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Proteolysis and Angiotensin-Converting Enzyme Inhibitory Activity of Peptide Fractions from Pigeon Pea Tempe

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Abstract: ***Pigeon pea seeds are considered as potential sources of angiotensin-converting enzyme (ACE) inhibitory peptides. During tempe fermentation, certain bioactive peptides including ACE inhibitory peptides can be generated due to proteolytic activity produced by tempe molds. The present study was conducted to investigate the proteolysis during pigeon pea tempe fermentation and determine the ACE inhibitory activity of peptide fractions from pigeon pea tempe. Commercial starter RAPRIMA® (0.02% w/w) consist of Rhizopus oligopsorus spores was used to fermented pigeon pea seeds for 0-96 h. Fractionation was completed by using dialysis tube with molecular weight cut-off of 1, 3.5, and 14 kDa. The longer fermentation time resulted in greater proteolysis in pigeon pea tempe. Proteolytic activity and peptide content were reached a maximum of 0.044 U/mL and 3.175 mg/100 g, consecutively after 96 h of fermentation. All of the resultant peptide fractions from pigeon pea tempe (48 h of fermentation) could inhibit ACE activity. Low-molecular-weight peptide fractions tended to have a higher ACE inhibitory activity than high-molecular-weight peptide fractions. Among the resultant peptide fractions, Peptide fraction of <1 kDa was found to be the most potent fraction to inhibit ACE activity with (87.98%) while peptide fractions of >14 kDa had the lowest ACE inhibitory activity (61.20%). In conclusion, peptide extract from pigeon pea tempe could become a promising source of ACE inhibitory peptides and could be used as a functional food or for other food applications.***

Keywords: ACE inhibitory, fermentation, pigeon pea, tempe

1. Introduction

Loads of attention are paid to bioactive peptides as an alternative for the prevention and treatment of certain chronic diseases including hypertension which has affected 1.13 billion people worldwide in 2015 [1]**.** Bioactive peptides could act as an inhibitor for angiotensin-converting enzyme (ACE), the main enzyme in the blood pressure regulator system. Thus, it could help to reduce elevated blood pressure.

The ACE inhibitory peptides are characterized by its low molecular weight, estimated at <10 kDa [2], [3]. The ability of the bioactive peptides to inhibit ACE activity is also related to the composition of its amino acids. Most ACE inhibitory peptide are consist of hydrophobic (Phe, Leu, Val, Ile, Pro, Ala, Trp) [3], [4], [5] and negatively charged amino acids [3], [6]. Pigeon pea (*Cajanus cajan)* seeds contain protein up to 17-32% (db) [7], [8] and have a relatively high amount of hydrophobic and negatively charged amino acids [9], [10], making it potential to generate ACE inhibitory peptides.

Tempe fermentation could be an effective way to produce ACE inhibitory peptides. Yet, tempe made from pigeon pea seeds is still rarely studied so the use of these seeds could be further explored. During tempe fermentation, proteolysis will occur due to the proteolytic activity of *Rhizopus* sp. Proteolysis will cause the hydrolysis of protein and polypeptide to produce peptides, as well as ACE inhibitory peptides. The proteolytic activity tends to change during tempe fermentation and generally increases with the duration of fermentation [11], [12].

Commercial tempe starter "Raprima®" is consists of *Rhizopus oligosporus* spores which have been reported to have high proteolytic activity [13], [14]. The high proteolytic activity allows more peptides to form. However, the proteolytic activity that occurs continuously during fermentation can also result in further hydrolysis of peptides to produces free amino acids so it could alter the capability of the peptides to inhibit ACE activity [15]. Our previous study showed that pigeon pea tempe could produce bioactive peptides that have the ACE inhibitory activity up to 76, 14% with an IC_{50} value of 0, 65 mg/mL [16]. However, as far as we know, the study about proteolysis during pigeon pea tempe fermentation in an attempt to produce ACE inhibitory peptides has never been done.

The peptide fractions obtained from the fractionation of germinated pigeon pea seeds were able to inhibit ACE activity [3]. The ACE inhibitory activity of the peptide fractions increased as the molecular weight decreased with a fraction of <3 kDa had the highest ACE inhibitory activity of 86.97%. As for the high-molecular-weight peptide (>10 kDa), the ACE inhibitory activity was only 3.06%. To date, there is no study on ACE inhibitory activity from peptide extract fractions of pigeon pea tempe. Our study was conducted to investigate the proteolysis during pigeon pea tempe fermentation and determine the ACE inhibitory activity of peptide fractions from pigeon pea tempe.

