Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

https://doi.org/10.17113/ftb.61.01.23.7635 original scientific paper

Jack Bean (*Canavalia ensiformis*) Tempehh: ACE-Inhibitory Peptide Formation during Absorption in the Small Intestine

Running title: Absorption of Jack Bean Peptides

Endah Puspitojati[1*], Muhammad Nur Cahyanto[2], Yustinus Marsono[2] and Retno Indrati[2]

[1]Polytechnic of Agricultural Development of Yogyakarta-Magelang, Jl. Kusumanegara, 55167 Umbulharjo, Yogyakarta, Indonesia
[2]Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Jl. Flora, 55281 Bulaksumur, Yogyakarta, Indonesia

Received: 18 May 2022
Accepted: 15 December 2022

SUMMARY

Research background. High blood pressure is the most significant cause of mortality globally. Some fermented foods include ACE inhibitor peptides that help fight this disease. The ability of fermented jack bean (tempehh) to inhibit ACE during consumption has not been demonstrated. This study identified and characterised ACE inhibitor peptides from jack bean tempehh produced by small intestine absorption using the everted intestinal sacs model.

Experimental approach. Sequentially, the protein extract of jack bean tempehh and unfermented jack bean was hydrolysed using pepsin-pancreatin for 240 min. The hydrolysed samples were then evaluated for the peptide absorption using three-segmented everted intestinal sacs (duodenum, jejunum, and ileum). The peptides absorbed from all intestine segments were mixed as the mixtures of peptides absorbed in the small intestine.

*Corresponding author:
Phone: +6289634548525
Fax: +62274375528
E-mail: endahpuspitojati2802@gmail.com

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Results and conclusion. The data showed that both jack bean tempehh and unfermented jack bean possessed the same peptide absorption pattern, with the highest percentage of peptide absorption in the jejunum, followed by the duodenum and ileum. The absorbed peptides of jack bean tempeh exhibited the same strong ACE inhibition activity in all intestine segments, while the unfermented jack bean showed strong activity only in the jejunum. The mixture of peptides that absorbed in the small intestine from jack bean tempeh possessed higher ACE inhibitory activity (81.09 %) than the unfermented jack bean (72.22 %). The peptides produced from jack bean tempeh were identified as pro-drug ACE inhibitors and possessed the mixed inhibition pattern. The mixture peptides consisted of seven types of the peptide with a molecular weight of 826,86 – 978,20 Da (DLGKAPIN, GKGRFVYG, PFMRWR, DKDHAEI, LAHLYEPS, KIKHPEVK, and LLRDTCK).

Novelty and scientific contribution. This study discovered that consuming jack bean tempeh generated more potent ACE inhibitory peptides during small intestine absorption than boiling jack beans. Absorbed tempeh peptides provide seven peptides with high ACE inhibitory action.

Keywords: ACE; ACE inhibitory peptides; small intestine absorption; Jack bean tempeh; inhibition pattern

INTRODUCTION

High blood pressure is familiar as the silent killer due to causing strokes, coronary infarction, mental degeneration, and premature death (*1*). The angiotensin I converting enzyme (ACE) influences blood pressure regulation in the renin-angiotensin system (RAS). This enzyme converted non-active angiotensin I to active angiotensin II (strong vasopressor) and inhibited the catalytic action of bradykinin (vasodilators), causing artery constriction and elevated blood pressure (*2*). Nowadays, studies about ACE inhibition peptides have been extensively reported. These peptides can prevent ACE capacities in converting angiotensin I to angiotensin II (*2*). Based on the changes of the inhibitory action, the ACE inhibition peptides were categorised into three types, (i) true-inhibitor peptides whose inhibitory activity is stable during digestion, (ii) substrate-type peptides which possessed weak inhibitory activity, and (iii) pro-drug peptides, which can be converted to the true–inhibitors peptides by ACE or digestive tracks proteases (*3*). Moreover, only true-inhibitor or pro-drug peptides could lower systolic blood pressure in hypertension rats (*3*).

Recently, several studies on food-derived ACE inhibition peptides have shifted to many sources of plant protein (*4–6*) which are the sources of protein chosen by vegetarians (*1*). Jack bean (*Canavalia ensiformis*) is a huge-seeded legume that has been hardly used as a food source. The seeds have great potential for food sources since they have rich in protein and have an adequate

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

amino acid composition (*7*). The investigations on jack bean as a possible source of ACE inhibitor peptide have been declared (*8,9*). The fermentation of jack bean using *Rhizopus oligosporus* for 72 h demonstrated the production of peptides with high capacities to inhibit ACE (*9*). Moreover, our previous study revealed that the action of ACE inhibition of both unfermented and fermented jack bean persists after digestion simulation using pepsin pancreatin (*10*).

The fate of food-derived ACE inhibitor peptides is the fundamental issue after passing through the gastrointestinal system in the human body. The peptides with significant ACE inhibitory capacity *in vitro* did not always have the same biological activity in vivo (*11*). The ACE inhibition peptides must be highly stable to maintain their bioactivity until they reach the bloodstream (*12*). Absorption from the digestive tract is critical in allowing ACE inhibitory peptides to enter the bloodstream and exert their bioactivity (*13*). Study in mice has shown that short peptides, the protein digestion products, were more quickly absorbed in the proximal segment of the small intestine (lower duodenum and upper jejunum). In contrast, some amino acids were more quickly absorbed in the end part of ileum (*14*). Studies about the absorption of ACE inhibitory peptides have been previously recorded on koro kratok (*Phaseolus lunatus*) (*6*), koro benguk (*Mucuna pruriens*) (*15*), casein (*16*), and pigeon pea (*Cajanus cajan*) (*17*).

