

Protein properties of mackerel viscera extracted by supercritical carbon dioxide

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Abstract: The extraction of mackerel viscera using supercritical carbon dioxide (SCO₂) was performed under the conditions of temperature range from 35 to 45°C, and constant pressure 25 MPa. The digestive enzyme activities were determined in comparison of untreated and treated SCO₂ and solvent treatment. Activities were maintained with high level compared to that of solvent extraction. Also from result of SDS-PAGE, the protein denaturation was minimized when using SCO₂ extraction. The major amino acids in the mackerel viscera were determined as glutamic acid, aspartic acid, glycine, leucine, lysine and the free amino acids were taurine, L-alanine, L-leucine, 1-methyl-L-histamine, 3-methyl-L-histidine.

Key words: Mackerel viscera, Supercritical carbon dioxide, Digestive enzymes, SDS-PAGE, Amino acids
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Introduction

Mackerel is representative dark fleshed fish with gizzard shad and sardine in Korea. Mackerel is caught in large quantities in the coastal region of Korea. It commonly used as fresh fish, also consumed as canned and salted types. Mackerel contain protein, digestive enzymes such as protease, lipase, and amylase and plenty of polyunsaturated fatty acids (PUFAs) such as EPA, DHA (Lee *et al.*, 1993; Kim *et al.*, 1980). So it is physiologically and nutritionally excellent food source, but it loses its freshness after catch. Moreover protein denaturation and lipid oxidation were occurred during industrial process (Lim *et al.*, 1997). Also mackerel viscera, by-product of fish, include useful materials such as PUFAs, digestive enzymes, but hardly ever used in general.

Protease is very important in digestion as they breakdown the peptide bonds in the protein foods to liberate the amino acids needed by the body. It constitutes the most important group of industrial enzymes used in the world today, accounting for about 50% of the total industrial enzyme market (Rao *et al.*, 1998). Lipase is the most widely used class of enzymes in the chemical industry (Guo and Sun, 2004; Reetz, 2002). They catalyze not only the hydrolysis but also the synthesis of long-chain acylglycerols (Jeon *et al.*, 1999; Kontkanen *et al.*, 2004; Wu and Song, 2002). α -amylase, the enzyme responsible for hydrolyzing starch mainly to maltose, has been detected in all fish species in which the activity has been studied (Cahu *et al.*, 2004; Krogdahl *et al.*, 2005; Moreau *et al.*, 2001; Munilla-Morán *et al.*, 1996; Natalia *et al.*, 2004 and Perez-Casanova *et al.*, 2006).

Supercritical fluid extraction (SFE) has been used for the determination of organic pollutants in environmental solids with variable success (Dean, 1998). In addition, SFE has been shown to produce equivalent or better results compared to other extraction techniques like Soxhlet, sonication and accelerated solvent extraction (Meyer, 1993; Berset *et al.*, 1999; Heemken *et al.*, 1997; Hawthorne *et al.*, 2000; Hartonen *et al.*, 2000; Assis *et al.*, 2000; Hawthorne

and Miller, 1994; Schantz *et al.*, 1998 and Bjorklund *et al.*, 1999). In the supercritical state, the distinction between the liquid and the gas phase has disappeared and the fluid can no longer be liquefied by raising the pressure nor can gas be formed on increasing the temperature. Thus, the physicochemical properties of a given fluid, such as density, diffusivity, dielectric constant and viscosity can be easily controlled by changing the pressure or the temperature without ever crossing phase boundaries (Sihvonen *et al.*, 1999). The main advantages of using supercritical fluids instead of conventional organic solvents are the minimal consumption of organic solvents, the exclusion of oxygen, and the reduction of heat. Modern SFE offers shorter extraction times, potentially higher selectivity and increased sample throughput (due to available automated instruments) compared to conventional solvent extraction techniques (Turner *et al.*, 2001). Several advantages are obtained when using CO₂ in SFE: selectivity, speed and efficiency, oxygen-free environment, minimal post-extraction manipulation, low operating temperature, and low toxicity (Raynie, 1997).

The aims of this study was to obtain a de-oiled protein powder from mackerel viscera after SCO₂ extraction at various conditions (from 35 to 45°C and at 25 MPa) and was to determine protein denaturation. The total and free amino acids in the mackerel viscera were also analyzed and compared with the digestive enzyme activities in mackerel viscera of untreated and treated SCO₂.

Materials and Methods

The mackerel viscera used in these experiments was from the East Sea in Korea. And used after vacuum freeze-dried (SFDSM 24 I, SamWon Freezing Engineering Co.), crushed (Philips, HR1727) and sieved (710 μ m, Chung Gye Sang Gong Sa, Korea). The samples were stored at -60°C in deep freezer (SW-UF-200, Samwon Freezing Engineering Co., Korea).

