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Biological activity of enzymatic hydrolysates and the membrane ultrafiltration fractions from perilla seed meal protein

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Abstract: The Perilla seed meal (PSM) protein was hydrolyzed with Flavourzyme; the hydrolysate was fractionated by an ultrafiltration and its physiological activity was measured. Peptides with low molecular weights exhibited higher antioxidant activity, except for the Fe^{2+} chelating activity, compared to peptides with a high molecular weight. The IC_{50} values of the α-amylase inhibitory activity ranged from 727.89 μg/ml to 757.18 μg/ml, the α-glucosidase inhibitory activity was highest in the < 1 kDa fraction. The < 1 kDa fraction exhibited the strongest angiotensin I-converting enzyme inhibitory activity. As a result, the peptides from PSM protein hydrolysates, particularly peptides < 1 kDa, exhibited excellent antioxidant, antidiabetic, and antihypertensive activities and thus were highly likely to be developed as a functional food material.

Keywords: agricultural by-products; antidiabetic activity; antioxidant activity; bioactive peptide

Peptides are intermediate products of the decomposition of proteins to amino acids. Peptides are those, in which various combinations of amino acids form a polymer by a dehydration condensation reaction between an amino group and carboxyl group, and generally have a molecular weight (MW) of 10 kDa less (Halim *et al*. 2016). The peptides can be liberated through hydrolysis by digestive enzymes and through the action of the proteolytic enzymes derived from microorganisms or plants (Korhonen & Pihlanto 2006). Peptides that have a positive effect on the body functions or conditions and may ultimately influence health are referred to as bioactive peptides. Bioactive peptides are more resistant to gastrointestinal digestion and have more opportunities to be absorbed through the intestinal membranes (Nimalaratne *et al*. 2015). Enzymatic hydrolysis is a promising technique for the production of bioactive peptides with higher functionality than their precursors (Ramadhan *et al*. 2017). The enzymatic hydrolysis of proteins has also been used to improve functional properties of protein, production of bioactive peptides, protein quality manipulation for specific diets, and reduction of protein allergenicity (Jamdar *et al*. 2010). The peptides derived from the enzymatic hydrolysates of food proteins can provide health benefits, such as antihypertensive, anticancer, antimicrobial, antioxidant, and anti-inflammatory activities (Halim *et al*. 2016; Agrawal *et al*. 2017; Jang *et al*. 2017a).

Perilla (*Perilla frutescens* var. *japonica* Hara) is cultivated widely in East Asian countries, and perilla seeds are used to flavour various foods because they contain a unique aromatic essential oil (Meng *et al*. 2009). Perilla seed meal (PSM), a by-product of the perilla seed oil product, contains a large amount of protein as well as many bioactive compounds. Nevertheless, only a fraction of the PSM is used as a protein source for animal food and bird feed, and its utilization as food is very low.

Enzymatic hydrolysates were produced from the PSM protein to improve the use of PSM as functional

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food materials and protein hydrolysate was divided into five fractions using an ultrafiltration (UF) system. In addition, the health-related properties, including the antioxidant activity, antidiabetic activity, and antihypertensive activity, of enzymatic hydrolysate and membrane fractions were measured.

MATERIAL AND METHODS

Material. The defatted PSM was provided by Queensbucket Co. (Korea) and ground and stored in a deep freezer (MDF-435; Japan) at – 42°C for future experiments. Flavourzyme (Novo Nordisk Co., Denmark) used in the preparation of PSM protein hydrolysate is a fungal protease/peptidase complex produced from *Aspergillus oryzae* and has endoprotease and exoproteidase activity. The protease activity of Flavourzyme was 5.0 U/g, and 1 U of activity is defined as the amount of enzyme that liberates 1 µmol of tyrosine from 0.1% casein substrate solution per min at pH 5.0 and 50°C (Jang *et al*. 2017b).

Preparation of PSM protein isolates. Distilled water (*DW*) was added at 1:10 (w/v) to the defatted PSM and stirred in a shaking water bath (BS-11; JeioTech, Korea) at 25°C for 1 hour. Subsequently, the pH was adjusted to 10.0, and seeds incubated at 25°C for 1 h to extract the proteins. After centrifugation, the pH of the supernatant was adjusted to 4.0, and left at 25°C for 30 min to precipitate protein. The precipitate was homogenized by adding the *DW*, and the pH of the mixture was adjusted to 7.0, and lyophilized.

