Thesis for the Degree of Doctor of Philosophy

Recovery and Characterization of Bioactive Compounds from Mackerel (*Scomber japonicus*) using Sub- and Supercritical Fluids

by

A. K. M. Asaduzzaman

Department of Food Science and Technology

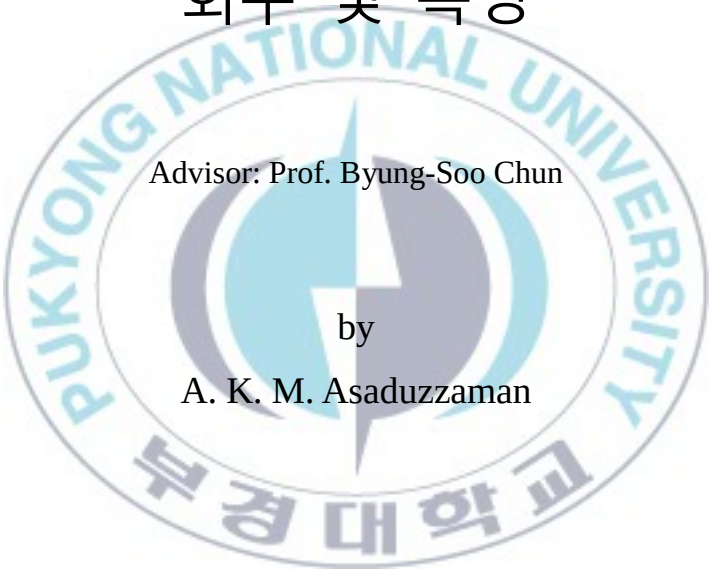
The Graduate School

Pukyong National University

August 2014

Recovery and Characterization of Bioactive Compounds from Mackerel (*Scomber japonicus*) using Sub- and Supercritical Fluids

아임계 및 초임계유체를 이용한 고등어(*S. japonicus*)로부터 생리활성 물질 회수 및 특성



Advisor: Prof. Byung-Soo Chun

by

A. K. M. Asaduzzaman

A thesis submitted in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

in Department of Food Science and Technology, The Graduate School,
Pukyong National University

August 2014

Recovery and Characterization of Bioactive Compounds from Mackerel (*Scomber japonicus*) using Sub- and Supercritical Fluids

A dissertation

by

A. K. M. Asaduzzaman

Approved by:

_____
Young-Mog Kim, Ph.D.
(Chairman)

_____
Yang-Bong Lee, Ph.D.
(Member)

_____
Eun-Woo Lee, Ph.D.
(Member)

_____
Kyoung-Ho Kim, Ph.D.
(Member)

_____
Byung-Soo Chun, Ph.D.
(Member)

August 22, 2014

CONTENTS

Chapter 4

Hydrolyzates produced from mackerel *Scomber japonicus* skin by the pressurized hydrothermal process contain amino acids with antioxidant activities and functionalities

List of Figures

List of Tables

Recovery and Characterization of Bioactive Compounds from Mackerel (*Scomber japonicus*) using Sub- and Supercritical Fluids

A. K. M. Asaduzzaman

Department of Food Science and Technology, The Graduate School,

Pukyong National University

Abstract

Mackerel (*Scomber japonicus*) is an important fish food which is consumed in many countries including Korea. A lot of mackerels are injured after catching by net which are non-merchantable. These are rich in biomolecules that are useful to living beings. The discarding of non-merchantable mackerel is a big problem in the environment. However, recovery of useful materials from non-merchantable mackerel will be beneficial economically and environmentally. In this study, the oil in mackerel muscle was extracted using an environmental friendly solvent, supercritical carbon dioxide (SC-CO_2) at a semi-batch flow extraction process and an *n*-hexane. The SC-CO_2 was carried out at temperature 45 ^{o}C and pressures ranging from 15 to 25 MPa. The flow rate of CO_2 (27 g/min) was constant at the entire extraction period of 2 h. The highest oil extracted residues after SC-CO_2 extraction was used for activity measurement of digestive enzymes. Four digestive enzymes were found in water soluble extracts after *n*-hexane and SC-CO_2 treated samples. Amylase, lipase and trypsin activities were higher in water soluble extracts after SC-CO_2 treated samples except protease. Among the four digestive enzymes, the activity of amylase was highest and the value was 44.57 uM/min/mg of protein. The water soluble extracts of SC-CO_2 and *n*-hexane treated mackerel sample showed the same alkaline optimum pH and pH stability for each of the digestive enzymes. Optimum temperatures of amylse, lipase, protease and trypsin were 40 ^{o}C,

50 ^{o}C, 60 ^{o}C and 30 ^{o}C, respectively of both extracts. More than 80% temperature stability of amylse, lipase, protease and trypsin were retained at mentioned optimum temperature in water soluble extracts of both treated samples. Based on protein patterns, prominent protein band showed in water soluble extracts after SC-CO_2 treated samples indicates no denaturation of protein than untreated and *n*-hexane.

Lecithin was isolated with ethanol from deoiled mackerel muscle after SC-CO_2 and hexane extraction. It was also isolated by SC-CO_2 with ethanol as co-solvent after oil removal by SC-CO_2 extraction. The main phospholipids of mackerel lecithin were phosphatidylcholine (PC) 20.11% and phosphatidylethanolamine (PE) 67.44%. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were found substantial amount in both phospholipids. After quality parameters checking, lecithin isolated by SC-CO_2 with ethanol from deoiled mackerel muscle showed better results compared to other systems. Oxidative stability and antioxidant activity of lecithin was high and significant differences were not found using those systems.

The production of amino acids with antioxidant activities and functional properties from protein hydrolyzates of freeze dried and SC-CO_2 deoiled mackerel skin by pressurized hydrothermal hydration (PHH) at different temperature (150-240 ^{o}C) and pressure (12-210 bar) was investigated. The highest yield of amino acid in freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzate was 121.93 ± 1.80 and 122.96 ± 2.84 mg/g, respectively, at 240 ^{o}C and 210 bar. Nine essential amino acids were identified in both skin hydrolyzates, of which histidine was the most abundant. All essential amino acids showed a temperature stability upto 240 ^{o}C with the exception of threonine and histidine. The antioxidant activity of the hydrolyzates, as demonstrated in the DPPH, ABTS, hydroxyl radical, Fe^{2+} chelating, and reducing power assays, increased with increases in temperature and pressure; it was high in both hydrolyzate at 240 ^{o}C and 210 bar. In terms of functional properties, hydrolyzate at different temperatures and pressures increased protein solubility to above 59% over a wide pH range (3.5-9.5). When the temperature and pressure increased, the emulsifying activity index, emulsion stability, foaming capacity, and foam stability of both hydrolyzates decreased,

possibly caused by the shorter peptide chain length. We conclude that protein hydrolyzate produced from mackerel skin can be used in food related industries as good additives.

Pepsin-solubilised collagen (PSC) was isolated from mackerel bone and skin and characterized with the aim of using these by-products resources more effectively. The yield of PSC (8.10%) from skin was considerably higher than that from bone (1.75%). Based on the protein patterns, both PSCs were type I, and consisted of two α-chains. Fourier-transform infrared spectra demonstrated that PSCs from the bone and skin exhibited a triple-helical structure. The denaturation temperatures (Td) of the PSCs from bone and skin were 27 ℃ and 30 ℃, respectively. Very low molecular weight peptides (<1650 Da) were generated from both PSCs after subcritical water hydrolysis treatment. Glycine accounted for 30% of the total amino acids identified in both PSC hydrolyzates. The antioxidant activities of both PSC hydrolyzates were significantly higher than those of the isolated PSCs. Therefore, PSC hydrolyzates can be used as a functional ingredient in the food, cosmetic and pharmaceutical industries.

Chapter 1

General introduction

1.1. Background

In 2011 , the annual global capture production of fish was about 91.8 million tons (FAO, 2012) but 35% of the capture fish by fishing net and hook is discarded (FAO, 1994). Discarding of these fishes creates two major problems. The first one is the underutilization of a huge amount of nutrients such as protein, vitamins, minerals, lipid and functional materials. Secondly, 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 utilize of these fish by efficient processing methods with the pollution control regulation (Choudhury and Bublitz, 1996). However, the processing of non-merchantable fish as resources may be benefited economically and also environmentally.

Mackerel is one of the important fish in the world which is caught annually over 5 million tons (FAO, 1994) and discarded 15% of captured fish which are non-merchantable. Like other marine fishes, no-merchantable mackerel can be unique source of lipid with polyunsaturated fatty acids (PUFAs), anti-aging collagen protein, amino acids, functional bioactive peptides and digestive enzymes.

1.2. Mackerel

Mackerel is a common name applied to a number of different species of pelagic fish, mostly but not exclusively from the family Scombridae. They are found in temperate and tropical seas, mostly living along the coast or offshore in the oceanic environment. In 2009, over five million tons were landed by commercial fishermen (FAO, 2014). Over thirty different species of Scombridae family are commonly referred as mackerel. The term "mackerel" means "marked" or "spotted" and derives from the old French *maquerel*, meaning a pimp or procurer. The connection is not altogether clear but mackerel spawn enthusiastically in shoals near the coast and medieval ideas on animal procreation were creative (OED, 2012). Mackerel typically have vertical stripes on their backs and deeply forked tails. Many species are restricted in their distribution ranges and live in separate populations or fish stocks based on geography. Some stocks migrate in large schools along the coast to suitable spawning grounds where they spawn in fairly shallow waters. After spawning they return in smaller schools to suitable feeding grounds often near an area of upwelling. From there, they may move offshore into deeper waters and spend the winter in relative inactivity. Other stocks migrate across oceans. Mackerel is prolific broadcast spawner. Individual females lay between 300,000 and 1,500,000 eggs (FAO, 2012), their eggs and larvae are pelagic that is they float free in the open sea. The larvae and juvenile of mackerel were fed by zooplankton. As adults, they have sharp teeth and hunt small crustaceans such as copepods as well as forage fish, shrimp and squid. In

turn they are hunted by larger pelagic animals such as tuna, billfish, sea lions, sharks and pelicans (Houttuyn, 1782; Cuvier, 1829). Table 1.1 shows size and estimated life span of some mackerel species.

1.2.1. *Scomber japonicus*

Scomber japonicus also known as the Pacific mackerel or Pacific chub mackerel (Houttuyn, 1782) which is closely resembles the Atlantic chub mackerel shown in Fig. 1.1. Chub mackerel is widespread in the Indo-Pacific. They are absent from the Indian ocean except for South Africa from KwaZulu-Natal to western cape and are replaced by the closely related Atlantic chub mackerel in the Atlantic. The chub mackerel is widely distributed usually found in the northwestern, southeastern and northeastern pacific. In the eastern pacific it can be found anywhere from central Mexico to southeastern Alaska. The chub mackerel is very abundant south of Point Conception, CA. It is regularly fished and canned for human consumption, pet food, bait or served fresh. From 1980-89, the recreational catch average in California was 1,462 tons. Since they have begun being fished, the chub mackerel reached its catching peak in 1978 with 3,412,602 tons. Since 1978, the catch ratings have continued to decrease but picked back up a little in 1995. Chub mackerel is caught all year round especially between June and November. It can be caught on both sides of North America, but the most important fisheries commercially are in California and Mexico (NOAA, 2013). *Scomber japonicus* is the most intensively fished scombroid mackerel, and they account for about half the

total capture production of scombroid mackerels (FAO, 2009) as shown in Fig. 1.2. As a species they are easily confused with Atlantic mackerel and migrate long distances in oceans and across the Mediterranean. According to nutrition specialists, chub mackerel is a very healthy meal and consumed worldwide. It is a fish that is very high in protein and rich in Omega-3 and unsaturated fatty acids which can prevent cardiovascular disease and cancer as well. Due to its high energy of protein intake and low carbohydrate value, is recommended in the diets of growing children and pregnant women (Bailly, 2013).

1.2.1.1. Habitat and biology of *S. japonicus*

Chub mackerel stay as an school, and their feeding habits same as Atlantic mackerel; eating the pelagic crustaceans, sagittae, copepods, amphipods, salps, appendicularians and young herring. They follow thrown bait as readily and bite quite as greedily as Atlantic mackerel do. Their breeding habits have not been studied. As larvae, chub mackerel feed mainly on copepods and rotifers and sometimes even smaller larvae of their own kind. Chub mackerel larvae can consume up to 87% of their dry body weight a day. As juveniles, chub mackerel mainly eat zooplankton. As adults, chub mackerels feed mysids and euphausids (ULPGC, 2013). Chub mackerel is generally found within 20 miles (37 km) off coast in waters between 50 to 72 ^{o}F (10-22 ℃). Young mackerel like to live around sandy beaches or kelp beds while adult is found in deeper water in shallow banks anywhere up to 1000 feet (300 m) of depth. During summer, chub mackerel likes travel to north and during winter they

travel to south (NOAA, 2014). Spawning between chub mackerel typically occurs at temperatures of 59 to 68 °F and spawning can happen from March through October, but spawning mostly happens from April through August. Sometimes in females, ripe translucent eggs appear simultaneously with unripe ova in early stages of development. Chub mackerel females lay an average of about 100,000 to 400,000 eggs during breeding season (Aquaticcommons, 2013). Seasonal reproductive cycle and spawning cycle of chub mackerel are shown in Fig. 1.3.

Table 1.1. Biological information of some mackerel species (FAO, 2009)

Common name	Scientific name	Maximum length (cm)	Common length (cm)	Maximum weight (kg)	Maximum age (year)
Short mackerel	*Rastrelliger brachysoma*	34.5	20	-	-
Indian mackerel	*R. kanagurta*	35	25	0.75	4
Blue mackerel	*Scomber australasicus*	44	30	1.36	7
Chub mackerel	*S. japonicus*	64	30	2.9	18
Atlantic mackerel	*S. scombrus*	66	32-36	1.0	12~18

(-) = data not found

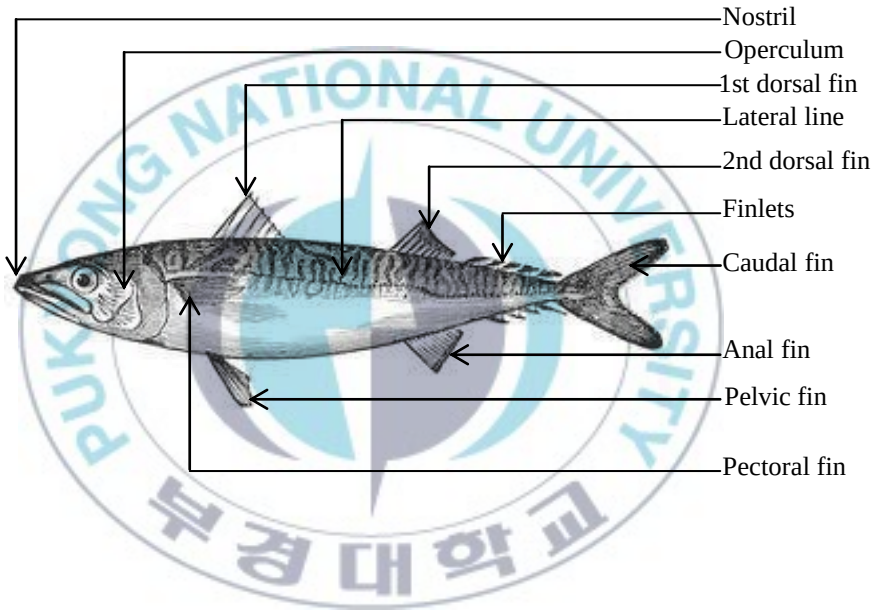


Fig. 1.1. Photograph and schematic representation of *S. japonicus* (Houttuyn, 1782)

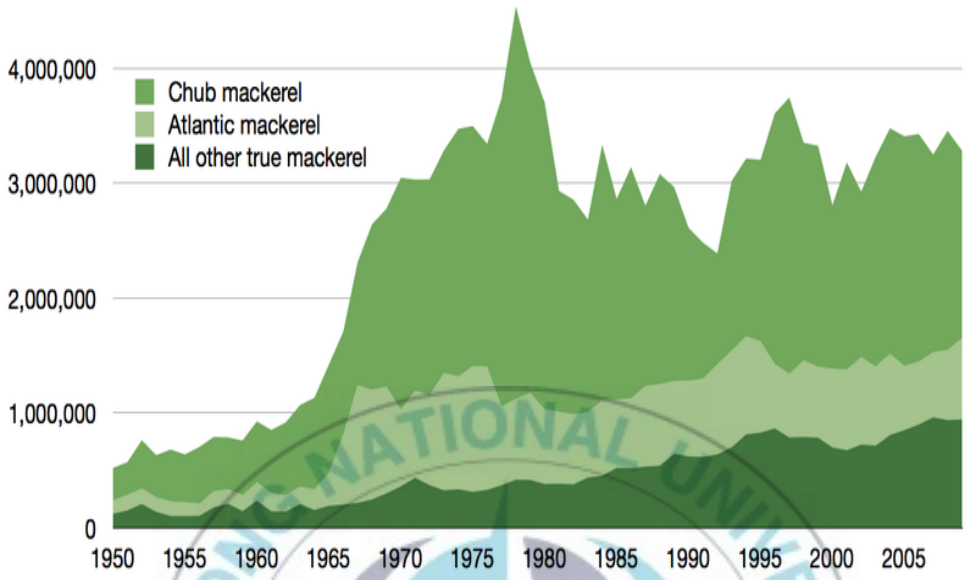


Fig. 1.2. Global capture production of scombroid mackerel (FAO, 2009)

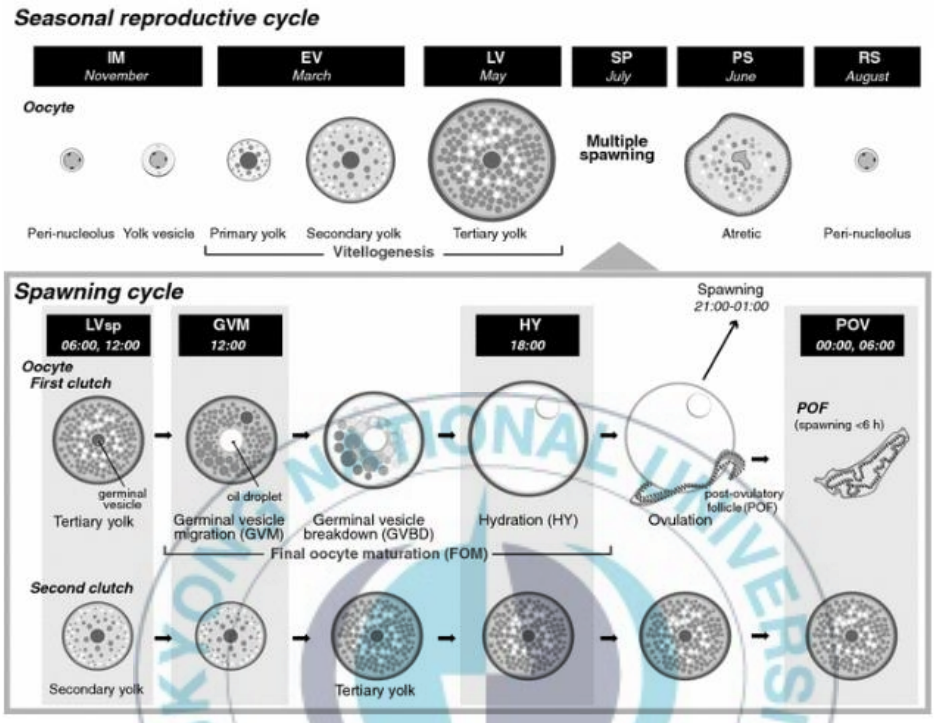


Fig. 1.3. Seasonal reproductive cycle and spawning cycle of *S. japonicus* (Nyuji et al., 2011)

1.3. Supercritical fluids (SFs)

A supercritical fluid (SF) is any substance at a temperature and pressure above its critical point, where distinct liquid and gas phases do not exist. It can effuse through solids like a gas, and dissolve materials like a liquid. In addition, close to the critical point, small changes in pressure or temperature result in large changes in density, viscosity and diffusivity.

Traditionally, lipids are extracted by hazardous organic solvent. Several methods have been reported for extracting lipids from fish. Extraction of lipid by conventional methods, such as hexane extraction, vacuum distillation, urea complexation or conventional crystalization have the disadvantages of requiring high-temperature processing that results in decomposition or degradation of the thermally labile compounds or employing toxic solvents that have adverse health effects (Hultin, 1994; Sahena et al., 2010). In recent year, the use of supercritical fluid extraction (SFE) for the removal of organic compounds from different liquid and solid matrices has been attracted much attention due to increasing awareness of environmental problems. This technique has some advantages over more conventional separation technique; largely due to the unique physical properties of SFs. Supercritical fluids are suitable as a substitute for organic solvents in a range of industrial and laboratory processes. Carbon dioxide and water are the most commonly used supercritical fluids, being used for extraction of lipids containing labile polyunsaturated fatty acids (PUFAs) and hydrolyzate production containing functional materials like peptide and amino acid, respectively.

1.3.1. Historical background of SFs

Baron Charles Cagniard de la Tour (1822) was first reported the observation of the occurrence of a supercritical phase. He mentioned that the gas-liquid boundary disappeared when the temperature of certain materials increased by heating each of them in a closed glass container. From these experimental works, he was first discovered critical point of a substance. Hannay and Hogarth (1879) were the first workers who demonstrated the solvating power of SFs for solids. They studied the solubilities of cobalt chloride, iron chloride, potassium bromide and potassium iodide in supercritical ethanol. They found that the concentrations of the metal chlorides in supercritical ethanol were much higher than their vapour pressures alone. They also found that the increasing pressure caused the solutes to dissolve and that the decreasing pressure caused the dissolved materials to precipitate like a snow. After a few years, Eduard Buchner (1906) reported that the solubilities of certain nonvolatile organic materials in CO_2 under supercritical conditions where solubilities were higher than that expected from vapour pressure alone.

In 1936, Wilson et al. (1936) devised a propane deasphalting process for refining lubricating oils. Selective separation of lube-oil feedstock into paraffin wax, asphalt, heavy ends, naphthenes, and purified light oil was achieved. Few years later, solexol process (Dickerson and Meyers, 1952) was developed for the purification and separation of vegetable and fish oils. The process concentrated polyunsaturated fatty acids in vegetable oils and vitamin A in fish oils using propane as a selective solvent.

A significant development in supercritical fluid extraction has been done by Zosel's paper which provided incentive for extensive future works (Zosel, 1964). In 1970, he reported the decaffeination of green coffee with CO_2. The process was accomplished by soaking the beans in water and then immersing them in supercritical CO_2. Since 1980, there has been quick development of supercritical fluid extraction in food and pharmaceutical areas e.g. the extraction of hops (Laws et al., 1980), cholesterol from butter (Krukonis, 1988), perfumes and flavours from natural products (Coenan et al., 1983), residual solvents and monomers from polymers (Krukonis, 1985) and unsaturated fatty acids from fish oils (Krukonis, 1988).

In 1990, the Joint Association for the Advancement of SF technology (JAAST) was formed in the United States to develop and disseminate knowledge regarding the application of SFs for cleaning purposes (Taylor, 1996). Since 1990, SFs has been extended to the field of green chemistry and engineering as integrating process. Now a day, SFs are used in different sector including food, cosmetics and pharmaceutical industries for specified functional materials.

1.3.2. Properties of SFs

The properties of SF can be explained by considering the density, viscosity, diffusivity, and solvating power which mainly depend on temperature and pressure. The density of a supercritical fluid is extremely sensitive to minor changes in temperature and pressure near the critical point. The physical stage

of a substance of fixed composition can be described by a phase diagram shown in Fig. 1.4. In pressure-temperature (PT) diagram, there are three lines describing the sublimation, melting and boiling processes which define the regions corresponding to the gas, liquid, and solid states. The vapor pressure (boiling) starts at the triple point (TP) and ends at the critical point (CP). The critical region has its origin at the critical point and there is only one phase and it possesses some of the properties of both a gas and liquid (Taylor, 1996).

Solvating power of the SF is highly dependent on its temperature and pressure. At low pressure, the solvent power of SF drastically decreases with rising temperature; whereas at high pressure, it increases with increasing temperature. If the parameter of ‘pressure’ is replaced by the parameter of ‘density’, the solubility-temperature relationship becomes much simpler. This anomaly happens because density decreases dramatically with an increase in temperature at low pressure; whereas at high pressure, changes in temperature have much less effect on density. Therefore, density is the first consideration regarding to the solvent power of SFs (Brogle, 1982).

A SF exhibits physicochemical properties of an intermediate between those of a liquid and a gas. SF has densities similar to that of liquids, while the viscosities and diffusivities are closer to that of gases as shown in Table 1.2. Thus, a supercritical fluid can diffuse faster in a solid matrix than a liquid, yet possess a solvent strength to extract the solute from the solid matrix. In addition, there is no surface tension in a supercritical fluid, as there is no liquid/gas phase boundary. By changing the pressure and temperature of the

fluid, the properties can be ‘tuned’ to be more liquid or more gas-like.

SF has good density, high diffusivity and low viscosity which show high solvating strength. This is the main advantages of SF in which their physical properties are similar to those of both liquids and gases. Additionally, the combination of low viscosities and high diffusion coefficients found in SF is a major advantage because low viscosity leads to good infiltration of the extraction materials, a small pressure drop, good mass transfer, and improved phase separation.

Table 1.3 shows critical properties of some solvents. Different solvent has different critical properties.

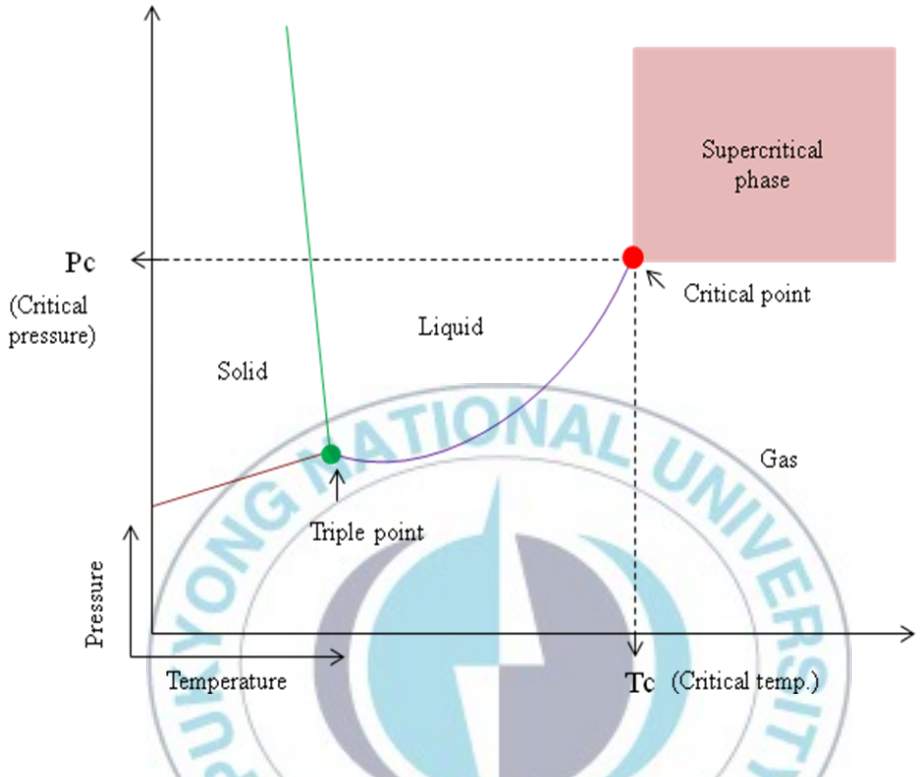


Fig. 1.4. Pressure-temperature phase diagram of a substance with critical temperature (Tc) and pressure (Pc)

Table 1.2. Physical properties of gases, compressed gases and liquids (Stah et al., 1988)

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

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

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

1.3.3. Supercritical carbon dioxide (SC-CO_2)

Supercritical carbon dioxide is a fluid state of carbon dioxide where it is held at above critical temperature of 31.1 ^{o}C and critical pressure of 73.8 bar. A SF separation process using CO_2 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 and high diffusivity (Lim et al., 2002). It has greater ability to diffuse through the ultra fine complex matrix and can be easily separated from the products by depressurizing process. Furthermore, low critical temperature of CO_2 means that the SC-CO_2 system could be operated at moderate temperature, preventing the degradation of the substance due to heat induction (Lopez et al., 2004; Machmudah et al., 2006; Krichnavaruk et al., 2008).

Fig. 1.5 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).

1.3.4. Comparison between SC-CO_2 and organic solvent extraction

Solvent extraction is a conventional method of lipid extraction. The advantages of SFE over other conventional processes such as extraction by

solvents and separation by distillation are automation, the reduction in operational steps, safe operations due to the use of nonorganic solvents and the use of moderate temperature in the critical range which are favorable for heat labile foods (Raventós et al., 2002). The most important advantage of SFE is the exceptional quality of the resulting product. Table 1.4 shows the comparison between SFE and organic solvent extraction. Several investigations have been done in order to compare SFE with conventional extraction methods. SFE yields were similar to those from a hot hexane extraction (Ikushima et al., 1986). Myer et al. (1992) reported that SC-CO_2 process recoveried extract from 97% to 100% of a soxhlet extraction in potato chips and puff-dried products. In fact, the main weakness is the cost of supercritical extraction equipment, incomplete lipid extractions under some conditions, and the extraction of nonfat material such as water (Dunford et al., 1997). Therefore, further development is needed since each biological system is distinctive. Conditions must be examined and improved in order to optimize yields for each kind of sample.

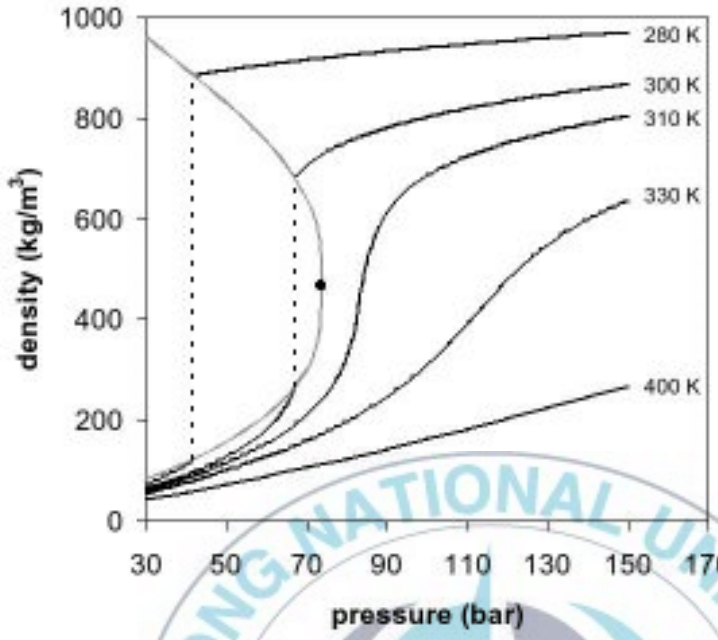


Fig. 1.5. Density of pure CO_2 at different temperatures and pressures

Table 1.4. Comparison between SC-CO_2 and organic solvent extraction (Sahena et al., 2009)

SC-CO_2 extraction	Solvent extraction
➢ Is totally free of solvents and hence very pure	➢ Solvent presence is unavoidable. The residual level of the solvent depends on the type of solvent used.
➢ Totally free of organic	➢ Inorganic salt content cannot be avoided
➢ CO_2 is highly selective and no chance of polar substances forming polymers exists.	➢ Polar substances get dissolved along with the liophilic substances from the raw material due to poor selectivity of the solvent
➢ Only non-polar colours can be extracted	➢ Both polar and non-polar colours are extracted
➢ Totally free of heavy metals since they are not extractable even if they are present in the raw material	➢ Heavy metal content is also unavoidable and depends on the solvent, the method of solvent recycling, the source of the raw material and the material used to construct the contact parts of the apparatus
➢ No extra unit operations needed and yield of useful material is very high	➢ Solvent removal requires extra unit operations resulting in higher cost and lower recovery of useful material

1.3.5. Subcritical water (SW)

Subcritical water is liquid water which is maintained in the subcritical state between its boiling point 100 ^{o}C at 1 bar and critical point 374 ^{o}C at 220 bar. Water is remained as liquid in the subcritical state due to apply the high pressure (King, 2000) shown in Fig. 1.6. Water in the subcritical states provides unique properties over water at ambient conditions. The two distinct advantages of subcritical water are lower dielectric constant as well as higher ion product shown in Fig. 1.7. The modification in the dielectric constant of subcritical water makes it a suitable solvent for dissolving organic compounds. The ions produced in the subcritical state are three orders of magnitude higher than ions in water at ambient conditions and therefore, it has potential to act as an acid or base like catalyst.

1.3.6. Comparison between subcritical water, enzyme and chemical hydrolysis

Subcritical water hydrolysis (SWH) is a clean and fast biomass hydrolysis method which can be used as an alternative acid, basic and enzymatic hydrolysis method with the advantages of high hydrolysis yield, shorter reaction time, less corrosion, lower residue generation, no use of toxic solvents, without pre-treatment and lower formation of degradation products (Zhao et al., 2011). However, acid and alkali hydrolysis needs violent reaction conditions which can create serious pollution of the environment. Enzyme hydrolysis is specific for macromolecule breakdown and it can take long time

for completing production cycle. Table 1.5 shows comparison between subcritical water, enzyme and chemical hydrolysis.

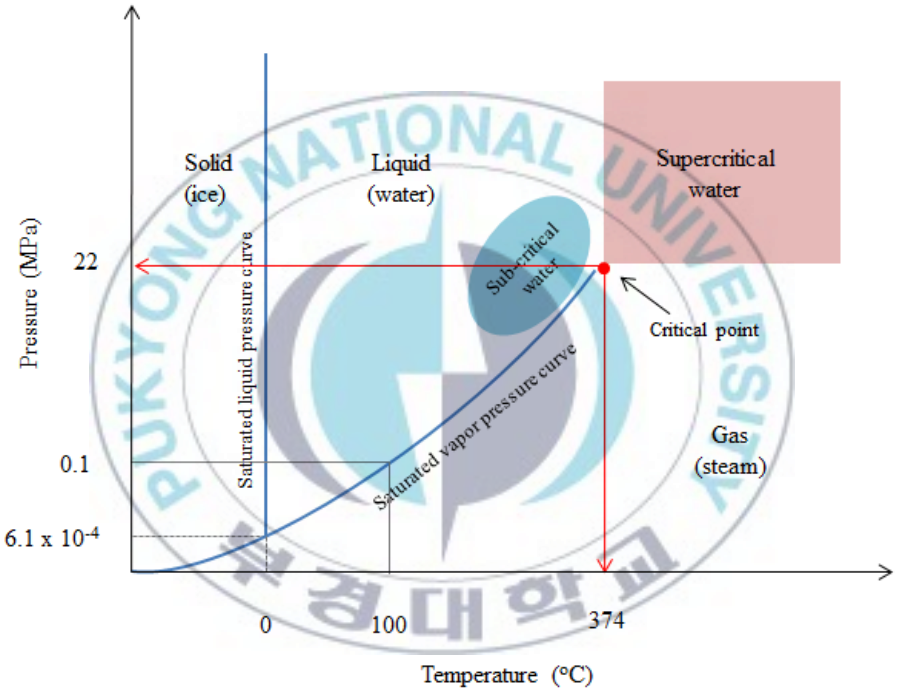


Fig. 1.6. Pressure-temperature phase diagram of subcritical water

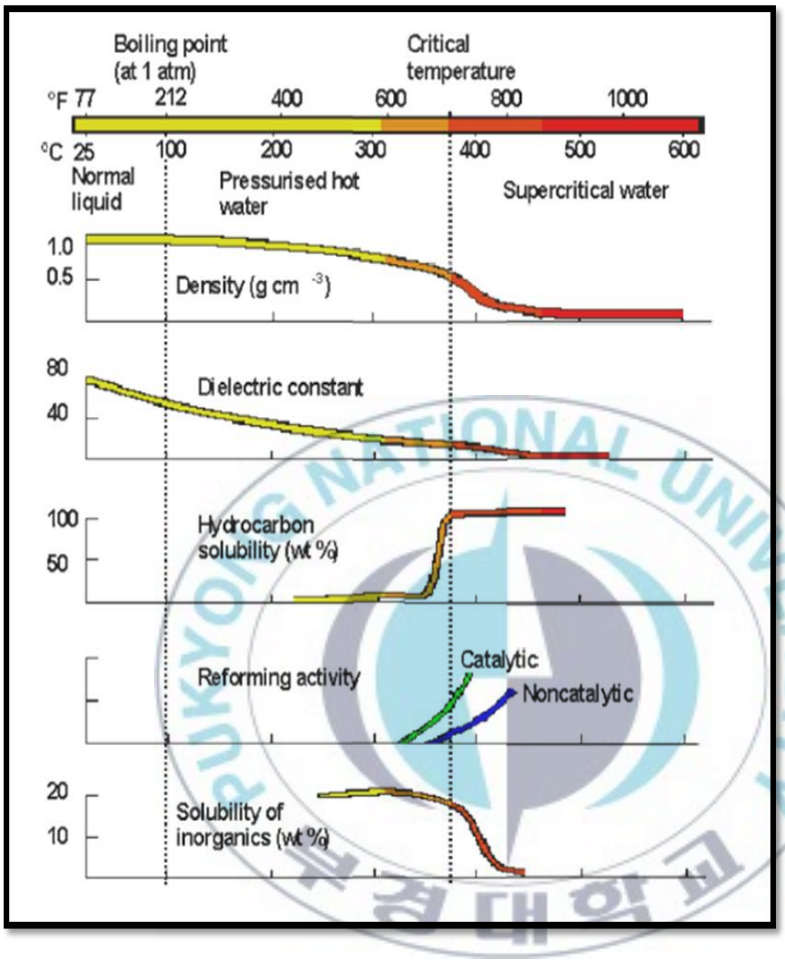


Fig. 1.7. Properties of normal, sub- and supercritical water (Laboratory of analytical chemistry, Finland)

Table 1.5. Comparison between subcritical, enzyme and chemical hydrolysis (Zhao et al., 2011; Taherzadeh and Karimi, 2007)

	Subcritical water hydrolysis	Enzyme hydrolysis	Chemical hydrolysis
Pretreatment	No	Yes	No
Solvent	Water	Water	Acid/base
Catalyst	No catalyst	Enzyme	No catalyst
Cost of catalyst	No	High	No
Hydrolysis conditions	Higher temperature and pressure	Medium temperature	Higher temperature
Reaction time	Shorter (1-3 min)	Higher (several days)	Shorter (3-5 min)
Hydrolysis yield	Higher	Medium	Higher
Residue generation	Lower	Medium	Lower
Product formation	Polar and non-polar compound	Specific	Polar and non-polar compound
Product inhibition	No	Yes	No
Product degradation	Lower	Lower	Higher

1.4. Polyunsaturated fatty acids (PUFAs)

Naturally, marine fishes are the unique source of PUFAs. Specially, PUFAs have omega-3 (ω-3) fatty acids. Now it has been very often subject of scientific studies. ω-3 Fatty acids have proven beneficial effects for human health. The health benefits of ω-3 fatty acids area as follows.