A schematic diagram of SFE process is shown in Fig. 2. A HP 7680T supercritical fluid extractor (Hewlett-Packard, USA) was



used for SFE. CO₂ with a purity of 99.99% was supplied by Air Liquide (Australia). CO₂ required for cooling different zones in the SFE apparatus, was used as cryo gas. Also all other reagents are analytical grade and HPLC grade supplied by Sigma Co. (USA).

One gram mackerel viscera samples were filled into the 7 ml stainless steel extraction vessels. This thimble was plugged first with filter paper? Then it was filled with sample and again plugged with filter paper. The caps at each end contain porous frits to hold the sample in place and form high-pressure seals when the extraction chamber closes. The CO₂ was pumped and allowed to pass through the vessel with various oven temperatures at the constant pressure (35°C, 24.7 MPa; 40°C, 25.0 MPa; 45°C, 25.3 MPa). The flow rate of CO₂ was set to 3 ml/min. The 30 seconds equilibrium time and 40 min dynamic time with 3 min interval were selected. The modifier is not used in this work. The components extracted were collected on an octadecylsilane (ODS) (Hewlett-Packard, USA) trap and were rinsed out to collection vials using 1.5 ml of *n*-hexane. The nozzle temperature was kept constant at 50°C and the trap temperature was kept at 45°C.

For determination of digestive enzyme activity, samples are crushed and sieved (710 μm), then added 0.05 M Tris-HCl buffer (pH 7.5) and centrifuged (3,000 rpm, 20 min). The supernatant was filtered (Advantec, No. 5A), and this was used as crude enzyme. Protease assay was carried out using modified Casein-Folin method (Oda and Muraio, 1974). Activity measured by using 1% casein solution in 12.5 mM sodium borate NaOH buffer (pH 10.5) as substrate. 0.5 ml of crude enzyme was mixed with 2.5 ml of substrate and incubated for 10 min at 37°C. The reaction was stopped by addition of 2.5 ml TCA solution and allowed to settle for 20 min. Then samples were centrifuged for 10 min at 2,000 rpm. 2 ml of supernatant mixed with 5 ml of 0.55 M Na₂CO₃. Thereafter 1 ml of 1 N Folin-Ciocalteu reagent was added, and absorbance was measured at 660 nm wavelength using UV-VIS spectrophotometer (UVIKON 933, Kontron, Germany). The protease unit is defined as the amount of enzyme which releases 1 mmol of tyrosine per minute from casein. Lipase assay was determined by the modified titrimetric method of Mukundan *et al.* (1985). 0.5 ml of enzyme solution was mixed with 1 ml of olive oil emulsified with polyvinyl alcohol as substrate (Yamada *et al.*, 1962) and 0.5 ml of McIlvaine buffer (McIlvaine, 1921) (pH 8.0). This mixture was incubated at 37°C for 4 hr. The reaction was terminated by the addition of 3 ml of 1:1 (v:v) ethanol-acetone solution. Blank determinations were conducted in a similar manner, using boiled enzyme instead of the enzyme solution. Lipase activity was estimated by titration with 0.01M NaOH using 1% phenolphthalein as indicator. One unit of lipase is expressed as the amount required liberating 1 μmol of fatty acid per minute. α-amylase assay was determined by the method of KFDA (2005) and determined by the number of reducing groups exposed during α-amylase hydrolysis. Potato starch solution (10 ml) was used as substrate and was incubated for 10 min at 37°C. And 1 ml of sample was added and incubated again. Then Fehling reagent

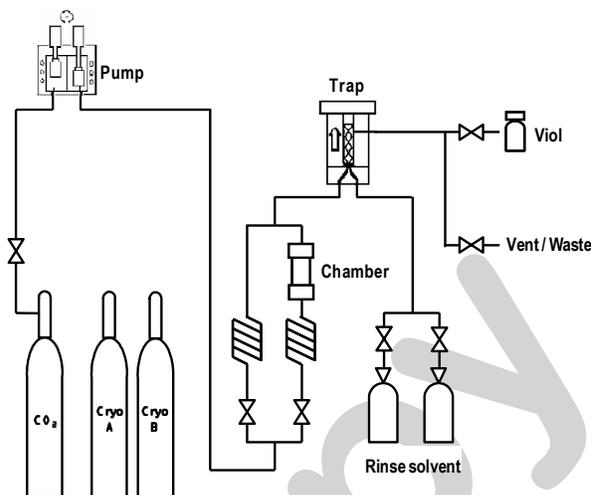


Fig. 1: A schematic diagram of supercritical fluid extraction (SFE) process using CO₂

