RESEARCH ARTICLE

Protein Hydrolysates from *Citrullus lanatus* Seed: Antiradical and Hydrogen Peroxide-scavenging properties and kinetics of Angiotensin-I converting enzyme inhibition

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Abstract: This study investigated the *in vitro* antihypertensive, antiradical and hydrogen peroxide-scavenging properties of protein hydrolysates from *Citrullus lanatus* (watermelon) seed (CSPHs) obtained through enzymatic digestion. Proteins from watermelon seeds were isolated and enzymatically hydrolyzed with non-specific (alcalase), moderately specific (pepsin) and highly specific (trypsin) proteases, mimicking human gastrointestinal digestion. The hydrolysates were investigated for inhibitory property against angiotensin-I-converting enzyme (ACE) activity. Using N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine as the substrate, CSPHs showed concentration-dependent ACE inhibition (IC₅₀ 1.377 - 1.757 mg/mL) with peptic CSPH having the strongest ACE-inhibition followed by tryptic CSPH. Kinetic analysis revealed that peptic CSPH inhibited ACE activity in a mixed-type inhibition pattern while alcalase and tryptic CSPHs exhibited non-competitive inhibition mode. Peptic CSPH demonstrated the strongest DPPH radical-scavenging activity while tryptic CSPH showed the highest H₂O₂-scavenging property. These results show that protein hydrolysates from watermelon seed possess bioactivities that could be exploited in the management of hypertension.

Keywords: Antihypertensive, antioxidant, watermelon, pepsin, trypsin, alcalase.

INTRODUCTION

Proteins from food sources usually contain specific peptide sequences which remain inactive as long as they are bonded to other amino acids within the primary structure (Aluko, 2015). Proteolysis by enzymes can release such peptide sequences from the proteins and can then be used as therapeutic agents (Li *et al.*, 2014; Aluko, 2015). Hypertension, a condition in which the blood pressure is abnormally high (Dufton,

2011), has been reported to be affecting about 0.9 to 1.0 billion people worldwide, and it is estimated that this number will rise to more than 1.6 billion by 2025 (Chockalingam *et al.*, 2006; WHO, 2013).

The renin-angiotensin system, known to perform an important role in the maintenance of blood pressure and associated cardiovascular diseases (CVDs), is chiefly controlled by two enzymes: renin and angiotensin-I converting enzyme (ACE) (Onuh *et al.*, 2013). Renin converts angiotensinogen to angiotensin-I which in turn is converted by ACE to angiotensin-II, a potent vasoconstrictor. ACE also degrades bradykinin, a vasodilator. Thus, inhibition of ACE is considered to be a useful procedure in the prevention and treatment of hypertension (Udenigwe *et al.*, 2009; Onuh *et al.*, 2013).

However, various synthetic ACE-inhibitory compounds that have been developed as antihypertensive drugs have shown undesirable side effects such as coughing, taste disturbances, skin rashes etc. (Acharya *et al.*, 2003; Lahogue *et al.*, 2010). Therefore, the search for safer and natural ACE inhibitors as alternatives to synthetic drugs has become important (Badyal *et al.*, 2003; Ghassem *et al.*, 2014). ACE-inhibitory peptides derived from food sources have no known side effects (Girgih *et al.*, 2011). Peptides with antihypertensive properties are the best known and most studied among the bioactive peptides (Onuh *et al.*, 2013).

Oxidative stress occurs as a consequence of a disproportion between the levels of reactive (oxygen and nitrogen) species and endogenously produced antioxidants (Hadi *et al.*, 2005;

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Harrison *et al.*, 2007). This imbalance is believed to be involved in many types of CVDs as there is a strong association between blood pressure and some oxidative stress-related parameters (Briones and Touyz, 2010; Baradaran and Rafieian-kopaei, 2014). For the purpose of designing preventive and curative strategies, reduction in the level of oxidative stress using natural antioxidants is important in the treatment of cardiovascular diseases (Bains and Hall, 2012; Maulik and Kumar, 2012).

