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Relationship between antioxidant capacity and angiotensin-converting enzyme inhibitory activity of papain-generated protein hydrolysates and peptides from palm kernel cake proteins

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Abstract

Palm kernel cake (PKC) proteins were hydrolysed in the presence of seven proteolytic enzymes. The antioxidant and antihypertensive activities of each protein hydrolysate were evaluated. Papain-generated protein hydrolysate showed the highest antioxidant effects measured by radical scavenging activity (65%), iron (Fe)(II)-chelating activity (65%), and antihypertensive activity (71%). The results revealed a strong correlation between the antioxidant activity of the protein hydrolysates with angiotensin-converting enzyme (ACE) inhibitory activity (R^2 = 0.69 - 0.98), except for trypsin-generated hydrolysate (R^2 = 0.22). The most potent protein hydrolysate was fractionated and profiled using reversed-phase high-performance liquid chromatography and isoelectric focusing; peptides were subsequently isolated and identified by tandem mass spectrometry. The individual peptides were evaluated for antihypertension potential. A positive correlation was identified between radical scavenging activity and Fe(II)-chelating activity together and ACE inhibitory activity with R^2 = 0.69 - 0.98. The findings indicate that there was a positive relationship between the antioxidant and antihypertensive activities of protein hydrolysates and bioactive peptides from PKC proteins.

Introduction

Palm kernel cake (PKC) is the main by-product of the palm oil extraction process and a rich source of protein. However, PKC has not been fully exploited as food protein even though the protein extraction technique has been established. In Malaysia, PKC is generated in large quantities, with approximately 2.5 million tons/annum produced by palm oil mills. As such, PKC may be considered a reliable and feasible raw material source of food protein. In addition to its use in the manufacturing of cellulose, hemicellulose, and lignin, the use of PKC is limited at present to being a source of fibres for the animal feed industry.

A number of epidemiological studies have indicated a potent inverse relationship between cardiovascular disease (CVD) and fruit- and vegetables-rich diets (Liu *et al.*, 2002; Weisburger, 2002; Dias, 2019). Other epidemiological studies have also shown evidence of an association between CVD and daily antioxidant intake (Gey *et al.*, 1993; Bacchetti *et al.*, 2019; Marhuenda *et al.*, 2019; Steinbrecher, 1997; Franzini *et al.*, 2012; Tinkel *et al.*, 2012). Furthermore, the findings of multiple observational epidemiological studies indicate that foods rich in antioxidants are correlated with reduced incidence of CVD (Stanner *et al.*, 2004; Núñez-Córdoba and Martínez-González, 2011).

Oxidative stress and hypertension are important areas of interest in research and the manufacturing of functional foods. Antioxidants protect the body against oxidative damage, a hypothetical etiologic factor in human aging, and age-related diseases such as CVD. Therefore, it has been suggested that antioxidants might play an important role in the prevention of CVD and other chronic age-related diseases. Basic research has elucidated a potential mechanism by which antioxidants may decrease the risk of atherosclerosis. However, it remains unclear whether these associations are only between

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antioxidants and CVD, or whether other dietary or lifestyle factors are also implicated. The available data from current researches are insufficient to determine the risk-to-benefit ratio of antioxidant use. Moreover, only one large-scale trial of more than 10 years has been conducted with respect to antioxidant use, and the benefits reported in this trial may have been attributable to prolonged exposure to antioxidants. Therefore, the data currently available can neither prove nor disprove the value of antioxidant use (Gaziano, 1996; Small *et al.*, 2018).

There is no evidence to date to support a correlation between antioxidant activity and angiotensin converting enzyme (ACE) inhibitory activity of peptides, although Benzie and Tomlinson (1998) reported the antioxidant activity of ACE inhibitors using ferric reducing antioxidant power (FRAP) *in vitro*. Based on these findings, only the ACE inhibitor captopril has been shown to exhibit significant antioxidant activity, and an association between concentration and antioxidant power of captopril was indicated in both aqueous and ethanolic solutions.

To the best of our knowledge, no study to date has established a relationship between antioxidant activity and ACE inhibitory activity of protein hydrolysates and bioactive peptides. Therefore, the main objective of the present work was to evaluate the relationship between antioxidant activity and ACE inhibitory activity of protein hydrolysate and bioactive peptides generated from PKC proteins.

Materials and methods

Chemicals

Palm kernel cake (PKC) was purchased from My-4-Seasons, Malaysia. Proteases were purchased from Novozyme Co. (Nottingham, UK) and Sigma Aldrich (Munich, Germany). Other chemicals used in the present work were obtained from Fisher Scientific Co. (Georgia, US).

