

Full Length Research Paper

# Antioxidant peptides from freshwater clam extract using enzymatic hydrolysis

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The enzymatic hydrolysate of freshwater clam (*Corbicula fluminea*) extract was prepared using commercially available proteases. The antioxidant activity of the hydrolysate was evaluated by reducing power and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid free radical decolorization assays. The hydrolysate, especially its fraction purified by gel filtration, had a good antioxidant activity. The Trolox equivalent antioxidant capacity of the fraction with the highest radical scavenging ability reached  $158.04 \pm 2.43 \mu\text{g}/\text{mg}$ , which was sevenfold higher than that of the original hydrolysate. Molecular weight distribution analysis revealed that peptides over 1000 Da in the hydrolysate had better abilities to donate electrons, whereas peptides below 1000 Da more effectively eliminated radicals. Therefore, peptides from freshwater clam extract could be employed as potential natural antioxidants, performing their activities via different mechanisms.

**Key words:** Antioxidant peptides, freshwater clam extract, enzymatic hydrolysate, molecular weight distribution.

## INTRODUCTION

Freshwater clam (*Corbicula fluminea*) is a widely consumed bivalve in Asia. In addition to traditional fresh or dry products, freshwater clam essences (hot water extracts) are currently being marketed as a nutritional supplement in Taiwan. Various physiological functions of freshwater clam have been recognized. Freshwater clam extracts have shown a hypocholesterolemic effect for accelerating cholesterol degradation as well as excreting neutral sterols and bile acids (Chijimatsu et al., 2008). The muscle protein hydrolysates of freshwater clam have demonstrated a high inhibitory effect on angiotensin I-converting enzyme *in vitro* and antihypertensive activity by oral administration *in vivo* (Tsai et al., 2006). Hence, more attention is being paid to the recovery of such bioactive substances from freshwater clam (Chijimatsu et al., 2009; Lin et al., 2010; Sun et al., 2011).

Many diseases such as diabetes, atherosclerosis, neurodegenerative disorders, etc. have been confirmed to be related with oxidative stress. Therefore, antioxidants

are widely used as ingredients in dietary supplements to maintain good health (Elias et al., 2008). The use of synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene is currently restricted to food because they can induce DNA damage and toxicity. Consequently, numerous efforts have been exerted on the search for safe and natural antioxidants from animal and plant sources. In the past few years, many peptides that possess antioxidant activities against reactive oxygen species and free radicals have been identified. Such peptides include those from milk (Pihlanto, 2006), porcine myofibrillar protein (Saiga et al., 2003), egg yolk protein (Sakanaka and Tachibana, 2006), canola (Cumby et al., 2008), and chickpea protein (Li et al., 2008). Peptides originating from various fish and shellfish have also exhibited good antioxidant activities in different oxidative systems (Je et al., 2005; Mendis et al., 2005; Kim et al., 2007). Jellyfish collagen peptides ranging from 400 to 1200 Da have displayed high superoxide

anion-scavenging ( $IC_{50} = 21.9 \mu\text{g/mL}$ ) and hydroxyl radical-scavenging ( $IC_{50} = 16.7 \mu\text{g/mL}$ ) activities in vitro (Zhuang et al., 2009). The heptapeptide His-Phe-Gly-Asp-Pro-Phe-His (MW = 962 Da), derived from fermented marine blue mussel (*Mytilus edulis*) could effectively scavenge superoxide, hydroxyl, carbon-centered, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. The related  $IC_{50}$  values are 21, 34, 52, and 96  $\mu\text{M}$ , respectively (Rajiapakse et al., 2005). However, studies on antioxidant peptides from freshwater clam are still limited.

