

Physiological properties of *Scomber japonicus* meat hydrolysate prepared by subcritical water hydrolysis

Jae-Suk Choi^{1,2}, Hye Eun Moon¹, Myong-Kyun Roh³, Yu-Mi Ha¹, Bo-Bae Lee¹, Kwang Keun Cho⁴ and In Soon Choi^{1,5*}

¹RIS Center, Industry-Academic Cooperation Foundation, Silla University, Busan 617-736, Republic of Korea

²Division of Bioindustry, College of Medical and Life Science, Silla University, Busan 617-736, Republic of Korea

³Department of R&D, Korea Bio & Engineering Co., Ltd, Nam-gu, Busan 608-736, Republic of Korea

⁴Department of Animal Resources Technology, Gyeongnam National University of Science and Technology, 150 Chilam-dong, Jinju, Gyeongnam, 660-758, Republic of Korea

⁵Department of Biological Science, Silla University, Busan 617-736, Republic of Korea

*Corresponding Author E-mail : ischoi@silla.ac.kr

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Abstract

The health-beneficial biological activities, including antioxidant and tyrosinase inhibitory activities, of *Scomber japonicus* muscle protein hydrolysates prepared by subcritical water hydrolysis were investigated. After 5 min of subcritical hydrolysis at 140°C, 59.76% of *S. japonicus* muscle protein was hydrolyzed, the highest degree of hydrolysis in all the groups were tested. According to the response surface methodology results, as the reaction temperature and reaction time became lower and shorter, the yield became higher. The highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity (90.63%) occurred in hydrolysates treated at 140°C for 5 min, and the highest tyrosinase inhibitory activity (65.54%) was identified in hydrolysates treated at 200°C for 15 min. Changes in the molecular weight distribution of *S. japonicus* muscle proteins after subcritical water hydrolysis were observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subcritical water hydrolysis is a suitable technique for obtaining *S. japonicus* muscle protein hydrolysates with useful biological activities, within a short time (5–15 min).

Key words

Antioxidant activity, Hydrolysate, *Scomber japonicus*, Subcritical water hydrolysis, Tyrosinase inhibitory activity

Introduction

Dietary proteins are a source of biologically active peptides with numerous physiological functions such as antihypertensive, opioid, immunomodulating, antithrombotic, antioxidant, anticancer, and antimicrobial actions (Hartmann and Meisel, 2007; Udenigwe and Aluko, 2012; Singha *et al.*, 2014; O'Loughlin *et al.*, 2014; Nongonierma *et al.*, 2015). Protein hydrolysates and peptides have been produced from pig, cow, and chicken by-products as relatively cheap and easily collectable sources (Kim *et al.*, 2001; Saiga *et al.*, 2003; Li *et al.*, 2007). However, outbreaks of mad cow disease, foot-and-mouth disease, and avian influenza as well as a ban on proteins from pig skin and bone

in some regions for religious reasons have made it necessary to find alternative sources (Trampuz *et al.*, 2004; Gómez-Guillén *et al.*, 2011). Various peptides or hydrolysates derived from fish or seafood muscle protein and waste possess better health benefits in comparison with those of crude materials (Hordur and Barbara, 2000; Choi *et al.*, 2003; Pádraigín and Richard, 2012; Huang *et al.*, 2012).

Scomber japonicus Houttuyn (Scombridae; Pacific chub mackerel or chub mackerel) is a mid-sized pelagic fish. It is essentially a near-coastal species with a vertical distribution ranging from depth of 0 to 300 m in the warm and temperate coastal transition areas and adjacent seas of the Pacific and northwest Indian Oceans (Collette and Nauen,

1983). Catches of *S. japonicus* reached 3.4 million tons in 1978, but decreased to a minimum of 1.3 million tons in 1991. Catches have recovered slightly to 1.6 million tons in 2010 (FAO, 2012). *Scomber japonicus* muscle has an excellent amino acid composition and is a unique source of nutrients and easily digestible protein (Oduro *et al.*, 2011). It is one of the most important fishing resources and is regarded as an economically important fish in Korea (Bae and Lim, 2012). In Korea, the estimated annual catch of *S. japonicus* in 2011 was 138,729 tons, including live fish (96 tons; 0.069%), fresh fish (136,759 tons; 98.58%), and frozen fish (1,874 tons; 0.0137%) (MOMAF, 2011).

