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Antihypertensive property of the peptic and chymotryptic hydrolysates derived from the crude protein extract of okra [*Abelmoschus esculentus* (L.) Moench] seeds

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Abstract

Bioactive peptides from okra seeds were successfully isolated, purified, and characterized for its antihypertensive properties. Okra seeds were decorticated, defatted, and suspended in 35 mM phosphate buffer (pH 7.6, with 0.4 M NaCl and 0.02% NaN_3) to isolate the okra seed crude protein extract (OSCPE). The protein content of OSCPE was estimated to be 13.8 ± 0.6 mg/mL. Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) revealed six major polypeptide bands with molecular weights of 11, 13, 16, 18, 25, and 49 kDa. Bioactive peptides from OSCPE were released through separate enzymatic hydrolysis, using pepsin and α-chymotrypsin, from 0 min to 24 h of incubation. OSCPE was more resistant to enzymatic hydrolysis with α-chymotrypsin than pepsin. The 12-h hydrolysate from both enzymes exhibited the highest angiotensin I-converting enzyme (ACE) inhibitory activity. Both hydrolysates were fractionated through anion-exchange chromatography (IEX) using a DEAE-Sephacel column (1.6 cm × 5.0 cm). Fractions from prominent peaks were subjected to ACE inhibition assay. The 17th peptic IEX fraction and the 15th chymotryptic IEX fraction showed the highest ACE inhibitory activities of 68.68 ± 2.91% and 71.89 ± 7.77%, with IC_{50} values of 8.62 ± 1.17 µg/mL and 2.44 ± 0.87 µg/mL, respectively. Aside from sustaining an individual's daily protein requirements, okra seeds also have the potential to further improve health by releasing bioactive peptides that can alleviate hypertension.

Introduction

Hypertension is considered a risk factor for cardiovascular diseases such as myocardial infarction, heart failure, stroke, atherosclerosis and end-stage diabetes (Hong *et al*., 2008; Boschin *et al*., 2014). It is defined by the World Health Organization (WHO) as the exceeding of 90 mm Hg for the diastolic arterial pressure and 140 mm Hg for the systolic pressure (Boshin *et al*., 2014). The disease currently affects a quarter of the world population (Hartmann and Meisel, 2007).

Antihypertensive peptides are the most extensively studied bioactive peptides. These peptides act by inhibiting the angiotensin I-converting enzyme (ACE). ACE participates in the renin-angiotensin system (RAS) by cleaving angiotensin I to generate angiotensin II, a potent vasoconstrictor; it also takes part in the kinin-nitric oxide system by catalyzing the inactivation of bradykinin, a vasodilator (Udenigwe and Aluko, 2012). Different synthetic ACE inhibitors have already been successfully created. However, because of their detrimental side effects, scientists have instead resorted to exploring the possibilities of using natural sources, like food-derived bioactive peptides, to inhibit ACE (Ryan *et al*., 2011; Boschin *et al*., 2014). Plant-derived ACE inhibitors have since been isolated from soybean (Kuba *et al*., 2005), sunflower (Megias *et al*., 2009), chickpea (Pedroche *et al*., 2002), while animal-derived ACE inhibitors include those isolated from egg (Majumder *et al*., 2015; Yousr and Howell, 2015), milk (Meisel, 1997; Jakala and Vapaatalo, 2010), and fish (Samaranayaka *et al*., 2010; Lee *et al*., 2011).

A common vegetable crop in the Philippines is okra [*Abelmoschus esculentus* (L.) Moench]. Okra is a plant that grows in subtropic and tropic regions of the world (Anwar *et al*., 2011). Aside from being consumed as vegetable, it is also used in the field of medicine as cure for various diseases such as gastrointestinal, genito-urinary, constipation, dysuria, chronic dysentery, spermatorrhoea, ulcers, diabetes, jaundice and relief from hemorrhoids (Alqasoumi, 2012; Hu *et al*., 2013; Pandey *et al*., 2013).

