Jack bean as tempe ingredients: The safety study and fate of protein against gastrointestinal enzymes

E Puspitojati¹,², R Indrati²,³, M N Cahyanto² and Y Marsono²

¹Magelang Agricultural Extension College, Jl. Kusumanegara, 55167, Umbulharjo, Yogyakarta, Indonesia
²Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Jl. Flora, Bulaksumur, 55281 Yogyakarta, Indonesia

E-mail: endahpuspitojati@gmail.com

Abstract. This study evaluated the change of hydrogen cyanide (HCN) content and concanavalin A (Con A) activity during the processing of jack bean tempe. In addition, the fate of protein of cooked tempe following in vitro gastrointestinal digestion was also investigated. The jack bean tempe was prepared by the step of the soaking-boiling-soaking-boiling-fermentation process. The mature tempe was harvested on 48 h of fermentation and followed by the cooking process for 10 min (T10) and 20 min (T20). The results showed the soaking, soaking-boiling, soaking-boiling-soaking, and soaking-boiling-soaking-boiling were able to decrease the HCN content of jack bean as much as 3.98; 85.88; 92.88 and 97.95%, respectively. It did not significantly change during fermentation with content for mature tempe was as low as 0.71 ppm, which is categorized as a safe level according to FAO. The haemagglutination assay of Con A showed the protein extracted from raw jack bean provided red blood cell clotting, while it was not found in the protein extracted from boiled jack bean and jack bean tempe. In the in vitro digestion model, both T10 and T20 samples showed that there was no significant change in peptides content before and after hydrolysis using pepsin (p<0.05). The action of gastrointestinal enzymes improved the released of the bioactive peptides which have the ability to inhibit angiotensin I– converting enzyme (ACE). It can be concluded that the jack bean was the safe protein source for consumption as tempe. Furthermore, the protein of cooked tempe was highly hydrolyzed following in vitro gastrointestinal digestion produced bioactive peptides with high ACE inhibitory activity.

1. Introduction
Jack bean (Canavalia ensiformis) is one of under-exploited legume that can grow in various ecological conditions. The utilization of jack bean as a food source is still very limited due to anti-nutritional substances such as concanavalin A (Con A), canavanine, canatoxine, polyamine, protease inhibitors, flatulence compounds, cyanogenic glycosides, tannins, saponins, urease, L-Dopa [1,2]. Most of these substances give rise to dangerous biological responses, while some are widely applied in pharmacologically active nutrition and agents [3]. Con A is a single-cell protein which binds to the non-reducing terminal α-D-mannosyl and α-D-glucosyl groups of sugars, glycoproteins, and glycolipids. It can agglutinate red blood cells by interacting with immunoglobulin glycol-peptides [4,5]. HCN is a toxic component which causes death if consumption reaches a dose of 0.5-3.5 mg
HCN/kg weight. It could be reduced by soaking, heat treatment, and fermentation during food processing [6].

Jack bean is a promising source of vegetable protein due to its high protein content. In addition, the essential amino acids (isoleucine, leucine, and tyrosine) were higher than legumes in general [2]. One of the uses of jack bean in Indonesia is in the production of tempe, although it is still restricted in a certain region [7,8]. Most people are still hesitant to consume it because they are still worried about the toxicity. Even though, it has been reported that jack bean protein during tempe fermentation produced bioactive peptides which found to have ACE inhibitory activity [9].

The bioactive peptides are able to undergo physiological changes during the digestive tract which determines its biological activity. The peptides that are hydrolyzed by digestive enzymes can lose their biological activity or even increase activity due to the formation of active fractions of new peptides [10]. The gastrointestinal enzymes such as pepsin, chymotrypsin, and peptidase produce peptides in various length that define their biological activities including ACE inhibitor peptides. The fate of protein and stability of ACE inhibitory peptides during in vitro gastrointestinal digestion have been widely reported in pea seeds, whey protein, peanut, and grape skin [11-14].

There is little information about the fate of jack bean tempe protein during human digestion. The information about the degradation of the toxic component of the jack bean during tempe processing was also very limited. The objective of this study was to evaluate the safety of tempe made from jack bean in the context of HCN content and Con A activity. Furthermore, in this study, tempe samples were cooked at various time according to the way people consumed tempe. The fate of protein of cooked tempe following in vitro gastrointestinal digestion was also investigated in terms of the production of the bioactive peptides.