Citullus lanatus (commonly called watermelon) belongs to the Cucurbitaceae family and is one of the most widely cultivated crops globally as it accounts for 6.8% of the world vegetable production area (Dane and Liu, 2007). Generally, the fleshy part of the fruit, known to have very low protein content (~2%), is consumed and the seeds which are rich in protein (30.63 – 43.6%) are usually discarded (Singh and Matta, 2010; Jacob *et al.*, 2015). *C. lanatus* has been reported traditionally and in several studies to have such therapeutic properties as antihypertensive, anti-inflammatory, antidiabetic, antioxidant, antimicrobial, anti-plasmodial, hepatoprotective and analgesic effects (Erhirhie and Ekene, 2013; Sani, 2014). However, the traditionally and locally reported antihypertensive potential of *C. lanatus* is yet to be fully verified (Honga *et al.*, 2015). Hence, this study investigated the inhibition of ACE by *C. lanatus* seed protein hydrolysates obtained through hydrolysis by pepsin, trypsin and alcalase, as well as their anti-radical and hydrogen peroxide-scavenging properties.

MATERIALS AND METHODS

Materials

Watermelon fruits were purchased from a local fruit store at Tanke area, Ilorin, Kwara state, Nigeria, and authenticated at the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria where a voucher no. UILH/001/1149 was deposited for record purposes. Trizma-base, n-hexane, bovine serum albumin, pepsin (from porcine gastric mucosa), trypsin (from bovine pancreas), alcalase (protease from *Bacillus licheniformis*), angiotensin-I converting enzyme (ACE; from rabbit lung), ACE substrate N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) and DPPH (1,1-diphenyl-2-picrylhydrazyl) were products of Sigma-Aldrich (USA).

Methods

Isolation of Watermelon Seed Proteins

C. lanatus seeds were dried and pulverized before being kept in an air-tight container at 4 °C. The powder was defatted using n-hexane as was previously described by Wani *et al.* (2011). The seed was defatted using Soxhlet apparatus ran for a period of five hours. The defatted meal was then de-solventized at room temperature and ground again to obtain a fine powder, termed defatted seed meal, which was then refrigerated until later use.

The protein component of the defatted meal was extracted using the method described by Alashi *et al.* (2014) with slight modifications. Defatted watermelon seed meal was suspended (1:10) in 0.1 M NaOH pH 12.0, stirred for 1 hr and centrifuged at 18 °C and 3000 rpm for 10 min. Two additional extractions of the residue were carried out and the supernatants pooled. The pH of the supernatant was adjusted to pH 4.0 using 1 M HCl solution and the precipitate formed was recovered by centrifugation. The precipitate was washed with distilled water, adjusted to pH 7.0 using 1 M NaOH, freeze-dried and the protein isolate termed CSPI was refrigerated until required for further analysis. Protein yield was determined as described by Arise *et al.* (2015).

Preparation of Watermelon Seed Protein Hydrolysates

The protein isolate was hydrolysed using the methods described by Udenigwe *et al.* (2009) with slight modifications. Hydrolysis was done using each of pepsin (pH 2.2, 37°C), trypsin (pH 8.0, 37°C) and alcalase (pH 8.0, 60°C). CSPI (5% w/v) was dissolved in the appropriate buffer (phosphate buffer, pH 8.0 for trypsin and alcalase; and glycine buffer, pH 2.2 for pepsin) and the enzyme was added at an enzyme-substrate ratio (E:S) of 1:100. Digestion was performed for 5 hr before the reaction vessel was immersed in boiling water for 15 min. The pH was adjusted to 4.0 followed by centrifugation at 4000 rpm for 30 min. The supernatant was collected, analysed for degree of hydrolysis and then freeze-dried. The dried hydrolysate termed CSPH was then refrigerated until further analysis. Degree of hydrolysis (DH) was determined according to the method described by Hoyle and Merritt, (1994). Percentage peptide

yield was determined using the method described by Girgih *et al.* (2011).