1.4.1. Cardiovascular health effects

Omega-3 fatty acids have beneficial effect the heart of healthy people, and those who have cardiovascular disease or at high risk. 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). Recent studies showed that ω-3 fatty acids decrease risk of arrhythmias (abnormal heartbeats) which can lead to sudden death and also decrease triglyceride levels, slow growth rate of atherosclerotic plaque, and slightly lower blood pressure (Masson et al., 2007). One of the important ω-3 fatty acids EPA has a unique role as the precursor to eicosanoids, a hormone-like substance which modulates several areas of cardiovascular health (Dyerberg et al., 1975).

1.4.2. Infant brain and vision development

DHA (docosahexaenoic acid) is one of the ω-3 fatty acids, which has important role in the development of the brain and vision development. 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 are of utmost importance at the early stages of life (Birch et al., 2007). Retinal photoreceptor membranes contain the body's highest concentrations of DHA. Recognition of this fact led to the hypothesis that diet-induced alterations in retinal fatty acid composition would lead to changes in retinal function (Neuringer, 2000). Benolken et al. (1973) were the first to show that such an effect was occurred. DHA takes 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. Long chain polyunsaturated fatty acids (LC-PUFA) composition of neural membranes is a key factor for brain development (Hogyes et al., 2003) and a normal adult brain contains more than 20 g of DHA (Paez et al., 2003). DHA is required for maintenance of normal brain functions in adults (Horrocks and Yeo, 1999). Maintaining concentration of PUFA is likely to favour enhanced cognitive, learning and memory functions (Youdim et al., 2000).

1.4.3. Other possible health effects

Without cardiovascular health benefit and infant brain and vision development, ω-3 PUFA research has expanded into other health issues such as cancer, arthritis, immunological disorder, diabetes, kidney disease, and skin

disorders (Sidhu et al., 1970; Jiang et al., 1998; Hering et al., 2007; Smyth and McGlynn, 2005; Moyad, 2005). In the other hand, food rich in PUFAs are highly susceptible to lipid peroxidation, which results in oxidative products that cause deterioration of food quality.

1.5. Digestive enzymes

Most raw foods contain their own digestive enzymes which assist their own digestion. When raw foods are heated much above body temperature, these enzymes are destroyed. Pasteurised foods and drinks, and most processed foods are enzyme-dead. The active enzymes in raw foods assist digestive system to extract all the nutrients from the food, and feel less hungry. Unless digestive system is in perfect condition (a rare occurrence), the food digests slowly and partially. Incompletely digested food provides a medium for harmful bacteria, yeasts and parasites to thrive. The fat that accumulates around the stomach, upper thighs, neck, major lymph nodes and other bulging parts of the body contains a high level of these partially digested fat-soluble toxins (Howell, 1985). Generally, fish contain very effective hydrolytic enzymes including protease, trypsin, amylase, nuclease and phospholipase, and so on. There is growing interest in the medical applications of digestive enzymes, which have been studied for use in treating ulcers and promoting wound healing due to their effective debridement of necrotic tissue (Westerhof et al., 1990). Digestive enzymes have also many other important applications for food industries which are used brewing and wine making, processed foods

and other commercially available food supplements (Kubrak and Lushchak, 2007).

1.6. Bioactive peptides

In recent years, a considerable amount of research has focused on the liberation of bioactive peptides which are encrypted within food proteins with a view to utilizie such peptides as functional food ingredients aimed at health maintenance. Bioactive peptides have been defined as food-derived components that in addition to their nutritional value exert a physiological effect in the body (Vermeirssen et al., 2007). Interestingly, within the parent protein sequence, the peptides are inactive and thus must be released to exert an effect. These bioactive peptides are usually 2~20 amino acid residues in length, although some one have been reported to be >20 amino acid residues. Bioactive peptides may be absorbed through the intestine where they subsequently enter the circulatory system intact to exert various physiological effects, or they may produce local effects in the digestive tract (Erdmann et al., 2008). Food-derived bioactive peptides have been shown to display a wide range of physiological functions including antihypertensive, antioxidative, opioid agonistic, immunomodulatory, antimicrobial, prebiotic, mineral binding, antithrombotic and hypocholesterolemic effects (Arihara, 2006). Fish proteins offer huge potential as novel sources of bioactive peptides. To date, bioactive peptides displaying antihypertensive, antioxidant, antimicrobial and anti-proliferative effects have been found in the hydrolyzates of fish proteins

(Cinq-Mars et al., 2008; Jang et al., 2008).

1.7. Objective of the thesis

Until now, there is a big problem in the fish processing industries to utilize non-merchantable mackerel for useful materials production. As far our knowledge, very few works have been conducted for producing useful materials from non-merchantable fish. No detail works have been carried out in which non-merchantable mackerel were properly used for recovery of bioactive and other useful materials. In this study, we have used SC-CO_2 and organic solvent to extract oil from non-merchantable mackerel muscle and the extracted residues have been used for recovery of other bioactive and useful materials. Additionally, other functional materials from mackerel bone and skin have been produced after subcritical water hydrolysis treatment. Therefore, to recover the maximum useful materials having good quality, the following tasks have been carried out:

- Characterization of digestive enzymes from deoiled mackerel muscle for applicability of SFs in the field of functional protein processing systems
- Isolation and characterization of lecithin from deoiled mackerel muscle
- Useful materials production and characterization from mackerel skin by subcritical water hydrolysis

- Isolation and characterization of collagen from mackerel bone and skin
- 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.

Chapter 2

Characterization of digestive enzymes from de-oiled mackerel (*Scomber japonicus*) muscle obtained by supercritical carbon dioxide and *n*-hexane extraction as a comparative study

2.1. Introduction

For higher efficiency of enzyme isolation, lipid removal is needed from the sample. Conventional methods for removal of oil from fish involve cooking, pressing, and/or liquid extraction. Removal of lipids with organic solvents causes protein denaturation and loss of functional properties (Pariser et al., 1978). Supercritical fluid extraction (SFE) is an efficient alternative for the extraction of natural substances from foods (Mendes et al., 2003; Sun and Temelli, 2006). In recent years, the use of SFE for the removal of organic compounds from different liquid and solid matrices has been attracted much attention. This technique has some advantages over more conventional separation techniques, largely due to the unique physical properties of SFs. 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). Supercritical carbon dioxide has been used for extraction of lipid from different marine organisms (Yamaguchi et al., 1986; Temelli et al., 1995; Park

*This work has been online published in Journal of Food Science and Technology (DOI:10.1007/s13197-014-1408-5).

et al., 2008). But attentions are getting increased on the protein after lipid separation using SC-CO_2 extraction.

Mackerel belongs to the family of *Scombridae*, and it is abundant in cold and temperate shelf areas. It contains a lot of protein, essential amino acid, lipid and many kinds of biological active compound. Various digestive enzymes are naturally present in fish. 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 of these enzymes in food processing (Shahidi and Janak-Kamil, 2001; Uddin et al., 2009; Abdelkarder et al., 2012).

Enzymes are large biological molecules which can function uniquely to control process time, enhance flavour, improve texture, extend shelf life and decrease the use of chemical food additives. Enzymes have been used as processing aids in various food related industries for a long time (Simpson et al., 1991; Vilhelmsson, 1997). Digestive enzymes have also many other important applications for different food related industries. Amylase is commonly used in brewing and wine making, processed foods and other commercially available food supplements (Kubrak, 2007). Lipolytic enzymes are currently attracting for their biotechnological potentiality. They constitute the most important group of biocatalysts for 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). Proteases execute a

large variety of functions and have important biotechnological applications and widely used in detergent industries, dairy industries and meat industries. The applications of proteases in medical industries are also growing widely (Djamel et al., 2009). Trypsin is a well-studied serine-protease having a lysine or arginine residue (Perona and Craick, 1995) which is used in the food industries as a baking enzyme to improve the workability of dough, to improve the texture of fish products and to control aroma formation in cheese and milk products.

Therefore, the aim of this study was to measure the digestive enzyme activities and characterizes the digestive enzymes from de-oiled mackerel muscle after lipid extraction using SC-CO_2 and *n*-hexane.

2.2. Materials and Methods

2.2.1. Materials

Mackerel was collected from Busan Cooperative Fish Market (Seo-gu, Busan, Korea). The muscle was separated by mechanically and washed thoroughly with cold distilled water in the laboratory. Pure carbon dioxide (99.99%) was supplied by KOSEM (Sangbuk-myeon Yangsan, Korea). All other chemicals used in this study were of analytical or HPLC grade.

2.2.2. Sample preparation

The mackerel muscle was dried in a freeze-dryer for about 72 h. The dried samples were crushed by a mechanical blender (PN, SMKA-4000, Ansan,

Korea). These samples called freeze dried mackerel muscle were then stored at -20 ^{o}C prior to SC-CO_2 and *n*-hexane extraction.

2.2.3. SC-CO_2 extraction

The set up of a laboratory scale of supercritical fluid extraction (SFE) process is shown in Fig. 2.1. Exactly, 20 g of freeze dried raw mackerel muscle was 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 (Milroyal, Milton Roy, USA) 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 (Shinagawa, Tokyo, Japan). After SC-CO_2 extraction, the mackerel muscle residues remaining in the vessel was stored at -20 ℃ until further use and analysis. Mackerel muscle was extracted at temperature 45 ℃ and pressure ranging from 15 to 25 MPa for 2 h using SC-CO_2. The flow rate of CO_2 was kept constant at 27 g/min for all extraction conditions.

2.2.4. *n*-Hexane extraction

The extraction was carried out using hexane as solvent. Exactly, 40 g of

freeze dried raw mackerel muscle with 200 mL hexane was placed into the beaker and stirred 20 h by a magnetic stirrer at 45 ℃ and 300 rpm. After extraction, the hexane solution was filtered by a filter paper and then evaporated in a rotary vacuum evaporator (EYELA N-1100, Tokyo, Japan) at 40 ℃. The remaining residue was dried using dry oven at 40 ℃ for 6 h and then residues and oil was stored at -20 ℃ until further use and analysis.

2.2.5. Water soluble extract preparation

The highest oil extracted mackerel muscle residues after SC-CO_2 extraction and residues after *n*-hexane extraction were homogenized in cold distilled water (1 g sample/7 mL of water) by mechanical stirring at 4 ℃ for 2 h. The samples were then centrifuged at 7,500 rpm for 15 m in at 4 ℃. The supernatants were collected and stored at -20 ℃. These samples called water soluble extracts which were used for protein estimation, digestive enzyme activity measurement and their characterization.

2.2.6. Measurement of protein content in water soluble extract

The protein content of the water soluble extract was assayed according to the method of Lowry et al. (1951). Bovine serum albumin (mg/mL) as a standard was used to construct a standard calibration curve shown in Fig. 2.2.

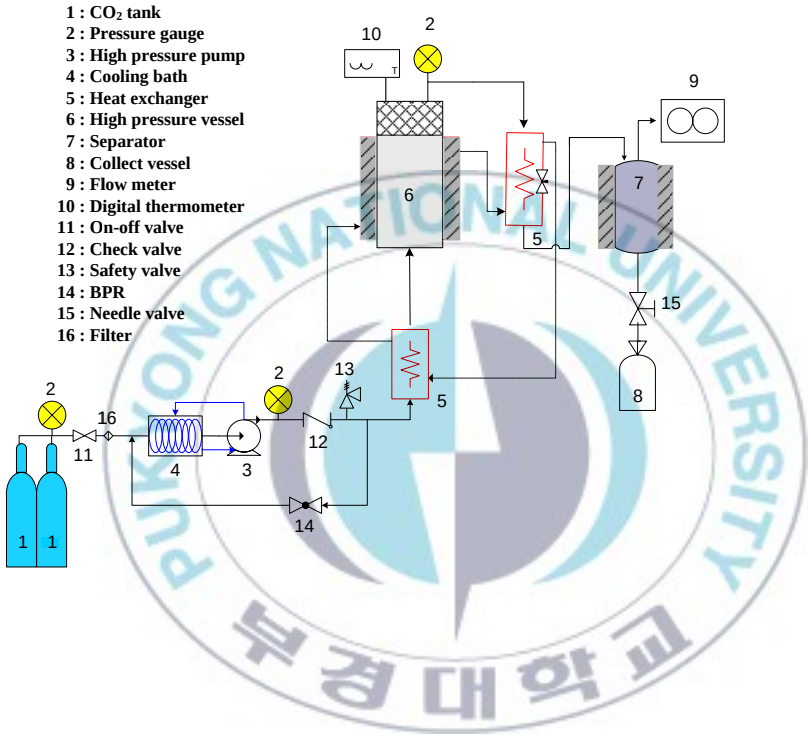


Fig. 2.1. Schematic diagram of SC-CO_2 extraction process

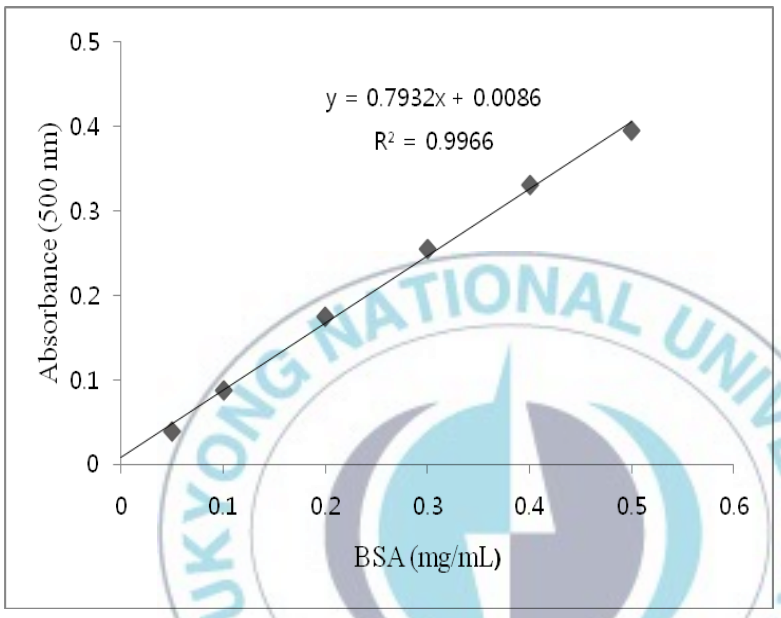


Fig. 2.2. Standard calibration curve of bovine serum albumin for protein estimation

2.2.7. Digestive enzyme assay of water soluble extract

2.2.7.1. Amylase assay

Amylase activity was determined by the dinitrosalicylic (DNS) acid method (Miller, 1959). The test tube containing 0.5 mL of water soluble extract which is equilibrated at 37 ^{o}C for 5 min and 0.5 mL of 1.0% (w/v) potato starch (Sigma Chemical Co., St. Louis, Mo., USA) in 0.016 M sodium acetate buffer (pH 6.0) was incubated at 37 ℃ for 10 min. After incubation, 1 mL of DNS solution was added in each tube. All tubes were heated at boiling water bath for 5 min to stop the reaction. After cooling, 10 mL of distilled water was added and mixed well. Absorbance was taken at 540 nm. One unit of amylase activity was defined as the amount of enzyme that released 1 μmol (uM) of reducing end groups per minute. D-glucose was used to construct a standard calibration curve.

2.2.7.2. Lipase assay

Lipase activity was assayed by the modified method of Uddin et al. (2009). 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 of 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 7.5) under intense vortexing. These emulsions were stable for 1 h at room temperature. 0.2 mL of water soluble extract was added to 3.2 mL of the substrate emulsion and the mixture was incubated for 10 min in a shaking

water bath at 37 ℃. The reaction was terminated by boiling for 5 min. After centrifugation at 3,000 rpm for 15 min, the absorbance of the clear supernatant was measured at 410 nm. The mixture with 0.2 mL of the inactivate enzyme extract (heated at 100 ℃ for 5 min) was used as a control. One unit of enzyme activity was defined as the amount of enzyme required for the liberation of 1 μmol (uM) *p*-nitrophenol from *p*-nitrophenyl laurate per minute under the assay conditions.

2.2.7.3. Protease assay

Protease activity was assayed by the casein Folin-Ciocalteau method (Oda and Murao, 1974) with slight modification. 1.14% casein solution in 0.1 M glycine-NaOH buffer (pH 9.6) was used as a substrate. Water soluble extract (0.5 mL) was mixed with 2.0 mL of substrate and it was incubated for 10 min at 37 ℃. The reaction was stopped by the addition of 2.5 mL of 0.44 M trichloroacetic acid (TCA) solution and settled down for 20 min. Then the sample was centrifuged for 10 min at 3,000 rpm. The supernatant was mixed with 2.5 mL of 0.5 M Na_2CO_3 and 0.5 mL of 2 N Folin-Ciocalteu reagents. The solution was kept in incubator at 37 ℃ for 20 min for colour developed and absorbance was measured at 660 nm (UVIKON 933, Kontron Instruments). One unit of protease activity was defined as the amount of enzyme required for liberating 1 μmol of tyrosine per min from casein. Tyrosine was used to construct a calibration curve.

2.2.7.4. Trypsin assay

Trypsin activity was measured by the modified method of Bergmeyer et al. (1974). 0.25 mM Nα-benzoyl-L-arginine ethyl ester in 67 mM sodium phosphate buffer, pH 7.6 was used as substrate. The water soluble extract (0.2 mL) was added to 3.0 mL of the substrate solution and the mixture was incubated at 35 ℃ for 10 min in a water bath. The reaction was terminated by boiling for 5 min. After cooling, the sample was centrifuged at 3,000 rpm for 10 min and the absorbance of the clear supernatant was measured at 253 nm. 0.2 mL of 1 mM hydrochloric acid solution instead of crude extract was used as a blank. One unit of enzyme activity was defined as the amount of enzyme required for the liberation of 1 μmol (uM) Nα-benzoyl-L-arginine from Nα-benzoyl-L-arginine ethyl ester per minute under the assay conditions.

2.2.7.5. Optimum pH and pH stability of amylase, lipase, protease and trypsin

Different buffers of wide range of pH values were used to evaluate the effect of pH on digestive enzyme activity. The buffers used were 0.1 M citric acid sodium citrate (pH 4~6), 0.1 M potassium phosphate (pH 7~8.5) and 0.1 M glycine-NaOH (pH 10~12). The pH stability was measured by 12 h pre-incubation of the water soluble enzyme extract in buffers that had the same ionic concentrations at different pH values ranging from 4.0 to 12.0 at 4 ^{o}C. The enzyme activities were measured immediately after this treatment with the standard methods as mentioned above.

2.2.7.6. Optimum temperature and temperature stability of amylase, lipase, protease and trypsin

For the effect of optimum temperature on digestive enzyme in water soluble extract used different buffers such as potassium phosphate (0.1 M, pH 8.5) buffer for amylase, lipase and protease and 0.1 M glycine-NaOH (pH 10) buffer for trypsin. Enzyme activity was determined by performing the standard assay as mentioned within a temperature range of 20~80 ^{o}C. Temperature stability was measured by incubation of water soluble enzyme extract at temperature ranging from 20~80 ^{o}C for 2 h in a constant temperature of water bath. After treatment, the residual enzyme activities were analyzed under standard assay conditions as mentioned above.

2.2.7.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of water soluble extracts were carried out by the method of Laemmli (1970) using 4.4% (w/v) stacking gel and 10% (w/v) separating gel. Water soluble extracts were mixed with sample buffer at the ratio of 1:2 (v/v). Then 10 µL of the sample was loaded in each well. Electrophoresis was performed using a Mini-Protein III cell module (Bio-Rad Laboratories, CA, USA) at a constant voltage (30 mA for 1.5 h). Molecular weight markers (Sigma Chemical Co., St. Louis, Mo., USA) were used to estimate the molecular weight of protein.

2.2.8. Statistical analysis

All experiments were carried out in triplicate. The data were expressed to analysis of variance (ANOVA) and the differences between means were evaluated by Duncan's multiple range test. SPSS statistics programme (SPSS version 15.0 for windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

2.3. Results and Discussion

2.3.1. Total oil extraction

The extraction curves of mackerel muscle oil by SC-CO_2 at temperature 45 ^oC and pressure ranging from 15 to 25 MPa are shown in Fig. 2.3. The highest oil obtained by SC-CO_2 extraction was 4.00 ± 0.11 g/20 g of mackerel muscle at temperature 45 ^oC and pressure 25 MPa. At constant temperature, the amount of oil extracted from mackerel muscle was increased with increasing pressure. Due to the increase in pressure, the density of 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). The total amount of oil obtained by SC-CO_2 extraction was 20.00 ± 0.54% at 45 ℃ and 25 MPa while the oil obtained from mackerel muscle by hexane was 25.62 ± 0.62% (data not shown). The observed difference in maximum yield may have been due to variations in the processing unit, operating conditions and so on.

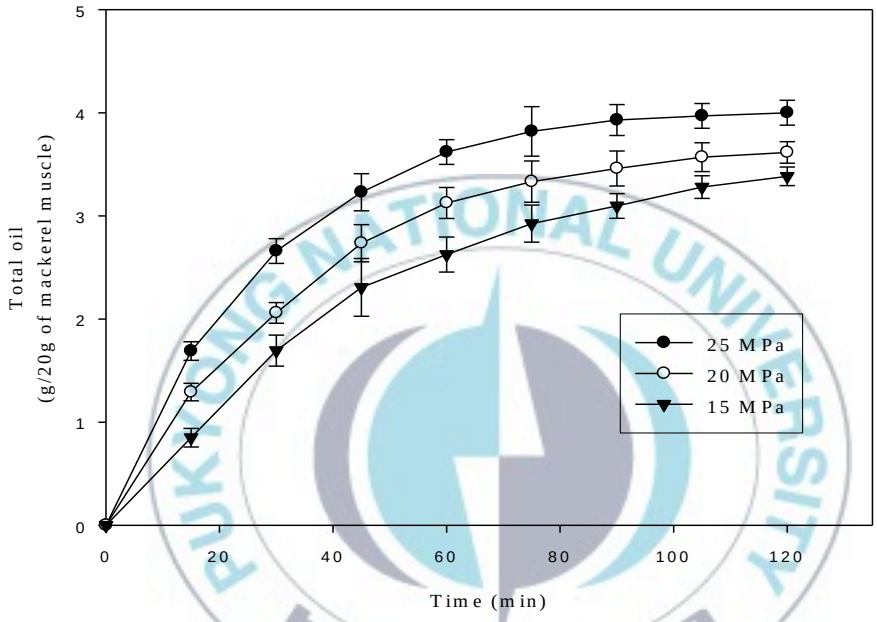


Fig. 2.3. SC-CO_2 extracted oil from mackerel muscle at temperature 45 ^{o}C and pressure ranging from 15 to 25 MPa. Bars represents the standard deviation (*n*=3)

2.3.2. Protein yield in water soluble extract

The protein content in water soluble extract from freeze dried, SC-CO_2 extracted at temperature 45 ^{o}C and pressure 25 MPa and hexane extracted residues are shown in Table 2.1. It was found that the water soluble extract of SC-CO_2 extracted residues contained more protein than freeze dried residues and hexane extracted residues. These occurrences can be happened that presence of lipid in the raw materials made them less accessible to water and due to the long time hexane extraction, protein concentration in residues may be reduced. Pariser et al. (1978) reported that protein denaturation is occurred by the removal of lipids with conventional liquid solvents. The protein yield in water soluble extract from freeze dried, SC-CO_2 and hexane extracted residues were 5.02 ± 0.07, 9.28 ± 0.09 and 6.53 ± 0.06 mg/mL, respectively.

Table 2.1. Protein yield of water soluble extract from freeze dried, SC-CO_2 extracted and hexane extracted residues

	Protein concentration (mg/mL)
Water soluble extract after freeze dried	5.02 ± 0.07^{c}
Water soluble extract after SC-CO_2 extraction	9.28 ± 0.09^{a}
Water soluble extract after hexane extraction	6.53 ± 0.06^{b}

Means ± SD (n=3). Different small letters in the same column indicate significant differences ($P < 0.05$)

2.3.3. Digestive enzyme activities

The activities of amylase, lipase, protease and trypsin of SC-CO_2 and hexane treated water soluble extracts of mackerel muscle are shown in Fig. 2.4 A~D. Among the four digestive enzymes, the activity of amylase was highest. Protease activity was slightly higher in *n*-hexane treated sample than SC-CO_2 and the activities of amylase, lipase and trypsin were higher in SC-CO_2 treated samples compared to *n*-hexane treated samples. This may have resulted from a loss of digestive enzyme activity in mackerel samples by the *n*-hexane treatment. This means enzymes are not stable in the presence of organic solvent and are susceptible to denaturation. Some authors reported similar observation (Ogino et al., 1994).

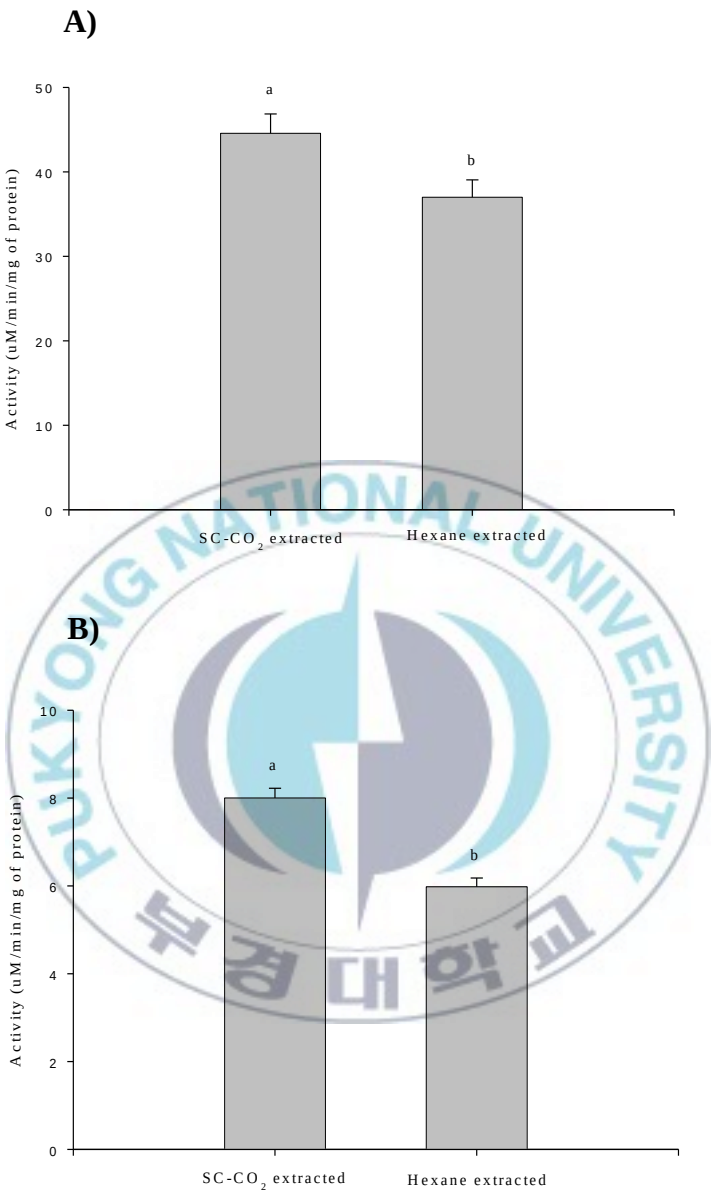
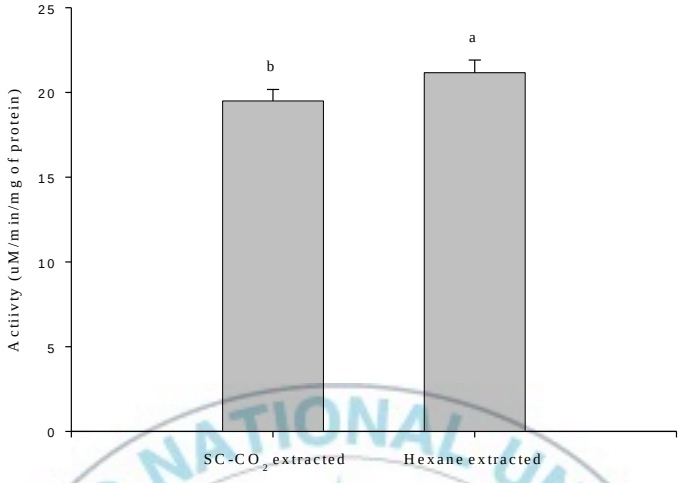


Fig. 2.4A-B. Activities of digestive enzymes in SC-CO_2 and *n*-hexane treated mackerel muscle residues extract (A) Amylase and (B) Lipase. Bars represents the standard deviation (*n*=3). Different small letters on the top of column in each figure indicate significant differences ($P < 0.05$)

C)



D)

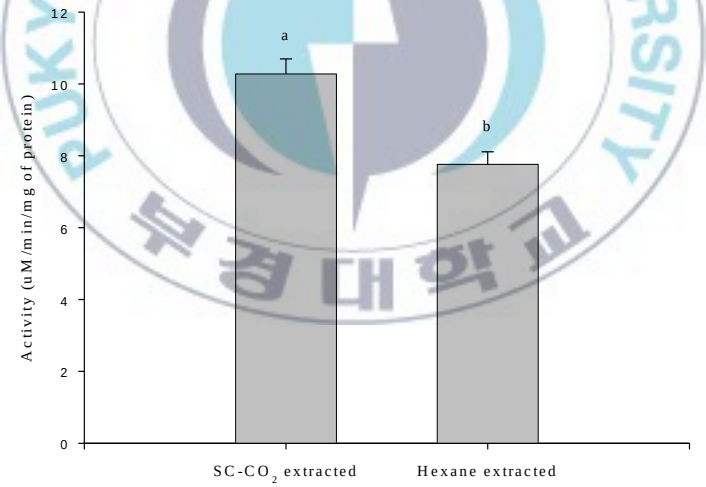


Fig. 2.4C-D. Activities of digestive enzymes in SC-CO_2 and *n*-hexane treated mackerel muscle residues extract (C) Protease and (D**)** Trypsin. Bars represents the standard deviation (*n*=3). Different small letters on the top of column in each figure indicate significant differences ($P < 0.05$)

2.3.4. Optimum pH

The optimum pH of amylase, lipase, protease and trypsin are shown in Fig. 2.5 A~D. The activities of amylase in the SC-CO_2 and hexane treated water soluble extracts of mackerel muscle were maximal at pH 8.5. An optimum pH of 7~11 was reported for amylase obtained from the siamese fighting fish (Karun et al., 2010). The water soluble extract of both treated mackerel was found optimum lipolytic activity at pH 8.5. The optimum pH 7~11 for lipase activities were reported for fish and other sources (Kumar et al., 2005; Uddin et al., 2009; Karun et al., 2010). The highest proteolytic activity of water soluble extracts after SC-CO_2 and hexane treated mackerel was found at alkaline pH 8.5. 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; Poonsuk and Thiraratana, 2008). Low protease activities were found in acidic pH. Among the acidic pH, the protease activity was high at pH 6. This indicates that the water soluble extracts of mackerel contained both acidic and alkaline proteases. Similar results were found by Natalia et al. (2004) from carnivorous ornamental fish and Uddin et al. (2009) from squid viscera. The trypsin in water soluble extracts showed maximal hydrolytic activity at alkaline pH 10 from both treated mackerel residues. The activity of trypsin decreased at pH 11 and 65% of activity was remained of that pH, probably as a result of the denaturation of enzyme. The alkaline proteinase, trypsin from the intestine of nile tilapia and bigeye snapper showed high activity at pH range of 7~10 and 8~11, respectively (Bezerra et al., 2005; Pham and Soottawat, 2006).

In the water soluble extracts of SC-CO_2 and hexane treated mackerel resdidues, pH differences effect on digestive enzyme activities were not significant.

2.3.5. pH stability

Fig. 2.6 A~D shows the pH stability of digestive enzymes of water soluble extract from SC-CO_2 and *n*-hexane treated mackerel residues. Amylase activity was retained more than 88% activity at pH 6.0 and 8.5-10.0 from mackerel extracts. Noman et al. (2006) and Uddin et al. (2009) reported that more than 80% and almost 90% amylase activity was retained at the pH range of 6.0~8.0 and 6.0~8.5, respectively of *Pachyrhizus erosus* L. tuber and squid viscera, which was almost similar to mackerel muscle residues. Lipase was stable within a pH range of 8.5-10.0 in water soluble mackerel extract, where more than 85% activity was retained. Kumar et al. (2005) and Aryee et al. (2007) reported that the stability of lipase was at pH range of 8.0~10.5 with 75% activity of *Bacillus coagulans* BTS-3 and pH of 7.0~10.0 with 70% activity of grey mullet, respectively. From water soluble extract of mackerel, protease activities were found more than 86% of its original value, in the range of pH 7.0~8.5 and then decreased with increasing pH. The pH stability of water soluble extracts of tuna and squid viscera were reported to be in the range of 9.0~11.0 and 8.0~10.5, where more than 90% activity was retained (Parsertsan et al., 2001; Uddin et al., 2009). Trypsin stability of mackerel extract was in alkaline pH range of 8.0~10.0, above 85% activity were retained of that pH. However, pH stability was decreased in acidic conditions.

At acidic pHs, the conformational changes of enzyme took place and enzyme could not bind to the substrate properly (Klomklao et al., 2006). Trypsin from pyloric caeca of bigeye snapper was stable in the neutral and alkaline pH range of 7~12 with more than 90% activity (Pham and Soottawat, 2006). However, pH stability of all digestive enzymes in mackerel muscle extract from SC-CO_2 and hexane treated was identical.