2. Materials and methods

2.1. Materials
Jack bean seeds (Canavalia ensiformis) and Raprima commercial inoculum were obtained from the local market. The human erythrocytes were provided by the Institute of Health Science, Yogyakarta, Indonesia. Angiotensin-I-converting enzyme from rabbit lung, HHL (Hippuryl-L-Histidyl-L-Leucine) from Sigma–Aldrich Company, O-phtaldialdehyde (OPA), KCN from Merck and Pepsin from porcine gastric mucosa (P7012), and Porcine pancreatin (P7545) were from Sigma–Aldrich Company. All other chemicals were of analytical grade.

2.2. Preparation of jack bean tempe
The making of jack bean tempe was adopted from the method of Puspitojati et al [9]. The inoculated seeds were wrapped by banana leaves and fermented at room temperatures (30°C) for 48 h. The matured tempe was cooked for 10 (T10) and 20 minutes (T20). The boiled jack bean was used as a control. Experimental samples were lyophilized for further analysis.

2.3. Protein extraction
Protein extraction was performed using the modified method of Rusdah and Puspitojati et al [9,15]. The protein extraction was conducted on the raw and boiled jack bean; the raw and cooked tempe. The protein was concentrated to 1 mg/mL (calculated by Lowry methods) and used for haemagglutination assay.

2.4. Simulated in vitro digestion
Enzymatic hydrolysis of jack bean tempe was conducted by the method of Mineskus et al and Sun et al [16,17] with slight modification. The sample was firstly hydrolyzed with pepsin (2000 U/mL) at 37°C for 120 min and followed by pancreatin (100 U/mL Trypsin) for 120 min. The sample was incubated in boiling water for 10 min for stopping the enzymatic reaction. The cool sample was centrifuged at 8000 xg, 4°C for 15 min (5424 R, Eppendorf, Germany). The supernatant was used for
further analysis.

2.5. Haemagglutination assay
Haemagglutination activity of Con-A was detected using human erythrocytes in the presence of anticoagulant according to the method of Marnedi [4]. The protein sample was serially diluted in a microtiter plate that was further mixed with 50 μl of the human erythrocytes. The positive control contained 50 μl of raw jack bean protein extract and 100 μl of blood, while negative control contained 50 μl of PBS solution and 50 μl of blood. Hemagglutination titers were examined after incubation for 60 s at room temperature.

2.6. Determination of HCN content
The HCN content was determined by the method of William [18]. The sample was pounded and mixed with the distilled water, then incubated for 4 h. 1 mL of filtrate was added by 1 mL of KOH 2%, followed by 5 ml of alkaline picrate. The solution was dipped in boiling water for 15 s. The absorbance was measured at 510 nm using a Spectrophotometer UV-VIS (Dynamica Scientific, Halo SB 10, UK) with potassium cyanide (KCN) used as a standard.

2.7. Assay of the peptide content and degree of hydrolysis
Determination of peptide content was performed by the method of Church [19], while the degree of hydrolysis was performed by the method of Lin et al [20]. Tryptone was used as a standard curve [21].

2.8. Determination of ACE-inhibitory activity
The ACE-inhibitory activity of jack bean tempe was calculated by Cushman and Cheung method with slight modifications [22].

2.9. Statistical analysis
The data were analyzed by analysis of one-way variance (ANOVA) and the average difference between treatments was analyzed by Duncan Multiple Range Tests (DMRT). The data from ACE inhibition were evaluated using an independent T-test. The statistical analysis was conducted using SPSS IBM 23 while the significant differences were determined with a 95% confidence interval.

3. Results and discussion

3.1. HCN content during jack bean tempe processing
The HCN content decreased significantly during the tempe pre-fermentation processes (p<0.05). It has been described in table 1. The pre-fermentation process was the stages before fermentation, included the first soaking, the first boiling, the second soaking, and the second boiling. The soaking of jack bean seeds for 24 h followed by boiling for 30 min resulted in the highest decrease of HCN content, with the value from 57.56 to 8.46 ppm. The addition of wood ash was conducted in this first boiling. It has been known that ash can be used as an absorbent [6]. Rice husk ash could be used as an adsorbent to remove cyanide, good scavenger, and low cost. The high reduction of HCN content using wood ash and rice husk as has been carried out in the previous studies on gadung (Discorea hispida) [23] and rubber seeds (Hevea brasiliensis) [6]. The effect of rice husk ash on the degradation of HCN as a function of contact time, pH, adsorbent concentration and temperature [6]. In addition, the boiling process itself was able to reduce HCN content because of its properties. HCN is a volatile compound and evaporates rapidly in the temperature over 28°C, it also dissolves readily in water. HCN content did not change significantly during tempe fermentation (p>0.05). Standard threshold levels of HCN produced by cyanogenic glycogen in plants (tubers, nuts, and seeds) is 50 ppm. The Codex Alimentarius Commission has developed and published standards for a product suitable for direct human consumption (edible cassava flour) with the total HCN content must
The HCN content of mature jack bean tempe in this study was 0.71 ppm, therefore, the final product is safe for consumption.