Very few studies have explored the benefits of okra as a potential source of bioactive peptides (Pshenichnov *et al*., 2005; Kondo and Yoshikawa, 2007). This study aims to demonstrate the potential

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of okra protein hydrolysates to generate ACE-inhibitory peptides at various incubation times using two proteases (pepsin and chymotrypsin). This is also the first report of antihypertensive bioactive peptides being prepared from okra seed proteins.

Materials and Methods

Summary of materials

Okra seeds (var. Smooth Green) were acquired from the seed bank of the Bureau of Plant Industry in Los Baños, Laguna, Philippines. Fresh pig lungs were obtained from the Los Baños Community Market, Los Baños, Laguna, Philippines. Captopril (Capomed™) tablets (25 mg/tablet) were bought from a local drugstore. All other reagents used in this experiment were analytical grade.

Isolation of soluble proteins from okra seeds

The method of extraction for okra seeds was adapted from Garcia *et al.* (2005), with some modifications. Okra seeds decorticated individually then crushed with mortar and pestle. The resulting finely ground solids were defatted with hexanes (1:2 w/v) by stirring the resulting mixture for 1 h, and then dried in the fumehood overnight. About 10.0 g of dried, defatted okra seed meal was obtained and placed in a solution containing 50 mL of extraction buffer [35 mM potassium phosphate buffer (pH 7.6) with 0.4 M NaCl and 0.02% NaN_3]. The resulting mixture was placed in an ice bath, and then stirred for 1 h using a magnetic stirrer. Then, the homogenate was filtered through four layers of cheesecloth. The resulting supernatant was further clarified through centrifugation (4°C) using an Allegra X-30R Refrigerated Centrifuge (Beckman Coulter, USA) at 9391.2 x g for 30 min. The clarified supernatant was considered the crude protein extract from okra seeds, which was kept in cold storage (-20°C) until use.

Determination of concentration of protein samples

Protein concentration was determined using a method described by Bradford (1976), with bovine serum albumin (BSA) as protein standard.

In vitro *enzymatic hydrolysis of the okra seed crude protein extract*

In vitro enzymatic hydrolysis of the crude protein extract was done separately using pepsin and α-chymotrypsin. Hydrolysis was done at different time intervals (0 min, 5 min, 15 min, 30 min, 1 h, 12 h and 24 h) according to specific conditions that optimize the activity of each enzyme as described by Contreras *et al.* (2006) and Marczak *et al.* (2003), with some modifications. The progress of enzymatic hydrolysis was monitored through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For pepsin, 200 µL aliquots of 1 mg/mL crude protein, suspended in 35 mM phosphoric acid-potassium dihydrogen phosphate buffer [(pH 2.0) with 0.4 M NaCl and 0.02% NaN_3], were made for each time interval. Then, 25 µL of 1 mg/mL of pepsin was added onto each aliquot. Hydrolysis was stopped by bringing the hydrolysates to neutralization using 1.0 M NaOH and heating the hydrolysates in a boiling water bath for 5 min. The hydrolysates were immediately stored in ice.

For α-chymotrypsin, 200 µL aliquots of 1 mg/mL crude protein were obtained, to which 25 µL of 1 mg/mL of the enzyme was added for each time interval. Hydrolysis was stopped by heating the hydrolysates in a boiling water bath for 5 min. The hydrolysates were immediately stored in ice.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was performed as described by the method of Laemmli (1970). Electrophoresis was accomplished on 11% discontinuous denaturing gels using a Mini-PROTEAN® Tetra Cell electrophoresis apparatus (Bio-Rad, USA). The electrophoresis set-up was run at 110 V for 1.5 h. After the run, gels were stained with an aqueous solution containing 0.25% (w/v) Coomassie Brilliant Blue R250, 50% (v/v) methanol and 10% (v/v) acetic acid. Then, the gels were destained sequentially with an aqueous solution of 50% (v/v) methanol and 10% (v/v) acetic acid, and an aqueous solution of 5% (v/v) methanol and 7% (v/v) acetic acid. Precision Plus Protein™ Unstained Standards (Bio-Rad, USA) for SDS-PAGE was used as molecular weight markers to determine the size of the proteins.