PKC protein isolate preparation

PKC was defatted with petroleum ether for 8 h in a Soxhlet extractor, and solvent was removed by rotary vacuum evaporator. Next, the defatted PKC was dried in a ventilator overnight at 20°C. PKC protein isolate (PKCPI) was produced following the method of Arifin *et al.* (2009), with slight modifications. PKCPI was obtained by dispersing 15 g DPKC in solution of NaOH (0.03 N) at a ratio of 1:30 (w/v) and extracted by shaking for 2 h in a water bath shaker. Following filtration through Whatman filter paper, the pH of the supernatant was adjusted to 3.5 (HCl 1 N), which is the isoelectric point of PKC protein. The precipitate was subsequently obtained by centrifugation at 10,000 *g* for 10 min, freeze-dried, and stored at -80°C until further use.

Preparation of PKC protein hydrolysates

PKC protein was hydrolysed following the method described by Zarei *et al.* (2015). PKC protein isolate was subsequently mixed with proteases including alcalase, papain, pepsin, trypsin, chymotrypsin, flavourzyme, and bromelain in a water bath shaker at the specific optimal conditions for each protease, and hydrolysed for 30 h under the conditions previously reported by Zarei *et al.* (2012). The reactions were terminated by heating the mixture in boiling water (100°C) for 10 min. After centrifugation (10,000 *g*, 4°C, 10 min), supernatants were obtained for antioxidant and ACE inhibitory activity assays.

DPPH• free radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity (RSA) assay was performed following the method described by Zarei *et al.* (2016) and Lee *et al.* (2010), with slight modifications. Briefly, peptide solution was mixed with deionised water and DPPH•. The mixture was kept in the dark, and the absorbance at 517 nm was measured using a 96-well microplate reader (Labomed, model UVD-2950, USA).

Iron-chelating activity assay

Iron-chelating activity was determined following the method of Decker and Welch (1990) and Zarei *et al.* (2014), with minor modifications.

ACE inhibition assay

ACE inhibition was determined following the method of Cushman and Cheung (1971) and Forghani *et al.* (2016), with slight modifications. Briefly, proteolysate or peptide solution was added to a mixture containing borate buffer and NaCl. Next, ACE was added to the mixture, which was then incubated at 37°C for 60 min. The enzymatic reaction was stopped by the addition of 1 N HCl. The hippuric acid released by the reaction was reacted with pyridine and benzenesulphonyl chloride (BSC). The mixture was vortexed for 1 min and placed in an ice bath, after which 200 µL was transferred to wells of a 96-well plate, and absorbance read at λ_{max} of 410 nm.

Fractionation and characterisation of PKC protein hydrolysates

Fractionation using reversed phase high performance liquid chromatography

Papain-generated PKC protein hydrolysate was fractionated following the method of Zarei *et al.* (2012) using a C_{18} reversed-phase high-performance liquid chromatography (RP-HPLC) column to fractionate based on the hydrophobicity of peptides.

Isoelectric focusing fractionation

The next step of fractionation was performed according to the isoelectric point (pI) of peptides following the method described by Zarei *et al.* (2015).

Peptide sequence identification using tandem mass spectrometry

Peptide sequences analysis was performed using a mass hybrid quadrupole-time of flight (Q-TOF) mass spectrometer following the method described by Zarei *et al.* (2014).

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's test to identify the differences between treatments at a 5% significant level, and Pearson's correlation to determine the correlation between biological activities. All analyses were performed using Minitab version 16 (Minitab Inc., State College, PA, USA).

Results and discussion

Bioactivities of papain-generated protein hydrolysates

PKC proteins were hydrolysed with papain, alcalase, pepsin, trypsin, chymotrypsin, bromelain, and flavourzyme to generate antioxidant and ACE inhibitory protein hydrolysates. Papain was added to the PKC protein buffer systems in three stages, at 0 h, 6 h, and 12 h with an enzyme-to-substrate ratio of 1:50. The Fe(II)-chelating activity and ACE inhibitory activity of all protein hydrolysates generated by papain markedly increased during the first 2 h of proteolysis, followed by a slow increase over the remaining hydrolysis time. The antioxidant activities and ACE inhibitory activities of the PKC protein hydrolysates were evaluated. As shown in Figure 1, papain-generated protein hydrolysates showed maximum antioxidant activity as measured by RSA, Fe(II)-chelating activity, and ACE inhibitory activity of 65%, 65%, and 71%, respectively, at 30 h