Determination of ACE-inhibitory Activity

The ACE-inhibitory activity of CSPH was determined according to the method of Holmquist *et al.* (1979) as reported by Udenigwe *et al.* (2009) using N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate. Briefly, aliquot amount (500 µL) of 0.5 mM FAPGG (dissolved in 50 mM Tris-HCl buffer containing 0.3 mM NaCl, pH 7.5) was mixed with 20 µL of ACE (0.1 U/mL; final activity of 2 mU) and 100 µL of CSPH (0.50 - 2.00 mg/mL) in 50 mM Tris-HCl buffer. The decreased absorbance at 345 nm, due to cleavage of the Phe-Gly peptide bond of FAPGG, was recorded at regular intervals for 3 min at room temperature. For the blank experiment, Tris-HCl buffer was used instead of CSPH. The activity of ACE was expressed as the rate of disappearance of FAPGG ($\Delta\text{Absorbance} \cdot \text{min}^{-1}$) and inhibitory activity calculated as shown:

$$\text{ACE Inhibition (100\%)} = \frac{S - B}{S} \times 100\%$$

where $S = (\Delta\text{Absorbance} \cdot \text{min}^{-1})_{\text{sample}}$ and $B = (\Delta\text{Absorbance} \cdot \text{min}^{-1})_{\text{blank}}$ are the reaction rates in the presence and absence of the hydrolysate, respectively.

The concentration of CSPH that inhibited ACE activity by 50% (IC_{50}) was interpolated from a non-linear regression plot of ACE inhibition *versus* sample concentrations using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). All experiments were performed in triplicates.

Determination of Kinetic Parameters of ACE Inhibition

The kinetic analysis of ACE inhibition was done using the method described by Girgih *et al.*, (2011). The kinetics of ACE-catalyzed conversion of FAPGG to N-[3-(2-furyl)acryloyl]-L-phenylalanine (FAP) was studied in the absence and presence of three different concentrations (0.50, 1.00 and 1.50 mg/mL) of CSPH with 0.0625, 0.125, 0.25 and 0.5 mM FAPGG. The mode of inhibition of ACE and the kinetic parameters (K_m , K'_m , V_{max} and V'_{max}) were determined using the Lineweaver-Burk plots. The catalytic efficiency (CE) in the

presence and absence of the inhibitor was also determined using the equation below:

$$\text{CE} = \frac{V_{max}}{K_m} \text{ or } \frac{V'_{max}}{K'_m}$$

K_m and V_{max} are the maximum reaction velocity and Michaelis constant respectively, whereas K'_m and V'_{max} represent their apparent values in the presence of the inhibitor. The inhibition constant (K_i) was determined as the intercept on the x-axis from the secondary plot of the slopes of the Lineweaver-Burk plots against inhibitor concentrations using Microsoft Office (Excel) version 2016.

Determination of DPPH Radical-scavenging Activity

The DPPH radical-scavenging activity of CSPHs was measured using the assay method described by Shimada *et al.*, (1992). DPPH (0.05 mM) was prepared in 95% ethanol and CSPH was dissolved in distilled water. Aliquot volume (0.60 mL) of CSPH (1.00 - 2.50 mg/mL) was added to 1.50 mL of 0.05 mM DPPH solution, shaken vigorously and incubated in the dark at room temperature for 30 min. Absorbance was read at 517 nm. Distilled water was used as blank and the control consisted of 0.60 mL distilled water and 1.50 mL DPPH solution. Percentage DPPH $\cdot$ inhibition was calculated as shown below.

$$\text{DPPH-SA(\%)} = \frac{AC - AS}{AC} \times 100\%$$

where SA=persentage of Scavenging Activity, AC=Absorbance_{control} and AS=Absorbance_{sample} respectively.

50% effective concentrations (EC_{50}) of CSPH and ascorbic acid that scavenged 50% DPPH radical were interpolated from a non-linear regression plot of DPPH $\cdot$ -scavenging activity *versus* CSPH concentrations. All experiments were performed in triplicates.

