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Angiotensin-I-Converting Enzyme Inhibitory (ACE-I) Peptide from Germinated Lamtoro Gung (*Leucaena leucocephala* ssp. *glabrata* (Rose) S. Zarate) Flour

(Angiotensin-I-Converting Enzyme Inhibitory (ACE-I) Peptida daripada Percambahan Tepung Lamtoro Gung (*Leucaena leucocephala* ssp. *glabrata* (Rose) S. Zarate))

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ABSTRACT

Hypertension is a significant public health problem worldwide, a leading risk factor for cardiovascular disease and cause of premature death. Angiotensin-I-converting enzyme (ACE) activity is one of the causes of hypertension. Lamtoro Gung has potential as an Angiotensin-I-converting Enzyme Inhibitory (ACE-I) due to the presence of peptide that able to inhibit its activity to prevent hypertension. The germination process was carried out to hydrolyse storage proteins and produce peptides that have a low molecular weight. This study investigated ACE-I activity from Lamtoro Gung seed during germination and evaluated the blanching effect on it. This experiment was conducted with a Completely Randomised Design (CRD), and the factor is the differences in germination duration (0, 12, 24, 36, 48, 60, and 72 h). Proteolytic activity and the degree of hydrolysis during germination were studied to know the correlation between germination and ACE-I activity. The highest ACE-I activity sample was blanched with three different durations (2, 4, and 6 s). The 48 h germinated Lamtoro Gung had the highest ACE-I activity (70.62%). This result was supported by the proteolytic activity (168.79 U/g protein dry matter) and degree of hydrolysis (23.26%). Forty-eight hours of germination of Lamtoro Gung resulted in the highest ACE-I activity. Blanching of germinated Lamtoro Gung for 2 s could hold the ACE-I activity, but the longer duration decreased it.

Keywords: ACE-I; blanching; germination; Lamtoro Gung

ABSTRAK

Hipertensi adalah masalah kesihatan awam utama di seluruh dunia, faktor risiko utama penyakit kardiovaskular dan punca kematian pramatang. Aktiviti Angiotensin-I-Angiotensin-I-Converting Enzyme adalah salah satu punca hipertensi. Lamtoro Gung berpotensi sebagai Angiotensin-I-Converting Enzyme Inhibitory kerana kehadiran peptida yang mampu menghambat aktivitinya untuk mencegah hipertensi. Proses percambahan boleh dijalankan untuk menghidrolisis protein simpanan dan menghasilkan peptida ringkas. Penyelidikan ini mengkaji aktiviti ACE-I daripada biji Lamtoro Gung semasa percambahan dan menilai kesan kukus kepadanya. Uji kaji ini dijalankan dengan Reka Bentuk Rawak Sepenuhnya (CRD) dan faktor penelitian yang digunakan iaitu perbezaan dalam tempoh percambahan (0, 12, 24, 36, 48, 60 dan 72 jam). Aktiviti proteolitik dan tahap hidrolisis semasa percambahan dikaji untuk mengetahui perkaitan antara percambahan dan aktiviti ACE-I. Sampel aktiviti ACE-I tertinggi kemudian dikukus dengan tiga tempoh didih yang berbeza (2, 4 dan 6 s). Lamtoro Gung yang bercambah 48 jam mempunyai aktiviti ACE-I tertinggi (70.62%). Keputusan ini disokong oleh aktiviti proteolitik (168.79 U/g protein bahan kering) dan tahap hidrolisis (23.26%). Lamtoro Gung yang bercambah dan dikukus selama 2 s boleh menahan aktiviti ACE-I, tetapi tempoh yang lebih lama mengurangkannya.

Kata kunci: ACE-I; Lamtoro Gung; pengukusan; percambahan

Introduction

The angiotensin-I-converting enzyme (ACE) plays an essential role in blood pressure regulation and cardiovascular function. It is best known to have a physiological process to convert inactive angiotensin I to active angiotensin II (Bünning & Riordan 1983), a potent vasoconstrictor. Furthermore, ACE is an target for preventing and treating hypertension and cardiovascular diseases.