In the current study, to evaluate the antioxidant activities of the peptides from freshwater clam, the enzymatic hydrolysate was prepared from the hot water extract of freshwater clam, and the corresponding antioxidant activities were evaluated by reducing power and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical decolorization assays. The antioxidant peptides were purified by gel filtration and reverse phase (RP) high-performance liquid chromatography (HPLC). The composition of the peptides was determined using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

## MATERIALS AND METHODS

The freshwater clams (*C. fluminea*) were purchased from a local fish market, transported on ice to the laboratory, and frozen at  $-20^{\circ}\text{C}$  until analysis. Crude protease G (from *Aspergillus oryzae*), peptidase R (from *Rhizopus oryzae*), and peptidase G (from *Aspergillus oryzae*) were supplied by Amano Enzyme Company (Naoya, Japan).

Folin-Ciocalteu's phenol reagent, bovine serum albumin (BSA), Leu-Gly,  $\beta$ -mercaptoethanol, o-phthalaldehyde (OPA), trichloroacetic acid, potassium ferricyanide ( $\text{K}_3\text{Fe}(\text{CN})_6$ ), ferric chloride ( $\text{FeCl}_3$ ), trifluoroacetic acid (TFA), ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analog), and Sephadex G-25 were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Merck Ltd. (Darmstadt, Germany).

### Preparation of the hydrolysate

Whole freshwater clams (1000 g) were added to tap water in a 1:1 (w/w) ratio and boiled for 40 min according to a commercial procedure. The resulting liquid was filtered and concentrated to 400 mL. After the pH adjusted to 6.0 using NaOH, the hydrolysate was directly prepared from this condensed hot water extract. Protease G, peptidase R, and peptidase G were added, with the ratio of each enzyme to the initial freshwater clams as 1:250 (w/w). The reaction mixture was then incubated at  $45^{\circ}\text{C}$  for 24 h and subsequently heated at  $90^{\circ}\text{C}$  for 10 min to inactivate the enzymes. The supernatant obtained by centrifugation at  $20\,000\times g$  for 20 min was lyophilized as the hydrolysate.

### Measurement of soluble protein content

The soluble protein content was measured by the Lowry method (Lowry et al., 1951) using BSA as a standard.

### Measurement of peptide content

The peptide content was measured by OPA using Leu-Gly as a standard according to the method of Church et al. (1983). Prior to the measurement, the sample solution (50 mg/mL) was pumped through a  $0.22 \mu\text{m}$  membrane and an ultrafiltration membrane with a molecular weight cutoff of 5000 (Millipore, Bedford, MA, USA).

### Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay was performed according to the method described by Re et al. (1999). The previously prepared  $\text{ABTS}^{+\cdot}$  solution was diluted to an absorbance of  $0.7 \pm 0.02$  at 734 nm using sodium phosphate buffer (20 mM, pH 7.8). After the sample (300  $\mu\text{L}$ ) was added to 2.7 mL of dilute  $\text{ABTS}^{+\cdot}$  solution, the resultant mixture was vigorously shaken and allowed to stand in the dark for 6 min at room temperature. The absorbance at 734 nm was recorded as

$A_{\text{sample}}$ . The blank and the control were prepared in the same way as the sample, using  $\text{ddH}_2\text{O}$  and corresponding solvents instead of the sample and the test reagents, respectively. The percentage of  $\text{ABTS}^{+\cdot}$  radical scavenging activity was calculated as follows:

$$\text{ABTS}^{\cdot+}\text{-radical scavenging activity (\%)} = \frac{A_{\text{blank}} - (A_{\text{sample}} - A_{\text{control}})}{A_{\text{blank}}} \times 100\%$$

A standard curve of Trolox ranging from 0  $\mu\text{g/mL}$  to 6  $\mu\text{g/mL}$  was also prepared in the same manner. The final  $\text{ABTS}^{+\cdot}$  radical scavenging activity of the samples was expressed as microgram Trolox equivalents per milligram.

