conditions. Data are the mean value of two replicates ± S.E.

2.3.6. Solubility correlation of astaxanthin

The correlations based on empirical 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. To correlate the solubility of astaxanthin in the SC-CO_2, Chrastil (1982) model was adopted. Despite its limitations, the usefulness of equation has proved in SFE work and is easy to use. The detailed plot of astaxanthin solubility vs. density was shown in Fig. 2.8. It clearly showed the isotherms and the effects of temperature and solvent density. Mole fractions of astaxanthin in carbon dioxide were calculated using the following equation

$$y_{astaxanthin} = \frac{n_{astaxanthin}}{n_{astaxanthin} + n_{CO_2}} \qquad (1)$$

where $y_{astaxanthin}$ represents the mole fraction of astaxanthin, and $n_{astaxanthin}$ and n_{CO_2} are moles of astaxanthin and carbon dioxide, respectively.

The density relationship in Chrastil model is based on the following equation (derived from Antoine)

$$y_{astaxanthin} = \rho_{CO_2}^{k} \exp\left(\frac{a}{T} + b\right) \qquad (2)$$

where $y_{astaxanthin}$ is the astaxanthin solubility (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 parameters obtained for astaxanthin are shown in Table 2.3. The solubility data of astaxanthin well fitted in Chrastil model because at a given temperature, almost a linear relation between solubility of astaxanthin and solvent density was obtained. Roh et al. (2008) reported that the solubility of another carotenoid, fucoxanthin in SC-CO_2 from brown sea weed was also fitted in Chrastil model.

2.4. Conclusions

In this study, squid viscera oil was extracted both in a high pressure apparatus using SC-CO_2 and soxhlet extraction using hexane. The highest yield of oil obtained by SC-CO_2 extraction was 87% comparing with the oil obtained by organic solvent extraction. The lipid compositions of oil showed a high amount of ω-3 fatty acids, especially EPA

and DHA. The oil obtained by SC-CO_2 extraction was more stable than organic solvent extraction. Squid viscera also contained moderate amount of astaxanthin that was extracted in highest amount at 25 MPa and 45ºC. Therefore, squid viscera oil obtained by SC-CO_2 extraction may be a good source of astaxanthin with PUFAs. SC-CO_2 extraction of oil was more efficient than organic solvent extraction in terms of oil quality and stability.

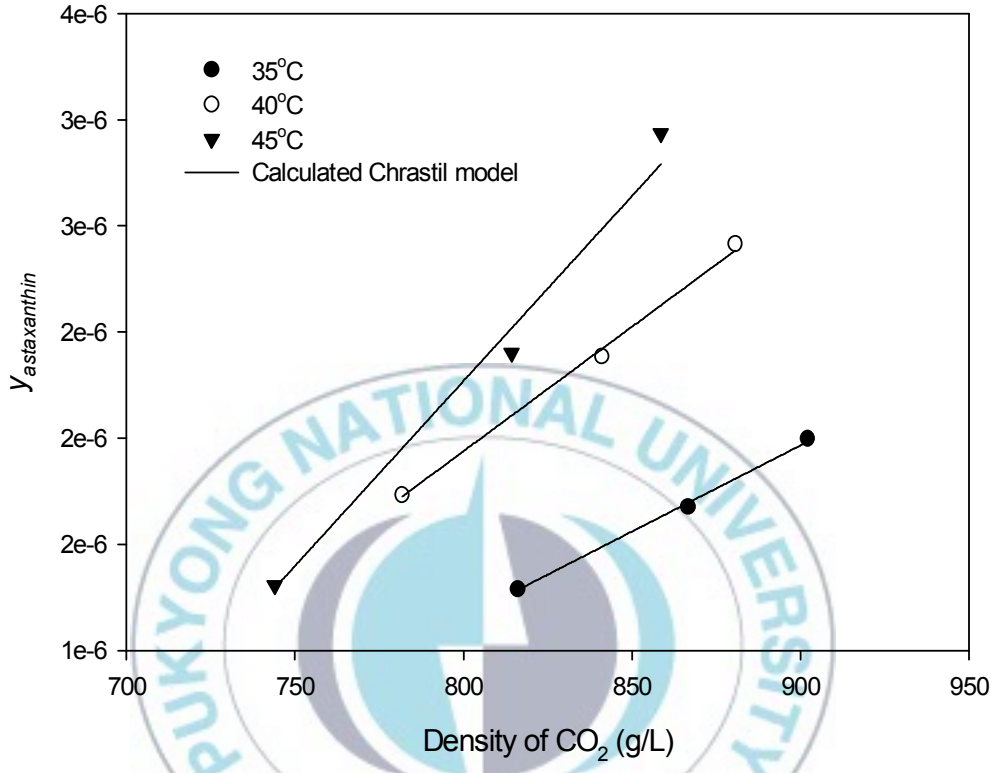


Fig. 2.8. Experimental data of astaxanthin solubility as a function of CO_2 density and calculated results from Chrastil model.

Table 2.3. Parameters in the Chrastil equation of astaxanthin mole fraction

Temperature (°C)	k	a	b
35	4.37	0.93	-42.86
40	4.37	0.86	-42.38
45	6.74	70.37	-33.06

2.5. References

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Chapter 3

Isolation and characterization of lecithin from supercritical carbon dioxide extracted squid viscera *

3.1. Introduction

Lecithin is sticky fatty substances composed of mainly phospholipid mixtures especially phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with small amount of glycerides, neutral lipids and other suspended matter. Lecithin, which occurs in egg yolk, animal and plant tissues, is used for its emulsifying properties in the food, pharmaceutical and cosmetic industries. Pharmacological use of lecithin includes treatment for hypercholesterolemia, neurologic disorders, and liver ailments. It has also been used to modify the immune system by activating specific and nonspecific defense systems (Budavari, 1989; Reynolds, 1996; Der Marderosian, 1988).

Commercially, lecithin comes from soybeans, egg yolk, or brain tissue (Budavari, 1989; Venturella, 1995). Until now, the soybean is the most frequent and studied source of lecithin. However, lecithin from soybeans is rich in saturated fatty acids with few lower unsaturated fatty acids. Lecithin from soybeans does not contain important PUFAs such as EPA and DHA. Egg yolk has also been used widely as a source of lecithin. Phospholipids from egg yolk are the major source used by the pharmaceutical industry for parenteral nutrition. For nutritional supplements egg phospholipids play no big role, because they have a relatively high cholesterol level and unfavorable fatty acid profile. In the current research it is understood now increasingly that essential fatty acids, like EPA and DHA demonstrate increased bioavailability, when they are integrated part of a phospholipid molecule. Lecithin from marine sources has several valuable nutritional benefits. Today it is well known that the most important ω-3 fatty acids, EPA and DHA are found in marine organisms. Marine phospholipids are valuable resources that can be applied to diverse areas such as nutrition, pharmacy, and medicine as well as

* This work has been accepted in Journal of Food Science.

basic research because they contain high levels of ω-3 fatty acids (Miniadis-Meimaroglou et al., 2008).

Lecithin is primarily extracted with solvents such as diethyl ether, hexane, chloroform, and ethanol. Acetone is also used to precipitate lecithin from other lipids. However, some of these solvents are considered undesirable because of environmental and health concerns (Sim, 1994; Nielson, 2001; Palacios & Wang, 2005). The use of less hazardous solvents to extract lecithin has a great importance to the environment. In second chapter, we have discussed in details about the SC-CO_2 (environmental friendly solvent) extraction of squid viscera oil at different temperatures and pressures. Since CO_2 is nonpolar, pure SC-CO_2 is not suitable for extraction of slightly polar solute due to low solute solubility (Krichnavaruk et al., 2008). Several reports also showed that SC-CO_2 is widely used to extract nonpolar lipids with lipid soluble bioactive compounds from different sources (Rubio-Rodriguez et al., 2009; Sahena et al., 2010; Sahena et al., 2010a; Davarnejad et al., 2008; Esquivel et al., 1997). However, SC-CO_2 as a non-polar solvent has extracted mainly non-polar lipid including lipid soluble bioactive compound from squid viscera. Some polar lipids might be present in the extracted residues. Therefore, the aim of this research was to isolate and characterize the lecithin from SC-CO_2 extracted residues of squid viscera. The quality of the extraction was evaluated to assess whether marine lecithin could be used in this way as functional lipids.

3.2. Materials and methods

3.2.1. Materials

Stored squid viscera residues obtained by SC-CO_2 extraction at 25 MPa and 45ºC were used for lecithin 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.

3.2.2. Isolation of lecithin

Lecithin was isolated from squid viscera obtained by SC-CO_2 extraction according to the method of Palacios and Wang (2005) with modifications shown in Fig. 3.1. Briefly,

100 mL of ethanol (95%) was added to 30 g of SC-CO_2 extracted squid viscera residues and stirred 12 hrs by a magnetic stirrer. The mixture was then centrifuged at 1900 g for 10 min. The supernatant containing mainly polar lipids with very small amount of neutral lipids was collected in a separatory funnel. The precipitate was again extracted with 100 mL of ethanol and after centrifugation the supernatant was added to the previous ethanol extract. Then, twice volume of hexane was mixed with the ethanol extract to separate the neutral lipids from polar lipids. The ethanol phase was evaporated in a rotary vacuum evaporator at 40ºC. The remaining lipid residue was dissolved in hexane. A fifth volume of chilled acetone (4ºC) was added to hexane mixture with slow stirring for precipitation of gummy material, lecithin. The mixture was placed on ice bath for 15 min and then centrifuged at 1500 g for 10 min. After discarding the supernatant, the collected precipitate called marine lecithin was stored at -40ºC until further analysis.

3.2.3. Characterization of squid viscera lecithin

3.2.3.1. Phospholipid content

The phospholipid content of lecithin from squid viscera 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 lecithin was dissolved in 2 mL of chloroform. Then, 1 mL of a solution prepared from ferric chloride (27 g/L) and ammonium thiocyanate (30 g/L) was added. After vortexing, the mixture was centrifuged at 1000 g for 15 min. The lower phase was collected and the absorbance was recorded at 488 nm by a spectrophotometer (UVIKON 933, Kontron Instruments). Phospholipid content was calculated by constructing a calibration curve of standard PC.

3.2.3.2. Measurement of hexane insoluble matter, acid value and peroxide value

Hexane insoluble matter of lecithin from squid viscera was determined by AOCS official method of Ja 3-87. This method is used to determine the substances insoluble in hexane. Acid value was measured according to AOCS official method of Ja 6-55 (AOCS, 1998a). The acid value was the mg of KOH necessary to neutralize the acids in 1 g of

sample. Peroxide value was measured by AOCS official method of Ja 8-87 (AOCS, 1998a). Peroxide value was expressed as milliequivalents peroxide/1000 g sample.

3.2.3.3. Free fatty acids

Free fatty acids were analyzed by the method of Bernardez et al., (2005). We have discussed this method in Chapter 2 for the determination of free fatty acids in viscera oil.

3.2.3.4. Major phospholipids quantification by HPLC

Major phospholipids of squid viscera lecithin were separated and quantified by Jasco HPLC equipped with controller, a 4-line degasser (DG-2080-54), a quaternary gradient unit (LG-2080-04), an intelligent HPLC pump (PU-2080 Plus), an evaporative light scattering detector (ELSD-Softa corporation, Model 400) and a silica column (5µm, 4.6 x 250 mm, Waters, USA). The analysis was carried out according to the method of Letter (1992) with modification of ELSD operation. Extracted lecithin was dissolved in chloroform and injected (20 µL) into injector. The mobile phase was isopropyl alcohol, hexane and water. The spray and drift tube temperature of ELSD was set to 70 and 60ºC, respectively. The pressure of nitrogen gas as a nebulizer was 50 psi. The quantification of phospholipids was performed based on the peak area of standard phospholipid, PC and PE. The millennium software was used to analyze the data obtained by HPLC.

3.2.3.5. Thin layer chromatography (TLC)

The phospholipids of lecithin from squid viscera were separated by TLC to know the fatty acid profile of PC and PE. Separation of individual lipids was performed by thin-layer chromatography, using Silica plates 60 (20 cm×20 cm, 0.2 mm thick, Macherey-Nazel, Duren, Germany). Lecithin was separated by modified method of Miniadis-Meimaroglou et al. (2008). The mobile phase was composed of chloroform:methanol:glacial acetic acid:water (50:25:6:2, v/v/v/v). Lecithin dissolved in chloroform:methanol (2:1, v/v) solvent was used for separation. Spots were visualized by iodine vapour. 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 phospholipid was again checked by TLC. PC and PE from the spots were again extracted as described above. This purified PC and PE were used for fatty acid compositions.

