Comparative Study of Digestive Enzymes of Krill (***Euphausia superba***) After Supercritical Carbon Dioxide and Organic Solvent Extraction

Ali-Nehari Abdelkader and Byung-Soo Chun*
International program of fisheries Sciences, Faculty of Fisheries Sciences
Pukyong National University, Pusan 608-737, Korea
E-mail: bschun@pknu.ac.kr Tel: +82-51-629-5830 Fax: +82-51-629-5824

ABSTRACT

In recent years, recovery and characterization of enzymes from fish and aquatic invertebrates has been achieved and some interesting and novel applications related to marine enzymes in food processing have emerged. In this study, Enzyme activities of krill were characterized before and after lipid extraction by supercritical carbon dioxide (SCO_2) and organic solvent, n-hexane and acetone. Krill SCO_2 extraction was performed under the conditions of temperature range from 35-45°C and pressure, 150-250 bar for 2.5 h with a constant flow rate of 22 g/min. Extraction yields of lipids increased with pressure and temperature. The digestive enzyme activities of protease, lipase and amylase of SCO_2 treated krill residues were slightly decreased comparing to organic solvent, n-hexane and acetone treated residues. In SCO_2 treated samples, all of the digestive enzymes showed slightly higher temperature stability. In the other hand the crude extracts of SCO_2 and n-hexane treated krill samples showed almost same optimum pH and pH stability for each of the digestive enzymes. It was also found in SDS-PAGE that there are no significant differences in protein patterns of the crude extracts of untreated and SCO_2 and n-hexane and acetone treated krill indicating no denaturation of proteins.

INTRODUCTION

Enzymatic methods have become an important and indispensable part of the processes used by the modern food and feed industry to produce a large and diversified range of products for human and animal consumption [1]. In general, enzyme technology has evolved to become an integral part of the food industry. Early advances focused on ways to control endogenous enzyme activities. Later on enzymes were developed as processing aids and as valuable tools for ingredient production [2]. Most enzymes from fish and aquatic invertebrates are also present in terrestrial organisms [3]. However, variations have been observed among these two groups due to differences in molecular weight, amino acid composition, pH optimum, temperature optimum, and stability, inhibition characteristics and kinetic properties [4]. Enzymes have also many other industrial applications. Proteases are enzymes that cleave peptide bonds with different specificities. They represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, pharmaceutical industry and bioremediation processes [5]. Because of their biotechnological potential, lipases are attracting an enormous attention. 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 [6]. Amylases constitute a class of industrial enzymes, which alone form approximately 25% of the enzyme market covering many industrial processes such as sugar, textile, paper, brewing, distilling industries and pharmaceuticals [7].

At present, the utilization of krill is limited to meat extracts, dried products, feed for fishing and culture fisheries, and a material for carotenoid pigments. Krill also offers the advantage of being high in the omega-3 polyunsaturated fatty acids [8]. Currently, a large number of methods are available to extract lipids from biological materials. Most of them use organic solvents, usually in mixtures containing chloroform and methanol, as in the procedures [9]. Removal of lipids with organic solvents causes protein denaturation and loss of functional properties [10]. And they are also harmful for human health as well as environment. The supercritical fluid extraction (SFE) method is very advantageous and environmentally friendly over other conventional either solvent or enzyme extraction methods for recovering natural oil. Use of SFE technology that offers suitable extraction and fractionation appears to be promising for the food and pharmaceutical industries [11]. Supercritical carbon dioxide (SCO_2) has been used for extraction of lipid from different marine organisms such as krill [12]. However, after extraction the leftover materials are rich sources of various enzymes that may have some unique properties of interest for both basic research and industrial applications. The present study was designed to investigate the digestive enzyme activities

and characterize the crude enzymes from krill after lipid extraction by SCO_2 and organic solvent, n-hexane and acetone.