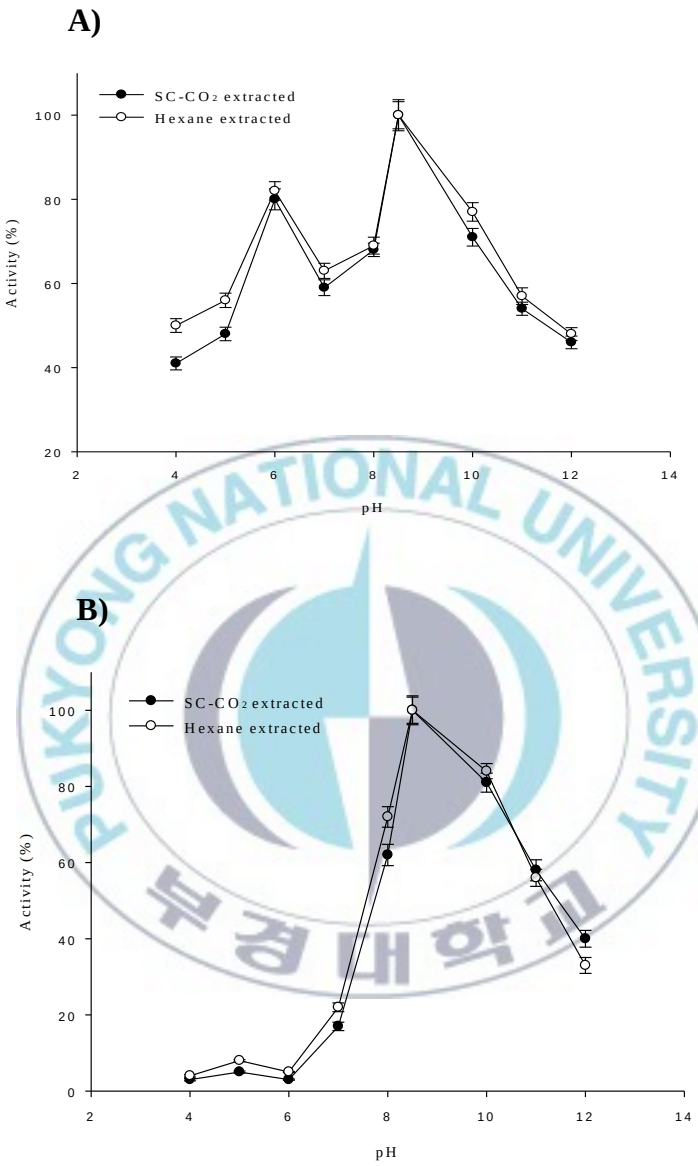
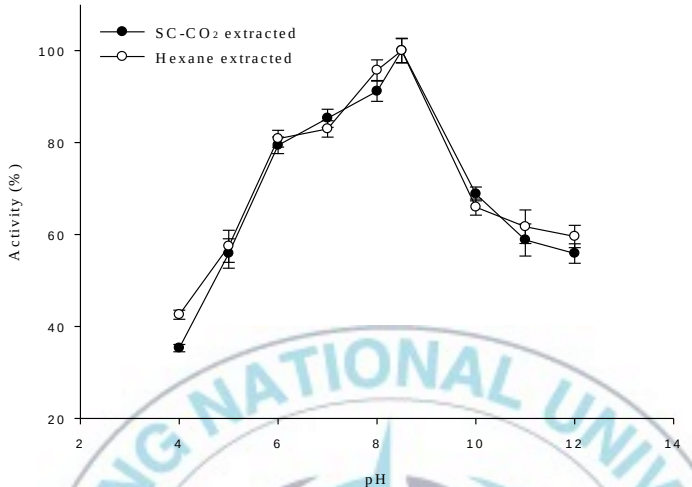


Fig. 2.5A-B. Optimum pH of digestive enzymes in SC-CO_2 and *n*-hexane treated mackerel muscle residues extract (A) Amylase and (B) Lipase. Bars represents the standard deviation (*n*=3)

C)



D)

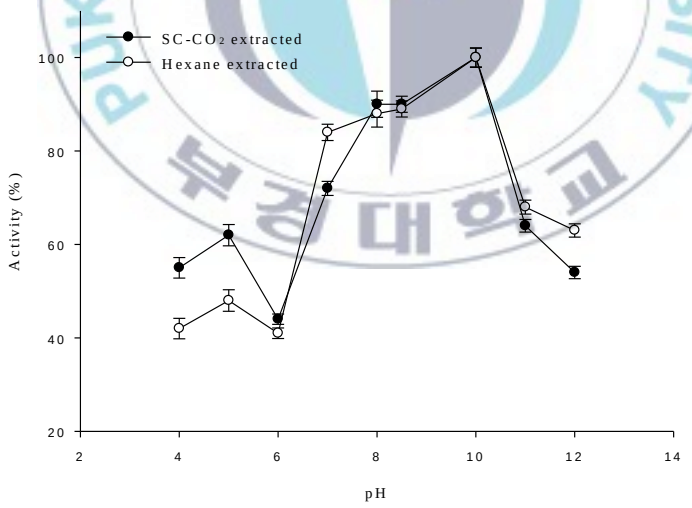
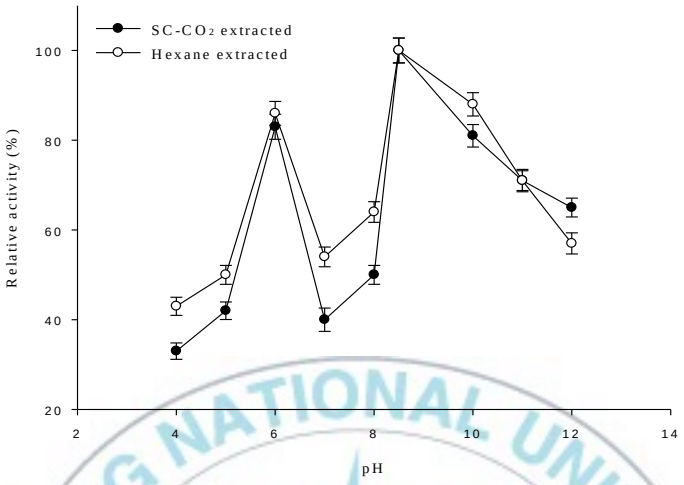


Fig. 2.5C-D. Optimum pH of digestive enzymes in SC-CO_2 and *n*-hexane treated mackerel muscle residues extract (C) Protease and (D) Trypsin. Bars represents the standard deviation (*n*=3)

A)



B)

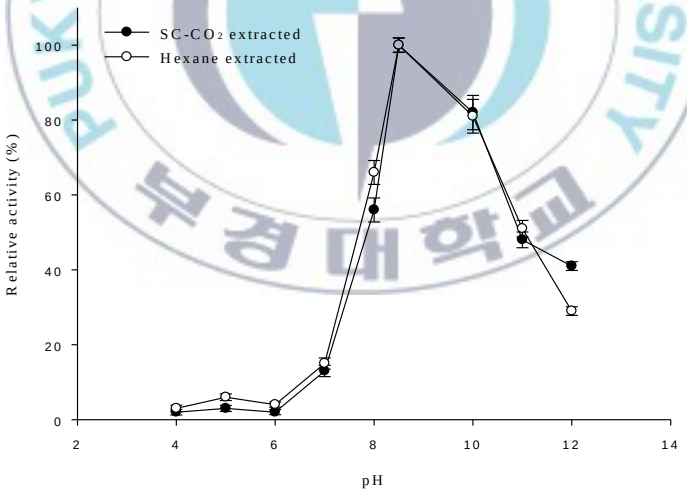


Fig. 2.6A-B. pH stability of digestive enzymes in SC-CO_2 and *n*-hexane treated mackerel muscle residues extract (A) Amylase and (B) Lipase. Bars represents the standard deviation (*n*=3)

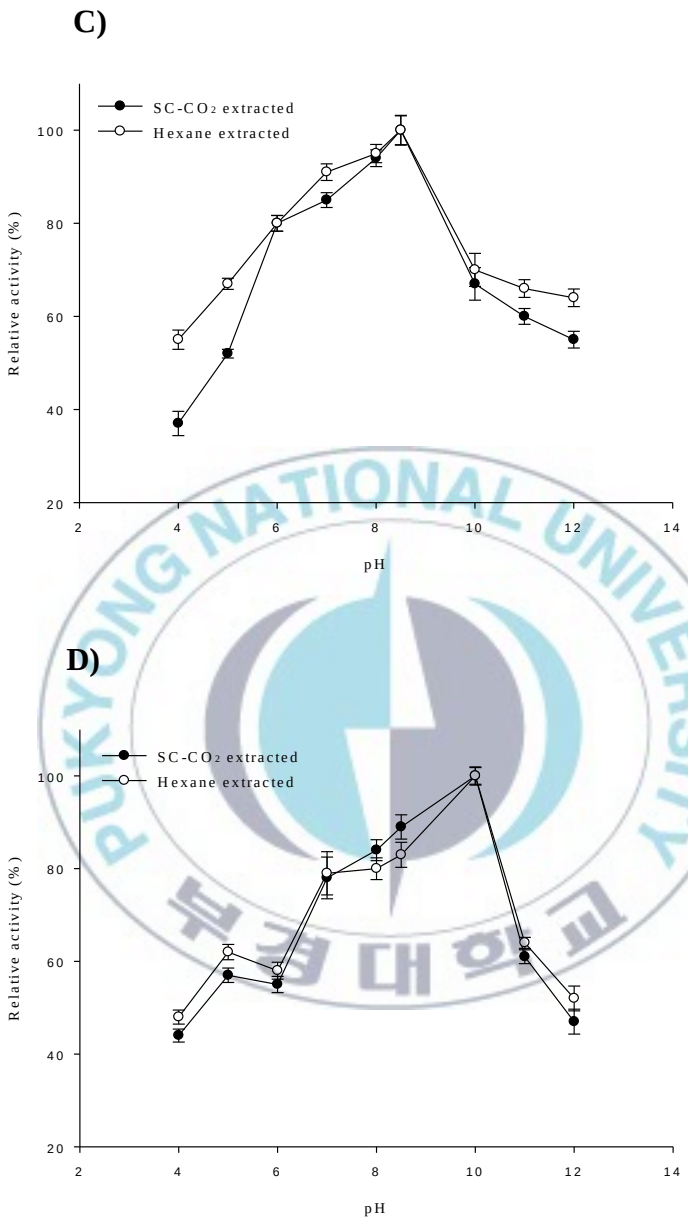


Fig. 2.6C-D. pH stability of digestive enzymes in SC-CO_2 and *n*-hexane treated mackerel muscle residues extract (C) Protease and (D) Trypsin. Bars represents the standard deviation (*n*=3)

2.3.6. Optimum temperature

The maximum activities of amylase, lipase, protease and trypsin in both SC-CO_2 and *n*-hexane treated mackerel water soluble extracts were found at the temperature of 40 ^{o}C, 50 ^{o}C, 60 ^{o}C and 30 ^{o}C, respectively shown in Fig. 2.7 A~D. The optimum temperature for amylase in the water soluble extract of tuber and squid viscera was 37 ^{o}C (Noman et al., 2006; Uddin et al., 2009) which was slightly lower than mackerel extract. Karun et al. (2010) reported that optimum lipase activity was found at 40 ^{o}C from siamese fighting fish which was lower than mackerel extract. The optimum temperature of protease from tampaqui waste was 60 ^{o}C (Espósito et al., 2009) which was similar compared to mackerel protease. The optimal temperature of trypsin in mackerel muscle was lower than those of cod, capelin, anchovy and sardine, which ranged from 40 to 45 ^{o}C (Murakami and Noda, 1981; Simpson and Haard, 1984; Martinez et al., 1988). The differences in temperature optima may be due to several factors including different living temperatures, extracts from different sources, the different substrates used for measurements since different substrate may exhibit temperature activity differences with enzymes.

2.3.7. Temperature stability

Temperature stability of amylase, lipase, protease and trypsin in SC-CO_2 and *n*-hexane treated mackerel extracts are shown in Fig. 2.8 A~D. For amylase, more than 90% of activities in mackerel extract were found up to 40 ^{o}C. Noman et al. (2006) and Uddin et al. (2009) reported that temperature

stability of α-amylase from tuber and squid viscera were 80% up to 40 ^{o}C which was lower compared to mackerel amylase. The water soluble extracts of mackerel retained above 90% lipase activity up to 50 ^{o}C. Above 70% of lipase activity in viscera of grey mullet was retained up to 50 ^{o}C (Aryee et al., 2007). At same temperature residual lipase activity was high in extract of mackerel residues than grey mullet. This might be happened due to very little change of conformation or properties of active site by temperature and high pressure during extraction by SC-CO_2. The protease activities of SC-CO_2 and *n*-hexane treated mackerel extracts were remained above 86% up to 60 ^{o}C. Uddin et al. (2009) and Espósito et al. (2009) reported that the protease from fish waste retained about 85% and 86% of activities up to 60 ^{o}C which was similar to mackerel residues. More than 80% trypsin activity was retained up to 40 ^{o}C in mackerel extracts but lost its activity rapidly at temperatures above 40 ^{o}C. At high temperature, the enzyme possibly underwent denaturation and lost its activity. Abdelkader et al. (2012) reported similar observation. Trypsin from the atlantic blue crab was stable at a temperature ranging from 30 to 50 ^{o}C for 30 min (Dendinger and O'Connor, 1990). Similar temperature stability of all digestive enzymes was observed from SC-CO_2 and hexane treated mackerel muscle extract.

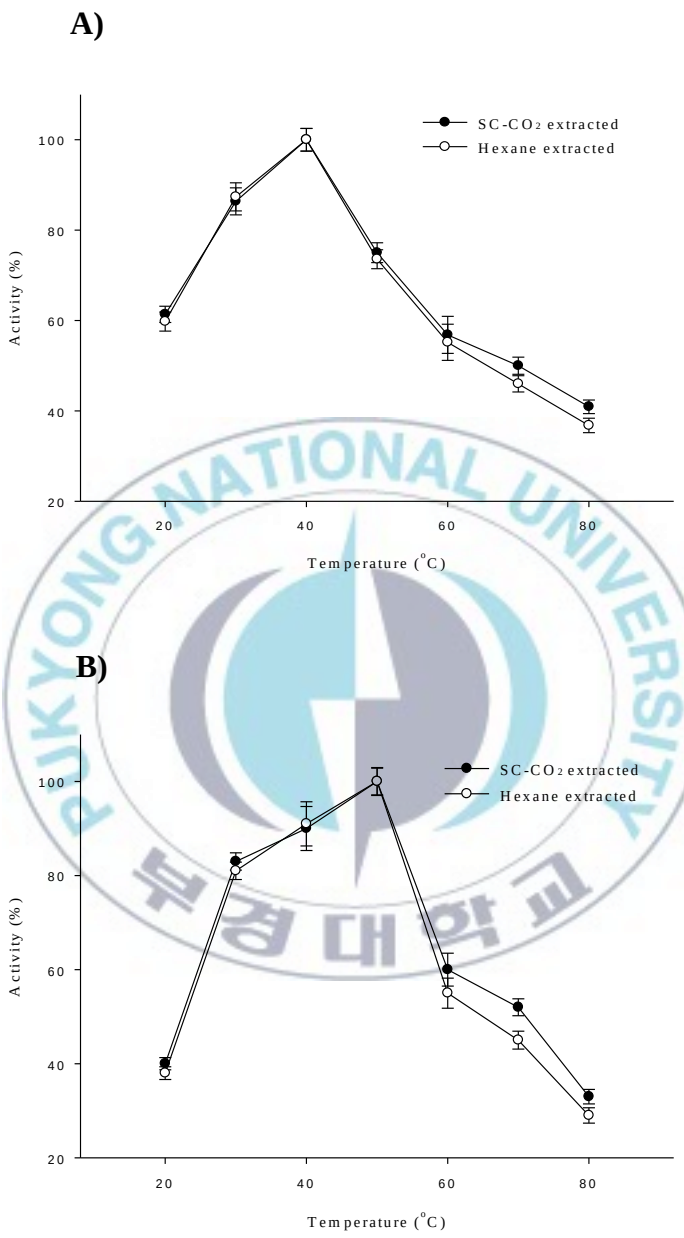
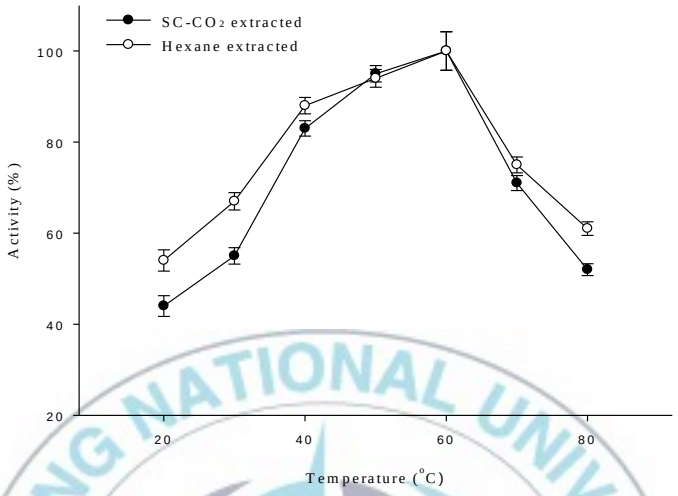


Fig. 2.7A-B. Optimum temperature of digestive enzymes in SC-CO_2 and *n*-hexane treated mackerel muscle residues extract (A) Amylase and (B) Lipase. Bars represents the standard deviation (*n*=3)

C)



D)

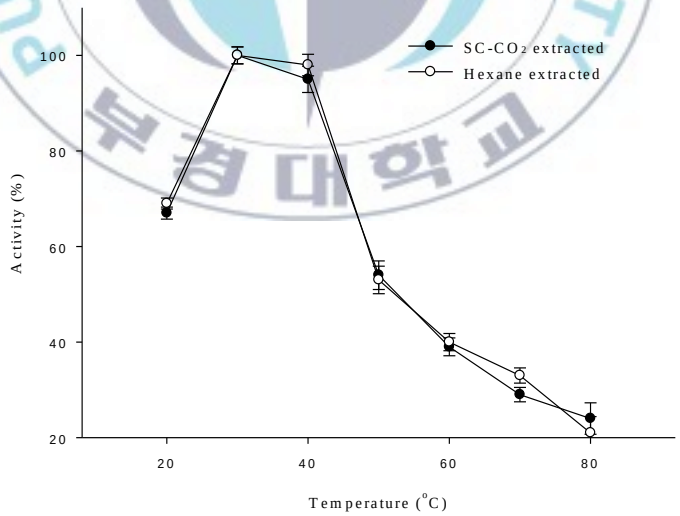


Fig. 2.7C-D. Optimum temperature of digestive enzymes in SC-CO_2 and *n*-hexane treated mackerel muscle residues extract (C) Protease and (D) Trypsin. Bars represents the standard deviation (*n*=3)

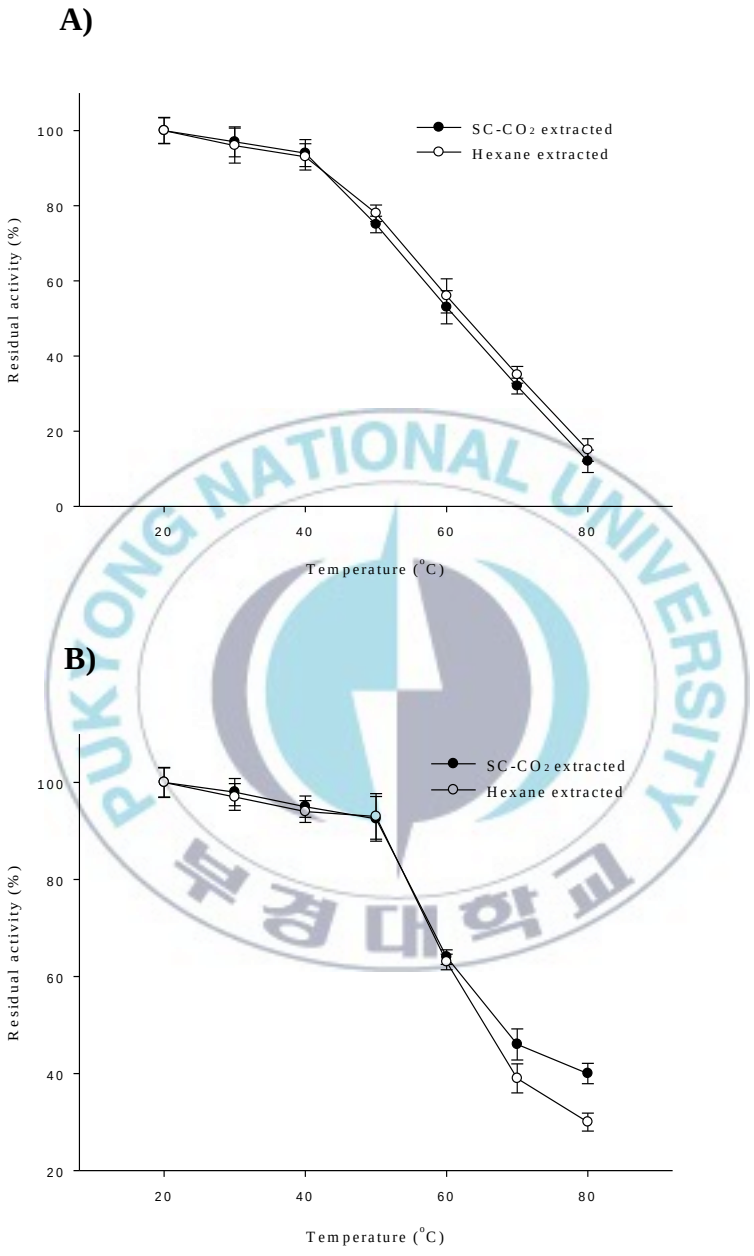
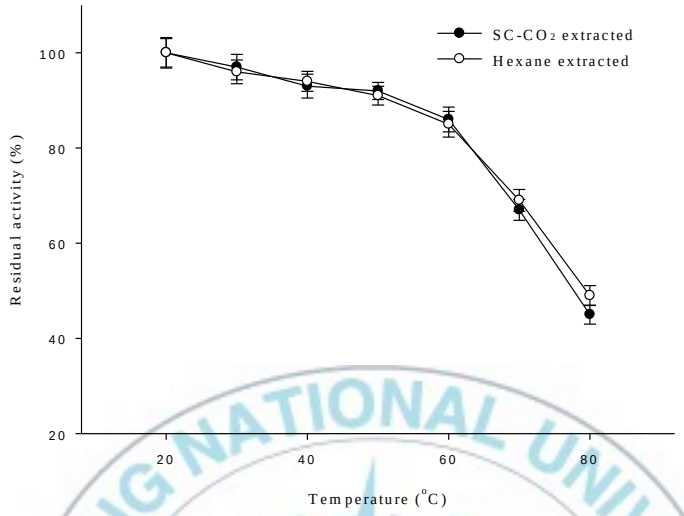


Fig. 2.8A-B. Temperature stability of digestive enzymes in SC-CO_2 and *n*-hexane treated mackerel muscle residues extract (A) Amylase and (B) Lipase. Bars represents the standard deviation (*n*=3)

C)



D)

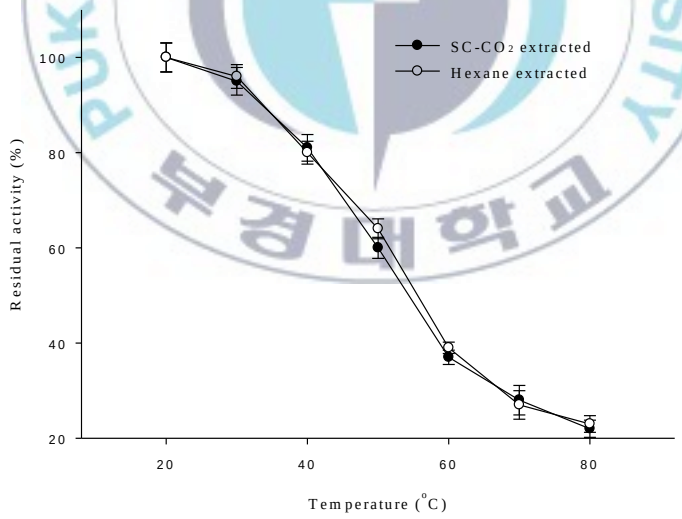


Fig. 2.8C-D. Temperature stability of digestive enzymes in SC-CO_2 and *n*-hexane treated mackerel muscle residues extract (C) Protease and (D) Trypsin. Bars represents the standard deviation (*n*=3)

2.3.8. SDS-PAGE

The gel electrophoresis of marker protein and the water soluble extract of freeze dried raw, SC-CO_2 and hexane treated mackerel muscle are shown in Fig. 2.9. Different size of molecular weight of protein was observed after SDS-PAGE. The proteins in freeze dried raw mackerel were almost similar in subunit composition to SC-CO_2 and hexane treated residues. After observing the gel banding patterns, protein band was more and prominent in SC-CO_2 treated samples than freeze dried raw and *n*-hexane treated mackerel residues. From this observation it can be concluded that protein concentration in the solution was high after removal of lipid using SC-CO_2 and protein denaturation was not found in SC-CO_2 treated mackerel sample. Similar results have been reported in SC-CO_2 extracted squid viscera residues and krill residues (Uddin et al., 2009; Abdelkader et al., 2012).

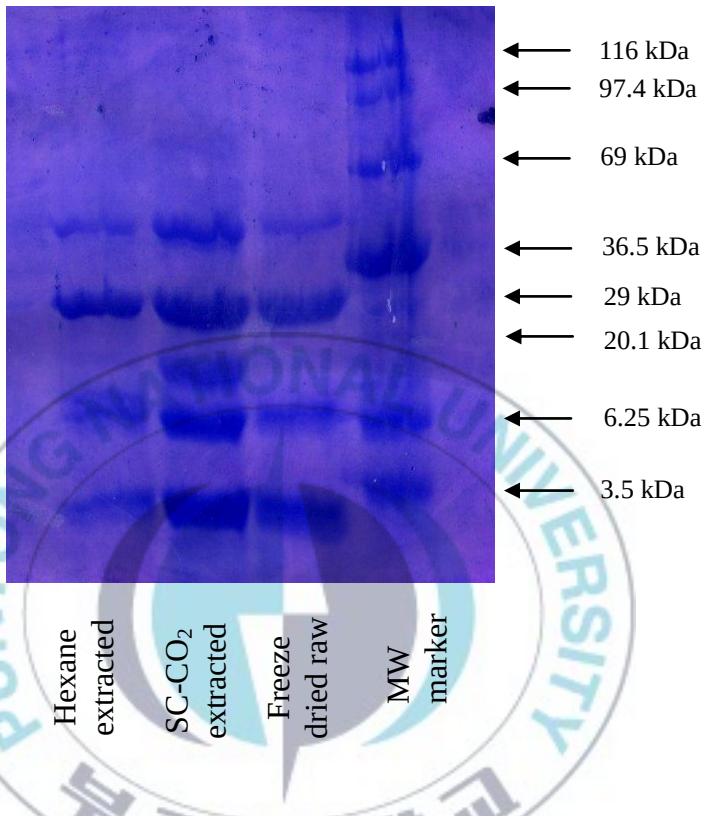


Fig. 2.9. SDS-PAGE electrophoresis of crude protein of freeze dried raw, SC-CO_2 and *n*-hexane extracted mackerel muscle residues extract

2.4. Conclusion

Protein concentration and digestive enzyme activities of SC-CO_2 treated mackerel muscle residues increased as compared to *n*-hexane treated residues. Enzyme activities were changed using different pH and temperature. Alkaline optimum pH and pH stability, high optimum tempearatue and higher temperature stability showed all of the digestive enzymes. Denaturation of protein was not found in SC-CO_2 treated samples. Thermally stable compounds are highly attractive for economical purposes. Therefore, SC-CO_2 treated digestive enzymes can be used as a food ingredient in the food related industries.

Chapter 3

Quality characteristics of lecithin obtained from deoiled mackerel (*Scomber japonicus*) muscle using various isolation methods

3.1. Introduction

Mackerel fish is an important food staff that is consumed worldwide (Croker and Symonds, 1933). As an oily fish, it is a rich source of omega-3 fatty acids especially eicosapentaenoic acid (EPA) and docosahexaenoicacid (DHA). Particular attention has been given to EPA which is decreases blood viscosity and the aggregation of platelates and promotes vaso-dilation (Harris, 1997) and DHA which promotes the sensorial and neuronal maturation in babies and it is often included as a supplement to the diet of pregnant women (O`Connor et al., 2001; Di Benedetto et al., 2007; Kraus-Etshcmann et al., 2007). Unlike fish oil, the EPA and DHA also contain in lecithin that has great potentially. Lecithin is composed of mainly phospholipid mixtures especially PC and PE with small amount of fatty acids, glycerols and other suspended matter (Erkkila et al., 2003; Lemaitre et al., 2003) and it is found in all living cells as a major component of cell membranes which regulate the nutrients entering and exiting the cell. Lecithin is also retailed as an emulsifying agent, allowing fats to be dispersed when mixed with water. This is a great aid in the production of foods such as margarine, mayonnaise, chocolate and baked

*This paper has been online published in Journal of Industrial and Engineering Chemistry (DOI: 10.1016/j.jiec.2014.03.029).

goods because it keeps foods from sticking to themselves and other surfaces. Lecithin is even used in the feed, pharmaceutical and cosmetic industries (Dunford and Temelli, 1995).

Many researchers have been isolated lecithin from soybean, canola and egg-yolk (Scholfield, 1981; Dunford and Temelli, 1995; Palacious and Wang, 2005) but commercially available lecithin is produced from soybean. Due to lack of favourable fatty acid in commercial lecithin, scientists have been much attention to isolate lecithin from marine organisms specially fishes. It is now proved that marine lecithin contain high concentration of important ω-3 fatty acids, EPA and DHA. Uddin et al. (2011) reported that the increase level of essential fatty acids, such as EPA and DHA, leads to increase bioavailability when they are an integrated part of a phospholipid molecule.

Generally, industrial-scale modification of crude lecithin is executed by physical, chemical and enzymatic hydrolysis (Van Nieuwenhuyzen, 1976). Commercially deoiled lecithin is obtained by treating crude lecithin with solvents such as diethyl ether, hexane, acetone and chloroform but some of these solvents are considered as an adverse because of environmental and health concerns (Sim, 1994; Nielson, 2001; Palacious and Wang, 2005). In recent years, the use of supercritical fluid extraction (SFE) for the removing of organic compounds from different liquid and solid matrices has been attracted much attention. As an environmental friendly and non toxic in food, supercritical carbon dioxide (SC-CO_2) is a promising process for the extraction and fractionation of edible lipids from different sources (Esquivel et

al., 1997; Davarnejad et al., 2008; Rubio-Rodriguez et al., 2008). Due to close chamber at SC-CO_2 extraction, outside air can not penetrate into the vessels. Therefore, recovered compound and inside remaining residues is occurred very less oxidation compared to traditional solvent extraction (Uddin et al., 2011). SC-CO_2 and hexane can extract mainly non polar lipids but remaining residues may contain some polar lipids like lecithin which potentiality is very high in food related industries. Since neat SC-CO_2 will not effectively solubilize lecithin, therefore a choice of suitable solvent must be based on thermodynamic considerations and with regard to its food safety. Prosise (1985) reported that ethanol is an excellent solvent for isolating lecithin for food use. Lecithin concentration is optimized when ethanol is used as co-solvent with SC-CO_2 (Manohar et al., 1995). Many researchers have done lecithin isolation by ethanol after oil removing by several extraction process (Palacious and Wang, 2005; Uddin et al., 2011; Lee et al., 2012) but as far we know lecithin isolation by SC-CO_2 with ethanol as co-solvent after oil removing by SC-CO_2 extraction process is very limited. Therefore, the aim of this study was to isolate and quality characteristics of lecithin using three systems (1) lecithin isolated with ethanol after hexane extraction (hexane/ethanol system), (2) lecithin isolated with ethanol after SC-CO_2 extraction (CO_2/ethanol system) and (3) lecithin isolated by SC-CO_2 with ethanol as co-solvent after oil removing by SC-CO_2 extraction (CO_2/CO_2 with ethanol system) from mackerel muscle as a comparative study.

3.2. Materials and Methods

3.2.1. Materials

Stored deoiled mackerel muscles obtained by SC-CO_2 extraction at 25 MPa and 45 ^{o}C and hexane extraction were used for lecithin isolation. PC, PE, trolox, ascorbic acid and oleic acid as standard purchased from Sigma-Aldrich, St. Luis, Mo., USA. In this study, all reagents and solvents as analytical or high performance liquid chromatography (HPLC) grade were used.

3.2.2. Isolation of lecithin by CO_2/ethanol and hexane/ethanol system

Lecithin was isolated with ethanol from mackerel muscle residues after SC-CO_2 and hexane extraction according to the modified method of our previous study (Lee et al., 2012). Exactly, 40 g of SC-CO_2 and hexane extracted mackerel muscle was mixed in 400 mL of HPLC grade ethanol and then it was stirred 12 h by a magnetic stirrer. After that, the mixture was filtered by a filter paper. The filtrate solution (supernatant) was collected and kept in a beaker. This solution containing mainly polar lipids and may have little amount of non polar lipids. The remaining non filtrate residue was again extracted with 400 mL of HPLC grade ethanol and then followed above steps. The two filtrate ethanol extract solution was mixed and non polar lipids were separated using twice volume of hexane. Afterwards, the polar containing ethanol extract was evaporated at 40 ℃ by a rotary vacuum evaporator (EYELA N-1100, Tokyo, Japan). A small amount of chilled acetone (4 ℃) was added in the remaining lipid residue with slow shaking for precipitation of

gummy material, lecithin. Subsequently, this solution was kept at 20 min on an ice bath. The supernatant was again separated by a filter paper and precipitate lecithin was collected. For further use and analysis it was stored at -20 ℃.

3.2.3. Isolation of lecithin by CO_2/CO_2 with ethanol system

A laboratory-scale of supercritical fluid extraction process was also performed for lecithin isolation. Accurately, 100 g of freeze dried raw mackerel samples were filled into the stainless steel extraction vessels. After oil removing with neat SC-CO_2 at 45 ^oC and 25 MPa for 2 h, lecithin was isolated with SC-CO_2 and ethanol as co-solvent at same temperature and pressure from remaining deoiled residues is shown in Fig. 3.1. During lecithin isolation, the flow rate of CO_2 (24.5 g/min) was constant at the entire extraction period of 2.5 h. Ethanol flow rate was 5 mL/min. The extract and ethanol mixture was collected in a glass tube. Ethanol was removed from the final extract using N_2 gas. The extracted lecithin was stored at -20 ℃ until further use and analysis.

3.2.4. Phospholipid measurement of lecithin

According to Lee et al. (2012) and Stewart (1980), the content of phospholipid from mackerel lecithin was measured by a colorimetric method. Exactly, 0.35 mg of mackerel lecithin was dissolved in 2 mL of chloroform. Then, 1 mL of a solution was added which was prepared by equal volume of 27 g/L ferric chloride and 30 g/L ammonium ferrothiocyanate. The mixture

was vortexed at 30 s and then it was centrifuged at 3,500 rpm for 15 min. After collecting the lower phase, the absorbance was read at 488 nm by a UV-VIS spectrophotometer (UVmini-1240, Shimadzu, Japan). Standard PC was used to make a calibration curve.

3.2.5. Acid value, peroxide value and free fatty acid content measurement

Acid value (AV) and peroxide value (POV) was measured according to the official method and recommended practices of the AOCS (Ja 6–55 & Ja 8-87) (1998). Free fatty acid (FFA) content of mackerel lecithin was analyzed according to the method of Bernardez et al. (2005). Calibration curve was made using oleic acid as the standard shown in Fig.3.2.

3.2.6. Analysis of major phospholipids

According to the method of Letter (1992) with modification and Lee et al. (2012), major phospholipids of mackerel lecithin were separated and quantitatively analyzed by HPLC-ELSD operation. A 4-line degasser (DG-2080-54) controller, a quaternary gradient unit (LG-2080-04), an intelligent HPLC pump (PU-2080 Plus), an evaporative light scattering detector (ELSD-Softa corporation, Model 400, London, UK) and a silica column (5 µm, 4.6 x 250 mm), Waters, Milford, Massachusetts, USA was used for phospholipid analysis. Isolated mackerel lecithin was dissolved in chloroform and methanol at 1:1 ratio and then 20 µL sample was injected into the HPLC injector. Three mobile phases such as isopropyl alcohol, hexane and water was used as a

gradient program and the flow rate of the mobile phase was 1 mL/min. In the ELSD operation, spray and drift tube temperature were set at 70 ℃ and 60 ℃, respectively. 0.345 MPa nitrogen gas was flowed as a nebulizer. Major phospholipids were identified and quantified by the checking of retention time and area of standard phospholipids, PC and PE. For data analyze, millennium software was used.