3.2.3.6. Gas chromatography for fatty acid compositions

GC analysis was carried out to determine the fatty acid compositions of lecithin, and purified PC and PE. A Hewlett Packard gas chromatograph (5890 Series II GC system) with an Agilent DB-Wax capillary column (30 m length x 0.250 mm internal diameter, 0.25 μm of film) was used. The fatty acid methyl esters were prepared firstly according to AOCS official method of Ce 2-66 (AOCS, 1998b). Nitrogen was used as a carrier gas (1.0 mL/min) 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 4ºC/min 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).

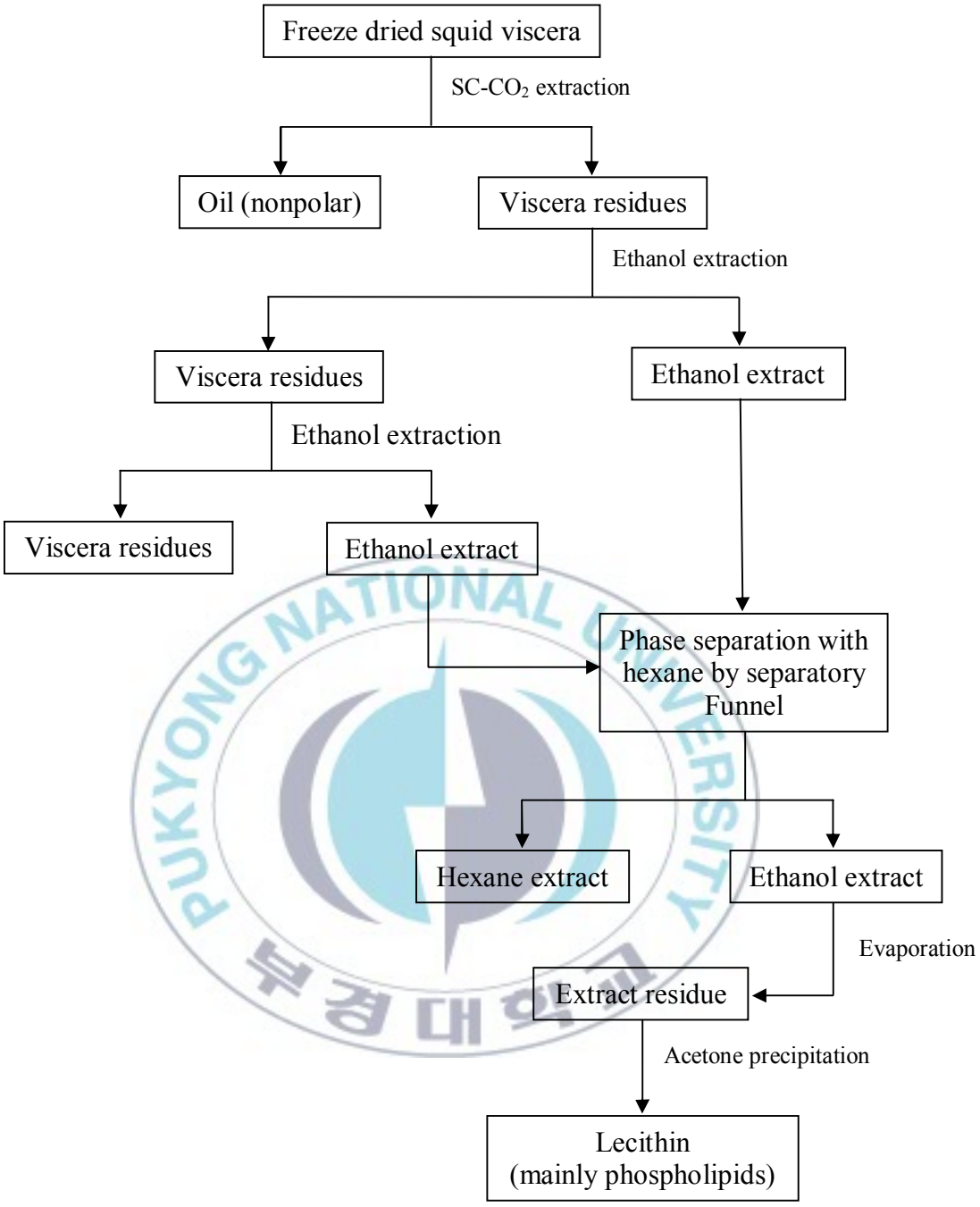


Fig. 3.1. Schematic representation of lecithin separation from squid viscera.

3.2.3.7. Oxidative stability

To measure the oxidative stability, emulsion of lecithin in water were oxidized at 37ºC. Three emulsions of lecithin in water (w/w) (linoleic acid 4%, lecithin 1%, water 95%; lecithin 5%, water 95%; trolox 1%, lecithin 4%, water 95%) were prepared. The deionized and degassed water was used for emulsion preparation. Linoleic acid and standard trolox were used to measure the oxidative stability of marine lecithin. The mixture was properly homogenized by a homogenizer. Oxidative stabilities were checked by thiocyanate (Mitsuda, et al., 1966) and thiobarbituric acid (Ottolenghi, 1959) method which were used to measure the antioxidant activity. In this study, these two methods were conducted to measure the quality in terms of oxidative stability.

3.2.3.7.1. Thiocyanate method

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. The absorbance was recorded at 24 hrs intervals during the incubation.

3.2.3.7.2. TBA method

TBA method is used for evaluating the extent of lipid peroxidation. Malonadehyde, the product of lipid breakdown caused by oxidative stress binds with TBA to form a red complex of thiobarbituric acid reactive substance (TBARS). 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 containing TBARS was measured at 532 nm.

3.3. Results and Discussion

3.3.1. Characterization of squid viscera lecithin

In this study, 4.25% (w/w) of lecithin was isolated from SC-CO_2 extracted squid viscera residues. Phospholipid content of squid viscera lecithin was 91.56% (w/w). Therefore, 3.89% of phospholipid (w/w) was found in squid viscera residues. Cho et al., (2001) reported that the phospholipid content of squid viscera extracted by chloroform-methanol was 3.8% of total lipid. This value was higher than that of phospholipid obtained in this study. Phospholipid content in squid viscera may vary depending on sample age, habitat, fishing time, extraction process and time, extracting solvents etc.

3.3.2. Hexane insoluble matter, FFA content, acid value and peroxide value

Hexane insoluble matter, FFA content, acid value and peroxide value of squid viscera lecithin are given in Table 3.1. These provided the quality index of marine lecithin. Hexane insoluble matter was less than 1%. However, this value was within the range of commercially-available soy lecithin. The FFA content, peroxide value and acid value are dependent on the nature of processing and such factors as the surrounding moisture, air, temperature, and more. The FFA content of isolated lecithin was 1.13±0.06%. Due to presence of moisture in lecithin, FFA may be liberated by its hydrolytic rancidity. Determination of FFA content was therefore an index of the quality of marine lecithin. Peroxide value provides the oxidation state of lipid. It is used to measure the rancidity which occurs by auto oxidation. Peroxide value of isolated lecithin was 3.77±0.26 milliequivalent/1000 g. In contrast, the acid value of squid viscera lecithin was 33.03±1.75 mg KOH/g of lecithin. Acid value was used to determine the acidity of lecithin. Generally, peroxide value and acid value of commercially available soy lecithin are found upto 12 milliequivalent/1000 g and 30 mg KOH/ g of lecithin, respectively. The lecithin isolated from squid viscera contained free fatty acids which increased its acid value.

3.3.3. Quantification of major phospholipids

The major phospholipids of lecithin obtained from squid viscera are shown in Table 3.2. The main phospholipid of squid viscera was PC and PE. PC and PE comprised 80.54±0.68 and 13.18±0.24% of total phospholipid, respectively. Cho et al. (2001) reported that phospholipid from squid viscera contained 79.2% of PE and 12.7% of PC. The composition of phospholipids (PC and PE) from squid viscera obtained by Cho et al. (2001) were almost opposite in trend to those values obtained in this study. However, Cho et al (2001) and Igarashi et al., (2001) also reported 3 to 5 times higher PC to PE content of phospholipids from muscle, mantle, fin, arm and integument of squid that was similar to this study. The phospholipid compositions may differ due to habitat, intake of food varieties, variation of isolation and quantification process, variation of seasons for harvesting etc.

3.3.4. Fatty acid compositions of lecithin, PC and PE

For fatty acid analysis, the individual phospholipids were separated by TLC (Fig. 3.2). The fatty acid compositions of lecithin, PC and PE from squid viscera are shown in Table 3.3. The percentage of the total PUFAs was higher in all lipid fractions (lecithin, PC and PE). The important PUFAs, EPA ranging from 10.38 to 16.35 and DHA ranging from 14.52 to 22.73 were found significantly in all lipid fractions. Among the monounsaturated fatty acids, C18:1 was present in higher amount. The most significant saturated fatty acids was C16:0 (ranging from 24.28 to 32.49) in all lipid fractions. DHA/EPA ratio for lecithin, PC and PE were 1.39, 1.74 and 1.08, respectively. DHA/EPA ratio for squid viscera phospholipids and salmon head lecithin were 0.90 and 1.65, respectively (Cho et al., 2001; Belhaj et al., 2010).

Table 3.1. Hexane insoluble matter, free fatty acid, acid value and peroxide value of lecithin as a quality index

Quality index	
Hexane insoluble matter (%)	0.96±0.02
Free fatty acid (%)	1.13±0.06
Acid value (mg KOH/g)	33.03±1.75
Peroxide value (milliequivalent/1000g)	3.77±0.26

Mean value of two replicates ± S.E.

Table 3.2. Major phospholipid compositions of squid viscera lecithin

Phospholipids (%)	**Squid viscera (In this study)**	**Cho et al. (2001)**	
		Squid viscera phospholipid	**Squid muscle phospholipid**
PC	80.54±0.68	12.7	71.7
PE	13.18±0.24	79.2	24.7
Others	6.28	8.1	3.6

Mean value of two replicates ± S.E.

PC PA PE Squid viscera lecithin

Fig. 3.2. Phospholipids separation by TLC.

Table 3.3. Fatty acid compositions of squid viscera lecithin, PC and PE

Fatty acids	Lecithin	PC	PE
C14:0	3.12	2.39	3.79
C16:0	32.49	25.46	24.28
C16:1	2.59	ND	ND
C18:0	6.77	7.08	4.71
C18:1n-9	10.62	11.51	9.23
C18:2n-6	5.22	3.19	8.93
C20:0	1.87	ND	1.37
C20:1	2.66	6.7	5.81
C20:3n-6	3.31	3.58	4.33
C20:4n-6	2.14	4.26	3.47
C20:5n-3 (EPA)	10.38	13.03	16.35
C22:6n-3 (DHA)	14.52	22.73	17.58
DHA/EPA	1.39	1.74	1.08

Results are the mean value of two replicates. S. E. of the fatty acid constituents were on the order of about ±2%. For lecithin, fatty acids showed which was present more than 1% of total fatty acids. ND- Not detected

3.3.5. Oxidative stability

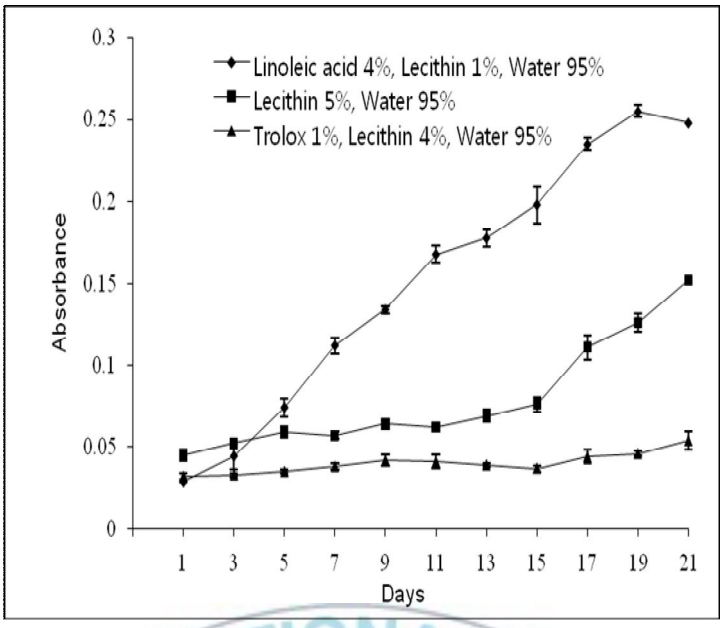
The oxidative stabilities of marine lecithin are shown in Fig. 3.3A and B. In this study oxidation trend was evaluated instead of determining the absolute state of oxidation of incubated sample. Lecithin with linoleic acid emulsion showed increase in absorbance values from the first day. The increase in absorbance value was an indicator of auto oxidation by formation of peroxides during incubation. Only the marine lecithin emulsion showed low absorbance value indicating low level of lipid peroxidation until 15 days. The marine lecithin showed significant increased oxidation after 20 days. On the other hand, marine lecithin emulsion with trolox showed high oxidative stability. Trolox, an antioxidant that inhibited the peroxide formation from lipid by peroxidation in a certain period. Initially, squid viscera lecithin emulsion showed slightly high absorbance comparing to lecithin with linoleic acid emulsion. This might be due to the presence of peroxide from the oxidation of neutral lipids of marine lecithin. In thiobarbituric acid method, the absorbance measured on 15^{th} day was also similar with lecithin and lecithin with trolox emulsion. However, this value was also high in the lecithin with linoleic acid emulsion indicating a low oxidative stability. On the other hand, a significant increase in absorbance was found on 20^{th} day for lecithin emulsion. EPA and DHA in lecithin which were the major part of unsaturated fatty acids were the most susceptible to oxidation. But marine lecithin showed high oxidative stability. In our previous study, it was found that squid viscera contained a natural antioxidant, astaxanthin. Lecithin from squid viscera may contain small amount of natural antioxidant that might be one of the causes for higher oxidative stability. Gogolewski et al., (2001) also reported that (LC-PUFAs) esterified with polar lipids had synergistic effect with antioxidant. High oxidative stabilities of lecithin from animal and plant sources were also reported by using different methods (Belhaj et al, 2010; Wang and Wang, 2008; Palacios and Wang, 2005).