Peptide utilisation in preventing hypertension is massively researched. These peptides are called ACE-I peptides. Its ability to avoid hypertension is evidenced by its ability to inhibit ACE activity. Various ACE-I peptides can be derived from legumes (Puspitojati et al. 2019), cereal (Li et al. 2007), marine (Ahn et al. 2012), dairy (Tavares et al. 2011), and animal products (Miguel & Aleixandre 2006).

ACE-I peptides have low molecular weight (MW) and consist of hydrophobic and negatively charged hydrophilic amino acid character (de Castro & Sato 2015; Lee, Jeon & Byun 2011). The MW peptides deal with the active site of ACE (Fan, Liao & Wu 2018). ACE has an intense active site, so the low MW peptides can enter the ACE's active site and obstruct its activity. Increasing the MW of peptides can lower its accessibility (Natesh et al. 2003).

Hydrophobic amino acids at the C terminal of peptides lead to hydrophobic interaction between the peptides and the amino acid residue at ACE's active site. This hydrophobic interaction triggers a conformation change of amino acid residue attached with Zn2+ (Fan, Liao & Wu 2018). Meanwhile, negatively charged hydrophilic amino acids, such as Asp and Glu, support ACE-I activity (Pebrianti, Nur Cahyanto & Indrati 2019). Both can create the electrostatic interaction with the Zn^{2+} at ACE's active site (Aluko 2015; Natesh et al. 2003).

Hydrolysis can be applied to obtain low MW peptides. Enzyme utilisation and fermentation process are often used. Germination can also be spread to produce small peptides. It is one of the easy and inexpensive ways to obtain ACE-I peptides from cereals and legumes (Mamilla & Mishra 2017). Proteases will be activated during germination and break the complex storage protein into a simple one. Next, the simple peptides will be used as energy sources for seedling development (Mayer & Poljakoff-Mayber 1979). Germination has been proven to improve the low MW peptides concentration (Mamilla & Mishra 2017) and significantly increase the ACE-I activity (Bamdad et al. 2009).

Lamtoro Gung (*Leucaena leucocephala* ssp. Glabrata (Rose) S. Zarate) belongs to the Leguminosae family and can be easily found in Indonesia. Lamtoro Gung is widely used in traditional Indonesian cuisine (Sayudi, Herawati & Ali 2015) because of its high protein content (30.99% DM) (Harifah 2017). Lamtoro Gung has many negatively charged hydrophilic amino acids, such as glutamic acid (10.84 g/100 g protein DM) and aspartic acid (8.9 g/100 g protein DM). It also has a total of hydrophobic amino acid 18.79 g/100 g protein DM (Supriyadi, Retno & Umar et al. 2021). Based on it, Lamtoro Gung is a potential source of ACE-I peptides. Germination application on Lamtoro Gung is expected to improve the Lamtoro Gung ACE-I through the protein breakdown into a simple peptide.

The use of Lamtoro Gung as ACE-I peptides have been slightly discovered. Some studies evaluate antimicrobial activity (Aderibigbe, Adetunji & Odeniyi 2011), germination profile (Obiazi 2015), tempeh production (Nursiwi et al. 2019, 2018), nutrition, and anti nutrition compounds (Harifah, Supriyadi & Umar 2018). Lamtoro Gung's ACE-I utilisation was not found yet, so it needs to be studied to prevent hypertension. Lamtoro Gung ACE-I peptides can be obtained by germination as a hydrolysis process. The blanching process will be applied as typical Lamtoro Gung processing in the community. This study aims to explore Lamtoro Gung ACE-I properties during germination and evaluate the impact of blanching on Lamtoro Gung ACE-I properties. By consuming the germinated Lamtoro Gung, the risk of hypertension is expected to prevent.

