Angiotensin I-Converting Enzyme (ACE) Inhibitory Activity of Peptides Isolated from Carabao Mango *(Mangifera indica)* Flesh

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ABSTRACT

Hypertension has been reported as the second major cause of morbidity in the Philippines. One of the mechanisms to control blood pressure is through the inhibition of the angiotensin I-converting enzyme (ACE). This study specifically focused on the determination of ACE inhibitory activities of peptides from 'Carabao' mango flesh of three shell colors (SC1, SC3, SC5). The bioactive peptides were obtained upon a series of extraction, purification, and enzymatic hydrolysis steps. Among the undigested and digested crude and purified samples, the highest *in vitro* ACE inhibition was exhibited by the three-hour digest of SC3 (83.28 ± 0.83%). The most prominent peak from the fractionation of the mixture of peptides in SC3 digest was evaluated for ACE inhibition, and the decrease in activity inferred the synergistic effect of the peptides in exhibiting the inhibitory function. This selected bioactive peptide was revealed to have alanine and phenylalanine as the components, which can possibly be AF – an ACE inhibitory peptide determined from *in silico* analyses. Meanwhile, assessment of the *in vivo* antihypertensive activity showed no significant results due to insufficiently administered doses of the samples. Overall, the measured activity of the ACE inhibitory peptides inferred the potential of mango as a functional food in dealing with hypertension.

Keywords: Angiotensin I-converting enzyme; Carabao mango; Bioactive peptides

INTRODUCTION

Hypertension or high blood pressure is a condition associated with the chronic increase in blood pressure, making it the major risk factor for cardiovascular diseases (Jao et al., 2012). The average of two or more measurements of blood pressure during outpatient visits is the basis for the

clinical diagnosis of hypertension. If the systolic blood pressure reading is ≥140 mmHg and/or the diastolic blood pressure is ≥90 mmHg, the patient is classified as hypertensive (Kotchen, 2015; World Health Organization, 2019). Globally, the burden of hypertension has reached an estimate of 1.13 billion people having a medical condition wherein two-thirds of this population is in the low- and middle-income countries (World Health Organization, 2019). In the analysis of worldwide data by Kearney and colleagues (2005), it was projected that by the year 2025, the number of adults with hypertension would reach 1.58 billion. The local impact of hypertension was seen in the 2015 report of the Department of Health on the Philippine health statistics stating that hypertension had become the second major cause of morbidity in the country. Data showed that around 596 deaths out of 100,000 population were attributed to hypertension.

The pathogenesis and the corresponding consequences of hypertension are complex thus, there exists a variety of treatment options for hypertensive disorders. In addition to lifestyle modifications, a pharmacologic approach is done to manage hypertension. The wide range of synthetic drugs commercially available includes β- blockers, alpha antagonists, sympatholytics, direct vasodilators, renin inhibitors, calcium antagonists, angiotensin II antagonists, and angiotensin-converting enzyme (ACE) inhibitors (Kotchen, 2015; Norris and FitzGerald, 2013). Such drugs have already been proven to be effective in the management of hypertension however, they pose adverse side effects including several allergic reactions, skin rashes, taste disturbances, and cough (Jao et al., 2012). Also, there exists a burden on the healthcare costs for hypertension medication (Geroy, 2012; Kirkland et al., 2018; Moran et al., 2015). It was previously reported that the cost of medicines in the Philippines was as high as that of international standards. Given that majority of the total costs for hypertension medication are paid without subsidy from the national government, the challenge of the cost still exists (Geroy, 2012). Because of these dilemmas, there has been extensive research on alternatives that can provide the same effect as that of the commercially available drugs yet are safer and more affordable.

Food-derived bioactive peptides have been the focus of several kinds of research for many years because of their potential as alternative therapeutics that are more cost-efficient and with less negative effects. Aside from the known nutritional value of the food source, the biologically active peptides exhibit drug-like activities that induce positive physiological responses (Norris and FitzGerald, 2013). These protein fragments become active when they are liberated upon proteolysis brought about by digestive enzymes, proteolytic microorganisms, or microorganism- or plant-derived proteolytic enzymes (Aluko, 2015; Kitts and Weiler, 2003; Korhonen and Pihlanto, 2006). Particularly, the antihypertensive capabilities of protein fragments have been explored having the same modes of action as those of the previously mentioned classes of hypertension medications.

One of the mechanisms to control blood pressure is through the inhibition of the angiotensin I-converting enzyme (ACE). ACE, which is highly expressed on the vascular endothelium, is one of the blood pressure regulators via the renin-angiotensin system (RAS) and the kinin-nitric oxide system (KNOS). In the RAS pathway, the conversion of liver-released angiotensinogen into angiotensin I is mediated by the plasma renin. Subsequently, the C-terminal dipeptide His-Leu is cleaved from angiotensin I to form angiotensin II in the lungs by the action of ACE. Angiotensin II is a vasoconstrictor, and an increase in the blood pressure results from its formation (Jao et al., 2012; Norris and FitzGerald, 2013). On the other hand, ACE inactivates the vasodilating peptides bradykinin and kallidin. If bradykinin remains inactive through ACE, it cannot bind to β-receptors thus decreasing intracellular Ca^{2+} levels. Decreased Ca^{2+} levels will then prevent the action of nitric oxide synthase to generate nitric oxide, a known vasodilator, from L-arginine (Norris and FitzGerald, 2013). Collectively, inhibition of ACE can indirectly reduce or control the rise of blood pressure.

Commercially available ACE inhibitors are captopril, lisinopril, ramipril, and enalapril (Jao et al., 2012; Kotchen, 2015). The first bioactive peptides with ACE inhibitory activity were isolated from

snake (*Brothrops jaraca*) venom, and different food sources have been evaluated thereafter to identify and isolate antihypertensive peptides (Murray and FitzGerald, 2007). The choice of the food source for studies on bioactive peptides considers the components of the daily diet, and its production and accessibility. Because of the known nutritional benefits from fruits, their necessity in the diet, and the limited local studies in mango fruit, it can be an ideal food source for bioactive peptide research.

Mango (*Mangifera indica* L.) belongs to the family Anacardiaceae, and over 1000 varieties of mango have been identified worldwide (Shah et al., 2010). The production of this tropical fruit increases every year due to increasing demand. Around 77% of the world's production of mangoes are from Asian countries (Jahurul et al., 2015). In the Philippines, it has been reported in 2007 that mango is the third fruit crop concerning export value and volume, next to banana and pineapple. Locally, the 'Carabao' variety is known to account for 73% of the mango production (Caparino et al., 2012). The Philippine Statistics Authority noted up to 558.07 thousand metric tons of mango produced for the second quarter of 2020, and 80.3% of which is the 'Carabao' variety. Generally, mango varieties in the country are available from May through August (Subramanyam et al., 1975).