3.4. Conclusions

In this study, marine lecithin was isolated from SC-CO_2 extracted squid viscera residues and characterized by measuring the FFA content, acid value and peroxide value. The major phospholipids of squid viscera lecithin were PC and PE. The phospholipids contained higher amounts of PUFAs especially EPA and DHA. The oxidative stability of

marine lecithin was also high. Therefore, it can be concluded that nonpolar lipids obtained by SC-CO_2 extraction may have different purposes and that the marine lecithin isolated from extracted samples provide PUFAs, which may be useful in the food industry as well as in the pharmaceutical industry.

A)



B)

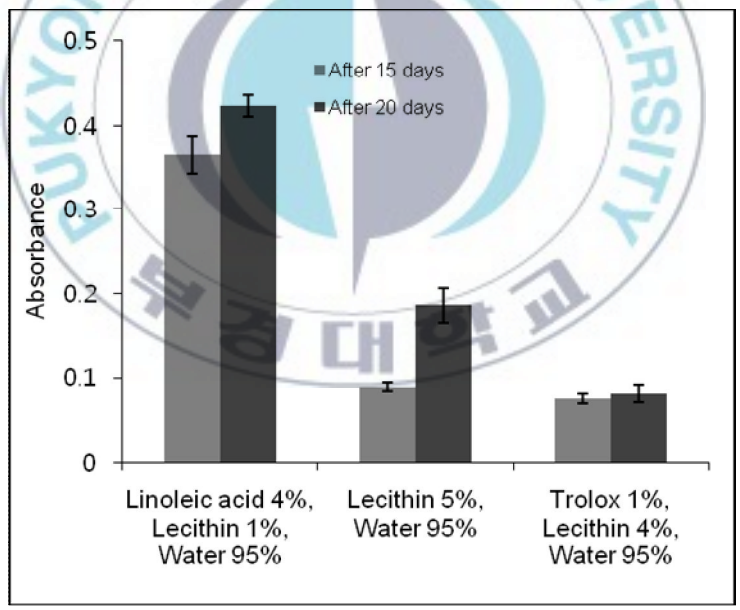


Fig. 3.3A-B. Oxidative stability of squid viscera lecithin A) Thiocyanate method and B) Thiobarbituric acid method. Results were the mean value of three replicates ± S.E.

3.5. References

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Chapter 4

Production of valued materials from squid viscera by subcritical water hydrolysis *

4.1. Introduction

Recently the requirement of biomass (aquatic, livestock, bird etc) products is enormously increasing around the world. Recognition of the limited biological resources and increasing environmental pollution have emphasized the need for better and more value-added utilization of the under-utilized by-products from the fishing industries. Traditionally, much of fish waste material has been converted to powdered fish meal by a combined process of cooking, separation of soluble from insoluble, concentration of the soluble and dehydration of the insoluble (Ferreira and Hultinn, 1994). The hydrolysis of waste into value added products (proteins, amino acids, reducing sugar etc.) is an alternative and effective way. Current industrial hydrolysis methods of biomass waste include chemical (acid, alkali or catalytic) hydrolysis and enzymatic hydrolysis. But the chemical hydrolysis needs violent reaction conditions and often brings serious pollution of the environment. Enzymatic hydrolysis takes long time for completing production cycle and is also expensive. Most of biomass waste is easily hydrolyzed in super- or subcritical water, which is structurally different from normal liquid water, and possesses some marvelous properties. Without any pollution, hydrolysis in sub- or supercritical water is environment-friendly technology (Cheng et al., 2008). Subcritical water is a promising medium for dissolution of biomass in water. The waste can be hydrolyzed into high value industrial raw material: amino acid, unsaturated fatty acid, oil and polysaccharide (Kang et al., 2001; Yoshida and Tavakoli, 2004; Tavakoli and Yoshida, 2006; Toyoji, 2001) hydrogen and methane (Levin et al., 2007) and so on. The thermal protein hydrolysis is gaining importance in economical as well as ecological aspects.

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In previous chapters, we have discussed the recovery of lipids from squid viscera. After extraction of lipid, the squid viscera residues still contain some other useful materials- mainly higher amount of proteins. However, value added materials may be recovered from viscera residues obtained by SC-CO_2 extraction. Therefore, the aim of this study was to produce valued materials by subcritical water hydrolysis of the squid viscera residues obtained after SC-CO_2 extraction. The efficiency of subcritical water hydrolysis for production of useful materials from SC-CO_2 extracted squid viscera was checked by comparing with that of freeze dried raw viscera sample.

4.2. Materials and Methods

4.2.1. Materials

The freeze dried raw and SC-CO_2 extracted (at 25 MPa and 45ºC) squid viscera samples were used for subcritical water hydrolysis. All reagents used in this study were of analytical or HPLC grade.

4.2.2. Proximate Composition

The moisture, ash and crude protein content were determined according to AOAC (1990) and lipid content was measured by conventional soxhlet extraction 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.

4.2.3. Subcritical water hydrolysis

Subcritical water hydrolysis unit are shown in Fig. 4.1. The subcritical hydrolysis was carried out in 80 mL of a batch reactor made of 276 Hastelloy with temperature control. The freeze dried raw and SC-CO_2 extracted squid viscera residues were suspended separately in distilled water (material to water ratio of 1:50) and charged into the reactor. The reactor was then closed and heated by an electric heater to the desired temperature (180-280ºC). The sample was stirred by stirrer at 140 rpm. The reaction time for each sample was 5 min. The short reaction time was considered to avoid the massive decomposition of amino acids into organic acids. The pressure in the reactor was

estimated to be between 0.101 MPa and 6.41 MPa based on saturated steam table for the temperature range studied. After cooling, the hydrolyzed sample from the reactor was collected and filtered. The liquid portion called hydrolyzate was analyzed for protein, amino acids and reducing sugars. All experiments were performed in duplicate.

4.2.4. Measurement of protein content of hydrolyzates

The protein content of the soluble product was assayed according to Lowry et al. (1951) using bovine serum albumin (BSA) as a standard. Protein content of hydrolyzate was measured on a calibration curve constructed from BSA standard (Fig. 4.2).

4.2.5. Measurement of reducing sugar content of hydrolyzates

Reducing sugars content was measured by dinitrosalicylic (DNS) acid method (Miller, 1959) using D-glucose as a standard. Briefly, 3 mL of the hydrolyzates were pipetted into test tubes and 3 mL of DNS reagent was added to each of the solution and mixed well. The test tubes were heated for 5 minutes in a boiling water bath. After the color has developed, 1 mL of 40% Rochelle salt solution was added to each of the tubes, when the contents of the tubes were still warm. 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 similarly. The spectrophotometric reading was recorded at 575 nm using a double beam UV/VIS spectrophotometer (UVIKON 933, Kontron Instruments). Reducing sugar content of hydrolyzate was measured on a calibration curve constructed from glucose standard (Fig. 4.3).

4.2.6. Analysis of amino acids

The quantitative determination of the amino acids was performed by an amino acid auto analyzer (Hitachi L-8900, Tokyo, Japan). The hydrolyzates of freeze dried raw and SC-CO_2 squid viscera were diluted to the protein concentration of 0.25 mg/mL by 0.02 N HCl. The diluted samples were then filtered and sent to central laboratory, Pukyong National University for amino acid analysis.

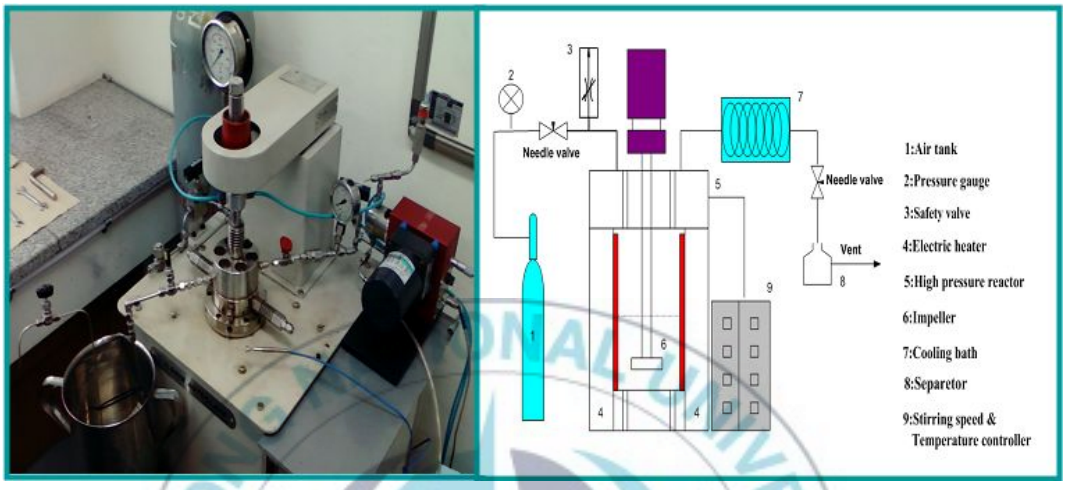


Fig. 4.1. Photograph and schematic diagram of subcritical water hydrolysis unit.

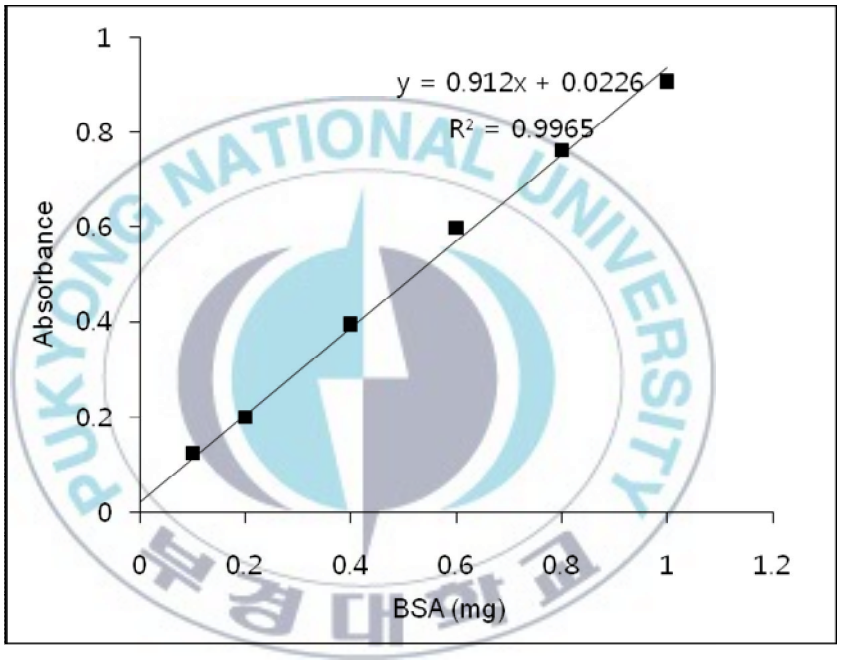


Fig. 4.2. Calibration curve of BSA for estimation of protein in hydrolyzate.

