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Effect of *in vitro* gastrointestinal digestion on the Angiotensin Converting Enzyme (ACE) inhibitory activity of pigeon pea protein isolate

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Abstract

The content of certain bioactive peptides in the gastrointestinal digestive products from legume proteins is expected to provide added value to the function of proteins (beyond the scope of nutrition). *In vitro* gastrointestinal digestion (GID) involves the hydrolysis of proteins by a mixture of proteases (pepsin, trypsin, and chymotrypsin) to produce protein hydrolysate. The present work aimed to evaluate the angiotensin converting enzyme (ACE) inhibitory activity of protein hydrolysate produced through a simulated *in vitro* GID of pigeon pea protein isolates, and to fractionate its bioactive peptide component. The protein content of pigeon pea protein isolate was 81.34%. The highest value of the degree of hydrolysis and the ACE inhibitory activity was obtained using $P_{120}TC_{120}$ treatment (120 min of pepsin followed by 120 min of trypsin-chymotrypsin mixture) with an IC_{50} value of 64.22 µg/mL. The fractionation of the protein hydrolysate using ultra-filtration method resulted in a peptide fraction with the molecular weight below 3 kDa as the most active fraction, which had an IC_{50} value of 11.76 µg/mL and contained 10 peptide components with molecular weight between 400-1,000 Da. These results indicated that the pigeon pea protein hydrolysate has the potential as an ACE inhibitory functional ingredient.

Introduction

Nowadays, peptides released in the enzymatic food protein hydrolysis known as bioactive peptide, receive attention from many food experts because they have many health benefits. These bioactive peptides are inactive in their parent protein sequence but can exert biological activities after being released through digestion process and food processing by protease activity (Korhonen *et al*., 1998). Various biological activities have been found in peptides released from food proteins, one of which is ACE inhibitory activity. Various types of ACE inhibitory bioactive peptides have been identified from food including casein, whey, ovalbumin, red algae, soy, gelatine, dried bonito, corn, rapeseed, potato, garlic and wheat germ (Pihlanto and Mäkinen, 2013). *In vitro* assay of ACE inhibitory activity can become the basis to indicate the potential of anti-hypertensive activity of protein hydrolysate and peptides.

ACE is a key enzyme in blood pressure regulation in a renin angiotensin system by releasing Angiotensin II (a vasoconstrictor hormone) which causes an increase in blood pressure. By inhibiting ACE activity using an inhibitory agent, the blood pressure is expected to be controlled. So, this concept has been applied in the treatment of hypertension, by giving oral ACE inhibitors. Various types of ACE inhibitory peptides have been known to reduce blood pressure *in vivo*. The food industry has recognised the potential of this natural anti-hypertensive agent to be applied as a functional ingredient that helps in the primary prevention and treatment of hypertension (Pihlanto and Mäkinen, 2013).

It has been known that once the food enters the digestive system, the proteins will be ready to be hydrolysed by various types of protease found in the gastrointestinal tract such as pepsin, trypsin, and chymotrypsin so that peptides with shorter chain

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size and free amino acids will be produced. Some of such peptides may exert biological function directly in the gastrointestinal tract, but other peptides can experience absorption to reach the target organs and tissues through the circulatory system. To determine the gastrointestinal protease effect on the release and breakdown of bioactive peptides from food, a GID simulation has been carried out on various protein sources, namely egg, meat, fish and vegetable protein. Hernández-Ledesma *et al*. (2007) identified peptides with ACE inhibitory and antioxidant activities in protein hydrolysates of several human milk samples and infant formula milk following digestion by pepsin and pancreatin in the simulation of infant gastrointestinal conditions. Bamdad *et al*. (2009) found that *in vitro* GID of lentil protein was able to exert ACE inhibitory activity of 75.5%. Lee *et al*. (2015) have found that water extract of pigeon pea and extract of B. subtilis-fermented pigeon pea significantly increased the systolic blood pressure (21 mmHg) and diastolic blood pressure (30 mmHg) in the animal model (spontaneously hypertensive rats).