Most previous research has been executed to understand the fate of ACE inhibition peptide activity after absorption using an everted intestinal model. In contrast, the identification of the produced peptides was not conducted. The research aimed to characterise ACE inhibitor peptides isolated from jack bean tempeh absorption using the everted intestinal sacs model, including their classification, inhibitory pattern, molecular weight, and amino acid sequence.

MATERIALS AND METHODS

Materials

Jack bean (*Canavalia ensiformis*) were collected from Yogyakarta (Indonesia), tempeh inoculum containing 10^6 CFU/g of *R. oligosporus* was obtained from a local market, pepsin (EC.3.4.23.1), pancreatin (EC.232-468-9), ACE (EC.3.4.15.1), hippuryl-L-histidyl-L-leucine (HHL) were procured from Sigma-Aldrich (St. Louis, Missouri, USA), *O*-phthaldialdehyde (OPA) was obtained from Merck (Kenilworth, New Jersey, USA), The male *Sprague-Dawley* rats were provided by The Study Center of Food and Nutrition (Universitas Gadjah Mada, Yogyakarta, Indonesia).

Samples preparation

Jack bean tempeh was prepared according to the method defined by Puspitojati *et al.* (*9*). The seeds were cleaned, steeped for 24 h in water, and then cooked for 30 min. After peeling and slicing,

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

the seeds were resoaked in tap water for 48 h. Soaking water was changed every 12 h. The water from boiling chopped seeds for 30 min was discarded. After draining, the seeds were cooled (30 °C).The cold seeds were inoculated with 0.02 % tempeh inoculums, equally mixed, covered in banana leaves, and fermented for 72 h (JF72). The unfermented jack bean was used as control (JF0). The resulting product was lyophilised. Both samples were hydrolysed using pepsin (EC.3.4.23.1, $2\cdot10^6$ mU/mL) and pancreatin (EC.232-468-9, $1\cdot10^5$ mU/mL) sequentially at 37 °C for 240 min (*10*). The simulation of digestion was performed by Minekus *et al.* with a slight alteration (*18*). The reaction process was ended by soaking the solution in 100 °C water for 15 min. The samples were then cooled and centrifuged at 8 000×*g* and 4 °C for 15 min. The supernatants were taken, lyophilised, and stored at −20 °C for further investigation. Each experiment had three replications.

Preparation of the everted intestinal sacs

Peptide absorption was carried out by the method of Amenta *et al.* (*19*). The evaluation used *Sprague-Dawley* male rats (11-12 weeks, ±250 g). The rats were fasted for 20-24 h and fed ad libitum water, then anaesthetised by intramuscular injection using ketamine (180 mg/kg). The experimental animals were placed on the operating table in the supine position. The incision began at the abdomen and proceeded to the left and right sides, cutting the skin and muscle. The incision continued toward the cranial and cut the costae to open the thoracic cavity. Furthermore, the intestinal organ was taken and divided into three segments. The duodenum was a U-form section of the intestine located close to the pancreas. The ileum was removed 1 cm from the cecum as the distal part of the small intestine, while the jejunum was segmented between the duodenum and the ileum. The inversion of small intestines was conducted as soon as possible and carried out in 0.9 % of sodium chloride. The animal experiment was performed according to the ethics committee approval Ref: KE/FK/1384/EC/2018 (Medical and Health Research Ethics Committee (MHREC), Dr. Sardjito General Hospital, Yogyakarta, Indonesia).

Absorption evaluation

The end of every segment of the small intestine was tied and filled with 1 mL of 0.9 % sodium chloride. The peptide samples were put in the tubes. Each small intestinal segment was put into a tube containing a peptide sample. The small intestine must be immersed in a sample-containing mucosal fluid, agitated constantly, and oxygenated at 100 bubbles/min. The experiment was performed at 37 °C for 120 min. The sample inside each everted sac was taken and centrifuged for 15 min at 13 500×*g* and 4 °C. The supernatant obtained from every segment of the small intestine was assayed for ACE inhibitory action and peptide content. The duodenum, jejunum, and ileum

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

supernatant were then mixed for further analysis as the mixture peptides were absorbed in the small intestine.

Assay of ACE inhibition

The ACE inhibition activity of the absorbed peptides was assayed by a bit of alteration of the Cushman and Cheung method using 8 mmol/L of Hip-His-Leu as the substrate and 25 mU/mL of ACE solution (*20*). The mixture was mixed for 120 s before being cold centrifuged for 15 min at 4000×*g*. The transparent upper layer was collected in a 1 mL amount and dried. 3 mL of distilled water was used to re-dissolve the residue. The samples were then read at 228 nm using a spectrometer UV-VIS (Dynamica Scientific Halo SB-10).

The following equation was used to calculate ACE inhibition activity:

$$ACE\ Inhibition\ Activity\ (\%) = \frac{A-I}{A-B} \times\ 100\% \qquad /1/$$

where A is the absorbance in the presence of ACE, B is the absorbance in the presence of both ACE and the peptide, and C is the absorbance of the reaction blank.

Assay of peptide content

OPA spectrophotometric assay was used for the determination of peptide content (*22*). A mixture of 12.5 mL of 100 mmol/L sodium tetraborate, 1250 µL of 20 % sodium dodecyl sulphate, and 550 µL of OPA reagent was made. For the OPA-based analysis, 1 mL of OPA and 20 µL of the hydrolysate were mixed together. The mixture was then quickly turned around and left to sit for 120 s in the dark. The sample and OPA reagents' absorbance mixture was evaluated at 340 nm using a UV-VIS spectrophotometer (Dynamica Scientific, Livingston, United Kingdom). This assay used tryptone as a standard curve.