Essential micronutrients, vitamins, phytochemicals, dietary fiber, carbohydrates, and proteins can be obtained from the mango fruit. To add to these known physiological benefits, this study aimed to isolate ACE inhibitory peptides, specifically from the 'Carabao' variety, and evaluate the *in vitro* and *in vivo* inhibiting activities.

METHODS

Materials and Equipment. For the purification procedures, ammonium sulfate was purchased from HiMEDIA, and the resin for gel filtration chromatography was Sephacryl S-200 from GE Healthcare. Boric acid for the extraction of ACE from pig lungs was from Thermo Fishers Scientific. Bradford reagent and protein marker for SDS-PAGE were obtained from Bio-Rad. For the ACE inhibition assay, Sigma-Aldrich was the source for the hippuric acid and hippuryl-histidyl-leucine (HHL). The enzymes for enzymatic hydrolysis, such as pepsin, chymotrypsin, trypsin, and thermolysin, were from Sigma-Aldrich.

The equipment used in this study include the refrigerated centrifuge from Hermle, SDS-PAGE setup, and GFC column from Bio-Rad, freeze dryer from LabConco, UV-Vis spectrophotometer from Thermo-Scientific, RP-UPLC unit from Waters, and HPTLC from CAMAG®. To measure the rat blood pressure, the MRBP system of IITC life science was used.

Sample Collection. 'Carabao' mango samples were obtained from the public market in Los Baños, Laguna. Based on the classification of shell colors by Castillo-Israel *et al*. (2014), shell colors 1, 3, and 5 were acquired for this study, as shown in Figure 1.

Isolation of Crude Proteins. Processing of the 'Carabao' mango flesh before protein extraction included the slicing of the flesh into thin strips which were subsequently subjected to freeze-drying. Around 200 g of the dried sample was homogenized with 300 mL of the combined 1:1 buffer system of 50 mM Tris-HCl (pH 8.5) and 10 mM phosphate buffer saline. After overnight extraction, coarse filtration using cheesecloth followed by centrifugation of the filtrate at 11,000 rpm and 4°C. The obtained supernatant liquid was carefully stored in stock and aliquot solutions in the freezer for further analyses.
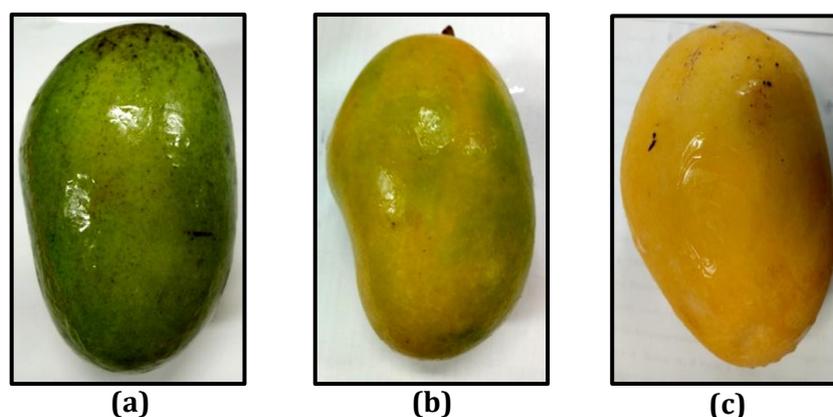
Figure 1. 'Carabao' mango samples in different shell colors as classified by Castillo-Israel *et al*. (2014). (a) shell color 1, (b) shell color 3, and (c) shell color 5.

Protein Purification. Targeting the major protein from the crude extract, two purification steps were performed – ammonium sulfate precipitation and gel filtration chromatography.

Ammonium Sulfate Precipitation. The crude extracts were subjected to precipitation in 0-80% and 80-90% saturation of ammonium sulfate. Precipitates collected were redissolved in a minimal amount of the extraction buffer before overnight dialysis in 14kDa cut off in water.

Gel Filtration Chromatography (GFC). Dialysates from ammonium sulfate precipitation were mixed with ethyl acetate in a 1:1 ratio and were centrifuged twice for 10 minutes (10,000 rpm, 4°C) in each run. The resulting supernatant was loaded to the GFC column with Sephacryl S-200 as the resin. The standard protocol for packing, cleaning, and equilibration of the column was followed. The equilibration and elution buffer used was the combined 1:1 buffer system of 50 mM Tris-HCl (pH 8.5) and 10 mM phosphate buffer saline. The sample of three to five mL volume was loaded into the column, with the flow rate maintained at around 0.7 mL/min and four mL collection volume. Elution profiles were plotted at 280 nm using a UV-Vis spectrophotometer. Corresponding peaks were subjected to further characterization.

Protein Quantification using Bradford Assay. The protein sample (5 µL) for analysis was mixed with 250 µL of the Bradford reagent in a microtiter plate. Dilution of protein isolates with extraction buffer was done if deemed necessary. UV-Vis spectrophotometric analysis measured the absorbance at 595 nm, and protein concentration values were identified using 1 mg/mL BSA as the standard.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Protein characterization via SDS-PAGE was done using the method of Laemmli (1970) with modifications. Samples were added with SDS reducing buffer in a 4:1 ratio. The resulting mixtures were heated in boiling water for four minutes and were allowed to cool to room temperature. In a pre-casted 11% resolving gel and 8% stacking gel, 20 µL or each sample was loaded, and the set-up was run at 110 V. Overnight staining using Coomassie brilliant blue R-250, and destaining using 5:4:1 methanol:water: acetic acid solution for two hours with shaking was performed.

Enzymatic Hydrolysis of Protein Isolates. The method of Pihlanto and colleagues (2008) was adopted with modifications. Both the crude and purified protein extracts were subjected to enzymatic hydrolysis with respective concentrations of 1 mg/mL and 0.1 mg/mL.

Digestion with pepsin was carried out at pH 2 for two hours, which was followed by simultaneous digestion using chymotrypsin, thermolysin, and trypsin at pH 7 for another one hour (3-hr

digest), two hours (4-hr digest), ten hours (12-hr digest) and 22 hours (24-hr digest). Samples were withdrawn at every reaction time from each mixture, followed by heating at 100°C for five minutes to stop the digestion and storing at -4°C. Prior to further analyses, the hydrolysates were made sure to be clear through centrifugation at 9,500 rpm, 4°C for two minutes.

Densitometric Analysis. The SDS-PAGE profiles obtained from the crude and purified isolates and their respective hydrolysates were further analyzed via densitometry using the TotalLab 1D software.