Determination of Hydrogen Peroxide-scavenging Activity

The ability of CSPH to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.* (1989) as described by Keser *et al.* (2012). H_2O_2 (4 mM) was prepared in 0.20 M phosphate buffer (pH 7.4). 1.00 mL CSPH (0.20 - 0.80 mg/mL) in distilled water

was added to 0.15 mL of H₂O₂ solution. Absorbance was read at 230 nm after 10 min against a blank (phosphate buffer without H₂O₂). H₂O₂ was taken as control and ascorbic acid was used as a standard antioxidant. The percentage scavenging capacity was calculated thus:

$$\text{H}_2\text{O}_2 - \text{SA}(\%) = \frac{AC - AS}{AC} \times 100\%$$

where SA=presentation of Scavenging Activity, AC=Absorbance_{control} and AS=Absorbance_{sample} respectively.

50% effective concentrations (EC₅₀) of CSPH and ascorbic acid were interpolated from a non-linear regression plot of H₂O₂-scavenging capacity versus CSPH concentrations using GraphPad Prism version 6.0. The experiments were performed in triplicates.

Statistical Analysis

Analyses were conducted in replicates as indicated above. Data are reported as mean of replicates $\pm$ standard deviation (SD), and were subjected to analysis of variance (ANOVA) and Tukey's multiple range tests using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

RESULTS

ACE-inhibitory Activity

The percentage ACE-inhibitory activity of CSPHs at different concentrations of peptic, tryptic and alcalase hydrolysates is shown in Figure 1. At lower concentrations of 0.50 – 1.50 mg/mL, the percentage ACE-inhibitory activity of tryptic CSPH was higher ($P < 0.05$) than those of peptic and alcalase CSPHs. At 2.00 mg/mL, ACE-inhibitory activity of peptic CSPH ($79.55 \pm 1.36\%$) was significantly higher than those of tryptic ($73.64 \pm 0.91\%$) and alcalase ($48.86 \pm 0.68\%$) CSPHs. The ACE-inhibitory activity of alcalase CSPH was consistently lower ($P < 0.05$) than those of peptic and tryptic CSPHs at all the studied concentrations. All the three hydrolysates exhibited concentration-dependent ACE-inhibitory activity. Figure 2 shows the 50% ACE-inhibitory concentrations (IC₅₀) of CSPHs.

The IC₅₀ of peptic and tryptic CSPHs were not significantly different but both were lower ($P < 0.05$) than that of alcalase CSPH.

Kinetics of ACE Inhibition

The effects of peptic, tryptic and alcalase CSPHs on the kinetic parameters of ACE-catalysed conversion of FAPGG to FAP were investigated (Figures 3-5 respectively). Kinetic parameters obtained from Lineweaver-Burk plots in the absence and presence of three different concentrations of each of peptic, tryptic and alcalase CSPHs are summarized in Table 1. The apparent Michaelis constant K'_m (mM) in the presence of peptic CSPH was higher than those of tryptic and alcalase CSPHs at almost all hydrolysate study concentrations. Similarly, the K'_m in the presence of tryptic CSPH was higher than that of alcalase CSPH at all hydrolysate concentrations studied. The presence of peptic CSPH showed a clear concentration-dependent increase in K'_m whereas the presence of tryptic and alcalase CSPHs appeared not to have an effect of K'_m .

The three CSPHs at all concentrations studied resulted in a decrease in the maximal rate V'_{max} (A/min). While V'_{max} in the presence of alcalase CSPH was lower than that of peptic CSPH, the value in the presence of tryptic CSPH was even lower than in the presence of both alcalase and peptic CSPHs. CE of ACE in the presence of all the hydrolysates at all studied concentrations was consistently reduced in a concentration-dependent manner. Tryptic CSPH caused the highest reduction in the CE of ACE followed by peptic CSPH. The enzyme-inhibitor dissociation constant K_i (mg/mL) of ACE inhibition by peptic CSPH ($K_i = 0.444$ mg/mL) was lower than those of tryptic (1.739 mg/mL) and alcalase (0.867 mg/mL) CSPHs. The modes of ACE inhibition were mixed type (peptic CSPH) and non-competitive (tryptic and alcalase CSPHs) mechanisms respectively. These modes of inhibition are also evident in the K'_m values obtained (Table 1) where peptic CSPH demonstrated a concentration-dependent increase in K'_m whereas tryptic and alcalase CSPHs caused no significant changes in the K'_m .