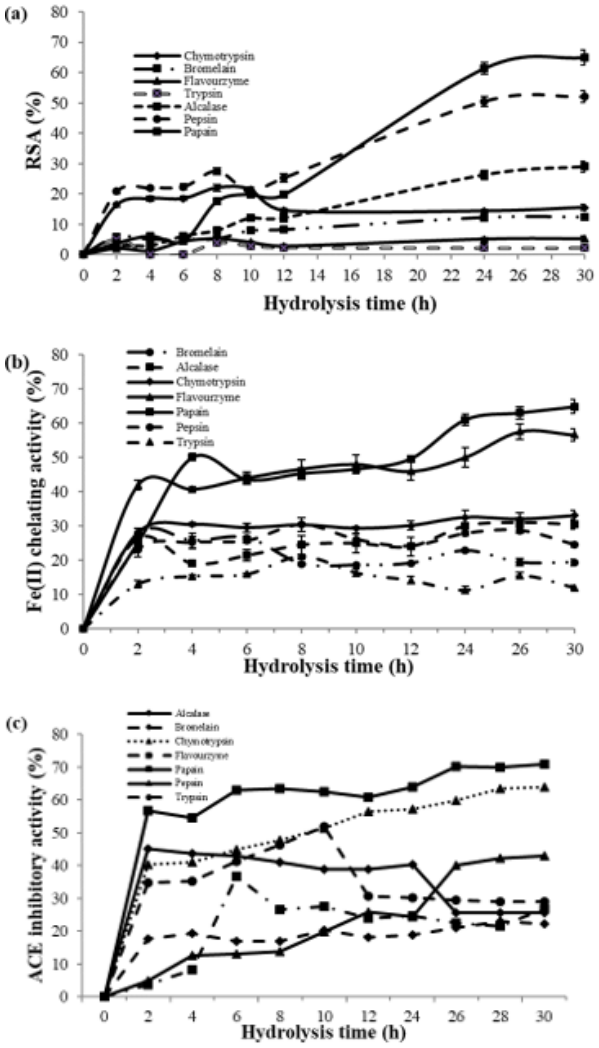

Figure 1. Bioactivities and time course of palm kernel cake protein hydrolysates generated by different enzymes: (a) radical scavenging activity; (b) Fe(II)-chelating activity; and (c) ACE inhibitory activity.

of hydrolysis time. ACE inhibitory activity greatly varied from 54% to 71% based on the hydrolysis time, thus reflecting variations in protein cleavage sites by hydrolysis time which could affect the resulting hydrolysate compositions and, therefore, the ACE inhibitory activity.

Correlation between ACE inhibitory activity and antioxidant activity of PKC protein hydrolysates

As shown in Figure 2, most of the RSA of PKC protein hydrolysates generated by different enzymes showed a positive correlation (R^2) with the ACE activity ranging between 0.69 and 0.98, except for trypsin generated protein hydrolysate which did not show a significant positive correlation (R^2 = 0.22), indicating that the bioactive peptides produced by trypsin are most likely not of low molecular weight (< 1,500 Da) and are not composed of hydrophobic