Densitometric analysis

Analysis of the percent composition of the different proteins was done on the scanned images of the electrophoretograms using the ImageJ® (Schneider *et al*., 2012) software.

Extraction of ACE from pig lungs

The method described by Yan *et al.* (2000) was used to extract ACE from pig lungs, with slight modifications. One hundred grams of fresh pig lungs were obtained, cut into pieces, and then minced. The minced tissue was defatted and then homogenized sequentially with acetone (1:2 w/v) and diethyl ether (1:2 w/v). The defatted tissue was air dried,

then ground into powder. The tissue powder was suspended in 100 mM sodium borate buffer (pH 8.3) at 1:5 (w/v), then mixed for 3 h at 4°C. The mixture was filtered through four layers of cheesecloth. The resulting supernatant was centrifuged at 9391.2 x g for 30 min, stored and labeled as the ACE extract.

ACE inhibition assay

The method for assessing the antihypertensive property of the peptic and chymotryptic protein hydrolysates was adapted from Cushman and Cheung (1971), as described by Dumandan *et al.* (2014), with slight modifications.

Assay mixtures containing 25 µL 100 mM phosphate buffer, 25 µL 300 mM NaCl, and 50 µL of 5 mM N-hippuryl-L-histidyl-L-leucine (HHL) (Sigma-Aldrich, USA), were placed in clean test tubes. A negative control was prepared by adding 125 µL 1.0 M HCl to an assay mixture. A blank was also prepared by adding 25 µL of the ACE extract to an assay mixture. Samples were prepared by adding 25 µL of the protein hydrolysate (from each time interval for each enzyme) to an assay mixture, and then 25 µL of the ACE extract were subsequently added. A positive control was also prepared using 1 mg/mL captopril. The addition of the ACE extract to the blank, samples, and control initiated the enzyme-catalyzed reaction.

The mixtures were then incubated at 37°C for 30 min. Except for the negative control, 125 µL of 1.0 M HCl was added into the mixtures to terminate the enzyme reaction. The hippuric acid (HA) formed was extracted by adding 750 µL of ethyl acetate. From each tube, a 500 µL aliquot of the ethyl acetate layer was collected and evaporated to dryness. Hippuric acid was dissolved in 500 µL distilled water and its absorbance was read at 228 nm.

The percent inhibition of the protein hydrolysates from ACE was calculated according to the equation described by Cushman and Cheung (1971).

$$\% \text{ Inhibition} = (A_{blank} - A_{sample})/(A_{blank} - A_{control}) \times 100\% \quad (1)$$

where A_{blank} is the absorbance of blank at 228 nm, A_{sample} is the absorbance of the sample at 228 nm, and $A_{control}$ is the absorbance of the negative control at 228 nm.

Anion-exchange chromatography (IEX) of peptic and chymotryptic hydrolysates

The protein hydrolysate exhibiting the highest ACE inhibition activity was purified through anion-exchange chromatography. One milliliter of the hydrolysate was added with 1 mL of buffer containing 35 mM potassium phosphate buffer (pH 7.6) with 0.02% NaN_3, and was loaded onto a DEAE-Sephacel column (1.6 cm × 5.0 cm) equilibrated with the same buffer described previously. After sample application the bound proteins were eluted with 10 column volumes of a NaCl step gradient from 0.2 M to 1.0 M in the same buffer, at a flow rate of 0.5 mL/min. Eluents, with a volume of 0.5 mL, were collected and kept in cold storage for subsequent procedures.

Determination of the IC_{50} of bioactive peptides

Selected fractions from IEX, exhibiting ACE inhibitory activities, were subjected to Bradford assay to estimate the amount of peptides. Aliquots of different concentrations of the peptides were prepared, with each aliquot being subjected to ACE inhibition assay to determine the IC50 at 95% confidence interval using GraphPad Prism® v. 6.01 (GraphPad Software Inc., USA) software. IC_{50} is defined as the inhibitor concentration needed to inhibit ACE activity by 50%.

