Low pressure supercritical carbon dioxide warm pasteurization to inactivate muscle protease and lipase of nile tilapia with emphasis on its fillet textural properties

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Abstract. Supercritical carbon dioxide warm pasteurization was applied to reduce the activity of muscle protease and lipase of nile tilapia. Combination between 75 bar and 80 bar with 40°C as well as 80 bar with 50°C were applied on tilapia fillet. Exposure time was set from 10 min to 25 min with an interval of 5 min. It was found that supercritical carbon dioxide have greater effect on muscle protease with lesser effect on muscle lipase. Increasing temperature at 80 bar was slightly increase inactivation rate of muscle enzymes, with the cost of severe degradation on fillet textural quality. The product of this warm pasteurization however, was aimed to be stored under chilled storage and not for direct human consumption without further processing.

1. Introduction
Supercritical carbon dioxide (SCCO₂) has been known to have effect on activity of enzymes[1]. The effect could be reducing activity or increasing activity of certain enzymes. For perishable foods such as raw meat and fish, product deterioration was induced by several types of enzymes. Protease and lipase were two of autolytic enzymes which responsible on quality degradation of raw product during storage, even under frozen storage. However, most studies of application of SCCO₂ to inactivate both protease and lipase were conducted on isolated enzymes. The implementation of SCCO₂ application for reducing activity of muscle protease and muscle lipase is hardly available. Most application of SCCO₂ on solid foodstuffs mostly focused on its bactericidal effects [2–7]. Therefore, this work was initiated to fill that void by investigating effect of low pressure SCCO₂ on activity of muscle protease and lipase of nile tilapia (Oreochromis niloticus). Since SCCO₂ also affects physical product appearance, the impact on its textural properties also investigated.

The independent variables of SCCO₂ application are pressure, temperature, and exposure time. This work was much more focused on exposure time since its operating pressure was within lower region of SCCO₂ pressure range. Typically, application of SCCO₂ is at 300 bar or lower [8]. Since it was low pressure, increasing residence time was expected to increase the rate of activity reduction of both muscle protease and muscle lipase. However, the drawback of high pressure gas or liquid application on fish foodstuffs is negative changes in physicochemical properties, particularly textural properties and color[9]. The objective of this study, therefore, was to search the optimum of exposure time on inactivation of muscle protease and lipase under selected combination of pressure and temperature, and its impact on textural properties of fish fillet.
In this study, tilapia was used as a solid food because tilapia is the second most produced freshwater fish after carp. From all aquacultured tilapias, nile tilapia (*Oerochromis niloticus*) was 69% of total world production of all tilapiine cichlids in 2015[10].

2. Material and Methods

Nile tilapia (individual size from 100 to 150 g each) were purchased alive from the local market. All fishes were killed, gutted, headed, and cleaned with tap water. The clean fishes were cut into two pieces, inserted into a polyethylene plastic pouch (20×15 cm), and subsequently placed into the pressure vessel. The plastic pouch seals were left in open position to allow CO$_2$ exchange and the tips were folded in order to prevent any unwanted solid or liquid entering the pouch container.

The pressure vessel was developed in Gadjah Mada University with gross volume of 2.01 L. Its lid was equipped with 180 bar analog pressure gauge with 5 bar resolution. A calibrated digital thermostat with a 10 kΩ negative-temperature-coefficient (NTC) thermistor was used to control barrel temperature of pressure vessel. Food grade CO$_2$ was supplied into the pressure chamber by inverting a 25 kg-type CO$_2$ cylinder. The schematic installation of the pressure vessel is shown in Fig. 1.

![Figure 1. Schematic installation of pressure vessel](image)

2.1. CO$_2$ pressurization

Liquid CO$_2$ was supplied into the pressure vessel by inverting the CO$_2$ cylinder. During the filling process, exhaust orifice was opened slightly to remove non-CO$_2$ gas and to reduce pressure chamber temperature. Since the critical pressure of CO$_2$ is 73.77 bar, critical temperature is 31.1°C, and critical density is 0.44 g/cm$^3$[11] ; SCCO$_2$ was obtained by supplying liquid CO$_2$ into pressure vessel and then warming the barrel of pressure vessel up to experimental temperature. In order to prevent overpressure, the pressure was maintained by releasing excess CO$_2$ through exhaust orifice when temperature and pressure approached experimental setting. The time required to raise the temperature
from 30°C to 40°C was ±10 min. After holding time was reached, exhaust orifice was opened slightly to release CO₂ slowly and initiate decompression stage. From preliminary study, sudden decompression would result destroyed fillet and exhaust orifice could be clogged by slurried fillet. Therefore, decompression time was set for 2–3 min.

2.2. Crude enzyme extract

The enzyme extraction process followed the method proposed by Lakshmanan et al. [11]. Ten gram of sample were homogenized by IKA Turax T25 S5 homogenizer (Janke and Kunkel GmbH) in 50 mL ice-cold deionized water for 2 min. Homogenate was allowed to stand for 30 min in ice with occasional stirring. After-then, the homogenate was centrifuged at 14600×g and 4°C for 20 min in refrigerated centrifuge (Beckman Alegra X30R). The supernatant was filtered by 0.45 μm polytetrafluoroethylene syringe filter (Merck milipore) and stored at -80°C before further analysis.