2. Materials and Methods

2.1. Materials

Black pigeon pea seeds (*Cajanus cajan*) were collected from a local farmer at Jepitu, Yogyakarta, Indonesia. Raprima® (Bandung, Indonesia) consisting of *Rhizopus oligosporus* spores was obtained from a local market at Sleman, Yogyakarta, Indonesia. The chemicals used include casein, tyrosine and tryptone standard, hippuryl-histidyl-leucine (HHL), and ACE (EC 3.4.1.5.1) were purchased from Sigma-Aldrich (St. Louis, MO, USA), O-phthaldialdehyde (OPA) was purchased from Merck (Darmstadt, Germany), and dialysis tube with molecular weight cutoff of 1, 3.5, and 14 kDa from *SPECTRA*/POR® (California, USA).

2.2. Preparation of Pigeon Pea Tempe Fermentation

The preparation of pigeon pea tempe was started with removing the physical contaminants from the seeds followed by washing and soaking for 24 h. The soaked seeds were rinsed and boiled for 30 min. The cooled seeds were then peeled and soaked again for 24 h. The second boiling was performed for 10 min followed by draining and cooling the seeds to room temperature. The preparation of pigeon pea tempe was completed by the inoculation of Raprima® starter at 0.02% (w/w) [16]. The seeds were wrapped in banana leaves and allowed it to ferment at room temperature for 0, 12, 24, 36, 48, 72, and 96 h.

2.3. Protease Extraction

Fresh samples from each of the fermentation time were used to extract the crude protease enzyme. Following the method of Elegado and Fujio [17] with some modifications, the extraction of protease was prepared by homogenizing 3 g sample with 30 mL of phosphate buffer (0.05 M, pH 6) for 3 min followed by incubation in a water bath shaker for 30 min (60 rpm, 30^{o}C). Afterward, the sample was centrifuged at 10000 rpm, 4^{o}C for 30 min. The supernatant was collected and termed as a crude protease enzyme solution.

2.4. Peptide Extraction

Samples from each of the fermentation times were first lyophilized and powdered before use for peptide extraction. A total of 1 g of dried powdered sample was dissolved in 30 mL of distilled water, homogenized for 3 min and incubated in a water bath shaker for 60 min (60 rpm, 30^{o}C). After that, the sample mixture was centrifuged at 14593 rpm for 15 min. The supernatant containing peptide extract was collected and saved for further analysis [18].

2.5. Fractionation of Peptide Extract from Pigeon Pea Tempe

Peptide extract from pigeon pea tempe was fractionated using a dialysis tube with a molecular weight cutoff (MWCO) of 1, 3.5, and 14 kDa according to the method by Pohl [19]. A total of 10 mL peptide extract was put into a dialysis tube, immersed in distilled water at 1:9 (v/v) then incubated for ±12 h in a cool room with gentle stirring. Fractionation will result in four peptide fractions with a molecular weight of <1 kDa, 1-3.5 kDa, 3.5-14 kDa, and >14 kDa which were then lyophilized and saved for further analysis.

2.5. Protein and Amino Acid Compostion

The analysis of pigeon pea seeds protein was determined according to the method by the association of official analytical chemists (AOAC 2005). The LC-MS/MS (The Water^{TM} *Xevo^{TM} TQD*®, Milford, USA) was used for the determination of amino acids in pigeon pea seeds.

2.6. Proteolytic Activity

The proteolytic activity was measured using casein as a substrate [20]. The proteolytic activity was monitored at 660 nm, one proteolytic activity unit (Unit) represented the amount of tyrosine equivalent released from casein per min. Proteolytic activity was calculated following equation 1:

$$\text{Proteolytic activity (U/mL)} = \frac{A \times Vtot}{T \times Ve} \quad (1)$$

Where A is µmol tyrosine released, V_{tot} is the total volume of all reagents include on the analysis, T is the incubation time, and V_e is the volume of enzyme solution.