Preparation of PSM protein hydrolysate and membrane fractions. The PSM protein hydrolysate was prepared following the method of Park and Yoon (2019). The PSM protein isolates (5% w/v) were suspended in 25 ml of 0.1 M phosphate buffer (pH 7.0), and hydrolyzed with 10 U of Flavourzyme at 50°C for 4 hours. After enzymatic hydrolysis, the reaction mixture was boiled at 95°C for 10 min to inactivate the enzyme and centrifuged. The supernatant containing the target peptides was used as PSM protein hydrolysate (PPH). The PPH was passed through UF membranes with a *MW* cut-off of 1, 3, 5 and 10 kDa using an Amicon stirred UF cell 8050 (Millipore, USA). The permeate obtained from each *MW* cut-off membrane was collected as < 1, 1–3, 3–5, 5–10 and > 10 kDa peptide fractions, respectively. The PPH and permeates were freeze-dried and stored at –42°C for further analysis, and then fractions were prepared at various concentrations and used for each analysis.

Measurement of antioxidant activity. The ability of PPH and its fractions to scavenge 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) radicals was measured according to Oh and Yoon (2018). Each sample (240 µl) was added to 80 µl of $ABTS^+$ solution, which was prepared by mixing 0.7 mM of ABTS stock solution with 2.45 mM potassium persulfate. The mixtures were incubated in the dark for 6 min at room temperature, and the absorbance was measured at 734 nm.

The hydroxyl radical scavenging activity was determined according to Jung *et al*. (2008). Two milliliters of 10 mM $FeSO_4 \cdot 7H_2O$, 2 ml of 10 mM EDTA, 0.2 ml of a 0.1 M 2-deoxyribose solution, 0.2 ml of each sample, and 0.1 M sodium phosphate buffer (pH 7.4) was prepared in a screw-capped test tube. A 200 µl of a 10 mM H_2O_2 solution was added to this reaction mixture and incubated at 37°C for 1 hour. After incubation, 1 ml each of 2.8% trichloroacetic acid and 1.0% thiobarbituric acid were added to the reaction mixture. The sample was heated to 95°C for 10 min, cooled on ice, and its absorbance was measured at 532 nm.

For measurement of ferrous ion (Fe^{2+}) chelating ability, 40 µl of each sample, and 60 µl of 2 mM ferric chloride and 700 µl of *DW* were mixed and reacted at room temperature for 10 min. A 5 mM ferrozine was added and reacted for about 5 min to induce the formation of the Fe^{2+}-ferrozine complex. The absorbance of the solution was then measured at 562 nm.

Measurement of the antidiabetic activity. The α-amylase inhibitory activity of samples was carried out the method reported by Oh and Yoon (2018). A reaction mixture containing 20 µl of the sample solution, 50 µl phosphate buffer (100 mM, pH 6.8), and 100 µl α-amylase (4-α-*D*-glucan glucanohydrolase, EC 3.2.1.1) (1 U/ml) was preincubated at 20°C for 5 minutes. Subsequently, 100 µl of 1% soluble starch was added as a substrate and incubated further for 5 min at 20°C. The amount of glucose produced through the reaction was measured by the 3,5-dinitrosalicylic acid (DNS) color reagent assay (Miller 1959). Briefly, a 100 µl aliquot of DNS reagent solution (96 mM DNS, 5.31 M sodium phosphate tartrate in 2 M NaOH) was then added and boiled at 95°C for 15 minutes. Finally, 900 µl of *DW* was added, vortexed, and the absorbance was measured at 540 nm.

The α-glucosidase inhibitory activity was measured using the method of Im and Yoon (2015).

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Briefly, 50 µl of the test sample was mixed with 50 µl of α-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) (0.2 U/ml) and pre-incubated at 37°C for 15 minutes. To initiate the enzyme reaction, 100 µl of 3 mM ρ-nitrophenol-α-D-glucopyranoside was added and incubated at 37°C for 10 minutes. The reaction was quenched by adding 50 µl of 0.1 M NaOH, and the absorbance was measured at 405 nm.