Table 1: Kinetic parameters of ACE-catalyzed FAPGG-FAP conversion in the absence and presence of *C. lanatus* seed protein hydrolysates

Catalytic Parameter	No Inhibitor	Peptic CSPH (mg/mL)			Tryptic CSPH (mg/mL)			Alcalase CSPH (mg/mL)		
		0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
K_m or K'_m (mM)	0.2957	0.3424	0.4257	0.7142	0.3469	0.3913	0.4016	0.2846	0.2547	0.2421
V_{max} or V'_{max} ^a	0.0181	0.0146	0.0135	0.0152	0.0062	0.0055	0.0050	0.0138	0.0080	0.0066
CE (A/min/mM)	0.0612	0.0428	0.0318	0.0213	0.0180	0.0141	0.0124	0.0485	0.0313	0.0274
K_i (mg/mL)	—	0.444			1.739			0.867		

^a - ($\mu\text{mol}/\text{mg}/\text{min}$); K_m or K'_m – Michaelis constant in the presence and absence of hydrolysate; V_{max} or V'_{max} – Maximum reaction rate in the presence and absence of hydrolysate; CE – Catalytic efficiency; K_i – Enzyme-inhibitor dissociation constant.

Figure 1: ACE-inhibitory activity of *C. lanatus* seed protein hydrolysates at different concentrations. Each bar represents mean of triplicate determinations $\pm$ SD. Bars at the same concentration but with different letters are significantly different ($P < 0.05$). Bars at different concentrations but with the same symbol (#) are not significantly different ($P > 0.05$).

Figure 2: 50% ACE-inhibitory concentration (IC₅₀) values of *C. lanatus* seed protein hydrolysates. Each bar represents the mean of triplicate determinations of IC₅₀ ± SD; bars with different letters are significantly different at $P < 0.05$.

R^2 - coefficient of determination

Figure 3: Lineweaver-Burk plot of ACE-inhibition by different concentrations of *C. lanatus* seed protein hydrolysate derived from peptic digestion

R^2 - coefficient of determination

Figure 4: Lineweaver-Burk plot of the inhibition of ACE by different concentrations of *C. lanatus* seed protein hydrolysate obtained from tryptic digestion

R^2 - coefficient of determination

Figure 5: Lineweaver-Burk plot of the inhibition of ACE by different concentrations of *C. lanatus* seed protein hydrolysate obtained from alcalase digestion

DPPH Radical-scavenging Activity

The percentage DPPH $^{\bullet}$ -scavenging activities of CSPHs are presented in Figure 6. DPPH $^{\bullet}$ -scavenging activity of ascorbic acid was significantly higher ($P < 0.05$) than those of CSPHs at all study concentrations, but all demonstrated concentration-dependent radical-scavenging activity. At 2.5 mg/mL, peptic CSPH exhibited the highest radical-scavenging power

($56.39 \pm 0.45\%$), followed by tryptic CSPH ($47.10 \pm 1.33\%$) while alcalase CSPH had the lowest radical-scavenging power ($43.22 \pm 0.77\%$). EC_{50} of CSPHs and ascorbic acid were estimated (Figure 7). EC_{50} of ascorbic acid ($0.303 \pm 0.047 \text{ mg/mL}$) was significantly lower than those of CSPHs. However, EC_{50} of peptic CSPH was lower ($P < 0.05$) than those of tryptic and alcalase CSPHs, while EC_{50} of tryptic CSPH was significantly lower than that of alcalase CSPH.

Figure 6: DPPH radical-scavenging activity of peptic, tryptic and alcalase protein hydrolysates derived from *C. lanatus* seeds. Each dot represents the mean of triplicate determinations $\pm$ SD. Dots belonging to different enzyme hydrolysates/samples at the same concentration but with different letters are significantly different at $P < 0.05$.