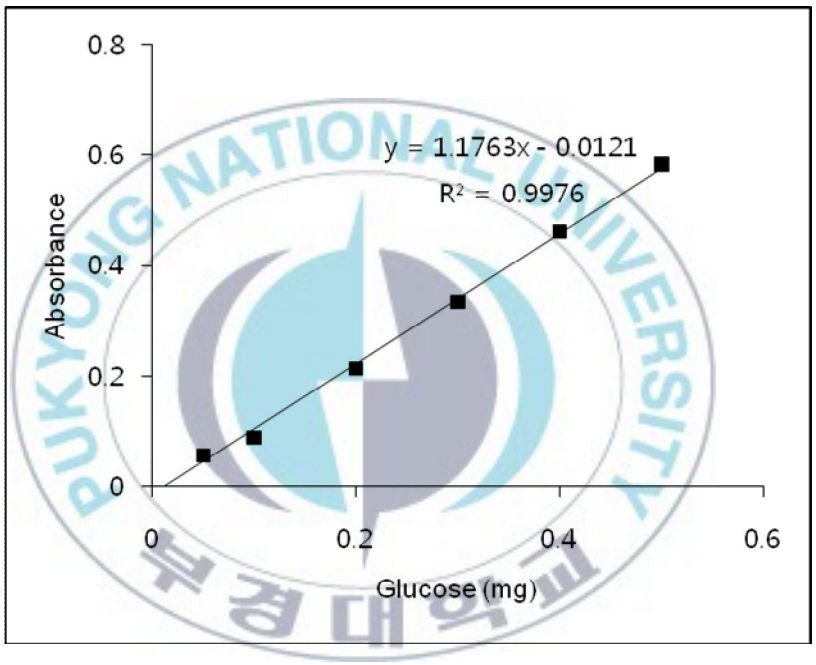


Fig. 4.3. Calibration curve of glucose for estimation of reducing sugar.

4.3. Results and Discussion

4.3.1. Compositions of freeze dried raw and SC-CO_2 extracted squid viscera

The compositions of freeze dried raw and SC-CO_2 extracted squid viscera are shown in Table 4.1. The squid viscera were dried in a freeze dryer for higher efficiency of SC-CO_2 extraction of lipid and lipid soluble bioactive materials. The moisture content of freeze dried raw and SC-CO_2 extracted sample was 3.5±0.2 and 2.51±0.15%, respectively. The moisture content was found to be decreased in SC-CO_2 extracted viscera residues. The moisture might be reduced by combining with CO_2. We have already discussed the SC-CO_2 extraction of lipid from squid viscera at different conditions in Chapter 2. The highest yield of oil by SC-CO_2 extraction was approximately 34%, where as, it was about 39% by conventional organic solvent extraction. Generally, SC-CO_2 can not extract all lipids from the sample. In our study, 5.05±0.21% lipid obtained by soxhlet extraction at hexane from SC-CO_2 extracted squid viscera residues. The protein content in freeze dried raw samples were 45.76±0.64%. After deoiling by SC-CO_2, the protein content of squid viscera increased to 71.12±0.69%. It was also found that the ash and non protein content was high in SC-CO_2 extracted squid viscera residues.

4.3.2. Protein yield in hydrolyzates

The protein content in squid viscera hydrolyzates at different temperatures are shown in Table 4.2. It was found that the hydrolyzate of SC-CO_2 extracted squid viscera contained more protein than that of raw sample hydrolyzate. The lipid present in the raw materials made them less accessible to water. The protein yield increased with the increase in temperature in subcritical water hydrolysis of SC-CO_2 extracted squid viscera. But Watchararuji et al. (2008) reported that the protein yield in subcritical water hydrolysis of soybean was found to decrease when temperature increased from 200 to 220ºC. The protein yields in raw and SC-CO_2 extracted squid viscera hydrolyzates were 340.55±6.25 and 660.58±2.94 mgg^{-1} at 180 and 280ºC, respectively. This result suggested that almost all protein could be recovered by the hydrolysis of SC-CO_2 extracted sample. Similar results were found in subcritical water hydrolysis of rice bran and soybean meal (Watchararuji et al. 2008). In general, at ambient temperature, protein normally has low

solubility in water due to strong aggregation through hydrophobic interactions. The solubility of protein in water increased at higher temperature. Moreover, the protein yield increased at elevated temperature due to the increased rate of hydrolysis caused by the increase in dissociation constant or ion product constant of water.

4.3.3. Reducing sugar yields

The reducing sugar content in freeze dried raw and SC-CO_2 extracted squid viscera hydrolyzates are shown in Fig. 4.4. Carbohydrate which reacts with hydronium and hydroxide ions produces reducing sugars. The amount of reducing sugar in both raw and SC-CO_2 extracted squid viscera increased with increasing temperature. The decomposition of reducing sugar did not occur within this temperature range (180-280ºC) for short reaction time. Similar results were found in subcritical water hydrolysis of rice bran and soybean meal (Watchararuji et al. 2008). The reducing sugar yield in hydrolyzate of SC-CO_2 extracted viscera was higher than that of raw viscera. This result also agreed with high content of non-protein substances in SC-CO_2 extracted squid viscera (Table 4.1).

Table 4.1. Proximate compositions of freeze dried raw and SC-CO_2 extracted squid viscera

Composition (%)	Freeze dried raw squid viscera	SC-CO_2 extracted squid viscera
Moisture	3.5±0.2	2.51±0.15
Ash	3.15±0.13	5.57±0.16
Protein	45.76±0.64	71.12±0.69
Lipid	39.24±1.03	5.05±0.21
Non protein	8.35	15.75

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

Table 4.2. Protein yield from freeze dried raw and SC-CO_2 extracted squid viscera by subcritical water hydrolysis at different temperatures

Temperature (ºC)	Freeze dried raw viscera hydrolyzate (mg/g)	SC-CO_2 extracted viscera hydrolyzate (mg/g)
180	340.55±6.25	439.35±3.12
200	324.25±4.89	434.25±5.79
220	303.12±2.55	460.21±1.69
240	302.22±5.66	468.56±2.19
260	296.22±1.76	616.5±3.55
280	275.76±5.22	660.58±2.94

Mean value of two replicates ± S.E.

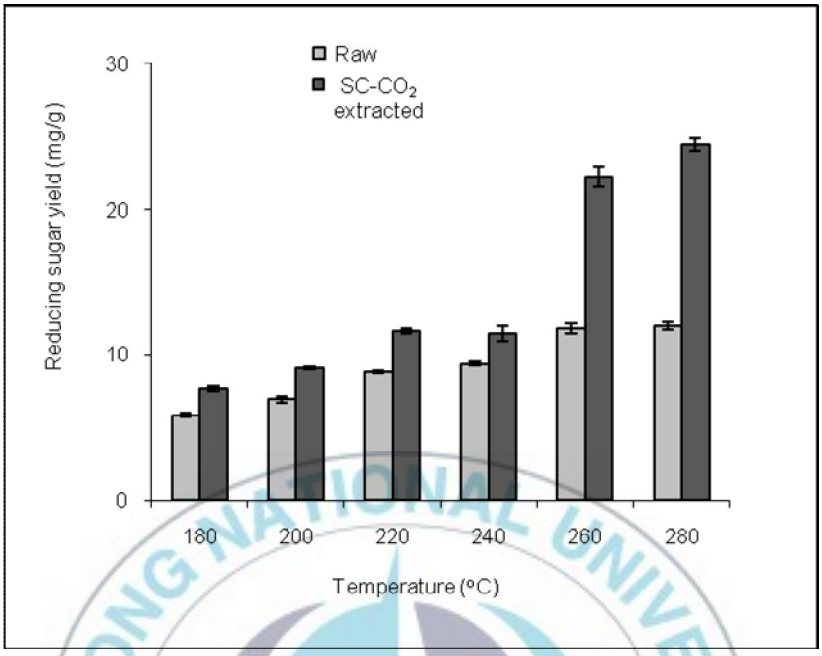


Fig. 4.4. Reducing sugar yield by subcritical water hydrolysis of raw and SC-CO_2 extracted squid viscera at different temperatures. Data were the mean value of two replicates ± S.E.

4.3.4. Amino acid yields

Amino acids play an important physiological role in all life-forms. Amino acids are relatively tasteless. Nonetheless, they contribute to the flavor of foods. Amino acids and protein hydrolyzates are therefore useful additives in food industry (Rogalinski *et al*., 2005). In this study, short reaction time had been used for subcritical water hydrolysis to decrease the decomposition of amino acids (Kang et al., 2001). Low ratio of material to water was used considering higher efficiency of subcritical water hydrolysis for amino acids yield. Lamoolphak et al. (2008) obtained highest amino acids yield by subcritical water hydrolysis at similar ratio of material to water. The total amino acid yield in raw and SC-CO_2 extracted squid viscera hydrolyzates are shown in Fig. 4.5. It was found that the total amino acid yield of SC-CO_2 extracted material hydrolyzates were higher than that of raw material hydrolyzates. This result agreed with the high protein yield of SC-CO_2 extracted squid viscera hydrolyzates comparing to raw material hydrolyzates. For SC-CO_2 extracted squid viscera, the amino acid yield increased with the increase in temperature. The highest yield of amino acids in SC-CO_2 extracted squid viscera hydrolyzate was 533.78±4.13 mg/g sample at 280ºC. Cheng et al. (2008) also reported that the yield of amino acids increases with increase in temperature to a certain degree.

In raw squid viscera hydrolyzates, the opposite result was found in which the total amino acid yield decreased with the increasing temperature. The highest amino acid yield in raw squid viscera hydrolyzates was 233.25±3.25 mgg^{-1} raw squid viscera at 180ºC. The total amino acid yield in raw squid viscera hydrolyzate was low at high temperature. Since the amino acid yield in SC-CO_2 extracted material was higher at high temperature, the oil present in the raw material may have interfered the breakdown of peptide bond by subcritical water hydrolysis at high temperature. This was also in agreement with low protein yield in raw squid viscera hydrolyzates at high temperature. For short reaction time at high temperature, oil may form complex with protein that may hinder the protein hydrolysis by subcritical water. Moreover, high temperature causes the decomposition of amino acids into organic acids or other products. Kang and Chun (2004) also observed a significant decrease in the amino acid production from a hydrothermal process of fish-derived wastes due to the decomposition of amino acids into organic acids or volatile

materials. In this study, the amino acid yield in raw squid viscera hydrolyzates was found more dominant at low temperature.

The yield of some individual amino acids from raw and SC-CO_2 extracted squid viscera by subcritical water hydrolysis at different temperatures are given in Fig 4.6A and B. For raw squid viscera hydrolyzates, the highest yield obtained within 180-220ºC. On the other hand, for SC-CO_2 extracted material it was found that the highest yield of amino acids obtained within the temperature of 260-280ºC. Cheng et al. (2008) reported that most of amino acids give maximum yield at the reaction temperature range of 200-290ºC. Some other works have been carried out in which the thermal degradation of amino acids occur at temperature above 250-300ºC, depending on the raw protein and corresponding contact time (Daimon et al., 2001; Yoshida et al., 1999; Quitain et al., 2001; Yoshida et al., 2003).

The highest recovery of amino acid from raw and SC-CO_2 extracted squid viscera by subcritical water hydrolysis were about 51 and 76%, respectively (Fig 4.7). The efficiency of subcritical water hydrolysis for amino acid yield was significantly higher in SC-CO_2 extracted material than raw material.

4.4. Conclusions

This study focused on the production of valued materials, especially amino acids, from squid viscera using subcritical water hydrolysis. Most proteins from SC-CO_2 extracted squid viscera were recovered in subcritical water hydrolysis at high temperature. In terms of protein hydrolysis, the highest amino acid yield from raw and SC-CO_2 extracted squid viscera hydrolyzates were found at low and high temperature, respectively. The recovery of the amino acids from raw sample hydrolyzate was lower than that of SC-CO_2 extracted sample hydrolyzate. Subcritical water hydrolysis was more effective for amino acid recovery from SC-CO_2 extracted material than raw material in short reaction time. Therefore, subcritical water hydrolysis may be a useful method for production of valued materials from squid viscera. This can act as source of food additives for living beings as well as a means to save the environment from pollution.