Extraction of Angiotensin I-Converting Enzyme (ACE) from Pig Lungs. Acetone lung powder was prepared from freshly purchased pig lungs following the procedure of Folk *et al*. (1960) with modifications. Defatting was done using acetone as the first solvent followed by hexane. The defatted tissue was dried for hours and was pulverized.

The angiotensin I-converting enzyme (ACE) was extracted from the acetone lung powder based on the method of Cushman and Cheung (1971). The powder was suspended in 100 mM sodium borate buffer (pH 8.3) and was mixed for three hours at 4°C. The ACE crude extract was obtained from the supernatant of the centrifuged mixture at 10,000xg for one hour at 4°C.

Determination of ACE Activity. Also based on the procedure of Cushman and Cheung (1971), the ACE activity was determined. The substrate, HHL, was initially prepared to a final concentration of 5 mM by dissolution in 0.1 M sodium phosphate (Na-P) buffer of pH 8.3 with 0.3 M NaCl. From the stock HHL solution, 100 μL was obtained and was added with 25 μL of the same solvent system. The incubation of the resulting solution was done at 37°C for four minutes. The solvent system, without HHL, served as the control solution. Then, 25 μL ACE was added to the solutions and was allowed to react for 30 mins at 37°C. The reactions were terminated with 125 μL 1 N HCl. Afterward, hippuric acid (HA) was extracted from the mixture by the addition of 0.75 mL ethyl acetate, followed by mixing using vortex and centrifugation at 3600 rcf for 5 min. Five hundred milliliters of the upper layer (organic phase) was transferred to a microcentrifuge tube, and ethyl acetate was removed via heat evaporation until dryness. Resulting HA was redissolved with 0.5 mL water and was briefly mixed using a vortex. Absorbance measurements were recorded at 228 nm using a UV-Vis spectrophotometer. The concentration of the obtained hippuric acid was determined through the interpolation of measured absorbance values to the constructed hippuric acid standard curve. All determinations were done in 5 replicates. The ACE activity was calculated using the formula shown below.

$$\text{Activity (U)} = \frac{\mu mol\ hippuric\ acid}{min} \qquad (1)$$

In vitro ACE Inhibition Assay. The test samples included 1 mg/mL of the crude protein hydrolysates and 0.1 mg/mL purified protein hydrolysates. Na-P buffer pH 8.3 with 0.3 M NaCl served as the control, while 1 mg/mL and 0.1 mg/mL captopril solutions as the positive control, and the extraction buffer as the blank. All the test solutions (25 μL) were pre-incubated at 37°C for four minutes with 25 μL 0.1 M Na-P buffer pH 8.3, 25 μL 0.3 M NaCl, and 50 μL HHL. Afterwards, 25 μL ACE was added to the resulting solutions except for the blank, which was added with 250 μL 1 N HCl instead.

Termination with the addition of 125 μL 1 N HCl was done after 30 minutes of incubation at 37°C. For the tube containing the blank, 25 μL ACE was added instead of HCl to complete the matrix. Extraction of HA and determination of absorbance readings were done as mentioned in the previous section. All determinations were performed in three trials. Inhibition rate (%) or percent ACE inhibitory activity was calculated using the following equation.

$$\text{ACE inhibition (\%)} = \left(\frac{B-A}{B-C}\right) \times 100 \qquad (2)$$

where   A = absorbance in presence of enzyme, substrate, and inhibitor
       (ACE + HHL + sample)
     B = absorbance with enzyme and substrate, but without the inhibitor
       (ACE + HHL)
     C = absorbance without enzyme and inhibitor
       (HHL only)

IC$_{50}$ Determination. The IC$_{50}$ is defined as the required concentration of the inhibitor to reduce 50% of ACE activity under the above assay condition. This was determined through regression analysis of the constructed plot of %ACE inhibition versus the equivalent logarithmic value of the hydrolysates at a varying concentration in µg/mL (Rinayanti et al., 2013). The IC$_{50}$ of the purified protein hydrolysates that showed the highest ACE inhibitory activity was determined.

Determination of Amino Acid Composition. The purified protein digests with the highest *in vitro* ACE inhibitory activity was subjected to amino acid composition determination through series of steps including further fractionation via reversed-phase ultra-performance liquid chromatography (RP-UPLC), total hydrolysis of the purified peptides, and thin layer chromatography (TLC) of the resulting hydrolysates.

Reversed Phase-Ultra Performance Liquid Chromatography (RP-UPLC). The procedure for fractionation was described by Silva et al. (2006). Sample aliquots (100 µl) were applied to a reversed-phase column (300 A pore size, 5 µm particle size). Elution was initiated at 40°C via mobile phase of 2 solvents – solvent A: 0.1% (v/v) TFA in water; and solvent B: 0.1% (v/v) TFA in 80% (v/v) aqueous acetonitrile – at a flow rate of 0.6 mL/min, starting with 98% (v/v) solvent A, passing through a linear gradient from 2 to 60% (v/v) solvent B over 45 mins., another linear gradient from 60 to 100% (v/v) solvent B for 2 mins, and ending with 2% (v/v) solvent B for 2 mins. The fractions were collected based on their peak assignments as measured at 280 nm. Lyophilization of pooled samples was done to concentrate the sample, and the resulting residues were reconstituted with water in minimal amount. The fraction corresponding to the highest peak in the chromatogram was assessed for its ACE inhibitory activity.

Total Hydrolysis (Acid and Base) of RP-UPLC Purified Peptides. To liberate the amino acid components of the bioactive peptide, acid and base hydrolysis reactions were performed. A volume of 50 µL sample was mixed with 50 µL 11.65 M HCl for acid hydrolysis, and another 50 µL was mixed with 11 N NaOH for base hydrolysis. All solutions were then autoclaved for 1 hr at 127°C with 17psi.

High Performance Thin Layer Chromatography (HPTLC). The hydrolyzed samples were subjected to HPTLC to determine the identity of possible amino acids present. The method of Qiu et al. (2010) was adapted with some modifications. The amino acid standards used were trans-alanyl glycine, glycine, leucine, isoleucine, valine, serine, threonine, methionine, cysteine, aspartic acid, glutamic acid, phenylalanine, tyrosine, tryptophan, lysine, histidine, and asparagine. Totally hydrolyzed purified hydrolysates (0.1 µL) along with amino acid standards (0.1 µL) were spotted on TLC plate. Pre-visualization of spots was done using the HPTLC visualizer. The plate was then developed in a chamber containing the optimized solvent system (2-butanol: acetic acid: water, 43:5:2, v/v/v) for 30 mins, followed by 5 mins of drying. This was followed by the soaking of the plate in 0.5% w/v ninhydrin solution and heating using a portable blower. HPTLC visualizer was used to visualizing the spots in the plates in a more enhanced manner. The Rf values of acid and base hydrolysates were determined and compared with the standard values.