Statistical analysis

Assays were done in duplicates, and subjected to ordinary one-way ANOVA followed by Tukey's test at 95% confidence interval using GraphPad Prism®. Assay results were expressed as mean activity ± standard deviation.

Results and Discussion

Extraction of soluble proteins from okra seeds

The proteins from okra seeds were effectively extracted using the buffer described above, yielding an estimated protein content of 13.8 ± 0.6 mg/mL. The SDS-PAGE revealed six major polypeptide bands, with apparent molecular weights of 11, 13, 16, 18, 25, and a prominent band observed with 49 kDa size (Figure 1a). The protein profile of OSCPE showed close similarity with that of vicilin in peas (Croy *et al.*, 1980) and mung bean (Viernes *et al*., 2012) in terms of the molecular weights of the component proteins.

In vitro *enzymatic hydrolysis of OSCPE*

Bioactive peptides from OSCPE were released through separate in vitro enzymatic hydrolysis of pepsin and α-chymotrypsin from 0 min to 24 h of incubation. The progress of enzymatic hydrolysis was monitored through SDS-PAGE. Figure 1b shows the progress of hydrolysis of OSCPE using pepsin. Because of the acidic environment by which pepsin is optimally active and stable, many of the OSCPE

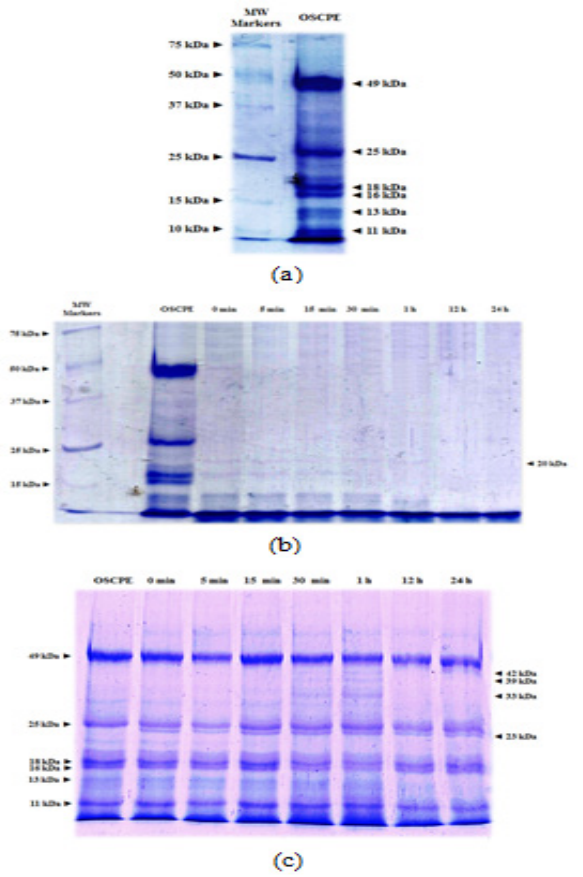


Figure 1. SDS-PAGE profile of OSCPE (a); enzymatic hydrolysis of OSCPE using pepsin (b), and α-chymotrypsin (c)

proteins were immediately denatured as seen by the reduced number of protein bands at 0 min. The 49 and 25 kDa polypeptides completely disappeared at 0 min while the 20 kDa polypeptide disappeared after 30 min of incubation. Densitometric analyses revealed that the 18 kDa polypeptide was relatively less susceptible to enzymatic hydrolysis than the 49 and 25 kDa polypeptides, with 35% of the polypeptide remaining at 0 min of incubation until it was essentially hydrolyzed after 1 h. The 13 kDa polypeptide was the least susceptible among the six major polypeptides, with 68% of the protein remaining at 0 min until it was essentially hydrolyzed after 1 h. All of the polypeptides, including the generated 20 kDa polypeptide, were essentially hydrolyzed starting from 12 h of incubation.