Legumes are a protein-rich food, and their consumption has been associated with the prevention of chronic diseases by their bioactive components. Cereals and legumes are key components of a healthy and balanced diet (Malaguti *et al*., 2014). Although some animal proteins have excellent functional and organoleptic properties, unfortunately, they needed a higher production cost and environmental impact than plant proteins, and also the risk of their cholesterol and fat content. Enzymatic hydrolysis strongly increases solubility of plant proteins and alters their functional properties. Therefore, research in plant protein functionality has increased over the last decades (Wouters *et al*., 2016).

Pigeon pea (*Cajanus cajan* (L.) Millsp.) is an important leguminous crop commonly grown in semi-arid tropical regions of the world. Unlike the major crops, orphan crops such as pigeon pea, have received only limited scientific investigation. The protein content of pigeon pea ranges from 15.5 to 28.8%, which are influenced by genetic and external factors such as fertilisation, season, the degree of maturity and location (Salunkhe, 1986). The results of Singh and Jambunathan (1982) study about the amino acid composition of the pigeon pea cotyledon protein fraction showed that it contained several types of amino acids which could potentially play a role in ACE inhibitory activity, especially hydrophobic amino acids. Proteins with a high abundance of hydrophobic amino acid are considered to be a good substrate for the production of ACE inhibitory peptides (Pihlanto and Mäkinen, 2013).

The present work thus aimed to evaluate the ACE inhibitory activity of pigeon pea protein hydrolysates produced through *in vitro* simulated GID, and to fractionate and characterise the bioactive peptide components contained in the peptide fractions.

Materials and methods

Isolation of the pigeon pea protein

The pigeon pea isolation process was carried out following the method of Bamdad *et al*. (2009). The pigeon pea flour used was prepared by grinding split pigeon pea (60 mesh). Next, 20 g pigeon pea flour was extracted with 200 mL 0.2% NaOH (to obtain pH 12), and shaken for 1 h. Then, the mixture was centrifuged at 5,000 rpm for 15 min at 4°C. Two additional extractions were carried out in the next hour using half the volume of 0.2% NaOH solution. Following alkaline extraction, the isoelectric precipitation step was then carried out through pH treatment of alkaline extraction filtrate to pH 4.5 with the addition of 0.1 N HCl. The purpose of this treatment was to precipitate all protein components that were previously dissolved in the filtrate resulting from alkaline extraction, due to the pH 4.5 region is the area of the isoelectric point of most protein molecules. The supernatant was collected, and the pH was adjusted to 4.5 and centrifugation was performed as previously mentioned. The precipitate (protein isolate) was washed with distilled water and re-centrifuged. The precipitate obtained was then freeze-dried and stored in -20°C prior to analysis, and called as pigeon pea protein isolate. The protein content of this pigeon pea protein isolate was then analysed by micro-Kjeldahl method (Jacobs, 1951).

In vitro GID on the pigeon pea protein isolate

The method used followed the procedure carried out by Bamdad *et al*. (2009) with modification. Physiological digestive conditions were based on gastrointestinal method carried out by Gauthier (1986). Simulated digestion of the stomach was carried out by using 100 mL 4% (b/v) pigeon pea protein solution adjusted to the pH of the gastric acid (pH 2.0) using 1 N and 10 N HCl with strong mixing (using magnetic stirrer until the pH was stable). Pepsin was added with the ratio of enzyme to substrate 1/250 (b/b) and the mixture was incubated in a water bath-shaker for 2 h at 37°C. The hydrolysis times were varied to 0, 30, 60, 90 and 120 min. After 2 h of hydrolysis using pepsin, the pH was adjusted to fit the conditions of the small intestine (pH 6.5), and a mixture of trypsin and chymotrypsin was added at a ratio of enzyme to substrate 1/250 (b/b). The solution

was then incubated again for 2 h at 37°C in the water bath shaker, and during the incubation period sampling was done at 0, 30, 60, 90 and 120 min. To stop the hydrolysis, each sample was immediately immersed in a boiling water for 10 min, and the pH was set to 5. The non-hydrolysed protein will be precipitated due to the pH 5 is close to the isoelectric point of protein (4.5). The clear supernatants of protein hydrolysate was obtained upon centrifugation at 5,000 rpm for 15 min at 4°C.