Figure 7: 50% effective concentration (EC₅₀) values of *C. lanatus* seed protein hydrolysate in scavenging DPPH radicals. Each bar represents the mean of triplicate determinations of EC₅₀ $\pm$ SD. Bars with different letters are significantly different at $P < 0.05$.

Hydrogen Peroxide-scavenging Capacity

H₂O₂-scavenging capacity of CSPHs was compared with that of ascorbic acid (Figure 8). All CSPHs demonstrated significantly higher ($P < 0.05$) H₂O₂-scavenging capacity than ascorbic acid with scavenging capacity of alcalase CSPH being significantly higher ($P < 0.05$) than others

at all study concentrations, followed by tryptic CSPH and then peptic CSPH. CSPHs as well as ascorbic acid exhibited concentration-dependent H₂O₂-scavenging capacity and they were all different ($P < 0.05$) at all the studied concentrations except peptic CSPH whose scavenging capacity at 0.80 mg/mL was not significantly different from that of ascorbic acid.

Figure 8: H_2O_2 -scavenging capacity of *C. lanatus* seed protein hydrolysates. Each dot represents the mean of triplicate determinations $\pm$ SD. Dots belonging to different enzyme/sample at the same concentration but with different letters are significantly different at $P < 0.05$.

Figure 9: 50% effective concentration (EC_{50}) values of *C. lanatus* seed protein hydrolysates in scavenging H_2O_2 . Each bar represents mean of triplicate determinations of $\text{EC}_{50} \pm$ SD. Bars with different letters are significantly different at $P < 0.05$.

Figure 9 shows the EC_{50} of CSPHs and ascorbic acid. EC_{50} of alcalase CSPH was lower ($P < 0.05$) than those of peptic and tryptic CSPHs as well as ascorbic acid. Whereas the EC_{50} of tryptic CSPH (0.373 ± 0.012 mg/mL) was significantly lower than those of peptic CSPH (0.945 ± 0.039 mg/mL) and ascorbic acid (0.880 ± 0.043 mg/mL), there was no significant CSPH and ascorbic acid. difference ($P > 0.05$) between the EC_{50} of peptic

DISCUSSION

ACE-inhibitory Activity

In this study, peptic and tryptic CSPHs exhibited remarkable concentration-dependent inhibitory effects against ACE activity. Alcalase CSPH also demonstrated concentration-dependent inhibitory effect against ACE but its response to increasing hydrolysate concentration was rather weak compared to the former. The strong inhibition of

ACE by peptic and tryptic CSPHs could be attributed to the type of amino acid residues often present in the peptides that resulted from their cleavage (i.e. hydrophobic/aromatic and acidic residues for pepsin and cationic residues for trypsin) (Naik, 2012). Studies have shown that ACE has binding preference for substrates or inhibitors having hydrophobic (aromatic and branched chain) amino acid residues at their C-terminal positions (Wu *et al.*, 2006; Alashi *et al.*, 2014). This also explains why peptic CSPH caused higher inhibition as many of these residues are targets of its cleavage.

The percentage ACE inhibition attained by peptic CSPH in this study is similar to the previously reported 81.2% and 79.62% for peptic-pancreatic and peptic hydrolysates from chicken thigh skin protein (Onuh *et al.*, 2013) and Australian canola protein (Alashi *et al.*, 2014) respectively; but higher than what was reported by Yoshie-Stark *et al.* (2006) for peptic hydrolysate from rapeseed protein. Peptic CSPH demonstrating the highest ACE inhibition in this study is comparable to the report of Kim and Byun, (2012) where peptic hydrolysate also caused the highest inhibition. The peptic CSPH IC_{50} obtained in this study is higher than the value reported for flaxseed peptic protein hydrolysate by Udenigwe *et al.* (2009) while tryptic CSPH IC_{50} was higher than values previously reported for tryptic hydrolysate from bovine milk (Haque and Chand, 2008) and flaxseed protein (Udenigwe *et al.*, 2009) respectively.