2.7. Protein Content

The method from Church *et al*. [21] was adapted to determine the peptide content. OPA solution was made by dissolving 40 mg of OPA in 1 mL of methanol and 100 mL of β-mercaptoethanol (100 mM). OPA reagent was then prepared by mixing OPA solution with 25 mL of sodium tetraborate (100 mM), 2.5 mL of SDS 20% (w/v in distilled water) and 21.4 mL of distilled water. A total of 30 µL of the sample was mixed evenly with 1 mL of OPA reagent then incubated in the dark for 2 min. The absorbance was measured at 340 nm and peptide content was determined based on a standard linear regression from the tryptone standard curve.

2.8. Inhibition of ACE Activity

The inhibition of ACE activity was done according to the modified method of Chusman and Cheng [22]. Substrate HHL will be hydrolyzed by ACE and produce hippuric acid which is then used for the determination of ACE inhibitory activity. Substrate HHL was first dissolved in 50 mM Hepes buffer (pH 8.3) containing 300 mM NaCl and then 50 mL of peptide extract (1 mg/mL protein) was added to 50 mL of HHL and preincubated for 10 min at 37 ^{o}C. The enzymatic reaction was started with the addition of 50 mL of ACE solution (25 mU/mL) and incubated for 30 min at 37 ^{o}C. Next, a total of 200 µL of HCl (1 M) was added to stop the enzymatic reaction. A total of 1.5 mL ethyl acetate was used to extract the hippuric acid released from the enzymatic reaction. The mixture of sample and ethyl acetate was shaken for 2 min and then centrifugated for 15 min (6526 rpm, 4^{o}C).

The upper layer of supernatant was collected, evaporated for 25 min at 100°C then dissolved and homogenized in 3 mL of distilled water. The absorbance was monitored at 228 nm and the ACE inhibitory activity was calculated following equation:

$$\text{ACE inhibitory activity (\%)} = \frac{A - B}{A - C} \times 100\% \quad (2)$$

Where A is the absorbance of the sample without inhibitory peptides, B is the absorbance of the sample in the presence of ACE and inhibitory peptides and C is the absorbance of the blank sample.

2.9. Statistical Analysis

The present study used a *completely* randomized *design using* three replications to obtain all the data. One-way ANOVA and Duncan's multiple range test analysis with a 95% confidence level were used to analyze all the data by using SPSS IBM version 22.0 software.

3. Results and Discussion

3.1 Protein Content and Amino acids Composition of Pigeon Pea Seeds

The protein content of pigeon pea seeds range from 17-25% (db) [8] and could reach up to 32% [7]. Result in the Table 1 showed that the protein content of pigeon pea seeds in the present study was 28.09%. The difference in the protein content is probably affected by the seed varieties, ecological diversity, different in the harvest and post-harvest handling, and storage conditions [8]. The higher protein content is considered more accessible to utilize and to form ACE inhibitory peptides than other sources with limited protein content [23]. The presence of certain amino acids such as hydrophobic or negatively charged amino acids was reported to have great potential for the formation of ACE inhibitory peptides. Pigeon pea seeds in the present study contained a good amount of hydrophobic (Phe, Leu, Ala, Val) and negatively charged amino acids (Glu, Asp). Hydrophobic amino acids could bind strongly to the active site of ACE [4], [24]. Meanwhile, negatively charged amino acids could make an electrostatic interaction with Zn^{2+} as a coenzyme of ACE [2], [6], [24]. This result indicated that protein of pigeon pea seeds has the potential to form ACE inhibitory peptides and fermentation would likely increase the formation of ACE inhibitory peptides.

Table 1: Protein content and amino acids composition

Amino acids	*Content (g/100 g)*
Hydrophobic amino acids	
Phenylalanine	5.40 ± 0.16
Leucine	4.45 ± 0.05
Alanine	3.36 ± 0.11
Valine	3.23 ± 0.02
Glycine	3.14 ± 0.32
Isoleucine	2.25 ± 0.04
Proline	2.19 ± 0.02
Methionine	0.40 ± 0.01
Cysteine	0.13 ± 0.03
Total	24.55
Hydrophilic amino acids	
Glutamatic acid (negatively charged)	15, 53 ± 0.67
Aspartic acid (negatively charged)	6, 19 ± 0.03
Arginine	4, 05 ± 0.49
Histidine	2, 84 ± 0.68
Threonine	3, 18 ± 0.15
Tyrosine	0, 38 ± 0.01
Lysine	5, 93 ± 0.01
Serine	3, 01 ± 0.03
Total	41.11

3.2 Proteolysis during Pigeon Pea Tempe Fermentation

Rhizopus sp. in known to produced various types of protease enzymes such as extracellular, intracellular and cell wall-bound protease including acidic, neutral and alkaline protease during fermentation of soybean tempe [13], [14]. The proteolysis during tempe fermentation is important and will greatly affect the formation and generation of ACE inhibitory peptides because it is related to the hydrolysis of proteins and polypeptides into peptides [15], [25], [26].