MATERIALS AND METHODS

Materials

Krill were collected from F & F Co., Busan, Korea. The sample were drained for 2-3 min and frozen within 5-15 min after catching. The krill blocks were stored at -80°C for no longer than 1 year before being used experimentally. The carbon dioxide (99.99% pure) was supplied by KOSEM, Korea. All other chemicals used in different analysis were of analytical or HPLC grade.

Sample preparation

The krill samples were dried in a freeze-drier for about 72 hours. The dried samples were crushed and sieved (700 ㎛) by mesh. These samples called raw krill were then stored at -80°C and used for oil extraction by SCO_2 and organic solvent.

Supercritical CO_2 extraction

A laboratory scale supercritical fluid extraction unit was used for extracting oil from krill. Forty five grams of freeze dried krill sample were loaded into 200 mL stainless steel extraction vessel containing cotton at the bottom. Before plugging with cap another layer of cotton was used at the top of the sample. CO_2 was pumped into the vessel by high pressure pump up to the desired pressure, which was regulated by a back pressure regulator. The vessel temperature was maintained by heater. Flow rates and accumulated gas volume passing through the apparatus were measured using a gas flow meter. The extracted oil was collected by a cyclone separating vessel. After SCO_2 extraction, the krill residues remaining in the vessel were stored at -80°C until further analysis.

Organic solvent extraction

Lipid extraction from krill samples were performed by n-hexane and acetone using soxhlet apparatus. The extraction time was 12 h. After extraction the krill residues remaining in soxhlet apparatus were stored at -80°C.

Digestive enzyme assay

Preparation of crude enzyme

The highest oil extracted residues of krill by SCO_2 , n-hexane and acetone treated krill residues were homogenized in cold distilled water (1g sample/6 mL water) by mechanical

stirring at 4°C for 2 h. The samples were then centrifuged at 9000 rpm for 15 min at 4°C. The supernatant were collected and stored at -20°C. These samples were used as crude enzyme extract and also for electrophoresis.

Protease assay

Protease activity was assayed by the casein Folin-Ciocalteau method [13] with slight modification. One percent casein solution in 0.0125 M sodium borate-NaOH buffer (pH 10.5) was used as substrate. Crude enzyme (0.5 mL) was mixed with 2.5 mL of substrate and incubated for 10 min at 37ºC. The reaction was stopped by addition of 2.5 mL of TCA solution and settled for 20 min. Then the sample was centrifuged for 10 min at 3000 rpm. The supernatant (2 mL) mixed with 5 mL of 0.55 M Na_2CO_3 and 1 mL of 1 N Folin-Ciocalteu reagent. The optical density of the colour developed at 37°C for 20 min was measured at 660 nm (UVIKON 933, Kontron Instruments). One unit of protease activity was defined as the amount of enzyme required for liberating 1 µmol of tyrosine per min from casein.

Lipase assay

Lipase activity was assayed by the modified method of Vorderwülbecke et al. [14] described by Hatzinikolaou *et al.* [15]. The substrate emulsions were prepared by drop wise addition of 0.2 mL solution A (40 mg of *p*-nitrophenyl-laurate was dissolved in 12.0 mL of isopropanol) into 3.0 mL solution B (0.4 g Triton X-100 and 0.1 g gum arabic were dissolved in 90 mL of 0.1 M potassium phosphate buffer, pH 7.0) under intense vortexing. These emulsions were stable for 1 h at room temperature. The crude extract (0.1 mL) was added to 3.2 mL of the substrate emulsion and the mixture was incubated for 20 min in a shaking water bath at 35°C. The reaction was terminated by boiling for 5 min. Following centrifugation (6000 rpm, 10 min) the absorbance of the clear supernatant was measured at 410nm. The mixture with 0.1 mL of the inactivate enzyme extract (heated at 100°C for 5 min) was used as control. One unit of enzyme activity was defined as the amount of enzyme required for the liberation of 1 µmol *p*-nitrophenol from *p*-nitrophenyl-laurate per minute under the assay conditions.