Determination of the angiotensin I converting enzyme (ACE) inhibitory activity. The ACE (EC 3.4.15.1)-inhibitory activity of samples was evaluated according to Cushman and Cheung (1971). Rabbit lung acetone powder (Sigma Chemical Co., Germany) was suspended 20-times in 0.1 M sodium borate buffer (pH 8.3), and the enzyme was extracted at 40°C for 24 hours. After centrifugation at 3000 *g* for 30 min, the supernatant was used as an ACE enzyme for the assay of the ACE inhibitory activity. Sample solution (50 µl) was mixed with 50 µl of an ACE solution (50 U/ml) extracted from rabbit lung powder and incubated at 37°C for 30 minutes. Subsequently, 50 µl of the substrate, hippuryl-L-histidyl-L-leucine solution in 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl was added and incubated at 37°C for 30 minutes. The reaction was stopped by adding of 1 N HCl, followed by 1.5 ml of ethyl acetate. After centrifugation, 1 ml of the supernatant was dried by heating at 80°C and the absorbance was measured at 228 nm.

Statistical analysis. The results are expressed as the mean ± standard deviation of triplicate experiments. Multivariate analysis of variance was conducted using SPSS ver. 21.0 (USA). Significant differences between the mean values were identified using Duncan's multiple range test. The level of significance was $P < 0.05$.

RESULTS AND DISCUSSION

Antioxidant activity. The < 1 kDa fraction was the most active (lowest IC_{50} value) against ABTS, followed by 1–3, < 3–5, 5–10 kDa, and PPH; the > 10 kDa fraction was the least active (Figure 1A). The ABTS radical scavenging activity of the peptide fractions from green tender sorghum protein hydrolysate has shown a significant increase in the < 3 kDa fraction with 54.71% inhibition than 3–10 kDa, > 10 kDa, and hydrolysate (Agrawal *et al*. 2017). Jang *et al*. (2016) reported that the < 3 kDa fraction from sandfish hydrolysates had the strongest antioxidant activity followed by 3–5 kDa, 5–10 kDa, and > 10 kDa. In addition, < 1 kDa fraction of the enzymatic hydrolysates of egg white protein possessed the strongest ABTS radical scavenging activity compared with other UF fractions (Lin *et al*. 2013), which was consistent with these results.

The < 1 kDa fraction exhibited the highest hydroxyl radical scavenging activity with a low IC_{50} value of 1.92 mg/ml, followed by 1–3, 3–5, 5–10 Da, and PPH; the > 10 kDa fraction had the lowest activity with a high IC_{50} value of 15.77 mg/ml (Figure 1B). Similar results were found in both sweet potato hy-

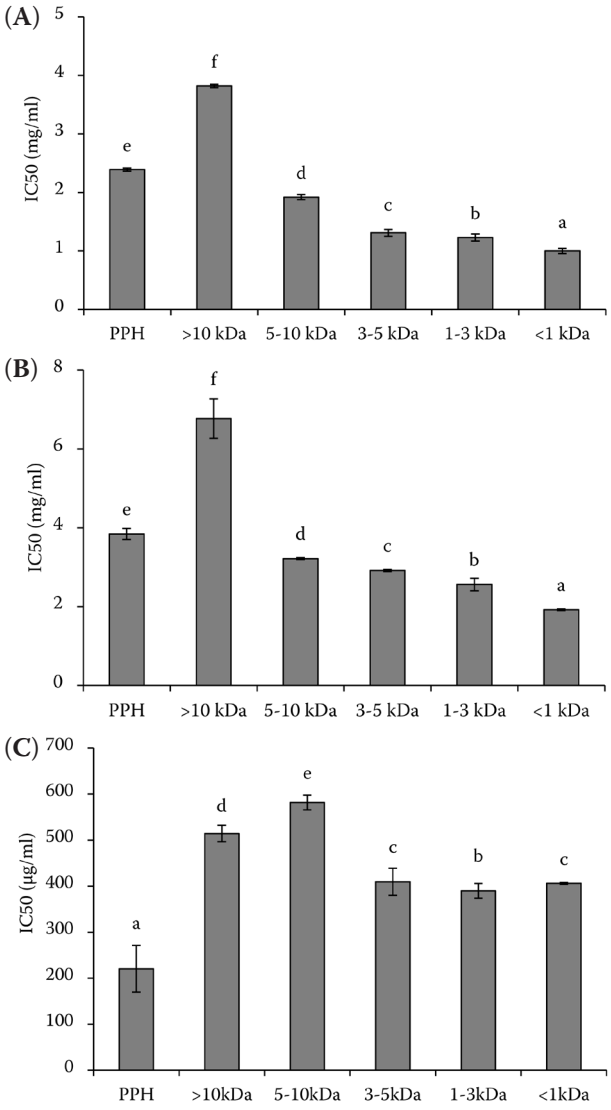

Figure 1. Antioxidant activity of the enzymatic hydrolysate and peptides obtained PSM protein: (**A**) ABTS, (**B**) hydroxyl scavenging activity; (**C**) Fe^{2+} chelating activity. PPH – PSM protein hydrolysate; bars represents mean ± sd ($n = 3$); different letters indicate significant differences ($P < 0.05$)