Assay of classification of ACE inhibition peptides

50 µL of the absorbed peptide sample solution was added to 50 µL of ACE solution (25 mU/mL). The mixture was incubated at 37 °C for 4 h and then added 50 µL of HHL solution to the mixtures. The samples were then incubated for 30 min. The next step followed the ACE inhibition activity analysis procedure (*20*).

The determination of the classification of peptides was according to the difference in ACE inhibition activity before and after ACE incubation. The classification of peptides was termed as a true-inhibitor peptide, for those peptides with no significant difference in ACE inhibition activity before and after ACE incubation; substrate peptides for those peptides with a decrease of ACE inhibition

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

activity after ACE incubation; and pro-drug peptides, for those peptides with an increase of ACE inhibition activity after ACE incubation (*3*).

Evaluation of ACE inhibition pattern

The pattern of ACE inhibition was evaluated using the Lineweaver-Burk plot (*23*). The experiment condition was the same as the calculation of ACE inhibition action. The activity of the enzyme was calculated using various concentrations of HHL (4, 8, and 12 mM) and inhibitor/peptide concentration (0, 0.25, 0.5 mg/mL). The type of inhibition was determined by data analysis using the Lineweaver-Burk method to obtain the Michaelis-Menten kinetics constant (K_m). It was calculated based on the regression equation:

$$y=a+bx \quad /2/$$

where x was presented as the reciprocal of substrate concentration [1/S] and y was presented as the reciprocal of velocity [1/V].

Determination of amino acid sequences

Amino acid sequences of absorbed peptides were evaluated using RSLC nano UPLC (Dionex™ Ultimate 3000, Thermo Scientific, MA, USA) coupled with HR-MS (Q Exactive^{TM}, Thermo Scientific). The lyophilised-absorbed peptide was dissolved in 0.1 % trifluoroacetic acid (TFA) and adjusted to the final pH of solution <4. Desalting of peptides was conducted using reversed-phase ZipTip C18 containing resin (Millipore, Sigma Aldrich). ZipTip C18 was placed on a 10 µL micropipette, then moistened using a wetting solution (0.1 % TFA in 50 % acetonitrile). The wetting solution was aspirated slowly and then discarded slowly. This procedure was repeated three times. Furthermore, ZipTip C18 was equilibrated by aspirating and discarding the equilibration solution (0.1 % TFA in water injection) slowly three times. The next step was the binding of the peptide in ZipTip C18. The peptide solution was slowly aspirated and then slowly removed using the same vial. This procedure was conducted repeatedly so that more peptides were bound to the resin. ZipTip C18 was then washed using a washing solution (0.1 % TFA in water injection) followed by peptides elution (0.1 % TFA in 50 % acetonitrile). The eluted peptides were then injected into the instrument. The mobile phase used in this study was nano pump A (0.01 % of formic acid in distilled water); nano pump B (0.1 % of formic acid in 80 % of acetonitrile); and loading pump (0.1 % of formic acid in distilled water). The analytical column used EASY-Spray column, 15 cm×75 µm ID, PepMap C18. The data obtained were then identified using Thermo Scientific Proteome Discoverer 2.2 software based on the Sequest HT database (*24*).

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Statistical analysis

The data were analysed using SPSS IBM 23 (IBM, Armonk, New York, USA) (*25*). One-way ANOVA with a 5 % significant difference was used to examine the data. Duncan's multiple range tests assessed treatment mean differences. The T-test was performed to compare the data classification of ACE inhibitory peptides.

RESULTS AND DISCUSSION

The absorption of peptides in small intestine's segments

The hydrolyzates of unfermented jack bean (JF0) and tempeh fermented for 72 h (JF72) were investigated on the absorption ability of peptides in the small intestine. L–leucine was used as the control in this study. The data demonstrated that the percentage of peptide absorption has a significant difference in each intestinal segment in all samples ($p<0.05$). Fig. 1 describes that JF0 and JF72 have the same peptide absorption pattern, with the highest proportion of jejunum, followed by the duodenum and ileum. In comparison, L-leucine possessed the highest absorption percentage in the ileum, with a value of 64.64 %.

The difference in absorption capacities of peptide samples was speculated due to the different characteristics among duodenum, jejunum, and ileum. The jejunum was reported to have a thicker mucosal layer with a larger diameter of 200 million microvilli every 1 mm^2 to increase the surface area of nutrient absorption by 14-40 folds (*26*). These results are also supported by Antunes *et al.* that the absorption of amino acids as short peptides was faster than free amino acids (*27*). Studies in mice showed that short peptides of protein digestion products were more quickly absorbed in the lower duodenum and upper jejunum. In contrast, some amino acids are more quickly absorbed in the ileum (*14,27*).

The percentage of absorbed peptides of JF0 and JF72 in the duodenum did not demonstrate significant differences ($p>0.05$), but it was significantly different in the jejunum ($p<0.05$) and ileum ($p<0.05$). JF72 exhibited a higher percentage of absorption compared to JF0 in jejunum and ileum. This condition was probably due to mould involvement during the tempeh fermentation of JF72, which exhibited proteolytic activity that could degrade the jack bean protein into shorter peptides and amino acids. Further, fermentation also increased protein solubility (*28*), suggesting that small intestine proteases were easier to break down the substrate to release short peptides and amino acids. Furthermore, JF2 can be absorbed greater than JF0. Previous studies reported that the fermentation process could improve the digestibility level of legume proteins by eliminating anti-nutrient components that can inhibit the activity of digestive enzymes (*29*). Preliminary studies have shown that JF72 contains more peptides of less than 3.5 kDa than JF0. This condition probably would affect

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

the action of the peptidase enzymes in the brush border membrane. These enzymes might digest the high molecular weight peptides slower than smaller peptides that consist of 6-20 amino acids (*26*).