Current methods for the hydrolysis of fish muscle and waste include chemical (acid, alkali, or catalytic) hydrolysis (Gao *et al.*, 2006), enzymatic hydrolysis (Ji *et al.*, 2002; Hsu *et al.*, 2009), and gamma irradiation hydrolysis (Choi *et al.*, 2011). Chemical hydrolysis requires violent reaction conditions and often results in environmental pollution. Enzymatic hydrolysis is difficult to control with respect to pH, time, and enzyme/substrate ratio, and is expensive with a long production cycle (Uddin *et al.*, 2010). Gamma irradiation hydrolysis demands large-scale facilities and higher costs.

Subcritical water technology, adapted for hydrothermal liquefaction, involves a reaction of biomass and water at elevated temperature and pressure and has been demonstrated for a range of biomasses such as carbohydrates, proteins and fatty acids (Toor *et al.*, 2011). When water is heated under pressure, its dielectric constant and density change, resulting in a change in its solvent and

reactant properties (Kruse *et al.*, 2007). Under these conditions, biomass is decomposed to form new products. The advantages of subcritical hydrolysis are that it is a simple process with a short hydrolysis time, it requires no additive catalysts, and simultaneous sterilization occurs. In addition, hydrolysis in subcritical water is regarded as an environmentally friendly technology (Uddin *et al.*, 2010).

Despite many advantages of subcritical hydrolysis, no attempt was made to subcritically hydrolyze muscle from *S. japonicus*. Therefore, this study determined the optimum conditions for subcritical hydrolysis of *S. japonicus* muscle and elucidated the biological activities of *S. japonicus* muscle hydrolysate, including antioxidant and tyrosinase inhibitory activities.

Materials and Methods

Sample: To exclude the effects of endogenous proteases, *S. japonicus* was purchased as a canned product of boiled *S. japonicus* (Dongwon F&B, Seoul, Korea) from a retail shop in Busan, Korea. The canned product was processed with a little salt (0.71 g/100 g as sodium) but without any other seasonings or food additives. After removing the skin and bone, the fish was homogenized in a homogenizer (Hanil, Seoul and Korea) for 5 min. The homogenized samples were stored at -20°C until used for following analyses.

Hydrolysis of fish muscle in subcritical water: The experimental apparatus included the batch process shown in Fig. 1. The hydrolysis was carried out in a batch-type reactor consisting of a 300-cm³ vessel, with a 5-cm inside diameter, made of Hastelloy C-276. The reactor was equipped with a temperature controller and quickly achieved heat that was quenched rapidly. A 2 g sample and 200 ml of distilled water were charged in the reactor at a ratio of 1:100 (w/v). To evaluate the bioactivity of *S. japonicus* subcritical hydrolysates, the applied temperatures (pressure) were 120°C (1.0 bar), 140°C (2.6 bar), 160°C (5.3 bar), 180°C (9.2 bar) and 200°C (15.0 bar). The reaction times were 0, 5, 10, 15 and 20 min.

Preparation of non-hydrolyzed sample: To compare non-hydrolyzed and hydrolyzed samples using subcritical water, a non-hydrolyzed sample was prepared by extracting 2 g of homogenized *S. japonicus* muscle with 200 ml of distilled water at 10°C for 1 d in a shaking incubator (150 rpm, VS-8480SR, Vision Scientific, Daejeon, Korea).

Protein content determination: The protein contents of the non-hydrolyzed and hydrolyzed samples were determined with Bradford reagent (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, CA, USA). The total protein content was expressed as micrograms of bovine serum albumin (Bio-