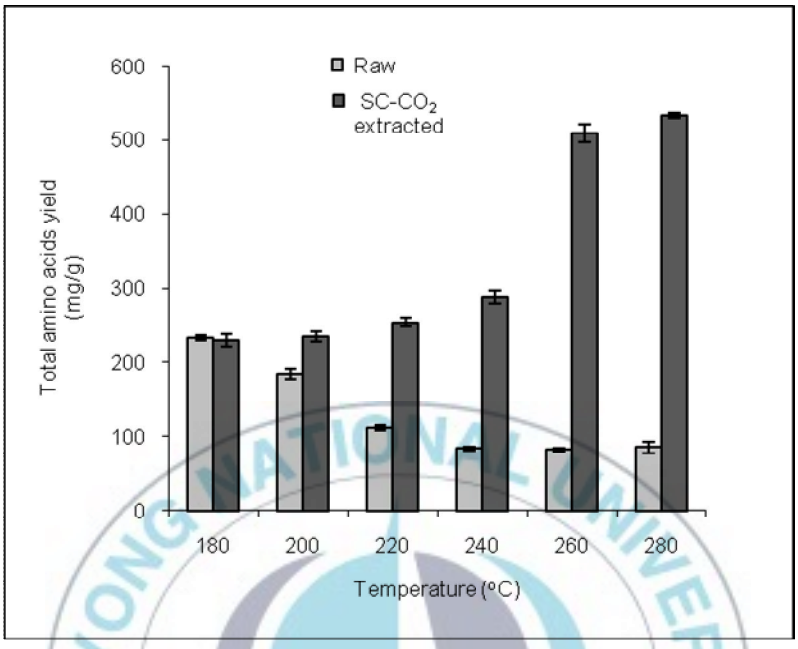
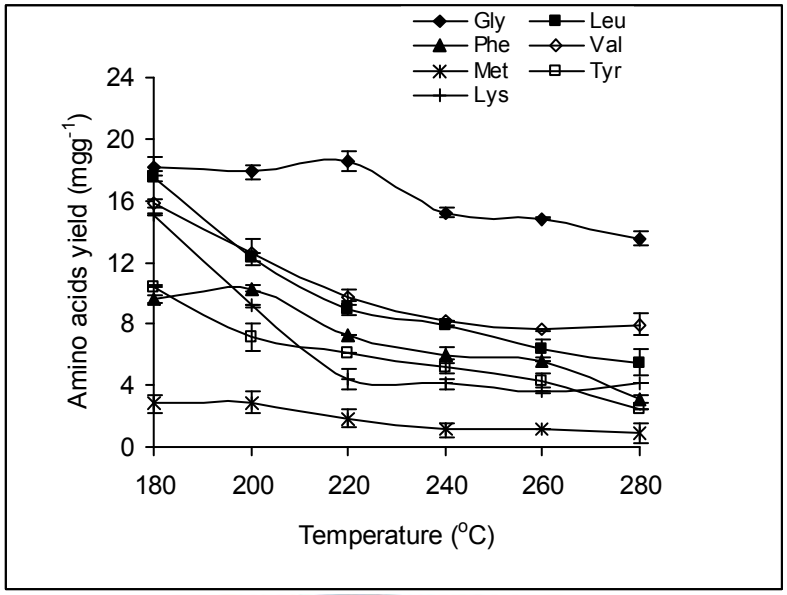


Fig. 4.5. Total amino acid yield by subcritical water hydrolysis of freeze dried raw and $SC\text{-}CO_2$ extracted squid viscera at different temperatures. Data are the mean value of two replicates ± S.E.

A)



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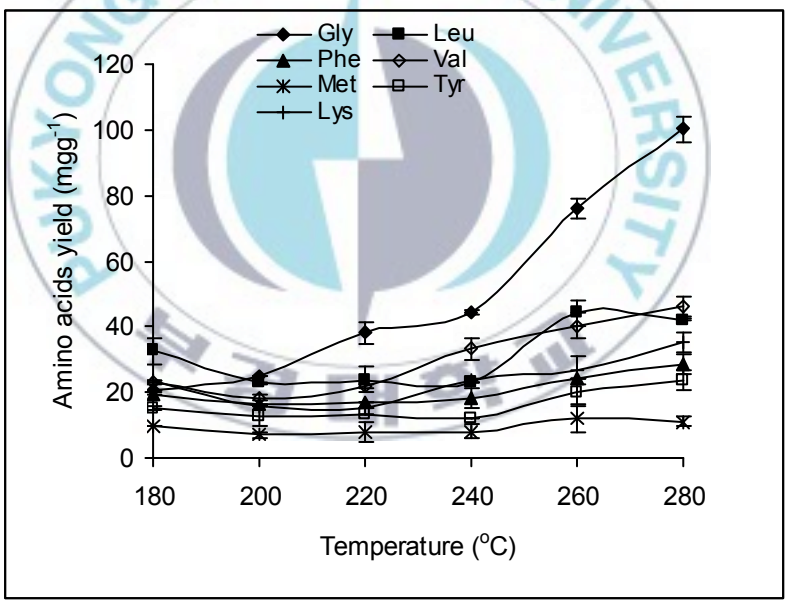

Fig 4.6A-B. Individual amino acid yield by subcritical water hydrolysis at different temperatures. A) Raw squid viscera, B) SC-CO_2 extracted squid viscera. Data were the mean value of two replicates ± S.E.

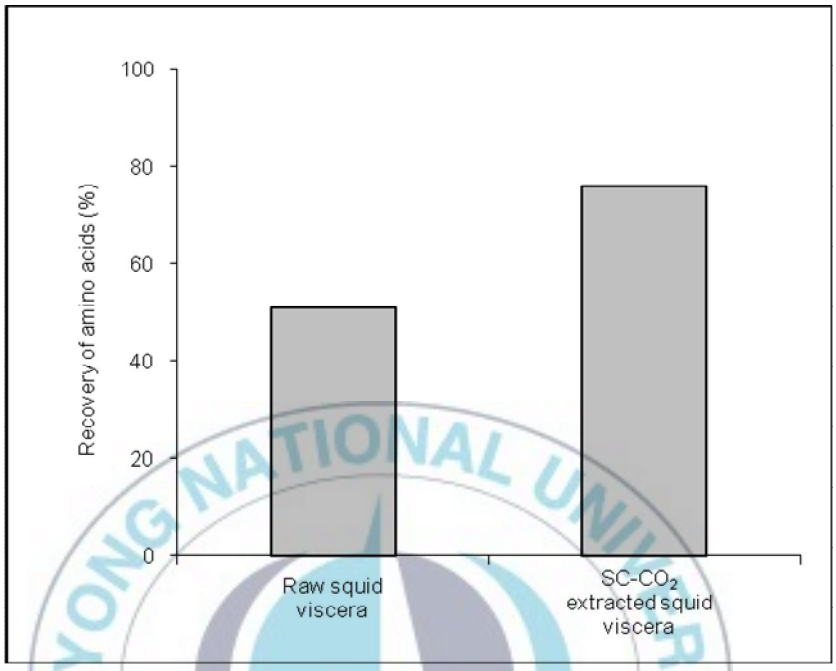

Fig 4.7. Recovery of amino acids from raw and SC-CO_2 extracted squid viscera by subcritical water hydrolysis.

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Chapter 5

Digestive enzymes activities of squid viscera followed by supercritical carbon dioxide and organic solvent extraction *

5.1. Introduction

Enzymes have been widely used as processing aids in various food related industries for a long time (Simpson et al., 1991; Vilhelmsson, 1997). The use of enzymes in the food industry is gaining interest, both for the production and conservation of food (Temiz et al., 2007). In general, enzyme technology has evolved to become an integral part of the food industry. Enzymes have also many other industrial applications. Proteases execute a large variety of functions and have important biotechnological applications. Proteases represent one of the three largest groups of industrial enzymes and are used in detergents, leather industry, pharmaceutical industry and bioremediation processes (Anwar and Saleemuddin, 1998). Lipolytic enzymes are currently attracting an enormous attention due to their wide spread use in the biotechnology industry. They constitute the most important group of biocatalysts in biotechnological applications. Some of the industrially important chemicals 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 are used for the preparation of sizing agents in textile industry, preparation of starch sizing pastes for use in paper industry and removal of spots in laundry.

The aquatic environment contains a wide variety of genetic material and, hence represents an enormous potential for discovering different enzymes (Raa, 1990). In recent years, recovery and characterization of enzymes from fish and aquatic invertebrates has gained importance and this has led to the emergence of some interesting new applications

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of these enzymes in food processing (Shahidi and Kamil, 2001). 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. This is mainly due to high tissue concentration of enzymes in these animals (Simpson et al., 1991) and partly due to a better understanding of enzymes and their commercial availability from marine sources (Stefansson & Steingrimsdottir, 1990; Han, 1993). Fish viscera are known to be a rich source of digestive enzymes. Squid viscera as a non-edible part are discarded by the fish processing industries. These materials like other fish viscera are rich sources of various enzymes that may have some unique properties of interest for both basic research and industrial applications (Simpson and Haard, 1999).

Lipids present in the sample prevent extraction and purification of enzymes. For purification of enzymes, lipid free sample is needed for higher efficiency. Removal of lipids with organic solvents causes protein denaturation and loss of functional properties (Pariser et al., 1978). In previous chapter we have discussed the SC-CO_2 extraction of lipid from squid viscera as an environmental friendly technology. After lipid extraction, the viscera residues contained functional proteins including other materials. However, it is plausible that SFE may also be used for the isolation of enzymes and production of a high quality protein meal from different sources.

Therefore, the aim of this study was to measure the digestive enzyme activities and characterize the crude enzymes from SC-CO_2 extracted squid viscera. The enzyme activities obtained from SC-CO_2 extracted sample was also compared with that obtained by organic solvent, hexane.

5.2. Materials And Methods

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

5.2.1. Materials

Freeze dried raw squid viscera and SC-CO_2 (at 25 MPa and 45ºC) and hexane extracted residues were used for this study. All other chemicals used in this study were of analytical or HPLC grade.

5.2.2. Digestive enzyme assay

5.2.2.1. Preparation of crude enzyme

The highest oil extracted residues of squid viscera by SC-CO_2 and hexane extracted viscera residues were homogenized in cold distilled water (1g sample/6 mL water) by mechanical stirring at 4°C for 2 hrs. The samples were then centrifuged at 9000 rpm for 15 min at 4°C. The supernatant were collected and stored at -20°C. These samples were used as crude enzyme extract and for electrophoresis.

5.2.2.2. Protease assay

Protease activity was assayed by the casein Folin-Ciocalteau method (Oda and Murao, 1974) with slight modification. One percent casein solution in 0.0125 M sodium borate-NaOH buffer (pH 10.5) was used as substrate. Crude enzyme (0.5 mL) was mixed with 2.5 mL of substrate and incubated for 10 min at 37ºC. The reaction was stopped by addition of 2.5 mL of TCA solution and settled for 20 min. The sample was then centrifuged for 10 min at 3000 rpm. The supernatant (2 mL) mixed with 5 mL of 0.55 M Na_2CO_3 and 1 mL of 1 N Folin-Ciocalteu reagent. The optical density of the colour developed at 37°C for 20 min was measured at 660 nm (UVIKON 933, Kontron Instruments). One unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol of tyrosine per min from casein. Tyrosine was used to construct a calibration curve.

5.2.2.3. Lipase assay

Lipase activity was assayed using the modified method of Vorderwülbecke et al. (1992) described by Hatzinikolaou et al. (1999). The substrate emulsions were prepared by drop wise addition 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 intense vortexing. These emulsions were stable for 1 hr at room temperature. The crude extract (0.1 mL) was added to 3.2 mL of the substrate emulsion and the mixture was incubated for 20 min in a shaking water bath at 35°C. The reaction was terminated

by boiling for 5 min. Following centrifugation (6000 rpm, 10 min) the absorbance of the clear supernatant was measured at 410 nm. The mixture with 0.1 mL of the inactivate enzyme extract (heated at 100°C for 5 min) was used as control. 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 assay conditions. A calibration curve was constructed using *p*-nitrophenol.

5.2.2.4. Amylase assay

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

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

The effects of pH on crude enzyme activity were determined using different buffers of wide range of pH values. The buffers used were 0.1 M citric acid-sodium citrate (pH 4.0-5.5), 0.1 M potassium phosphate (pH 6.0-8.0) and 0.1 M glycine-NaOH (pH 8.5-12). The pH stability was tested by 5 hrs pre-incubation of the crude enzyme extract in buffers that had the same ionic concentrations at different pH values ranging from 3.0 to 12.0 at 0°C. The enzyme activities were measured immediately after this treatment using the standard methods as mentioned above.

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

Potassium phosphate (0.1M, pH 7.5) buffer for amylase and 0.1 M glycine-NaOH (pH 8.5) buffer for protease and lipase were used at different temperatures (20-80°C) for the determination of optimal temperature for enzyme activities. Temperature stability of the crude enzyme was tested by pre-incubating the enzyme extract at different

temperatures (20-80°C) for 1 hr and the residual enzyme activities were assayed under standard assay conditions.