### Table 1. HCN content during tempe processing.

| Treatment | HCN content (ppm) | HCN reduction (%) |
|-----------|-------------------|-------------------|
| Initial   | 59.95±2.67<sup>e</sup> | 3.98±1.15        |
| S         | 57.56±0.69<sup>d</sup> | 85.88±0.32       |
| SB        | 8.46±0.19<sup>e</sup>  | 92.16±0.14       |
| SBS       | 4.70±0.08<sup>b</sup>  | 97.95±0.06       |
| SBSB      | 1.23±0.03<sup>a</sup>  | 98.19±0.05       |
| SBSBF24   | 1.08±0.03<sup>a</sup>  | 98.86±0.11       |
| SBSBF48   | 0.71±0.06<sup>a</sup>  | 98.6±0.11        |

Data are the mean ± SD of n=3, Different letters in the same column indicate significant statistical differences (p < 0.05). Initial is raw jack bean; S is jack bean seeds soaked for 24 h; SB is S followed by boiling for 30 min; SBS is SB followed by soaking for 48 h; SBSB is SBS followed by boiling for 30 min; SBSBF24 is SBSB followed by fermentation for 24 h or tempe 24 h; and SBSBF48 is SBSB followed by fermentation for 48 h or tempe 48 h.

3.2. Haemagglutination activity during jack bean tempe processing

The agglutination activity of the jack bean protein was tested with the human red blood cells. Figure 1 shows the protein extracted from raw jack bean had blood agglutination activity (line 1). The previous study investigated that all the *Canavalia* spp. were found to have high hemagglutination activity against cattle and human red blood cells [2]. That's why we used the protein extracted from raw jack bean as a positive control in this study. It was noted that red jack bean (*Canavalia gladiata*) had more ability to agglutinate human blood than other *Canavalia* spp.

![Figure 1. Agglutination activity of protein observed in microtiter plate from the bottom (A) and the top (B); Lane 1: positive control; lane 2: negative control; lane 3: boiled jack bean; lane 4-5: jack bean tempe 24 and 48 h; lane a-c: protein content 50, 25, and 10 µg.](image)

It was found in the previous study that cracking the seeds into 2-7 pieces helped to eliminate Con A. In addition, cooking for 2 h or pressure-cooking for 45 min was effective in the elimination of hemagglutination activity in whole seeds [5]. The jack bean seed was sliced into 4-6 pieces during tempe processing in this study. The combination of cracking and cooking twice for each 30 min did not show any red blood cell clotting, it also found in tempe 24 h and 48 h. Jack bean tempe could be categorized as safe for consumption in terms of Con A content.

3.3. The peptide content and degree of hydrolysis following in vitro digestion
The fate of protein from tempe cooked for 10 and 20 min following gastrointestinal digestion was investigated. The peptides content in both samples increased during digestion simulation (figure 2). However, hydrolysis using pepsin did not show any significant increase. Pepsin breaks down large protein molecule into small peptides and converts almost all structural protein into the component with soluble small molecules [25]. Furthermore, it has a certain preference to cleave peptide bonds that contain the amine group of aromatic amino acids [26]. Tempe is a fermented product that has naturally undergone a process of protein hydrolysis during fermentation. In the previous study, it was reported that tempe fermented for 48 h only had less than 22% protein with a molecular weight more than 14 kD, the rest was a relatively small molecular weight peptide (unpublished data). Table 2 shows the degree of hydrolysis of jack bean tempe following in vitro digestion model. The level of protein hydrolysis using pepsin was very low in both samples. T20 had lower hydrolysis degree than T10. The time of cooking affected the type of peptides produced, which resulted in a difference in pepsin action.