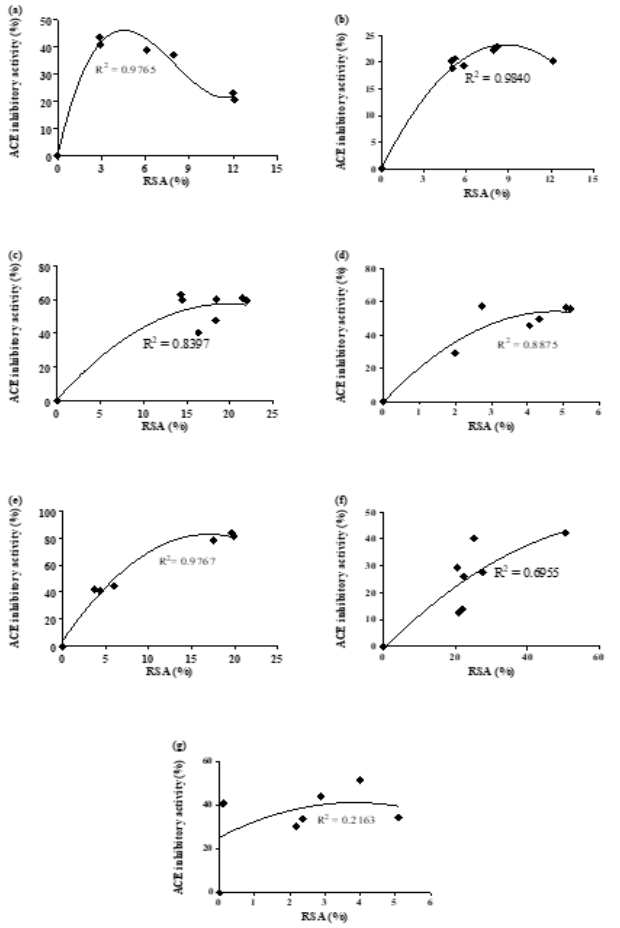

Figure 2. Function of radical scavenging activity (RSA) versus ACE inhibitory activity of PKC protein hydrolysates produced by different enzymes: (a) alcalase; (b) bromelain; (c) chymotrypsin; (d) flavourzyme; (e) papain; (f) pepsin; and (g) trypsin.

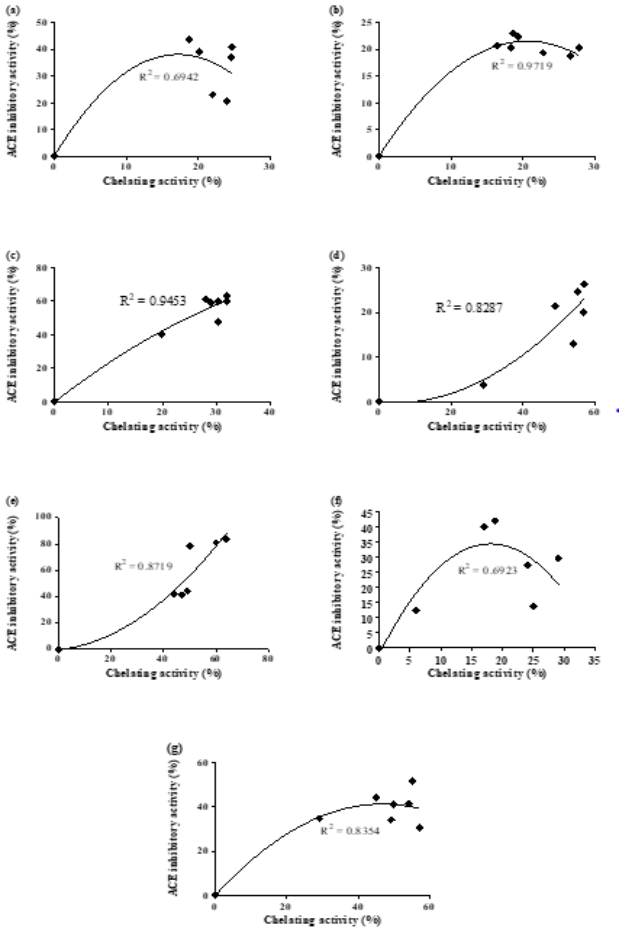

Figure 3. Function of Fe(II)-chelating activity versus ACE inhibitory activity of PKC protein hydrolysates produced by different enzymes: (a) alcalase; (b) bromelain; (c) chymotrypsin; (d) flavourzyme; (e) papain; (f) pepsin; and (g) trypsin.

residues. Figure 3 shows the correlation between ACE inhibitory activity and chelating activity of PKC protein hydrolysates produced by different proteases. As shown, a positive correlation was identified between the ACE inhibitory activities and chelating activities, with R^2 = 0.69 - 0.97 (Figures 3a - g). Among all of the protein hydrolysates, the ACE inhibitory activities of papain, flavourzyme, and chymotrypsin protein hydrolysates (Figures 3c - e) increased almost linearly with extended chelating activity. The other protein hydrolysates exhibited the same correlation, although they reached a plateau at different degrees of hydrolysis.

Few studies to date have examined the correlation between ACE inhibitory activity and antioxidant activity of protein hydrolysates generated from different protein sources *in vitro* and *in vivo*. However, some studies have demonstrated a relationship between CVD and antioxidant intake. In one report, individuals with a diet rich in fruit and vegetables or having a high plasma concentration of antioxidant micronutrients had a lower risk of stroke and CVD. The results of these studies indicate that high-risk populations with a high incidence of CVD, coronary heart disease, and stroke should increase their daily intake of dietary antioxidants. Furthermore, populations with low intakes of fruit and vegetables who follow this recommendation have subsequently been shown to have significantly increased plasma antioxidant concentrations (Szeto *et al.*, 2004).

In another study, antioxidants from pomegranate juice were shown to reduce blood pressure in hypertensive patients due to serum ACE inhibition, as these antioxidants can increase the activity of serum paraoxonase which can protect against lipid peroxidation (Aviram *et al.*, 2001). Moreover, the common carotid intima–media thickness, blood pressure, and LDL oxidation were markedly reduced in these patients after pomegranate juice consumption for three years (Aviram *et al.*, 2004).

