Comparison of protease properties from skipjack tuna viscera after defatting by supercritical carbon dioxide and organic solvent

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Abstract
The characterization of protease from tuna viscera were carried out after lipid extraction by supercritical carbon dioxide (SCO_2) and organic solvents, n-hexane. Supercritical extraction process for lipid isolation was performed at different temperatures (35-45°C) and pressures (15-25 MPa) for 2.5 h. The mass flow rate of carbon dioxide was 22g/min entire the extraction process. The amount of oil extracted by SCO_2 was increased with the temperature and pressure. The highest oil extracted residues of tuna viscera were used for characterization of protease. The organic solvent treated residue of tuna viscera showed higher enzyme activity than that of SCO_2 treated residue. The crude extracts of SCO_2 and n-hexane treated tuna viscera retained the almost similar optimum pH and pH stability for protease. The optimum temperature for protease was also same in both the crude extracts of SCO_2 and n-hexane treated tuna viscera. But thermal stability was slightly higher in SCO_2 treated residue comparing to n-hexane treated residue of tuna viscera. The crude extracts of untreated and SCO_2 and n-hexane treated tuna viscera showed no differences in protein pattern at SDS-PAGE.

INTRODUCTION

Enzymes have been used as processing aids in various food related industries for a long time [1]. In general, enzyme technology has evolved to become an integral part of the food industry. Enzymes have also many other industrial applications. Proteases execute a large variety of functions and have important biotechnological applications. Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, pharmaceutical industry and bioremediation processes [2].

Tuna viscera are non-edible parts produced in large quantities in Korea by the fish processing industry as a by-product. The disposal of these by-products is a major problem for organized industries as well as for instant processing in market places. These materials like other fish, however, are rich sources of various enzymes that may have some unique properties of interest for both basic research and industrial applications [3].

For higher efficiency of enzyme isolation and purification, lipid must be removed from the sample. Conventional methods for removal of oil from proteins involve cooking, pressing and/or liquid extraction. Removal of lipids with organic solvents causes protein denaturation and loss of functional properties [4]. Organic solvents are also harmful for human health as well as environment. Supercritical fluid extraction (SFE) technology is used as an alternative for lipid extraction to organic solvent extraction. Supercritical carbon dioxide (SCO_2) has been used for extraction of lipid from different marine organisms [5-6]. But attentions have

been given on the supercritical fluid extracted essential oils for polyunsaturated fatty acids and other bioactive materials. The aim of this study was to characterize the crude protease from tuna viscera after lipid extraction by SCO_2 and organic solvent, n-hexane.

MATERIALS AND METHODS

Materials

Tuna viscera were supplied by Dongwon Co. Ltd, Busan, Korea. The visceral waste was brought to the laboratory in iced condition. 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 tuna viscera samples were dried in a freeze-drier for about 72 h. The dried samples were crushed and sieved (700 ㎛) by mesh. These samples 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 tuna viscera (Fig. 1). 25 g of freeze dried tuna viscera 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 (15-25 MPa), which was regulated by a back pressure regulator. The vessel temperature (35-45ºC) was maintained by heater. Flow rates and accumulated gas volume passing through the apparatus were measured using a gas flow meter. The flow rates of CO_2 were kept constant at 22 g/min for 2.5 h in all extraction conditions. The extracted oil was collected by a cyclone separating vessel. After SCO_2 extraction, the tuna viscera residues remaining in the vessel were stored at -80°C until further analysis.

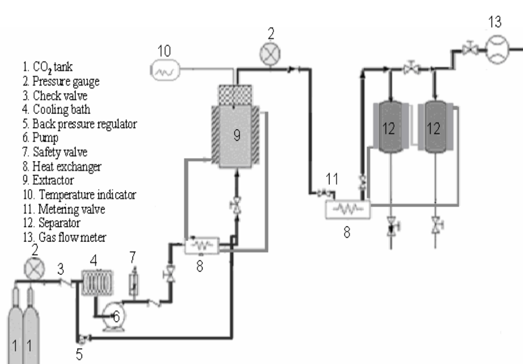

Fig. 1: Schematic diagram of supercritical carbon dioxide extraction process.

Organic solvent extraction

Lipid extraction from tuna viscera samples were performed by n-hexane using soxhlet apparatus. The extraction time was 12 hours. After extraction the viscera residues remaining in thimble were stored at -80°C.

Enzyme assay

Preparation of crude enzyme

The highest oil extracted residues of tuna viscera by SCO_2 and n-hexane treated viscera 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 [7]. One unit of protease activity was defined as the amount of enzyme required for liberating 1 μmol of tyrosine per min from casein.

Effect of pH and pH stability

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

Effect of temperature and temperature stability

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

Electrophoresis

Sodium dodecyl sulfate-polyacrylamaide gel electrophoresis (SDS-PAGE) of crude extracts were carried out by the method of Laemmli [8] using 5% (w/v) stacking gel and 12% (w/v) separating gel.