In silico Analyses. The amino acid sequences of the proteins from *Mangifera indica* with molecular weights based on the SDS-PAGE results were obtained from National Center for Biotechnology Information (NCBI). Resulting sequences were subjected to identification of reported ACE inhibitory peptides using the BIOPEP database (Minkiewicz et al., 2019).

In vivo Antihypertensive Activity Assay. All *in vivo* procedures were approved by the Institutional Animal Care and Use Committee, University of the Philippines Los Baños (UPLB).

Spontaneously hypertensive rats (SHR) and Sprague-Dawley rats (SD) were acquired and were placed in separate cages for acclimatization for two weeks inside a room maintained at 20-22°C at the Department of Basic Veterinary Sciences, College of Veterinary Medicine, UPLB. The test animals were consistently given around 30 g of feeds and 50 mL of water each day.

After the acclimatization period, the blood pressure values of SHR and SD were read using the Mouse and Rat Tail Cuff Method Blood Pressure (MRBP) System. The standard protocol provided by the instrument was followed in determining the blood pressure of the rat samples. Crude mango protein extract (8.35 mg/mL) and a commercially available mango puree were administered to the test rats. The systolic, diastolic, and arterial blood pressures were measured at different time points.

Statistical Analyses. Results of the assays were expressed as means ± standard deviation and were analyzed using Microsoft Office Excel 2013 and GraphPad Prism 6.0. Data were analyzed using one-way analysis of variance (ANOVA), $p < 0.5$ indicating statistical significance.

RESULTS AND DISCUSSION

Major storage protein isolation and characterization. Mango flesh samples from three different shell colors (Figure 1) were prepared, as mentioned, for crude protein extraction. Quantification of the protein extract was done via Bradford assay. As stated in Table 1, the crude protein concentration significantly increased as the maturity of the samples progressed, as represented by the three shell colors. Upon the purification of the crude extracts, a significant decrease in concentration was recorded that can be accounted for the removal of impurities and other protein components considering the limits of the performed purification methods. Besides, the only target for further analyses is the major storage protein of each sample.

Table 1. Protein concentrations of the crude and purified mango protein extracts.

Sample[1]	Protein Concentration[1], mg/mL	
	Crude	Purified
Shell Color 1	0.33 ± 0.06[a,a]	0.19 ± 0.03[b,a]
Shell Color 3	1.36 ± 0.23[a,b]	0.21 ± 0.03[b,a]
Shell Color 5	1.95 ± 0.49[a,c]	0.20 ± 0.07[b,a]

[1] The mean ± standard deviation values of nine determinations across the rows (1st entry letter) and down the column (2nd entry letter) if followed by the same letters are not significantly different at 5% level of significance using unpaired T-test and one-way ANOVA.

The elution profile, as shown in Figure 2, obtained after the gel filtration chromatography showed two distinct peaks for the SC1 sample, in which the highest peak (SC1a) corresponding to higher molecular weight proteins was not common to SC3 and SC5. The second peak of the SC1 purified proteins (SC1b) was noted to coincide with the peaks of SC3 and SC5 inferring that the molecular weight values of these isolates were relatively close to each other. Also, the SC1b peak height, which can be indicative of the concentration, was lower than that of the peaks of SC3 and SC5.
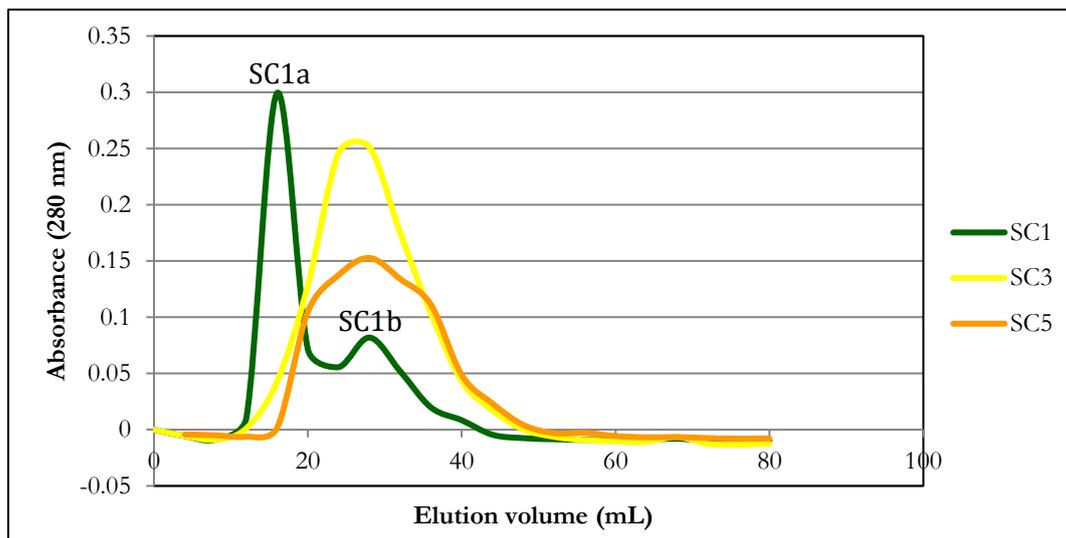
Figure 2. Elution profile of mango samples of varying shell colors upon gel filtration chromatography.