On the contrary, the polypeptides were fairly resistant against hydrolysis with α-chymotrypsin (Figure 1c). At 24 h of incubation 48, 62, and 79% remained of the 49, 25, and 11 kDa polypeptides, respectively. The 16 and 18 kDa polypeptides were analyzed as one component, wherein 80% of the polypeptides remained at 24 h of incubation. The 13 kDa polypeptide was essentially hydrolyzed at 12 h of incubation. It was also observed that 42, 39, and 33 kDa polypeptide bands appeared at 30 min of incubation. These polypeptides were being generated up to one h of incubation, but essentially hydrolyzed at 12 h of incubation. A 23 kDa polypeptide was also detected at 0 min of incubation, and remained even at 24 h of incubation.

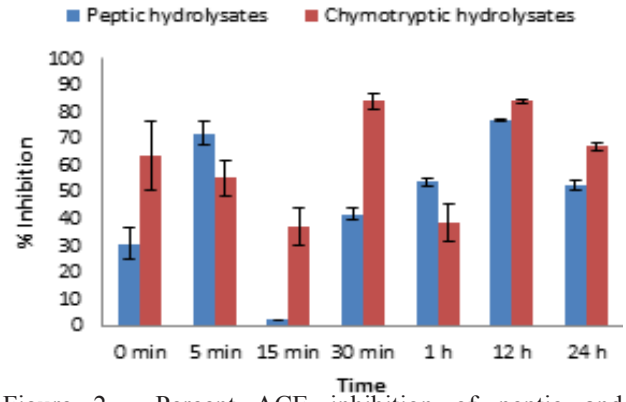
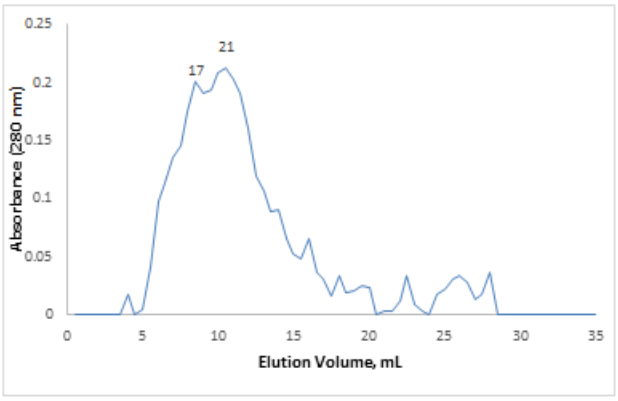


Figure 2. Percent ACE inhibition of peptic and chymotryptic hydrolysates of OSCPE digested at different time intervals

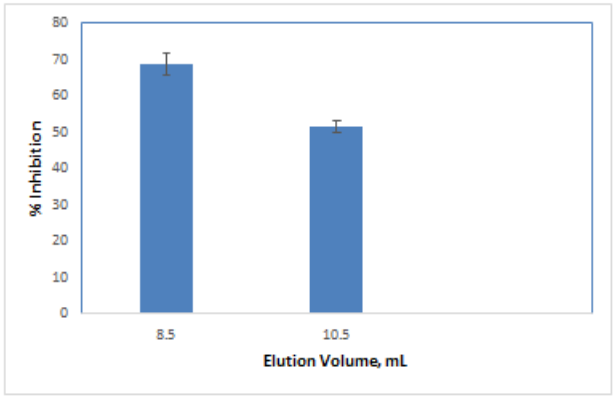
In general, OSCPE is more resistant to enzymatic hydrolysis with α-chymotrypsin than pepsin, with most of the major polypeptides still present even at 24 h of incubation. On the other hand, digestion of OSCPE with pepsin showed that most of these polypeptides were hydrolyzed even at short contact time (0 min of incubation) and were all essentially hydrolyzed at 12 h of incubation. These differences in susceptibility of the proteins to digestion may be attributed to a lesser number of cleavage sites for chymotrypsin than pepsin. Pepsin is relatively a non-specific protease although it does have a preference for cleaving the amino-terminal side of the aromatic amino acid residues (tyrosine, tryptophan, and phenylalanine). Chymotrypsin cleaves preferentially on the carboxyl side of aromatic amino acid residues (tyrosine, tryptophan, and phenylalanine) and some other bulky nonpolar residues (leucine and methionine). Different proteins are hydrolyzed to varying degrees depending on the quantity of cleavage sites present, as well as on other factors like enzyme-to-substrate ratio, optimum temperature, and hydrolysis time (Wu *et al*., 2012). Greater degree of hydrolysis often results to smaller and more effective ACE-inhibitory peptides (Li *et al.*, 2004; Hernandez-Ledesma *et al*., 2011).