2.3. Residual protease activity measurement

Protease assay followed the method of Amano Enzyme Inc[12]. One mL of crude enzyme extract was added into 5 mL 0.2% casein solution (Merck CAS 9000-71-9) in 0.02 M phosphate buffer at pH 8.0. The mixture was incubated at 37±0.5 °C for 10 min and shaken at 300 rpm. The enzymatic digestion was terminated by addition of 5 mL TCA solution and incubated for 10 min at 37±0.5°C for 10 minutes and shaken at 300 rpm. The reaction solution was centrifuged at 10000×g at 25°C for 10 min. One mL of supernatant was added to 5 mL mixture of 3-folds folin reagent and 0.55 M Na₂CO₃ (1 : 5). The reaction solution was vortexed and allowed to stand at 37±0.5°C for 30 min. Two-hundreds μL of the solution was pipetted into 96 wells microplate and its absorbance was read at 660 nM in ELISA reader (Bio-Tek μQuant). The residual activity (A/A₀) of protease is stated as a percentage of activity of treated samples (A) to the activity of untreated samples (A₀).

2.4. Residual lipase activity measurement

Lipase assay followed the manual of Asahi Kasei Enzymes [13] with slight modification. The emulsifier, Adekatol (Adeka, SO–120) was replaced by Polyvinil alcohol (PVA) (Merk, CAS-No 9002-89-5). The substrate mixture was prepared from 25% consumer grade olive oil, 1.5% PVA, and deionized water; boiled and mixed rigorously. The substrate must be cooled adequately before further usage. Five mL of substrate was added with 4 mL of 0.05 M buffer tris HCl (pH 7.7). One mL of crude lipase extract was added into the substrate and incubated at 37±0.5°C for 20 min and shaken at 150 RPM. The enzymatic digestion was terminated by adding 5 mL mixture of 50% ethanol and 50% aceton reaction stopper. Two drops of phenolphthalein indicator was added into the mixture and shaken carefully. Eventually, the mixture was titrated with 0.05 M NaOH until flash pink color appeared. The residual activity (A/A₀) of lipase is stated as a percentage of activity of treated samples (A) to the activity of untreated samples (A₀).

2.5. Texture readings

TA.XT plus texture analyzer (Stable Microsystem Inc.) with 50 kg load-cell was used to measure textural property of fish chunks. Fish chunk with 40 mm width, were compressed with 36 mm diameter cylindrical probe. The compression speed was 2 mm/s. The readings were recorded with an interval of 0.005 s. The compression travel was half of the original thickness of fish chunk. The hardness of fillet, which is maximum force recorded during compression, represented textural property of fillet. The unit of hardness was kg.
2.6. Experimental design
At 40°C, the critical pressure of SCCO₂ is at 74 bar and critical density is 0.22407 g/mL. At 50°C the critical pressure of SCCO₂ is 74 bar and critical density is 0.18931 g/mL. The option of 75 and 80 bar at 40°C were taken as SCCO₂ treatments to find out the effect of pressure increase and density increase on enzymatic activity. Eighty bar at 50°C was taken in order to find out the effect of increase of temperature while pressure was maintained and CO₂ density was decreased. All treatments combined with exposure time, from 10 to 25 min with an interval of 5 min.

2.7. Statistical analysis
Statistical analysis was carried out on textural parameters of fillet, in order to determine the significant changes of subjected fillet to its original texture. One way Analysis of Variance (ANOVA) was carried out and followed by New Duncan Multiple Range Test pairwise comparison of each treatment. The results are from triple replications ± standard deviation. Each replication was from individual fish.

3. Result and Discussion
3.1. Effect of SCCO₂ on muscle protease and lipase activity

![Figure 2](image)

**Figure 2.** Effect of exposure time on residual activity of muscle protease (a) and muscle lipase (b) at certain combination of pressure and temperature

As shown by Fig. 2, all treatments reduced the activity of muscle protease and muscle lipase. However, the impact of SCCO₂ was much greater on muscle protease rather than on muscle lipase. The inactivation of enzyme activity is typically follow first order kinetic,

\[ \log(A/A_0) = -kt \]