### Reducing power assay

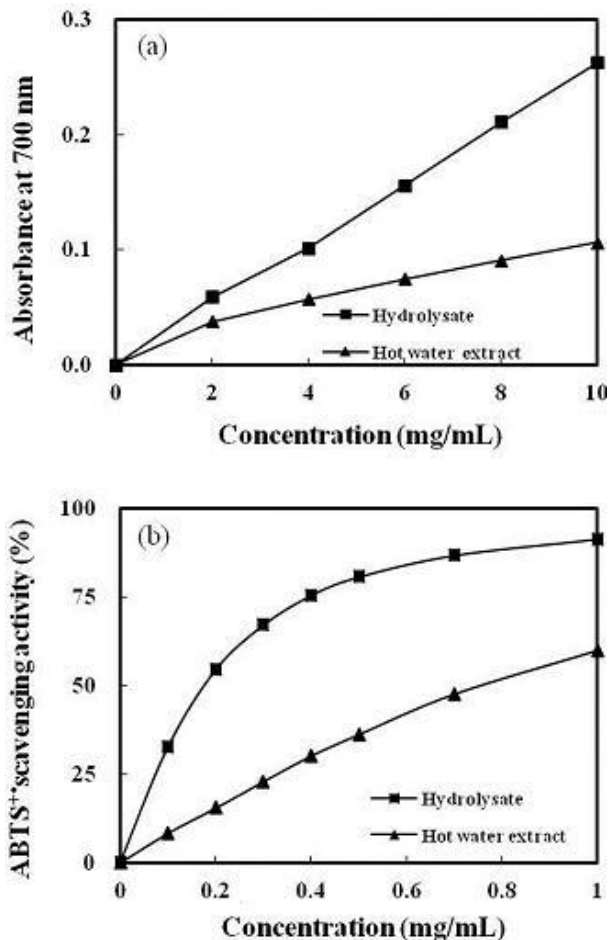
The reducing power was determined according to the method of Oyaizu (1986). The sample (1 mL) was mixed with 1 mL of 20 mM sodium phosphate buffer (pH 7.0) and 1 mL of 1%  $\text{K}_3\text{Fe}(\text{CN})_6$ . The mixture was incubated in a water bath at  $50^{\circ}\text{C}$  for 20 min, and trichloroacetic acid (10%, 0.5 mL) was added. After centrifugation at  $750 \times g$  for 10 min at room temperature, 2 mL of supernatant was collected, to which 2 mL of deionized water and 400  $\mu\text{L}$  of 0.1%  $\text{FeCl}_3$  were added. The absorbance of the reaction mixture at 700 nm was measured and recorded as  $A_{\text{sample}}$ . The reducing power (A) was calculated from the increase in the absorbance at 700 nm, as follows:

$$A = A_{\text{sample}} - A_{\text{blank}} - A_{\text{control}}$$

### Purification of antioxidant peptides in the hydrolysate

The peptides in the hydrolysate were purified by gel filtration chromatography using a fast protein liquid chromatography AKTA explorer 10S (GE Healthcare, Uppsala, Sweden). After filtration through a  $0.22 \mu\text{m}$  membrane, the hydrolysate (10 mg/mL, 200  $\mu\text{L}$ ) was loaded on a Sephadex G-25 column (16  $\times$  25 mm) and eluted by deionized water at a flow rate of 1 mL/min. Each 1.0 mL fraction was collected until no protein was monitored by the absorbance at 280 nm.

The fraction obtained from the gel fraction, which possessed the highest  $\text{ABTS}^{+\cdot}$  radical scavenging activity, was further separated using an RP-HPLC system (Jasco intelligent HPLC model 2080, Inc., Jasco, Tokyo, Japan) based on the absorbance at 220 nm. The lyophilized hydrolysate fraction (20 mg) was dissolved in 2 mL of 0.1% TFA. About 20  $\mu\text{L}$  of this sample was injected into a TSK-gel ODS-80Ts column (5  $\mu\text{m}$ , 4.6 mm  $\times$  250 mm, TosohBioscience



**Figure 1.** Reducing power and ABTS<sup>++</sup> radical scavenging activity of the hydrolysate from freshwater clam extract at various concentrations (a) Reducing power; (b) ABTS<sup>++</sup> radical scavenging activity.