3.2.7. Identification of phospholipids by thin layer chromatography (TLC)

The phospholipids were identified from lecithin by TLC to determine the fatty acid profiles according to the modified method of Miniadis-Meimaroglou et al. (2008). Silica plate 60 (10 cm x 10 cm, 0.2 mm thick, Macherey-Nazel, Duren, Germany) was used for phospholipid identification. Chloroform, methanol, glacial acetic acid and water were used as a mobile phase at a ratio of 50:8:8:2 (v/v/v/v). As a sample preparation, lecithin was dissolved in a mixture of chloroform and methanol at a ratio of 1:1 (v/v). Spots were visualized by iodine vapour and it was compared with standard PC and PE. After that, spots were scraped off in screw cap tube separately. The separated sample was then dissolved in the chloroform and ethanol (2:2, v/v) composed solvent. The chloroform phase was separated by a filter paper and then evaporated by vacuum rotary evaporator (Eyela N-1100, Tokyo, Japan). Next, each phospholipid was used for analysis of fatty acid composition.

3.2.8. Fatty acid compositions analysis

Fatty acid compositions of lecithin and purified phospholipids were analyzed by gas chromatography (GC) according to the method of Lee et al. (2012). An 6890 Agilent Technologies (Wilmington, DE, USA) gas chromatograph with a fused silica capillary column (length, 100 m; internal diameter, 0.25 mm; length of film, 0.2 μm), Supelco (Bellefonte, PA, USA) was used. Before running the sample in the GC, the sample was prepared methyl ester according to the official method and recommended practices of the AOCS (Ce 2-66) (1998). The flow rate of helium was 1.0 mL/min as a carrier gas. The oven temperature was programmed starting at a constant temperature of 130 ℃ for 3 min, and then it was increased to 240 ℃ at a rate of 4 ℃/min and hold at 240 ℃ for 10 min. 250 ℃ temperature was fixed for injector and detector. Fatty acid methyl esters were identified by comparison of retention time with standard fatty acid methyl esters mixture (Supelco).

3.2.9. Oxidative stability of lecithin

Oxidative stability was checked by oxidation of lecithin in water at 37 ℃ by the thiocyanate method Mitsuda et al. (1966) with modification. Three types of sample in deionized and degassed water (w/w) (oleic acid 4%, water 96%; lecithin 4%, water 96%; trolox 2%, water 98%) were prepared. Oleic acid and standard trolox were used to compare the oxidative stability of mackerel lecithin. Firstly, 4.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate solution was taken in the test tube and then 0.1 mL of

each lecithin solution was added. After 3 min, 0.1 mL of 0.02 M ferrous chloride in 3.5% HCl was added to the reaction mixture and it was appeared red colour in the solution. The absorbance of red colour was measured at 500 nm. Oxidative stability was checked by measuring the absorbance at 2 days intervals after 1 day incubation.

3.2.10. Antioxidant activity of lecithin

3.2.10.1. DPPH free radical scavenging assay

The scavenging effects of mackerel lecithin were determined by the method of Yen and Chen (1995) with slight modification. Briefly, 2.0 mL of 0.1 mM DPPH solution (in methanol) was added to the test tube containing 1.0 mL aliquot of sample (1 g lecithin was dissolved in 10 mL of methanol for aliquot preparation). The mixture was vortexed for 1 min and kept at room temperature for 50 min in the dark. The absorbance of all the sample solutions was measured at 517 nm. The percentage of DPPH free radical scavenging activity was calculated using this formula:

DPPH free radical scavenging activity (%) = (1- [As/Ac]) x 100%

Where, As = absorbance of sample and control; Ac = absorbance of blank. The samples of blank and control (0.1 M standard ascorbic acid) were performed according to the method.

3.2.10.2. $ABTS^+$ free radical scavenging activity

$ABTS^+$ assay was performed by the method of Zheleva-Dimitrova et al. (2010) with a slight modification. ABTS was dissolved in water to make a concentration of 7 mM/L. $ABTS^+$ was produced by reacting the equal volume of ABTS stock solution with 2.45 mM/L potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. For the test of samples, the $ABTS^+$ stock solution was diluted with 80% methanol to an absorbance of 0.70 ± 0.02 at 734 nm. After the addition of 3.9 mL of diluted $ABTS^+$ to 1 mL of samples (1 g lecithin was dissolved in 10 mL of 80% methanol), the mixtures were kept in dark environment at room temperature for 6 min. The absorbance of all the sample solutions was measured at 734 nm. The percentage of $ABTS^+$ free radical scavenging activity was calculated using this formula:

ABTS free radical scavenging activity (%) = (1- [As/Ac]) x 100%

Where, As = absorbance of sample and control; Ac = absorbance of blank. The samples of blank and control (0.5 mg/mL standard trolox) were analyzed as described above.

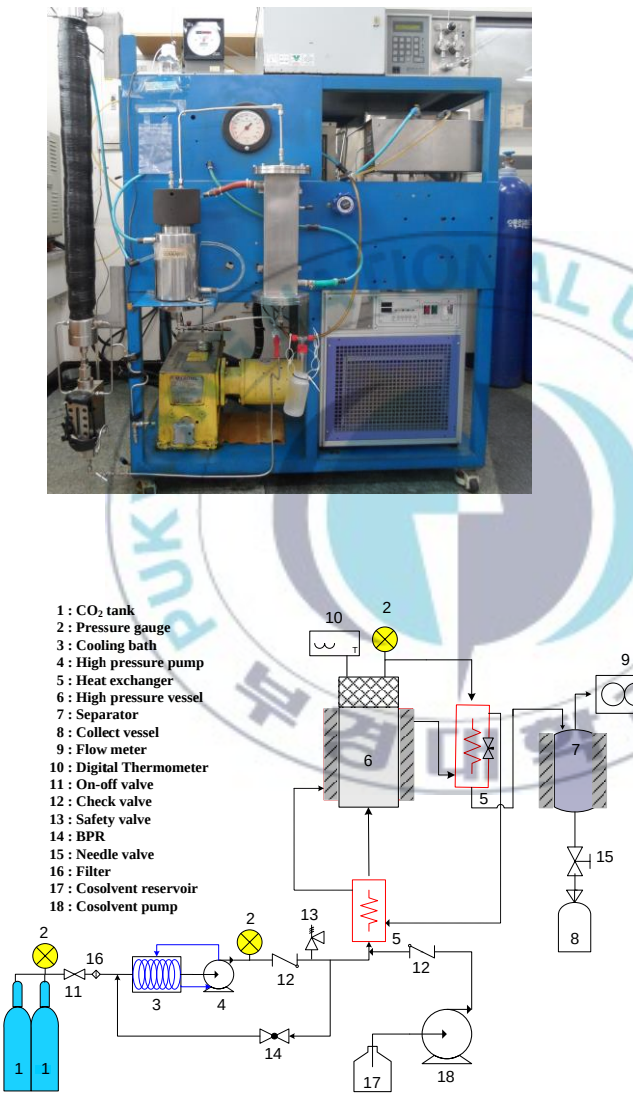


Fig. 3.1. Photograph and schematic diagram of the SC-CO_2 extraction process with cosolvent

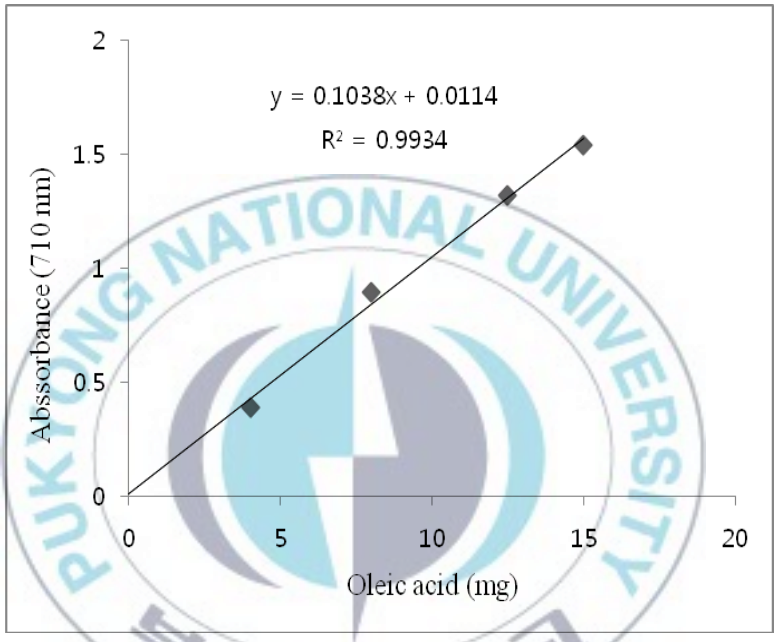


Fig. 3.2. Calibration curve of oleic acid for estimation of free fatty acids in mackerel muscle lecithin

3.3. Results and Discussion

3.3.1. Phospholipid content in mackerel lecithin

In the present study, 9.75 ± 0.08%, 8.93 ± 0.06% and 12.24 ± 0.09% (w/w) of lecithin was isolated from deoiled mackerel residues by three different systems (hexane/ethanol system, CO_2/ethanol system and CO_2/CO_2 with ethanol system) shown in Table 3.1. Among the three systems, the percentage of lecithin was higher in CO_2/CO_2 with ethanol system. Manohar et al. (1995) reported that the use of CO_2 with ethanol as a co-solvent increased lecithin concentration of deoiled soya which was similar compared to our obtained results. Phospholipid content in mackerel lecithin was 82.15 ± 1.95%, 84.10 ± 2.12% to 68.57 ± 1.70% (w/w) by hexane/ethanol system, CO_2/ethanol system and CO_2/CO_2 with ethanol system shown in Table 3.1. Therefore, 8.01 ± 0.08%, 7.51 ± 0.06% and 8.39 ± 0.09% of total phospholipids (w/w) were found in mackerel residues using above systems. Uddin et al. (2011) and Lee et al. (2012) reported that the phospholipid content of squid viscera and anchovy extracted by the SC-CO_2 were 3.89% and 16%, respectively. Phospholipids content of mackerel by three systems was medium compared to squid viscera and anchovy.

3.3.2. Acid value, peroxide value and free fatty acid content

The AV, POV and FFA content provide quality index of mackerel lecithin are given in Table 3.2. These parameters are depends on the nature of processing and the factors such as the surrounding moisture, air, temperature,

etc. AV was used to determine the acidity of lecithin. POV provides the oxidation state of lipid and it was used to measure the rancidity that occurred by autoxidation. AV and POV of different extracted lecithin (hexane/ethanol system, CO_2/ethanol system and CO_2/CO_2 with ethanol system) were 37.60 ± 0.42, 23.00 ± 0.36 and 21.32 ± 0.21 mg KOH/g and 1.60 ± 0.10, 1.40 ± 0.08 and 1.10 ± 0.06 meq/1,000 g, respectively. FAO/WHO recommended that AV and POV ranges up to 36 mg KOH/g and 10 meq/1,000 g, respectively for food grade lecithin (Nieuwenhuyzen and Tomas, 2008). AV of hexane extracted lecithin (hexane/ethanol system) was little higher compared to FAO/WHO recommendation and much higher compared to CO_2/ethanol system and CO_2/CO_2 with ethanol system. Essien et al. (2012) reported that high AV indicates low oxidative stability. POV of hexane extracted lecithin (hexane/ethanol system) was also higher compared to CO_2/ethanol system and CO_2/CO_2 with ethanol system. Due to open system (hexane/ethanol system), high exposure of oxygen was contacted during extraction and isolation of lecithin that's why higher oxidation was occurred. FFA content of different extracted mackerel lecithin (hexane/ethanol system, CO_2/ethanol system and CO_2/CO_2 with ethanol system) was 5.41 ± 0.09, 5.05 ± 0.07 and 2.28 ± 0.04 g/100 g, respectively. AV, POV and FFA content of lecithin provide better results in CO_2/CO_2 with ethanol system than hexane/ethanol and CO_2/ethanol system. It can be happened that isolated lecithin by CO_2/CO_2 with ethanol system occurred less oxidation because of oxygen can not penetrate into the vessel of SC-CO_2 extraction process due to close chamber of whole extraction

period.

Table 3.1. Lecithin and phospholipid content in mackerel muscle

Sample name	Lecithin isolation	Lecithin (%)	Phospholipid content in lecithin (%)	Total purified phospholipid (%)
Mackerel muscle	hexane/ethanol system	9.75 ± 0.08	82.15 ± 1.95	8.01 ± 0.08
	CO_2/ethanol system	8.93 ± 0.06	84.10 ± 2.12	7.51 ± 0.06
	CO_2/CO_2 with ethanol system	12.24 ± 0.09	68.57 ± 1.70	8.39 ± 0.09

Means ± SD (n = 3)

Table 3.2. Acid value, peroxide value and free fatty acid content of mackerel lecithin as quality index

Lecithin isolation	Quality index		
	Acid value (mg KOH/g)	Peroxide value (meq/1,000 g)	Free fatty acids (g/100 g)
Hexane/ethanol system	37.60 ± 0.42	1.60 ± 0.10	5.41 ± 0.09
CO_2/ethanol system	23.00 ± 0.36	1.40 ± 0.08	5.05 ± 0.07
CO_2/CO_2 with ethanol system	21.32 ± 0.21	1.10 ± 0.06	2.28 ± 0.04

Means ± SD (n = 3)

3.3.3. Fatty acid composition of lecithin

The fatty acid compositions of lecithin in different extraction system (hexane/ethanol system, CO_2/ethanol system and CO_2/CO_2 with ethanol system) from mackerel muscle residues obtained by GC are shown in Table 3.3. The percentages of the total unsaturated fatty acids (UFAs) were high in all extracted lecithin. Among the unsaturated fatty acids, oleic acid was present in higher amounts in all extracted mackerel lecithin. The important PUFAs were EPA ranging from 3.98 ± 0.07% to 8.00 ± 0.09% of total fatty acids and DHA ranging from 10.47 ± 0.15% to 17.34 ± 0.18% of total fatty acids in all extracted lecithin. Among the systems, EPA and DHA were higher in CO_2/ethanol system than hexane/ethanol and CO_2/CO_2 with ethanol system. Lee et al. (2012) reported that EPA and DHA were found 3.70% and 10.19% from anchovy which was lower than mackerel. The most significant saturated fatty acid was palmitic acid ranging from 22.14 ± 0.56% to 28.90 ± 0.68% of total fatty acids in extracted mackerel lecithin.

3.3.4. Major phospholipids quantification

For containing high EPA and DHA, SC-CO_2 extracted lecithin (CO_2/ethanol system) was used for phospholipid identification and quantification. Individual phospholipids of lecithin were identified by HPLC shown in Fig. 3.3. The major phospholipids of lecithin obtained from mackerel lecithin are shown in Table 3.4. PC and PE were main phospholipids of mackerel lecithin and they are included 20.11 ± 0.07% and 67.44 ± 0.20%

of the total phospholipids, respectively. Squid viscera contained 12.7% of PC and 79.2% of PE which has been reported by Cho et al. (2001). Lee et al. (2012) also reported that phospholipid from anchovy contained 68.0% of PC and 29.0% of PE. Total percentages of PC and PE of mackerel phospholipid were almost similar than squid viscera and lower than anchovy. Sources, habitat, intake varieties of food, variation of isolation, and so on can be responsible for phospholipids composition differences.

Table 3.3. Fatty acid compositions of mackerel lecithin

Fatty acid compositions	Fatty acid (%)		
	Hexane/ethanol system	CO_2/ethanol system	CO_2/CO_2 with ethanol system
Myristic acid (C14:0)	6.11 ± 0.12	5.07 ± 0.10	4.49 ± 0.08
Palmitic acid (C16:0)	28.90 ± 0.68	22.14 ± 0.56	24.40 ± 0.62
Palmitoleic acid (C16:1)	4.45 ± 0.07	4.18 ± 0.08	4.03 ± 0.06
Stearic acid (C18:0)	8.93 ± 0.14	6.79 ± 0.11	8.05 ± 0.16
Oleic acid (C18:1)	20.31 ± 0.32	18.98 ± 0.28	22.21 ± 0.26
Elaidic acid (C18:1)	5.74 ± 0.09	5.15 ± 0.08	5.39 ± 0.10
Linoleic acid (C18:2)	2.12 ± 0.06	2.92 ± 0.05	2.24 ± 0.04
Eicosenoic acid (C20:1)	2.80 ± 0.05	2.58 ± 0.06	3.87 ± 0.07
Eicosadienoic acid (C20:2)	2.21 ± 0.04	2.77 ± 0.04	2.07 ± 0.05
Eicosatrienoic acid (C20:3)	2.24 ± 0.08	1.95 ± 0.09	3.44 ± 0.10
Tricosanoic acid (C23:0)	1.01 ± 0.03	1.44 ± 0.04	0.95 ± 0.02
EPA (C20:5)	3.98 ± 0.07	8.00 ± 0.09	5.49 ± 0.06
Nervonic acid (C24:1)	0.73 ± 0.01	0.68 ± 0.01	1.59 ± 0.03
DHA (C22:6)	10.47 ± 0.15	17.34 ± 0.18	11.77 ± 0.14

Means ± SD (n = 3)

Table 3.4. Major phospholipid compositions of mackerel lecithin by CO_2/ethanol system

Phospholipid composition	Total phospholipid (%)
Phosphatidylcholine (PC)	20.11 ± 0.07
Phosphatidylethanolamine (PE)	67.44 ± 0.20
Others	12.45 ± 0.06

Means ± SD (n = 3)

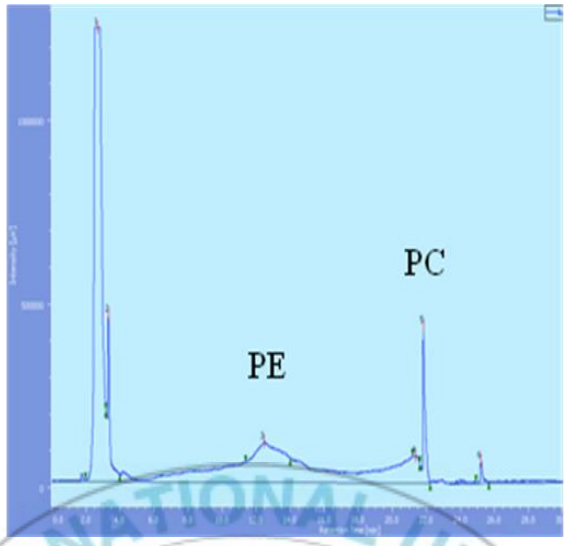


Fig. 3.3. Identification of phospholipids from mackerel lecithin by HPLC

Fig. 3.4. Identification of phospholipids from mackerel lecithin by TLC

3.3.5. Fatty acid compositions of PC and PE

Individual phospholipids, PC and PE were also identified from mackerel lecithin by TLC is shown in Fig. 3.4. Fatty acid compositions of PC and PE were found from mackerel lecithin which is presented in Table 3.5. EPA (7.98 ± 0.11% & 8.53 ± 0.14%) and DHA (19.56 ± 0.30% & 18.00 ± 0.28%) were found in identified PC and PE. DHA/EPA ratio in PC and PE were 2.45 ± 0.06 and 2.11 ± 0.05, respectively. From squid viscera and anchovy, DHA/EPA ratio for PC were 1.74 & 2.12 and PE were 1.08 & 1.50, respectively (Uddin et al., 2011; Lee et al., 2012). DHA/EPA ratio of mackerel PC and PE were comparatively higher than squid viscera and anchovy.

Table 3.5. Fatty acid compositions of PC and PE

Fatty acid compositions	Fatty acid (%)	
	PC	PE
Myristic acid (C14:0)	ND	7.52 ± 0.22
Palmitic acid (C16:0)	27.10 ± 0.88	24.35 ± 0.82
Stearic acid (C18:0)	13.22 ± 0.32	9.71 ± 0.24
Oleic acid (C18:1)	18.70 ± 0.44	18.01 ± 0.52
Elaidic acid (C18:1)	7.78 ± 0.12	7.19 ± 0.15
Linoleic acid (C18:2)	5.67 ± 0.09	6.70 ± 0.10
EPA (C20:5)	7.98 ± 0.11	8.53 ± 0.14
DHA (C22:6)	19.56 ± 0.30	18.00 ± 0.28
DHA/EPA	2.45 ± 0.06	2.11 ± 0.05

Means ± SD (n = 3). ND-not detected

3.3.6. Oxidative stability of lecithin

Oxidative stability of lecithin from mackerel muscle by three different systems is shown in Fig. 3.5. In this study oxidation was evaluated by checking the absorbance value of incubated sample. The increasing absorbance value was an indicator of autoxidation which occurred due to formation of peroxides during incubation (Uddin et al., 2011). Absorbance value was increased from third day when oleic acid was mixed with water. Three different extracted lecithin (hexane/ethanol system, CO_2/ethanol system and CO_2/CO_2 with ethanol system) in water showed low absorbance value which indicates that low level of lipid peroxidation was occurred until 11 days. It has been shown that oxidation of lecithin was increased rapidly after 11 days. Among the three extracted lecithin, oxidative stability was slightly higher in hexane extracted lecithin (hexane/ethanol system). It might be happened due to low contain of unsaturated fatty acids. On the other hand, standard trolox in water showed high oxidative stability compared to mackerel lecithin. Trolox inhibited the peroxide formation of the lipids over a certain period. Lecithin from mackerel may contain small amounts of natural antioxidative compounds that might be one of the causes of its higher oxidative stability. Many researchers reported that lecithin has high oxidative stability (Palacious and Wang, 2005; Wang and Wang, 2008; Belhaj et al., 2010; Uddin et al., 2011; Lee et al., 2012).

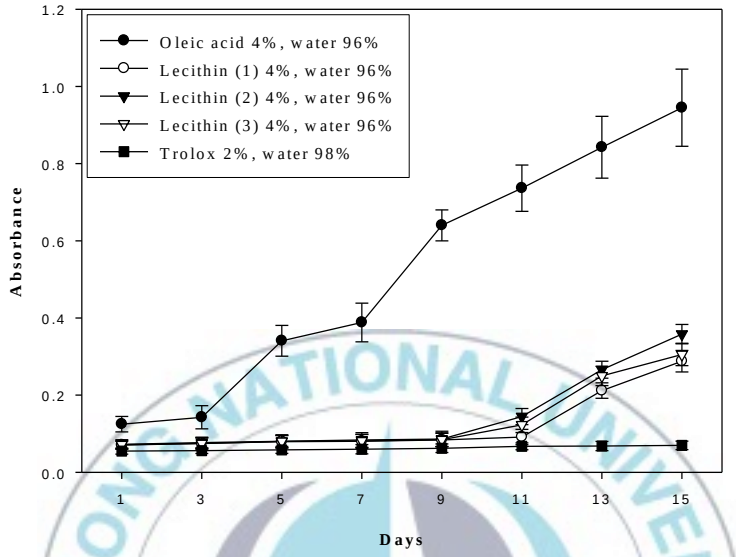
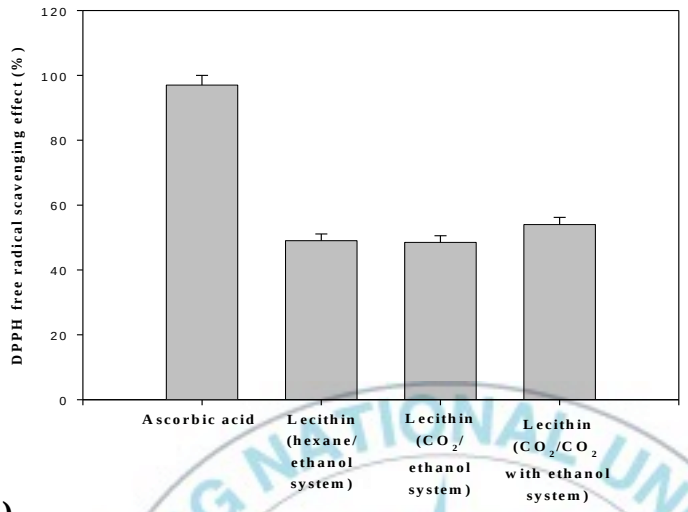


Fig. 3.5. Oxidative stability of mackerel lecithin. Lecithin (1) isolated with hexane/ethanol system; Lecithin (2) isolated with CO_2/ethanol system; Lecithin (3) isolated with CO_2/CO_2 with ethanol system. Means ± SD (n = 3)

3.3.7. Antioxidant activity of lecithin

The DPPH and ABTS methods are used to evaluate the antioxidant activity of different extracted lecithin (hexane/ethanol system, CO_2/ethanol system and CO_2/CO_2 with ethanol system). Lecithin is an emulsifying agent which has hydrophobic and hydrophilic properties. For that reason we have done antioxidant activity of lecithin by DPPH and ABTS assay. In this sense, it is important to remember that DPPH has been described as more specific for hydrophobic antioxidant and ABTS has been described as hydrophobic and hydrophilic antioxidant (Prior et al., 2005). The results of antioxidant activity obtained from each lecithin of mackerel are shown in Fig. 3.6A and 3.6B. Almost 50% DPPH radical-scavenging activity was shown in extracted lecithin. A high antiradical activity (over 90%) was observed with ABTS in the extracted lecithin. Among all the extracted lecithin, antioxidant activity of lecithin was slightly higher using CO_2/CO_2 with ethanol system. Scholfield (1981) reported that some amount of tocopherol was contained in lecithin which can be reason for antioxidative properties. Another researcher Ramadan (2008) reported that phospholipids, which are composed of highly unsaturated fatty acid chains in addition to the polar heads responsible for their antioxidative properties. So far numerous studies have been focused on the antioxidant properties of lecithin. It is likely that antioxidant activity in lecithin may differ according to the wide variance in functional groups, structures, their fatty acid pattern and the chemical composition of the lipid system etc. (Ramadan et al., 2003; Ramadan and Moersel, 2004).

(A)



(B)

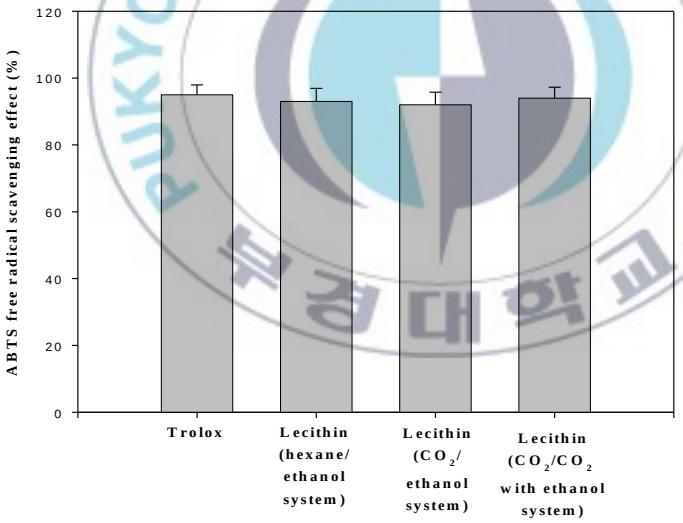


Fig. 3.6. Antioxidant activity of mackerel lecithin. (A) DPPH free radical scavenging effect and (B) ABTS free radical scavenging effect. Means ± SD (*n* = 3)

3.4. Conclusion

AV, POV and FFA content of mackerel lecithin from different extraction methods were measured for quality determination and it was found that values were lower in CO_2/CO_2 with ethanol system than other systems. The major phospholipids of mackerel lecithin were PC and PE. The phospholipids were contained higher amount of PUFAs especially EPA and DHA. Oxidative stability of mackerel lecithin was high and there were no significant differences within all systems. Mackerel lecithin also exhibited antioxidant activity and it was little higher in CO_2/CO_2 with ethanol system but not significant different. Therefore, we can say that quality of lecithin was better in CO_2/CO_2 with ethanol system than other systems. Moreover, all extracted lecithin provide PUFAs which may be considered as valuable additives in the food industries as well as in the pharmaceutical and cosmetic industries.

Chapter 4

Hydrolyzates produced from mackerel *Scomber japonicus* skin by the pressurized hydrothermal process contain amino acids with antioxidant activities and functionalities

4.1. Introduction

Every year, thousands of tons of fish by-products of high nutrient content are discarded by fish processing plants throughout the world. Discarding by-products creates two major problems. First, is the underutilization of a huge amount of functional materials such as peptide and amino acid. 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 and Bublitz, 1996). Hydrolysis processes have been developed to convert fish by-products into functional materials, which can be widely used in food rather than as animal feed or fertilizer (Benjakul and Morrissey, 1997).

Current industrial hydrolysis methods 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. As a new green conversion method, reactions in

*This work has been published in Journal of Fisheries Science, 80:369-380 (2014)

pressurized hydrothermal have been gaining more attention of late. Pressurized hydrothermal can provide a new reaction medium in many chemical reactions. Changes in physical and chemical properties of water under pressurized hydrothermal conditions, especially in hydrogen bond, ion product and dielectric constant, could facilitate reactions with a wide range of organic compounds resulting in many valuable functional materials (Tomita and Oshima, 2004). The chemical properties of pressurized hydrothermal are similar with acetone, and its ionic product is over thousand fold that of normal water. So, it plays the role of catalyst as acid or alkali without any environmental pollution (Wang and Zhu, 2005). Most of biomass is easily hydrolyzed in pressurized hydrothermal, which is structurally different from normal liquid water. Without any pollution, hydrolysis in pressurized hydrothermal is environment friendly technology (Cheng et al., 2008). Recently growing attention has led to extensive research activities using pressurized hydrothermal for hydrolysis and conversion of biomass to useful compounds (Yoshida et al., 1999; Tavakoli and Yoshida, 2006; Salak Asghari and Yoshida, 2007; Uddin et al., 2010). The thermal protein hydrolysis is gaining in importance in economical as well as ecological aspects.

Protein hydrolyzates using enzyme from different fish sources such as mackerel, yellow stripe trevally, ornate threadfin bream (Wu et al., 2003; Klompong et al., 2007; Nalinanon et al., 2011) have been found to possess antioxidant activity. Levels and compositions of free amino acids and peptides were reported to determine the antioxidant activities of protein hydrolyzates

(Wu et al., 2003). Functional properties (solubility, emulsifying activity index, emulsion stability, foaming capacity and foam stability) of protein can be improved by pressurized hydrothermal hydrolysis at different temperature and pressure. Hydrolysis potentially influences the molecular size, hydrophobicity and polar groups of the hydrolyzate and produces a decrease in peptide size, which can modify functional characteristics of the proteins and improve their quality (Petersen, 1981). However, antioxidant activities and functional properties related work from fish-byproduct hydrolyzate using pressurized hydrothermal is very limited particularly mackerel *Scomber japonicus* skin is not reported. Therefore, this study was aimed to produce functional material especially amino acids from the mackerel skin hydrolyzate using pressurized hydrothermal and to study their antioxidant activities and functional properties.

4.2. Materials and Methods

4.2.1. Materials

The freeze dried raw and SC-CO_2 deoiled mackerel skin samples were used for prerssurized hydrothermal hydrolysis. All reagents used in this study were of analytical or HPLC grade.

4.2.2. Sample preparation

Mackerel muscles with skin were dried in a freeze-drier. After completely dried, skin was separated manually from muscle and then crushed by a

mechanical blender. After that sample was sieved by mesh size 3 mm. Supercritical carbon dioxide (SC-CO_2) at 55 ^oC and 250 bar was applied for getting oil free containing mackerel skin. These samples were then stored at -20 ^oC prior to pressurized hydrothermal hydrolysis.

4.2.3. Proximate composition analysis

The moisture, ash and crude protein content were determined according to the method of AOAC (1990) and lipid content was measured by conventional soxhlet extraction using hexane as solvent for 24 h. Non protein content was estimated by subtracting the sum of weight of moisture, ash, protein and lipid from total weight.

4.2.4. Pressurized hydrothermal hydrolysis (PHH)

The set up of a laboratory scale of PHH process is shown in Fig. 4.1. PHH was carried out in 200 mL of a batch reactor made of 276 Hastelloy with temperature controller. 6.6 g of freeze dried raw and supercritical carbon dioxide (SC-CO_2) deoiled mackerel skin residues were put in the reactor and closed. High pressure pump was applied to flow water from tank to reactor for getting initial pressure 4 bar where material to water ratio was 1:30 (w/v). The reactor was heated by an electric heater which was previously heated to the desired temperature (150-240 ^oC) and pressure (12-210 bar). Heating time was 26 to 52 min to reach the desiered temperature. The sample was stirred by stirrer at 150 rpm. The temperature and pressure in reactor of each experiment

was measured by temperature controller and pressure gauge, respectively. The reaction time for each sample was taken 5 min. After cooling to room temperature, the hydrolyzed sample from the reactor was collected and filtered using a filter paper (Advantec No. 5A, Tokyo, Japan). After liophilization, the hydrolyzed samples were stored at 4 ^{o}C for further analysis.

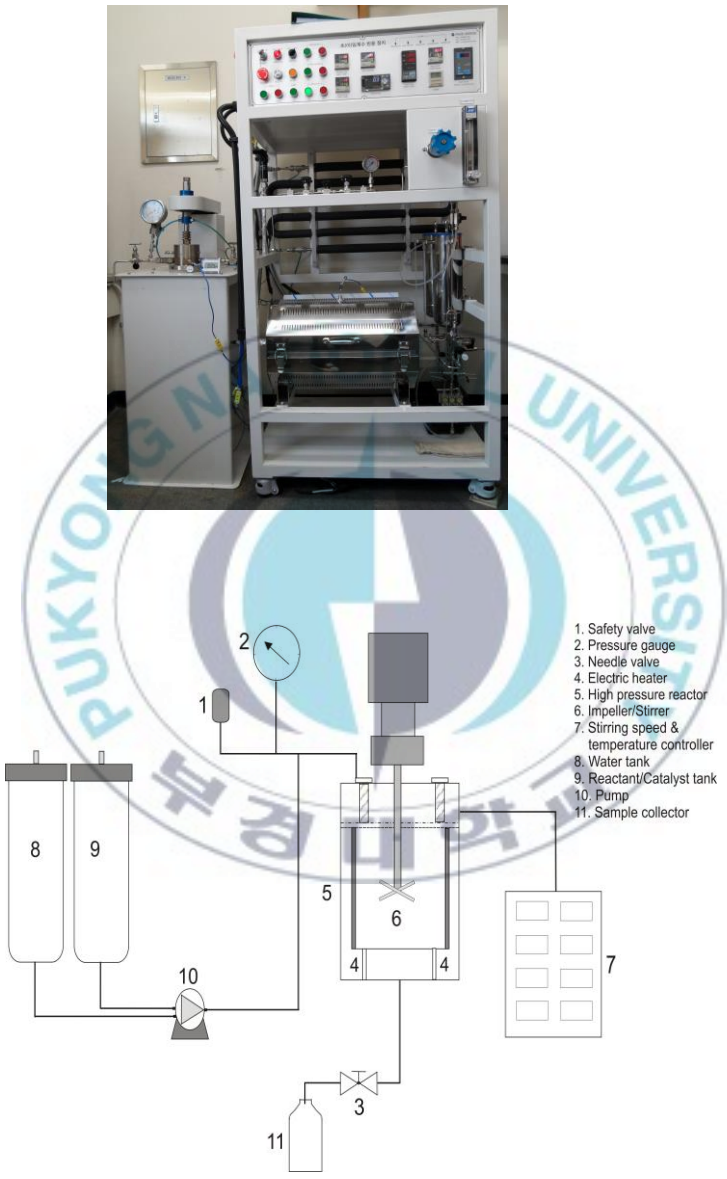


Fig. 4.1. Photograph and schematic diagram of pressurized hydrothermal hydrolysis experimental apparatus

4.2.5. Amino acid composition analysis

Hydrolyzates of mackerel skin obtained by pressurized hydrothermal hydrolysis were filtered and loaded onto a S430 (SYKAM) amino acid auto analyzer for amino acid analysis. Cation separation column LCA K07/Li (4.6 x 150 mm), column temperature (37-74 ^{o}C), buffer pH of 2.90-7.95 were used for amino acid analysis. The mobile phase was 5 mM of *p*-toluenesulfonic acid solution at a flow rate of 0.45 mL/min. A mixture of 5 mM *p*-toluenesulfonic acid, 20 mM of bis-tris and 100 mM of EDTA was used as post column reagent at a flow rate of 0.25 mL/min. Excitation and emission wavelength was kept at 440 and 570 nm for both operational conditions.