Materials and Methods

Biomaterials and Chemicals

Lamtoro Gung seeds (Figure 1) were bought from the traditional market. They were from Prujakan Village, Sinduharjo, Ngaglik District, Sleman Regency, Yogyakarta, Indonesia. The seeds' length and width of approximately 1 and 0.5 cm. They were dry form, brown to dark brown, and mature from their tree. The maturation was indicated with a dry condition in its tree. The chemicals are pro analysis grades such as Folin-Ciocalteu's phenol reagent (Merck #109001), bovine serum albumin, Potassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$), casein from bovine milk (Sigma C3400), trichloroacetic acid (TCA), L-Tyrosine, L-tryptophane, Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), Disodium hydrogen phosphate (Na_2HPO_4), N-Hippuryl-His-Leu (HHL) (Sigma 859052), HEPES sodium salt (sigma

H7006), Angiotensin-I-Converting Enzyme (ACE) (Sigma A6778), Hydrochloric acid (HCl), ethyl acetate, sodium dodecyl sulfate, Sodium tetraborate ($Na_2B_4O_7$), β-mercaptoethanol (Merck 805740), *o*-Phthaldialdehyde (OPA) (Merck 111452), and some chemicals liquid chromatography grades.

FIGURE 1. Lamtoro Gung seed before imbibition

GERMINATION PROCESS

The germination process followed patent P00202103659 (Fitriani et al. 2021a). Five hundred grams of seed were washed and soaked in 60±1.5 °C water for 30 min in a plastic bowl. Then, they were rinsed and soaked (1:5, w/v) in ambient temperature water for 24 h. Imbibed seeds were rinsed, drained, placed on wet tissue paper on a tray box, and held a damp cloth over it. Germination takes place in a closed environment using a black plastic box. The seeds were germinated at room temperature and in dark conditions. Water was sprayed every 4 h during germination to prevent seeds from drying out. The germination duration varies every 12 h (0, 12, 24, 36, 48, 60, and 72 h). The germination process was done in three repetitions. The resulting sprouts were washed, rinsed, and frozen (-20±2.0 °C). Then, the seeds were freeze-dried (-40±2.0 °C) under vacuum conditions for 48 h. The dried sprouts were ground to pass through a 60 mesh sieve. It was a germinated Lamtoro Gung flour for further analysis.

PEPTIDE EXTRACTION

Peptide extraction followed previously described methods (Pertiwi, Yustinus & Retno 2019) slightly modified. Each sample powder was macerated in distilled water (1:10; w/v) (30±2.0 °C, 1 h) and centrifuged (Sorvall™ ST 8 Centrifuge, Thermo Fisher Scientific, Massachusetts, US) at 3000×g and 4±0.5 °C for 20 min. The supernatant was separated and called the peptide extract, then stored at -20±2.0 °C for further analysis.

CRUDE PROTEASE EXTRACTION

The crude protease extraction was prepared by dissolving 3 g sample powder into 30 mL sodium phosphate buffer (50 mM; pH 6.5) (Gonçalves et al. 2016). Next, the solution was homogenated (4000 rpm; 1 minute) using ULTRA-TURRAX (IKA Dispersers T 50 digital ULTRA-TURRAX, Malaysia) and followed by incubation (Memmert Water bath WNB 29, Germany) for 2 hours (24±2.0 °C; 80 rpm). Last, centrifugated (3000×g; 4±0.5 °C; 30 min). The resulting supernatant is called crude protease extract.

SOLUBLE PROTEIN DETERMINATION

The soluble protein content of the samples was determined according to the modified Lowry method developed by Hartree (1972). One mL of protein extract was poured into the reaction tube, and 0.9 mL Lowry A was added. The solution was incubated at a water bath shaker (50±2.0 °C; 10 min; 80 rpm), cooled to room temperature, and treated with 0.1 mL Lowry B. The mixture was incubated at room temperature (10 min). Finally, 3 mL of Lowry C was added and forced (Maxi Mix® II Vortex Mixer M37600 Thermo Fisher Scientific, United Kingdom) to ensure mixing within 1 s.

The solution was incubated (50±2.0 °C; 10 min; 80 rpm) and cooled to room temperature. The absorbance was read in a 3 mL cuvette at 650 nm (Thermo Scientific GENESYS 10S UV-Visible Spectrophotometers, US). Bovine serum albumin (BSA) 100 mg/L was used as a standard stock solution.