LLC, Tokyo, Japan) and eluted using a two-solvent system [solvent A: 0.1% (v/v) TFA in deionized water; solvent B: 0.1% (v/v) TFA in 80% acetonitrile] at a flow rate of 0.8 mL/min. The gradient elution was as follows: 98% A in 0 min to 5 min, 98% to 50% A in 5 min to 55 min, 50% to 5% A in 55 to 70 min, and 5 to 100% A in 70 to 90 min.

### Molecular mass analysis

Molecular mass analysis was performed using a MALDI-TOF-MS system (AutoFlex, Bruker Daltonics, Bremen, Germany). The mass spectrometer was operated in the reflector and positive ion modes. External mass calibration was performed using a standard peptide mixture (Bruker Daltonics). The sample was diluted with 0.1% TFA aqueous solution, and  $\alpha$ -cyano-4 hydroxycinnamic acid (CCA) was used as the matrix.

### Statistical analysis

All analyses were carried out in triplicate, and results are reported as the mean  $\pm$  standard deviation (SD). Significant differences were

analyzed by two-way ANOVA using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL). Differences at  $p < 0.05$  were considered significant.

## RESULTS AND DISCUSSION

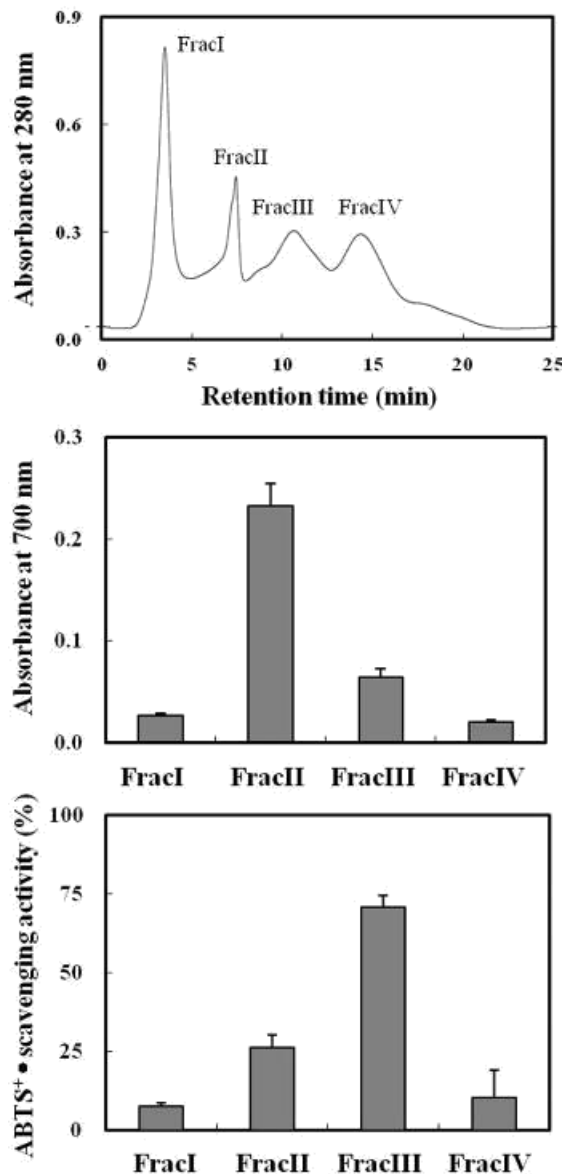
### Enzymatic hydrolysate of freshwater clam extract

The recovery of proteins in the hot water extract and enzymatic hydrolysate were calculated as  $2.22 \pm 0.06\%$  and  $2.13 \pm 0.12\%$ , respectively. The peptide contents were also determined as  $0.12 \pm 0.003$  and  $0.46 \pm 0.01$  mM/(mg·mL). The low recovery of proteins from raw freshwater clam was found not only in the current study, but also in other previous ones. Chijimatsu et al. (2011) have obtained approximately 1.5% (w/w) proteins from raw freshwater clam by hot water extraction. Tsai et al. (2006) have also pointed out that residual meat after hot water extraction still accounts for over 80% of the total weight of freshwater clam meat. In the present study, the recovery of proteins had a weak loss after hydrolysis. However, the peptide content remarkably improved for a wide variety of smaller peptides, and free amino acids were generated by enzyme effects.