5.2.5. Electrophoresis

Sodium dodecyl sulfate-polyacrylamaide gel electrophoresis (SDS-PAGE) of crude extracts were carried out by the method of Laemmli (1970) using 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 h). 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) were used.

5.3. Results And Discussion

5.3.1. Digestive enzyme activities

Fig. 5.1A-C shows the activities of protease, lipase and amylase of crude enzyme extracts of squid viscera. Among the three classes of digestive enzymes, the activity of lipase was highest. The activities of protease, lipase and amylase were higher in hexane extracted squid viscera samples than in SC-CO_2 extracted samples. This may have resulted from a loss in digestive enzyme activity of squid viscera samples by the SC-CO_2 treatment. The loss of enzyme activity after SC-CO_2 extraction has been observed in prior studies and this phenomenon has been attributed to interactions between CO_2 and the enzyme. This means CO_2 may form covalent complexes with free amino groups on the surface of the enzyme. Some authors reported similar observation (Kamat et al., 1995; Habulin and Knez, 2001). Due to formation of covalent complex, the activities of enzymes were reduced.

5.3.2. Optimum pH of protease, lipase and amylase

The optimum pH of protease, lipase and amylase were shown in Fig. 5.2A-C. The highest proteolytic activities of crude extract in both SC-CO_2 and hexane extracted squid viscera were found at alkaline pH 9. High protease activities at pH ranging from 8.0 to 10.0 have also been reported in several fish species (Eshel et al., 1993; Hidalgo et al., 1999; Prasertsan et al., 2001). Low protease activities were also found in acidic pH. This indicated that the crude extracts of squid viscera contained both the acidic and alkaline proteases. Similar results were found by Natalia et al. (2004) for carnivorous ornamental fish.

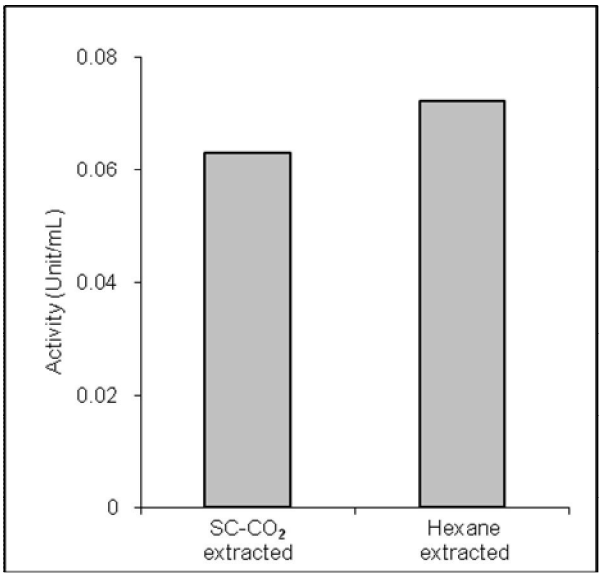
The crude enzyme of squid viscera exhibited optimum lipolytic activity at pH 8.5. An optimum pH of 7-9 for lipase activities were reported from fish and other sources (Gjellesvik et al., 1992; Raso and Hultin, 1988; Mukundan et al., 1985; Kumar et al., 2005). The activities of amylase in the crude extracts of squid viscera were maximal at pH 7.5. An optimum pH of 7-7.5 was reported for amylase obtained from the gut of seabream and turbot (Munilla-Mordn and Saborido-Rey, 1996) and tuber (Noman et al., 2006). Lipase and amylase activities were very low at an acidic pH due to the presence of

acidic lipase and amylase. In the crude extracts of SC-CO_2 and hexane extracted squid viscera, the difference in the pH effect on digestive enzyme activities were not significant.

5.3.3. pH stability

pH stability of protease, lipase and amylase in the crude extracts of SC-CO_2 and hexane extracted squid viscera are shown in Fig. 5.3A-C. In the pH range of 8-10.5, the protease activities were more than 90% of its original value and then decreased with increasing pH. The pH stability of crude enzyme extracts of tuna viscera were reported to be in the range of 9-11, where more than 90% activity was retained (Prasertsan et al., 2001). The instability of acidic proteases towards the alkaline pH region contrasts with the behavior of gastric proteases obtained from many of the lower vertebrates species (Yamamoto, 1975). The crude extract lipase was stable within a pH range of 7-9.5 with 90% activity. Kumar et al. (2005) and Aryee et al. (2007) reported that lipases were stable within a pH range of 8-10.5 and 7-10, respectively. The stability of amylase of squid viscera extract was in pH range of 6-8.5 (almost 90% activity). Noman et al. (2006) reported the α-amylase stability at the pH range of 6-8. The differences in the stable pH range for the crude extract of SC-CO_2 and hexane extracted squid viscera were not significant.

A)



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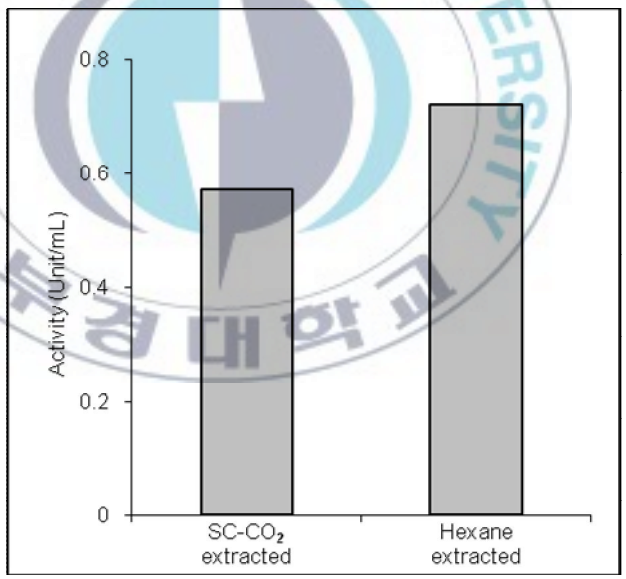


Fig. 5.1A-C. Digestive enzyme activities of crude extracts of SC-CO_2 and hexane extracted squid viscera. A) Protease, B) Lipase and C) Amylase.

C)

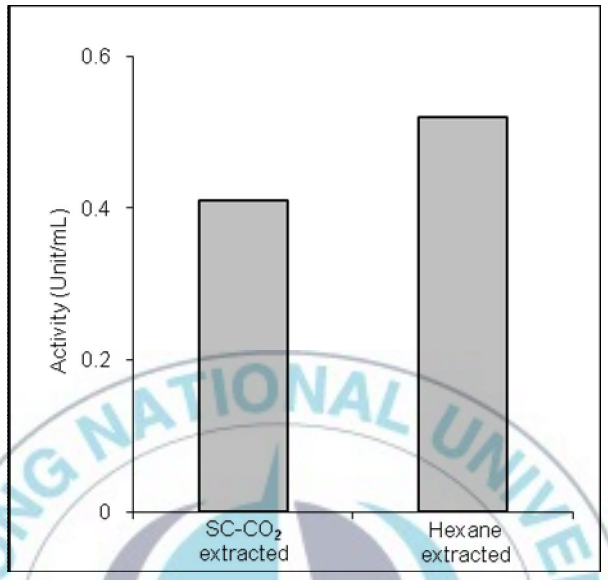
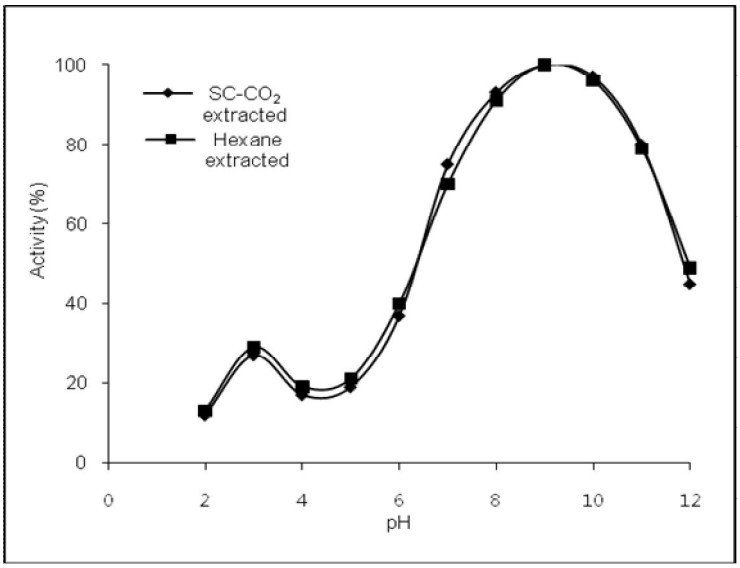


Fig. 5.1A-C. Digestive enzyme activities of crude extracts of SC-CO_2 and hexane extracted squid viscera. A) Protease, B) Lipase and C) Amylase.

A)



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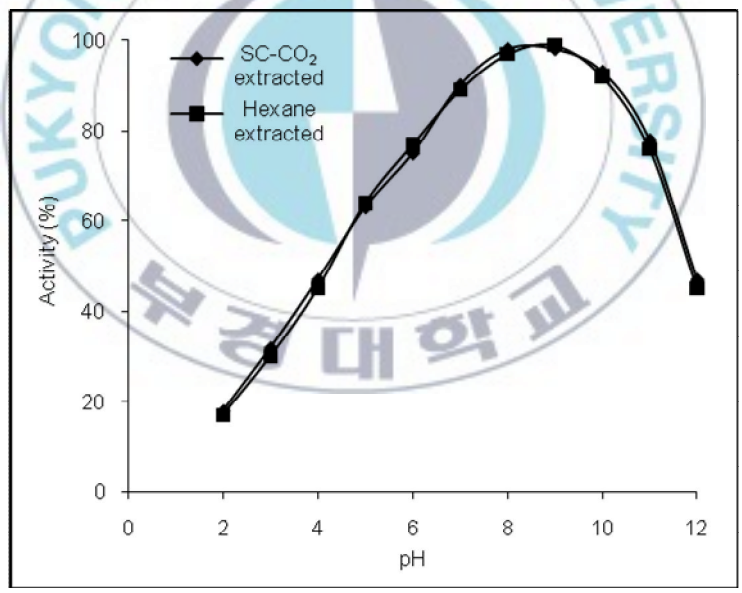


Fig. 5.2A-C. Optimum pH of digestive enzymes in crude extracts of SC-CO_2 and hexane extracted squid viscera. A) Protease, B) Lipase and C) Amylase.

C)

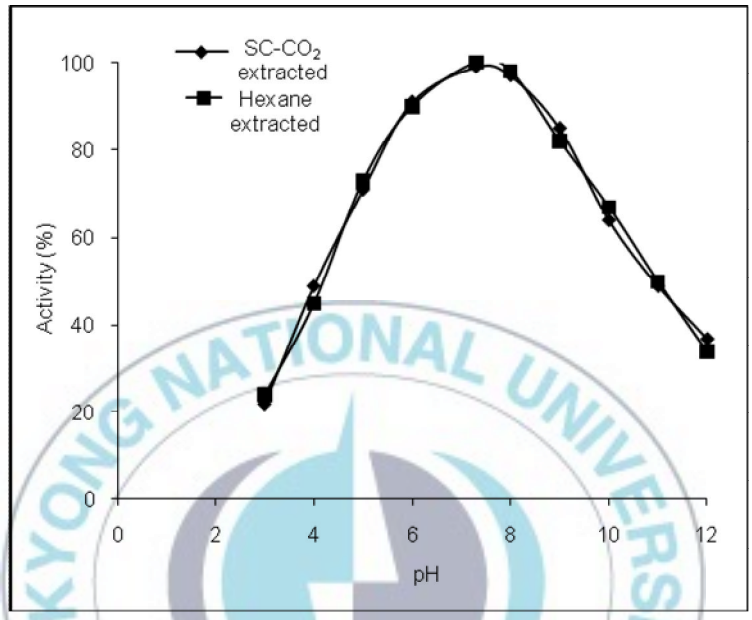
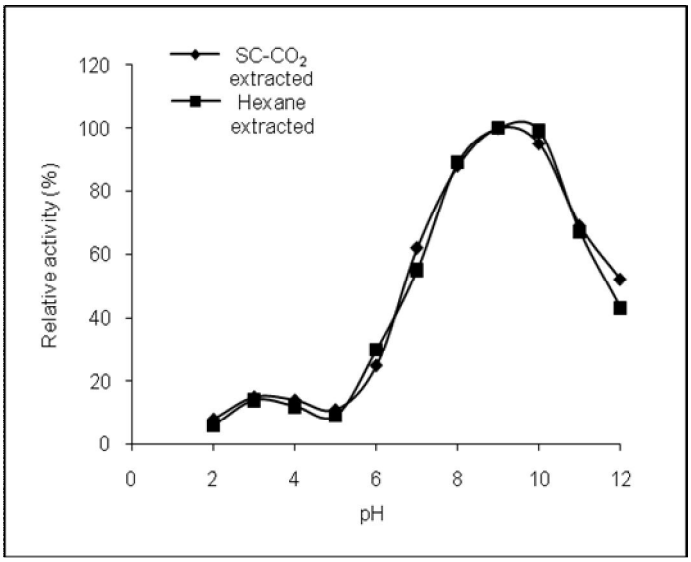


Fig. 5.2A-C. Optimum pH of digestive enzymes in crude extracts of SC-CO_2 and hexane extracted squid viscera. A) Protease, B) Lipase and C) Amylase.