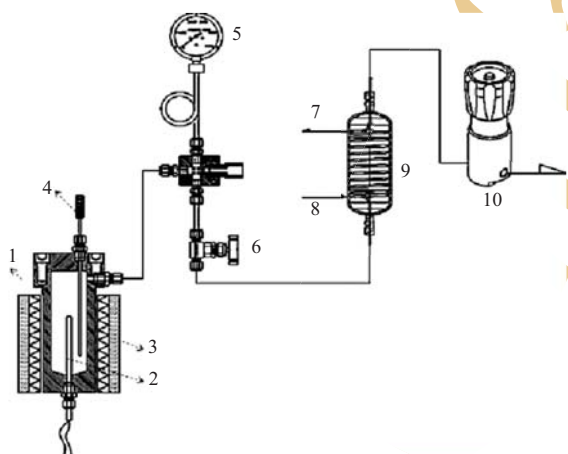


Fig. 1 : Schematic of the lab-scale supercritical water reaction apparatus used in this study: (1) reactor, (2) cartridge heater, (3) water jacket, (4) temperature indicator, (5) pressure gauge, (6) ball valve, (7) cool water out-flow (8) cool water in-flow, (9) heat exchanger, and (10) back pressure regulator

Rad Protein Assay Standard II, Bio-Rad, CA, USA) equivalents per mg of sample.

Measurement of the degree of hydrolysis (DH): Degree of hydrolysis of hydrolyzed protein was determined as a ratio of the amount of α -amino acids released during hydrolysis to the maximum amount of α -amino acids in the homogenized *S. japonicus* muscle, following method of Benjakul and Morrissey (1997). The absorbance of the samples was measured at 420 nm to determine the α -amino acid content, and α -amino acids were expressed in terms of L-leucine. DH was calculated as follows: $DH (\%) = [(L_t - L_0)/(L_{max} - L_0)] \times 100$, where L_t is the amount of α -amino acids released at time t ; L_0 is the amount of α -amino acids in homogenized *S. japonicus* muscle; L_{max} is the maximum amount of α -amino acids in hydrolyzed *S. japonicus* muscle (Beak and Cadwallader, 1995).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging effect: The stable DPPH radical was purchased from Sigma-Aldrich (St. Louis, MO, USA). DPPH radical-scavenging capacity was determined by the method of Blois (1958) with slight modifications (Kuda *et al.*, 2005) and was calculated using the formula: Scavenging ability (%) = $[1 - (\text{sample absorbance} - \text{sample color absorbance})/\text{control absorbance}] \times 100$. Ascorbic acid (Sigma, A5960) was used as positive control.

2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical-scavenging effect: ABTS radical-scavenging activity was determined according to the method of Marklund and Marklund (1975) with some modifications (Re *et al.*, 1999). The antioxidant activity of the samples was calculated as: Scavenging ability (%) = $[(Ac - At)/Ac] \times 100$, where At and Ac were the absorbance of the sample and blank, respectively. Ascorbic acid was used as positive control.

Measurement of superoxide dismutase (SOD)-like activity: SOD-like activity was assayed by the method of Marklund and Marklund (1975) with some modifications (Jung *et al.*, 2008) and calculated using the following equation: SOD-like activity (%) = $[(A - B)/A] \times 100$, where A is the increase in absorbance of the control and B is the increase in absorbance of the sample. Ascorbic acid was used as the positive control.

Inhibitory effect on angiotensin-converting enzyme (ACE): ACE activity was assayed by the method of Cushman and Cheung (Cushman and Cheung, 1971). Captopril (Sigma, C4042) was used as positive control.

Tyrosinase inhibitory activity : Tyrosinase activity was assayed by the method of Yagi *et al.* (1986). The inhibitory effect of the sample on tyrosinase activity was calculated

using the equation: tyrosinase inhibitory activity (%) = $[1 - (\text{sample absorbance}/\text{control absorbance})] \times 100$, where control refers to the absorbance of distilled water instead of the sample. Ascorbic acid was used as positive control (Iwai *et al.*, 2004).

Color determination : The color of the samples was measured using a color-difference meter (CM-700d spectrophotometer; Konica Minolta Sensing, Tokyo, Japan) with a 1-cm-diameter cuvette. Color was expressed using the L (lightness), a (redness), and b (yellowness) Hunter system values, which were monitored by a computerized system using Spectra Magic ver. 2.11 (Minolta Cyberchrom, Osaka, Japan). The overall color difference (ΔE) was calculated using the equation $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$.