TOTAL AMINO ACID

The total amino acid was determined using LC-MS. Five hundred mg of sample was put in a 50 mL screw test tube. Twenty mL HCl 6 N was added and hydrolysed (110±1.0 °C; 12 h). Six N NaOH was used to neutralise the solution and adjust the volume with distilled water to 50 mL. It was diluted and filtered (0.22 µM). Five µL solution was injected into LC-MS. The mobile phase used are A solution (0.1% pentadecafluorooctanoic acid (PDFOA): water; 99.5%:0.5%) and B solution (0.1% PDFOA: water; 10%:90%). Mobile phase flow was set at 0.6 mL/min with a gradient elution system.

PROTEOLYTIC ACTIVITY

The proteolytic activity of crude protease was determined according to Cupp-Enyard (2008). L-tyrosine was used as standard (1.1 mM). Distilled water was used as a blank. Casein solution (0.65%; w/v) for a substrate was made by dissolving casein into potassium phosphate buffer (50 mM; pH 7.5). Next, 5 mL of casein was poured into the reaction tube and incubated with a water bath shaker (37±2.0 °C; 5 min; 60 rpm). Next, 1 mL crude proteases (sample) was added except for the blank and then incubated (37±2.0 °C; 1 min; 60 rpm). Then, 5 mL 110 mM TCA was added to stop the reaction. Next, one mL of crude protease was added to the blank tube to balance the volume of the solution. The solution was incubated (37±2.0 °C; 30 min; 60 rpm). It was finally filtered with filter paper.

Two mL of filtrate (test solution, standard solution, and blank) was prepared. Then, 5 mL Na_2CO_3 500 mM and 1 mL Folin Ciocalteu 50 mM were added, respectively, and incubated in a water bath shaker (37±2.0 °C; 30 min; 60 rpm). Finally, the absorbances were read at 660 nm with spectrophotometry UV-Vis. The absorbance was used to calculate the amount of L-tyrosine produced during the enzymatic reaction. According to the equation below, the L-tyrosine concentration was used to calculate the protease activity.

$$\text{U/mL enzyme: } \frac{(\mu mole\ L\text{-}tyrosine\ obtained)\ x\ (11)}{(1)\ x\ (10)x\ (2)}$$

where (11) is the total volume (mL) of assay; (1) is the time of assay (minute) as per unit definition; (10) is the volume of the enzyme (mL) of enzyme used; and (2) is the volume (mL) used in colorimetric determination.

Proteolytic activity (U/mL) was defined as the amount of tyrosine (µmole/mL) that was released from the substrate (casein) per minute per mL protease at pH 7.5 and 37 °C.

DEGREE OF HYDROLYSIS AND PEPTIDE CONCENTRATION

The evaluation of the degree of hydrolysis and peptide concentration according to Charoenphun et al. (2013). OPA reagent was used as a derivatisation agent, and L-tryptophane (10 mg/mL) was used as a standard. OPA reagent made by dissolving 40 mg OPA to 1 mL methanol and 100 µL β-mercaptoethanol. Then, 25 mL 100 mM $Na_2B_4O_7$ (sodium tetra borate) and 2.5 mL 20% SDS (sodium dodecyl sulphate) were added to the OPA reagent. Distilled water was added and adjusted to 50 mL with a volumetric flask.

Three mL of OPA reagent was added to the 0.4 mL prepared sample. The mixture was incubated at room temperature (dark condition; 20 minutes). The absorbance was read at spectrophotometer UV-Vis (340 nm). The degree of hydrolysis was calculated according to the equation as follows.

$$\%DH = \frac{[(NH2)Tx - (NH2)T0]}{[(NH2)Tot - (NH2)T0]} \times 100$$

where (NH_2)Tx is the hydrolysed sample at x time (test sample); (NH_2)T0: hydrolysed sample at 0 h; and (NH_2) Tot is the fully hydrolysed sample.

A fully hydrolysed sample was obtained by hydrolysing 250 mg sample with 10 mL 6 M HCl containing 0.5% phenol for 4 h at 110 °C, neutralised with 10 mL 6 M NaOH. The solution was centrifuged (3000 rpm; 15 min), and the remaining supernatant was used.