The proteolysis during pigeon pea tempe fermentation was described by the changes in proteolytic activity and peptide content. As shown in Figure 1, the proteolytic activity of 0.0057 U/mL was first detected after 12 h of fermentation even though it still very low. Similar results have been reported by Puspitojati *et al.*, [15] and Ruiz-Teran and Owens [11] during the fermentation of jack bean and soybean tempe, respectively. At the beginning of fermentation, mold growth has just begun to enter the initial phase so that the production of enzymes is estimated still very low [11]. As the mycelium of mold appeared thicker and the texture of pigeon pea tempe became compact, the proteolytic activity also continued to increase significantly ($P<0.05$) along with fermentation time. The same pattern of proteolytic activity was also reported by Ruiz-Teran and Owens [11] in soybean tempe, Starzynska-Janiszewska *et al*. [12] in grass pea tempe fermented by *Rhizopus microsporus* var. *Chinensis* and *Aspergillus oryzae*, and Puspitojati *et al.* [15] in jack bean tempe fermented by commercial starter "Raprima" containinig *Rhizopus oligosporus*.

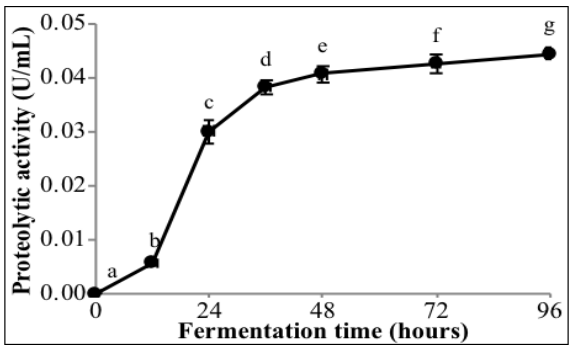

Figure 1: Proteolytic activity during pigeon pea tempe fermentation. Different letters are significantly different ($p<0.05$)

The proteolytic activity reached a maximum of 0.0444 U/mL after 96 h of fermentation. However, after 48 h of

fermentation, the increase in proteolytic activity began to slow down. It is thought to be related to the mold growth that has entered the end of the log phase or the early stationary phase. The ratios of immature and mature molds are associated with the proteolytic activity produced during tempe fermentation. Mature molds produced fewer enzymes than immature molds, so the proteolytic activity tends to be slower when entering the end of fermentation [27].

An increment in proteolytic activity was positively correlated with an increase in peptide content. Protease enzymes produced by *Rhizopus* sp. are known to be an endopeptidase, which cleavage peptide bonds from the inside of the polypeptide chain and released peptides and free amino acids [13], [14]. Peptide content detected before fermentation (0 h) was 0.181 mg/100 g and slightly increased insignificantly to 0.186 mg/100 g ($P<0.05$) as shown in Figure 2. Peptide content of 2.539 mg/100 g was found at 48 h of fermentation when pigeon pea tempe reached its compact texture with the typical smell of tempe.

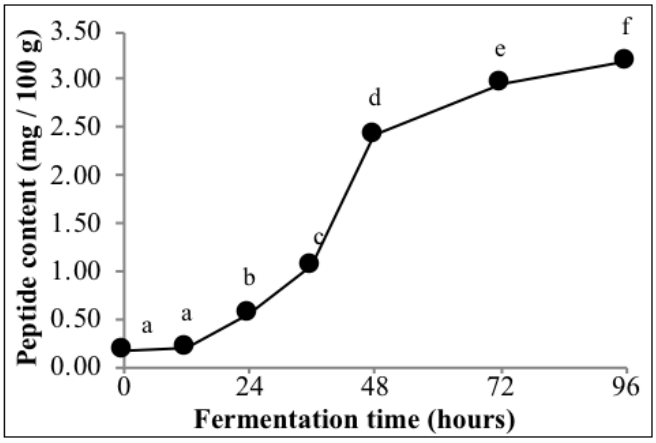

Figure 2: Changes in peptide content during pigeon pea tempe fermentation. Different letters are significantly different ($P<0.05$)