Response surface method (RSM) : A regression analysis using a central composite design was performed using RSM to determine the optimal conditions for subcritical water hydrolysis of *S. japonicus* muscle. Minitab statistical software, ver. 16 (Minitab, State College, PA, USA), was used to generate the design of experiments, perform statistical analysis, and create regression model. The variables in this study included two numerical factors, temperature (X_1) and hydrolysis time (X_2). Subcritical water hydrolysis was performed at 140, 150, 160, 170, and 180°C for 0, 5, 10, 15, and 20 min, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): All reagent and electrophoresis kits were purchased from Bio-Rad (Hercules, CA, USA). The protein load was adjusted to 20 μg protein/lane. Electrophoresis was performed using precast 4–20% Mini-PROTEAN[®] TGX[™] bis-Tris gels and a Mini-PROTEAN Tetra cell (cat. #1658004) at 100 V in a Tris/glycine/SDS running buffer system (cat. #161-0732) according to the manufacturer's instructions. Standard molecular weight markers (protein standard; cat. #161-0374) were loaded in the first lane.

Statistical analysis: All experiments were performed independently at least three times. The significance of the results was calculated using analysis of variance with SPSS ver. 10 (SPSS, Chicago, IL, USA). Results were considered significant at ^a $p < 0.001$, ^b $p < 0.01$, and ^c $p < 0.05$ as compared with control.

Results and Discussion

At all tested temperatures, the hydrolysis of homogenized *S. japonicus* muscle with subcritical water proceeded at a high rate during the initial 5 min, and thereafter the proteins were further cleaved (Fig. 2a). Thus, the maximum cleavage of peptides occurred within the initial 5 min of hydrolysis. As temperature increased, the DH

decreased: the highest DH occurred at 140°C, and the lowest DH occurred at 200°C, probably due to decomposition of amino acids owing to the excess hydrolysis rate at high

temperature and pressure (Toor *et al.*, 2011). No subcritical water hydrolysis of *S. japonicus* muscle occurred at 120°C. DH at 140 and 160°C was highest at 5 min (59.76 and