ACE INHIBITORY ACTIVITY

The ACE-I activity assay followed Cushman and Cheung (1971). The peptide extract was used as an ACE-I agent. Fifty µL of the sample (1 mg/mL) and 50 µL substrate solution (50 mM Hipp-His-Leu) were mixed in a 2 mL Eppendorf tube. The distilled water was used to replace the sample for blank and control. At the initial step, the blank solution was added to 200 µL HCl 1 M. For the positive control, captopril, the hypertensive drug, was used. The mixture was incubated in a water bath shaker (37±2.0 °C; 10 min; 50 rpm). Next, 50 µL ACE solution (92.5 mU/mL) was added and incubated for 30 min at a water bath shaker (37±2.0 °C; 50 rpm). The

reaction was terminated with the addition of 200 µL HCl 1 M. Hyppuric acid as a product was extracted with 1.5 mL ethyl acetate. The tube was shaken with a vortex at maximum speed. The tube was centrifuged at 14000 × g for 20 min. One mL of supernatant was removed and placed in other reaction tubes. The supernatant was evaporated using boiling water to dry (± 15 min). Then, 3 mL bidistilled water was added and mixed. The absorbance was read at 228 nm with a spectrophotometer UV-Vis. The inhibitory activity of the sample could be calculated according to the equation below:

$$\%ACE - I\ activity = \frac{(A-B)}{(A-C)} \text{x } 100$$

where A is the absorbance of control solution; B is the absorbance of sample solution; and C is the absorbance of blank solution.

EFFECT OF BLANCHING ON ACE-I ACTIVITY

The ACE-I activity from the highest ACE-I activity sample was evaluated. The water blanched method was used. The sprouts were placed in boiling water (1:3, w/v) for 2, 4, and 6 s. Then, they were rinsed with water to stop the heating process and drained well. Freeze-dried was applied to prepare the sample before ACE-I activity determination.

STATISTICAL ANALYSIS

This study applied a one-factor, completely randomised design with the variation of germination duration as a factor. Every unit experiment was done in three experimental and analysis replications. Furthermore, the data were statistically analysed (IBM® SPSS® Statistics 22.0 software) using ANOVA (Analysis of Variance). Finally, if the factor had a significant effect, post hoc testing was carried out by DMRT (Duncan's Multiple Range Test) (Bower 2013).

RESULTS AND DISCUSSION

SOLUBLE PROTEIN

Figure 2 shows the soluble protein content. The soluble protein content of germinated Lamtoro Gung flour significantly (P<0.05) increased from 24.22±0.81 % DM at the beginning to 34.35±0.69 % DM at 48 h germination. The storage proteins were hydrolysed into small peptides and affected the increase of soluble protein utilised in the seedling development (Kesari & Rangan 2011). The changes insoluble protein might be due to a series of proteolytic activities. Proteases that were activated in the imbibition phase triggered the hydrolysis process of the storage protein, causing the increase of soluble protein (Ratnayani et al. 2019). This result is supported by the proteolytic activity (Figure 3) and the degree of hydrolysis (Figure 4) data.

During the imbibition, the hydration process triggered the activation of proteases to provide the energy sources for seedling development (Ali & Elozeiri 2017; Mayer & Poljakoff-Mayber 1979). The protein breakdown occurred mainly at protein bodies and vacuoles (Shutov and Vaintraub 1987). This finding is in agreement with field beans germination studies. The nitrogen protein decrease, whereas the soluble protein increase on the 4th day of germination (Lichtenfeld et al. 1979). This result is in accord with the previous reports (Kırmızı & Güleryüz 2006), which explained that soluble protein increased initially and peaked on the 3rd day of germination, then decreased on the 7th day.