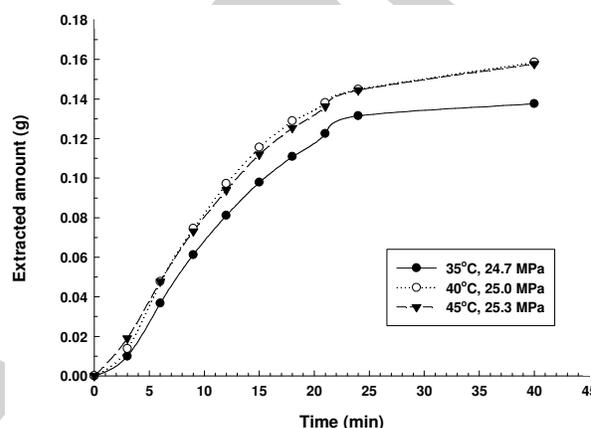


Fig. 2: Effect of temperatures and pressures on the amount of oil extracted by SCO₂ from mackerel viscera

was added and this was placed in a boiling water bath for 15 min and then cooled. Two ml of concentrated potassium iodide solution was added followed 2 ml of diluted sulfuric acid. Blank determinations were conducted in a similar manner, using water instead of the substrate. α-amylase activity was determined by titration with 0.05 N sodium thiosulfate using starch solution as indicator. One unit is defined as the amount of enzyme increasing reducing power corresponds to 1 mg of glucose per minute.

SDS-PAGE analysis was performed. The untreated and treated SCO₂ samples of mackerel viscera were used. Protein solutions were initially prepared by mixing 0.1 g of sample with 1 ml of distilled water. Then each sample was centrifuged and supernatant was collected. 500 μl 1:1 (v:v) of sample of SDS-PAGE disruption mix: 0.5 M Tris-HCl (pH 6.8) / 2-mercaptoethanol/10% SDS / 50% glycerol, containing a little bromophenol blue was added. Then sample was incubated for 5 min at 100°C and analyzed by reducing SDS-PAGE electrophoresis in 12% polyacrylamide gels. Electrophoresis was performed using a Mini-Protein III cell module

Table 1. Amino acid composition of mackerel viscera untreated and treated with SCO_2 (mg/100 g)

	Mackerel viscera	
	Untreated	SCO_2 treated
Essential amino acids -	15.47	22.63
Arginine	2.24	3.32
Histidine	1.29	1.91
Isoleucine	1.52	2.29
Leucine	2.72	3.96
Lysine	2.58	3.77
Phenylalanine	1.49	2.21
Threonine	1.62	2.32
Valine	2.01	2.85
Non-essential amino acids -	18.21	26.21
Alanine	2.61	3.72
Aspartic acid	3.47	5.02
Glycine	2.99	4.09
Glutamic acid	4.55	6.63
Proline	1.93	2.52
Serine	1.68	2.50
Tyrosine	0.98	1.73
Total	33.68	48.84

(Bio-Rad Laboratories, CA, USA) at a constant voltage (100 V for 2 hr). The gels were stained with 0.1% Coomassie Brilliant blue R-250. The destaining was performed in methanol/acetic acid solution. SigmaMark™ Wide Molecular Weight Range (Sigma Co., USA) was used as molecular weight standards from 6.5 to 205 kDa for SDS-PAGE.

The untreated and treated SCO_2 samples of mackerel viscera were used for their amino acids compositions. For the analysis of total amino acids, the samples were crushed and mixed with 50 ml of sodium citrate buffer solution (pH 2.2), and then concentrated using 6 M HCl. Similarly, samples were prepared to analyze the free amino acids, centrifuged and filtered and then concentrated by 75% ethanol treatment. The amount of free amino acids was determined using a S433 amino acid analyser (Sykam, Gilching, Germany) under the following conditions: column size 4 mm i.d. × 150 mm, lithium form resin, analysis cycle time 160 min, reactor temperature 130°C, reactor size 15 m, flow rates 0.45 ml/min for buffer and 0.25 ml/min for ninhydrin.

Results and Discussion

The extraction curves of mackerel viscera are illustrated in Fig. 2. These curves are obtained by summing the extracted oil magnitude of the samples over time. They were acquired during extractions from 35 to 45°C and at the 25 MPa. The extraction time of at least about 24 min can be considered sufficient to extract oil from the samples. Extracted oil amount was increased with an increasing temperature. The extracted amounts at 40 and 45°C are similar, and indicated low extracted amount at 35°C. But there were no remarkable differences in extraction amounts.