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drolysate and tuna dark muscle protein by-product hydrolysate (Saidi *et al*. 2014; Zhang *et al*. 2014). Sun *et al*. (2011) also reported that < 3 kDa peptide fraction from porcine hemoglobin hydrolysate showed the highest hydroxyl radical scavenging activity, which could be attributed to the combined effects of donating of hydrogen atoms and scavenging oxygen. This result suggests that the < 1 kDa peptides from PPH exert protection against oxidative damage by scavenging hydroxyl radicals.

Transition metal ions involved in redox reactions play a role in catalyzing the lipid oxidation process, and metals, such as Fe^{3+} and Cu^{2+}, in foods promote the production of radicals. Therefore, the catalytic action of the lipid oxidation process can be reduced by inhibiting the formation of free radicals through chelation of metal ions (Valko *et al*. 2006). PPH exhibited the strongest chelating ability with an IC_{50} value of 120.34 µg/ml, followed by 1–3, < 1, 3–5, and > 10 kDa; the 5–10 kDa fraction possessed weak Fe^{2+} chelating ability (Figure 1C). He *et al*. (2013) reported that the unfractionated hydrolysate and high *MW* peptide (5–10 kDa) had the strongest chelating activity with low IC_{50} values, which was consistent with the present results. Tang *et al*. (2009) also reported that increasing the peptide chain length could lead to a higher iron chelating effect. In addition, the amino acid composition of peptides effects on antioxidant activity. For example, peptides with a high proportion of hydrophobic amino acids such as Ala, Val, Ile, Leu, Phe, Try, Pro, Met and Cys have higher antioxidant activity than peptides containing a large amount of hydrophilic acid (Zou et al 2016). Among the nanofiltration fractions extracted from the dark muscular by-products of tuna, 1–4 kDa showed the highest reducing power, which 30.3% of total amino acids were composed of hydrophobic acid (Saidi *et al*. 2014). It is therefore assumed that a 1–3 kDa fraction contains a higher percentage of hydrophobic amino acids than < 1 kDa.

As a result, peptides with a low MW showed higher antioxidant activity except for the Fe^{2+} chelating activity than peptides with a high *MW*. In general, the action as electron donors and reactions with free radicals increase with decreasing *MW* of the peptides, making them more stable substances that stop chain reactions (Chi *et al*. 2014). The < 1 kDa fraction from PPH exhibited good antioxidant activity, which showed that it could serve as an antioxidant for reducing or eliminating the damage induced by free radicals in foods and biological systems.

Antidiabetic inhibitory activity. The α-amylase or α-glucosidase inhibitory activity is an index for inhibiting the increase in blood glucose level because α-amylase and α-glucosidase are enzymes involved in blood glucose elevation in non-insulin dependent diabetes mellitus, obesity and hyperglycemia (Cetto *et al*. 2000). The IC_{50} values of the α-amylase inhibitory activity ranged between 727.89–757.18 µg/ml (Figure 2A). The α-glucosidase inhibitory activity was the highest in the < 1 kDa (IC_{50} value of 54.51 µg/ml), whereas the inhibitory activity of > 10 kDa was the lowest (IC_{50} value of 1119.00 µg/ml) (Figure 2B). The IC_{50} values against the α-glucosidase activity of < 3 kDa peptides and the purified peptide from *Aspergillus oryzae* N159-1 were 7.7 and 3.1 mg/ml, respectively (Kang *et al*. 2013). The IC_{50} values against α-amylase and α-glucosidase of the Chinese giant salamander protein hydrolysate obtained using trypsin were 25.0 and 375.0 mg/ml, respectively (Ramadhan *et al*. 2017). Compared to the results of previous studies, the peptides obtained from the PSM had very high inhibitory activity on α-amylase and α-glucosidase. These results indicate that the peptides from PSM may be potential therapeutic agents