These results from GFC were further characterized by SDS-PAGE to check the purified protein profiles. Eluted samples respective to the peaks were pooled and concentrated, and equal amounts of the samples were loaded to eliminate the concentration bias in the interpretation of the SDS-PAGE profiles, which are shown below.
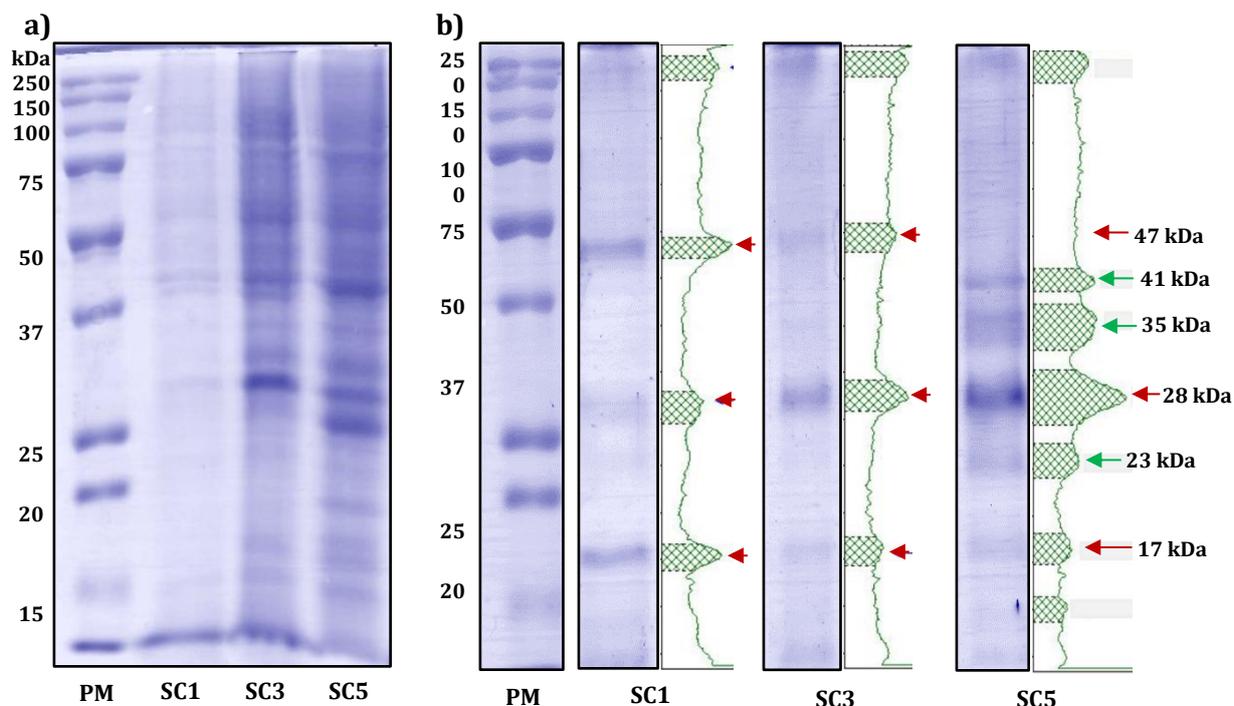

Figure 3. SDS-PAGE profiles of (a) crude and (b) purified protein samples (with corresponding results from densitometric analysis) in different shell colors. (PM=protein marker; SC=shell color)

Apparent differences of the band intensities among the SDS-PAGE profiles of different shell colors can be interpreted based on the possible protein identities with the corresponding estimated molecular weights (MW). The prominent bands at ~47 kDa and ~17 kDa in the SC1 sample that appeared as faint bands in the SC3 and SC5 samples can be associated with the first eluted proteins from SC1 (Figure 2, peak SC1a). Since the GFC elution only showed a single peak and two bands were observed from SDS-PAGE, it is suggested that the peak corresponded to a protein, with MW of ~64kDa, having two subunits of different molecular weights that were separated due to the denaturing conditions of the electrophoretic method applied. Using the available protein database for *Mangifera indica* and the estimated MW, the possible identity of this protein is the auxin efflux carrier protein with the reported MW of around 66 kDa. From available data, the protein corresponds to a single coding sequence but with two functional regions, one of which has a theoretical MW of ~18kDa. Auxin is a plant hormone that is highly involved during fruit maturation processes. Studies showed that ripening process can be triggered by decrease in auxin levels (Li et al., 2017; Liu et al., 2005). The study of Paciorek and colleagues (2005) may imply that decrease in auxin levels can decrease the amount of auxin efflux regulators in the plasma membrane. In a different study in the maturation of apple fruit, it was reported that the reducing auxin level activated the pathway for ethylene biosynthesis, wherein ethylene is known to be produced at the onset of ripening. Also, the expression of the gene for auxin efflux carrier (PIN) showed up-regulation after treatment of the fruit with exogenous auxin (Shin et al., 2015). To date, there are no reports yet on the gene expression profiling during the maturation of mango.

Another observation from the protein profiles was the increasing band intensity of the ~28kDa-protein (Figure 3) as supported by the increasing peak height from SC1 (peak SC1b) to SC5 (Figure 2). This protein can possibly be the light harvesting complex (LHC), which has a theoretical molecular weight of ~28 kDa. From the name itself, LHC determines and regulates the amount of sunlight that can be captured and transmitted (Blankenship and Chen, 2013). From the review of Zoratti et al. (2014), the biosynthesis of flavonoids in fruits is greatly influenced by surrounding light conditions. Flavonoids are determinants of fruit quality as they are involved in the pigmentation. The accumulation of anthocyanin, which is associated with the flavonols during the first stages of fruit development, indicates ripening as a response to light stimuli. Moreover, ethylene biosynthesis is involved during ripening in climacteric fruits, and it was previously studied that blue light exposure up-regulated ethylene biosynthetic genes. Blue light also induced the ethylene signaling pathway that paved way to the increased ethylene production and fruit softening (Gong et al., 2015). Overall, these infer that as light is favorable for fruit maturation, LHCs may have increasing concentration as the fruit matures.

In vitro ACE inhibitory activities. Upon the purification of the crude extracts and before determination of *in vitro* ACE inhibitory activities, enzymatic hydrolysis of the crude and purified extracts of varying shell colors were performed using the enzyme pepsin followed by the combination of enzymes chymotrypsin, thermolysin, and trypsin to simulate the human intestinal digestion. The digestion procedure was successful as analyzed by the densitometry of electrophoretograms generated from the SDS-PAGE of the digests (data not shown).

The resulting hydrolysates were examined for their inhibitory activities against the angiotensin I-converting enzyme (ACE) *in vitro*. Previously isolated ACE from fresh pig lungs was determined to have an enzymatic activity of $6.60 \times 10^{-3} \pm 0.55$ U, and this enzyme was used for the ACE inhibition assay. For all the inhibition assays conducted, Captopril, which is a known ACE inhibitor, was used as the positive control.

Figure 4 shows the comparison of percent ACE inhibition exhibited by the undigested and digested crude extracts in various shell colors. All samples were found to have significantly lower activities than Captopril (99.84 ± 0.39%). The undigested crude proteins of the SC1 and SC3 samples showed significantly higher inhibition values in comparison to their respective