In addition, in a study performed by Benzie *et al.* (2002) on the antioxidant power of ACE inhibitors, captopril was shown to have significant antioxidant activity. The findings also demonstrated

Table 1. Antioxidant and ACE inhibitory activity of palm kernel cake peptides.

Sequence	No. of residues	Molecular weight (g/mol)	Hydrophobic residues content (%)	DPPH• radical scavenging activity (%)	Iron-chelating activity (%)	ACE inhibitory activity (%)
YLLLK	5	648.85	80	41 ± 1.11^{E}	53 ± 1.00^{AB}	100 ± 2.99^{A}
YGIKVGYAIP	10	1080.29	60	44 ± 1.10^{DE}	50 ± 1.42^{BC}	100 ± 3.00^{A}
GGIF	4	392.46	50	31 ± 0.69^{F}	56 ± 1.56^{A}	97 ± 2.18^{A}
GIFE	4	462.52	50	31 ± 1.01^{F}	41 ± 0.83^{D}	40 ± 1.00^{E}
WAFS	4	509.56	75	47 ± 1.10^{CD}	38 ± 0.84^{D}	55 ± 1.81^{D}
GVQEGAGHYALL	12	1,214.34	50	36 ± 0.66^{F}	41 ± 0.58^{D}	70 ± 1.15^{C}
WAF	3	422.48	100	$56^{B} \pm 1.71^{B}$	47 ± 1.05^{C}	94 ± 2.36^{A}
AWFS	4	509.56	75	71 ± 2.22^{A}	41 ± 1.08^{D}	86 ± 2.58^{B}
LPWRPATNVF	10	1,200.41	60	50 ± 1.93^{C}	48 ± 1.05^{BC}	100 ± 3.00^{A}
Glutathione	-	-	-	83 ± 2.81	-	-
EDTA	-	-	-	-	96 ± 2.4	-
Captopril	-	-	-	-	-	98 ± 2.73

Data are presented as mean ± SD of three replications (n = 3). Mean values within each row indicated with different superscript letters A–F are significantly different ($p < 0.05$).

the concentration-dependent antioxidant activity of captopril in both aqueous and ethanolic solutions. Furthermore, a correlation was identified between PKC protein hydrolysates with higher DHs and ACE inhibitory activity and antioxidant activity with higher R^2. This finding indicated that PKC protein hydrolysates, including peptides with lower molecular weights and more hydrophobic residues, could simultaneously exhibit ACE inhibitory and antioxidant activities.

Identification and characterisation of bioactive peptides from papain-generated protein hydrolysate

Bioactive peptide sequences

Table 1 shows the antioxidant and ACE inhibitory activity of PKC peptides generated from PKC proteins. All peptides showed antioxidant and ACE inhibitory activities that varied from 31% - 71% for RSA, 38% - 56% for Fe(II)-chelating activity, and 40% - 100% for ACE inhibitory activity. The peptides AWFS and GGIF showed the highest radical scavenging and Fe(II)-chelating activity (71% and 56%, respectively) while peptides YLLLK, YGIKVGYAIP and LPWRPATNVF showed the highest ACE inhibitory activity (all 100%). As shown, papain-generated bioactive peptides showed higher and more potent ACE inhibitory activities as compared to their antioxidant activities.

Correlation between radical scavenging activity, Fe(II)-chelating activity, and ACE inhibition of bioactive peptides

The antioxidant properties of peptides are attributable to their free-radical-scavenging, metal-ion-chelating, and singlet-oxygen-quenching activities (Kitts and Weiler, 2003). The results of the structure–function relationships described in previous sections showed a close relationship between RSA, Fe(II)-chelating activity, and ACE inhibitory activity.

Figure 4 shows the relationship between the radical-scavenging activity and metal-chelating activity of biopeptides generated from PKC. As shown, the RSA increased with increasing metal-chelating activity. All bioactive peptides followed a second-order polynomial model with R^2 in the range of 0.74 - 0.97.

The mechanism of correlation between radical-scavenging activity and iron-chelating activity has not been previously reported, but it seems that the structure-function relationships of peptides could be the reason for the relationship between RSA and Fe(II)-chelating activity. As previous studies have shown (Xia *et al.*, 2012; Guo *et al.*, 2014), peptides with Phe, Tyr and Trp, Lys and Arg in their sequences show high chelating activity, and bioactive peptides having these amino acids in their structure (Wang and De Mejia, 2005) also show potent RSA. Furthermore, it has been previously reported that small peptides (< 12 kDa) showed higher chelating activity and RSA than larger peptides, although smaller bioactive peptides (< 500 Da) had higher iron-chelating activities than peptides > 500 Da (Torres-Fuentes *et al.*, 2012). Therefore, similarities in peptide structure appears to result in similar radical scavenging and metal-chelating activities.