where A is activity after SCCO₂ exposure at t, A₀ is initial activity, k is reaction constant, and t is exposure time in minutes after experimental setting is achieved. The reaction constants (k) of muscle protease inactivation were 1.39·10⁻²/min for 75 bar at 40°C with coefficient of determination (R²) was 0.92, 2.6·10⁻²/min for 80 bar at 40°C with R²= 0.93, and 2.81·10⁻²/min for 80 bar at 50°C with R² = 0.93. The reaction constants of muscle lipase inactivation were 2.9·10⁻³/min for 75 bar at 40°C with R² = 0.88, 4·10⁻³/min for 80 bar at 40°C with R² 0.88, and 5.2·10⁻³/min for 80 bar at 50°C with R² = 0.93. The residual protease activity after 25 min exposure was 49.86% for 75 bar-40°C, 28.34% for 80 bar-40°C, and 25.19% for 80 bar-50°C. The residual activity of lipase after 25 min exposure were 85.53% of 75 bar-40°C, 81.82% of 80 bar-40°C, and 76.03% of 80 bar-50°C. This is in agreement with the study of Ishikawa et.al [14] , that reported the inactivation of alkaline protease and lipase solution after subjected to microbubble supercritical CO₂. Microbubble SCCO₂ was produced by 10 μM microfiltration when SCCO₂ was pumped into the pressure chamber. Complete inactivation of alkaline protease was achieved at pressure above 150 bar while lipase was able to retain 80% of its activity after exposed to the same conditions.
Protease is an enzyme that capable to withstand against high pressure processing. Bilbao-Sáinz et al.\[15\] reported 70% residual activity of milk protease after subjected to High Hydrostatic Pressure (HHP) application at 6000 bar, 15 min, and 60°C. Leite Júnior et al.\[16\] reported no change of protease activity from Rhizomucor miehei after subjected to 1900 bar and 25°C. In this work, inactivation of protease could be achieved at much lower pressure. Therefore, pressure was not the only factor that could reduce the activity of enzyme in SCCO₂ application. The presence of SCCO₂ dan its penetration into cell and muscle tissue is the mandatory factor that affect inactivation of enzymes. The mechanism on how SCCO₂ inactivate enzymes was discovered by Ishikawa et al\[17\]. It was reported that microbubble SCCO₂ decomposed α-helix structures and left 31.3% α-helix structures of alkaline protease and 62.9% α-helix structures of lipase after exposed to microbubble SCCO₂ at 350 bar and for 30 min. The decomposition of α-helix would result the inactivation of the enzymes. In this work, liquid CO₂ was injected in its subcritical form and SCCO₂ was developed during preheating stage. Since the cell size is within 1-100 μM\[18\], the size of developed SCCO₂ inside the cell must not be larger than cell interior.

3.2. Effect of SCCO₂ on textural properties of tilapia fillet

Although protease inactivation of protease and lipase between 80 bar–40°C and 80 bar–50°C was slightly difference (Fig. 2), the effect of SCCO₂ on texture of fillet was very noticeable, as shown by Table 1. Until 20 min exposure, the texture of fillet that exposed to 80 bar at 40°C was not significantly difference than its original texture. At this point, residual activity of protease was 29.04% and lipase at 82.23%. Meanwhile, fillet that subjected to 80 bar at 50°C was softened severely since 10 min and getting worse when the exposure time was prolonged. Therefore, 50°C is not considered suitable to reduce autolytic enzymes while simultaneously retaining most of its original texture. For 75 bar and 40°C application, fillet texture changed noticeably since 20 min of SCCO₂ exposure and the effect of enzyme inactivation was not significant compared to its initial activity.

| Exposure time (min) | Hardness (kg) |
|--------------------|---------------|
|                    | 75 bar-40°C   | 80 bar - 40°C | 80 bar - 50°C |
| Untreated          | 15.86±3.03 ab | 13.40±5.95 bc | 4.27±0.76 ef |
| 10                 | 16.21±3.37 a  | 13.40±5.95 bc | 4.27±0.76 ef |
| 15                 | 11.15±0.19 bcd| 12.9±1.97 bcd | 4.3±1.78 ef |
| 20                 | 9.39±0.4 d    | 12.61±3.56 bc | 2.55±0.89 f |
| 25                 | 7.64±1.20 de  | 10.41±3.24 cd | 1.84±0.54 f |

a-f means with the same superscript letters were not significantly different

The hardness of 10 min exposure of 75 bar that was slightly harder than control might be attributed to initial hardness of samples prior to SCCO₂. Hardness test is destructive test, therefore, the samples could not be checked their initial hardness prior to SCCO₂ exposure, except for those of untreated samples. Immediately after death, all fishes experienced rigor mortis. Since it was not possible to pasteurize all samples at once, the stage of rigor mortis was not uniform on all fishes. Prior to rigor mortis, the muscle are soft and limp. During rigor mortis, the muscle begin to stiffen and harden. After rigor mortis stage is passed, the muscle is soft and limp again \[20\]. This uniformity could be addressed by allowing the fishes to pass rigor mortis completely. Rigor mortis depends on the handling temperature and the size of fish. Rigor mortis of nile tilapia could be achieved completely after 15 hr at ambient temperature \[21\]. In this study; the slaughtering, preparation of pasteurization, and four batches of SCCO₂ pressurization were completed before 8 hr. However, pasteurizing the fish...
after the completion of rigor mortis will result time delay of processing and additional storage requirement, which should be avoided in order to save time and other resources.

4. Conclusion
The increase in SCCO$_2$ density was found to have greater effect on protease rather than lipase. The increase of temperature to compensate decrease of SCCO$_2$ density was found to deteriorate textural properties of Nile tilapia fillet. Therefore, 40°C was found to be upper limit for application of SCCO$_2$ for preserving raw fish for chilled storage. Combination of 80 bar and 40°C for 20 min was found to be the optimum treatment to reduce muscle protease and lipase while simultaneously retain most of its original textural properties.

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