hydrolysates. On the contrary, an enhanced inhibition was generally observed upon the digestion of the SC5 samples. Comparing the shell colors for each digestion time also revealed the difference in the ACE inhibitory activities in which the SC1 and SC3 samples were significantly more inhibiting when remained undigested, while SC5 samples exhibited higher inhibition after enzymatic hydrolysis. These results may infer that the ACE inhibition of the crude SC1 and SC3 samples was because of the synergistic effect of the intact proteins. In the case of SC5 samples, the peptides released after the digestion can be the major contributors to the activity.
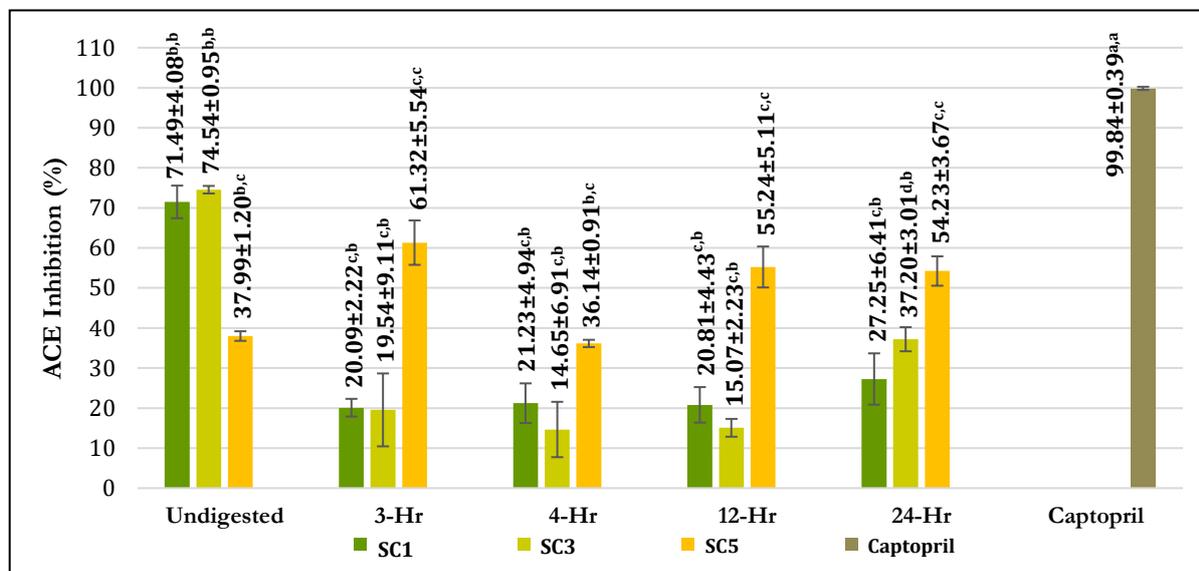

Figure 4. ACE inhibitory activities (%) of crude protein extracts and hydrolysates from 'Carabao' mango samples of different shell colors. The 1st entry superscript letters indicate comparison among values of various digestion times of each shell color, and 2nd entry superscript letters indicate comparison among values of different shell colors of the same digestion time. All comparisons were respective to the positive control, Captopril. The reported mean ± SD values of three determinations if followed by the same letters are not significantly different at 5% level of significance using one-way ANOVA.

The ACE inhibition of undigested and digested purified protein extracts were also determined (Figure 5). Similar to the crude samples, all purified proteins had significantly lower ACE inhibitory activity compared to the positive control (97.58 ± 0.24%). An apparent observation from the presented data was that the purified SC1 and SC3 samples – undigested and digested – were generally more inhibiting to ACE activity than the SC5 samples, except for the undigested SC1 and SC5 samples which revealed no significant difference. Also, the digestion of the purified SC1 and SC3 samples did not show a consistent effect on the inhibitory activities. For SC5 samples, enzymatic hydrolysis significantly decreased the bioactivity, which may imply that the activity was due to the intact purified proteins. Connecting these results to the purified protein profiles from SDS-PAGE in Figure 3, the higher inhibition exhibited by the SC3 sample compared to SC1 may not solely be attributed to the increased levels of the 28 kDa-protein. With this premise, the SC5 sample should have shown higher inhibitory effects than SC3 however, the opposite was noted. Despite the 28 kDa-protein being the most prominent in SC5, the inhibition was lower. Possible reason for such observations is that for the SC1 and SC3 samples, the activities were brought by synergistic effects of the purified protein isolates rather than 28 kDa alone. In the case of the purified SC5 sample, from Figure 3, there were additional isolated proteins indicated by the bands emphasized by the green arrows. There can be numerous protein candidates based on the MW estimates. Such proteins potentially triggered other pathways that eventually decreased ACE-inhibiting activities. They may also contain amino acids that posed antagonistic effects

against the bioactive components of the protein. Somatic ACE is comprised of two active domains that differ in substrate specificity hence, the variety of interactions of different amino acids with the binding sites may induce changes in the enzyme activity (Guang and Phillips, 2009; Daskaya-Dikmen et al., 2017).
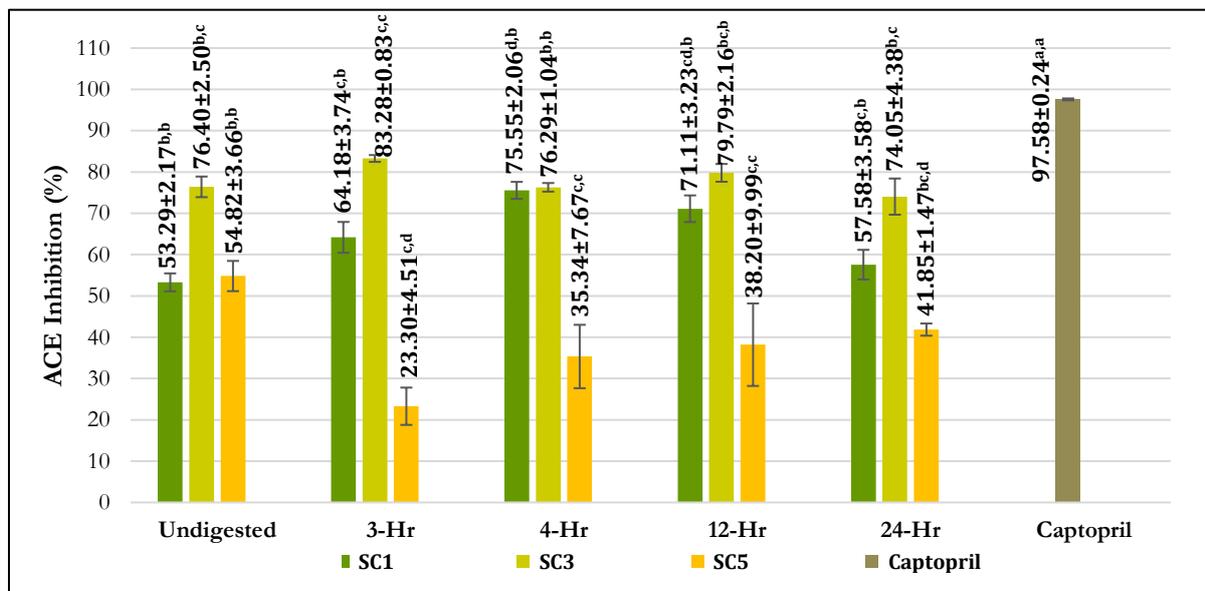

Figure 5. ACE inhibitory activities (%) of purified protein extracts and hydrolysates from 'Carabao' mango samples of different shell colors. The 1st entry superscript letters indicate comparison among values of various digestion times of each shell color, and 2nd entry superscript letters indicate comparison among values of different shell colors of the same digestion time. All comparisons were respective to the positive control, Captopril. The reported mean ± SD values of three determinations if followed by the same letters are not significantly different at 5% level of significance using one-way ANOVA.