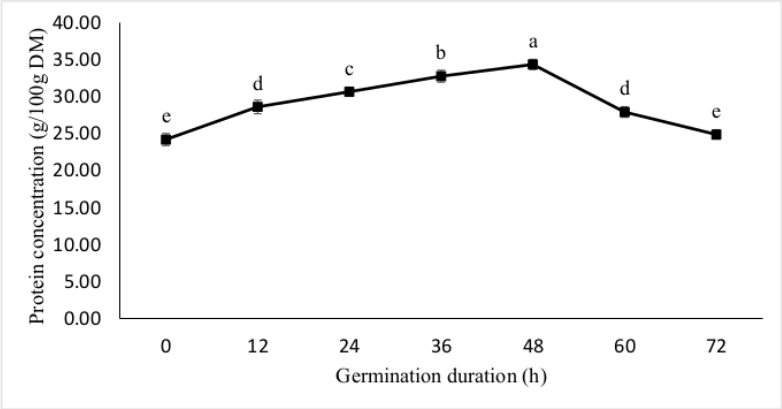

FIGURE 2. Soluble protein content of germinated Lamtoro Gung flour

At the end of germination duration, the soluble protein is reduced. It indicates that soluble protein had been used for seedling development. Kesari and Rangan (2011) reported that 45 days after the imbibition period, the Pongamia pinnata soluble protein content decreased. Its result displayed a similar trend. These results confirm previous findings that the soluble protein content decreased with more extended germination periods (Urbano et al. 2005).

AMINO ACID CONTENT

Lamtoro Gung amino acid changed during germination. At the beginning of germination duration, amino acid concentration was decreased, and the 36 h germinated Lamtoro Gung had the highest value. Table 1 shows the concentration of two groups of amino acids, the hydrophobic amino acid and the negative charge hydrophilic amino acid. The presence of these amino acids

TABLE 1. Amino acid concentration of germinated Lamtoro Gung flour

Amino acid	Amino acid concentration (g/100 g protein DM) during germination						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
Hydrophilic amino acid							
L-Aspartic Acid	6.97±0.33^{a}	0.22±0.00^{e}	0.27±0.01^{e}	5.55±0.17^{c}	5.29±0.05^{c}	4.89±0.14^{d}	0.05±0.00^{e}
L-Glutamic Acid	10.33±0.21^{a}	0.18±0.00^{g}	0.19±0.00^{g}	8.53±0.20^{b}	6.93±0.02^{c}	6.08±0.03^{d}	0.13±0.00^{g}
L-Arginine	10.30±0.37^{a}	7.56±0.15^{b}	7.12±0.03^{c}	2.28±0.03^{g}	6.54±0.08^{d}	4.99±0.14^{f}	0.14±0.02^{g}
L-Cystein	0.16±0.00^{a}	0.13±0.00^{c}	0.12±0.00^{d}	0.12±0.00^{d}	0.11±0.00^{ef}	0.11±0.00^{fg}	0.15±0.00^{b}
L-Glysine	3.44±0.19^{a}	1.12±0.06^{g}	1.12±0.08^{g}	2.83±0.05^{b}	1.99±0.04^{cd}	2.16±0.06^{c}	1.86±0.02^{de}
L-Threonine	2.46±0.07^{a}	1.55±0.03^{c}	2.11±0.07^{b}	2.07±0.01^{b}	1.57±0.00^{c}	1.38±0.03^{de}	0.07±0.00^{e}
L-Thryptophane	0.01±0.00^{a}	0.01±0.00^{a}	0.08±0.00^{b}	0.01±0.00^{d}	0.004±0.00^{e}	0.005±0.00^{e}	0.007±0.00^{cd}
L-Serine	3.26±0.01^{a}	1.33±0.05^{g}	2.34±0.08^{c}	2.58±0.03^{b}	2.32±0.03^{c}	2.06±0.03^{e}	0.02±0.00^{h}
L-Tyrosin	0.18±0.02^{a}	N.D.	N.D.	0.11±0.01^{b}	0.05±0.00^{c}	0.04±0.01^{cd}	0.02±0.01^{de}
L-Histidine	3.61±1.17^{a}	2.02±0.53^{bc}	1.08±0.30^{c}	2.51±0.79^{ab}	1.83±0.34^{bc}	1.62±0.59^{bc}	1.24±0.40^{bc}
L-Lysine	2.69±0.52^{a}	1.86±0.28^{b}	1.08±0.12^{cd}	1.54±0.33^{bc}	1.12±0.23^{cd}	0.79±0.16^{de}	0.31±0.13^{e}
Total negative charge hydrophilic amino acids	17.30	0.40	0.46	14.08	12.23	10.97	0.18
Total hydrophilic amino acids	43.43	15.98	15.46	28.13	27.77	24.14	4.00
Hydrophobic amino acid							
L-Isoleucine	1.50±0.15^{a}	1.10±0.07^{b}	0.80±0.06^{c}	0.74±0.04^{cd}	0.57±0.06^{ef}	0.49±0.00^{efg}	0.61±0.08^{de}
L-Leucine	1.12±0.11^{a}	0.80±0.01^{b}	0.53±0.06^{c}	0.59±0.00^{c}	0.42±0.00^{d}	0.30±0.00^{e}	0.36±0.04^{de}
L-Proline	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-Methionine	0.07±0.00^{a}	N.D.	0.003±0.00^{de}	0.06±0.05^{a}	0.04±0.00^{a}	0.02±0.00^{c}	0.009±0.00^{d}
L-Phenylalanine	0.61±0.05^{a}	N.D.	N.D.	0.42±0.01^{b}	0.25±0.00^{c}	0.17±0.01^{d}	0.23±0.03^{c}
L-Alanine	0.36±0.00^{a}	N.D.	N.D.	0.22±0.00^{cd}	0.21±0.00^{d}	0.28±0.02^{b}	ND.
L-Valine	1.65±0.21^{a}	1.11±0.14^{b}	0.75±0.07^{c}	0.66±0.08^{cd}	0.53±0.06^{de}	0.46±0.04^{d}	0.52±0.00^{def}
Total hydrophobic amino acid	4.9	3.01	2.07	2.69	1.80	1.44	1.73
Total	48.69	18.99	17.52	30.82	29.78	25.86	5.73