A)



B)

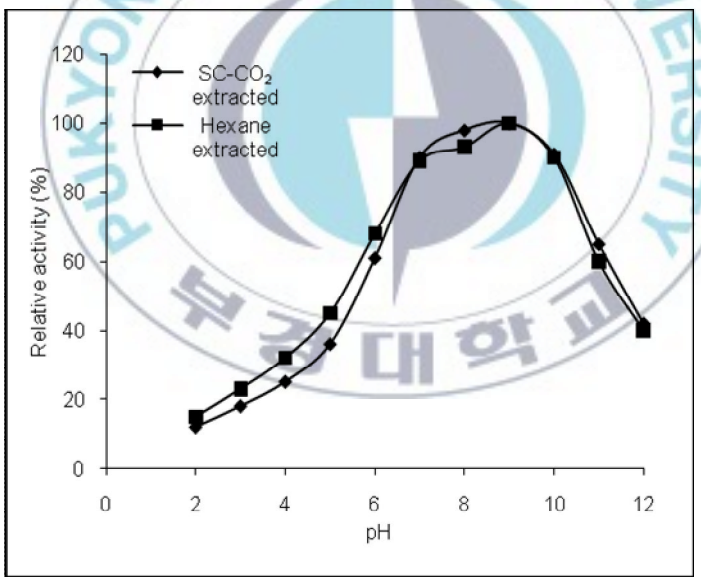


Fig. 5.3A-C. pH stability of digestive enzymes in crude extracts of SC-CO_2 and hexane extracted squid viscera. A) Protease, B) Lipase and C) Amylase.

C)

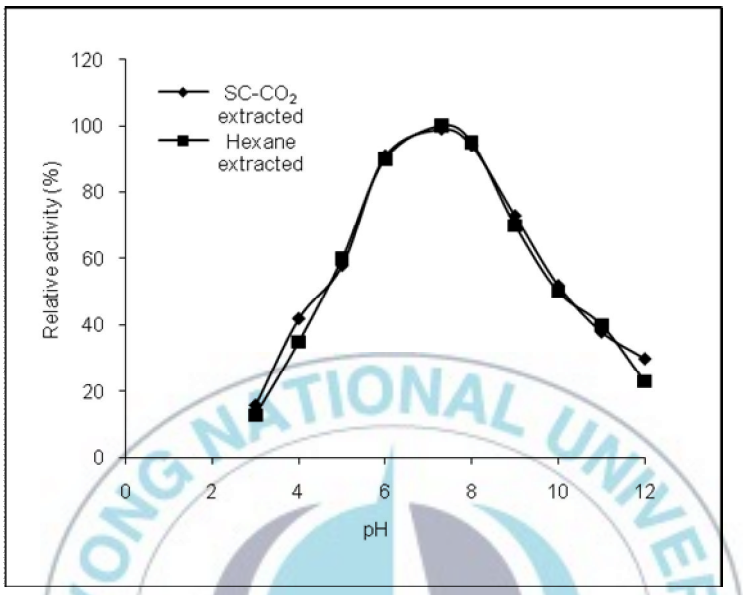


Fig. 5.3A-C. pH stability of digestive enzymes in crude extracts of SC-CO_2 and hexane extracted squid viscera. A) Protease, B) Lipase and C) Amylase.

5.3.4. Optimum temperature of protease, lipase and amylase

The optimum temperatures of different digestive enzymes are given in Fig. 5.4A-C. The maximum protease, lipase and amylase activity in both crude extracts of SC-CO_2 and hexane extracted squid viscera were found at 60, 50 and 37°C, respectively. The optimum temperature for protease in the crude extract of tambaqui waste was to be 60°C reported by Esposito et al. (2009). Similar results for the optimum temperature of amylase were described in tuber (Noman et al., 2006). The optimum temperature for lipase from grey mullet was 55°C which is slightly higher than the tuna viscera lipase. The differences in optimal temperature may be due to several factors including the varying mechanical properties of the homologous lipases and the different substrates used for measurements, which has been shown to affect the temperature sensitive activity of enzymes (Aryee et al., 2007).

5.3.5. Temperature stability

Fig. 5.5A-C shows the temperature stability of digestive enzymes of the crude extract of SC-CO_2 and hexane extracted squid viscera. The protease activities of SC-CO_2 and hexane extracted squid viscera residues remained above 85% up to 60°C. Esposito et al. (2009) reported that the protease from fish waste retained about 86% of its activity after 30 min incubation at 60°C. When the temperature was increased above 60°C the activity declined sharply. However, the SC-CO_2 extracted sample retained approximately 80% of its protease activity at 65°C where as the hexane extracted sample retained 66% of its activity. The crude extracts of squid viscera retained more than 75% of lipase activity up to 50°C. However, at higher temperatures the activity rapidly decreased. More than 70% of lipase activity in the viscera for the grey mullet was retained up to 50°C (Aryee et al., 2007). The crude extract of SC-CO_2 extracted squid viscera showed slightly higher temperature stability of lipase at temperatures greater than 50°C. For amylase, more than 80% of its activity was retained up to 40°C. Noman et al. (2006) observed a similar temperature stability of α-amylase from tuber. At 45°C, the SC-CO_2 extracted sample contained higher amylase activity than that of hexane extracted. Protease, lipase and amylase of SC-CO_2 extracted squid viscera showed slightly higher temperature stability relative to hexane extracted viscera. This may have occurred because of very

slight changes in the conformation or properties of the active site by temperature and high pressure during extraction by SC-CO_2.

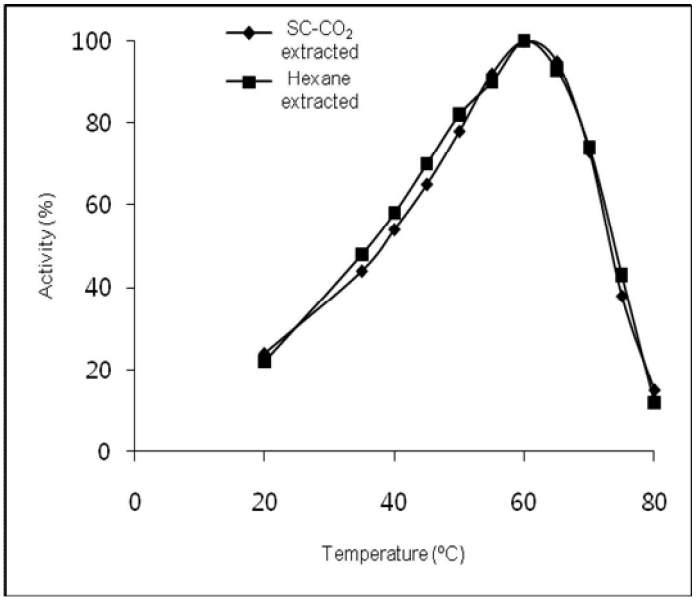
5.3.6. Electrophoresis

The slab gel electrophoresis of marker protein and the crude extract of freeze dried raw, SC-CO_2 and organic solvent extracted squid viscera are shown in Fig. 5.6. The proteins in freeze dried raw squid viscera were very similar in subunit composition to SC-CO_2 and organic solvent extracted samples. There was no any change in the intensity of protein bands since the gel banding patterns observed were almost identical. From this observation it can be concluded that protein denaturation was not found in SC-CO_2 extracted squid viscera sample. Stahl et al. (1984) also reported very little protein denaturation in SC-CO_2 extracted seed residues.

5.4. Conclusions

The digestive enzyme activities of SC-CO_2 extracted squid viscera residues were slightly lower compared to organic solvent, hexane extracted residues. In SC-CO_2 extracted samples, all of the digestive enzymes showed slightly higher temperature stability. Based on the electrophoretic patterns, no protein denaturation was found in SC-CO_2 extracted samples. The use of carbon dioxide for lipid extraction is environment friendly. Therefore, squid viscera after lipid extraction by SC-CO_2 might be used for the isolation and purification of different digestive enzymes. 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)



B)

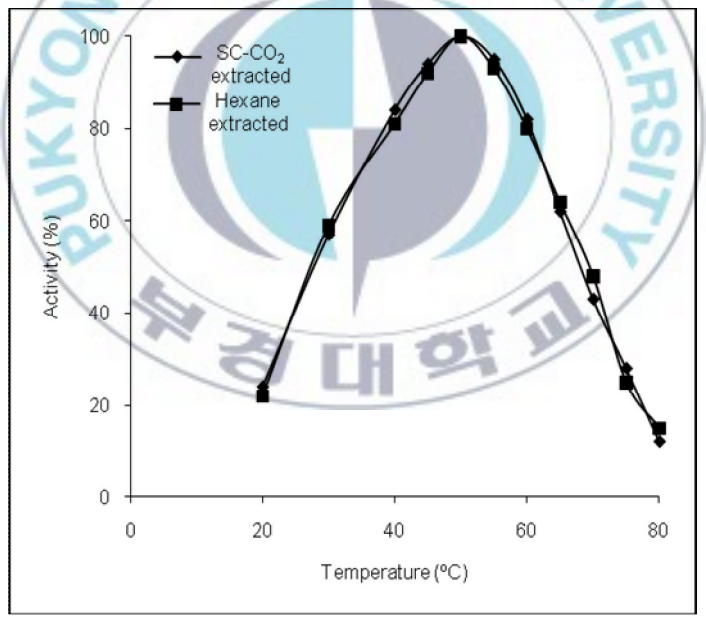


Fig. 5.4A-C. Optimum temperature of digestive enzymes in crude extracts of SC-CO_2 and hexane extracted squid viscera. A) Protease, B) Lipase and C) Amylase.

C)

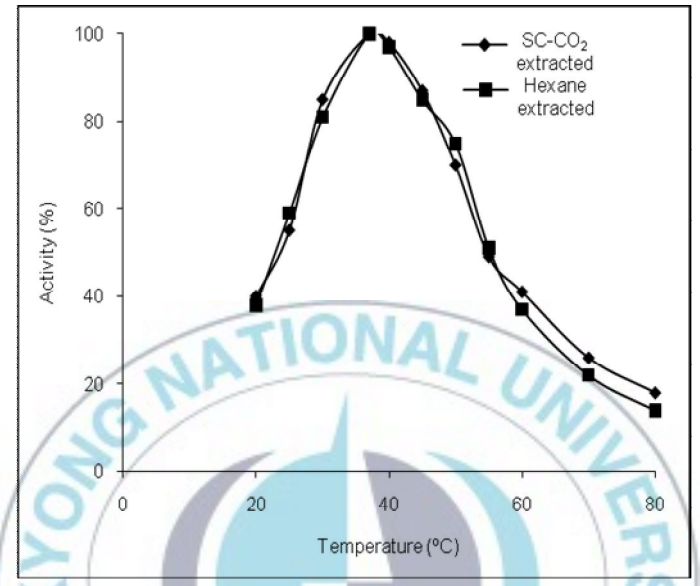
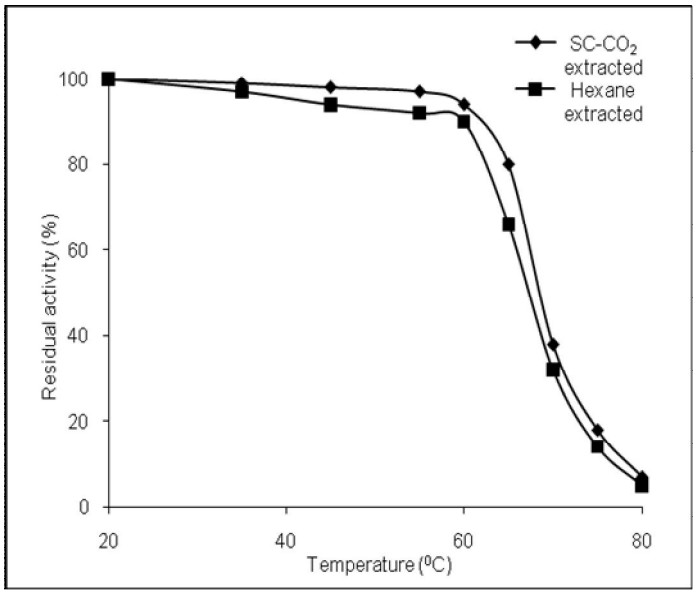


Fig. 5.4A-C. Optimum temperature of digestive enzymes in crude extracts of SC-CO_2 and hexane extracted squid viscera. A) Protease, B) Lipase and C) Amylase.