ACE Inhibition assay

Preliminary ACE inhibition assay of the peptic and chymotryptic hydrolysates indicated presence of antihypertensive peptides, as shown in Figure 2. It was expected that pools of different bioactive peptides were generated due to various degrees of

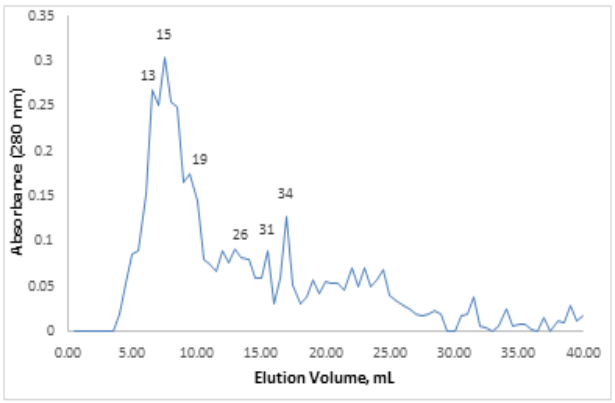


(a)

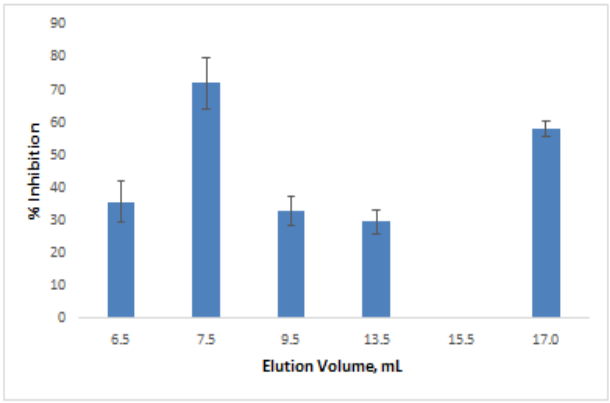


(b)

Figure 3. (a) IEX elution profile of the 12-h peptic hydrolysate. Fraction numbers – 17 and 21; and (b) ACE inhibitory activities of IEX fractionated 12-h peptic hydrolysates



(a)



(b)

Figure 4. (a) IEX elution profile of the 12-h chymotryptic hydrolysates. Fraction numbers – 13, 15, 19, 26, 31, and 34; (b) ACE inhibitory activities of IEX fractionated 12-h chymotryptic hydrolysates

hydrolysis with pepsin at different time intervals. Each hydrolysate exhibited different ACE inhibitory activities, showing no direct relationship between digestion time and ACE inhibitory activity.

For the hydrolysis using pepsin, the 12-h hydrolysate exhibited the highest ACE inhibitory activity, at 77.01 ± 0.14%. Statistical analysis indicated that there were significant differences among the ACE inhibitory activities at different time intervals. The 5-min and 12-h hydrolysates, which exhibited the highest inhibition against ACE, were not significantly different. Nonetheless, the 12-h hydrolysate was subjected to IEX fractionation for the reason that ACE inhibitory peptides that are generated from near completion of enzymatic hydrolysis are observed to be more effective (Dumandan *et al.*, 2014).