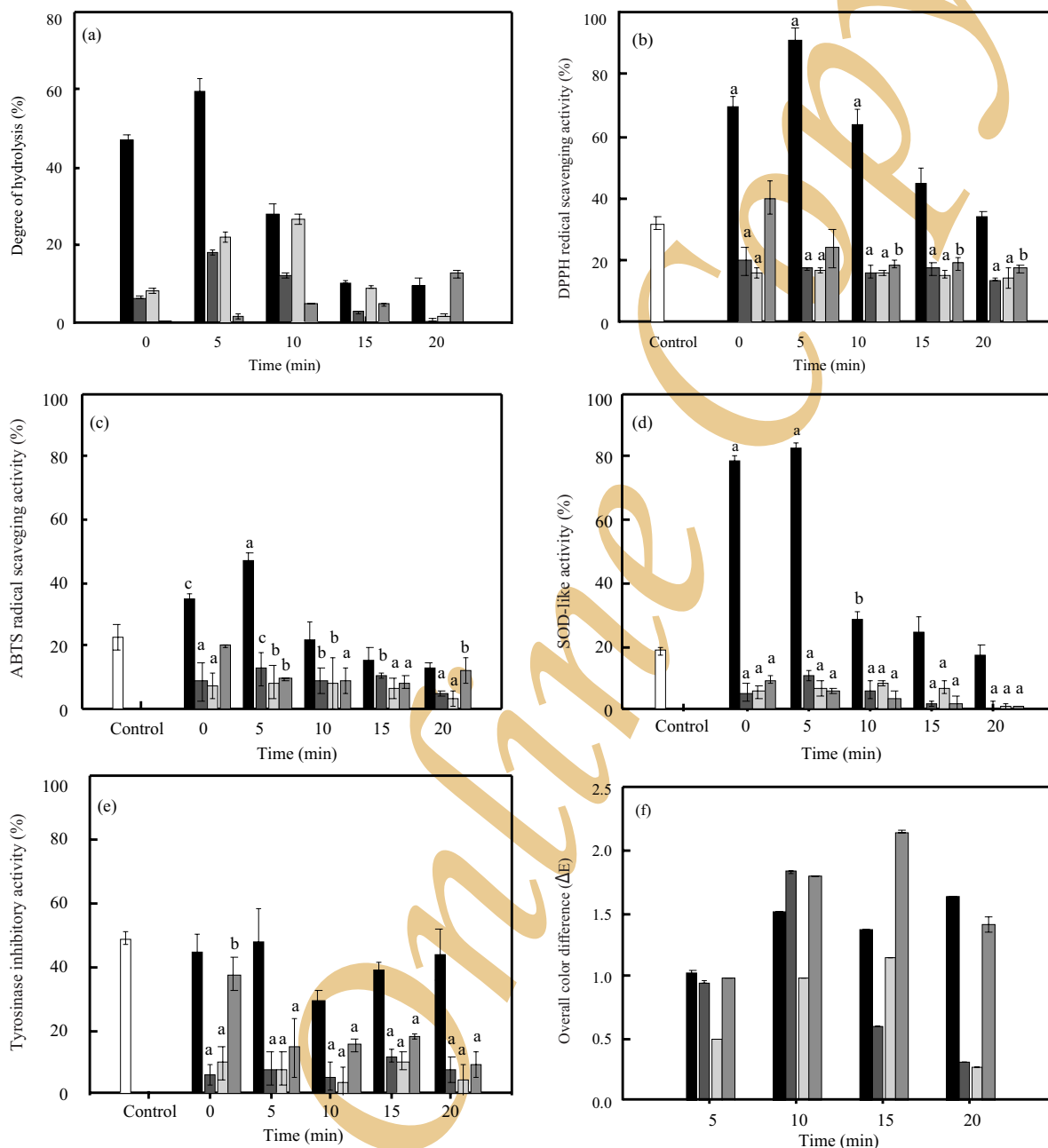


Fig. 2 : Physiological properties of *Scomber japonicus* muscle hydrolysate prepared by subcritical water hydrolysis. (a) Degree of hydrolysis (DH), (b) DPPH radical scavenging activity, (c) ABTS radical scavenging activity, (d) superoxide dismutase (SOD)-like activity, (e) tyrosinase inhibitory activity, and (f) overall color difference (ΔE) of *Scomber japonicus* hydrolysate following subcritical water hydrolysis at 140 (■), 160 (■), 180 (■) and 200°C (■) for 0, 5, 10, 15, and 20 min, respectively. All measurements were performed in triplicate, and values are means of three replicates. Statistical significance is indicated as ^a $p < 0.001$, ^b $p < 0.01$ and ^c $p < 0.05$ as compared with control

18.25%, respectively); at 180°C, it was highest at 10 min (26.67%). The α -amino acid content of *S. japonicus* muscle treated by acid hydrolysis was $294.29 \mu\text{g g}^{-1}$.

To determine the DPPH radical scavenging activity, 5- μg protein samples were used. In *S. japonicus* muscle not treated with subcritical water hydrolysis, DPPH radical-scavenging activity was 32.12%. Among the samples treated with subcritical water hydrolysis, the sample treated at 140°C for 5 min had the highest DPPH radical-scavenging activity of 90.63% (Fig. 2b). Samples treated at 160, 180, and 200°C, the activity was $\leq 40\%$. DPPH radical-scavenging activity of $0.25 \mu\text{g ml}^{-1}$ L-ascorbic acid was $56.67 \pm 1.87\%$.

To determine the ABTS radical-scavenging activity, 0.5- μg protein samples were used. The ABTS radical-scavenging activity was 22.89% in the sample not treated with subcritical water hydrolysis. The sample treated with subcritical water hydrolysis at 140°C for 5 min showed highest ABTS radical-scavenging activity of 47.44%. In the samples treated at 160, 180, and 200°C, ABTS radical-scavenging activity was $\leq 12\%$ (Fig. 2c). ABTS radical-scavenging activity of $10.0 \mu\text{g ml}^{-1}$ L-ascorbic acid was $65.27 \pm 4.79\%$.

To determine SOD-like activity, 3 μg protein samples were used. In *S. japonicus* muscle not treated with subcritical water hydrolysis, SOD-like activity was 5.64%. Among the samples treated with subcritical water hydrolysis, the sample treated at 140°C for 5 min had the highest SOD-like activity (15.76%). The samples treated at 160, 180, and 200°C had

SOD-like activity of $\leq 2.1\%$ (Fig. 2d). SOD-like activity of $25.0 \mu\text{g/ml}$ L-ascorbic acid was 65.32%.