Kinetics of ACE Inhibition

Kinetic parameters are important in understanding the effectiveness of the inhibitory potential of peptides against activities of enzymes. Kinetic plots also give a rough estimate of the amount of peptides (inhibitor) required to inhibit the activities of the enzymes as reflected by the affinity to bind to the active site of the enzyme. K_i (enzyme-inhibitor dissociation constant) defines the binding strength of inhibitor to enzyme to form enzyme-inhibitor complex (Barbana and Boye, 2011; Girgih *et al.*, 2015).

The K_m of ACE in the absence of CSPHs in the study was 0.2957 mM which is very similar to previously reported values of 0.30mM (Holmquist *et al.*, 1979), 0.292 mM (Hou *et al.*, 2003) and 0.3058 mM (Udenigwe *et al.*, 2009). As depicted by the Lineweaver-Burk plots, the

mode of peptic CSPH inhibition was mixed type inhibition. This is evident in the concentration-dependent increase in K'_m of ACE which implies a reduction in the affinity of ACE for FAPGG with increasing concentrations of peptic CSPH (Berg *et al.*, 2002). This implies that the peptides contained in peptic CSPH could combine with ACE molecule to form a non-productive complex, regardless of whether a substrate molecule is bound to the enzyme active site or not. This is similar to what was reported for hemp seed peptic-pancreatic protein hydrolysate (Girgih *et al.*, 2011).

The mechanism of inhibition exhibited by tryptic and alcalase CSPHs was non-competitive type as indicated by the maintenance of nearly constant K'_m values in the presence of these hydrolysates. Similar to what was reported for lentil tryptic and alcalase protein hydrolysates (Barbana and Boye, 2011), this implies that the peptides contained in tryptic and alcalase CSPHs did not bind to the active site of ACE, but might have bound to other sites on ACE molecule to produce an inactive (enzyme-inhibitor or enzyme - substrate - inhibitor) complex, irrespective of substrate binding.

The concentration-dependent reduction in V_{max} and CE caused by CSPHs is typical of mixed and non-competitive modes of inhibition. Despite having similar IC_{50} , peptic CSPH had K_i several folds lower (indicating a stronger affinity for ACE) than tryptic and peptic CSPHs, thus translating to a lower CE of ACE. The K_i (0.444 mg/mL) of peptic CSPH obtained in this study is lower than the reported 2.55, 3.96 and 4.74 mg/mL for hemp seed peptic-pancreatic protein hydrolysate and its peptide fractions (Girgih *et al.*, 2011), but higher than 0.16 mg/mL reported for lentil protein hydrolysate (Barbana and Boye, 2011). It could be concluded that peptic CSPH is a better ACE inhibitor than tryptic and alcalase CSPHs because of the said parameters.

DPPH Radical-scavenging Activity

The relatively stable DPPH radical has been extensively employed to examine the free radical scavenging or hydrogen donating ability of compounds; and thus to assess their antioxidant activity. These antioxidants donate hydrogen to free radicals, leading to non-toxic species and therefore to inhibition of the propagation phase of lipid oxidation (Jao and Ko, 2002). Naqash and Nazeer, (2013) reported a DPPH'-

scavenging activity of 48% for yellow fin sole protein peptic hydrolysate (at 3.0 mg/mL) which is lower than $56.39 \pm 0.45\%$ DPPH $\cdot$ -scavenging activity of peptic CSPH (at 2.5 mg/mL) achieved in this study. Similar to what was obtained in this study, Jun *et al.*, (2004), Sun *et al.*, (2011) and Naqash and Nazeer, (2013) reported peptic hydrolysate from various animal sources to have exhibited the highest radical-scavenging activity than hydrolysates produced by other proteases used. The higher DPPH $\cdot$ -scavenging activity of peptic CSPH in the present study may be attributed to the presence of acidic residues in the peptides generated since pepsin is known to cleave polypeptides at sites containing acidic and hydrophobic amino acid residues (Kageyama, 2002; Naik, 2012), exposing these proton donors which could react with DPPH $\cdot$, donating hydrogen and transforming them to more stable products, thus terminating the radical chain reaction. It has also been reported that the presence of hydrophobic residues enhances the radical scavenging properties of peptides (Sarmadi and Ismail, 2010).