Degree of hydrolysis

A series of standard amino acid solutions (represented by leucine) were prepared by diluting a 0.2 mM leucine stock solution using the same solvent used to prepare the protein sample solution, and 0.2 mL ninhydrin reagent was added. A total of 0.2 mL ninhydrin reagent was also separately added to 1 mL protein hydrolysate sample, 1 mL blank solution and 1 mL non-hydrolysed protein solution. All the mixtures were then heated in water bath (85°C) for 1 min, and the absorbance was read at 570 nm. The number of amino groups released (*h*) was determined by reducing the control value that was not hydrolysed on the corresponding standard curve after the blank was calculated. This gave the equivalent number of hydrolysed peptide bonds (*h*), which were expressed as milliequivalents per gram of protein using Equation 1 (Adler-Nissen, 1986):

$$h = (A \times b) / m \quad \text{(Eq. 1)}$$

where A = absorbance at 570 nm, $b = y$ intercept, and m = slope of the calibration curve.

The degree of hydrolysis was calculated using Equation 2:

$$DH = h / h_{total} \times 100\% \quad \text{(Eq. 2)}$$

where htotal = total number of peptide bonds (obtained from the reconstitution of protein samples (% b / v).

ACE inhibitory activity

The ACE inhibitory activity of samples was measured following the method described by Cushman and Cheung (1971) with slight modification in the buffer composition used in the reaction and the enzyme/substrate ratio. For each measurement, 20 µL samples and 50 µL 100 mM HHL (contained 300 mM NaCl) were incubated with 10 µL ACE 100 mU/mL at 37°C for 30 min. The reaction was stopped by addition of 100 µL 1 N HCl, then 1 mL of ethyl acetate was added and homogenised using vortex for 30 s, and then centrifuged at 3,000 g for 10 min. A total of 750 µL supernatant (top layer) was taken and dried at 140°C for 10 min. The residue obtained from the drying product was dissolved in 2 mL 1 M NaCl, and the absorbance was read at 228 nm.

Fractionation of ACE inhibitory peptide of the most active protein hydrolysate

The ACE inhibitory peptide contained in the protein hydrolysate was fractionated by a series of sequential filtration based on the size of the molecular weight of the peptide using ultra-filtration method described by Siow and Gan (2013) with slight modification in the pore size of membrane filter. The ultra-filtration method was performed using a membrane filter (Amicon, Millipore) with MWCO 10 kDa and 3 kDa, and three types of peptide fraction distinguished by its molecular weight size was obtained namely fraction > 10 kDa, fraction 3-10 kDa and fraction < 3 kDa.

Total amino acid composition

The total amino acid composition of the sample was analysed based on the procedure described by Waters Corporation (2008). The sample was analysed by hydrolysing the sample with 6 N HCl, then the hydrolysis results were derivatised with AccQ Fluorine Reagent Kit. A total of 5 µL derivatised sample was then injected into the ALLIANCE HPLC system (USA), using two mobile phases (AccQ•Tag Eluent A and acetonitrile), AccQ•Tag Waters column and fluorescence detector. The HPLC measurement conditions were as follows: column temperature 37°C, column length 250-395 nm, gain 10, mobile phase, flow rate 1.05 mL/min.

Molecular weight profiling of peptide

The peptide fraction with the highest ACE inhibitory activity was then analysed by the UPLC-MS-TOF method (Model Xevo G2-S QT, Waters, USA). A total of 5 µL samples were injected into the ACQUITY UPLC @BEH C18 (1.7 µm 2.1 × 50 mm) column. The mobile phase used was solvent A (acetonitrile and 0.05% formic acid) and solvent B (water and formic acid 0.05%), with a flow rate of 0.2 mL/min (step gradient). Positive ions were recorded with a mass range of 50-3,000 m/z.

Results and discussion

Isolation of the pigeon pea protein from pigeon pea flour

The results of protein content analysis of the pigeon pea protein isolates using the micro-Kjeldahl method indicated that the pigeon pea protein isolates had high protein content which reached 81.34 ± 0.12%. This high protein content of pigeon pea protein isolates indicated that the protein isolate obtained was a good starting material in the production of pigeon pea protein hydrolysate due to it having low amounts of undesirable compounds such as soluble sugars, lipids, phenols or fibre (Villanueva *et al*., 1999).