Figure 5 shows the function of ACE inhibitory activity of peptides generated from PKC proteins versus RSA. The relationship between ACE

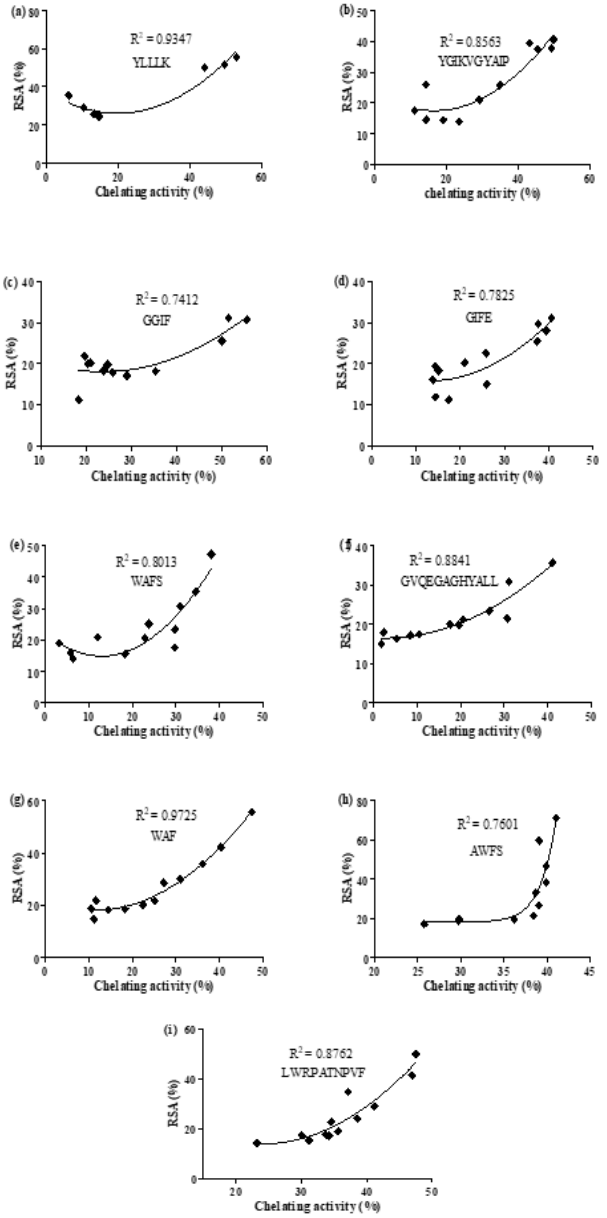

Figure 4. Function of radical scavenging activity (RSA) versus Fe(II)-chelating activity of bioactive peptides: (a) peptide 1; (b) peptide 2; (c) peptide 3; (d) peptide 4; (e) peptide 5; (f) peptide 6; (g) peptide 7; (h) peptide 8; and (i) peptide 9.