### Antioxidant activity of the hydrolysate

Antioxidants play roles in multiple reaction mechanisms. An efficient way of assessing antioxidants is to take into account their oxidation-reduction potentials, i.e., measuring their reduction powers. Another generally applicable method is to examine their free radical scavenging activities (Jiménez-Escrig et al., 2001). In the current study, the antioxidant activity of the hot water extract and its hydrolysate was evaluated via reducing power and TEAC assays with the ABTS<sup>++</sup> radical. As shown in Figure 1a, the reducing power of the extracts with and without subsequent hydrolysis both increased within the concentration range of 0 to 10 mg/mL. The reducing power of the hydrolysate was much stronger than that of the hot water extract (named as nonhydrolysate). The TEAC assay results (Figure 1b) were similar. The ABTS<sup>++</sup> radical scavenging activity of the two extracts increased with their concentration (0 mg/mL to 1 mg/mL), and the increasing trend of radical scavenging effect was more noticeable in the enzymatic hydrolysate. Using the standard curve of Trolox, the TEAC values of the nonhydrolysate and enzymatic hydrolysate were calculated as 5.20

$\pm 0.28$  and  $21.00 \pm 0.72$   $\mu\text{g}/\text{mg}$ , respectively. The enhanced antioxidant activity of the hydrolysate resulted from the opening and exposure of active amino acid residues, which were electron donors and reacted with free radicals to terminate the radical chain reaction. The TEAC value of the hydrolysate from freshwater clam was relatively lower than that from Pacific hake (*Merluccius productus*:



**Figure 2.** Gel-filtration chromatography of antioxidant peptides from the hydrolysate of freshwater clam extract. The separation was carried out on a Sephadex G-25 column using deionized water as the eluent. The antioxidant activities of the eluted peaks were determined by reducing power and ABTS<sup>•+</sup> radical scavenging activity assays.

65.58  $\mu\text{g}/\text{mg}$ ) (Samaranayaka and Li-Chan, 2008). However, the antioxidant activity of the hydrolysate could be markedly improved via the purification of peptides.

#### Antioxidant peptides purified by gel-filtration chromatography

The hydrolysate was fractionated using Sephadex G-25. As shown in Figure 2, four major peaks were detected by the absorbance at 280 nm. The fractions associated with