In vitro GID of pigeon pea protein isolates

In this stage, the pigeon pea protein isolates obtained were then hydrolysed using pepsin (mimicking the digestive process in the stomach) and followed by a mixture of trypsin and chymotrypsin (mimicking the digestion process of protein in the intestine). The pH, temperature, and enzyme-substrate ratio were adjusted to mimic the situation in the GID (Gauthier *et al*., 1986). The variation of hydrolysis time was carried out by taking samples from the mixture every 30 min. To simulate the process in the stomach using pepsin, the variation of hydrolysis time was limited to 120 min, followed by treatment in the intestine which was also limited to the hydrolysis time of 120 min. This hydrolysis time refers to the standard protocol for *in vitro* digestion methods on food which is an international consensus (Minekus *et al*., 2014). Each type of endoprotease has a different specificity in choosing the position of the peptide bond to be cut (what type of amino acid flanks the peptide bond). For example trypsin will choose a peptide bond formed by lysine (Lys) or arginine (Arg) on the left side of the peptide bond, whereas the chymotrypsin has a broader specificity, namely selecting the amino acid tyrosine, phenylalanine, leucine, isoleucine, valine, tryptophan and histidine (Matthews and Van Holde, 1996). The wider specificity of chymotrypsin is expected to increase the degree of hydrolysis value in the hydrolysis process of the pigeon pea protein. The degree of hydrolysis is one of the basic parameters which needs to be controlled because the characteristics of the protein hydrolysate are closely related to the degree of hydrolysis. This control is necessary because the hydrolytic power of an enzyme can vary based on the source and substrate used.

Based on Table 1, it can be observed that the hydrolysis treatment using pepsin, trypsin and chymotrypsin increased the value of the degree of hydrolysis. It can also be observed that there was an increase in the degree of hydrolysis value with increasing hydrolysis time. The same pattern of the degree of hydrolysis was also found in the study on protein hydrolysis of okara (a low value by-product of soybean obtained from soymilk processing) (Sbroggio *et al*., 2016). Degree of hydrolysis is independent of five variables: substrate concentration, enzyme/substrate ratio, pH, temperature and time (Adler-Nissen, 1982). Theoretically, the longer the hydrolysis time, the more chance of contact between the active site of the enzyme with the protein substrate so that the peptide bond is increasingly broken. Degree of hydrolysis measures the percentage of peptide bonds hydrolysed during protein hydrolysis (Adler-Nissen, 1976).

Table 1. Effect of hydrolysis time on the degree of hydrolysis and ACE inhibitory activity of pigeon pea protein hydrolysate.

Hydrolysis Time (min)	Degree of Hydrolysis (%)	ACE Inhibitory Activity (%)
P_{30}	2.50 ± 1.49^{a}	38.01 ± 0.75^{a}
P_{60}	8.75 ± 1.15^{b}	43.34 ± 1.57^{b}
P_{90}	22.3 ± 1.85^{c}	46.60 ± 0.54^{b}
P_{120}	28.74 ± 0.91^{d}	47.58 ± 0.43^{b}
$P_{120}TC_{30}$	40.23 ± 0.64^{e}	55.62 ± 0.44^{c}
$P_{120}TC_{60}$	45.09 ± 0.54^{f}	58.43 ± 2.77^{c}
$P_{120}TC_{90}$	48.71 ± 1.63^{g}	65.42 ± 3.27^{d}
$P_{120}TC_{120}$	53.58 ± 1.06^{h}	70.51 ± 1.13^{e}

Data are means ± SD. Different superscripts in the same column indicate significant difference at $p < 0.05$.

ACE inhibitory activity assay of pigeon pea protein hydrolysate

The results of the ACE inhibitory activity assay on the pigeon pea protein hydrolysate with hydrolysis time variations is shown in Table 1, in which an increase in ACE inhibitory activity with increasing hydrolysis time can be noted and reached the highest peak in the $P_{120}TC_{120}$ treatment (120 min hydrolysis by pepsin and followed by 120 min of trypsin and chymotrypsin mixture). It is also apparent from Table 1 that the increase in the ACE inhibitory activity was directly proportional to the increase in the degree of hydrolysis value (highest in the $P_{120}TC_{120}$ treatment).