Amylase assay

Amylase activity was determined by measuring the formation of reducing sugars released during starch hydrolysis. The reaction mixture containing 0.5 mL of enzyme extract and 0.5 mL of 1.0% (w/v) potato starch (Sigma) in 100 mM acetate buffer (pH 6.5) was incubated at 37°C for 10 min. The amount of liberated reducing sugar was determined by the dinitrosalicylic (DNS) acid method [16]. One unit of amylase activity was defined as the amount of enzyme that released 1 µmol of reducing end groups per minute. D-Glucose was used to construct a standard curve.

Effect of temperature and temperature stability of enzymes

For the determination of optimal temperature for enzyme activities; potassium phosphate (0.1M, pH 7.5) buffer for amylase and 0.1 M glycine-NaOH (pH 8.5) buffer for protease and lipase were used at different temperatures (20-80°C). Temperature stability of the crude enzyme was tested by pre-incubating the enzyme extract at different temperatures (20-80°C) for 1 h and the residual enzyme activities were assayed under standard assay conditions.

Electrophoresis

According to the method of Laemmli [17] sodium dodecyl sulfate-polyacrylamaide gel electrophoresis (SDS-PAGE) of crude extracts were carried out by using 5% (w/v) stacking gel and 12% (w/v) separating gel. Electrophoresis was performed using a Mini-Protein III cell module (Bio-Rad Laboratories, CA, USA) at a constant voltage (100 V for 2 h). After electrophoresis the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 for 2 h and destained in 50% methanol (v/v) and 10% (v/v) acetic acid solution for a couple of hours; and washed with destaining solution containing 40% methanol and 10% acetic acid. The SDS-PAGE gels included standard proteins as molecular weight markers (Sigma).

RESULTS

SCO_2 extraction

The extraction curves of krill oil by SCO_2 at different temperatures (35, 40 and 45°C) and pressure (15, 20 and 25 MPa) are shown in Figure1. Depending on the pressure and temperature, the amount of extracted oil was increased with the increasing of CO_2 mass. The amount of extracted krill oil per solvent (CO_2) mass used was increased constantly over the entire extraction period, until almost all the oil was extracted. After SCO_2 extraction at low pressure and low temperature, krill residues contained still oil. At constant temperature, the amount of oil extracted from krill was increased with the pressure. Due to the increase in pressure, the density of the SCO_2 was increased and hence the solvating power.

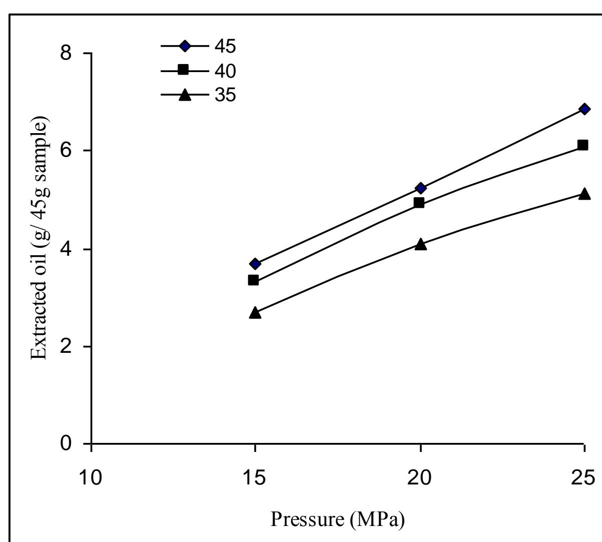


Figure 1: SCO_2 extraction of oil at different temperatures and pressure.

The effect of pressure can be attributed to the increase in solvent power and by the

strengthening of intermolecular physical interactions [18-19]. The amount of oil extracted was highest at 45°C as compared to other conditions.

Digestive enzyme activities

The activities of protease, amylase and lipase of crude enzyme extracts of krill are represented in the Figure 2. Among the three classes of digestive enzymes, the activity of lipase was highest. The highest activities of protease, lipase and amylase were found in n-hexane treated krill samples comparing to acetone and SCO_2 treated. The digestive enzyme activities of krill samples might be lost due to the treatment by SCO_2. Prior research has been showed that the loss of enzyme activity after SCO_2 treatment attributed to the interactions between CO_2 and the enzyme. This means CO_2 may form covalent complexes with free amino groups on the surface of the enzyme [20, 21].