ACE inhibition activity of the absorbed peptides

Fig. 2 presents the ACE inhibitory activity from the tempeh peptides that absorbed by small intestine. JF0 and JF72 showed significantly different ACE inhibitory activity in the duodenum, ileum, and small intestine. The absorbed peptides in both samples exhibited strong ACE inhibitory activity. This phenomenon indicated that not all peptides that pass through the brush border membrane were hydrolysed into amino acids. This condition was speculated that there were still short peptides that could be absorbed in the intact form. Some studies stated that di-, tri-, and tetra-peptides resistant to hydrolysis of cytoplasmic peptidase could be transported intact to blood circulation (*26*). Fan *et al.* (*29*) added that peptides composed of less than six amino acids could pass through enterocytes with decreased absorption ability with increasing chain length.

Peptide transport systems were strongly influenced by peptide structure, including chain length, hydrophobicity, and charge. Both unfermented and fermented jack beans contained a high concentration of hydrophobic amino acids, including phenylalanine, leucine, proline, and isoleucine (*10*). In addition, positively charged amino acids, including arginine and lysine, were found in jack beans. Hydrophobic amino acid residues were reported can strengthen interactions between peptides and dependent peptide transporters (PepT1) (*30,31*). Short peptides containing arginine amino acid residues can be transported intact through paracellular diffusion via tight junctions (TJs) (*32*). The resulting peptides produced by the intestinal sacs model were speculated to have arginine residue due to the high arginine concentration in the jack bean protein parent.

The best ACE inhibition activity of JF0 was obtained in the jejunum with a value of 79.80 %. At the same time, JF72 showed having the same strong ACE inhibition activity in all segments of the small intestine ($p>0.05$). This condition was thought to be caused by differences in the composition of amino acid composition from the two samples' raw materials, which caused the difference in the rate of hydrolysis of peptides in the brush border membrane.

Our previous study showed that JF0 possessed higher leucine, aspartic acid, and glutamate acid than JF72 (*10*). The condition probably affected aminopeptidase A, aminopeptidase N, and carboxypeptidase G activity in the brush border membrane. Aminopeptidase A was reported that have the specificity to cleave amino acid residues of glutamate acid at the N terminal, whereas aminopeptidase N cleaved alanine residues at the same terminal (*33*). Moreover, it was also described that carboxypeptidase G was located in the brush border membrane, which had the specificity of cleaving the γ-glutamyl bond at the C terminal. Glutamate carboxypeptidase II in the

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

brush border membrane played a role in the hydrolysis of Asp-Glu and Glu-Glu peptide bonds in the C terminal (*34*). Some endopeptidase enzymes such as meprin A and meprin B also played a role in the hydrolysis of peptides with a specificity of extensive cleaving of hydrophobic amino acid residues (*33,34*). The presence of these enzymes was speculated to cause boiled jack bean (unfermented jack bean) to undergo hydrolysis more quickly in the jejunum and produce short peptides, which among them have stronger ACE inhibition capacity than other segments of the small intestine

The classification of ACE inhibition peptides of absorbed peptides

Fig. 3 demonstrates that the absorbed peptide (the mixture of peptides solution from duodenum, jejunum, and ileum) promoted a significant increase in ACE inhibition activity after ACE incubation ($p<0.05$). As a result of these findings, the peptide might be classed as a pro-drug inhibitor peptide (*3*). The increase in ACE inhibition activity in peptides that have been pre-incubated by ACE was speculated caused by two possibilities.

The first possibility was that the short peptides that have residues of amino acid corresponding to the active site of ACE would interact with this enzyme. The length of incubation time resulted in more peptides that have the opportunity to interact with ACE. This condition would result in a higher ACE inhibitory activity. The second possibility is associated with the broad specificity of ACE, which cleaved the dipeptides His-Leu, Tyr-Asp / Glu, and Phe-Leu at the C terminal (*1*). The absorbed peptides derived from jack bean tempeh in this study were a mixture of peptides with different chain lengths and amino acid sequences. Some of them might consist of amino acid sequences that fulfil the specificity of ACE cleaving, and hence ACE could hydrolyse the peptides to produce shorter peptides and amino acids. These peptides probably had an excellent affinity to the active site of ACE so the peptide can ultimately inhibit the activity of ACE.

ACE inhibitory pattern

The Lineweaver-Burk plot of the ACE inhibition pattern of absorbed peptides is presented in Fig. 4. This figure indicated that the absorbed peptide has a pattern close to uncompetitive inhibition because no intersection points were found on either the x or y-axis. However, when seen from the v_{max} and K_m values in the Lineweaver-Burk plot, it demonstrated that the presence of tempeh peptides as ACE inhibitors could reduce v_{max} values from 35.59 µmol/min to 14.68 µmol/min and increase K_m values from 0.49 to 1.37 (Table 1). The decreasing v_{max} and increasing K_m indicated that the tempeh peptide had a mixed inhibition mechanism (*35*). The pattern of mixed inhibition was indicated by differences in the small intestine peptide structure. JF72 peptides absorbed in the small intestine still consisted of a mixture of peptides with different characteristics. Peptides that acted as ACE inhibitors

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

also exhibited other inhibitory characteristics due to their affinity for enzymes.

In a mixed inhibition system, the inhibitor does not have the same structure as the substrate but can be bound to free enzymes or the substrate enzyme complex. The mixed inhibition pattern could indicate that the peptide could be attached to the active or non-active site to reduce the catalytic activity of ACE (*36*). In previous studies, several peptides have been reported to have mixed inhibitory patterns, such as peptide EPNGLLLPQY from walnut protein (*36*) and peptides KAQYPYV, KIIIYN, and KILIYG from coconut (*37*).