![Figure 2](image_url)

**Figure 2.** The peptide content of jack bean tempe following in vitro digestion model.

**Table 2.** The degree of hydrolysis of jack bean tempe following in vitro digestion model.

| Sample | Degree of hydrolysis |
|--------|-----------------------|
|        | Pepsin                | Pepsin-pancreatin |
| T10    | 4.34±0.61             | 54.44±2.01        |
| T20    | 0.45±0.20             | 54.44±1.18        |

Data are the mean ± SD of n=3

T10 is tempe cooked for 10 min; T20 is tempe cooked for 20 min

Furthermore, the peptide content and hydrolysis degree were observed to have increased sharply in both samples after the hydrolysis using pancreatin. Pancreatin is mixtures enzymes that have wide specificity of hydrolytic action. It has been reported to have a greater hydrolytic action in lysine or arginine, tryptophan or tyrosine, leucine, alanine, and phenylalanine [27]. The wide specificity of pancreatin caused the high degree of protein hydrolysis, therefore, resulting in more small size peptides. The increase of peptides content was almost 5-fold after tempe protein was hydrolyzed using pancreatin (figure 2).

3.4. *ACE inhibitory activity*

The ACE inhibitory of peptides increased following in vitro gastrointestinal digestion (figure 3). The protein degradation during gastrointestinal digestion may release new peptides with stronger ACE inhibition. The protein hydrolysis using pepsin-pancreatin resulted in more small-size peptides than using pepsin. The peptide size is associated with the nutritional value of hydrolyzed protein [28].
ACE-inhibitory peptides were generally short chain peptides with 3-20 amino acids [10]. The protein hydrolysis using pepsin-pancreatin produced more small-size peptides which influenced the increase in ACE inhibitory activity. The activity of ACE inhibition was significantly different from both T10 and T20 in each stage of hydrolysis (p<0.05). The strongest of ACE inhibition activity was found to T10 after sequential hydrolysis using pepsin-pancreatin, with the value as high as 86.85%.

![Figure 3. ACE inhibitory activity of jack bean tempe following in vitro digestion model.](image)

**Figure 3.** ACE inhibitory activity of jack bean tempe following in vitro digestion model.

### 4. Conclusion
The decrease of HCN occurred during the processing of jack bean tempe due to the treatment such as soaking and boiling. The resulting tempe had a very low HCN content and did not show agglutination activity when reacting with red blood cells. It can be concluded that the jack bean protein was safe to consume in the form of tempe. Furthermore, the protein of cooked tempe was highly hydrolyzed by pepsin-pancreatin following in vitro gastrointestinal digestion. The increase of bioactive peptides with ACE inhibitory activity was found during digestion. The highest inhibition was obtained in cooked tempe for 10 min with a value of 86.85%.

### Acknowledgments
The authors acknowledge the financial support of the Doctoral Program from the Indonesia Endowment Fund for Education (Lembaga Pengelola Dana Pendidikan), the Ministry of Finance. The authors also appreciate the Ministry of Research, Technology and Higher Education for supporting this project through Hibah Tim Pascasarjana 2018 on behalf of Dr. Ir. Retno Indrati, M.Sc.