RESULTS AND DISCUSSION

SCO_2 extraction

The extraction curves of tuna viscera oil at different temperatures (35, 40 and 45°C) and pressure (15, 20 and 25 MPa) are shown in Fig. 2. Oil extraction yield increased with increasing extraction pressure and temperature. The amount of oil extracted was highest at 45°C and 25 MPa. At constant temperature, the amount of oil extracted from tuna viscera was increased with the pressure. Due to the increase in pressure, the density of the SCO_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. The amount of oil extracted was highest at 45°C as compared to other conditions. Despite of

the decreasing of solvent's density, the oil extraction yield was increased with the temperature which can be attributed to the increase of the oil components vapour pressure. The effect of the increase of solute vapour pressure seems to have dominated over solvent's density.

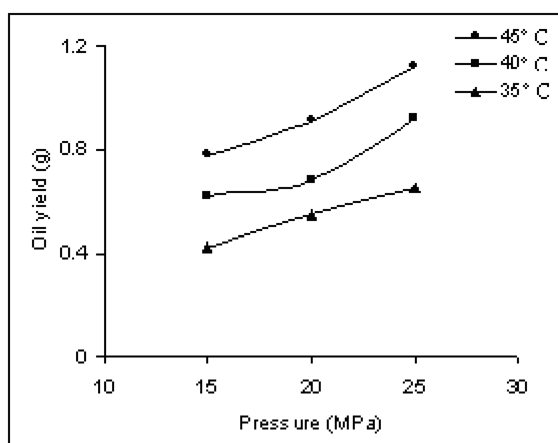

Fig. 2: SCO_2 extraction of oil from tuna viscera

Protease activity

Fig. 3 shows the specific activity of protease in crude extracts of tuna viscera. The highest activities were found in n-hexane treated tuna viscera comparing to SCO_2 treated. The digestive enzyme activities might be lost due to the treatment by SCO_2. The loss of enzyme activity after SCO_2 treatment has been argued prior research that attributed to the interactions between CO_2 and the enzyme.

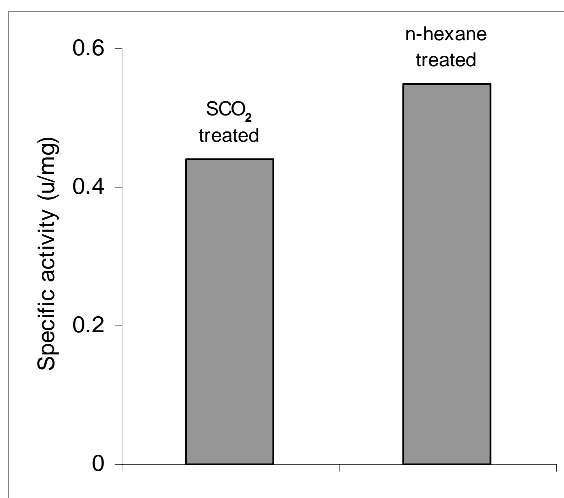

Fig. 3: Specific activity of protease in crude extract of tuna viscera

Optimum pH and pH stability

The highest proteolytic activities in both SCO_2 and n-hexane treated tuna viscera extracts were found at alkaline pH 9.5. In the pH range 8-10.5, the protease activities were more than 90% of its original and then decreased with increasing pH (data not shown). The changes of pH stability range in SCO_2 and n-hexane treated tuna viscera crude extracts were not significant.

Optimum temperature and temperature stability

The optimum temperature of protease in both SCO_2 and n-hexane treated tuna viscera extracts were 55ºC (data not shown). Fig. 4 shows the temperature stability of crude protease of SCO_2 and n-hexane treated tuna viscera. The protease activities of SCO_2 and n-hexane treated tuna viscera residues were remained almost 90% up to 55°C. Above temperature of 55°C the activities declined sharply. But SCO_2 treated sample retained above 80% of protease activity at 60°C where as n-hexane treated samples contained 72% activities. Protease of SCO_2 treated tuna viscera showed slightly higher temperature stability comparing to n-hexane treated viscera. 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.

Fig. 4: Temperature stability of protease

Electrophoresis

The slab gel electrophoresis of marker protein and untreated (non-extracted) and SCO_2 and organic solvent treated tuna viscera extracts are shown in fig. 5. 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 tuna viscera sample.

Fig. 5: SDS-PAGE electrophoresis of untreated and SCO_2 and n-hexane treated tuna viscera extracts.

CONCLUSION

Protease activities of SCO_2 treated tuna viscera residues were slightly decreased comparing to organic solvent, n-hexane treated residues. In SCO_2 treated samples, protease showed slightly higher temperature stability. By electrophoretic patterns, no denaturation of proteins was found in SCO_2 treated samples. The use of carbon dioxide for lipid extraction is environment friendly. So, tuna viscera after lipid extraction by supercritical carbon dioxide in place of organic solvent might be used for isolation and purification of protease. Thermostable biocatalysts are highly attractive in economic purposes. So, further study by using SCO_2 at different extraction conditions may also help to obtain high quality and high thermally stable functional proteins.

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