The ACE inhibitory activity of protein hydrolysate depends on the protein substrate, the proteolytic enzymes used to break it down, and the condition of hydrolysis process (Pihlanto and Mäkinen, 2013) because these will affect the structure of the bioactive peptide products contained in the protein hydrolysate. Malaguti *et al*. (2014) stated that ACE inhibitory activity was determined by molecular size, composition and amino acid sequence of the

peptide. The treatment of $P_{120}TC_{120}$ hydrolysis was found able to produce peptide extracts with the highest ACE inhibitory activity possibly due to the protein hydrolysate contained the bioactive peptide components with molecular weight, composition, and amino acid sequence characteristic of an ACE inhibitor.

A similar study on lentil found that the treatment of 2 h hydrolysis using pepsin followed by a mixture of trypsin and chymotrypsin for 2.5 h was able to produce lentil protein hydrolysate with the highest ACE inhibitory activity (Bamdad *et al*., 2009). Also, similar study of pigeon pea protein hydrolysate has been carried out by Olagunju *et al*. (2018), who found that pigeon pea protein hydrolysate had ACE inhibitory activity of 61.82%, and had a strong anti-hypertensive effect after *in vivo* activity assay in spontaneously hypertensive rats (SHR) which was able to reduce systolic blood pressure (-26.12 mmHg) after 2 h orally. The best hydrolysis condition found in the present work was using the pepsin enzyme for 2 h followed by a mixture of trypsin-chymotrypsin for 2 h at the enzyme to substrate ratio of 1:250. However, Olagunju *et al*. (2018) used a mixture of pepsin and pancreatin with 2 h of hydrolysis time (substrate-enzyme ratio 1:20), and did not vary the hydrolysis time, peptide fractionation, IC_{50} value and LCMS analysis of their protein hydrolysates.

In the present work, the IC_{50} analysis was also carried out to compare the IC_{50} values of protein hydrolysate before and after fractionation. The results of the IC_{50} values determination of the pigeon pea protein hydrolysate ($P_{120}TC_{120}$) was 64.22 µg/mL. The IC_{50} value was quite high compared to the results of a study conducted on common beans (Phaseolus vulgaris), which found that a total treatment of 1 h hydrolysis with pepsin (1/2 h) and pancreatin (1/2 h) capable of producing IC_{50} value of 250 µg/mL (Chel-Guerrero *et al*., 2012).

Fractionation of ACE inhibitory peptides from pigeon pea protein hydrolysate

The pigeon pea protein hydrolysate ($P_{120}TC_{120}$) was then fractionated based on their molecular size by centrifugal ultra-filtration method so that three types of fractions were obtained namely: fraction > 10 kDa, 3-10 kDa and < 3 kDa. The value of ACE inhibitory activity of each fraction is shown in Table 3. Based on Table 3, it can be concluded that the fraction < 3 kDa had the highest ACE inhibitory activity as compared to the other two fractions. The results of this fractionation had similar pattern to the results of fractionation of the protein *Kluyveromyces marxianus* hydrolysate using the ultra-filtration method, which also found that the fraction < 3 kDa had the highest activity (Mirzaei *et al*., 2018). A similar result was also found in research on fractionation of *Ginkgo biloba* seed protein hydrolysate. The protein hydrolysate was ultra-filtered to obtain fraction with different molecular weights (< 1 kDa, 1-3, 3-5, and 5-10 kDa). The < 1 kDa fraction showed better ACE inhibitory activity with an IC_{50} value of 0.2227 mg/mL (Ma *et al*., 2019).

Table 3. ACE inhibitory activity of ultra-filtration-based fractions of pigeon pea protein hydrolysate ($P_{120}TC_{120}$).

Peptide Fraction	ACE Inhibitory Activity (%)
> 10 kDa	51.47 ± 8.23a
10-3 kDa	59.65 ± 5.26a
< 3 kDa	86.08 ± 4.56b

Data are means ± SD. Different superscripts in the same column indicate significant difference at $p < 0.05$.