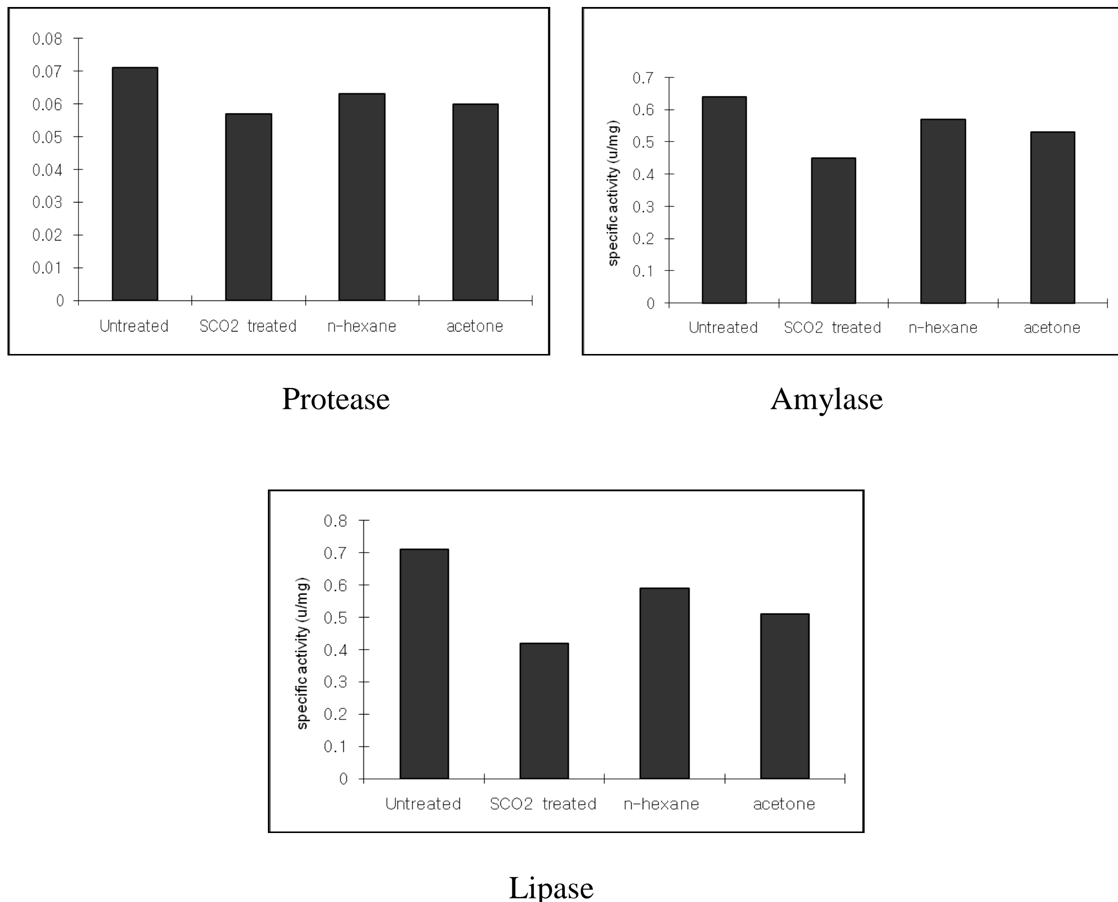


Figure 2: Activities of digestive enzymes of SCO_2, n-hexane treated and acetone treated krill

Optimum pH of protease, lipase and amylase

The highest proteolytic activities in all treated krill extracts were found at pH 8.5. High protease activities at pH ranging from 8.0 to 10.0 have also been reported in several fish species [22]. Low protease activities were also found in acidic pH. This means that the crude extracts of krill contain both the acidic and alkaline proteases. Similar results were found by Natalia *et al.* [23] from carnivorous ornamental fish.