From both results of the undigested and digested crude and purified protein samples, it can be inferred that there was an increase in the inhibitory activities of the SC1 and SC3 samples upon purification. This may indicate that the purification steps could have removed unnecessary impurities and other proteins with sequences interacting with the angiotensin-I converting enzyme in a manner that might decrease the inhibitory effects of the ACE inhibitory peptides. In a possibly similar mechanism, the undigested purified SC5 proteins were determined to have an improvement in activity compared to the undigested crude samples. However, the digestion of the crude SC5 proteins increased the percent inhibition and the digestion of the purified proteins decreased the activity. This contradiction might have occurred as some of the intact proteins that reduced the efficiency of inhibition might potentially have the bioactive peptides. Such proteins could have been eliminated during the series of purification steps hence, decreasing the resulting ACE inhibitory activities of the hydrolyzed purified samples.

The sample with the highest percent inhibition was identified to be the purified 3-hour digest of SC3 (83.28 ± 0.83%). This hydrolysate was prepared in different concentrations to determine the IC_{50} value. Determination of the IC_{50} value of an inhibitory peptide provides information on the amount of potential inhibitor needed to exert an activity at half maximal efficiency. For the selected sample, the IC_{50} identified was 74.55 µg/mL (inhibition plot not shown).

Isolation of ACE-inhibitory peptides. It should be noted that the obtained digests from the enzymatic hydrolysis were mixtures of peptides resulting from cleavage at different positions in the protein depending on the action of the enzymes used. Further purification via reversed phase

ultra-performance liquid chromatography (RP-UPLC) was done to separate the peptides of the hydrolysate previously determined to have the highest ACE inhibitory activity among all the purified samples. For this reason, the SC3 3-hr digest was selected for purification. Assessment of the ACE inhibitory activity of the sample corresponding to the highest peak in the chromatogram (Figure 6) was conducted.
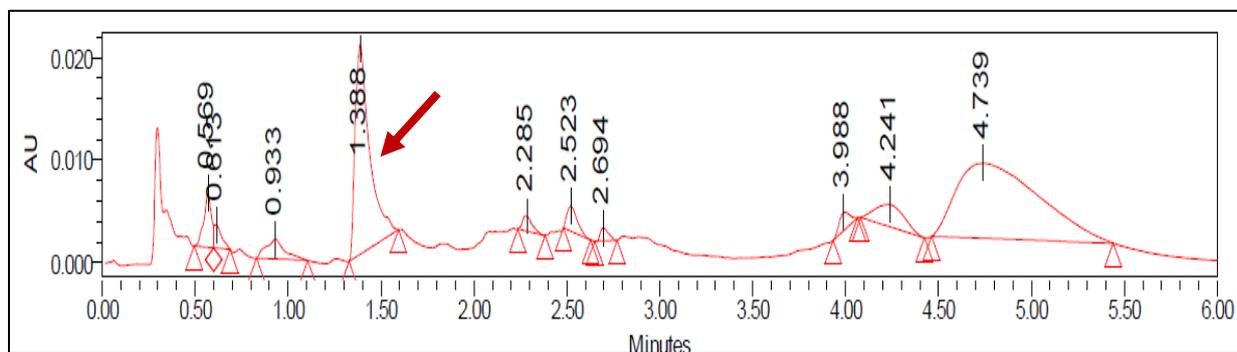

Figure 6. RP-UPLC chromatogram of the SC3 3-hr digest. The peaks were measured at 280 nm. Peak marked with arrow represented the selected fraction for further analyses.

Based on the results of the ACE inhibition assay for the selected RP-UPLC fraction, the activity was 44.95 ± 1.42%, which was significantly lower than the SC3 3-hr digests not subjected to RP-UPLC and Captopril. This suggests that the peptides exhibited a synergistic effect in functioning as ACE inhibitors when present as a mixture in the SC3 digest.

The selected RP-UPLC fraction was then subjected to acid and base hydrolysis to produce the amino acid components in the peptide. The hydrolyzed sample was subjected to identification of the possible amino acids by high performance thin layer chromatography (HPTLC) analysis. Table 2 summarizes the Rf values of the spots developed in the chromatogram of the amino acid standards (Figure 7a) as the basis for the amino acid identity of the samples (Figure 7b).
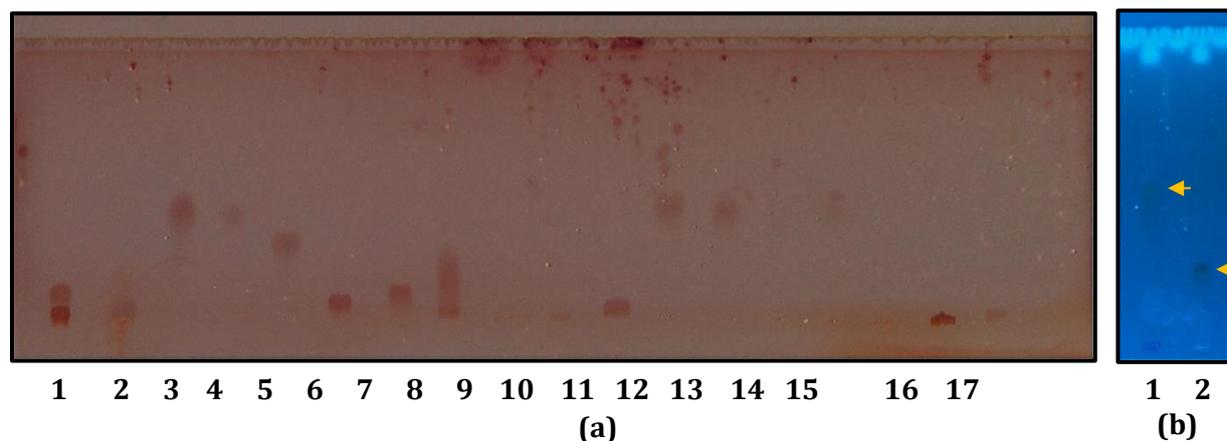

Figure 7. HPTLC chromatogram of the (a) amino acid standards and (b) the hydrolyzed RP-UPLC fraction. Visualization was aided by 0.5% w/v ninhydrin solution and the HPTLC visualizer.

Table 2. Rf values of the amino acid standards and the hydrolyzed RP-UPLC fraction.