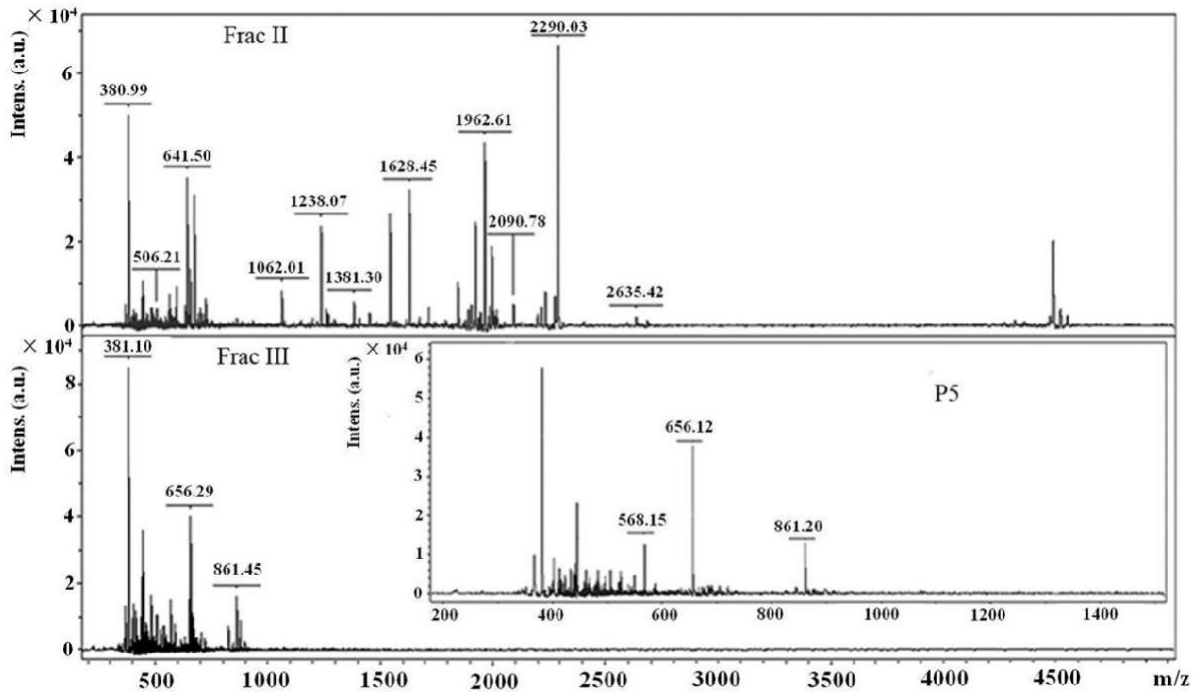
each peak (named as Frac I, II, III, and IV) were collected and lyophilized. Their antioxidant activities were evaluated by reducing power and ABTS<sup>•+</sup> radical scavenging activity assays. Among these four fractions, Frac II had the strongest reducing power, whereas Frac III had the highest ABTS<sup>•+</sup> radical scavenging activity. The absorbances of Fracs II and III at 700 nm were 0.233 and 0.064, respectively, at the peptide concentrations of 0.62 and 0.86 mM/ mM/(mg·mL), respectively. The TEAC values of Fracs II and III were measured as  $58.84 \pm 2.43 \mu\text{g}/\text{mg}$  and  $158.04 \pm 2.43 \mu\text{g}/\text{mg}$ , respectively. In addition, the IC<sub>50</sub> value of Frac III against ABTS<sup>•+</sup> was measured as 21.33  $\mu\text{g}/\text{mL}$  and was sevenfold higher than that of the hydrolysate.

Using MALDI-TOF-MS, the molecular weight distributions of Fracs II and III were analyzed (Figure 3). Most peptides in Frac II were greater than 1000 Da, mainly ranging from 1000 to 2500 Da, whereas those in Frac III were below 1000 Da. These findings indicated that short peptides (between two and 8 amino acid residues) were the main components of Frac III. Considering the different mechanisms of the reducing power and ABTS<sup>•+</sup> radical scavenging activity assays, peptides greater than 1000 Da evidently possessed better abilities to donate electrons, whereas those below 1000 Da more effectively eliminated free radicals. The higher radical scavenging activity of small peptides may be induced by steric effects given that smaller peptides have better access to the radicals. Similar observations have been obtained by other researchers. Wu et al. (2003) have found that mackerel hydrolysate with a molecular weight of 1400 Da possesses a stronger antioxidant activity than fractions whose molecular weights are 900 and 200 Da. Among enzymatic hydrolysates from purple sea urchin (*Strongylocentrotus nudus*) gonad, fractions below 1 kDa exhibit the highest DPPH radical scavenging capacity, whereas those ranging from 1 to 3 kDa show the highest reducing capacity (Qin et al., 2011).

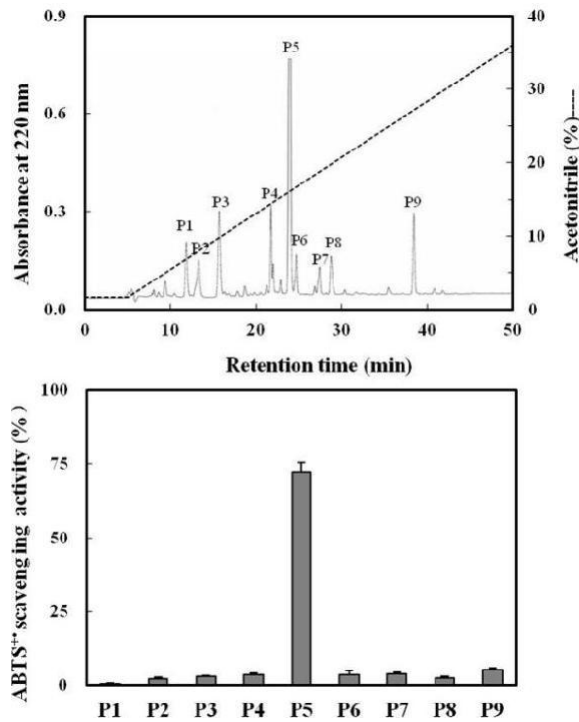
#### Antioxidant peptides further purified by RP-HPLC