4.2.6. Antioxidant activity measurement

4.2.6.1. DPPH free radical scavenging assay

The scavenging effects of hydrolyzate samples were determined by the method of Yen and Chen (1995) with slight modification. Briefly, 3.9 mL of 0.1 mM DPPH solution in methanol was added to the test tube containing 0.2 mL (5.5 mg hydrolyzate/mL distilled water) of hydrolyzate sample. We have discussed this method in chapter 3. Sample blank and control sample (trolox 0.5 mg/mL distilled water) was performed according to the method.

4.2.6.2. $ABTS^{+}$ free radical scavenging assay

For $ABTS^{+}$ assay, the procedure was followed by the method of Zheleva-Dimitrova et al. (2010) with slight modification. 4 mL of diluted $ABTS^{+}$ was

added to 0.05 mL (5.5 mg hydrolyzate/mL distilled water) of hydrolyzate samples. This method also has discussed in chapter 3. Sample blank and control sample (trolox 0.4 mg/mL distilled water) was performed according to the method.

4.2.6.3. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was determined according to the method of Beara et al. (2009) with some modification. 1 mL (5.5 mg hydrolyzate/mL distilled water) of sample solution, 1 mL of ortho-phenanthroline (7.5 mM\L), 5 mL of phosphate buffer (0.2 M, pH 6.6), 1 mL of ferrous sulfate (7.5 mM\L) and 1 mL of H_2O_2 (0.1%) were mixed and diluted to 25 mL with distilled water. After incubation at room temperature for 30 min, the absorbance was measured at 510 nm. The scavenging percentage (*P%*) was calculated as following equation:

$$P\% = ((A\text{-}A_1)/(A_2\text{-}A_1)) \times 100$$

where A, A_1 and A_2 are the absorbance value of the system with all solution including H_2O_2 and the sample solution, the system without sample solution, and the system without H_2O_2 and the sample solution, respectively. Control sample (ascorbic acid 0.5 mg/mL distilled water) was performed according to the method.

4.2.6.4. Fe^{2+} chelating assay

The chelating activity on Fe^{2+} was determined using the method of Decker

and Welch (1990). 0.5 mL (5.5 mg hydrolyzate/mL distilled water) of sample solution was mixed with 3.7 mL of distilled water. The mixture was then reacted with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 20 min at room temperature. The absorbance was read at 562 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. Chelating activity (%) was then calculated as follows:

$$\text{Chelating activity (\%)} = (1 - \frac{A_{562} \text{ of sample}}{A_{562} \text{ of control}}) \times 100$$

4.2.6.5. Ferric reducing power assay

Ferric reducing power was determined by the method of Oyaiza (1986). 1 mL (5.5 mg hydrolyzate/mL distilled water) sample solution was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ^{o}C for 20 min. An aliquot (2.5 mL) of 10% trichloroacetic acid was added to the mixture, and followed by centrifugation at 3,000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with 2.5 mL of distilled water and 2.5 mL of 0.1% ferric chloride and the absorbance was measured at 700 nm. A rise in absorbance reflected an increasing reducing power.

4.2.7. Functional properties

4.2.7.1. Solubility

For protein solubility determination, hydrolyzates samples (20 mg) were

dispersed in 18 mL of deionised water and pH of the mixture was adjusted to 3.5, 5.5, 7.5 and 9.5 with either 2 M HCl or 2 M NaOH. The mixture was stirred at room temperature for 20 min. The volume of solutions was made up to 20 mL by distilled water, previously adjusted to the same pH as the sample solution, prior to centrifugation at 3,000 rpm for 10 min at 4 ^{o}C. Protein content in supernatant was measured using the lowry method (Lowry et al., 1951). Total protein content was determined by extracting 20 mg of hydrolyzate samples in 20 mL of 0.5 M NaOH. Protein solubility was calculated using the following equation:

$$\text{Solubility (\%)} = \frac{\text{protein content in supernatant}}{\text{total protein content}} \times 100$$

4.2.7.2. Emulsifying properties

Emulsifying properties were determined according to the method of Pearce and Kinsella (1978) with slight modification. Soybean oil (2 mL) and hydrolyzates samples (0.10%, 0.25% and 0.50%) in 6 mL water were homogenized using a homogenizer (Ultra-Turax T25, IKA Labortechnik, Staufen, Germany) at 20,500 rpm for 2 min. An aliquot of the emulsion (50 µl) was pipette from the bottom of the container at 0 and 10 min after homogenization and mixed with 5 mL of 0.1% sodium dodecyl sulphate solution. The absorbance of the solution was measured at 500 nm. The absorbance measured immediately 0 min (A_0) and 10 min (A_{10}) after emulsion formation were used to calculate the emulsion activity index (EAI) and

emulsion stability index (ESI) as follows:

$$EAI = 2T/\varphi C$$

Where, T is the turbidity (T=2.303A_{500}/l; A_{500} is absorbance at 500 nm; l is path length; φ is oil volume fraction (0.25) and C is protein concentration in aqueous phase.

$$\text{EAI } (m^2/g) = \frac{2 \times 2.303 \times Abs_{500}}{0.25 \times \text{protein weight (g)}}$$

$$\text{ESI (min)} = \frac{A_o \times \Delta t}{\Delta A}, \text{ Where, } \Delta A = A_0 - A_{10} \text{ and } \Delta t = 10 \text{ min}$$

4.2.7.3. Foaming properties

Foaming capacity (FC) and foam stability (FS) of mackerel skin hydrolyzates were determined according to the method of Sathe and Salunkhe (1981). 20 mL hydrolyzate samples (0.1%, 0.25% and 0.50% in water) were homogenised at 13,500 rpm to incorporate the air for 1 min at room temperature. The whipped sample was immediately transferred into a 50 mL cylinder and the total volume was recorded within 10 s. The following capacity and stability were expressed as:

$$\text{FC (\%)} = \frac{\text{(volume after whipping - volume before whipping}}{\text{volume before whipping}} \times 100$$

$$\text{FS (\%)} = \frac{\text{residual foam volume after 3 min}}{\text{total foam volume}} \times 100$$

Total foam volume was the volume measured immediately after whipping.

4.3. Results and Discussions

4.3.1. Proximate analysis of mackerel skin

Proximate analysis was done to determine the approximate amounts of substances within a material. The proximate compositions of freeze dried and SC-CO_2 deoiled mackerel skin are shown in Table 4.1. The protein content was 49.49 ± 1.52% and 68.25 ± 1.80%, respectively in freeze dried and SC-CO_2 deoiled mackerel skin. Significant differences were found in protein content between freeze dried and SC-CO_2 deoiled mackerel skin. Moisture, ash, lipid and non-protein content in freeze dried and SC-CO_2 deoiled mackerel skin were 4.60 ± 0.06%, 4.40 ± 0.04%; 4.50 ± 0.05%, 6.40 ± 0.07%; 29.47 ± 1.26%, 8.18 ± 0.84% and 11.94 ± 0.76%, 12.77 ± 0.66%, respectively. Due to oil removing from freeze dried mackerel *Scomber japonicas* skin by SC-CO_2 protein content was increased. This procedure was done to increase hydrolysis yield at high temperature and pressure because lipid present in the raw materials made them less accessible to water. Sampath Kumar et al. (2012) reported that protein content in horse mackerel *Magalaspis cordyla* skin was 13%, which was lower than freeze dried and SC-CO_2 deoiled mackerel *Scomber japonicus* skin.

Table 4.1. Proximate compositions of mackerel skin

Proximate composition (%)	Freeze dried mackerel skin	SC-CO_2 deoiled mackerel skin
Moisture	4.60 ± 0.06a	4.40 ± 0.04a
Ash	4.50 ± 0.05a	6.40 ± 0.07b
Lipid	29.47 ± 1.26a	8.18 ± 0.84b
Protein	49.49 ± 1.52a	68.25 ± 1.80b
Non protein	11.94 ± 0.76a	12.77 ± 0.66a

Means ± SD (n = 3). Different small letters in the same row indicate significant differences ($P < 0.05$)

4.3.2. PHH conversion yield

In Fig. 4.2, hydrolysis yield at different temperatures and pressures after PHH from freeze dried and SC-CO_2 deoiled mackerel skin are shown. It was found that hydrolysis yield increased with the increase in temperature and pressure in the vessel. The highest hydrolysis yield in freeze dried and SC-CO_2 deoiled mackerel skin was 84.25 ± 0.46% and 86.15 ± 0.49%, respectively at 240 ℃ and 210 bar. Protein usually has low solubility in water at ambient temperature and pressure and strong aggregation occur through hydrophobic interactions. However, the solubility of new material such as protein in water increases at higher temperature and pressure. In addition, at high temperature and pressure, the hydrolysis yield increased due to the raise in water ionization constant. Similar result was reported by Park et al. (2012) for subcritical water hydrolysis of *Laminaria japonica*.

4.3.3. Total and essential amino acid yield

Total amino acid yield in freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates at different temperatures and pressures are shown in Fig. 4.3. It was found that amino acid yield in SC-CO_2 deoiled mackerel skin hydrolyzates was higher than freeze dried. The highest yield of amino acids in the freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates were 121.93 ± 1.80 and 122.96 ± 2.84 mg/g at 240 ^{o}C and 210 bar, respectively. Amino acid yield was increased with increasing temperature and pressure up to 240 ^{o}C and 210 bar.

In the present study, nine essential amino acids were found in freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates (Fig. 4.4). Among them histidine was highest and it was 7.50 ± 0.06 and 9.32 ± 0.16 mg/g, respectively at 150 ^{o}C and 150 bar. All essential amino acids concentration was increased due to increases the temperature at 240 ^{o}C and pressure 210 bar except threonine and histidine.

Amino acids and protein hydrolyzates are useful as additives in food industries. In this study, we had applied short reaction time for pressurized hydrothermal hydrolysis in order to decrease the decomposition of amino acids. We got highest amount of amino acid by applied high temperature and pressure. Cheng et al. (2008) reported the same increase of amino acids yield with the increase in temperature to a certain degree.

Essential amino acids can not be produced by the body and it should be derived from food. Without these, body can not maintain proper metabolism as a result cause many serious physiological problem and in some extreme cases, cause death. Mackerel skin hydrolyzate contains nine essential amino acids which can meet nutritional value for human body metabolism. Most of the essential amino acids content was increased upto temperature at 240 ^{o}C and pressure 210 bar compare to other experimental operational conditions. By this condition, protein breakdown was almost completely occurred and produced more amino acids. Cheng et al. (2008) and Uddin et al. (2010) reported that most of amino acids gave maximum yield at the reaction temperature range of 180 to 220 ^{o}C and 200 to 290 ^{o}C. It may be differ due to the different sample

sources, processing and experimental operational conditions. Some other works have been carried out in which thermal degradation of amino acids occured at temperature above 250 to 300 ^{o}C depending on the raw protein and corresponding contact time (Yoshida et al., 2003). Among the identified essential amino acids from mackerel skin, histidine and threonine showed temperature sensitivity. Espinoza and Morawicki (2012) reported that the greatest concentration of histidine was found below 200 ^{o}C by subcritical water hydrolysis and after increasing the temperature, concentration was decreased. Abdelmoez et al. (2007) found that threonine was sensitive to temperature around 230 ^{o}C during subcritical water hydrolysis.

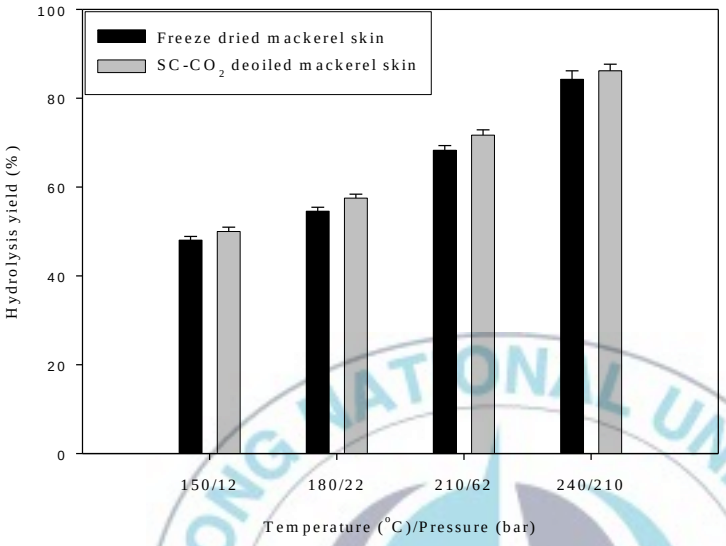


Fig. 4.2. Comparison of hydrolysis yield obtained from freeze dried and SC-CO_2 deoiled mackerel skin by pressurized hydrothermal at different temperature and pressure. Means ± SD (n = 3)

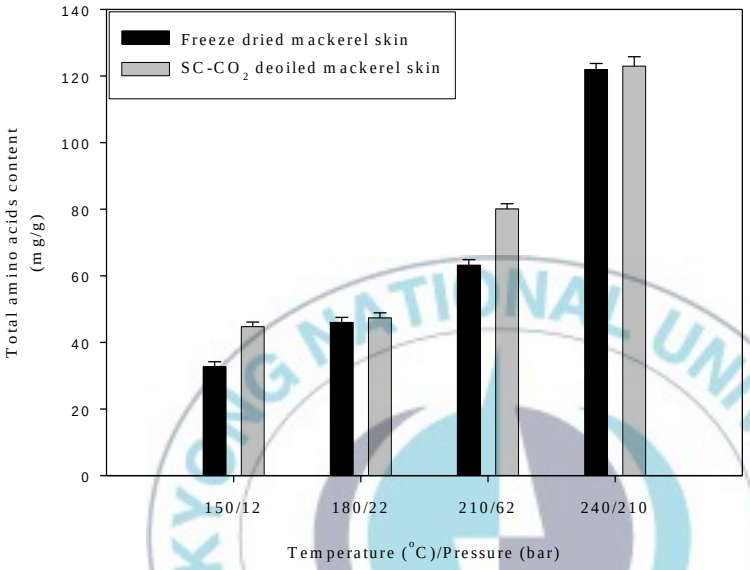


Fig. 4.3. Total amino acids yield from freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates at different temperature and pressure. Means ± SD (n = 3)

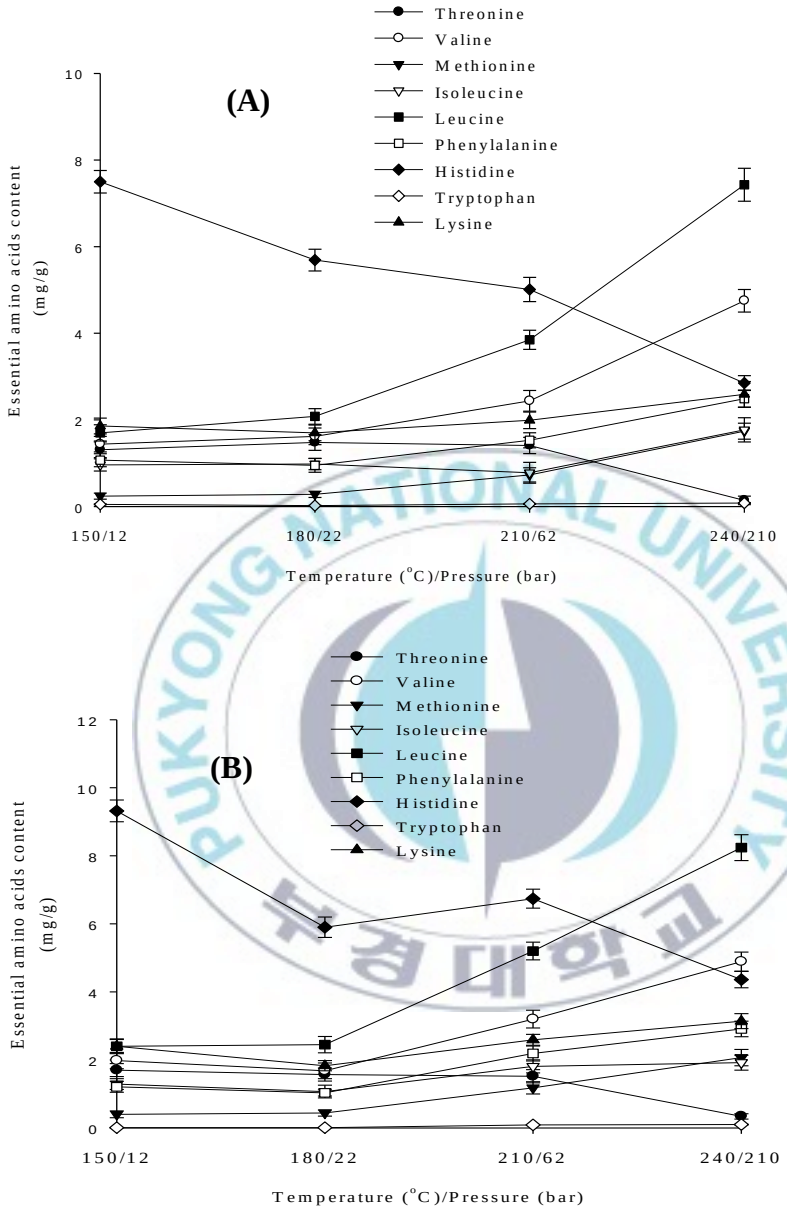


Fig. 4.4. Essential amino acids yield from freeze dried mackerel skin hydrolyzate (A) and SC-CO_2 deoiled mackerel skin hydrolyzate (B) at different temperature and pressure. Means ± SD (n = 3)

4.3.4. Antioxidant activities

All the hydrolyzates were found to differ significantly from each other in their DPPH scavenging ability (Fig. 4.5A). The maximum scavenging activity was found 80.50 ± 1.05% and 80.68 ± 0.82%, respectively at 240 ^{o}C and 210 bar in freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzate. SC-CO_2 deoiled mackerel skin hydrolyzates were little higher DPPH radical scavenging activity compared to freeze dried. Trolox (0.5 mg/mL) which was used as positive control showed higher activity (96.98 ± 0.82%) than hydrolyzates.

$ABTS^{+}$ assay is an excellent tool for determining the antioxidant activity of hydrogen-donating compounds (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavenger of lipid peroxyl radicals) (Binsan et al., 2008). The scavenging activities of the different hydrolyzates for the ABTS radical were measured and compared (Fig. 4.5B). As can be seen, all hydrolyzates were found to change significantly from each other in their $ABTS^{+}$ radical scavenging ability ($P < 0.05$). The hydrolyzate at 240 ^{o}C and 210 bar was higher $ABTS^{+}$ radical scavenging activity in freeze dried and SC-CO_2 extracted mackerel skin hydrolyzate which was 98.58 ± 1.25% and 98.89 ± 1.25%, respectively. By comparison with all hydrolyzates, SC-CO_2 deoiled mackerel skin hydrolyzates were higher ABTS radical scavenging activity than freeze dried. Trolox (0.4 mg/mL) which was used as positive control showed lower activity (87.80 ± 1.10%) than the hydrolyzates at temperature 240 ^{o}C and pressure 210 bar.

The hydroxyl radical scavenging activity of the various hydrolyzates and standard ascorbic acid was investigated (Fig. 4.5C). All hydrolyzates exhibited high scavenging for the hydroxyl radical. Freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates at 240 ^{o}C and 210 bar was found to be the most powerful scavenger of the hydroxyl radical with an inhibition of up to 78.26 ± 0.70% and 84.04 ± 0.76%, respectively which was higher than standard ascorbic acid (0.5 mg/mL) 60.70 ± 0.68%. The weakest scavenger of the hydroxyl radical was found in freeze dried and SC-CO_2 extracted mackerel skin hydrolyzates at 150 ^{o}C and 12 bar which inhibition was 40.64 ± 0.72% and 37.25 ± 0.79%, respectively.

The chelation of Fe^{2+} was used to determine the ability of hydrolyzates in Fe^{2+} chelating. Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agent, the complex formation is disrupted, resulting in the decrease in colour formation (Thiansilakul et al., 2007b). Metal chelating activity in the mackerel skin hydrolyzates are shown in Fig. 4.5D. Freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates at 240 ^{o}C and 210 bar exhibited the highest Fe^{2+} activity which was 54.78 ± 1.42% and 54.83 ± 1.25%, respectively but chelating activity was not found in the hydrolyzates at 150-180 ^{o}C and 12-22 bar.

Reducing power is one symbol of antioxidant ability and may serve as a significant indicator of potential antioxidant activity. Several studies have indicated that the antioxidant effect is related to the development of reductones (Yen and Duh, 1993). In the present study, all hydrolyzate samples showed

higher reducing power (Fig. 4.5E). Hydrolyzate at 240 ^{o}C and 210 bar, reducing power was very high than other hydrolyzates. However, reducing power of SC-CO_2 deoiled all mackerel skin hydrolyzates was higher compared to freeze dried.

Many researchers reported that fish protein hydrolyzate contained antioxidant activity (Klompong et al., 2007; Nalinanon et al., 2011) but our study was focused that mackerel skin hydrolyzate contained a wide range of antioxidant activity. Therefore, we had done antioxidant activity in hydrolyzate by DPPH, ABTS, hydroxyl radical, Fe^{2+} chelating and ferric reducing power assay. Hydrolyzate at 240 ^{o}C and 210 bar showed high antioxidant activity by different assay. During hydrolysis at high temperature and pressure, a wide variety of smaller peptides (data not shown) and free amino acids is generated which were hydrogen and electron donors and could react with radicals to convert them to more stable products, thereby terminating the radical chain reaction. Similar observation was found in peptide and amino acid fraction of skipjack tuna hydrolyzate (Nalinanon et al., 2011). Changes in size, level and composition of free amino acids and small peptides affect the antioxidant activity (Wu et al., 2003).

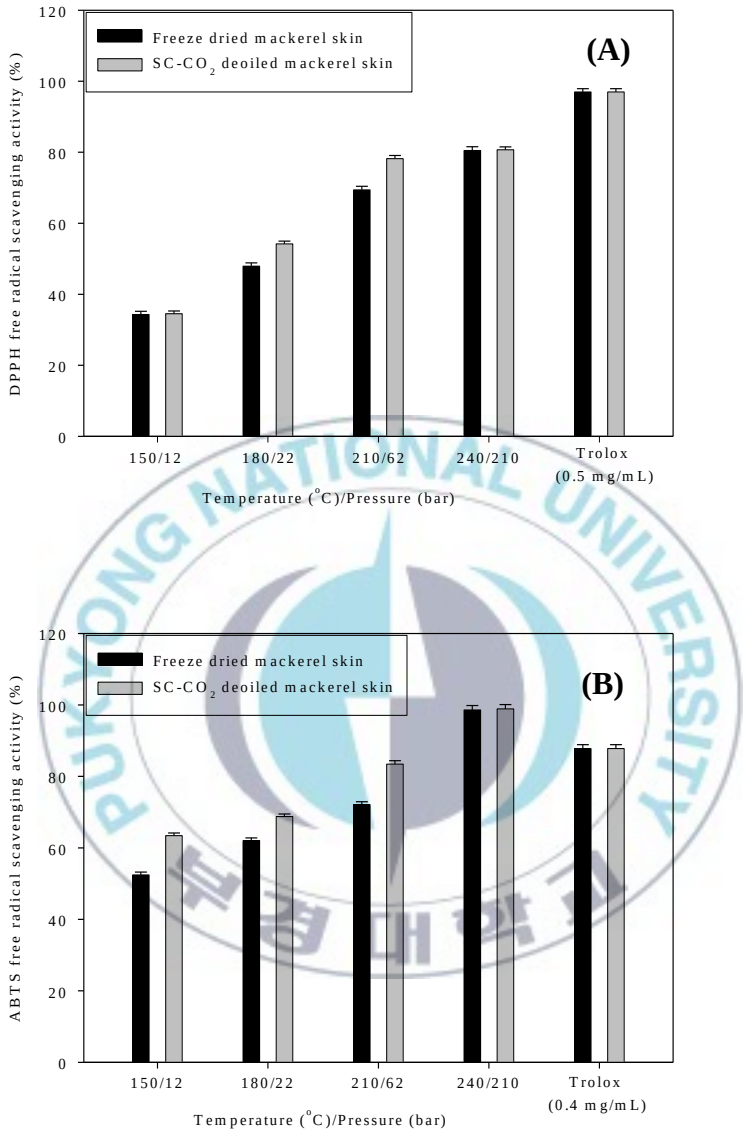


Fig. 4.5A-B. Antioxidant activities measurement from freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates at different temperature and pressure by DPPH free radical scavenging assay (A) and ABTS free radical scavenging assay (B). Means ± SD (n = 3)

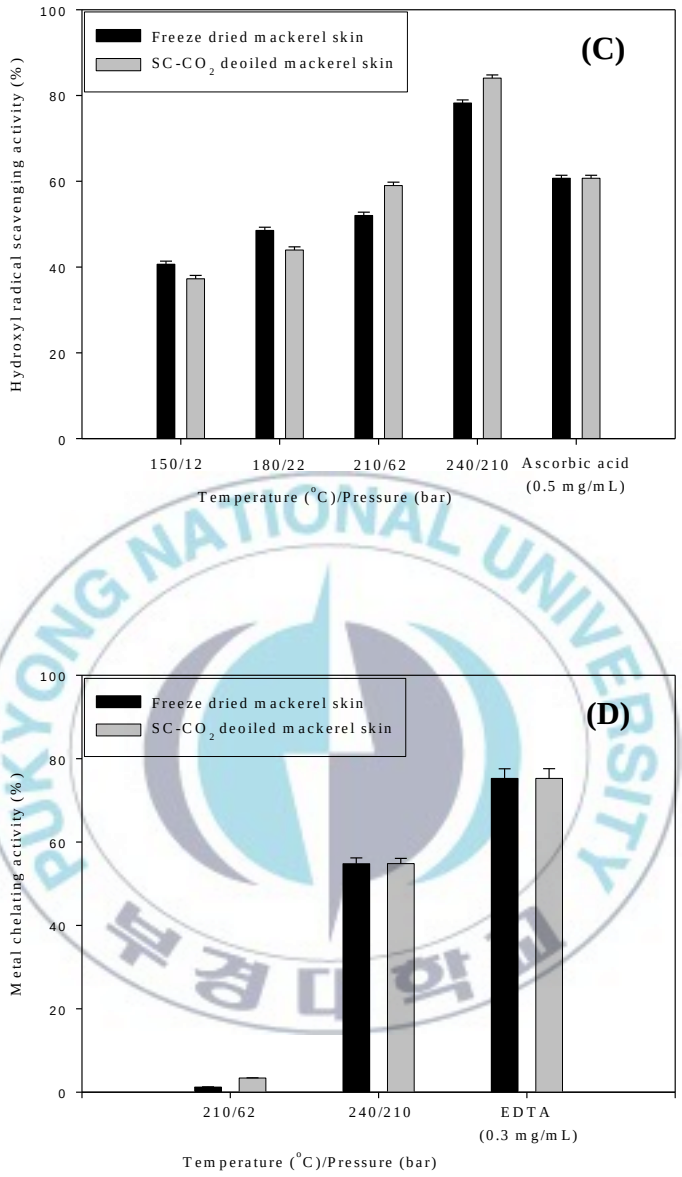


Fig. 4.5C-D. Antioxidant activities measurement from freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates at different temperature and pressure by hydroxyl radical scavenging assay (C) and Fe^{2+} chelating assay (D). Means ± SD (n = 3)

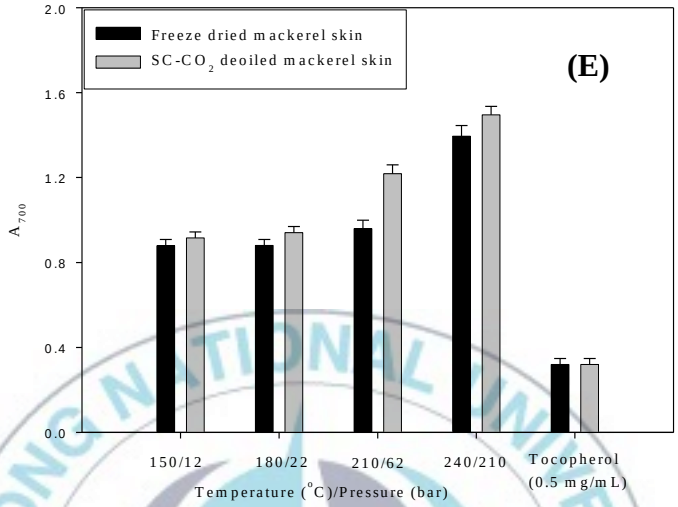


Fig. 4.5E. Antioxidant activities measurement from freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates at different temperature and pressure by ferric reducing power assay (E). Means ± SD (n = 3)

4.3.5. Functional properties

4.3.5.1. Solubility of protein hydrolyzates

The solubilities of hydrolyzates from freeze dried and SC-CO_2 deoiled mackerel skin with different temperature and pressure at various pHs (3.5, 5.5, 7.5 and 9.5) are shown in Table 4.2. All hydrolyzates were soluble over a wide pH range, in which more than 59% solubility was obtained. Generally, freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates at 210-240 ^{o}C and 62-210 bar showed higher solubility at all pH values compared to hydrolyzates at 150-180 ^{o}C and 12-22 bar. The highest solubilities in freeze dried and deoiled extracted mackerel skin hydrolyzates were found at 5.5 and 7.5 pHs. Protein solubility in SC-CO_2 deoiled mackerel skin hydrolyzates at pH 7.5 was higher compared to freeze dried one.

Protein solubility of mackerel skin hydrolyzate was high at 5.5 and 7.5 pH compared to other experimental pHs. Many researchers have been found highest protein solubility in basic and acidic conditions (Nalinanon et al., 2011; Barac et al., 2012). This pH differences for solubility may be due to sample processing. The smaller peptides from proteins have more polar residues which have the ability to form hydrogen bonds with water and augment solubility (Gbogouri et al., 2004). The molecular size and hydrophobicity, as well as polar and ionisable groups of protein hydrolyzates are potentially affected by pressurized hydrothermal hydrolysis. There is another crucial influence on the solubility of protein hydrolyzate is the balance of hydrophilic and hydrophobic forces of peptide (Gbogouri et al., 2004). As a

result, the solubility differences of hydrolyzates with different temperature and pressure might be determined during pressurized hydrothermal process by the size of peptides, the hydrophobic-hydrophilic balance, as well as the charge group of the peptides formed.

4.3.5.2. Emulsifying properties of protein hydrolyzates

Emulsifying activity index (EAI) and emulsion stability index (ESI) of freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates with different temperature and pressure at various concentrations (0.10%, 0.25% and 0.50%) are shown in Table 4.3. Generally, both EAI and ESI of hydrolyzates with different temperatue and pressure varied, particularly when the protein concentration increased. EAI and ESI of freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates at 240 ^{o}C and 210 bar was lowest than other hydrolyzates. However with the same hydrolyzate, EAI and ESI decreased with increasing concentrations.

Emulsifying properties of mackerel skin protein hydrolyzate was varied when applied different temperature and pressure. Mutilangi et al. (1996) reported that higher contents of higher MW peptides contribute to the stability of the emulsion. In contrast, the loss of emulsifying properties occurs by excessive hydrolysis (Klompong et al., 2007; Gbogouri et al., 2004). Protein adsorption at the oil-water interface is diffusion controlled at low protein concentrations and at high protein concentration, the activation energy barrier does not allow protein migration to take place in a diffusion dependent manner,

leading to the accumulation of proteins in the aqueous phase (Lawal, 2004). Thus, proteins or peptides were most likely localized in the aqueous phase and a lower amount of proteins or peptide migrated to the interface (Thiansilakul et al., 2007a). The increase in protein-protein interaction resulted in a lower protein concentration at the interface, in which a thinner film stabilizing the oil droplet is formed. Therefore, emulsifying properties of hydrolyzates were formed by the size of peptides and the concentration used.

4.3.5.3. Foaming properties of protein hydrolyzates

Foaming capacity (FC) and foam stability (FS) of freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates with different temperature and pressure at various concentrations (0.10%, 0.25% and 0.50%) are shown in Table 4.4. FC and FS in freeze dried and SC-CO_2 deoiled mackerel skin hydrolyzates at 150-180 ^{o}C and 12-22 bar was more higher compare to hydrolyzates at 240 ^{o}C and 210 bar. However with the same hydrolyzate, FC was increased with increasing protein concentration and FS was not significantly different after increasing the protein concentration ($P < 0.05$).

At different temperature and pressure, FC and FS of mackerel skin hydrolyzate was different. Protein breakdown was lower when applied at low temperature and pressure compared to high temperature and pressure. High molecular weight peptides are generally positively related to foam stability of protein hydolysates (van der Ven C et al., 2002). Hydrophobicity of unfolded proteins has been shown to correlate with foaming characteristics (Townsend

and Nakai, 1983). Foam formation is governed by three factors, including transportation, penetration and reorganization of molecules at the air-water interface. To exhibit good foaming, a protein must be capable of migrating rapidly to the air-water interface, unfolding and rearranging at the interface (Halling, 1981). Foam stability depends on the structure of the film and reflects the extent of protein-protein interaction within the matrix (Mutilangi et al., 1996). Foam stability is enhanced by flexible protein domains, which enhance viscosity of the aqueous phase, protein concentration and film thickness (Phillips et al., 1994). Therefore, hydrolyzates at different temperature and pressure might be produced different size of peptides as a result showed different foaming properties.