The crude enzyme of krill exhibited optimum lipolytic activity at pH 8. The optimum pH 7-9 for lipase activities were reported for fish and other sources [24]. The activities of amylase in the crude extracts of krill were maximal at pH 7.5. Optimum pH 7-7.5 for amylase was reported in gut of seabream and turbot [25]. Very small activities of lipase and amylase were found in acidic pH due to the presence of acidic lipase and amylase. The differences of pH effect on digestive enzyme activities were not significant in all treated crude extracts.

PH stability

The results show that in the pH range 8.5-10.5, the protease activities were more than 90% of its original and then decreased with increasing pH. Investigation on pH stability of crude enzyme extracts of tuna viscera were in the range of 9-11 with more than 90% activity [26]. The crude extract lipase was stable within a pH range of 7-9.5 with 90% activities. Previous studies reported the stability of lipase at pH range of 8-10.5 and 7-10, respectively. The stability of amylase of krill extract was in pH range of 6-8.5 (almost 90% activity). The changes of pH stability range in SCO_2 and organic solvents treated krill crude extracts were not significant.

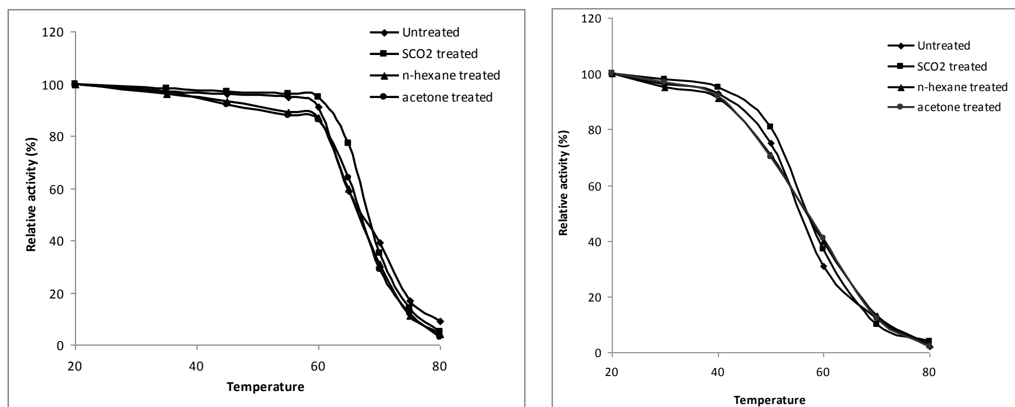
Optimum temperature of protease, lipase and amylase

The enzyme protease, lipase and amylase in both SCO_2 and organic solvents treated krill extracts maintained above 50% activity over a temperature range of 20–60 °C with the optimum at the temperature of 60, 45 and 37°C, respectively. These results are in accordance with those previously reported from S. oryzae [27]. The differences in temperature optima may be due to several factors including the varying mechanical properties of the homologous lipases, the different substrates used for measurements since different substrates may and do exhibit temperature activity differences with enzymes.

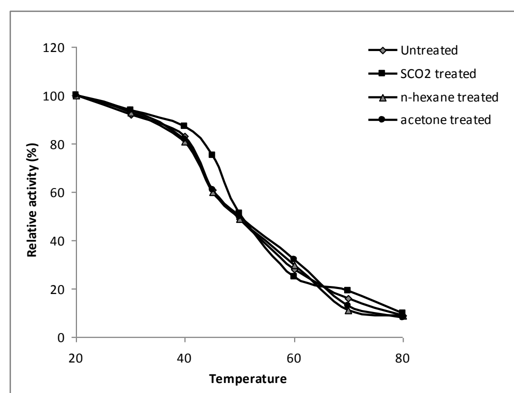
Temperature stability

The temperature stability of digestive enzymes of crude extract of SCO_2 and organic solvents treated krill are showed in Figure 3.