The increase in peptide content continued until the end of fermentation with 3.175 mg/100 g. However, similar to the result of proteolytic activity, the increase of peptide content began to slow down after 48 h of fermentation. The high proteolytic activity can cause more protein or polypeptide hydrolysis to occur and increasing peptide content. The higher frequencies of protein hydrolysis, the more peptides are produced. However, hydrolysis can also alter the composition of amino acid of the peptides, thereby changing the effectiveness of peptides to inhibit ACE activity. Therefore, high peptide content does not always guarantee to have the highest ACE inhibitory [15], [16].

3.3 Inhibition of ACE Activity by Peptide Fractions from Pigeon Pea Tempe

Tempe is considered ready for consumption after fermented for 36-48 hours. It is defined by the firm and compact texture with the typical smell of tempe without an ammonia-like odor. Our previous study showed that pigeon pea tempe fermented for 48 h had developed the compact mycelium texture with a tempe-like odor. Also, pigeon pea tempe (48 h) had the highest ACE inhibitory activity of 76, 14% [16]. Therefore, pigeon pea tempe fermented for 48 h was used in the present study to further explored through fractionation of its peptide extract as an inhibitor of ACE activity.

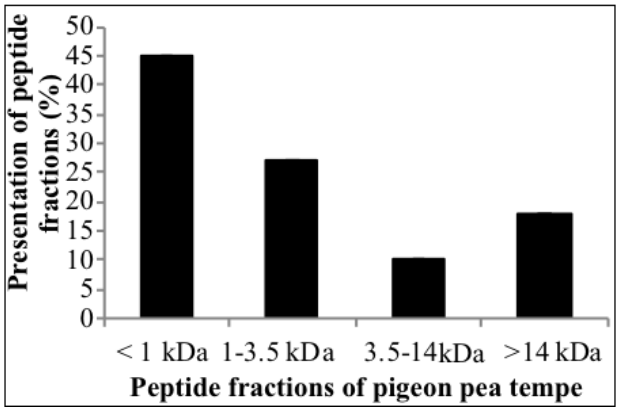

Figure 3: Distribution of peptide fractions from pigeon pea tempe fermented for 48 h.

As shown in Figure 3, peptide fraction of <1 kDa dominated the distribution of peptides in pigeon pea tempe, followed by peptide fraction of 1-3.5 kDa, peptide fraction of >14 kDa and the lowest distribution came from peptide fraction of 3.5-14 kDa. The large percentage of peptide fraction with low-molecular-weight (<1kDa and 1-3.5 kDa) in pigeon pea tempe is the result of proteolytic activity that degrades protein during fermentation to produce peptides with lower molecular weights. The presence of peptide fraction with high-molecular-weights (>14 kDa) showed that the protein hydrolysis that occurred during pigeon pea tempe fermentation did not only produced low-molecular-weight peptides.

The ACE inhibitory activity of four peptide fractions resulting from the fractionation of pigeon pea tempe is shown in Figure 4. Low molecular weight fractions significantly ($p<0.05$) have greater ACE inhibitory activity compared to high molecular weight fractions. The ACE inhibitory activity increased as the molecular weight of the peptide fractions decreased. Peptide fraction of <1 kDa had the highest ACE inhibitory activity of 87.98%, followed by the fraction of 1-3.5 kDa (80.87%), fraction 3.5-14 kDa (78.96%) and fraction >14 kDa which had the lowest ACE inhibitory activity of 61.20%. This result also suggested that pigeon pea tempe fermented for 48 h were possibly dominated by low molecular weight peptides less than 14 kDa.