Characterisation of < 3 kDa peptide fraction

The < 3 kDa fraction as a peptide fraction with the highest ACE inhibitory activity from the pigeon pea protein hydrolysate was further characterised for IC_{50} value, amino acid composition and its peptide molecular weight profile. The IC_{50} value (inhibitor concentration which causes 50% ACE inhibition) was used to estimate the effectiveness of various ACE inhibitor.

The IC_{50} value

The results of the analysis showed that the IC_{50} value of the < 3 kDa peptide fraction (after fractionation) was 11.76 µg/mL. This value was much lower than the IC_{50} value of the pigeon pea protein hydrolysate before fractionation, which was 64.22 µg/mL. This indicated that the fractionation process had successfully reduced the IC_{50} value by 81.75% as compared to without fractionation. This low IC_{50} value of the fraction < 3 kDa also indicated that the peptide components that contributed to ACE inhibitory activity in the pigeon pea protein hydrolysate were dominated by short peptides of less than 3 kDa size. After going through the fractionation process, the larger proteins and peptides have been eliminated through the molecular sieving process by the ultra-filtration membrane.

The amino acid composition

The results of the analysis of amino acid composition by HPLC (Table 2) indicated that the peptide fraction was dominated by hydrophobic amino acid groups as compared to other groups. In the hydrophobic group, leucine (Leu) and proline (Pro) dominated as compared to other amino acids. The literature study revealed that the presence of

hydrophobic amino acids, especially the branching ones such as proline (Pro) in the peptide plays an important role in determining ACE inhibitory activity (Ryan *et al*., 2011). The high presence of these hydrophobic amino acids is likely to contribute to the ACE inhibitory activity of the peptide fraction.

Table 2. The amino acid composition of the < 3 kDa fraction (g/100 g).

	g/100g
Acid Group	
Asx (D)	6.4
Glx (E)	15.8
Total:	22.2
Polar Group	
Ser (S)	5.2
Tyr (Y)	2.6
Thr (T)	12.6
Total:	20.4
Basic Group	
Arg (R)	5
His (H)	1
Lys (K)	6.2
Total:	12.2
Hydrophobic Group	
Ala (A)	3.6
Val (V)	1.4
Phe (F)	0.4
Ile (I)	5.2
Leu (L)	7.4
Pro (P)	5.6
Gly (G)	4.6
Total:	28.2

Molecular weight profiling using the LCMS method

Based on Table 4, it can be seen that the LCMS analysis was able to detect 10 peptides with a molecular weight below 1 kDa (molecular weight between 400-1,000 Da). This short size of the peptide is favourable for ACE inhibitory activity which agrees with the results of Natesh *et al*. (2003) which concluded that the active site of ACE cannot accommodate large peptide molecules. The ACE inhibitory peptides contain usually between 2-12 amino acid residues, even though larger peptides may also exhibit such an activity (López-Fandiño *et al*., 2006).

The study about the ability of fermented defatted walnut residues using *Bacillus subtilis* in producing ACE inhibitory peptides found that < 1 kDa fraction presented the greatest ACE inhibitory activity which contained two types of peptide component confirmed as VQTL (459.351 Da) and LGYEN (594.276 Da) by UPLC-ESI-MS/MS (Zheng *et al*., 2017). Ma *et al*. (2019) studied the purification and identification of *Ginkgo biloba* protein hydrolysate by Sephadex G15 gel chromatography and LC-MS/MS, giving three new potential ACE inhibitory peptides i.e. TNLDWY, RADFY, RVFDGAV.

By using *in vitro* GID method in producing protein hydrolysate, proteins undergo degradation similar to that in the human digestive system, which releases peptides that are expected to be resistant to digestion *in vivo* (Vermeirssen *et al*., 2005). Anti-hypertensive peptides have major potential as functional ingredients aiding in the prevention and management of hypertension. Although these peptides have been found to be less potent than anti-hypertensive synthetic drugs, as part of the daily diet, they could play an important part as natural and safe blood pressure control agents (Pihlanto and Mäkinen, 2013).