Amino acid sequences of the absorbed peptides

The identification of peptides using Thermo Scientific™ Proteome Discoverer 2.2 software demonstrated that absorbed peptides consisted of seven types of peptides with molecular mass of 826.86–978.20 Da, with amino acid sequences DLGKAPIN, GKGRFVYG, PFMRWR, DKDHAEI, LAHLYEPS, KIKHPEVK, and LLRDTCK. Surprisingly, two of the seven peptides produced were fragments of the Cow Pea Severe Mosaic Virus (CPSMV) polyprotein RNA1. CPSMV was most commonly found in cowpea, but Lima *et al.* reported that the virus was also isolated from jack bean (*38*). All peptides produced were tested for toxicity prediction using a toxin prediction program (http://crdd.osdd.net/raghava/toxinpred/index.html), which revealed that all peptides (HF72) absorbed by the small intestine were non-toxic.

Table 2 shows that the peptides absorbed by the small intestine were short peptides consisting of 6-8 amino acids. This latest study was supported by Fernandes-Musoles *et al.*, who stated that only short peptides resistant to brush border membrane peptidase could be transported intact into the bloodstream (*32*). Specific peptides containing 5-9 amino acids, such as RPPGFSPFR, YAEER, KPVAAP, and VLPVPQK, have also been shown to be resistant to brush peptidase hydrolysis and capable of being carried intact across monolayers of Caco-2 cell (*30,39,40*).

The peptide mixture absorbed by the small intestine showed ACE inhibition activity of 81.09 %. According to BIOPEP analysis on the potential profile of its biological activity, all peptides absorbed have the potential as precursors for ACE inhibitor peptides. According to the A score, peptides GKGRFVYG, LAHLYEPS, and DLGKPIN were speculated to have a substantial role in the strong ACE inhibition in the peptide mixtures. RF, HL, and VY were identified as potential ACE inhibitors by molecular docking analysis of jack bean protein (*8*). The three peptide fragments were found in the GKGRFVYG and LAHLYEPS peptides in this study. In the peptide absorption test using the everted intestinal method, peptides GKGRFVYG and LAHLYEPS are proven to be resistant to the hydrolysis of peptidase. The small intestine absorbs them in intact form.

CONCLUSIONS

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

In an everted intestinal sacs model, both unfermented jack bean (JF0) and tempeh (JF72) retained ACE inhibitory activity after intestinal absorption. In this study, the peptides of both samples were appropriately absorbed in the small intestine, especially in the jejunum. The jack bean tempeh released seven peptides (DLGKAPIN, GKGRFVYG, PFMRWR, DKDHAEI, LAHLYEPS KIKHPEVK, and LLRDTCK). The mixture peptides exhibited a mixed inhibition pattern with ACE inhibitory activity of 81.09 %. In addition, the released peptides were classified as pro-drug inhibitory peptides.

ACKNOWLEDGEMENTS

The author would like to thank the technician of The Study Center of Food and Nutrition (Universitas Gadjah Mada, Yogyakarta, Indonesia), who provided experimental animals for the absorption evaluation of the peptide.

FUNDING

Indonesia Endowment Fund for Education funded this research, the Ministry of Finance of the Republic of Indonesia through the doctoral program of the Indonesia Education Scholarship (Batch PK.42).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

E. Puspitojati contributed to the experimental design, the concept of the manuscript, and data analysis. M.N. Cahyanto and Y. Marsono gave a constructive discussion. R. Indrati contributed as a supervisor and finalised the manuscript.

ORCID ID

E. Puspitojati https://orcid.org/0000-0002-0719-0674

M.N. Cahyanto https://orcid.org/0000-0002-1448-876X

Y. Marsono https://orcid.org/0000-0002-4346-4679

R. Indrati https://orcid.org/0000-0002-2809-5887

REFERENCES

1. Hernández-Ledesma B, Del Mar Contreras M, Recio I. Antihypertensive peptides: Production, bioavailability and incorporation into foods. Adv Colloid Interface Sci. 2011;165(1):23–35.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

https://doi.org/10.1016/j.cis.2010.11.001

2. Lee SY, Hur SJ. Antihypertensive peptides from animal products, marine organisms, and plants. Food Chem. 2017;228:506–17. https://doi.org/10.1016/j.foodchem.2017.02.039

3. Fujita H, Yokohama K, Yoshikawa M. Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. J Food Sci. 2000;65(4):564–69. https://doi.org/10.1111/j.1365-2621.2000.tb16049.x

4. Pinciroli M, Aphalo P, Nardo AE, Añón MC, Quiroga AV. Broken rice as a potential functional ingredient with inhibitory activity of renin and angiotensin-converting enzyme (ACE). 2019;405–13.

5. Ari M, Nadia S, Shyan C, Ebrahimpour A. High angiotensin-I converting enzyme (ACE) inhibitory activity of Alcalase-digested green soybean (*Glycine max*) hydrolysates. Food Res.Int. 2018;106(2018):589–97. https://doi.org/10.1016/j.foodres.2018.01.030

6. Pertiwi MGP, Marsono Y, Indrati R. *In vitro* gastrointestinal simulation of tempeh prepared from koro kratok (*Phaseolus lunatus* L.) as an angiotensin-converting enzyme inhibitor. J Food Sci Technol. 2020;57(5):1847–55. https://doi.org/10.1007/s13197-019-04219-1

7. Sridhar KR, Seena S. Nutritional and antinutritional significance of four unconventional legumes of the genus *Canavalia* - A comparative study. Food Chem. 2006;99(2):267–88. https://doi.org/10.1016/j.foodchem.2005.07.049

8. Harvian ZA. *In silico* proteolysis and molecular docking for the evaluation of jack bean (*Canavalia ensiformis*) protein as precursors of bioactive peptides with dual dipeptidyl peptidase-4 and angiotensin-I-converting enzyme inhibitors. Universitas Gadjah Mada; 2019.