To determine the inhibitory effect on ACE activity, 10 μg protein samples were used. According to the result of Do (2000), *S. japonicus* muscle protein peptides degraded by the enzyme papain had ACE inhibitory activity ($\text{IC}_{50} = 967 \mu\text{g}$). However, no ACE inhibition was observed in any of the samples including *Scomber japonicus* hydrolysate following subcritical water hydrolysis at 140, 160, 180, and 200°C for 0, 5, 10, 15 and 20 min, respectively. Captopril ($1 \mu\text{g ml}^{-1}$) produced 51.17% inhibition of ACE activity.

To determine the inhibitory effect on tyrosinase activity, 0.5- μg protein samples were used. *S. japonicus* muscle not treated with subcritical water hydrolysis inhibited tyrosinase activity by 29.02%. Among the samples, the one treated with subcritical water hydrolysis at 200°C for 15 min exhibited highest inhibition of tyrosinase activity, (65.54%). The sample treated at 140°C for 5 min inhibited 48.59% of tyrosine activity. Tyrosinase inhibition in the samples treated at 160 and 180°C was $\leq 48.31\%$ (Fig. 2e). Tyrosinase inhibitory activity of $100.0 \mu\text{g ml}^{-1}$ L-ascorbic acid was 64.02%.

From the perspective of color, there were no significant differences among *S. japonicus* muscle hydrolysates obtained at different temperature and time (Fig. 2f). The color of *S. japonicus* muscle hydrolysates was light beige to the naked eye.

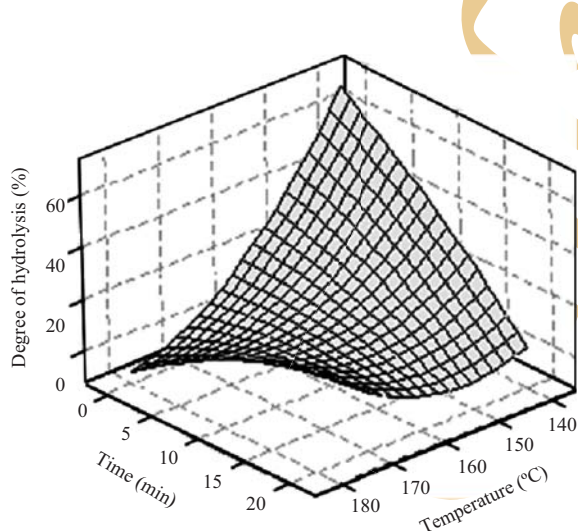


Fig. 3 : Response surface plot of the effect of temperature and hydrolysis time on the yield of hydrolyzed *Scomber japonicus* muscle protein. Subcritical water hydrolysis was performed at 140, 150, 160, 170 and 180°C for 0, 5, 10, 15 and 20 min, respectively

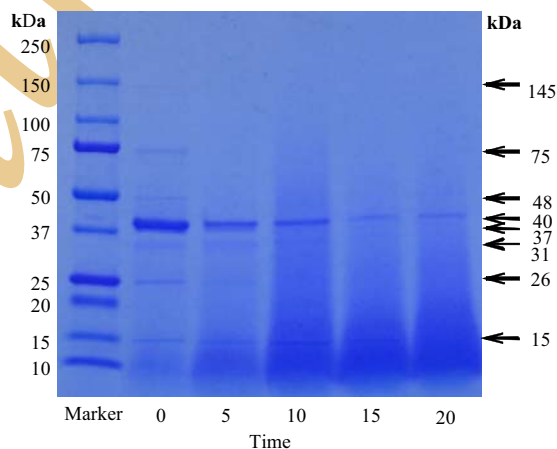


Fig. 4 : The effect of subcritical water hydrolysis on the molecular weight of *Scomber japonicus* muscle proteins. Molecular weights were investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 0, 5, 10, 15 and 20 min after subcritical water hydrolysis at 140°C. The left side indicates the molecular weights (kDa) of the standard marker. Arrows show some high-molecular-weight proteins that changed on subcritical water hydrolysis

The predicted RSM model using a quadratic equation with factors for temperature (X_1) and hydrolysis time (X_2) was as follows: Y (hydrolysis rate; %) = $12.3551 - 6.6067X_1 - 3.8061X_2 + 6.3833X_1X_2 + 3.8016X_{11} - 0.9754X_{22}$. According to RSM, the yield increased as the reaction temperature and reaction time became lower and shorter, respectively. As the hydrolysis reaction progressed, the yield decreased at all reaction temperatures (Fig 3).