Table - 2: Free amino acid composition of mackerel viscera untreated and treated with SCO_2 (mg/100 g)

Free amino acids	Mackerel viscera	
	Untreated	Treated
Phosphoserine	0.023	0.027
Phosphoserine	0.023	0.027
Taurine	0.476	0.789
Phosphoethanolamine	ND	ND
Urea	ND	ND
L-Aspartic acid	ND	ND
L-Threonine	ND	ND
L-Serine	ND	ND
Asparagine	ND	ND
L-Glutamic acid	ND	ND
L- α -Aminoadipic acid	ND	ND
L-Proline	0.161	0.254
L-Glycine	0.075	0.116
L-Alanine	0.365	0.627
L-Citrulline	ND	ND
L- α -Aminobutyric acid	ND	ND
L-Valine	0.206	0.342
L-Cystine	ND	ND
L-Methionine	ND	0.083
L-Isoleucine	0.162	0.294
L-Leucine	0.351	0.606
L-Tyrosine	0.183	0.429
L-Phenylalanine	0.171	0.334
β -Alanine	ND	ND
DL- β -Aminoisobutyric acid	ND	ND
γ -Aminobutyric acid	ND	ND
L-Ornithine	0.157	0.278
L-Lysine	0.032	0.061
1-Methyl-L-Histidine	0.322	0.611
L-Histidine	ND	ND
3-Methyl-L-Histidine	0.372	0.511
L-Carnosine	ND	ND
L-Arginine	ND	ND
Total	3.056	5.362

ND - means quantity not detected

The activities of digestive enzyme are shown in Fig. 3. In Fig. 3(a), the change of activity after SCO_2 and solvent extraction of protease is shown. Mackerel viscera maintain 67.3% after SCO_2 extraction. Also mackerel viscera maintain 36.3% after chloroform extraction and 39.2% after ethyl ether extraction. Lipase activity is shown in Fig. 3(b). Lipase activity of mackerel viscera was 83.3% after SCO_2 extraction, 57.7% after chloroform extraction and 55.1% after ethyl ether extraction. In Fig. 3(c), α -amylase activity of SCO_2 treatment, solvent extraction is shown. α -amylase activity of mackerel viscera was 84.1% after SCO_2 extraction, 9.5% after chloroform extraction and 22.2% after ethyl ether extraction. These results indicated that using SCO_2 , digestive enzyme denaturation was minimized than solvent extraction. But there was a loss of enzyme activity after SCO_2 extraction. This has been attributed to the interactions between CO_2 and the enzyme (Knez and Habulin, 2002; Kamat *et al.*, 1995a; Habulin and Knez, 2001; Kamat *et al.*, 1995b).