9. Puspitojati E, Cahyanto MN, Marsono Y, Indrati R. Production of angiotensin-I-converting enzyme (ACE) inhibitory peptides during the fermentation of jack bean (*Canavalia ensiformis*) tempeh. Pakistan J Nutr. 2019;18(5):464–470. https://doi.org/10.3923/pjn.2019.464-470

10. Puspitojati E, Cahyanto MN, Marsono Y, Indrati R. Changes in amino acid composition during fermentation and its effects on the inhibitory activity of angiotensin-I-converting enzyme of jack bean tempeh following *in vitro* gastrointestinal digestion. J Food Nutr Res. 2019;58(4):319–27.

11. Zeng M, Zhao Y, Liu Z, Dong S. ACE-inhibitory activities of marine proteins and peptides. Mar Proteins Pept Biol Act Appl. 2013;431–40.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

https://doi.org/10.1002/9781118375082.ch20

12. Vermeirssen V, Van Camp J, Verstraete W. Bioavailability of angiotensin I converting enzyme inhibitory peptides. Br J Nutr. 2004;92(3):357–66. https://doi.org/10.1079/BJN20041189

13. Chakrabarti S, Guha S, Majumder K. Food-derived bioactive peptides in human health: Challenges and opportunities. Nutrients. 2018;10(11):1738–55. https://doi.org/10.3390/nu10111738

14. Matthews DM, Adibi SA. Peptide absorption. Gastroenterology. 1976;71(1):151–61. https://doi.org/10.1016/s0016-5085(76)80117-5

15. Rizkaprilisa W, Marsono Y, Indrati R. Bioactive peptide tempeh made from *Mucuna pruriens* (l) dc as an inhibitor of angiotensin-I-converting enzyme (ACE) in a digestion simulation. Prev Nutr Food Sci. 2020;25(1):93–7.

16. Xue H, Han J, He B, Yi M, Liu X, Song H, *et al.* Bioactive peptide release and the absorption tracking of casein in the gastrointestinal digestion of rats. Food Funct. 2021;12(11):5157–70. https://doi.org/10.1039/d1fo00356a

17. Putra ID, Marsono Y, Indrati R. Effect of simulated gastrointestinal digestion of bioactive peptide from pigeon pea (*Cajanus cajan*) tempeh on angiotensin-I converting enzyme inhibitory activity. Nutr Food Sci. 2020;51(2):244–54 https://doi.org/10.1108/NFS-03-2020-0071

18. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, *et al.* A standardised static in vitro digestion method suitable for food – an international consensus. Food Funct. 2014;5(6):1113–24. https://doi.org/10.1039/C3FO60702J

19. Amenta F, Buccioni M, Ben DD, Lambertucci C, Navia AM, Ngouadjeu Ngnintedem MA, *et al.* *Ex-vivo* absorption study of lysine R-lipoate salt, a new pharmaceutical form of R-ALA. Eur J Pharm Sci. 2018;118(2018):200–07. https://doi.org/10.1016/j.ejps.2018.03.025

20. Cushman. D.W HSC. Spectrophotometric assay and properties of the angiotensin converting enzymes from rabbit lung. Biochem Pharmacol. 1971;20:1637–48.

21. Wenno MR, Suprayitno E, Aulanni'Am A, Hardoko. The physicochemical characteristics and angiotensin converting enzyme (ACE) inhibitory activity of skipjack tuna (K*atsuwonus pelamis*) "bakasang." J Teknol. 2016;78(2016):112–24

22. Church FC, Swaisgood HE, Porter DH, Cantignani GL. Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J Dairy Sci.

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

1983;66(6):1219–27.
https://doi.org/10.3168/jds.S0022-0302(83)81926-2

23. Rawendra RDS, Aisha, Chang CI, Aulanni'am, Chen HH, Huang TC, *et al.* A novel angiotensin converting enzyme inhibitory peptide derived from proteolytic digest of Chinese soft-shelled turtle egg white proteins. J Proteom. 2013;94(2013):359–69.
https://doi.org/10.1016/j.jprot.2013.10.006

24. Nawaz KAA, David SM, Murugesh E, Thandeeswaran M, Kiran KG, Mahendran R, *et al.* Identification and in silico characterisation of a novel peptide inhibitor of angiotensin converting enzyme from pigeon pea (*Cajanus cajan*). Phytomedicine. 2017;36(17):1–7.
https://doi.org/10.1016/j.phymed.2017.09.013

25. IBM Corp. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.2015

26. Picariello G, Ferranti P, Addeo F. Use of brush border membrane vesicles to simulate the human intestinal digestion. Food Res Int. 2016;88(Part B):327–35.
https://doi.org/10.1016/j.foodres.2015.11.002

27. Antunes F, Andrade F, Ferreira D, Nielsen HM, Sarmento B. Models to predict intestinal absorption of therapeutic peptides and proteins. Curr Drug Metab. 2013;14(1):4–20.
https://doi.org/10.2174/138920013804545160

28. Puspitojati E, Indrati R, Cahyanto MN, Marsono Y. Formation of ACE-inhibitory peptides during fermentation of jack bean tempeh inoculated by usar *Hibiscus tiliaceus* leaves starter. IOP Conf Ser Earth Environ Sci. 2019;292:012022.
https://doi.org/10.1088/1755-1315/292/1/012022

29. Çabuk B, Nosworthy MG, Stone AK, Korber DR, Tanaka T, House JD, *et al.* Effect of fermentation on the protein digestibility and levels of non-nutritive compounds of pea protein concentrate. Food Technol Biotechnol. 2018;56(2):257–64.
https://doi.org/10.17113/ftb.56.02.18.5450