SDS-PAGE was used to examine the molecular weight distributions of proteins from *S. japonicus* muscle treated with subcritical water hydrolysis at 140°C for 0, 5, 10, 15 and 20 min. Hydrolyzed *S. japonicus* muscle consisted of proteins with diverse molecular weights. The main proteins had molecular weight of 145, 75, 48, 40, 37, 31, 26 and 15 kDa, with 40-kDa proteins being most abundant. All of these proteins appeared to be degraded by subcritical water hydrolysis (Fig. 4). Protein degradation was dependent on hydrolysis time, such that high-molecular-weight proteins were degraded to a greater degree with increased hydrolysis time. The protein bands tended to disappear at hydrolysis time longer than 15 min, probably because of protein decomposition from excess hydrolysis. Based on the DH measurements, predicted RSM model, and SDS-PAGE, it took a relatively short time (< 15 min) to prepare *S. japonicus* muscle hydrolysates, using subcritical water hydrolysis.

Previous research reported the antioxidative activity (Yeum and Kim, 1994) and angiotensin I-converting enzyme (ACE) inhibitory activity (Do, 2000) of enzymatic hydrolysates of *S. japonicas*. According to Yeum and Kim (1994), the peroxide values (POV) of each *S. japonicus* muscle protein hydrolysates containing 5 mg of protein were 40–90 POV (mEq kg⁻¹) as antioxidant activities. The DPPH- and ABTS-radical scavenging activities and SOD-like activity of *S. japonicus* muscle protein hydrolysates and *S. japonicus* muscle not treated with subcritical water hydrolysis were compared in this study as antioxidant activities. The results of the study showed that, the antioxidant activities of *S. japonicus* muscle protein hydrolysates were superior to those of *S. japonicus* muscle not treated with subcritical water hydrolysis (Fig. 2. b-d). Although the experimental methods used by Do (2000) and in this study for estimating antioxidant activities differed, a similar tendency was observed.

Do (2000) reported that the ACE inhibitory activity of *S. japonicas* muscle enzymatic hydrolysates was 967 µg of IC₅₀. Otherwise, In this study no ACE inhibition was observed in all the tested *S. japonicus* hydrolysate samples following subcritical water hydrolysis. The mismatch between the ACE inhibitory activities probably results from the difference between the profile and content of each

compound(s) in both the hydrolysates and its ACE inhibitory activity.

Although the tyrosinase inhibitory activity of *Thunnus thynnus* irradiated hydrolysate has been reported by Choi et al. (2011), to our knowledge, this report is the first on the tyrosinase inhibitory activity of *S. japonicus* hydrolysate. Comparing the tyrosinase inhibitory activity of *S. japonicus* hydrolysate and *S. japonicus* muscle not treated with subcritical water hydrolysis, similar values were observed (Fig. 2e).

Both the predicted RSM model (Fig. 3) and molecular weight distribution of proteins by SDS-PAGE (Fig. 4) in subcritical water hydrolyzed *S. japonicus* muscle; the yield increased as the reaction temperature became lower and reaction time shorter. This probably occurred because the amino acid decomposition rate exceeded the protein hydrolysis rate (Toor et al., 2011). Consequently, to adapt subcritical water hydrolysis to fish protein hydrolysis, the appropriate reaction temperature and time might be essential.

In conclusion, the subcritical water hydrolysis technique showed potential for the preparation of *S. japonicus* muscle proteins with health-benefiting biological activities, including antioxidant and tyrosinase inhibitory activities. These results suggest that subcritical water hydrolysis, with its benefits of being a simple and quick process requiring no additive catalysts or large-scale facilities, could be applied in various food industry areas.

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