For further purification, Frac III with the highest radical scavenging ability was subjected to RP-HPLC and divided into nine major portions (P1 to P9) by the gradient elution of acetonitrile (0 to 36%) containing 0.1% TFA. Accordingly, the ABTS<sup>•+</sup> radical scavenging ability of the nine major portions was evaluated. As shown in Figure 4, P5 exhibited a noticeable free radical scavenging effect, whereas the others had weak efficacies.

The molecular weight of P5 analyzed by MALDI-TOF-MS was similar to that of Frac III, especially in the low-mass region below  $m/z$  500 (Figure 3, inset). This similarity can be attributed to the used matrix of CCA, considering that ions produced from the matrix of CCA generally appear in this low-mass region and interfere with the acquisition of useful data (Langley et al., 2007). The peak at  $m/z$  568 may also correspond to a multimer



**Figure 3.** MALDI-TOF-MS spectra of Fracs II and III isolated from the hydrolysate of freshwater clam extract. Inset: MALDI-TOF-MS spectrum of P5 purified from Frac III.



**Figure 4.** RP-HPLC chromatograms (TSK-gel ODS-80Ts column) of antioxidant peptides from Frac III. The elution was performed with the linear gradient of acetonitrile (0% to 36%) containing 0.1% TFA. The antioxidant activities of the eluted peaks were determined by the ABTS<sup>•+</sup> radical scavenging assay.

matrix ion (3HCCA+H)<sup>+</sup> (Sönksen and Roepstorff 2001). Consequently, two major peptides in P5 were observed at the [M+H]<sup>+</sup> *m/z* values of 656 and 861 Da. The molecular weights of these peptides excellently agreed with previous studies. One such report is that on Ile-Glu-Phe-Phe-Thr-NH<sub>2</sub> isolated from the Australian red tree frog *Litoria rubella* (Steinborner et al., 1996), and another is that on a bradykinin-related peptide, Thr-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg, obtained from *Phyllomedusa hypochondrialis* (Brand et al., 2006).

The antioxidant activity of a peptide depends not only on its molecular size, but also on its chemical properties, such as hydrophobicity and the electron transferring ability of its amino acid residues. Although the antioxidant activities of the two reported peptides from frogs have not been studied, their amino acids Pro, Leu, and Arg have been confirmed to possess obvious antioxidant activities in other peptides (Chen et al., 1996). Phe has also been considered as an auxiliary antioxidant because it can render active oxygen stable via direct electron transfer, and donate protons to electron-deficient radicals (Jumeri, 2011). Based on this, the fraction of P5 purified from the enzymatic hydrolysate of freshwater clam ex-tracts was expected to exhibit a strong antioxidant ability.

## Conclusion

An enzymatic hydrolysate from freshwater clam extract was prepared and purified. Compared with the crude hot water extract, the hydrolysate exhibited a remarkably improved antioxidant activity based on reducing power and ABTS<sup>•+</sup> radical scavenging capacity assays. The anti-oxidant activity of the hydrolysate was further improved by more than sevenfold via gel filtration purification and RP-HPLC.

The MALDI-TOF-MS results indicated that higher-molecular weight (1000 to 2500 Da) peptides in the hydrolysate had better reducing powers, whereas peptides below 1000 Da more efficiently scavenged free radicals. Therefore, antioxidant peptides could be prepared from freshwater clam extracts via enzymatic hydrolysis, and may be used as ingredients of food and nutraceuticals.

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