The 12-h hydrolysate also exhibited the highest ACE inhibitory activity among the chymotryptic hydrolysates, at 84.14 ± 0.85%. Statistical analysis showed that there were significant differences in terms of ACE activities between time intervals. However, there were no significant differences between time intervals that exhibited high ACE inhibitory activities, namely 0 min, 30 min, 12 h, and 24 h. The 12-h hydrolysate was chosen for further purification.

Purification of okra seed protein hydrolysates using anion-exchange chromatography

The 12-h hydrolysates of pepsin and α-chymotrypsin were purified using anion-exchange chromatography (IEX). The IEX chromatogram of 12-h peptic hydrolysate is shown in Figure 3a. Two prominent peaks were observed on the chromatogram, occurring at elution volumes of 8.5 and 10.5 mL. Fractions 17 and 21, corresponding to elution volumes of 8.5 and 10.5 mL respectively, were subjected to ACE inhibition assay to check for ACE inhibitory activity. Fraction 17 showed higher inhibitory activity against ACE than fraction 21 at 68.68 ± 2.91% and 51.45 ± 1.43%, respectively (Figure 3b).

On the other hand, there were four prominent peaks observed on the IEX chromatogram of the 12-h chymotryptic hydrolysate (Figure 4a), with fraction

Table 1. ACE inhibitory activities of the hydrolysate IEX fractions.

Sample (Volume of eluent, mL)	% Inhibition	IC_{50} (µg/mL)
8.5 (IEX peptic fraction)	68.68 ± 2.91	8.62 ± 1.17
7.5 (IEX α-chymotryptic fraction)	71.89 ± 7.77	2.44 ± 0.87
captopril	91.80 ± 5.28	0.004*

*Ondetti *et al.* (1977)

15 showing the highest ACE inhibitory activity of 71.89 ± 7.77% (Figure 8). Fraction 31 was devoid of any activity. For both hydrolysates, it has been observed that the fractions exhibited ACE inhibitory activities to a lesser extent than the hydrolysate as a whole. Nevertheless, it can be said that the peptides contained in these fractions may demonstrate synergistic effects to achieve an ACE inhibitory activity similar to the hydrolysates in their entirety (Dumandan *et al*., 2014).

Determination of IC_{50} of the IEX hydrolysate fractions against ACE

IC_{50} values were calculated for IEX fractions 17 and 15 of the 12-h hydrolysates of pepsin and α-chymotrypsin, respectively. The IC_{50} values (Table 1) indicated that bioactive peptides derived from OSCPE, released through digestion with pepsin and α-chymotrypsin, exhibited ACE inhibition activities relatively to the same extent. However, both fractions are inferior to captopril in terms of their potency in inhibiting ACE.

IC_{50} values reflect the inhibitor potency, expressed in terms of inhibitor concentration, that can effect a 50% reduction of enzymatic activity (Copeland, 2005). This implies that captopril concentration required to inhibit ACE is much lower in contrast with the needed peptide concentrations. Although the individual fractions are not as effective as captopril, the synergistic effect of all ACE inhibitory peptides may still give a positive impact on health by preventing and lowering the incidence of hypertension.

Conclusion

The okra is a vegetable crop that contains seed proteins capable of releasing ACE inhibitory peptides when subjected to chymotrypsin and pepsin hydrolysis. The proteins were more resistant to hydrolysis with chymotrypsin than pepsin. Nevertheless, both enzymes produced peptides with high inhibitory activities at 12-h hydrolysis time. The 12-h hydrolysates were successfully fractionated using anion exchange chromatography, with several fractions exhibiting ACE inhibitory activities. The chromatographic fractions that demonstrated ACE activities were still inferior to captopril in terms of their potency to inhibit ACE. Regardless, the study reveals that okra contains bioactive ACE inhibitory peptides that may be favorable to health by preventing and alleviating hypertension.

Acknowledgements

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