Table 4. Peptide component detected on the < 3 kDa fraction by LCMS.

No	RT (min)	m/z	Molecular Weight (Da)
1	3.833	717.3962	716.825
2	4.313	724.3502	723.771
3	4.427	617.3659	616.359
4	4.479	816.4590	815.956
5	4.582	846.4712	845.982
6	4.828	645.3605	644.718
7	5.010	457.3395	456.619
8	5.376	991.5118	991.095
9	6.108	736.4017	735.870
10	6.394	764.4332	763.923

Conclusion

The present work evaluated the effect of *in vitro* GID of pigeon pea protein isolate (protein content of 81.34%). GID treatment was demonstrated able to increase ACE inhibitory activity of the pigeon pea protein isolates, which reached the highest value using the $P_{120}TC_{120}$ treatment (120 min pepsin followed by 120 min trypsin and chymotrypsin). The $P_{120}TC_{120}$ treatment produced pigeon pea protein hydrolysate with an IC_{50} value of 64.22 µg/mL. The ultra-filtration result of the protein hydrolysate showed that the fraction below 3 kDa had the highest ACE inhibitory activity at IC_{50} value 11.76 µg/mL, and consisted of relatively high hydrophobic amino acid, and contained 10 peptides with a molecular weight below 1 kDa.

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References

Adler-Nissen, J. 1976. Enzymatic hydrolysis of proteins for increased solubility. Journal of Agricultural and Food Chemistry 24(6): 1090-1093.

Adler-Nissen, J. 1982. Limited enzymic degradation of proteins: a new approach in the industrial application of hydrolases. Journal of Chemical Technology and Biotechnology 32: 138-156.

Adler-Nissen, J. 1986. Enzymic hydrolysis of food proteins. London: Elsevier Applied Science Publishers Ltd.

Bamdad, F., Dokhani, S., Keramat, J. and Zareie, R. 2009. The impact of germination and *in vitro* digestion on the formation of Angiotensin Converting Enzyme (ACE) inhibitory peptides from lentil proteins compared to whey proteins. International Journal of Nutrition and Food Engineering 3(1): 109-119.

Chel-Guerrero, L., Domínguez-Magaña, M., Martínez-Ayala, A., Dávila-Ortiz, G. and Betancur-Ancona, D. 2012. Lima bean (*Phaseolus lunatus*) protein hydrolysates with ACE-I inhibitory activity. Food and Nutrition Sciences 3: 511-521.

Cushman, D. W. and Cheung, H. S. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochemical Pharmacology 20(7): 1637-1648.

Gauthier, S. F., Vachon, C. and Savoie, L. 1986. Enzymatic conditions of an *in vitro* method to study protein digestion. Journal of Food Science 51(4): 960-964.

Hernández-Ledesma, B., Quirós, A., Amigo, L. and Recio, I. 2007. Identification of bioactive peptide after digestion of human milk and infant formula with pepsin and pancreatin. International Dairy Journal 17(1): 42-49.

Jacobs, M. B. 1951. Micro-Kjeldahl method for biologicals. Journal of the American Pharmaceutical Association (Scientific ed.) 40(3): 151-153.

Korhonen, H., Pihlanto-Leppäla, A., Rantamäki, P. and Tupasela, T. 1998. Impact of processing on bioactive proteins and peptides. Trends in Food Science and Technology 9(8-9): 307-319.

Lee, B.-H., Lai, Y.-S. and Wu, S.-C. 2015. Antioxidation, angiotensin converting enzyme inhibition activity, nattokinase, and antihypertension of *Bacillus subtilis* (natto)-fermented pigeon pea. Journal of Food and Drug Analysis 23(4): 750-757.

López-Fandiño, R., Otte, J. and van Camp J. 2006. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. International Dairy Journal 16(11): 1277-1293.

Ma, F.-F., Wang, H., Wei, C.-K., Thakur, K., Wei, Z.-J. and Jiang, L. 2019. Three novel ace inhibitory peptides isolated from Gingko biloba seeds: purification, inhibitory kinetic and mechanism. Frontiers in Pharmacology 9: article ID 1579.