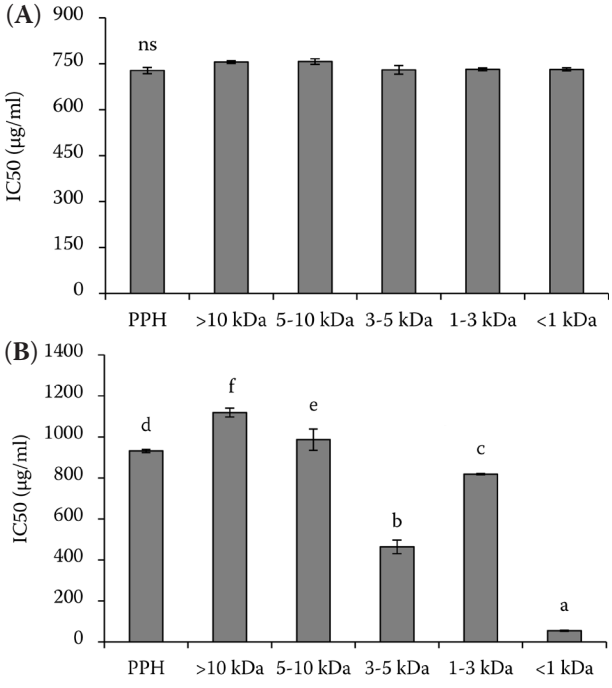

Figure 2. Antidiabetic inhibitory activity of the enzymatic hydrolysate and peptides obtained from PSM protein: (**A**) α-amylase; (**B**) α-glucosidase.

PPH – PSM protein hydrolysate; bars represents mean ± d (n = 3); different letters indicate significant difference (P < 0.05); ns – not significant

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for the management of postprandial hyperglycemia. In particular, the < 1 kDa fraction that exhibited α-glucosidase inhibitory activity could be considered as a potential α-glucosidase inhibitor agent.

ACE inhibitory activity. The ACE is a central component of the renin-angiotensin system and is a key enzyme influencing the regulation of blood pressure (Ngo *et al*. 2016). The < 1 kDa showed the strongest ACE inhibitory activity (IC_{50} value of 3.48 mg/ml), whereas 5–10 kDa showed the lowest activity (IC_{50} value of 8.57 mg/ml) (Figure 3). PPH (4.47 mg/ml), > 10 kDa (4.62 mg/ml), and 1–3 kDa (4.48 mg/ml) had similar inhibitory activity and there was no significant difference among these fractions. Similar results were observed by NGO *et al*. (2016) on Pacific cod skin gelatin hydrolysate with a report showing that the pectic hydrolysate < 1 kDa exhibited the strongest ACE inhibitory activity. Mane and Jamdar (2017) fractionated poultry viscera protein hydrolysate into three fractions by *UF*, and the < 1 kDa fraction exhibited the strongest ACE inhibitory activity. Most food protein-derived peptides with ACE inhibitory abilities have relatively low molecular masses, generally ranging from dipeptides to pentapeptides with MWs of 150–800 Da (Daskaya-Dikmen *et al*. 2017). These results are consistent with those reported in a previous study of ACE inhibitory peptides, in which low MW peptides had more potent ACE inhibitory activity than high *MW* peptides.

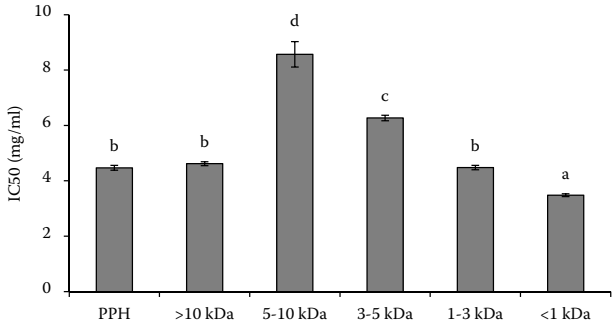

Figure 3. ACE inhibitory activity of the enzymatic hydrolysate and peptides obtained from PSM protein.
PPH – PSM protein hydrolysate; bars represents the mean ± sd (n = 3); different letters indicate significant difference (P < 0.05)

CONCLUSTIONS

The peptides fractionated by *UF* were found to have various physiological functions such as antioxidant, antidiabetic and antihypertensive activities. In particular, the < 1 kDa fraction exhibited the strongest ABTS and hydroxyl radicals scavenging activity and α-glucosidase and ACE inhibitory activity, and 1–3 kDa fraction showed the highest Fe^{2+} chelating ability. Therefore, peptides from PSM, and particularly < 1 kDa peptides, are expected to be used not only as a functional food but also as a pharmaceutical material due to excellent physiological activity. For this purpose, it is necessary to separate and purify bioactive peptides and confirm the amino acid composition and sequence of peptide depending on the purpose of each application.

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