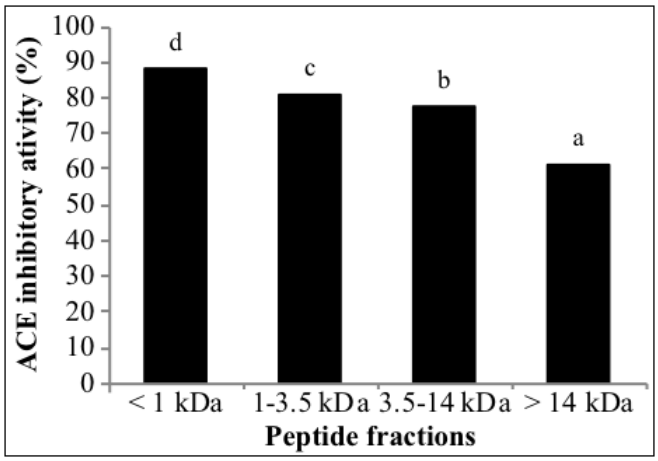

Figure 4: Inhibition of ACE activity by peptide fractions from pigeon pea tempe fermented for 48 h. Different letters are significantly different ($p<0.05$)

Similar results have been reported by Tuz and Campos [2] in peptide fractions from velvet bean hydrolysate and Ratyanani *et al.* [3] in peptide fractions from germinated pigeon pea seeds. Low-molecular-weight peptides are considered to be easier to bind to the narrow side of the ACE active site. Meanwhile, high-molecular-weight peptides tend to have long polypeptide chains, making it difficult to access the active site of the ACE [6], [24]. The low-molecular-weight peptides exhibited greater ACE inhibitory activity than the high-molecular-weight peptides so it is a more potential candidate to be an antihypertensive agent.

4. Conclusion

Pigeon pea tempe fermented for 48 h were likely to consist of low-molecular-weight peptides less than >14 kDa. The fraction of <1 kDa from peptide extract of pigeon pea tempe had the highest ACE inhibitory activity of 87.98%. These results suggest that fermentation and fractionation of peptide extract from pigeon pea tempe can be used as a functional food or other alimentary applications such as additional ingredients for hypertension treatment. The study of the fate of ACE inhibitory peptide from pigeon pea tempe in the digestive tract is needed to be done.

5. Conflict of Interest

There is no conflict of interest in this study

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References

[1] Zhou, D., Xi, B., Zhao, M., Wang, L., & Veeranki, S. P. (2018). Uncontrolled hypertension increases risk of all-cause and cardiovascular disease mortality in US adults; the NHANES III Linked Mortality Study. *Scientific Reports*, 8, 1-7.

[2] Tuz, M. A. O., & Campos, M. R. S. (2017). Purification of *Mucuna pruriens* (L) peptide fractions and evaluation of their ACE inhibitory effect. *Biocatalysis and Agricultural Biotechnology*, 10, 390-295.

[3] Ratnayani, K., Suter, I. K., Antara, N. S., & Putra, I. N. K. (2019). Angiotensin Converting Enzyme (ACE) inhibitory activity of peptide fraction of germinated pigeon pea (*Cajanus cajan* (L) Milsp.). *Indonesia Journal of Chemistry*, 19(4), 900-906.

[4] Nawaz, K. A. A., David, S. M., Murugesh, E., Thandeeswaran, M., Kiran, K. G., Mahendran, R., Palaniswamy, M., & Angayarkanni, J. (2017). Identification and in silico characterization of a novel peptide inhibitor of angiotensin converting enzyme from pigeon pea (*Cajanus cajan*). *Phytomedicine*, 36, 1-7.

[5] Daliri, E. B. M., Lee, H. B., Jong-Hak, K., & Deog-Hwan, O. (2018). Novel angiotensin I-converting enzyme inhibitory peptides from soybean protein isolate fermented by *Pediococcus pentosaceus* SDL1409. *LWT-Food Science and Technology*, 9, 88-93.

[6] Aluko, R. E., Girgih, A. T., He, R., Malono, S., Li, H., Offengenden, M., & Wu, J. (2015). Structural and functional characterization of yellow field pea seed (*Pisum sativum* L.) protein-derived antihypertensive peptides. *Food Research International*, 77(1), 10-16.

[7] Saxena, K. B., & Sawargaonkar, S. L. (2015). Generic enhancement of seed proteins in pigeonpea – methodologies, accomplishment, and opportunities. *International Journal od Scientific Research*, 4(5), 3-7.

[8] Kachare, D. P., Satbhai, R. D., Rathod, D. B., & Naik, R. M. (2019). Evaluation of pigeon pea (*Cajanus cajan* L.) genotypes for nutritional quality. *Legume Research*, 42(4), 485-489.