Table 4.2. Protein solubility of mackerel skin hydrolyzates at different temperature and pressure

Temperature (^{o}C)/Pressure (bar)	Protein solubility (%)							
	Freeze dried mackerel skin				SC-CO_2 deoiled mackerel skin			
	pH							
	3.5	5.5	7.5	9.5	3.5	5.5	7.5	9.5
150/12	65.52 ± 1.02aA	86.53 ± 1.12aC	74.33 ± 1.04aB	62.50 ± 1.72aA	83.84 ± 0.88aA	85.94 ± 1.20aA	90.43 ± 1.22aB	74.06 ± 0.88aC
180/22	67.59 ± 0.90aA	81.02 ± 1.52bC	72.93 ± 0.92aB	59.13 ± 1.06aA	83.60 ± 1.20aA	90.72 ± 2.02bB	95.04 ± 1.42bC	86.63 ± 0.92bA
210/62	77.43 ± 1.22bA	97.46 ± 0.78cC	86.19 ± 0.88bB	77.45 ± 2.02bA	87.21 ± 1.42bA	92.41 ± 1.60bB	99.86 ± 0.40cC	96.83 ± 1.05cC
240/210	91.22 ± 1.40cA	96.65 ± 1.10cB	97.81 ± 0.60cB	90.57 ± 1.52cA	91.02 ± 1.00cA	97.92 ± 1.32cB	97.93 ± 1.54cB	90.10 ± 1.62bA

Means ± SD (n = 3). Different small letters in the same column indicate significant differences ($P < 0.05$). Different capital letters in the same row indicate significant differences ($P < 0.05$)

Table 4.3. Emulsifying properties of mackerel skin hydrolyzates at different temperature and pressure

Hydrolyzate sample concentrations (%)	Temperature (^{o}C)/Pressure (bar)	Freeze dried mackerel skin		SC-CO_2 deoiled mackerel skin	
		EAI (m^2/g)	ESI (min)	EAI (m^2/g)	ESI (min)
0.10	150/12	124.05 ± 1.50a	16.03 ± 0.50a	110.54 ± 1.65a	21.69 ± 0.32a
0.25		70.75 ± 1.10b	14.40 ± 0.42b	83.15 ± 1.12b	18.96 ± 0.26b
0.50		67.68 ± 1.15b	11.69 ± 0.35c	59.02 ± 1.02c	18.41 ± 0.28b
0.10	180/22	129.89 ± 1.30a	17.41 ± 0.45a	171.65 ± 1.72a	25.29 ± 0.41a
0.25		91.01 ± 1.18b	17.21 ± 0.42a	118.28 ± 1.55b	24.99 ± 0.32a
0.50		48.27 ± 1.00c	16.52 ± 0.28a	81.99 ± 1.10c	24.97 ± 0.25a
0.10	210/62	80.45 ± 1.40a	15.20 ± 0.60a	99.45 ± 1.26a	15.13 ± 0.50a
0.25		53.43 ± 1.25b	15.16 ± 0.40a	54.54 ± 1.05b	14.19 ± 0.38a
0.50		39.00 ± 1.20c	12.07 ± 0.40b	59.45 ± 1.10b	11.51 ± 0.27b
0.10	240/210	71.81 ± 0.92a	14.44 ± 0.30a	93.04 ± 1.60a	14.64 ± 0.30a
0.25		41.27 ± 1.22b	14.36 ± 0.20a	47.41 ± 1.10b	10.93 ± 0.25b
0.50		28.31 ± 0.70c	13.60 ± 0.22a	28.13 ± 0.60c	10.02 ± 0.20b

Means ± SD (n = 3). Different small letters in the same column at constant temperature and pressure with different hydrolyzate concentration indicate significant differences ($P < 0.05$)

Table 4.4. Foaming properties of mackerel skin hydrolyzates at different temperature and pressure

Hydrolyzate sample concentrations (%)	Temperature (^{o}C)/Pressure (bar)	Freeze dried mackerel skin		SC-CO_2 deoiled mackerel skin	
		Foaming capacity (%)	Foaming stability (%)	Foaming capacity (%)	Foaming stability (%)
0.10	150/12	82.86 ± 2.68a	66.67 ± 2.20a	96.90 ± 2.04a	92.31 ± 2.11a
0.25		97.43 ± 2.84b	73.33 ± 2.43b	123.81 ± 3.30b	84.62 ± 1.80b
0.50		100.00 ± 3.05c	52.38 ± 2.15c	133.33 ± 3.22c	89.29 ± 1.42c
0.10	180/22	91.90 ± 2.10a	69.23 ± 2.32a	95.24 ± 2.60a	85.00 ± 2.15a
0.25		109.52 ± 2.95b	73.91 ± 2.40b	123.81 ± 3.10b	82.08 ± 2.10b
0.50		138.10 ± 3.12c	82.76 ± 2.52c	142.86 ± 3.40c	83.33 ± 2.45b
0.10	210/62	85.71 ± 2.75a	63.33 ± 2.73a	80.95 ± 2.48a	74.35 ± 2.18a
0.25		90.48 ± 2.80b	68.95 ± 2.41b	104.76 ± 2.10b	75.82 ± 2.20a
0.50		95.52 ± 2..65c	73.91 ± 2.44c	128.57 ± 2.92c	78.89 ± 2.52b
0.10	240/210	38.09 ± 1.50a	57.50 ± 2.18a	52.38 ± 1.25a	72.82 ± 2.42a
0.25		47.62 ± 1.58b	50.00 ± 1.50b	61.90 ± 1.30b	77.62 ± 2.12b
0.50		52.38 ± 1.60c	45.45 ± 1.11c	61.90 ± 1.36b	76.92 ± 1.78b

Means ± SD (n = 3). Different small letters in the same column at constant temperature and pressure with different hydrolyzate concentration indicate significant differences ($P < 0.05$)

4.4. Conclusions

The result presented in this study demonstrated that pressurized hydrothermal hydrolysis technique has great potential for practical application. It was shown not only to be energy efficient, environmentally friendly, and cost effective but also to produce functional material, especially amino acids with zero emission from mackerel skin. The protein hydrolyzates produced from the freeze dried and SC-CO_2 deoiled mackerel skin exerted good antioxidant activities and functional properties and varied with the temperature and pressure applied. Therefore, we conclude that mackerel skin protein hydrolyzates can be incorporated as a multifunctional ingredient into foods.

Chapter 5

Characterization of pepsin-solubilized collagen recovered from mackerel (*Scomber japonicus*) bone and skin using subcritical water hydrolysis

5.1. Introduction

Mackerel (*Scomber japonicus*) is an important food source that is consumed worldwide. The Korean population consumes a large amount of mackerel daily as sashimi, or in fried and boiled foods. The production of these foods requires the removal of fish by-products, such as bone, skin and viscera. During this processing, a large amount of by-products are generated from the original raw materials (Kittiphattanabawon et al., 2005) in fishmongers and processing factories. Improper disposal of these by-products might cause serious environmental pollution, due in part to the resulting offensive odour. However, by-product is of high nutritional value, and so the proper utilization of this by-product can both prevent environment pollution and be commercially beneficial.

Collagen is a vertebrate structural protein that it is generally found in bone, skin and other connective tissues (Balian and Bowes, 1977). Traditionally, collagen has been isolated from bones and skins of land-based animals such as cows, bovine and pigs. It is widely used in food, cosmetic, biomedical and pharmaceutical industries due to its excellent biocompatibility,

biodegradability and weak antigenicity (Liu et al., 2012). In recent years, an increasing number of consumers have been concerned regarding the safety of collagen produced from land-based animals due to outbreaks of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), foot-and-mouth disease (FMD) and avian influenza (AI) (Jongjareonrak et al., 2005). In addition, collagen obtained from pigs cannot be used as a component of certain foods for religious reasons. Therefore, there is a need to develop alternative collagen sources. Because collagen produced from fish by-products such as the bones and skin is likely not to be associated with BSE, TSE, FMD and AI, and is not subject to religious restrictions, it has received increasing attention as an alternative source of collagen (Gomez-Guillen et al., 2002).

Obtaining effective functional materials requires the breakdown of macromolecules. Subcritical water hydrolysis (SWH) is a clean and rapid method of hydrolysing macromolecules that is an alternative to acid, base and enzymatic hydrolysis methods. It has the advantages of a shorter reaction time, no use of toxic solvents and the reduced formation of degradation products (Zhao et al., 2011). In contrast, acid- and alkali-based hydrolysis requires extreme reaction conditions that can cause significant environmental pollution. Although enzymatic hydrolysis is specific for the macromolecule being broken down, it can involve long production cycles.

Now a day consumers are increasingly aware of the benefits of nutritionally enhanced foods. Dietary supplements containing collagen

hydrolyzates are considered to have the potential to improve tendon or joint regeneration and reduce activity-related joint pain (Wu et al., 2004). Collagen hydrolyzates have the nutraceutical and/or functional properties that are necessary to create novel, innovative and healthy food products. Low-molecular-weight peptides are easier for the body to absorb and utilise than whole proteins. In addition, high-molecular-weight collagen mixed with cosmetic products cannot penetrate deeply into human skin, and so production of low-molecular-weight collagen peptides (<3 kDa) is of great importance to the cosmetic industry (Collagen diagram-reborn, website). Collagen hydrolyzates are also a good source of amino acids for individuals suffering from anorexia and anaemia, and for vegetarians. Glycine and proline which perform an important function in the formation of connective tissue are highly enriched in collagen hydrolyzates (PB Gelatin, website). In addition, it was recently reported that smaller peptides and amino acids in protein hydrolyzates exhibit antioxidant activities (Zhao et al., 2011).

Recently, collagens from several fish species have been isolated and characterized (Jongjareonrak et al., 2005; Liu et al., 2012; Zhang et al., 2014; Zhang et al., 2007). Interestingly, collagen produced from diverse fish species, habitats and tissues exhibited different properties significantly. Therefore, the aim of this study was to isolate collagen from bones and skin of mackerel and to characterize their potential in commercial applications to make more effective use of the by-products generated during fish processing.

5.2. Materials and Methods

5.2.1. Materials

Freeze dried mackerel bone and skin were used for collagen isolation. Pepsin, protein marker, standard collagen from calf skin and bovine achilles tendon were purchased from Sigma-Aldrich. All other reagents and solvents used in this study were of analytical or HPLC grade.

5.2.2. Proximate analysis of mackerel bone and skin

The moisture, ash, crude lipid and protein contents of mackerel bone and skin were determined according to the AOAC method (AOAC, 1990).

5.2.3. Isolation of collagen from mackerel bone and skin

Collagen was isolated from mackerel bone and skin according to the methods described by Liu et al. (2012), Matmaroh et al. (2011) and Ogawa et al. (2004) with slight modifications. All experimental procedures were performed at 4 ℃.

To remove non-collagenous substances, crushed bones were treated with 0.1 M NaOH at a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred continuously for 24 h using a magnetic stirrer at 250 rpm; the alkaline solution was changed every 6 h. Next, samples were washed with cold distilled water until the washing water reached a neutral pH, and then lyophilized (EYELA FDV-2100, Rikakikai Co. Ltd., Tokyo, Japan). Subsequently, the insoluble bone was decalcified with 0.5 M

ethylenediaminetetraacetic acid (EDTA, pH 7.5) at a sample/EDTA solution ratio of 1:10 (w/v) for 4 days; the EDTA solution was changed daily. The residue was then washed with cold distilled water, and fat was removed with 10% (v/v) butyl alcohol at a sample/solvent ratio of 1:10 (w/v) for 24 h. The residue was then washed again using cold distilled water. Subsequently, the remaining residue was extracted using 0.57 M acetic acid containing 0.1% (w/v) pepsin for 3 days at a sample/solution ratio of 1:10 (w/v) followed by centrifugation at 12,000 rpm for 50 min. The residue was then re-extracted with the same solution for 3 days, and centrifuged under identical conditions. Each viscous solution was mixed and salting out by adding NaCl to a final concentration of 2.0 M. The solutions were incubated for 24 h, and precipitation was assessed. The precipitates were harvested by centrifugation at 12,000 rpm for 30 min, and then dissolved in 0.57 M acetic acid. The solutions were dialyzed against 0.1 M acetic acid and distilled water in a dialysis bag for 2 days, and then lyophilized.

The crushed mackerel skin was treated with 0.1 M NaOH for 24 h at a sample/alkaline solution ratio of 1:35 (w/v), and mixed using a magnetic stirrer. The solution was changed four times in 1 day, and then lyophilized after washing with cold distilled water. Next, the lyophilized samples were de-fatted with 10% (w/v) butyl alcohol for 24 h at the above ratio. The defatted residues were washed with cold distilled water, and then mixed with 0.57 M acetic acid containing 0.1% (w/v) pepsin for 3 days at a sample/solution ratio of 1:35 (w/v). Further procedures were carried out according to those

described above for collagen isolation from mackerel bone.

5.2.4. Characterization of PSCs

5.2.4.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analyzed by SDS-PAGE according to the method of Laemmli (1970) using a 3.0% stacking gel and a 5.0% resolving gel. PSC samples (2 mg) were dissolved in 1.0 mL of 0.02 M sodium phosphate buffer (pH 7.2), and then mixed with sample buffer (1 M Tris-HCl, pH 6.8, containing 10% SDS, 25% glycerol, 2% bromophenol blue and 5% 2-mercaptoethanol) at a 1:1 ratio (v/v). Then, 20 µL of each sample (20 µg protein) were loaded onto the polyacrylamide gels, and next procedure has been discussed in chapter 2. Collagens from calf skin and bovine achilles tendons were isolated according to the above procedures and used as standards for comparison with the collagen harvested from mackerel bone and skin.

5.2.4.2. Fourier-transform infrared (FT-IR) spectra analysis

The lyophilzed PSCs from mackerel bone and skin FT-IR spectra were obtained using a Perkin Elmer (USA), Spectrum X. Spectra were measured over the frequency range 4000–650 cm^{-1}, with a resolution of 4 cm^{-1}.

5.2.4.3. Measurement of viscosity

A Brookfield DVII + Pro viscometer (Brookfield Engineering Laboratories,

Inc., Middleboro, MA 02346 USA) was used to measure the viscosity of PSCs from mackerel bone and skin according to the method described by Ogawa et al. (2004) with slight modifications. 8 mL of 0.1% (w/v) PSC in 0.1 M acetic acid was incubated at 10 ℃ for 20 min, and then placed in a vessel. Spindle SC4-18 with agitation at 150 rpm was then used to measure viscosity, which was expressed as centipose (cP).

5.2.4.4. Denaturation temperature (Td) determination

The denaturation temperature (Td) was measured according to the method of Nagai et al. (2008) with a little modification. 8 mL of 0.1% (w/v) PSC in 0.1 M acetic acid was heated from 10–40 ℃ at intervals of 3 ℃. The solution was held at the designated temperature for 20 min prior to the measurement of viscosity. The thermal denaturation curve of the PSC was determined by plotting the fractional change in viscosity against temperature. Fractional viscosity was calculated for each temperature as follows: fractional viscosity = (measured viscosity/maximum viscosity). The Td was determined to be the temperature at which fractional viscosity was close to 0.5.

5.2.4.5. Collagen hydrolyzate production using subcritical water hydrolysis (SWH)

PSCs (0.5 g) and water at a ratio of 1:200 (w/v) were placed in the reactor, and the cover was closed. The reactor was heated using an electric heater at the desired temperature (200–250 ℃) and pressure (30–70 bar). Heating time

was 36 to 54 min to reach the desired temperature. Next procedure has been discussed in chapter 4.

5.2.4.6. Peptide molecular weight analysis

1 µL of PSC hydrolysate samples from mackerel bone and skin was spotted onto a polished steel 384 target plate, mixed with 1 µL of 2,5-dihydroxybenzoic acid (DHB) matrix solution containing 1% THF and dried. Each sample was analyzed using matrix-assisted laser desorption ionisation time-of-flight mass spectrum (MALDI-TOF MS) on an Ultraflex III mass spectrometer (Bruker Daltonics, Germany) equipped with a 337 nm pulse nitrogen laser. Measurements in the m/z range of 700–6000 were acquired in the positive ionisation and reflection modes by accumulating data from 200 laser shots.

5.2.4.7. Analysis of amino acid composition

We have discussed this method in chapter 4.

5.2.4.8. Antioxidant activities

5.2.4.8.1. DPPH free radical scavenging assay

The scavenging effects of PSC hydrolyzates from mackerel bone and skin were determined by the method of Yen and Chen (1995) with slight modifications. Briefly, 3.95 mL of 0.1 mM DPPH solution in methanol were added to test tubes containing 0.1 mL of PSC samples at various concentrations before and after hydrolysis. Next step has been discussed in

chapter 3. The samples of blank and control (various concentrations of trolox in methanol) were analyzed as described above.

5.2.4.8.2. $ABTS^{+}$ free radical scavenging assay

$ABTS^{+}$ free radical scavenging assays were performed as described by Zheleva-Dimitrova et al. (2010) with a slight modification. 3.95 mL of diluted $ABTS^{+}$ was added to 0.05 mL of PSC samples at different concentrations before and after hydrolysis. Next procedure has been discussed in chapter 3. The sample blank and control (various concentrations of trolox in 80% methanol) were analyzed as described above.

5.2.4.8.3. Ferric reducing power assay

The Fe^{3+} reducing power of PSC samples at various concentrations were determined by the method of Oyaiza (1986) with a slight modification which has been discussed in chapter 4.

5.2.4.8.4. Fe^{2+} chelating assay

The ability of samples to chelate Fe^{2+} was determined using the method of Decker and Welch (1990) with modifications. PSC samples (0.1 mL) at various concentrations before and after hydrolysis were mixed with 3.0 mL of distilled water; followed step has been discussed in chapter 4. Various concentrations of EDTA were used as control samples. Chelating activity (%) was calculated as follows:

$$\text{Chelating activity (\%)} = \left(1 - \frac{A_{562} \text{ of sample}}{A_{562} \text{ of control}}\right) \times 100$$

5.3. Results and Discussion

5.3.1. Proximate composition of mackerel bone, skin and PSCs

Protein was the major component of freeze-dried mackerel bone and skin, accounting for 47.25 ± 1.25 and 49.49 ± 1.52% of the content, respectively; they also contained moisture (6.68 ± 0.07 and 4.60 ± 0.06%), ash (6.89 ± 0.06 and 4.50 ± 0.05%), lipids (17.59 ± 1.10 and 29.47 ± 1.26%) and non-protein substances (21.59 ± 1.10 and 11.94 ± 0.76%) shown in Table 5.1. When mackerel bone and skin were compared directly, the protein and lipid content was higher in the skin, whereas the moisture, ash and non-protein contents were higher in bone. The PSC yields from mackerel bone and skin were 1.75 ± 0.07 and 8.10 ± 0.12%, respectively, as shown in Table 5.2. Generally, pepsin can cleave intermolecular cross-links at the telopeptide region, leading to increased solubility (Balian and Bowes, 1977). Therefore, pepsin was used during collagen isolation. The yield of collagen (wet-weight) from fish bone was 1.6% from bigeye snapper and 1.3% from bighead carp (Kittiphattanabawon et al., 2005; Liu et al., 2012). Conversely, the wet-weight yield from fish skin was 7.6% from unicorn leatherjacket, 7.1% from bigeye snapper (*Priacanthus macracanthus*) and 7.7% from striped catfish (Ahmad and Benjakul, 2010; Benjakul et al., 2010; Singh et al., 2011). The differences in PSC yield could be attributed to the differences in fish species, biological

conditions and preparative methods. These observations are consistent with Liu et al. (2012). The protein contents of PSCs isolated from mackerel bone and skin were comparable, at 90.05 ± 2.34 and 86.89 ± 2.48%, respectively. In addition, PSCs from mackerel bone and skin contained similar moisture (6.28 ± 0.12 and 7.48 ± 0.10%) and ash (3.48 ± 0.09 and 5.37 ± 0.06%) contents, and a lower fat content (0.19 ± 0.02 and 0.26 ± 0.03%, respectively), as shown in Table 5.2. Similar results were reported for collagen isolated from blacktip shark (Kittiphattanabawon et al., 2010).

Table 5.1. Proximate composition of freeze dried bone and skin of mackerel

Sample name	Moisture (%)	Ash (%)	Crude lipid (%)	Crude protein (%)	Non-protein (%)
Bone	6.68 ± 0.07	6.89 ± 0.06	17.59 ± 1.10	47.25 ± 1.25	21.59 ± 1.28
Skin	4.60 ± 0.06	4.50 ± 0.05	29.47 ± 1.26	49.49 ± 1.52	11.94 ± 0.76

Means ± SD (n = 3)

Table 5.2. Yield and proximate composition of collagen from bone and skin of mackerel

	Collagen	
	Bone	Skin
Yield (% dry weight)	1.75 ± 0.07	8.10 ± 0.12
Protein (% dry weight)	90.05 ± 2.34	86.89 ± 2.48
Moisture (% dry weight)	6.28 ± 0.12	7.48 ± 0.10
Ash (% dry weight)	3.48 ± 0.09	5.37 ± 0.06
Lipid (% dry weight)	0.19 ± 0.02	0.26 ± 0.03

Means ± SD (n = 3)

5.3.2. Analysis of PSCs by SDS-PAGE

The subunit composition of PSCs from mackerel bone and skin are shown in Fig. 5.1. Both PSC mainly contained two α-chains (α_1 and α_2), similar to many other fish species (Liu et al., 2012; Wang et al., 2007; Zhang et al., 2014). The approximate MWs of both PSCs were identical: 116 kDa for α_2 and 126 kDa for α_1, similar to the standard collagen isolated from calfskin and bovine achilles tendons. Electrophoresis mobility and subunit composition analyses suggested that the PSCs from mackerel bone and skin were mainly composed of type-I collagen, a heterotrimer containing two identical α_1-chains and one α_2-chain in the molecular form of $[\alpha_1(I)]_2\alpha_2(I)$. This is consistent with previous reports of skin collagen from deep-sea redfish (Wang et al., 2007), skin, scale and bone collagen from carp (Duan et al., 2007) and fins, scales, skins, bones and swim bladders collagen from bighead carp (Liu et al., 2012). In contrast, Saito et al. (2001) reported that collagen from the skin and muscles of rainbow trout contained three non-identical α-chains, $\alpha_1(I)\alpha_2(I)\alpha_3(I)$, whereas Zhang et al. (2014) reported that collagen from jellyfish mainly consisted of homotrimeric α-chains, $[\alpha_1(I)]_3$. The molecular form of collagen thus likely differs among species. Moreover, PSCs from mackerel bone and skin contained one β-chain with a MW of ~205 kDa.

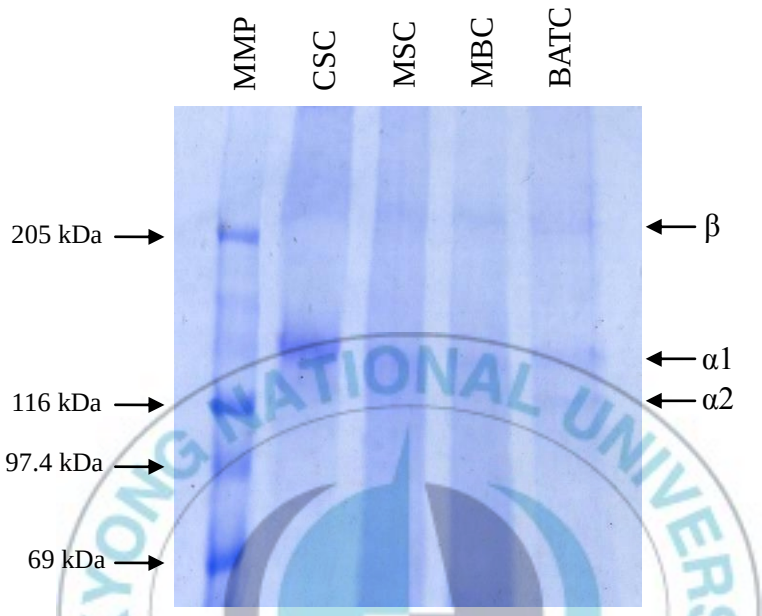


Fig. 5.1. SDS-PAGE pattern of collagens. MMP-molecular weight marker protein, CSC-calf skin collagen, MSC-mackerel skin collagen, MBC-mackerel bone collagen and BATC-bovine achilles tendon collagen

5.3.3. FT-IR spectra of PSC from mackerel bone and skin

The FT-IR spectra of PSCs from mackerel bone and skin are shown in Fig. 5.2. The amide A bands of PSCs from mackerel bone and skin were detected at 3283 and 3285 cm^{-1}, respectively, suggesting the presence of N-H stretching vibration and hydrogen bonds. Doyle et al. (1975) reported that a free N-H stretching vibration occurs commonly in the range 3400–3440 cm^{-1}, and that this position is shifted to a lower wavenumber when the NH group of a peptide forms a hydrogen bond. The amide B band of both PSC collagens was observed at 2922 cm^{-1}, which is related to the asymmetrical stretch of CH_2 (Muyonga et al., 2004; Nagai et al., 2008).

Heu et al. (2010) reported that the wavenumbers of the amide I, amide II and amide III bands were directly associated with the configuration of the collagen. The amide I band (1600–1660 cm^{-1}) is associated with the stretching vibrations of carbonyl groups (C=O) in peptides, and is the most important factor when investigating the secondary structure of a polypeptide (Muyonga et al., 2004). Amide II (~1550 cm^{-1}) is associated with N-H bending and C-N stretching vibrations, and a shift to lower wavenumbers suggests the existence of hydrogen bonds (Ahmad and Benjakul, 2010). Amide III (1220–1320 cm^{-1}) is related to C-N stretching vibration and N-H deformation, and an absorption ratio of 1 between the amide III and 1453–1455 cm^{-1} bands indicates an intact triple helical structure (Ahmad and Benjakul, 2010). PSCs from mackerel bone and skin exhibited amide bands at wavenumbers of 1650 and 1651 cm^{-1} for amide I, 1537 and 1548 cm^{-1} for amide II and 1237 and 1238 cm^{-1} for

amide III, respectively. Ratios of 0.98 and 0.99 were obtained for the PSCs from mackerel bone and skin, respectively, suggesting that the triple-helical structure was not affected by pepsin digestion during collagen isolation.

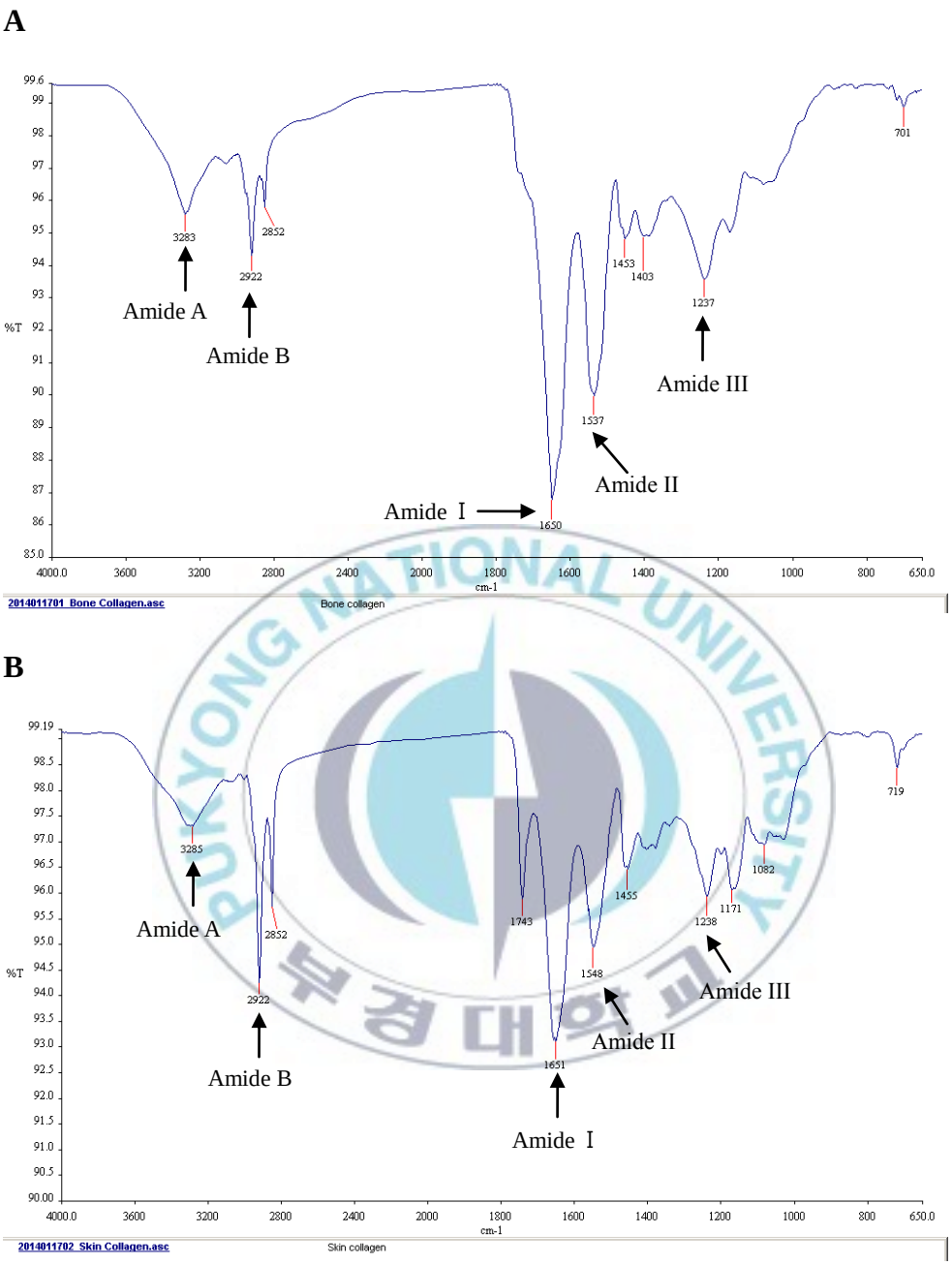


Fig. 5.2. FT-IR spectra of collagen from (A) bone and (B) skin of mackerel

5.3.4. PSC viscosity and its thermal behaviour

High viscosity is an important characteristic of collagen. The viscosities of PSCs from mackerel bone and skin were 18.34 ± 0.25 and 20.26 ± 0.21 cP, respectively shown in Table 5.3. However, the viscosity of PSC from mackerel skin was slightly higher than that from bone, possibly due to the high proportion of collagen polymers, which results in a higher mean molecular weight.

Fig. 5.3 shows the transition curve of the denaturation temperatures (Td) of PSCs from mackerel bone and skin. The Td of the PSCs was determined from the viscosity changes upon heating. The viscosity of both PSCs decreased as temperature increased from 10 ℃~40 ℃. The Tds of PSCs from mackerel bone and skin were 27 ℃ and 30 ℃, respectively shown in Table 5.3. This is higher than those reported for arabesque greenling (15.4 ℃) and deep sea redfish (16.1 ℃) (Nalinanon et al., 2010; Wang et al., 2007), but lower than those for bigeye snapper (*P. tayenus*; 31.3 ℃), blacktip shark (34.37 ℃) and striped catfish (39.6 ℃) (Kittiphattanabawon et al., 2005; Kittiphattanabawon et al., 2010; Singh et al., 2011). The Td values of collagen from various sources correlated with the imino acid content, the temperature of the normal habitat, the season, and age (Matmaroh et al., 2011; Singh et al., 2011).

Table 5.3. Viscosity and denaturation temperature of collagen from bone and skin of mackerel

	Collagen	
	Bone	Skin
Viscosity (cP)	18.34 ± 0.3	20.26 ± 0.2
Denaturation temperature (^{o}C)	27	30

Means ± SD (n = 3)

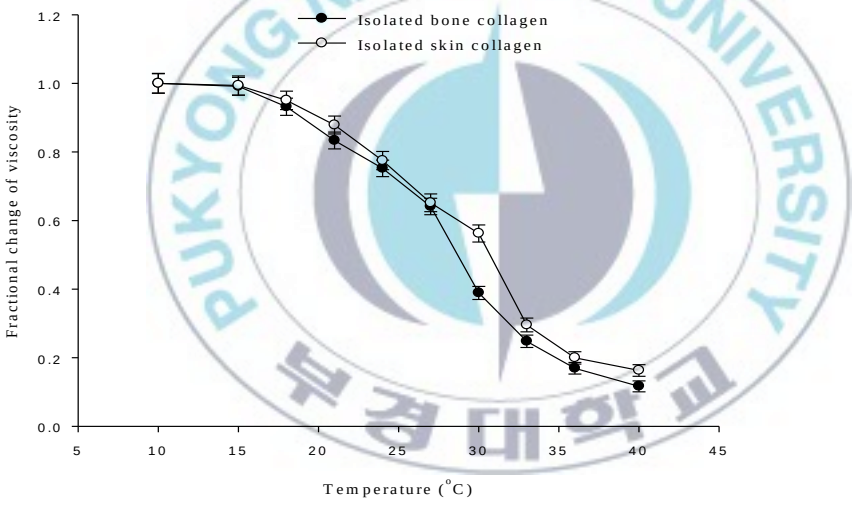


Fig. 5.3. Thermal denaturation curve of collagen from the bone and skin of mackerel. Means ± SD (n = 3)

5.3.5. Hydrolysis PSC yield

PSCs from mackerel bone and skin were hydrolyzed completely at temperatures of 200–250 ℃ and pressures of 30–70 bar.

5.3.6. Peptide MW and the predicted peptide sequence of PSC hydrolyzates

Table 5.4 shows molecular weight and peptide content of each PSC from the bone and skin of mackerel at temperatures of 200–250 ℃ and pressures of 30–70 bar. MALDI-TOF mass spectra data are shown in Fig. 5.4. A MW range of 759.12–988.20 Da was obtained from the PSC hydrolyzate from mackerel bone at a temperature 200 ℃ and pressure of 30 bar. The peptide intensity (3335.74) was particularly high at a MW of 984.21 Da. No peptide peak was identified in mackerel bone PSC hydrolyzates treated at 250 ℃ and 70 bar within the selected MALDI-TOF MS m/z data range (700–6000 Da). Treatment of PSC hydrolyzates at 250 ℃ and 70 bar likely results in generation of lower-MW peptides (<700 Da) and an increased amount of organic compounds. Nine predicted peptides were identified from PSC hydrolyzates at 200 ℃ and 30 bar; these had the following amino acid sequences: KTKATLARM, KRMDLARI, KVTFNRKQ, KATLARMARG, RMARGAMVRF, RFVFIYQH, MKIIIAPAKK, MADAELEAIRQ and KTLWHCSDKL, as determined using the Mascot database.

PSC hydrolyzates from mackerel skin obtained using temperatures of 200–250 ℃ and pressures of 30–70 bar yielded MW ranges of 789.36–1632.92 and

952.30–1368.13 Da. The highest peptide intensities were 2173.88 for 1216.11 Da at 200 ℃ and 30 bar, and 5328.47 for 952.30 Da at 250 ℃ and 70 bar. Smaller-MW products with a higher intensity were produced due to the application of high temperature and pressure. The amino acid sequences of the predicted peptides from mackerel skin PSC hydrolyzates at 200 ℃ and 30 bar were RSDGSRIRF, MIQMQTKLK, KDAAADKAEDVKD, KEGLGKLTGNEKL, RRYANIGDVIKY, KQLDTLGNDKGRL, KDAVEDKVEDAKE, KKGDVYDAVVVRT and RSAQFMKIVSLAPEVL, compared with RHAEVVASIKA, RSVDPGSPAARS, MKTAQELRV and K.NLLTGSASESVYK.A at 250 ℃ and 70 bar. Based on the peptide MW, the amino acid sequences and composition of PSC hydrolyzates from mackerel skin and bone were different according to the applied temperature and pressure. Kittiphattanabawon et al. (2010) and Liu et al. (2012) reported previously that the MW of collagen was reduced to maximums of 48.8 and 20 kDa using V8-protease from blacktip shark and bighead carp; these values were greater than those of PSC hydrolyzates from mackerel bone and skin. This suggests that SWH enables the production of lower-MW peptides.