30. Miguel M, Dávalos A, Manso MA, De La Peña G, Lasunción MA, López-Fandiño R. Transepithelial transport across Caco-2 cell monolayers of antihypertensive egg-derived peptides. PepT1-mediated flux of Tyr-Pro-Ile. Mol Nutr Food Res. 2008;52(12):1507–13.
https://doi.org/10.1002/mnfr.200700503

31. Gleeson JP, Brayden DJ, Ryan SM. Evaluation of PepT1 transport of food-derived antihypertensive peptides, Ile-Pro-Pro and Leu-Lys-Pro using *in vitro*, *ex vivo* and *in vivo* transport models. Eur J Pharm Biopharm. 2017;115:276–84.
https://doi.org/10.1016/j.ejpb.2017.03.007

32. Fernández-Musoles R, Salom JB, Castelló-Ruiz M, Contreras M del M, Recio I, Manzanares

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

P. Bioavailability of antihypertensive lactoferricin B-derived peptides: Transepithelial transport and resistance to intestinal and plasma peptidases. Int Dairy J. 2013;32(2):169–74. https://doi.org/10.1016/j.idairyj.2013.05.009

33. Donowitz M, Singh S, Salahuddin FF, Hogema BM, Chen Y, Gucek M, *et al.* Proteome of murine jejunal brush border membrane vesicles. J Proteome Res. 2007;6(10):4068–79. https://doi.org/10.1021/pr0701761

34. McConnell RE, Benesh AE, Mao S, Tabb DL, Tyska MJ. Proteomic analysis of the enterocyte brush border. Am J Physiol Liver Physiol. 2011;300(5): 914–26. https://doi.org/10.1152/ajpgi.00005.2011

35. Jahanbani R, Ghaffari M, Vahdati K, Salami M, Khalesi M, Sheibani N, *et al.* Kinetics Study of protein hydrolysis and inhibition of angiotensin converting enzyme by peptides hydrolysate extracted from walnut. Int J Pept Res Ther. 2017:1–9. https://doi.org/10.1007/s10989-017-9594-4

36. Wang C, Tu M, Wu D, Chen H, Chen C, Wang Z, *et al.* Identification of an ACE-inhibitory peptide from walnut protein and its evaluation of the inhibitory mechanism. Int J Mol Sci. 2018;19(4): 1155–65 https://doi.org/10.3390/ijms19041156

37. Zheng Y, Li Y, Li G. ACE-inhibitory and antioxidant peptides from coconut cake albumin hydrolysates: Purification, identification and synthesis. RSC Adv. 2019;9(11):5925–36. https://doi.org/10.1039/c8ra10269d

38. Booker HM, Umaharan P, McDavid CR. Effect of Cowpea severe mosaic virus on crop growth characteristics and yield of cowpea. Plant Dis. 2005;89(5):515–20. https://doi.org/10.1094/PD-89-0515

39. Gallego M, Grootaert C, Mora L, Aristoy MC, Van Camp J, Toldrá F. Transepithelial transport of dry-cured ham peptides with ACE inhibitory activity through a Caco-cell monolayer. J Funct Foods. 2016;21:388–95. https://doi.org/10.1016/j.jff.2015.11.046

40. Vij R, Reddi S, Kapila S, Kapila R. Transepithelial transport of milk derived bioactive peptide VLPVPQK. Food Chem. 2016;190:681–88. https://doi.org/10.1016/j.foodchem.2015.05.121

Table 1. v_{max}, K_m, and K_i of the absorbed peptides

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

γ(inhibitor)/(mg/mL)	A	B	v_{max}/(µmol/min)	K_m	K_i
0	0.0281	0.0138	35.587	0.491	-
0.256	0.0365	0.0353	27.397	-	0.967
0.512	0.0681	0.0932	14.684	-	1.368

v_{max}=represents the maximum reaction rate of ACE, K_m=Michaelis-Menten constant, A=1/v_{max}, B=K_m/v_{max}

Table 2. Amino acid sequences of absorbed peptides

Amino acid sequence	BM (Da)	Frequency (A)
DLGKAPIN	826.95	0.500
GKGRFVYG	883.02	0.750
PFMRWR	892.09	0.333
DKDHAEI	826.86	0.149
LAHLYEPS	929.04	0.625
KIKHPEVK	978.20	0.375
LLRDTCK	848.03	0.143

A=represents the frequency of bioactive fragments in a peptide

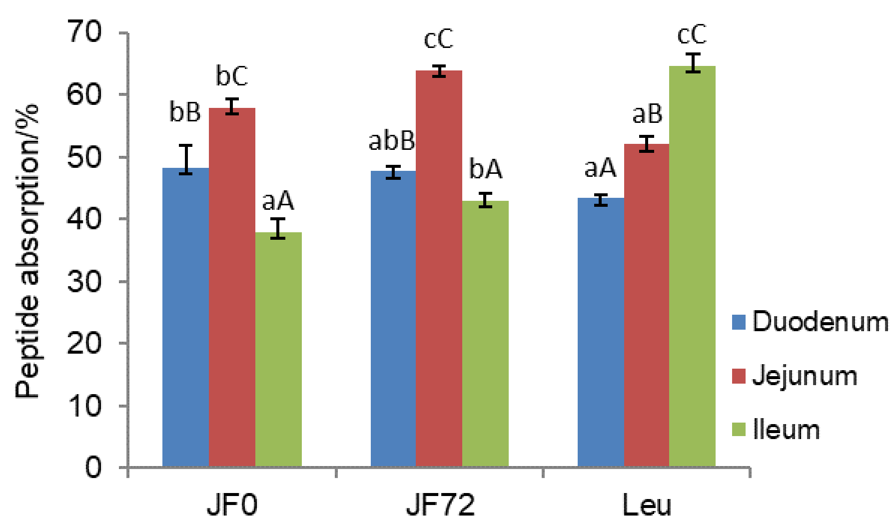

Fig. 1. Percentage of absorbed peptides in the small intestine. Mean values with small alphabet superscripts described significant differences in the same segments and different samples, while those with capital alphabet superscripts illustrated significant differences in the same samples and different segments ($p<0.05$). JF0=hydrolyzate of unfermented jack bean, JF72=hydrolyzate of jack bean tempeh