Malaguti, M., Dinelli, G., Leoncini, E., Bregola, V., Bosi, S., Cicero, A. F. and Hrelia, S. 2014. Bioactive peptides in cereals and legumes: agronomical, biochemical and clinical aspects. International Journal of Molecular Sciences 15: 21120-21135.

Matthews, C. K. and van Holde, K. E. 1996. Biochemistry. 2nd ed. California: Benjamin Cumming Publishing Company.

Minekus, M., Alminger, M., Alvito, P., Balance, S., Bohn, T., Bourlieu, C., … and Brodkorb, A. 2014. A standardised static *in vitro* digestion method suitable for food - an international consensus. Food and Function 5(6): 1113-1124.

Mirzaei, M., Mirdamadi, S., Ehsani, M. R. and Aminlari, M. 2018. Production of antioxidant and ACE-inhibitory peptides from *Kluyveromyces marxianus* protein hydrolysates: purification and molecular docking. Journal of Food and Drug Analysis 26(2): 696-705.

Natesh, R., Schwager, S. L., Sturrock, E. D. and Achraya, K. R. 2003. Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. Nature 421: 551-554.

Olagunju, A. I., Omoba, O. S., Enujiugha, V. N., Alashi, A. M. and Aluko, R. E. 2018. Antioxidant properties, ACE/renin inhibitory activities of pigeon pea hydrolysates and effects on systolic blood pressure of spontaneously hypertensive rats. Food Science and Nutrition 6(7): 1879-1889.

Pihlanto, A. and Mäkinen, S. 2013. Antihypertensive properties of plant protein derived peptides. In Hernández-Ledesma, B. and Hsieh, C.-C. (Eds). Bioactive Food Peptides in Health and Disease. United Kingdom: InTech Open.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F. and Stanton, C. 2011. Bioactive peptide from muscle sources: meat and fish. Nutrients 3(9): 765-791.

Salunkhe, D. K., Chavan, J. K., Kadam, S. S. and Reddy, N. R. 1986. Pigeon pea as an important food source. C R C Critical Reviews in Food Science and Nutrition 23(2): 103-145.

Sbroggio, M. F., Montilha, M. S., Figueiredo, V. R. G. D., Georgetti, S. R. and Kurozawa, L. E. 2016. Influence of the degree of hydrolysis and type of enzyme on antioxidant activity of okara protein hydrolysates. Food Science and Technology 36(2): 375-381.

Singh, U. and Jambunathan. R. 1982. Distribution of seed protein fractions and amino acids in different anatomical parts of chickpea (*Cicer arietinum* L.) and pigeon pea (*Cajanus cajan* L.). Plant Foods for Human Nutrition 31(4): 347-354.

Siow, H.-L. and Gan, C.-Y. 2013. Extraction of antioxidative and antihypertensive bioactive peptide from *Parkia speciosa* seeds. Food Chemistry 141(4): 3435-3442.

Vermeirssen, V., Augustijns, P., Morel, N., van Camp, J., Opsomer, A. and Verstraete, W. 2005. *In vitro* intestinal transport and antihypertensive activity of ACE inhibitory pea and whey digests. International Journal of Food Sciences and Nutrition 56(6): 415-430.

Villanueva, B. A., Vioque, J. Sánchez-Vioque, S., Clemente, A., Bautista, J. and Millán, F. 1999. Production of an extensive sunflower protein hydrolysate by sequential hydrolysis with endo- and exo-proteases. Grasas y Aceites 50(6): 472-476.

Waters Corporation. 2008. ACCQ fluorescence reagent kit (care and use manual). Retrieved from website: https://www.waters.com/webassets/cms/support/docs/wat0052881.pdf

Wouters, A. G. B., Rombouts, I., Fierens, E., Brijs, K. and Delcour, J. A. 2016. Relevance of the functional properties of enzymatic plant protein hydrolysates in food systems. Comprehensive Reviews in Food Science and Food Safety 15(4): 786-800.

Zheng, X., Li, D. S. and Ding, K. 2017. Purification and identification of angiotensin I-converting enzyme inhibitory peptides from fermented walnut residues. International Journal of Food Properties 20(Suppl. 3): S3326-S3333.