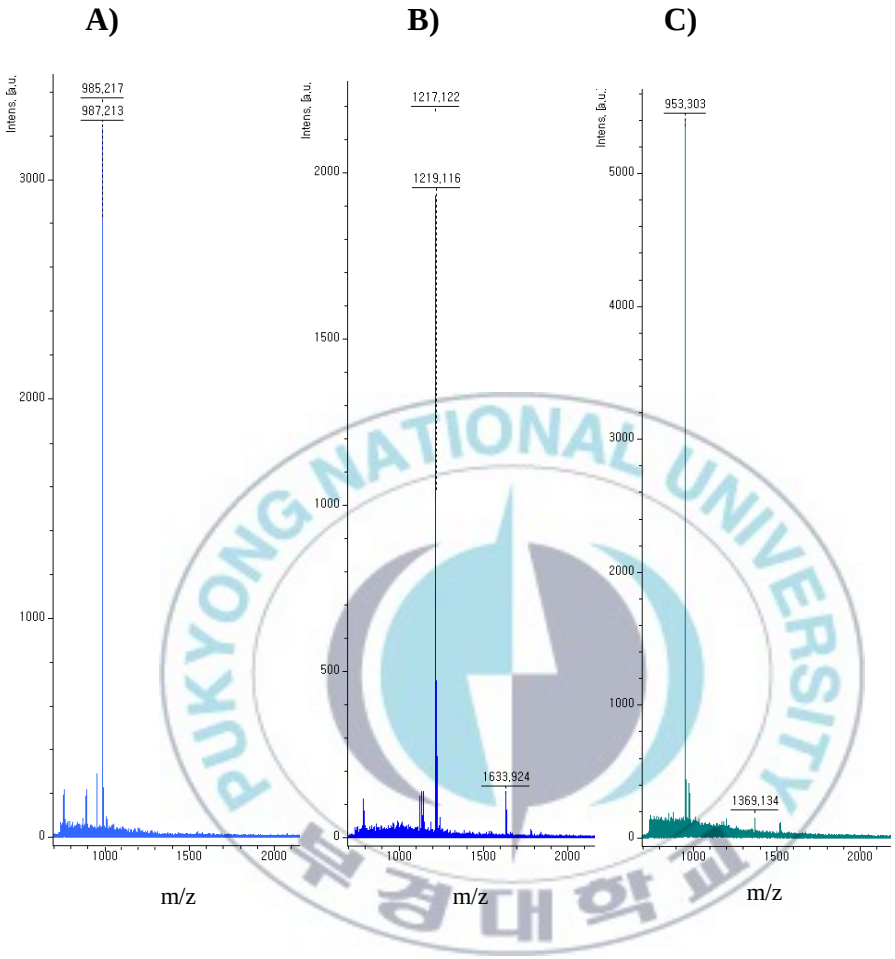


Fig. 5.4. Mass spectra chromatogram of (A) bone collagen hydrolyzate (200 ^{o}C, 30 bar), (B) skin collagen hydrolyzate (200 ^{o}C, 30 bar) and (C) skin collagen hydrolyzate (250 ^{o}C, 70 bar)

Table 5.4A-C. Molecular weight and peptide identification of PSC hydrolyzate from the bone and skin of mackerel by MALDI-TOF mass spectra data

(A) PSC hydrolyzate (200 ^{o}C, 30 bar) from the bone of mackerel

m/z	Molecular weight (dalton)	Predicted peptide*	Intensity
760.13	759	KTKATLARM	144.83
762.12	761	KRMDLARI	212.60
764.14	763	KVTFNRKQ	129.75
889.63	888	KATLARMARG	181.04
891.62	890	RMARGAMVRF	178.46
953.29	952	RFVFIYQH	282.55
985.22	984	MKIIIAPAKK	3335.74
987.21	986	MADAELEAIRQ	2814.04
989.21	988	KTLWHCSDKL	987.93

(B) PSC hydrolyzate (200 ^{o}C, 30 bar) from the skin of mackerel

m/z	Molecular weight (dalton)	Predicted peptide*	Intensity
790.36	789	RSDGSRIRF	114.43
1121.27	1120	MIQMQTKLK	91.13
1133.11	1132	KDAAADKAEDVKD	150.02
1146.10	1145	KEGLGKLTGNEKL	138.77
1148.09	1147	RRYANIGDVIKY	70.98
1217.12	1216	KQLDTLGNDKGRL	2173.88
1219.12	1218	KDAVEDKVEDAKE	1036.45
1221.11	1220	KKGDVYDAVVVRT	169.52
1633.92	1632	RSAQFMKIVSLAPEVL	120.54

Abbreviations: A-alanine, C-cysteine, D-aspartate, E-glutamate, F-phenylalanine, G-glycine, H-histidine, I-Isoleucine, K-lysine, L-leucine, M-methionine, N-asparagine, P- Proline, Q-glutamine, R-arginine, S-serine, T-threonine, V-valine, W-tryptophan, Y-tyrosine. *Predicted peptide identified from mascot database (http://www.matrixscience.com)

Table 5.4A-C. Molecular weight and peptide identification of PSC hydrolyzate from the bone and skin of mackerel by MALDI-TOF mass spectra data

(C) PSC hydrolyzate (250 ^{o}C, 70 bar) from the skin of mackerel

m/z	Molecular weight (dalton)	Predicted peptide*	Intensity
953.30	952	RHAEVVASIKA	5328.47
955.30	954	RSVDPGSPAARS	1027.45
976.27	975	MKTAQELRV	398.92
1369.13	1368	KNLLTGSASESVYKA	163.41

Abbreviations: A-alanine, C-cysteine, D-aspartate, E-glutamate, F-phenylalanine, G-glycine, H-histidine, I-Isoleucine, K-lysine, L-leucine, M-methionine, N-asparagine, P- Proline, Q-glutamine, R-arginine, S-serine, T-threonine, V-valine, W-tryptophan, Y-tyrosine. *Predicted peptide identified from mascot database (http://www.matrixscience.com)

5.3.7. Amino acid composition of PSC hydrolyzates

The amino acid composition of collagen from mackerel bone and skin, expressed as grams of amino acid per 1,000 g collagen, is shown in Table 5.5. The concentrations of amino acids differed mackerel bone and skin. In addition, the used temperature and pressure affected the concentration of amino acids recovered from mackerel bone. Among the amino acids identified in both PSC hydrolyzates at 200–250 ℃ and 30–70 bar, glycine was the major component, forming 27.90–29.44% of the recovered amino acids from mackerel bone PSCs and 32.38–35.25% of those from mackerel skin. The glycine content among the recovered amino acids was 30.8% from jellyfish (Zhang et al., 2014), 35.7% from deep-sea redfish (Wang et al., 2007), 33.2–34.1% from bighead carp (Liu et al., 2012) and 32.30% from blacktip shark (Kittiphattanabawon et al., 2010), which are comparable with the values reported here for mackerel bone and skin.

The total imino acid (proline and hydroxyproline) contents of both PSC hydrolyzates from mackerel bone and skin at 200–250 ℃ and 30–70 bar were 13.11–14.07% and 17.41–17.46%, respectively, which are comparable with those reported for other cold water fish species (16–18%) (Jongjareonrak et al., 2005; Muyonga et al., 2004; Wang et al., 2007). Ikoma et al. (2003) reported that a lower imino acid content and denaturation temperature directly correlated with the thermal stability of proteins. Therefore, the thermal stability of PSC from mackerel skin was likely higher than that from bone.

Mackerel bone and skin PSC hydrolyzates at 200 ℃ and 30 bar were both

rich in alanine, valine, phenylalanine, leucine, isoleucine, tyrosine and histidine. When temperature and pressure were increased, the content of most recovered amino acids slightly decreased. Cheng et al. (2008) reported that the maximum yield of most amino acids was achieved at temperatures of 180–220 ℃. Arginine and serine were not detected in either PSC hydrolyzate at 250 ℃ and 70 bar; this is consistent with both the thermal instability of several amino acids and the increased production of organic compounds in both hydrolyzates at elevated temperatures. Abdelmoez et al. (2007) reported that arginine was sensitive to temperatures of ~230 ℃ during SWH. Most essential amino acids except threonine and tryptophan were detected in PSC hydrolyzates from mackerel bone and skin. Other studies reported that cysteine and tryptophan were not detected in fish collagen hydrolyzates (Muyonga et al., 2004; Wang et al., 2007; Zhang et al., 2014). These differences might be due to use of diverse fish species and various hydrolyzate production processes.

Table 5.5. Amino acid composition of different PSC hydrolyzate from the bone and skin of mackerel (g/1,000 g)

Amino acid composition	Bone collagen hydrolyzate		Skin collagen hydrolyzate	
	Temperature (°C)/Pressure (bar)			
	200/30	250/70	200/30	250/70
Phosphoserine	0.32 ± 0.03[a]	0.27 ± 0.02[a]	0.20 ± 0.01[b]	0.16 ± 0.01[b]
Taurine	0.40 ± 0.02[a]	0.48 ± 0.03[a]	0.18 ± 0.01[b]	0.26 ± 0.02[b]
Aspartic acid	2.28 ± 0.05[a]	1.89 ± 0.04[a]	0.99 ± 0.04[b]	1.01 ± 0.03[b]
Hydroxyproline	5.99 ± 0.09[a]	5.33 ± 0.07[b]	5.37 ± 0.08[b]	4.71 ± 0.06[c]
Serine	1.67 ± 0.06[a]	ND	1.08 ± 0.04[b]	ND
Glutamic acid	1.17 ± 0.04[a]	1.24 ± 0.04[a]	0.73 ± 0.02[b]	0.64 ± 0.03[b]
Proline	13.20 ± 0.12[b]	11.52 ± 0.17[c]	15.36 ± 0.11[a]	11.92 ± 0.15[c]
Glycine	40.86 ± 1.80[a]	35.26 ± 1.12[b]	38.56 ± 1.15[a]	33.59 ± 1.25[b]
Alanine	24.82 ± 0.88[a]	21.19 ± 0.82[b]	24.83 ± 0.92[a]	19.36 ± 0.78[c]
α-aminobutyric acid	1.09 ± 0.04[a]	1.15 ± 0.03[a]	0.59 ± 0.03[b]	0.31 ± 0.02[b]
Valine	4.44 ± 0.15[b]	4.07 ± 0.18[c]	5.27 ± 0.16[a]	3.89 ± 0.11[c]
Cysteine	2.61 ± 0.09[a]	2.28 ± 0.08[a]	1.70 ± 0.08[b]	1.36 ± 0.07[b]
Methionine	2.29 ± 0.07[a]	1.35 ± 0.08[b]	0.64 ± 0.03[c]	0.59 ± 0.02[c]
Cystathionine	3.34 ± 0.10[a]	2.80 ± 0.07[b]	1.42 ± 0.06[c]	0.88 ± 0.04[d]
Isoleucine	4.36 ± 0.15[a]	3.77 ± 0.12[a]	1.78 ± 0.09[b]	1.42 ± 0.08[b]
Leucine	8.05 ± 0.18[a]	4.07 ± 0.11[c]	5.33 ± 0.15[b]	4.47 ± 0.12[c]
Tyrosine	5.34 ± 0.16[a]	4.53 ± 0.11[b]	1.90 ± 0.08[c]	1.63 ± 0.09[c]
Phenylalanine	6.03 ± 0.13[a]	5.89 ± 0.11[a]	2.30 ± 0.10[b]	2.02 ± 0.08[b]
β-alanine	0.01 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
Aminoisobutyric acid	0.18 ± 0.02	0.12 ± 0.02	0.10 ± 0.01	0.11 ± 0.01
γ-aminobutyric acid	0.03 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Histidine	5.46 ± 0.11[a]	3.50 ± 0.10[b]	1.93 ± 0.08[c]	1.73 ± 0.07[c]
Hydroxylysine	0.13 ± 0.01[b]	0.55 ± 0.02[a]	0.06 ± 0.01[b]	0.43 ± 0.02[a]
Ornithine	1.09 ± 0.05[b]	1.01 ± 0.06[b]	1.91 ± 0.06[a]	0.83 ± 0.04[b]
Lysine	7.08 ± 0.12[a]	6.79 ± 0.10[a]	4.21 ± 0.11[b]	3.64 ± 0.09[b]
Ethanolamine	0.79 ± 0.03[a]	0.62 ± 0.02[b]	0.46 ± 0.04[c]	0.28 ± 0.01[d]
Arginine	3.42 ± 0.09[a]	ND	2.11 ± 0.08[b]	ND

Means ± SD (n = 3). Different small letters in each row indicate significant differences ($P < 0.05$)

5.3.8. Antioxidant activities of PSC hydrolyzates

5.3.8.1. DPPH free radical scavenging activity

In the present study, PSC hydrolyzates from mackerel bone and skin isolated at 200–250 ℃ and 30–70 bar were effective scavengers of DPPH radicals, as shown in Fig. 5.4A. In contrast, no DPPH free radical scavenging activity was found in either PSC. Increasing concentrations of both PSC hydrolyzates exhibited elevated DPPH free radical scavenging activity. When DPPH encounters a hydrogen-donating substance, the radical is scavenged, resulting in a reduction in absorbance (Shimada et al., 1992). During hydrolysis of both PSCs, a wide range of smaller peptides and amino acids with the capacity to function as hydrogen donors were generated; these could then react with DPPH free radicals, convert them into more stable products and terminate the radical chain reaction. The IC_{50} values of PSC hydrolyzates at 200–250 ℃ and 30–70 bar were 8.71 ± 0.14 and 8.38 ± 0.13 mg/mL from mackerel bone, and 9.57 ± 0.12 and 7.58 ± 0.10 mg/mL from mackerel skin, compared with 0.35 ± 0.05 mg/mL for trolox as shown in Table 5.6. The PSC hydrolyzate produced from mackerel skin at 250 ℃ and 70 bar showed a reduced IC_{50} value, compared with the hydrolyzate from mackerel bone.

5.3.8.2. $ABTS^+$ free radical scavenging activity

An ABTS radical cation is relatively stable, and its green colour is reduced by the presence of antioxidant compounds with electron-donating capacity (Nalinanon et al., 2011). The $ABTS^+$ free radical scavenging activities of

various PSC concentrations before and after hydrolysis are shown in Fig. 5.4B. The ABTS free radical scavenging activities of both PSC hydrolyzates were significantly higher than those of the isolated PSCs. This suggests that the antioxidant compounds were most likely hydrophilic (Nalinanon et al., 2011). The IC_{50} values of PSC from mackerel bone were 10.77 ± 0.11 mg/mL (isolated), 4.18 ± 0.07 mg/mL (hydrolyzate at 200 ℃ and 30 bar) and 2.61 ± 0.05 mg/mL (hydrolyzate at 250 ℃ and 70 bar). The corresponding values from mackerel skin were 12.12 ± 0.13 mg/mL (isolated), 4.05 ± 0.08 mg/mL (hydrolyzate at 200 ℃ and 30 bar) and 2.50 ± 0.05 mg/mL (hydrolyzate at 250 ℃ and 70 bar) as shown in Table 5.6. In contrast, the IC_{50} value of standard trolox was 0.25 mg/mL, which was lower than both PSCs before and after hydrolysis. Antioxidant activity is influenced by changes in the size, quantity and composition of free amino acids and smaller peptides (Wu et al., 2003).

5.3.8.3. Ferric reducing power activity

As shown in Fig. 5.4C, Fe^{3+} was transformed to Fe^{2+} in the presence of PSCs both before and after hydrolysis. Various concentrations of PSC hydrolyzates from mackerel skin obtained at 250 ℃ and 70 bar exhibited greater ferric reducing activity, compared with those from bone. This suggests that PSC hydrolyzates function by donating electrons to free radicals, consistent with Klompong et al. (2007).

5.3.8.4. Fe^{2+} chelating activity

The Fe^{2+} chelating activities of various concentrations of mackerel bone and skin PSC hydrolyzates at 200–250 ℃ and 30–70 bar are shown in Fig. 5.4D. The isolated PSCs did not exhibit Fe^{2+} chelating activity. In contrast, both PSC hydrolysates exerted similar Fe^{2+} chelating effects. However, the Fe^{2+} chelating activities of both PSC hydrolyzates were increased at elevated temperatures and pressures. The PSC hydrolyzates might contain diverse peptides of varying sizes and amino acid sequences with different modes of action. This observation is consistent with Nalinanon et al. (2011). Therefore, PSC hydrolyzates could function as Fe^{2+} chelators. The IC_{50} values of PSCs from mackerel bone were 8.21 ± 0.15 mg/mL (hydrolyzate at 200 ℃ and 30 bar) and 7.27 ± 0.14 mg/mL (hydrolyzate at 250 ℃ and 70 bar), compared with 7.91 ± 0.11 mg/mL (hydrolyzate at 200 ℃ and 30 bar) and 7.01 ± 0.12 mg/mL (hydrolyzate at 250 ℃ and 70 bar) from mackerel skin as shown in Table 5.6. The IC_{50} value of EDTA was 0.34 ± 0.06 mg/mL, showing a lower value than those of the PSC hydrolyzates.

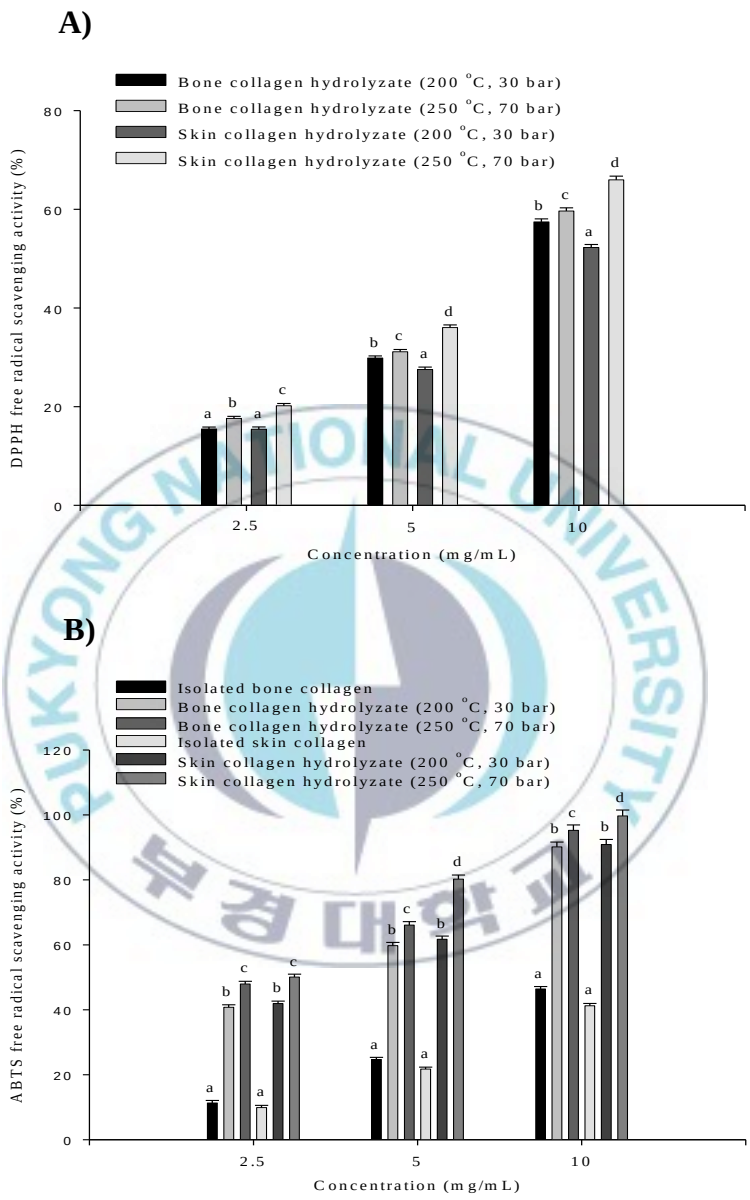


Fig. 5.5A-B. Antioxidant activities of collagen before and after hydrolysis from the bone and skin of mackerel. (A) DPPH free radical scavenging activity and (B) ABTS free radical scavenging activity. Means ± SD (n = 3). Different small letters on column bar with different concentration indicate significant differences ($P < 0.05$)

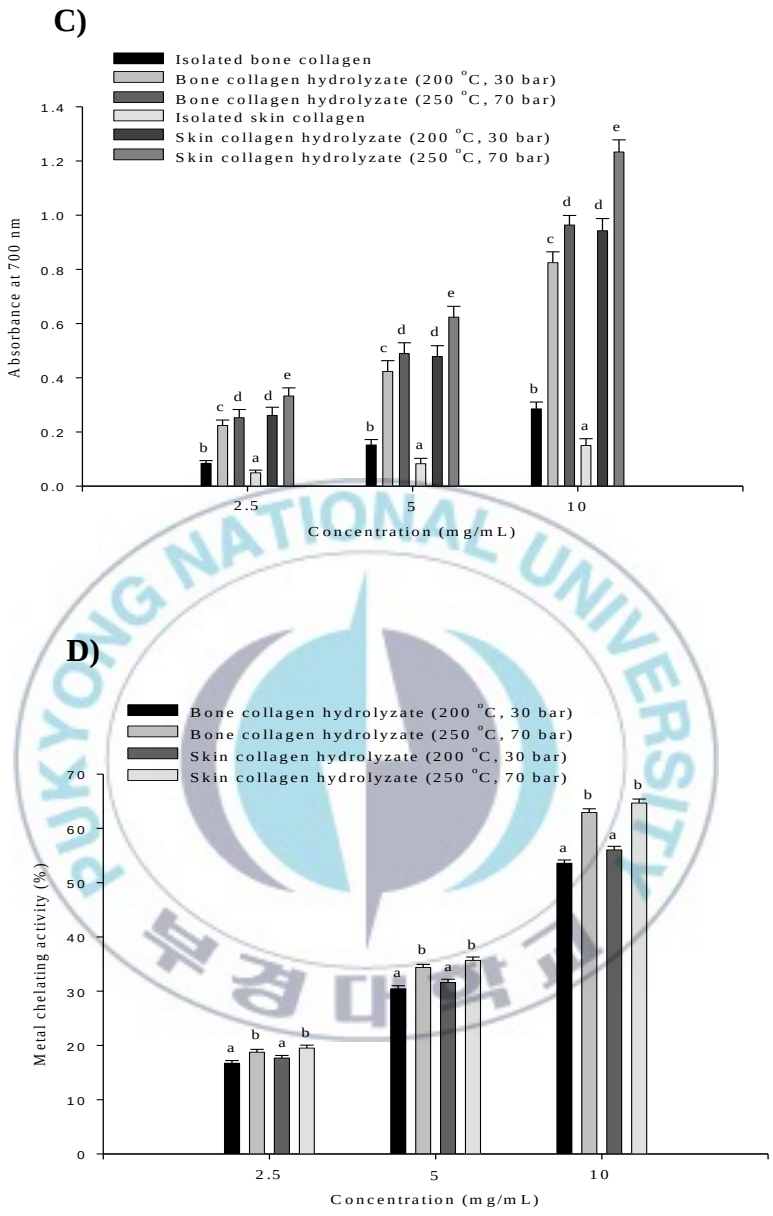


Fig. 5.5C-D. Antioxidant activities of collagen before and after hydrolysis from the bone and skin of mackerel. (C) Ferric reducing power activity and (D) Fe^{2+} chelating activity. Means ± SD (n = 3). Different small letters on column bar with different concentration indicate significant differences (P < 0.05)

Table 5.6. Scavenging of DPPH and ABTS and metal chelating activity (IC_{50} values) of collagen before and after hydrolysis from the bone and skin of mackerel and reference compounds

Activity	Collagen/Reference	IC_{50} (mg/mL)
DPPH free radical scavenging	Bone collagen hydrolyzate (200 ^{o}C, 30 bar)	8.71 ± 0.14^{c}
	Bone collagen hydrolyzate (250 ^{o}C, 70 bar)	8.38 ± 0.13^{c}
	Skin collagen hydrolyzate (200 ^{o}C, 30 bar)	9.57 ± 0.12^{d}
	Skin collagen hydrolyzate (250 ^{o}C, 70 bar)	7.58 ± 0.10^{b}
	Trolox	0.35 ± 0.05^{a}
ABTS free radical scavenging	Isolated bone collagen	10.77 ± 0.11^{d}
	Bone collagen hydrolyzate (200 ^{o}C, 30 bar)	4.18 ± 0.07^{c}
	Bone collagen hydrolyzate (250 ^{o}C, 70 bar)	2.61 ± 0.05^{b}
	Isolated skin collagen	12.12 ± 0.13^{d}
	Skin collagen hydrolyzate (200 ^{o}C, 30 bar)	4.05 ± 0.08^{c}
	Skin collagen hydrolyzate (250 ^{o}C, 70 bar)	2.50 ± 0.05^{b}
	Trolox	0.25 ± 0.03^{a}
Fe^{2+} chelating	Bone collagen hydrolyzate (200 ^{o}C, 30 bar)	8.21 ± 0.15^{c}
	Bone collagen hydrolyzate (250 ^{o}C, 70 bar)	7.27 ± 0.14^{b}
	Skin collagen hydrolyzate (200 ^{o}C, 30 bar)	7.91 ± 0.11^{c}
	Skin collagen hydrolyzate (250 ^{o}C, 70 bar)	7.01 ± 0.12^{b}
	EDTA	0.34 ± 0.06^{a}

Means ± SD (n = 3). Different small letters in the same column with specific activity indicate significant differences ($P < 0.05$)

5.4. Conclusions

PSCs were successfully isolated from bone and skin of mackerel; the yield from skin was considerably higher. Both PSCs were most likely type I collagen, and maintained their triple helical structure. The thermal stability of PSC from skin was slightly higher than that from bone. Diverse and small MW-containing peptides were produced from both PSCs after SWH treatment; these exhibited multifunctional antioxidant activities greater than that of the isolated PSC. Therefore, PSC hydrolyzates from mackerel bone and skin have the potential to serve as an alternative source of isolated collagen.

References

Abdelkarder A.N., Kim S.B., Lee Y.B., Chun B.S. (2012) Digestive enzymes characterization of krill (Euphausia superba) residues deoiled by supercritical carbon dioxide and organic solvents. *Journal of Industrial and Engineering Chemistry*, 18: 1314-1319.

Abdelmoez W., Nakahasi T., Yoshida H. (2007) Amino acid transformation and decomposition in saturated subcritical water conditions. *Industrial and Engineering Chemistry Research*, 46: 5286-5294.

Ahmad M., Benjakul S. (2010) Extraction and characterization of pepsin solubilised collagen from the skin of unicorn leatherjacket (*Aluterus monocerous*). *Food Chemistry*, 120: 817-824.

American Oil Chemists Society (AOCS) (1998). *Official methods of analysis*, Volume I, II & III, Champaign, IL, USA.

Aquaticcommons (2013) Maturation and growth of pacific mackerel. http://aquaticcommons.org/660/1/Technical

Arihara K. (2006) Strategies for designing novel functional meat products. *Meat Science*, 74: 219-229.

Aryee A.N.A., Simpson B.K., Villalonga R. (2007) Lipase fraction from the viscera of grey mullet (Mugil cephalus): Isolation, partial purification, and some biochemical characteristics. *Enzyme and Microbial Technology*, 40: 394-402.

Association of Official Analytical Chemists (AOAC) (1990) Official Methods of Analysis, 15th edn., Washington, DC, USA.

Bailly N. (2013) *Scomber japonicus* Houttuyn, 1782. In *Fish Base*, R. Froese, D. Pauly (Ed.). Accessed through: World Register of Marine Species at http://www.marinespecies.org/aphia.php

Balian G., Bowes J.H. (1977) The structure and properties of collagens. In *The science and technology*, A.G. Ward, A. Courts (Ed.), Academic Press, London, pp. 1-31.

Barac M., Cabrilo S., Stanojevic S., Pesic M., Pavlicevic M., Zlatkovic B., Jankovic M. (2012) Functional properties of protein hydrolyzates from pea (Pisum sativum, L) *seeds. International Journal of Food Science and Technology*, 47: 1457-1467.

Beara I.N., Lesjak M.M., Jovin E.D., Balog K.J., Anackov G.T., Orcić D.Z., Mimica-Dukić N.M. (2009) Plantain (*Plantago* L.) species as novel sources of flavonoid antioxidants. *Journal of Agricultural and Food Chemistry*, 57: 9268-9273.

Belhaj N., Arab-Trhrany E., Linder M. (2010) Oxidative kinetics of salmon oil in bulk and in nanoemulsion stabilized by marine lecithin. *Process Biochemistry*, 45: 187-195.

Benjakul S., Morrissey M.T. (1997) Protein hydrolyzates from Pacific whiting solid wastes. *Journal of Agricultural and Food Chemistry*, 45: 3423-3430.

Benjakul S., Thiansilakul Y., Visessanguan W., Roytrakul S., Kishimura H., Prodprand T., et al. (2010) Extraction and characterization of pepsin solubilised collagen from the skin of bigeye snapper (*Priacanthus tayenus* and *Priacanthus macracanthus*). *Journal of the Science of Food and*

Agriculture, 90: 132-138.

Benolken R.M., Anderson R.E., Wheeler T.G. (1973) Membrane fatty acids associated with the electrical response in visual excitation. *Science*, 182: 1253-1254.

Bergmeyer H.U., Gawehn K., Grassi M. (1974) In *methods of enzymatic analysis*, H.U. Bergmeyer (Ed.), Academic Press, Inc., New York, pp 515-516.

Bernardez M., Pastoriza L., Sampedro G., Hettera J.J.R., Cabo M.L. (2005) Modified method for the analysis of free fatty acids in fish. *Journal of Agricultural and Food Chemistry*, 53: 1903-1906.

Bezerra R.S., Lins E.J.F., Alencar R.B., Paiva P.M.G., Chaves M.E.C., Coelho L.C.B.B., Carvalho L.B. (2005) Alkaline proteinase from intestine of nile tilapia (*Oreochromis niloticus*). *Journal of Process Biochemistry*, 40: 1829-1834.

Binsan W., Benjakul S., Visessanguan W., Roytrakul S., Tanaka M., Kishimura H. (2008) Antioxidative activity of Mungoong, an extract paste, from the cephalothorax of white shrimp (*Litopenaeus vannamei*). *Food Chemistry*, 106: 185-193.

Birch E.B., Garfield S., Castañeda Y., Hughbanks-wheaton D., Uauy R, Hoffman D. (2007) Visual acuity and cognitive outcomes at 4 years of age in a double-blind, randomized trial of long-chain polyunsaturated fatty acid-supplemented infant formula. *Early Human Development*, 83: 279-284.

Brogle H. (1982) Supercritical fluid CO_2 extract. *Chemistry and Industry*, 12: 385-390.

Brunner G. (1994) Gas extraction- an introduction to the fundamentals of supercritical fluids and the application to separation processes, Springer, New York, USA.

Buchner E.H. (1906) *Zeitschrift für Physikalische Chemie*, 54: 665.

Cagniard de la tour B.C. (1822) *Annales de Chimie et de Physique*, 21: 127, 178.

Cheng H., Zhu X., Zhu C., Qian J., Zhu N., Zhao L., Chen J. (2008) Hydrolysis technology of biomass waste to produce amino acids in subcritical water. *Journal of Bioresource Technology*, 99: 3337-3341.

Cho S.Y., Joo D.S., Choi H.G., Nara E., Miyashita K. (2001) Oxidative stability of lipids from squid tissues. *Journal of Fisheries Science*, 67: 738-743.

Choudhury G.S., Bublitz C.G. (1996) Computer-based controls in fish processing industry. In *computerized control systems in the food industry*, G.S. Mittal (Ed.), Marcel Dekker, Inc., New York, pp 513-538.

Cinq-Mars C.D., Hu C., Kitts D.D., Li-Chan E.C.Y. (2008) Investigations into inhibitor type and mode, simulated gastrointestinal digestion, and cell transport of the angiotensin I converting enzyme-inhibitory peptides in pacific hake (Merluccius products) fillet hydrolysate. *Journal of Agricultural and Food Chemistry*, 56: 410-419.

Coenan H., Hagen R., Knuth M. (1983) U.S. patent no. 4, 400, 398.

"Collagen diagram-reborn". http://rebornasia.com/Sondno%20Collagen%20Peptide.pdf.

Croker R., Symonds (1933) *The California mackerel fishery*, Academic Press, California, USA, pp. 9-10.

Cuvier C.C. (1829) Règne Animal, 2nd ed., 2: 200 (based on Marcgrav's Guarapucu from Brazil).

Davarnejad R., Kassim K.M., Zainal A., Sata S.A. (2008) Extraction of fish oil by fractionation through supercritical carbon dioxide. *Journal of Chemical Engineering*, 53: 2128-2132.

Decker E.A., Welch B. (1990) Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry*, 38: 674-677.

Dendinger J.E., O'connor K.L. (1990) Purification and characterization of a trypsin-like enzyme from the midgut gland of the Atlantic blue crab, *Callinectes sapidus*. *Comparative Biochemistry and Physiology B*, 95: 525-530.

Di Benedetto R., Attorri L., Salvati S., Leonardi F., Di Biase A. (2007) Eicosapentaenoic acid stimulates leptin receptor gene expression in the hypothalamus of newborn rats. *Nutrition Research*, 27: 367-371.

Dickerson N.L., Meyers J.M. (1952) *Journal of American Oil Chemist Society*, 29: 235-239.

Djamel C., Ali T., Nelly C. (2009) Acid protease production by isolated species of penicillium. *European Journal of Scientific Research*, 25: 469-477.

Doyle B.B., Bendit E.G., Blout E.R. (1975) Infrared spectroscopy of collagen and collagen-like polypeptides. *Biopolymers*, 14: 937-957.

Duan R., Zhang J.J., Du X.Q., Yao X.C., Konno K. (2009) Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*). *Food Chemistry*, 112: 702-706.

Dunford N.T., Temelli F. (1995) Extraction of phospholipids from canola with supercritical carbon dioxide and ethanol. *Journal of American Oil Chemist`s Society*, 72: 1009-1015.

Dunford N.T., Temelli F., Leblanc E., (1997) Supercritical CO_2 extraction of oil and residual proteins from Atlantic mackerel (*Scomber scombrus*) as affected by moisture content. *Journal of Food Science*, 62: 289-294.

Dyerberg J., Bang H.O., Hjorne N. (1975) Fatty acid composition of the plasma lipids in greenland eskimos. *The American Journal of Clinical Nutrition*, 28: 958-966.

Erdmann K., Cheung B.W.Y., Schroder H. (2008) The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry*, 19: 643-654.

Erkkila A.T., Lehto S., Pyorala K., Uusitupa M.I. (2003) Fatty acids (n-3) and 5-y risks of death and cardiovascular disease events in patients with coronary artery disease. *American Journal of Clinical Nutrition*, 78: 65-71.

Eshel A., Lindner P., Smirnoff P., Newton S., Harpaz S. (1993) Comparative study of proteolytic enzymes in the digestive tracts of the European sea

bass and hybrid striped bass reared in freshwater. *Comparative Biochemistry and Physiology A*, 106: 627-634.

Espinoza A.D., Morawicki R.O. (2012) Effect of additives on subcritical water hydrolysis of whey protein isolate. *Journal of Agricultural and Food Chemistry*, 60: 5250-5256.

Espósito T.S., Amaral I.P.G., Buarque D.S., Oliveira G.B., Carvalho Jr L.B., Bezerra R.S. (2009) Fish processing waste as a source of alkaline proteases for laundry detergent. *Food Chemistry*, 112: 125-130.

Esquivel M.M., Bandarra N.M., Fontan I., Bernardo-Gil M.G., Batista I., Nunes M.L., Empis J.A. (1997) Supercritical carbon dioxide extraction of sardine *Sardinapilchardus* oil. *LWT-Food Science and Technology*, 30: 715-720.

Essien E.A., Umoren S.A., Essien E.E., Udoh A.P. (2012) Preparation and evaluation of *Cucumeropsis mannii* Naud seed oil metallic soaps as driers in gloss paint. *Journal of Materials and Environmental Science*, 3: 477-484.

FAO (1994) Corporate document repository: A global assessment fisheries by-catch and discards. http://www.fao.org/docrep/003/t4890e/t4890e03.htm

FAO (2009) Based on data sourced from the relevant FAO Species Fact Sheets, United Nations.

FAO (2012) Biological characteristics of tuna FAO Fact Sheets, United Nations.

FAO (2012) www.fao.org/fishery/topic/16140/en

Gbogouri G.A., Linder M., Fanni J., Parmentier M. (2004) Influence of hydrolysis degree on the functional properties of salmon byproduct hydrolyzates. *Food Science*, 69: 615-622.

Ge Y., Ni Y., Chen Y., Cai T. (2002) Optimization of the supercritical fluid extraction of natural vitamin E from wheat germ using response surface methodology. *Journal of Food Science*, 67: 239-243.

Gomez-Guillen M.C., Turnay J., Fernandez-Diaz M.D., Ulmo N., Lizarbe M.A., Montero P. (2002) Structural and physical properties of gelatin extracted from different marine species: A comparative study. *Food Hydrocolloids*, 16: 25-34.

Halling P.J. (1981) Protein stabilized foams and emulsions. *CRC Critical Reviews of Food Science*, 12: 155-203.

Hannay J.B., Hogarth J. (1887) *Proceedings of the Royal Society*, London, UK, 29: 324.

Harris W.S. (1997) *n*-3 fatty acids and serum lipoproteins human studies. *American Journal of Clinical Nutrition*, 65: 1645-1654.

Herring J., Garrean S., Dekoj T.R., Razzak A., Saied A., Trevino J., Babcock T.A., Espat N.J. (2007) Inhibition of proliferation by omega-3 fatty acids in chemoresistant pancreatic cancer cells. *Annals of Surgical Oncology*, 14: 3620-3628.

Heu M.S., Lee J.H., Kim H.J., Lee S.J., Lee J.S., Jeon Y J., et al. (2010) Characterization of acid-and pepsin-soluble collagens from flatfish skin. *Food Science and Biotechnology*, 10: 27-33.

Hidalgo M.C., Urea E., Sanz A. (1999) Comparative study of digestive enzymes in fish with different nutritional habits: Proteolytic and amylase activities. *Journal of Aquaculture*, 170: 267-283.

Högyes E., Nyakas C., Kiliaan A., Farkas T., Penke B., Luiten P.G.M. (2003) Neuroprotective effect of development docosahexaenoic acid supplement against excitotoxic brain damage in infant rats. *Neuroscience*, 119: 999-1012.

Horrocks L.A., Yeo Y.K. (1999) Health benefits of docosahexaenoic acid (DHA). *Pharmacological research*, 40: 211-225.

Houttuyn M. (1782) Beschryving van eenige Japanese visschen, en andere zee-schepzelen. *Verhandelingen de Hollandsche Maatschappye der Weetenschappen te Haarlem*, 20: 311-350.

Howell E. (1985) *Enzyme Nutrition-the food enzyme concept*, Avery Publishing Groups, Inc., Wayne, New Jersey, USA.

Hultin H.O. (1994) Oxidation of lipids in seafoods. In *Seafoods: Chemistry, processing technology and quality*, F. Shahidi, J.R. Botta (Ed.), Chapman & Hall, London, UK, pp. 49-74.

Ikoma T., Kobayashi H., Tanaka J., Walsh D., Mann S. (2003) Physical properties of type 1 collagen extracted from fish scales of Pagrus and Oreochromis. *International Journal of Biological Macromolecule*, 32: 199-204.