The protease activities of SCO_2, n-hexane and acetone treated krill residues were remained above 80% up to 60°C. Above temperature of 60°C the activities declined sharply. But SCO_2 treated sample retained about 80% of protease activity at 65°C where as n-hexane treated samples contained 66% activities. The crude extracts of krill retained above 75% of lipase activity up to 50°C. The crude extract of SCO_2 treated krill showed slightly higher temperature stability of lipase after 50°C. For amylase, more than 80% of activities were found up to 40°C. Previous studies reported the similar temperature stability of α-amylase. At 45°C, the SCO_2 treated sample contained higher amylase activity than that of n-hexane and acetone treated. Protease, lipase and amylase of SCO_2 treated krill showed slightly higher temperature stability comparing to organic solvent treated krill. This might be happened due to very little change of conformation or properties of active site by temperature and high pressure during extraction by SCO_2.



Protease Lipase



Amylase

Figure 3: Temperature stability of digestive enzymes

Electrophoresis

The SDS–PAGE of marker protein and untreated and SCO_2 and organic solvent treated krill extracts are shown in Figure 4. The proteins in untreated krill were almost similar in subunit composition to SCO_2 and organic solvent treated krill. Results obtained by substrate SDS-PAGE confirm that there was no any change in intensity of protein bands since the gel banding patterns observed were almost identical. From this observation it can be concluded that protein denaturation was not found in SCO_2 treated krill sample. Stahl *et al.* [28] reported very little denaturation of protein in SCO_2 treated seed residue.

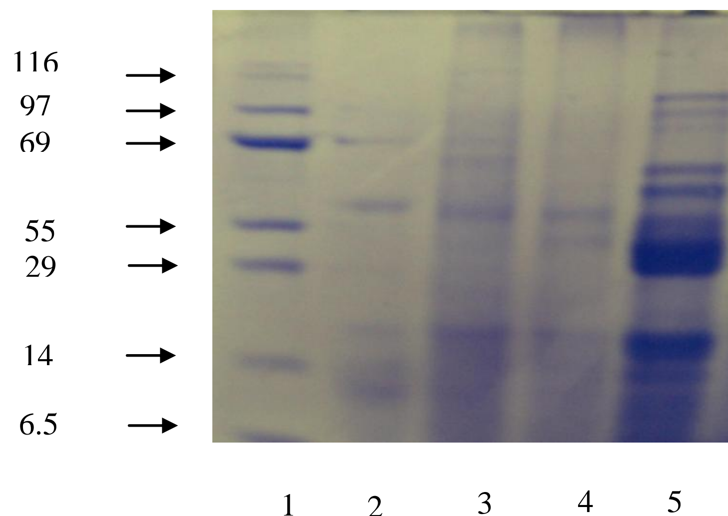


Figure 4. SDS-PAGE electrophoresis of crude protein of untreated and SCO_2 n-hexane, and acetone treated krill extracts.Lane 1: Molecular Weight Marker (MWM en kDa). Lane 2: SCO_2 treated krill extract. Lanes 3: n-hexane treated krill extract. Lanes 4: acetone treated krill extracct. Lanes 5: untreated krill extract.

CONCLUSION

The study indicates that the supercritical carbon dioxide removed almost all lipids from krill at pressure, 25 MPa and temperature, 45°C. The digestive enzyme activities of SCO_2 treated krill residues were slightly decreased comparing to organic solvent treated residues. In SCO_2 treated samples, all of the digestive enzymes showed slightly higher temperature stability. No denaturation of proteins was found in SCO_2 treated samples. The use of carbon dioxide for

lipid extraction is environment friendly. Results obtained in the present work point to the possibility to use krill after lipid extraction by supercritical carbon dioxide in place of organic solvent , for isolation and purification of different digestive enzymes. In addition, the unique pressure dependence of supercritical fluid physical properties presents opportunities for rational control of enzyme activity, specificity, and stability.

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