[9] Oshodi, A. A., Olaofe, O., & Hall, G. M. (1993). Amino acids, fatty acid and mineral composition of pigeon pea (*Cajanus cajan*). *International Journal of Food Sciences and Nutrition*, 43, 187-191.

[10] Akande, K. E., Abubakar, M. M., Adegbola, T. A., Bogoro, S. E., & Dona, U. D. (2010). Chemical evaluation of the nutritive quality of pigeon pea [*Cajanus cajan* (L.) Millsp.]. *International Journal of Poultry Science*, 9(1), 63-65.

[11] Ruiz-Teran, F., & Owens, J. D. (1996). Chemical and enzymic changes during the fermentation of bacteria-free soya bean tempe. *Journal of the Science of Food and Agriculture*, 71, 523-530.

[12] Starzynska-Janiszewska, A., Stodolak, B., & Wikiera, A. (2015). Proteolysis in tempeh-type products obtained with *Rhizopus* and *Aspergillus* strains from grass pea (*Lathyrus sativus*) seeds. *Acta Science Polonorum Technologia Alimentaria*, 14(2), 125–132.

[13] Baumann, U., & Bisping, B. (1995). Proteolysis during tempe fermentation. *Food Microbiology*, 12, 39-47.

[14] Heskamp, M. L., & Barz, W. (1998). Expression of protease by *Rhizopus* species during tempeh of fermentation of soybean. *Molecular Nutrition & Food Research*, 42(1), 23-28.

[15] Puspitojati, E., Cahyanto M. N., Marsono, Y., & Indrati, R. (2019). Production of angiotensin-I-converting enzyme (ACE) inhibitory peptides during the fermentation of jack bean (*Canavalia ensiformis*) tempe. *Pakistan Journal of Nutrition*, 18(5), 464-470.
[16] Pebrianti, S. A., Cahyanto, M. N., & Indrati, R. (2020). Angiotensin i-converting enzyme (ACE) inhibitory activity of ACE inhibitory peptides produced during the fermentation of pigeon pea (*Cajanus cajan*) Tempe. *Journal of Indonesian Food and Nutrition Progress*, 16(2), 47-52.
[17] Elegado, F. B., & Fujio, Y. (1993). Growth of *Rhizopus* strains on soybean and their protease formation. *Journal of Faculty of Agriculture*, 37, 315-324.
[18] Rusdah, R., Suhartono, M.T., Palupi, N.S., & Ogawa, M. (2017). Tingkat kelarutan peptide tempe dengan bobot molekul kecil pada berbagai jenis pelarut. AgriTECH, 37 (3): 327-333.
[19] Pohl, T. (1990). Concentration of protein and removal of solutes. *Methods in Enzymology*, 182, 68-83.
[20] Cupp-Enyard, C. (2008). Sigma's non-specific protease activity assay - casein as a substrate. *Journal of Visualized Experiment*, 19, 1-2.
[21] Church, F. C., Porter, D. H., Catignani, G. L., & Swaisgood, H.E. (1985). An *o*-Phthalaldehyde *Spectrophotometric Assay* for proteinases. *Analytical Biochemistry*, 146, 343-348.
[22] Chusman, D. W., & Cheung, H. S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology*, 20, 1637-1648.
[23] Udenigwe, C. C & Aluko, R. E. (2012). Food protein-derived bioactive peptides: production, processing, and potential health benefits. Journal of Food Sciences, 71(1), 11-24.
[24] Wu, Q., Du, J., Jia, J., & Kuang, C. (2016). Production of ACE inhibitory peptides from sweet sorghum grain protein using alcalase: Hydrolysis kinetic, purification and molecular docking study. *Food Chemistry*, 199, 140-149.
[25] Okamoto, A., Hanagata, H., Matsumoto, E., Kawamura, Y., Koizumi, Y., & Yanagida, F. (1995). Angiotensin I converting enzyme inhibitory activities of various fermented foods. *Journal of Bioscience, Biotechnology, and Biochemistry*, 59(6), 1147-1149.
[26] Gibbs, B. F., Zougman, A., Masse, R., & Mulligan, C. (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy fermented food. *Journal of Food Research International*, 37, 123–131.
[27] Handoyo, T., & Morita, N. (2007). Structural and functional properties of fermented soybean (tempeh) by using *Rhizopus oligosporus. International Journal of Food Properties*, 9(2), 347-355.