Ikushima Y., Saito N., Hatakeda K., Ito S., Asano T., Goto T. (1986) A supercritical carbon dioxide extraction from mackerel (*Scomber japonicus*)

powder: experiment and modelling. *Bulletin of the Chemical Society of Japan*, 59: 3709-3713.

Jang A., Jo C., Kang K.S., Lee M. (2008) Antimicrobial and human cancer cell cytotoxic effect of synthetic angiotensin-converting enzyme (ACE) inhibitory peptides. *Food Chemistry*, 107: 327-336.

Jiang W.G., Bryce R.P., Horrobin D.F. (1998) Essential fatty acids: molecular and cellular basis of their anti-cancer action and clinical implications. *Critical Reviews in Oncology/Hematology*, 27: 179-209.

Jongjareonrak A., Benjakul S., Visessanguan W., Nagai T., Tanaka M. (2005) Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of brown stripe red snapper (*Lutjanus vitta*). *Food Chemistry*, 93: 475-484.

Karun T., Uthaiwan K., Arunee E., Krisna R.T. (2010) Temperature and pH characteristics of amylase and lipase at different developmental stages of siamese fighting fish (*Betta splendens* regan, 1910). *Kasetsart Journal of Natural Science*, 44: 210-219.

King J. (2000) Advances in critical fluid technology for food processing. *Food Science and Technology Today*, 14: 186-191.

Kittiphattanabawon P., Benjakul S., Visessanguan W., Nagai T., Tanaka M. (2005) Characterization of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). *Food Chemistry*, 89: 363-372.

Kittiphattanabawon P., Benjakul S., Visessanguan W., Shahidi F. (2010) Isolation and properties of acid-and pepsin-soluble collagen from the skin

of blacktip shark (*Carcharhinus limbatus*). *European Food Research and Technology*, 230: 475-483.

Klomklao S., Benjakul S., Visessnguan W., Kishimura H., Simpson B.K. (2006a) Purification and characterization of trypsin from the spleen of tongol tuna (*Thunnus tonggol*). *Journal of Agricultural and Food Chemistry*, 54: 5617-5622.

Klompong V., Benjakul S., Kantachote D., Shahidi F. (2007) Antioxidative activity and functional properties of protein hydrolyzate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry*, 102: 1317-1327.

Kraus-Etshcmann S., Shadid R., Campoy C., Hoster E., Demmelmair H., Jimenez M. (2007) Effect of fish oil and folate supplementation of pregnant women on maternal and fetal plasma concentration of docosahexaenoic acid and eicosapentaenoic acid: a European randomized multicenter trial. *American Journal of Clinical Nutrition*, 85: 1392-1400.

Krichnavaruk S., Shotipruk A., Goto M., Pavasant P. (2008) Supercritical carbon dioxide extraction of astaxanthin from Haematococcus pluvialis with vegetable oils as co-solvent. *Bioresource Technology*, 99: 5556-5560.

Krukonis V.J. (1985) *Polymer News*, 11: 7.

Krukonis V.J. (1988) *ACS Symposium Series*, 366: 30.

Kubrak O.I. (2007) Microbe amylases: characteristic, properties and practical use. *Journal of Microbiology Z*, 69: 56-76.

Kubrak O.I., Lushchak V.I. (2007) Microbe amylases: characteristic,

properties and practical use. *Journal of Microbiology Z*, 69(6): 56-76.

Kumar S., Kikon K., Upadhyay A., Kanwar S.S., Gupta R. (2005) Production, purification, and characterization of lipase from thermophilic and alkaliphilic Bacillus coagulans BTS-3. *Protein Expression and Purification*, 41: 38-44.

Laemmli U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.

Lawal O.S. (2004) Functionality of African locust bean (*Parkia biglobossa*) protein isolate: effects of pH, ionic strength and various protein concentrations. *Food Chemistry*, 86: 345-355.

Laws D.R.J., Bath N.S., Wheldon A.G. (1980) U.S. patent no. 4, 218, 491.

Leaf A., Xiao Y.F., Kang J.X., Billman G.E. (2003) Prevention of sudden cardiac death by n-3 polyunsaturated fatty acids. *Pharmacology and Therapeutics*, 98: 355-377.

Lee S.M., Asaduzzaman A.K.M., Chun B.S. (2012) Charecterization of lecithin isolated from anchovy (*Engraulis japonica*) residues deoiled by supercritical carbon dioxide and organic solvent extraction. *Journal of Food Science*, 77: 773-778.

Lemaitre R.N., King I.B., Mozaffarian D., Kuller L.H., Tracy R.P., Sisovick D.S. (2003) Polyunsaturated fatty acids (n-3), fatal ischemic heart disease, and nonfatal myocardial infarction in older adults: the cardiovascular health study. *American Journal of Clinical Nutrition*, 77: 319-325.

Letter W.S. (1992) A rapid method for phospholipids class separation by HPLC using an evaporative light-scattering detector. *Journal of Liquid Chromatography*, 15: 253-266.

Lim G.B., Lee S.Y., Lee E.K., Haam J.S., Kim W.S. (2002) Separation of astaxanthin from red yeast Phaffia rhodozyma by supercritical carbon dioxide extraction. *Biochemical Engineering Journal*, 11: 181-187.

Liu D., Liang L., Regenstein J.M., Zhou P. (2012) Extraction and characterisation of pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of bighead carp (*Hypophthalmichthys nobilis*). *Food Chemistry*, 133: 1441-1448.

Lopez M., Arce L., Garrido J., Rios A., Valcarcel M. (2004) Selective extraction of astaxanthin from crustaceans by use of supercritical carbon dioxide. *Talanta*, 64: 726-731.

Lowry O.H., Roserbrough N.J., Farr A.W., Randall R.J. (1951) Protein measurements with pholin phenol reagent. *Journal of Biological Chemistry*, 193: 265-275.

Machmudah S., Shotipruk A., Goto M., Sasaki M., Hirose T. (2006) Extraction of astaxanthin from haematococcus pluviaris using supercritical CO_2 and ethanol as entrainer. *Industrial and Engineering Chemistry Research*, 45: 3652-3657.

Makrides M., Neumann M., Simmer K., Pater J., Gibson R. (1995) Are long chain polyunsaturated fatty acids essential nutrients in infancy? *The Lancet*, 345: 1463-1468.

Manohar B., Began G., Sankar K.U. (1995) Response surface methodology applied to fractionation of deoiled soy lecithin obtained by supercritical carbon dioxide extraction. *LWT-Food Science and Technology*, 28: 218-221.

Martinez A., Olsen R.L., Serra J.L. (1988) Purification and characterization of two trypsin-like enzymes from the digestive tract of anchovy *Engraulis encrasicholus*. *Comparative Biochemistry and Physiology B*, 91: 677-684.

Masson S., Latini R., Tacconi M., Bernasconi R. (2007) Incorporation and washout of n-3 polyunsaturated fatty acids after diet supplementation in clinical studies. *Journal of Cardiovascular Medicine*, 8: S4-S10.

Matmaroh K., Benjakul S., Prodpran T., Encarnacion A.B., Kishimura H. (2011) Characteristics of acid soluble collagen from scale of spotted golden goatfish (*Parupeneus heptacanthus*). *Food Chemistry*, 129: 1179-1186.

Mendes R.L., Nobre B.P., Cardoso M.T., Pereire A.P., Palavre A.F. (2003) Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. *Inorganic Chimica Acta*, 356: 328-334.

Miller G.L. (1959) Use of dinitrosalicyclic acid reagent for the determination of reducing sugar. *Journal of Anal Chemistry*, 31: 426-429.

Miniadis-Meimaroglou S., Kora L., Sinanoglou V.J. (2008) Isolation and identification of phospholipid molecular species in wild marine shrimp *Penaeus kerathurus* muscle and cephalothorax. *Chemisty and Physics of Lipids*, 152: 104-112.

Mitsuda H., Yasumoto K., Iwami K. (1966) Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Eiyo to Shokuryo*, 19: 210-214.

Mitsuo N., Tetsuro S., Sethu S., Vu V.I., Hajime K., Akihiko Y., Kumiko O., Shizumasa O., Akio S., Michiya M. (2011) Immunoreactive changes in pituitary FSH and LH cells during seasonal reproductive and spawning cycles of female chub mackerel Scomber japonicus. *Fisheries Science*, 77: 731-739.

Morita A., Kajimoto O. (1990) Solute solvent interaction in nonpolar supercritical fluid: a clustering model and size distribution. *Journal of Physical Chemistry*, 94: 6420-6425.

Moyad M.A. (2005) An introduction to dietary/supplemental omega-3 fatty acids for general health and prevention: Part II. Urologic Oncology: Seminars and Original Investigations, 23: 36-48.

Murakami K., Noda M. (1981) Studies on proteinases from the digestive organs of sardine-purification and characterization of three alkaline proteinases from the pyloric ceca. *Journal of Biochimica et Biophysica Acta B*, 65: 17-26.

Mutilangi W.A.M., Panyam D., Kilara A. (1996) Functional properties of hydrolystes from proteolysis of heat-denatured whey protein isolate. *Food Science*, 61: 270-274.

Muyonga J.H., Cole C.G.B., Duodu K.G. (2004) Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*).

Food Chemistry, 85: 81-89.

Myer L., Dmian J., Liescheski P., Tehrani J., (1992) Étude comparative d'extractions par un fluide a phase supercritique et par la méthode de soxhlet. *Spectra 2000*, 165: 57-63.

Nagai T., Suzuki N., Nagashima T. (2008) Collagen from common minke whale (*Balaenoptera acutorostrata*) unesu. *Food Chemistry*, 111: 296-301.

Nalinanon S., Benjakul S., Kishimura H. (2010) Collagens from the skin of arabesque greenling (*Pleurogrammus azonus*) solubilized with the aid of acetic acid and pepsin from albacore tuna (*Thunnus alalunga*) stomach. *Journal of the Science of Food and Agriculture*, 90: 1492-1500.

Nalinanon S., Benjakul S., Kishimura H., Shahidi F. (2011) Functionalities and antioxidant properties of protein hydrolyzates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. *Food Chemistry*, 124: 1354-1362.

Natalia Y., Hashim R., Ali A., Chong A. (2004) Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue Scleropages for mosus (Osteoglossidae). *Journal of Aquaculture*, 233: 305-320.

Neuringer M. (2000) Infant vision and retinal function in studies of dietary long-chain polyunsaturated fatty acids: methods, results, and implications. *The American Journal of Clinical Nutrition*, 71: 256S-267S.

Nielson H. (2001) In situ solid phase extraction of phospholipids from heat coagulated egg yolk by organic solvents. *LWT-Food Science and*

Technology, 34: 526-532.

Nieuwenhuyzen W.V., Tomas M.C. (2008) Update on vegetable lecithin and phospholipid technologies. *European Journal of Lipid Science and Technology*, 110: 472-486.

NOAA (2013) Fish watch: Pacific mackerel. http:// www.fishwatch.gov/seafood_profiles/species

NOAA (2014) Fish watch seafood facts: Pacific mackerel. http://www.fishwatch.gov/seafood_profiles/species/mackerel/species_pages/pacific

Noman A.S.M., Hoque M.A., Sen P.K., Karim M.R. (2006) Purification and some properties of amylase from post-harvest *Pachyrhizus erosus* L. tuber. *Food Chemistry*, 99: 444-449.

O`Connor D.L., Hall R., Adamkin D., Castillo M., Connor W.E., Connor S.L., et al. (2001) Growth and development in preterm infants fed long-chain polyunsaturated fatty acids: a prospective, randomized controlled trial. *Pediatrics*, 108: 359-371.

Oda K., Murao S. (1974) Purification and properties of carboxyl proteinase in basidiomycetes. *Journal of Agricultural and Biological Chemistry*, 38: 2435-2437.

Ogawa M., Portier R.J., Moody M.W., Bell J., Schexnayder M.A., Losso J.N. (2004) Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*). *Food Chemistry*, 88: 495-501.

Ogino H., Miyamoto K., Ishikawa H. (1994) Organic solvent tolarent bacteria which secretes organic solvent stable lipolytic enzyme. *Applied and Environmental Microbiology*, 60: 3884-3886.

Online Etymology Dictionary (OED) (2012) http://www.etymonline.com/bio.php

Oyaiza M. (1986) Studies on products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine. *Journal of Nutrition*, 44: 307-315.

Paez B.C., Medina A.R., Rubio F.C., Moreno P.G., Grima E.M. (2002) Production of structured triglycerides rich in n-3 polyunsaturated fatty acids by the acidolysis of cod liver oil and caprylic acid in a packed-bed reactor: equilibrium and kinetics. *Chemical Engineering Science*, 57: 1237-1249.

Palacious L.E., Wang T. (2005) Egg-yolk lipids fractionation and lecithin characterization. *Journal of American Oil Chemist`s Society*, 82: 571-578.

Pariser E.R., Wallerstei M.B., Corkery C.J., Brown N.L. (1978) *Fish protein concentrate: panacea for world malnutrition*, MIT Press, Boston, ISBN: 10: 0262160692, pp 256.

Park J.N., Ali-Nehari A., Woo H.C., Chun B.S. (2012) Thermal studies of polyphenols and fatty acids in *Laminaria japonica* hydrolyzates produced using subcritical water. *Korean Journal of Chemical Engineering*, 29: 1604-1609.

Park J.Y., Lee M.K., Uddin M.S., Chun B.S. (2008) Removal of off flavors

and isolation of fatty acids from boiled anchovies using supercritical carbon dioxide. *Biotechnology and Bioprocess Engineering*, 13: 298-303.

Parsertsan P., Jitbunjerdkul S., Trairatananukoon, Prachumratana T. (2001) Production of enzyme and protein hydrolyzate from fish processing waste. In *new horizons in Biotechnology,* S. Roussos, C.R. Soccol, A. Pandey, C. Augur (Ed.), Kluwer Academic Publisher, India, pp 63-72.

"PB Gelatin". http://www.pbgelatins.com. Accessed 07.08.09

Pearce K.N., Kinsella J.E. (1978) Emulsifying properties of proteins: evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry*, 26: 716-723.

Perona J.J., Craick S.C. (1995) Structural basis of substrate specificity in the serine proteases. *Protein Science*, 4: 337-360.

Petersen R.B. (1981) The impact of the enzymic hydrolysis process on recovery and use of proteins. In *enzymes and food processing*, G.G. Birch, N. Blakebrough, K.J. Parker (Ed.), Applied Science Publication Ltd., London, pp 296-299.

Pham V., Soottawat B. (2006) Purification and characterization of trypsin from pyloric caeca of bigeye snapper (*pricanthus macracanthus*). *Journal of Food Biochemistry*, 30: 478-495.

Phillips L.G., Whitehead D.M., Kinsella J.E. (1994) Protein stabilized foams. In *structure-function of food proteins*, L.G. Phillips, D.M. Ehitehead, J.E. Kinsella (Ed.), Academic Press, New York, USA, pp 131-152.

Poonsuk P., Thiraratana P. (2008) Properties of protease and lipase from whole

and individual organ of viscera from three tuna species. *Songklanakarin Journal of Science and Technology*, 30: 77-86.

Prior R.L., Wu X., Schaich K. (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 53: 4290-4302.

Prosise W.E. (1985) Commercial lecithin products: Food use of soybean lecithin. In Lecithins, B.F. Szuhaj, G.R. List (Ed.), *American Oil Chemist`s Society*, Champaign, IL, USA, pp. 163-182.

Ramadan M.F. (2008) Quercetin increases antioxidant activity of soy lecithin in a triolein model system. *LWT- Food Science and Technology*, 41: 581-587.

Ramadan M.F., Kroh L.W., Moersel J.T. (2003) Radical scavenging activity of black cumin (*Nigella sativa* L.), coriander (*Coriandrum sativum* L.) and niger (*Guizotia abyssinica* Cass.) crude seed oils and oil Fractions. *Journal of Agricultural and Food Chemistry*, 51: 6961-6969.

Ramadan M.F., Moersel J.T. (2004) Oxidative stability of black cumin (*Nigella sativa* L.), coriander (*Coriandrum sativum* L.) and niger (*Guizotia abyssinica* Cass.) upon stripping. *European Journal of Lipid Science and Technology*, 106: 35-43.

Raventós M., Duarte S., Alarcón R., (2012) Application and possibilities of supercritical CO_2 extraction in food processing industry: an overview. *Food Science Technology International*, 8: 269-284.

Reid R.C., Prausnitz J.M., Poling B.E. (1987) The properties of gases and liquids, McGraw-Hill, New York, USA.

Rubio-Rodriguez N., De Diego S.M., Beltran S., Jaime I., Sanz M.T., Rovira J. (2008) Supercritical fluid extraction of the omega-3 rich oil contained in hake *(Merlucciuscapensis-Merluccicus paradoxus)* by-products: study of the influence of process parameters on the extraction yield and oil quality. *Journal of Supercritical Fluids*, 47: 215-226.

Sahena F., Zaidul I.S.M., Jinap S., Yazid A.M., Khatib A., Norulaini N.A.N. (2010) Fatty acid compositions of fish oil extracted from differents parts of Indian mackerel (*Rastrelliger kanagurta*) using various techniques of supercritical CO_2 extraction. *Food Chemistry*, 120: 879-885.

Saito M., Takenouchi N.Y., Kunisaki N., Kimura S. (2001) Complete primary structure of rainbow trout type I collagen consisting of $\alpha_1(I)\alpha_2(I)\alpha_3(I)$ heterotrimers. *European Journal of Biochemistry*, 268: 2817-2827.

Salak Asghari F., Yoshida H. (2007) Kinetics of the decomposition of fructose catalyzed by hydrochloric acid in subcritical water: formation of 5-hydroxymethylfurfural, levulinic, and formic acids. *Industrial and Engineering Chemistry Research*, 46: 7703-7710.

Sampath Kumar N.S., Nazeer R.A., Jaiganesh R. (2012) Purification and identification of antioxidant peptides from the skin protein hydrolyzate of two marine fishes, horse mackerel (*Magalaspis cordyla*) and croaker (*Otolithes ruber*). *Journal of Amino Acids*, 42: 1641-1649.

Sathe S.K., Salunkhe D.K. (1981) Functional properties of the Great Northern Bean (*Phaseolus vulgaris* L.) proteins: emulsion, foaming, viscosity and gelation properties. *Food Science*, 46: 71-74.

Scholfield C.R. (1981) Composition of soybean lecithin. *Journal of American Oil Chemist`s Society*, 58: 889-892.

Shahidi F., Janak-Kamil Y.V.A. (2001) Enzymes from fish and aquatic invertebrates and their application in the food industry. *Trends in Food Science and Technology*, 12: 435-64.

Shimada K., Fujikawa K., Yahara K., Nakamura T. (1992) Antioxidative properties of xanthan on the antioxidation of soy bean oil in cyclodextrin emulsion. *Journal of Agriculture and Food Chemistry*, 40: 945-948.

Sidhu G.S., Montgomery W.A., Holloway G.L., Johnson A.R., Walker D.M. (1970) Biochemical composition and nutritive value of krill (*Euphausia superb Dana*). *Journal of the Science of Food and Agriculture*, 21: 293-296.

Sim J.S. (1994) New extraction and fractionation method for lecithin and neutral oil from egg yolk. In *Egg uses and processing tecgnologies*, J.S. Sim, S. Nakai (Ed.), CAB International, Wallingford, UK, pp. 128-138.

Simpson B.K., Haard N.F. (1984) Trypsin from Greenland cod, *Gadus ogac*. Isolation and comparative properties. *Comparative Biochemistry and Physiology B*, 79: 613-622.

Simpson B.K., Smith J.P., Haard N.F. (1991) Marine enzymes. In *encyclopedia of food science and technology*, Y.H. Hui (Ed.), John Wiley

and Sons, New York, pp 1645-1653.

Singh P., Benjakul S., Maqsood S., Kishimura H. (2011) Isolation and characterization of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*). *Food Chemistry*, 124: 97-105.

Smyth A.L., McGlynn H. (2005) Modifications of the radiosensitivity of a renal cancer cell line as a consequence of polyunsaturated fatty acid supplementation. *Nutrition Research*, 25: 65-67.

Stahl E., Quirin K.W., Gerard D. (1988) Dense gases for extraction and refining, translated by M.R.F. Ashworth, Springer-Verlag, New York, USA, p.176.

Stewart J.C.M. (1980) Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Journal of Anal Biochemistry*, 104: 10-14.

Sun M., Temelli F. (2006) Supercritical carbon dioxide extractions of carotenoids from carrot using canola oil as a continuous co-solvent. *Journal of Supercritical Fluids*, 37: 397-408.

Taherzadeh M.J., Karimi K. (2007) Enzyme based hydrolysis processes for ethanol from lignocellulosic materials: A review. *Bioresources*, 2(4): 707-738.

Tavakoli O., Yoshida H. (2006) Conversion of scallop viscera wastes to valuable compounds using sub-critical water. *Green Chemistry*, 8: 100-106.

Taylor L.T. (1996) Supercritical fluid extraction, John Wiley & Sons, Inc., New York, USA.

Temelli F., Leblanc E., Fu L. (1995) Supercritical carbon dioxide extraction of oil from Atlantic Mackerel (*Scomber scombrus*) and protein functionality. *Journal of Food Science*, 60: 703-706.

Thiansilakul Y., Benjakul S., Shahidi F. (2007a) Compositions, functional properties and antioxidative activity of protein hydrolyzates prepared from round scad (*Decapterus maruadsi*). *Food Chemistry*, 103: 1385-1394.

Thiansilakul Y., Benjakul S., Shahidi F. (2007b) Antioxidative activity of protein hydrolyzate from round scad muscle using alcalase and flavourzyme. *Food Biochemistry*, 31: 266-287.

Tomita K., Oshima Y. (2004) Stability of manganese oxide in catalytic supercritical water oxidation of phenol. *Industrial and Engineering Chemistry Research*, 43: 2345-2348.

Townsend A.A., Nakai S. (1983) Relationships between hydrophobicity and foaming characteristics of food proteins. *Food Science*, 48: 588-594.

Uddin M.S., Ahn H.M., Kishimura H., Chun B.S. (2009) Comparative study of digestive enzymes of squid (*Todarodes pacificus*) viscera after supercritical carbon dioxide and organic solvent extraction. *Biotechnology and Bioprocess Engineering*, 14: 338-344.

Uddin M.S., Ahn H.M., Kishimura H., Chun B.S. (2010) Production of valued materials from squid viscera by subcritical water hydrolysis. *Journal of Environmental Biology*, 31: 675-679.

Uddin M.S., Kishimura H., Chun B.S. (2011) Isolation and charecterization of lecithin from squid (*Todarodes pacificus*) viscera deoiled by supercritical

carbon dioxide extraction. *Journal of Food Science*, 10: 217-222.

ULPGC (2013) Diet of larvae. http://www.acceda.ulpgc.es/bitstream/10553/327/1/551.pdf

van der Ven C., Gruppen H., de Bont D.B.A., Voragen A.G.J. (2002) Correlations between biochemical characteristics and foam-foaming and -stabilizing ability of whey and casein hydrolyzates. *Journal of Agricultural and Food Chemistry*, 50: 2938-2946.

Van Nieuwenhuyzen W. (1976) Lecithin production and properties. *Journal of American Oil Chemist`s Society*, 53: 425-427.

Vermeirssen V., Camp J.V., Verstraete W. (2007) Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition*, 92: 357-366.

Vilhelmsson O. (1997) The state of enzyme biotechnology in the fish processing industry. *Trends in Food Science and Technology*, 8: 266-270.

Vulfson E.N. (1994) Industrial applications of lipases. In *lipases*, P. Wooley, S.B. Petersen (Ed.), Cambridge University Press, Cambridge, pp 271-288.

Wang G., Wang T. (2008) Oxidative stability of egg and soy lecithin as affected by transition metal ions and pH in emulsion. *Journal of Agricultural and Food Chemistry*, 56: 11424-11431.

Wang L., An X., Xin Z., Zhao L., Hu Q. (2007) Isolation and characterization of collagen from the skin of deep sea redfish (*Sebastes mentella*). *Journal of Food Science*, 72: 450-455.

Wang Q., Zhu X. (2005) Toluene oxidization to benzaldehyde in sub-critical water. *Journal of Chemical Engineering of Chinese University*, 19: 503-506.

Westerhof W., van Ginkel C.J., Cohen E.B., Mekkes J.R. (1990) Prospective randomized study comparing the debriding effect of krill enzymes and a non-enzymatic treatment in venous leg ulcers. *Dermatologica Sinica*, 181: 293-297.

Wilson R.E., Keith P.C., Haylett R.E. (1936) Liquid-propane use in dewaxing, deasphalting, and refining heavy oils. *Industrial and Engineering Chemistry*, 28: 1065-1078.

Wu H.C., Chen H.M., Shiau C.Y. (2003) Free amino acids and peptides as related to antioxidant properties in protein hydrolyzates of mackerel (*Scomber austriasicus*). *Food Research International*, 36: 949-957.

Wu J., Fujioka M., Sugimoto K., Mu G., Ishimi Y. (2004) Assessment of effectiveness of oral administration of collagen peptide on bone metabolism in growing and mature rats. *Journal of Bone and Mineral Metabolism*, 22: 547-553.

Yamaguchi K., Murakami M., Nakano H., Konosu S., Kokura T., Yamamoto H., Kosaka M., Hata K. (1986) Supercritical carbon dioxide extraction of oils from Antarctic krill. *Journal of Agricultural and Food Chemistry*, 34: 904-907.

Yen C., Duh P.D. (1993) Antioxidative properties of methanolic extracts from peanut hulls. *Journal of American Oil Chemist's Society*, 70: 383-386.

Yen G.C., Chen H.Y. (1995) Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of Agricultural and Food Chemistry*, 43: 27-32.

Yoshida H., Takahashi Y., Terashima M. (2003) A simplified reaction model for production of oil, amino acids and organic acids from fish meat by hydrolysis under subcritical and supercritical conditions. *Journal of Chemical Engineering Japan*, 36: 441-448.

Yoshida H., Terashima M., Takahashi Y. (1999) Production of organic acids and amino acids from fish meat by sub-critical water hydrolysis. *Biotechnology Progress*, 15: 1090-1094.

Youdim K.A., Martin A., Joseph J.A. (2000) Essential fatty acids and the brain: possible health implications. *International Journal of Developmental Neuroscience*, 18: 383-399.

Zhang J., Duan R., Huang L., Song Y., Regenstein J.M. (2014) Characterisation of acid soluble and pepsin-solubilised collagen from jellyfish (Cyanea nozakii Kishinouye). *Food Chemistry*, 150: 22-26.

Zhang Y., Liu W., Li G., Shi B., Miao Y., Wu X. (2007) Isolation and partial characterisation of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). *Food Chemistry*, 103: 906-912.

Zhao J., Huang G.R., Zhang M.N., Chen W.W., Jiang J.X. (2011) Amino acid composition, molecular weight distribution and antioxidant stability of shrimp processing by-product hydrolyzates. *American Journal of Food Technology*, 6, 904-913.

Zhao Y., Wang H., Lu W.J., Wang H.T. (2011) Combined supercritical and subcritical conversion of cellulose for fermentable hexose production in a flow reaction system. *Chemical Engineering Journal*, 166: 868-872.

Zheleva-Dimitrova D., Nedialkov P., Kitanov G. (2010) Radical scavenging and antioxidant activities of methanolic extracts from hypericum species growing in Bulgaria. *Pharmacognosy Magazine*, 6: 74-78.

Zosel K. (1964) German patent no. 1, 493, 190.

Summary

The activities of principal digestive enzymes of SC-CO_2 deoiled mackerel muscle residues were increased slightly compared to hexane deoiled muscle residues, it proved the applicability of SFs in enzyme processing. Lecithin obtained by SC-CO_2 with ethanol from SC-CO_2 deoiled mackerel muscle residues showed good oxidative stability and high antioxidant activity. The main phospholipids compositions of deoiled mackerel muscle lecithin were PC and PE with higher amounts of PUFAs mainly EPA and DHA. Most of the protein from SC-CO_2 deoiled mackerel skin residues were recovered as amino acids by SWH showed good antioxidant activities and functionalities. Based on protein patterns, isolated PSC from mackerel bone and skin were type I and maintained triple-helical structure. Lower molecular weight peptides were produced from both PSC after SWH treatment showed high antioxidant activities. Finally, it can be concluded that the bioactive compounds such as digestive enzymes, lecithin, proteins as amino acids and peptide might be obtained from mackerel by environmental friendly solvent, SC-CO_2 and SWH for using in the food industry as well as in the pharmaceutical and cosmetic industry.

아임계 및 초임계유체를 이용한 고등어(*Scomber japonicus*)로부터 생리활성 물질 회수 및 특성

A. K. M. Asaduzzaman

부경대학교 대학원 식품공학과

요약

본 연구에서는 고등어 육 내의 지질을 추출하기 위해 환경 친화적인 용매인 초임계 이산화탄소 (SC-CO_2)를 반 회분식 공정과 및 *n*-헥산을 사용하여 하였다. SC-CO_2는 온도 45°C 에서, 압력은 15-25 MPa 범위에서 수행하였다. CO_2 유량은 27 g/min 로하여 총 추출 시간인 2 시간 동안 지속적으로 흐르게 하였다. SC-CO_2추출 후, 오일 제거 수율이 가장 조건에서 회수된 추잔물을 이용하여 소화 효소의 활성 측정을 위해 사용되었고, *n*-헥산 및 SC-CO_2 추출한 후에 회수된 고등어 추잔물로부터 수용성 추출물을 분리 회수하여 네가지 소화 효소에 대한 활성을 측정하였다. SC-CO_2 처리 후 회수된 추잔물의 수용성 추출물에서 아밀라아제, 리파아제와 트립신 활성이 높았으며, n-헥산 처리 추잔물에서는 단백질 분해 효소가 비교적 높은 활성을 나타내었다. 4 가지 소화 효소 중에서 아밀라아제의 활동이 가장 높았고 그 값은 44.57 uM/min/mg 을 보였다. SC-CO_2와 n-헥산 처리한 고등어 육 추잔물의 수용성 추출물은 소화 효소의 각각에 대해

동일한 알칼리 최적 pH 와 산도 안정성을 보였다. 또한 두 시료에 대한 아밀라제, 리파제, 프로테아제 및 트립신의 최적 온도는 각각 40°C, 50°C, 60°C, 30°C 이었다. 언급 한 최적의 온도에서 두 가지 시료의 수용성 추출물에 대한 아밀라제, 리파제, 프로테아제 및 트립신은 80% 이상 안정성을 유지했다. 단백질 패턴으로 볼 때, SC-CO_2 후 고등어 육 시료의 수용성 추출물은 단백질 밴드의 변형이 없었으며, 이것은 n-헥산 처리한 군과 처리하지 않은 군 보다 단백질의 변성이 일어나지 않은 것을 나타낸다.

레시틴은 초임계 이산화탄소 (SC-CO_2)와 에탄올을 사용한 방법과 헥산 추출법으로 오일이 제거된 고등어 육으로부터 추출, 분리되었다. 고등어 레시틴의 주요 인지질은 포스파티딜콜린 (PC) 20.11%와 포스파티딜 에탄올 아민 (PE) 67.44%였다. Eicosapentaenoic acid (EPA) 와 docosahexaenoic acid (DHA) 는 두 인지질에서 높은 함량을 나타내었다. 품질에 영향을 주는 인자를 검사한 결과, SC-CO_2 에와 에탄올을 사용하여 지질을 제거한 고등어 근육으로부터 분리된 레시틴은 다른 시스템에서 분리된 레시티에 비해 우수한 결과를 보였다. SC-CO_2 에와 에탄올을 사용한 시스템에서 레시틴의 산화 안정성과 항산화 활성은 유의한 차이를 찾을 수 없었다.

고등어 껍질을 동결 건조한 시료와 초임계 이산화탄소 (SC-CO_2)로 처리하여 지질을 제거한 시료를 아임계 수의 온 150-240°C 와

압력 12-210 bar 에서 처리하여 회수된 가수분해물에 대한 항산화활성의 특성, 아미노산의 생산 및 조성, 단백질 가수분해물의 기능성 특성을 조사하였다. 가장 높은 아미노산 수율은 240°C 와 210 bar 조건에서 SC-CO_2 처리하여 지질을 제거한 고등어 육의 가수분해물의 경우 122.96 ± 2.84 ㎎/g, 동결 건조한 121.93 ± 1.80 ㎎/g 을 나타내었다. 가수분해 한 두 시료에서 모두 필수 아미노산 9 종이 확인되었고 그 중에 히스티딘의 함량이 가장 높았다. 모든 필수 아미노산은 트레오닌과 히스티딘을 제외하고 240 °C 까지의 온도 안정성을 보였다. 가수 분해물의 항산화 활성은 DPPH, ABTS, 히드록실 라디칼, 금속 킬레이트, 전력 분석법, 환원력 실험에서 온도 및 압력의 증가에 따라 증가하는 것으로 나타났다. 240°C, 210 bar 에서 두 가수분해물의 항산화 활성이 모두 높았다. 기능성 관점에서, 서로 다른 온도 및 압력에서 회수된 가수 분해물을 분말화시킨 후, 그 분말 단백질의 물에 대한 용해도는 pH 3.5 - 9.5 범위에서 59% 이상으로 증가하였다. 온도 및 압력이 증가 할 때, 유화제 활동 지수, 에멀젼 안정성, 거품 용량 면에서 두 가수분해물의 기능이 모두 줄어들었다. 이것은 가수분해에 의해 짧은 펩티드 사슬이 생성된 것으로 예상된다. 본 연구의 고등어 껍질로부터 생산된 단백질 가수 분해물은 우수한 첨가제로 식품 관련 산업에 이용 될 수 있다고 예상된다.

부산물 또는 비상품성 자원을 효과적으로 사용하는 목적으로 PSC (pepsin solubilized collagen)을 고등어 뼈와 껍질로부터 분리 정제되었다. PSC 의 수율은 껍질에서 8.10%, 뼈에서 1.75%를 보였다. 단백질 패턴에 기초하여, PSC 는 타입 I 과 두 개의 α-사슬 모두 이루어져 있었다. Fourier-transform 적외선 스펙트럼은 뼈와 피부로부터 PSC 의 트리플-나선 구조를 나타낸 것으로 보였다. 뼈와 피부에서 PSC 의 변성 온도 (TD)는 각각 27 과 30°C 였다. 매우 낮은 분자량의 펩타이드 (<1650 Da)가 아임계 물을 가수 분해 처리한 모든 PSC 에서 생성되었다. 글리신은 모든 PSC 가수 분해물에서 확인되었으며, 전체 아미노산의 30%를 차지하였다. 두 PSC 가수 분해물의 항 산화 기능은 가수분해 전의 정제된 PSC 보다 높게 나타났다. 따라서 고등어 뼈와 껍질 유래 PSC 가수 분해물은 식품, 화장품 및 제약 산업의 기능성 소재로 응용될 수 있을 것으로 사료된다.

Acknowledgements

It is a pleasure to convey my deepest gratitude and sincere thanks to Almighty God, who always gives me strength, encourage, hopes and endurance during my lifetime, without his help this dissertation would not have been possible. I am heartily thankful to my esteemed supervisor, Prof. Byung-Soo Chun, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject. All possible conditions necessary for a good work were provided by him without restraints. My gratitude is also expressed to my supervisor family, especially his respected wife, for treating me as a member of their family.

It is my honor to thanks to my thesis evaluation committee, Prof. Young-Mog Kim, Prof. Yang-Bong Lee, Prof. Eun-Woo Lee and Prof. Kyoung-Ho Kim, for their fruitful evaluations, reviews, criticisms and suggestions to improve my thesis. I would like to express my sincere thanks to all professors and friends in Department of Food Science and Technology, who helped me in numerous ways to complete my doctoral dissertation a successful event.

I offer my regards and blessings to all of those who supported me in any respect during the completion of the project. I would like to express my cordial thanks to all of my lab members. I have been blessed with a friendly and cheerful environment in lab: A.S.M. Tanbirul Haque and Yin Shipeng.

My heartfelt thanks go to my wife for her tremendous support, sacrifices and constant encouragement and finally, to my wonderful parents and beloved siblings for their great love, huge support and compassion. I know that there is no way to treat them back during my life for what they did for me, but I am sure they will be in my heart throughout my lifetime. I also extend my cordial thanks to all of my family members, teachers and friends in Bangladesh who inspired me every moment to reach the ultimate destination. I would like to mention some of them in this small space; Md. Enamul Haque, Dr. Md. Salim Uddin and Sifat Rahi.