Sample	Lane	Amino Acid	Rf
Standards	1	Trans-alanyl glycine (Alanine)	0.2075
		Trans-alanyl glycine (Glycine)	0.1321
	2	Glycine	0.1132
	3	Leucine	0.4623
	4	Isoleucine	0.4528
	5	Valine	0.3679
	6	Serine	0.1698
	7	Threonine	0.1887
	8	Methionine	0.1509
	9	Cysteine	0.1226
	10	Aspartic acid	0.1415
	11	Glutamic acid	0.1604
	12	Phenylalanine	0.4906
	13	Tyrosine	0.4623
	14	Tryptophan	0.4151
	15	Lysine	0.4340
	16	Histidine	0.1226
	17	Asparagine	0.1321
RP-UPLC fraction	1	Acid-hydrolyzed	0.2759
	2	Base-hydrolyzed	0.5000

Given the measured retention factors, the probable identities of the sample spots were alanine (Ala, A) for the acid-hydrolyzed sample and phenylalanine (Phe, F) for the base-hydrolyzed. This infers that the RP-UPLC fraction possibly contained the amino acids ala and phe. Performing *in silico* analyses of proteins present in *Mangifera indica* based on the experimentally identified molecular weights of the SDS-PAGE bands for SC3 (Figure 3), it was revealed that reported ACE inhibitory peptide with ala and phe was AF only. The *in silico* analyses on the proposed identity of the purified proteins for SC3, which were the auxin efflux carrier protein and the light harvesting complex, also revealed that these proteins contained the reported peptide AF. However, in this research, the exact amino acid sequence cannot be determined. Rather, these reported peptides can possibly be the peptides resulting to the observed activity. There is also the possibility that a novel peptide sequence was isolated, but only amino acid sequencing methods can confirm.

Studies showed that the interaction of substrate or competitive inhibitors containing hydrophobic, such as aromatic or branched-side chains, amino acid residues towards ACE is often favored. Reported tripeptides and dipeptides with high potent inhibitory activity contain phenylalanine, tyrosine, proline, leucine, or tryptophan at their C-terminal (Li et al., 2004; Ondetti and Cushman, 1982; Maruyama et al., 1987; Saito et al., 1994). Many studies also mentioned the importance of the hydrophilic-hydrophobic property of the peptide in its inhibitory activity (Kohmura et al., 1989; Kobayashi et al., 2008; Katayama et al., 2004). Kobayashi and colleagues (2008) suggested that the bulky aromatic residue may sterically block the substrates to the active site of the ACE. This is consistent with the reported study of Cushman and colleagues (1982). In their proposed binding interaction model of venom peptide analog to the active sites of the ACE, the Phe residue of the drug is suggested to potentially interact at the subsite (S1) of the ACE.

In vivo antihypertensive activity. In this study, the *in vivo* ACE inhibitory activities of the samples were indirectly measured by antihypertensive activities because of the lack of measurable parameters for ACE inhibition *in vivo*. The response to test samples was assessed by the

monitoring of blood pressure of spontaneously hypertensive rats (SHRs) and Sprague-Dawley rats (SDRs). Following the acclimatization period and serving as the baseline blood pressure readings, the average values (systolic/diastolic) for SHRs and SDRs were 163/128 and 92/82, respectively. These measurements validated the characteristics of the distributed rats.

For the actual *in vivo* antihypertensive activity assay, the crude mango protein extract as well as the commercially available mango extract were given to the test rats. The preliminary dose-response efficacy test protocol using 1 gram/kg body weight and 500 mg/kg body concentration was not followed since the highest concentration of samples that could be administered to the rats was only 8.35 mg/mL considering the low protein content of the mango and viscosity of the extract. As a result, no decrease in systolic/diastolic pressure readings of rats was observed. The administered dose was insufficient for the model organisms to induce bioactivity.

Overall, the study had several limitations including the identification of amino acids of the only peptide of the highest ACE inhibitory activity, which was obtained from the 3-hr digest of SC3 samples. The results of the SDS-PAGE of the purified SC1, SC3, and SC5 samples and the corresponding ACE inhibitory activities revealed differences in the protein components as well as a possible mechanism of inhibition, respectively. It could be that the peptides identified for SC3 were not the same functioning bioactive peptides for SC1 and SC5. The peptide sequence was not specifically determined; instead, the amino acid components were identified. Further research can be conducted employing amino acid sequencing methods. Additionally, for the *in vivo* antihypertensive activity assay, the actual identified ACE inhibitory peptide can be synthesized at a higher concentration to meet the minimum requirement for the dose-response efficacy test. Methods to improve the peptide delivery and bioavailability in an *in vivo* system can also be explored.

CONCLUSIONS

Biologically active peptides, commonly known as bioactive peptides, are broadly recognized to exhibit positive physiological effects on body function specifically when being liberated from the parent protein upon gastrointestinal digestion aided by enzymes. One group of bioactive peptides are antihypertensive peptides, which particularly inhibit angiotensin I-converting enzyme (ACE). In this study, ACE inhibitory peptides were isolated from 'Carabao' mango flesh samples.

The maturity of the mango samples was based on their shell color as classified Castillo-Israel *et al*. (2015). Shell colors (SC) 1, 3, and 5 were selected for protein extraction. It was determined that the crude protein concentration was the highest for SC5 samples and the lowest for SC1. Purification of the crude extract revealed that the proteins expressed in mango samples varied among the shell colors as observed in the intensity of bands in SDS-PAGE profiles. The *in vitro* ACE inhibitory assay of the enzymatically digested and undigested crude and purified samples were conducted against a known ACE inhibitor – Captopril. All samples exhibited ACE inhibition, however, significantly lower than that of Captopril. The highest activity was recorded for the SC3 3-hour digest. Further fractionation of the digest to its peptides and assessment of the ACE inhibitory activity inferred that the sample might have exhibited a synergistic effect for its inhibition. Moreover, the total hydrolysis of the peptide corresponding to the highest peak after the fractionation liberated alanine and phenylalanine as the component amino acids of the bioactive peptide. The hydrophobicity of these amino acids could have resulted in their inhibitory activity like the previously studied ACE inhibitory peptides. Also, several proteins present in mango were already reported to have AF as an ACE inhibitory peptide. Assessment as antihypertensive peptides *in vivo* using spontaneously hypertensive rats as the animal models showed no significant reduction in blood pressure. This can be primarily attributed to the insufficient dose of the samples administered to the organisms.

Further research can be conducted to optimize the extraction procedures and to determine the amino acid sequence of the bioactive peptides. Improvements in the peptide delivery and bioavailability in a living organism can also be explored. Nonetheless, it has been shown in this study that mango has eminent potential as a functional food particularly in dealing with hypertension.

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