A)



B)

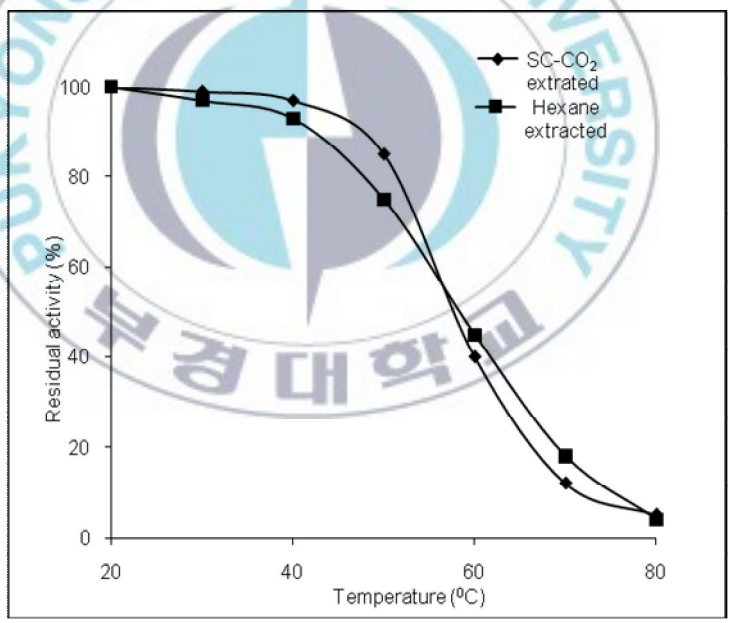


Fig. 5.5A-C. Temperature stability of digestive enzymes in crude extracts of SC-CO_2 and hexane extracted squid viscera. A) Protease, B) Lipase and C) Amylase.

C)

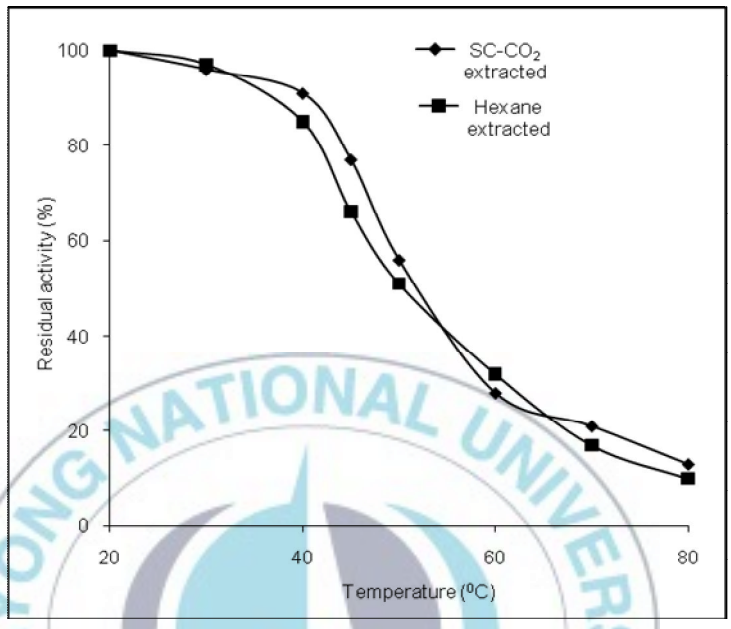


Fig. 5.5A-C. Temperature stability of digestive enzymes in crude extracts of SC-CO_2 and hexane extracted squid viscera. A) Protease, B) Lipase and C) Amylase.

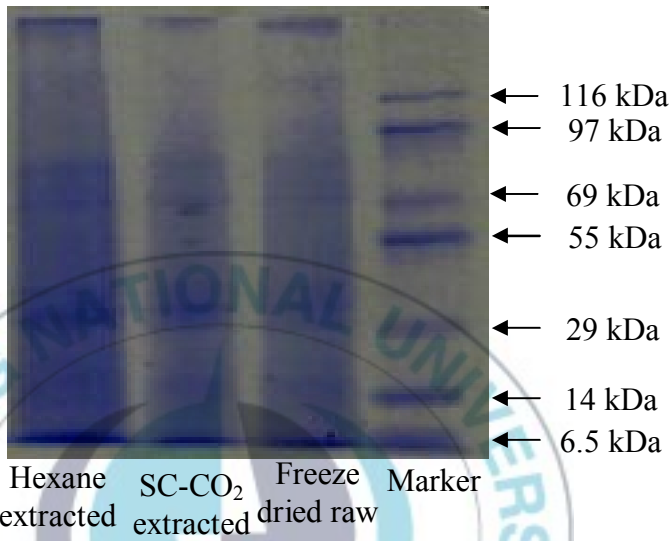


Fig. 5.6. SDS-PAGE electrophoresis of crude protein of freeze dried raw and SC-CO_2 and hexane extracted squid viscera.

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Summary

Squid viscera oil with lipid soluble bioactive compounds was extracted using SC-CO_2 and hexane. The deoiled viscera residues were used to recover other useful and functional compounds. Therefore, it can be summarized as follows

- Squid viscera 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 was more stable than organic solvent, hexane extracted oil.

- The marine lecithin obtained from SC-CO_2 extracted squid viscera residues showed high oxidative stability.

- The major phospholipids in squid viscera lecithin were PC and PE containing higher amounts of PUFAs especially EPA and DHA.

- Most of the proteins from SC-CO_2 extracted squid viscera were recovered as amino acids by subcritical water hydrolysis.

- Though the activities of principal digestive enzymes of SC-CO_2 extracted viscera residues were reduced slightly compared to hexane extracted residues, it proved the applicability of SFs in enzyme processing.

Finally, it can be concluded that the quality oil with bioactive compound would be extracted by environmental friendly solvent, SC-CO_2. Marine lecithin, proteins as amino acids and enzymes might be recovered from SC-CO_2 extracted viscera residues for using in the food industry as well as in the pharmaceutical and cosmetic industry.

아임계 및 초임계 유체를 이용한 오징어 (*Todarodes pacificus*) 내장으로부터 유용성분 회수

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요 약

오징어는 한국과 일본을 포함한 많은 나라에서 대중적으로 섭취되고 있는 식품이다. 현재 수산가공산업에 있어서 많은 양의 오징어 내장 등의 부산물이 폐기되고 있는 실정이나, 이렇게 폐기되고 있는 부산물에는 인체에 유용하고 생리활성을 가지는 물질들이 풍부하다. 수산가공산업에 있어서는 가공 중 생기는 폐기물들을 처리하는 것이 큰 문제이지만, 오징어 내장에 함유되어 있는 유용한 물질들의 재이용은 경제적으로나 환경적 측면으로 볼 때, 이익을 창출하게 해줄 것이다. 본 연구에서는 오징어 (*Todarodes pacificus*) 내장에서 친환경적 공정인 초임계 이산화탄소 (SC-CO_2) 추출법과 hexane 을 이용한 유기용매 추출법을 이용해 오일을 추출하였다. SC-CO_2 추출은 온도 범위 35 - 45℃, 압력 15 – 25 MPa 범위에서 실행하였으며 CO_2 유량은 22g/min 로서 총 추출 시간인 2 시간 30 분동안 지속적으로 흐르게 하였다. 오일의 최대 추출 수율은 온도와 압력이 높을수록 높았다. 오일의 지방산 분석을 위해 gas chromatography (GC)를 이용하였고, 그 결과 SC-CO_2 를 이용하여 추출한 오일에 고도불포화지방산 (PUFAs)의 함량이 높았으며, 그 중 특히 eicosapentaenoic acid (EPA) 와 docosahexaenoic acid (DHA) 의 함량이 높았다. SC-CO_2 를 이용하여 추출한 오징어 내장유와 hexane 을 이용한 유기용매법으로 추출한 오징어 내장유의 품질을 비교해 본 결과 SC-CO_2 추출 오일이 유기용매 추출 오일보다 높은 안정성을 보였다. 또한 high performance liquid chromatography (HPLC) 를 이용해 오징어 내장유에 함유되어 있는 astaxanthin 을 정량 분석하였고 각각 다른 추출조건으로 비교해보았다. 그 결과 25MPa, 45℃ 추출 조건에서 오징어 내장유의 astaxanthin 의 수율이 가장 높음이 확인되었다.

오징어 내장을 SC-CO_2 추출로 추출하였고, 지질이 제거된 추잔물로부터 레시틴을 분리, 특성을 파악하였다. 오징어 내장 레시틴의 주요 인지질은 HPLC 를 이용하여 정량 분석하였으며. Phosphatidylcholine (PC- 80.54±0.68%) 와 phosphatidylethanol-amine (PE- 13.18±0.24%) 의 결과값을 얻었다. .각각의 인지질 정제는 Thin layer chromatography (TLC)로 실험하였고, 레시틴과 PC, PE 의 지방산 조성을 GC 로 분석하였다. 그 결과 PC 와 PE 의 인지질 모두 상당한 양의 EPA 와 DHA 가 함유되어 있음이

확인되었다. 레시틴은 균질기를 이용하여 O/W 에멀젼으로 만들었고, 오징어 내장 레시틴의 산화안정성을 측정한 결과, 긴 사슬 불포화지방산 (LC-PUFAs) 함량이 높음에도 불구하고 산화안정성은 높았다.

아임계 가수분해는 수산가공산업에서 활용도가 낮은 오징어 내장을 고부가가치 소재로 이용되는 공정이다. 동결 건조 된 오징어내장 원료와 SC-CO_2 추출물의 가수분해 반응은 5 분 동안 180°C 에서 280°C 의 조건하에 수행되었다. 가수분해하기 위해 오징어 내장과 물의 비율을 1:50 으로 하였고, 그 결과 SC-CO_2 오징어내장 추출물의 주요 단백질은 높은 온도에서 회수되었으나 동결 건조 된 오징어내장 원료 가수분해물의 단백질 수율은 온도의 증가에 따라 감소하는 것을 확인할 수 있었다. 아임계 가수분해시 환원당 수율은 SC-CO_2 처리한 오징어 내장 추출물과 동결 건조 된 오징어내장 원료 모두 고온에서 더 높게 나타났다. SC-CO_2 처리 전, 후 오징어 내장 가수분해물의 아미노산 수율은 180 과 280°C 에서 각각 233.25±3.25 과 533.78±4.13 mg/g 로 가장 높게 나타났다. SC-CO_2 처리 전 아미노산 수율은 180 과 220°C 에서 가장 높게 나타났고, SC-CO_2 처리 후는 260-280°C 에서 가장 높게 나타났다. 아미노산 회수율은 SC-CO_2 처리 전 보다 처리 후에 1.5 배 더 높게 나타났다.

그리고 SC-CO_2 추출법이 효소정제체계에 대한 적용가능성을 확인해보았다. 오징어 내장으로부터 SC-CO_2 추출법, hexane 을 이용한 유기용매 추출법으로 추출한 오일에 함유되어 있는 3 가지 주요 소화효소의 특성에; 대한 연구를 수행하였다. 가장 많은 오일이 추출된 25MPa, 45°C 조건에서 추잔물에 함유되어 있는 소화효소들의 특성을 알아본 결과, hexane 을 이용한 오징어 내장추출물의 프로테아제, 리파아제, 아밀라아제 활성이 가장 높았고, SC-CO_2 처리된 추출물에서 활성이 가장 낮음을 확인 할 수 있었다. 소화효소의 최적 pH 와 pH 안정성은 SC-CO_2 와 hexane 을 이용한 오징어 내장 조추출물에 유의적인 차이가 없었다. 또한 프로테아제, 리파아제, 아밀라아제의 최적온도도 SC-CO_2 와 hexane 을 처리한 추출물이 거의 유사함을 확인할 수 있었다. 그러나 SC-CO_2 처리된 오징어 내장 소화효소의 열 안정성은 hexane 을 이용한 추출물보다 약간 높게 나타났다. SDS-PAGE 를 이용한 결과, 동결 건조 된 시료와 SC-CO_2, hexane 을 이용한 오징어 내장 추출물에서 단백질 패턴의 큰 차이가 없었고, 이는 단백질 변성이 일어나지 않았음을 입증할 수 있었다.

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