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

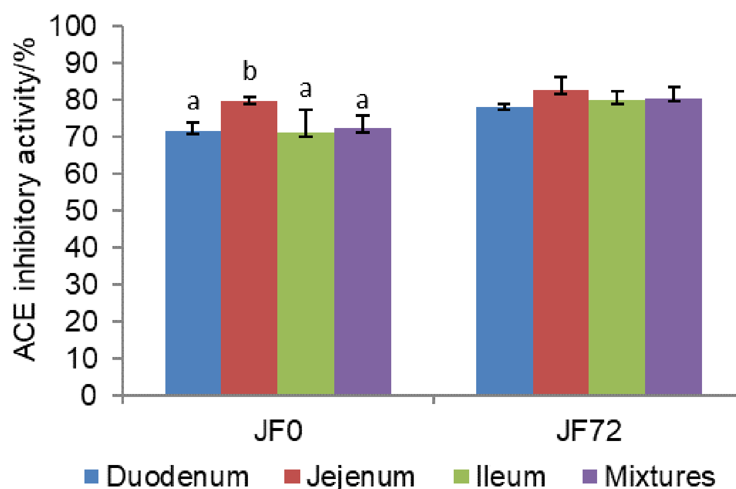

Fig. 2. ACE inhibition activity of the absorbed peptides. Mean values with different superscripts are significantly different ($p<0.05$). Mixtures=mixture of peptide solution from duodenum, jejunum, and ileum, JF0=hydrolyzate of unfermented jack bean, JF72=hydrolyzate of jack bean tempeh

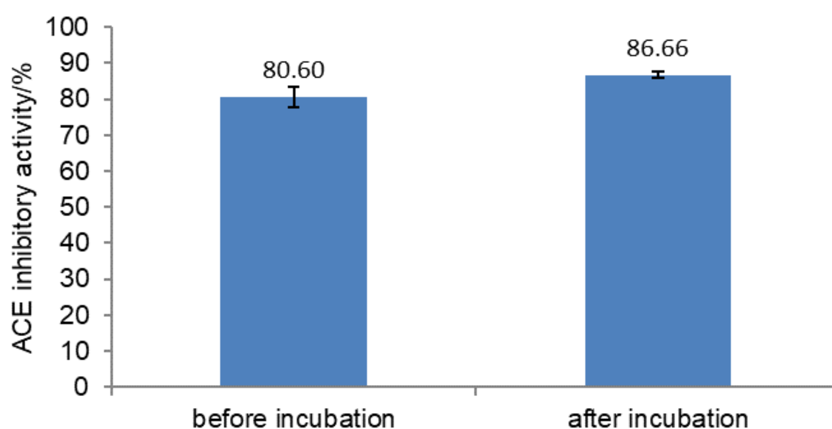

Fig. 3. Classification of ACE inhibitory peptides that are absorbed in the small intestine

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

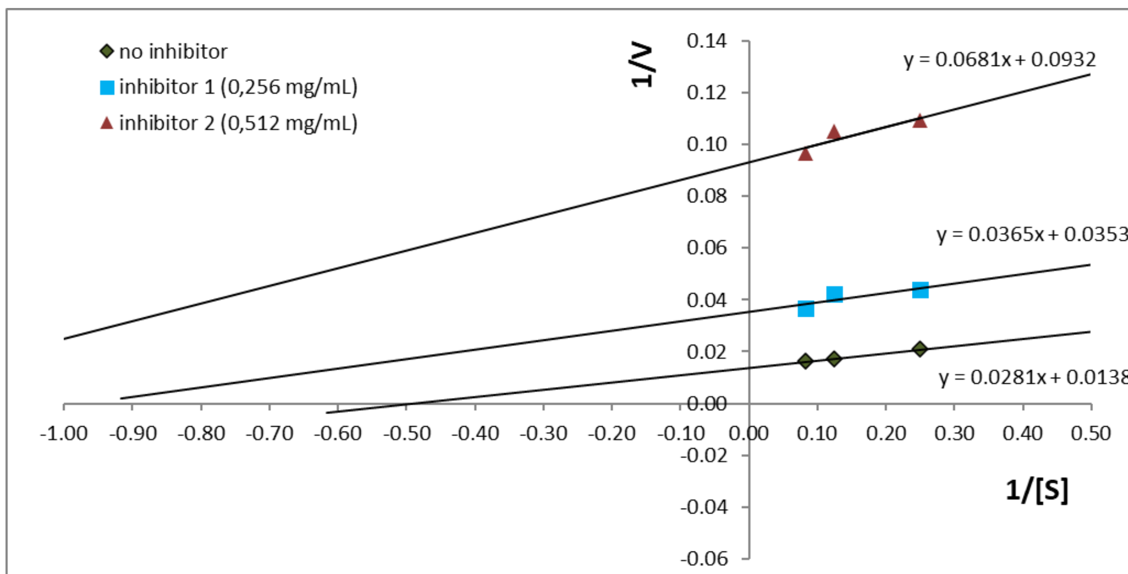

Fig. 4. ACE inhibitory pattern of the absorbed peptides