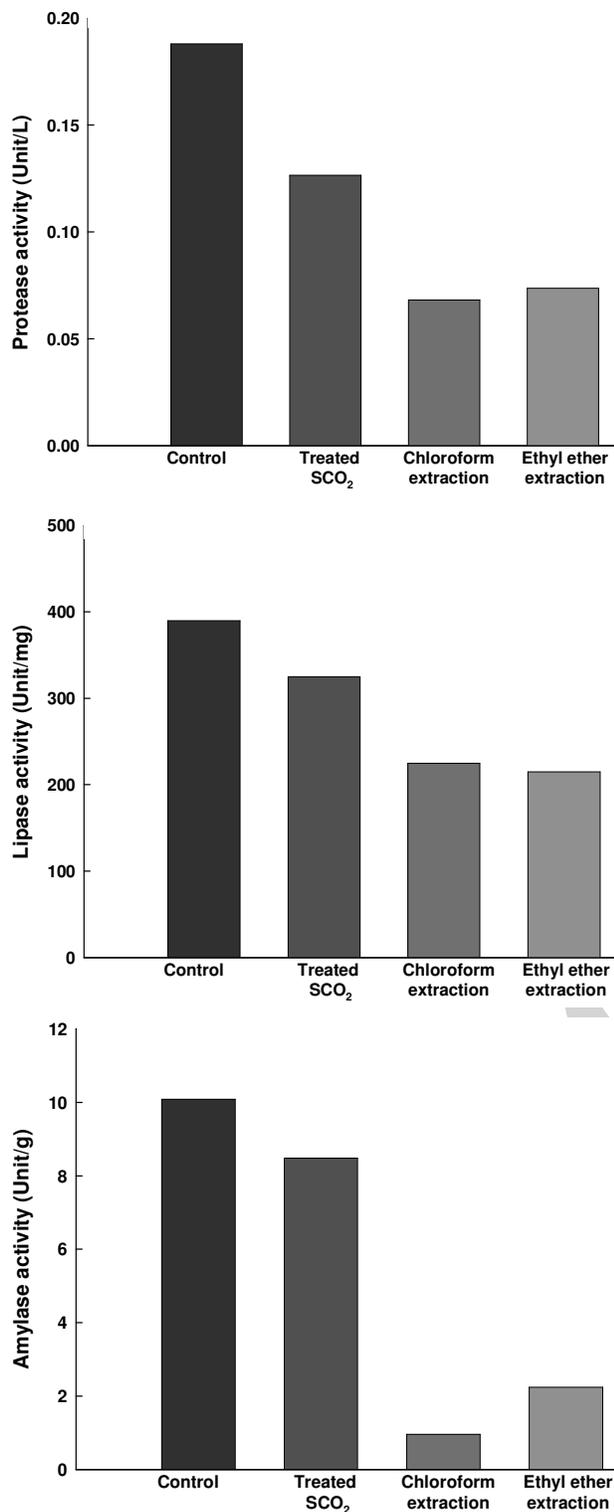


Fig. 3: Digestive enzyme activity of mackerel viscera untreated and treated with SCO₂ (a) protease activity, (b) lipase activity, (c) α -amylase activity

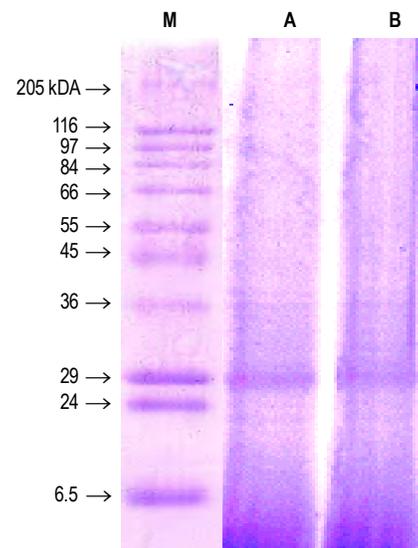


Fig. 4: SDS-PAGE pattern of mackerel viscera untreated and treated with SCO₂ (M; molecular weight standard, a; untreated mackerel viscera, b; SCO₂ treated mackerel viscera)

This means CO₂ may form covalent complexes with free amino groups on the surface of the enzyme.

The electrophoretic patterns of SCO₂ untreated and treated mackerel viscera were compared in Fig. 4. A main single band of mackerel viscera was estimated to be approximately 29 kDa and shows no change in intensity after treatment with SCO₂. In this result, due to moderate temperature used in SCO₂ extraction procedure denaturation of protein was minimized.

The total contents of total and free amino acids are shown in Table 1, 2. As shown in Table 1, 15 amino acids of mackerel viscera were identified. Among the total amino acids, eight essential (arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, and valine) and seven non-essential (alanine, aspartic acid, glycine, glutamic acid, proline, serine, and tyrosine) amino acids were determined. Glutamic acid (4.55%) was the highest contained component of mackerel viscera, followed by aspartic acid (3.47%), glycine (2.99%), leucine (2.72%) and lysine (2.58%). This is in agreement with Toppe *et al.* (2007). Three major components such as aspartic acid, glycine and glutamic acid are known to play a key role in the process of wound healing (Chyun and Griminger, 1984). Especially glutamic acid and glycine were commonly recognized to be taste-active in seafoods (Fuke and Konosu, 1991). Essential amino acids contain 15.47% in total amino acids. After SCO₂ extraction, essential amino acids composition increased to 22.63%. Moreover total amino acids amounts were increased from 33.68% to 48.84% after SCO₂ extraction. Table 2 shows the results of the free amino acid analyses. The total content of free amino acids in the SCO₂ treated mackerel viscera was higher than in the untreated

material. The predominant free amino acids were taurine, L-alanine, L-leucine, 1-methyl-L-histamine, 3-methyl-L-histidine. Taurine has been tested medically in the treatment of congestive heart failure, cystic fibrosis, diabetes, epilepsy and several other conditions and is also one of the ingredients commonly found in energy drinks (Kendler, 1989). Free amino acids contain 3.056% in raw material and it increased to 5.362% after SCO_2 extraction. Therefore, SCO_2 treated mackerel viscera seems to be a proper source than untreated raw material as fish-protein.

In SCO_2 extracted mackerel viscera samples, the protein denaturation was minimized. Therefore SFE technology is useful method to manufacturing functional materials using fish by-products. Furthermore from the viewpoint of protein-sourced materials, SCO_2 extraction would be a better substitute for the conventional solvent extraction.

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