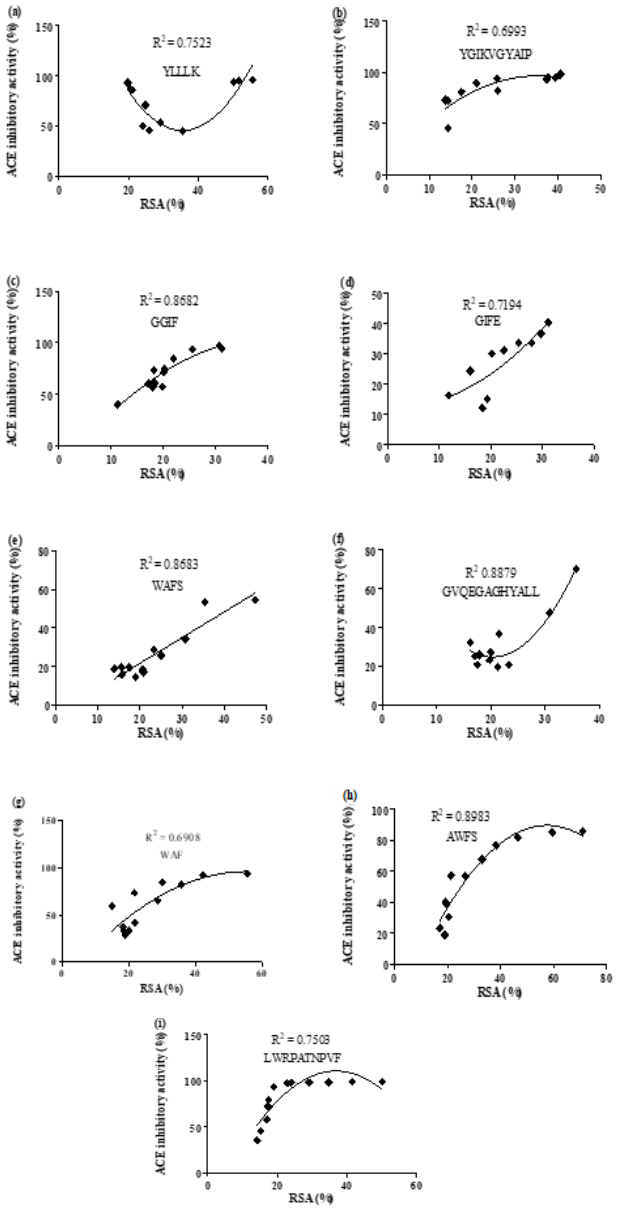

Figure 5. Function of ACE inhibitory activity versus radical scavenging activity (RSA) of bioactive peptides: (a) peptide 1; (b) peptide 2; (c) peptide 3; (d) peptide 4; (e) peptide 5; (f) peptide 6; (g) peptide 7; (h) peptide 8; and (i) peptide 9.

inhibitory activity and RSA also followed a second-order polynomial model, with R^2 between 0.69 and 0.89. The ACE inhibitory activity of bioactive peptides increased with increasing RSA, although the ACE inhibitory activity of peptide YLLLK decreased with increasing RSA up to 40%, after which the ACE inhibitory activity gradually increased.

It has been suggested that amino acid composition, peptide structure, and hydrophobicity are the most important factors affecting the antioxidant properties and ACE inhibitory activity of bio-peptides. Moreover, it has been indicated that cysteine, tryptophan, tyrosine, lysine, and methionine in the peptide sequence or at the C-terminal and Val or Leu at the N-terminal contribute to antioxidant potential and ACE inhibitory activity. Furthermore, aromatic residues are thought to enhance the antioxidant and ACE inhibitory activity of bio-peptides (Kitts and Weiler, 2003).

The ACE inhibitory activity of antioxidant peptides can be attributed to the hydrophobic and aromatic residues of antioxidant bioactive peptides obtained from PKC proteins, which can bind with the active site of ACE as a hydrophobic active site.

In comparison, previous studies have suggested that diets with high consumption of fruits and