DPPH $\cdot$ -scavenging activity of alcalase CSPH attained in this study is similar to the previously reported 44.31% of chicken pea alcalase hydrolysate fraction (Li *et al.*, 2008). However, the previously reported DPPH $\cdot$ -scavenging activity of tryptic whey protein hydrolysate is higher than what was obtained in this study (Kamau and Lu, 2010). The scavenging activity attained by each of peptic, tryptic and alcalase CSPHs is higher than the 25.7% obtained from the sequential tryptic, peptic and chymotryptic digestion of lysozyme from hen egg white as reported by Rao *et al.* (2012).

EC₅₀ value is often employed in the evaluation of antioxidant, antiradical and reduction efficiencies. This is because it depicts the concentration of hydrolysate needed to cause 50% inhibition of the oxidant (Razali *et al.*, 2015). Low EC₅₀ is desirable as lower values indicate better effectiveness. The EC₅₀ values of CSPHs were in agreement with their corresponding radical scavenging activities. EC₅₀ of alcalase CSPH obtained in the present study is lower than the EC₅₀ (4.42, 3.71 and 3.58 mg/mL) of all alcalase hydrolysate fractions obtained from cobia skin gelatin and *Arca subcrenata* protein reported in previous studies (Song *et al.*, 2008; Razali *et al.*, 2015).

Hydrogen Peroxide-scavenging Capacity

Hydrogen peroxide is a biologically relevant non-radical oxidizing species which may be formed in tissues through oxidative processes. Hydrogen peroxide itself is not very reactive, but it can be toxic to cell because of its ability to give rise to hydroxyl radicals ($\cdot\text{OH}$) in cells (Halliwell, 1991; Rahman *et al.*, 2012). Since antioxidant mechanisms are diverse, the antioxidant activities of hydrolysates may vary with assay methods based on their mechanisms of activity (Moure *et al.*, 2006). Thus, differences may exist in the anti-radical and reducing activities of individual hydrolysate (Sroka and Cisowski, 2003).

Despite the seemingly inefficient radical-scavenging activity of alcalase CSPH, it exhibited significantly higher H₂O₂-scavenging power than peptic and tryptic CSPHs both of which demonstrated higher antiradical property. This is similar to the trend of results reported by Sroka and Cisowski, (2003). This could be attributed to certain amino acid residues present in alcalase CSPH which could have scavenging effect against H₂O₂ otherwise not exhibited against other oxidants. Alcalase is a generally non-specific endopeptidase that cleaves polypeptides at random sites. However, studies have described certain amino acid residues (which include Trp, Tyr, Phe and Leu) that may sometimes be target sites of alcalase degradation (Motyan *et al.*, 2013). EC₅₀ of CSPHs (Figure 9) correspond to their individual H₂O₂-scavenging property. EC₅₀ value of alcalase CSPH obtained in this study is lower than the reported EC₅₀ of 19.09 mg/mL for *Arca subcrenata* alcalase hydrolysate (Song *et al.*, 2008).

CONCLUSION

The results obtained in this study revealed that each of the hydrolysates possess antioxidant property expressed through different mechanisms of anti-radical and reducing abilities. Peptic CSPH showed the highest anti-radical property whereas tryptic CSPH demonstrated the strongest reducing ability. Hydrolysates obtained from the three proteases all possess potent ACE-inhibitory activity. However, peptic CSPH demonstrated the strongest ACE inhibition followed by tryptic CSPH. The results generally suggested that peptic CSPH would make a good candidate for combined antioxidant and antihypertensive agent as it demonstrated both

antiradical and reducing abilities as well as ACE inhibition. The use of watermelon seeds for therapeutic purpose will contribute towards increased value-added utilization of watermelon seeds whilst providing the highly sought alternative therapy for the menace of hypertension.

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