### References
[1] Widaningrum, Sukasih E and Purwani E Y 2015 Introductory study on processing of fermented jack bean J. Penelit Pasca Panen Pertan 12 129-36
[2] Sridhar K R and Seena S 2006 Nutritional and antinutritional significance of four unconventional legumes of the genus Canavalia - A comparative study Food Chem. 99 267-88
[3] Cheeke P R 1989 Toxicants of Plant Origin (CRC Press) Boca Raton Florida United States
[4] Marndi R 2012 Isolation and characterization of concanavalin a from the seeds of Canavalia ensiformis (Departement of Life Science, National Institute of Technology, India) p 44
[5] Udedibie A B and Carlini C 1998 Crack and cook: A simple and quick process for elimination of concanavalin A (Con A) from Canavalia seeds Anim. Feed Sci. Technol. 74 179-84
[6] Fortuna D, Rahimsyah A and Puspitasri Y 2015 Degradation of acid cyanide poison in rubber seed (Hevea brasiliensis) after treatment with rice husk ash Int. J. Adv. Sci. Eng. Inf. Technol. 5 291-3
[7] Suciati A 2012 Pengaruh lama perendaman dan fermentasi terhadap kandungan HCN pada tempe kacang koro (Canavalia ensiformis L) (Food Sci. Technol. Dep.Universitas, Hasanudin) p 83
[8] Bintari S H and Nugraheni K 2017 The Potential of tempeh as a chemopreventive and chemotherapeutic agent targeting breast cancer cells Pakistan J. Nutr. 16 743-9
[9] Puspitojati E, Cahyanto M N, Marsono Y and Indrati R 2019 Production of angiotensin-I-converting enzyme (ACE) inhibitory peptides during the fermentation of jack bean (Canavalia ensiformis ) tempe Pakistan J. Nutr. 18 464-70
[10] Escudero E, Mora L and Toldrá F 2014 Stability of ACE inhibitory ham peptides against heat treatment and in vitro digestion Food Chem. 161 305-11
[11] Jakubczyk A, Karas M, Baraniak B and Pietrzak M 2013 The impact of fermentation and in vitro digestion on formation angiotensin converting enzyme (ACE) inhibitory peptides from pea proteins Food Chem. 141 3774-80
[12] Luo Q, Boom R M and Janssen A E M 2015 Digestion of protein and protein gels in simulated gastric environment Food Sci. Technol. 63 161-8
[13] Quist E E, Phillips R D and Saalia F K 2009 Angiotensin converting enzyme inhibitory activity of proteolytic digests of peanut (Arachis hypogaea L.) flour Food Sci. Technol. 42 694-9
[14] Fernández K and Labra J 2013 Simulated digestion of proanthocyanidins in grape skin and seed extracts and the effects of digestion on the angiotensin I-converting enzyme (ACE) inhibitory activity Food Chem. 139 196-202
[15] Rusdah 2016 Antioxidative of tempe peptides from Indonesia (Department Food Sci. Technol. Bogor. Agric. Univ.) p 50
[16] Minekus M et al 2014 A standardised static in vitro digestion method suitable for food – an international consensus Food Funct. 5 1113-24
[17] Sun Y et al 2016 An investigation into the gastrointestinal stability of exenatide in the presence of pure enzymes, everted intestinal rings and intestinal homogenates Biol. Pharm. Bull. 39 42-8
[18] Williams H J and Edwards T G 1980 Estimation of cyanide with alkaline picrate J. Sci. Food Agric. 31 15-22
[19] Church F C, Swaisgood H E, Porter D H and Cantignani G L 1983 Spectrophotometric assay using o-phthalaldehyde for determination of proteolysis in milk and isolated milk proteins J. Dairy Sci. 66 1219-27
[20] Lin H and Alashi A M 2017 Antihypertensive properties of tilapia (Oreochromis spp.) frame and skin enzymatic protein hydrolysates Food Nutr. Res. 61 1-11
[21] Wulandari R D, Rahayu E, Marsono Y ad Utami T 2017 Aktivitas antioksidan dan angiotensin-I converting enzyme inhibitor oleh yogurt dengan ekstrak daun ficus glomerata Roxb Agritech 37 246-55
[22] Cushman, D W H S C 1971 Spectrophotometric assay and properties of the angiotensin converting enzymes from rabbit lung Biochem. Pharmacol. 20 1637-48
[23] Alma’arif A L, Wijaya A and Murwono R P D 2012 Penghilangan racun asam sianida (HCN) dalam umbi gadung dengan menggunakan bahan penyerap abu J. Teknol. Kim. Dan. Ind. 1 14-20
[24] Codex 2018 Discussion paper on maximu level for hydrocyanic acis and mycotoxin contamination in cassava and cassava-based products Joint FAO/WHO Food Standards Programme 12th Session Utrecht (The Netherlands) 12 - 16 March 2018
[25] Lowe J S and Anderson P G 2015 Stevens & Lowe’s Human Histology 4th Edi Elsevier Ltd. New York United States
[26] Ahn J, Cao M-I and Engen J R 2013 Assessing the reproducibility and specificity of pepsin and other aspartic protease Biochim. Biophys. Acta 1834 1222-9
[27] Evinin L B, Vasquez J R and Craik C S 1990 Substrate specificity of trypsin investigated by using a genetic selection Biochemistry 87 6659-63
[28] Silvestre M P C, Morais H A, Silva V D M and Silva M R 2013 Degree of hydrolysis and peptide profile of whey proteins using pancreatin *Nutrire* **38** 278-90