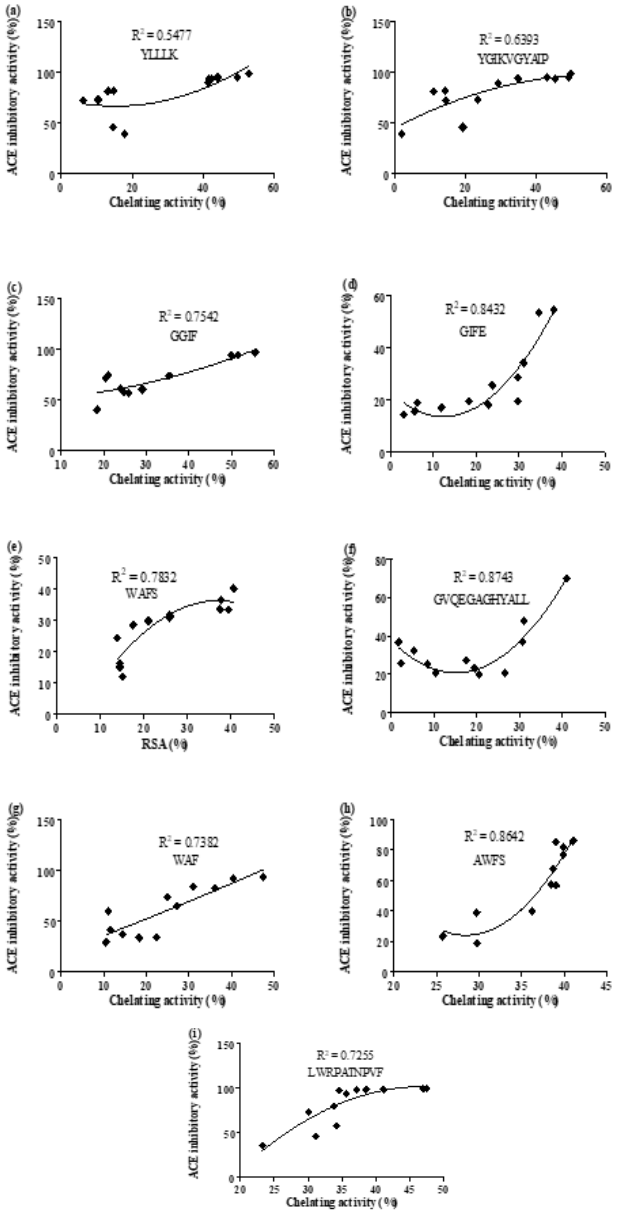

Figure 6. Function of ACE inhibitory activity versus Fe(II)-chelating activity of bioactive peptides: (a) peptide 1; (b) peptide 2; (c) peptide 3; (d) peptide 4; (e) peptide 5; (f) peptide 6; (g) peptide 7; (h) peptide 8; and (i) peptide 9.

vegetables are associated with moderately lower overall mortality rates and lower death rates from CVD and some types of cancer. The 'antioxidant hypothesis' proposes that vitamin C, carotenoids, vitamin E, and other antioxidants significantly increase protection against chronic diseases by reducing oxidative stress (Stanner *et al.*, 2004).

Moreover, a number of epidemiological studies have indicated that the risk of CVD is markedly decreased with a diet rich in plant-based foods such as fruits and vegetables (Liu *et al.*, 2002; Weisburger, 2002). Several epidemiological studies have also shown evidence of a positive correlation between CVD and antioxidant intake (Gey *et al.*, 1991; 1993), and multiple studies and reviews about the relationship between CVD and antioxidants have been published (Steinbrecher, 1997; Tinkel *et al.*, 2012). Furthermore, a number of observational epidemiological studies support the hypothesis that the incidence of CVD in individuals with high daily antioxidant intake is meaningfully lower than in those with low daily antioxidant intake (Stanner *et al.*, 2004).

Although no study to date has described a correlation between antioxidant activity and ACE inhibitory activity of bioactive peptides, Benzie and Tomlinson (1998) evaluated the antioxidant power of ACE inhibitors using FRAP *in vitro*. Based on their findings, only captopril was found to exhibit significant antioxidant power, and this effect was observed in both aqueous and ethanolic solutions.

Figure 6 shows the function of ACE inhibitory activity versus Fe(II)-chelating activity of bioactive peptides generated from PKC proteins. The correlation between ACE inhibitory activity and metal-chelating activity followed a second-order polynomial model, with an R^2 between 0.5477 and 0.8743. The ACE inhibitory activity of peptides increased with increasing chelating activity of bioactive peptides. However, the ACE inhibitory activity of the peptides GIFE and GVQEGAGHYALL slightly decreased as chelating activity increased but gradually increased with a sharp slope (Figure 6d) and (Figure 6f).

Inhibitors of ACE include metal-chelating agents such as ethylenediaminetetraacetic acid, *o*-phenanthroline, and 8-OH-quinoline, and a number of synthetic pharmaceuticals and bioactive peptides (Wyvratt and Patchett, 1985; Murray and FitzGerald, 2007). The high ACE inhibitory activity of some bioactive peptides might presumably relate to their ability to chelate metal in the zinc ligand of ACE (Kang *et al.*, 2003; Qian *et al.*, 2007). The results obtained in the present work thus confirm that there is a positive correlation between DPPH• RSA and metal-chelating activity with ACE inhibitory activity.

Conclusion

Seven enzymatic-protein hydrolysates were generated from PKC proteins; and were found to exhibit ACE inhibitory and antioxidant activities. Papain-generated protein hydrolysate showed the most potent ACE inhibitory and antioxidant activities among the various protein hydrolysates. Bioactive peptides were isolated and identified from papain-generated PKC protein hydrolysate by tandem MS, and their respective antioxidant and antihypertensive

activities were evaluated. The results showed a positive correlation between ACE inhibition and radical scavenging and Fe(II)-chelating activities, with $R^2 = 0.69 - 0.98$ and $R^2 = 0.69 - 0.97$, respectively. Furthermore, the ACE inhibitory and antioxidant activities of bioactive peptides were also shown to have a positive correlation. ACE inhibitory activity of antioxidant peptides appeared to be attributable to the hydrophobic and aromatic residues of antioxidant bioactive peptides obtained from PKC proteins, which could bind to the active site of ACE as a hydrophobic active site.

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