*Results are expressed as mean values±standard deviations. Means in a row with different superscripts are significantly different (P≤0.05). N.D.: Not detected

affects the ACE-I activity of Lamtoro Gung. Negative charge hydrophilic amino acids, such as aspartic acid and glutamic acid, are essential to inhibit ACE activity. Those amino acids can build electrostatic interaction with Zn^{2+} located in the catalytic site of ACE and disturb its activity (Durak et al. 2013; Natesh et al. 2003). In addition, hydrophobic amino acids can form hydrophobic interactions with some hydrophobic amino acid residues on the active site of ACE (Fan, Liao & Wu 2018). Accordingly, ACE activity may be decreased.

Thirty-six h germinated Lamtoro Gung flour has the highest value of negative charge amino acids (14.08 g/100 g of protein), followed by the 48 h germinated (12.23 g/100 g of protein). These findings are similar to germinated lentils. The glutamic acid increased during lentils germination, but the aspartic acid decreased (Kuo et al. 2004). Germinated Ethiopian and Syrian black cumin showed the same trend. Aspartic and glutamic acid increased during germination for 2 - 6 days (Mariod et al. 2012). The 36 h germinated Lamtoro Gung flour has 2.69 g/100 g protein hydrophobic amino acids, and the 48 h has 1.81 g/100 g protein. Storage protein degradation by proteolytic enzymes caused the increase of total amino acids during germination. But, decreasing amino acids might be due to amino acid utilisation in seedling growth and development (Mariod et al. 2012).

PROTEOLYTIC ACTIVITY

Figure 3 shows the proteolytic activity of germinated Lamtoro Gung flour. At the beginning (0 h) of germination, proteolytic enzymes were activated, equal to 85.55±1.18 U/g protein. Moreover, the proteolytic activity increased as germination progressed. The imbibition step initiates germination. During imbibition, the seeds are hydrated and reactivate the proteolytic enzymes. Proteolytic activation is intended to hydrolyse storage protein to small peptides used as energy sources during seedling development (Ali & Elozeiri 2017; Mayer & Poljakoff-Mayber 1979). Lamtoro Gung reached the maximum proteolytic activity at 48 h germination (168.79±0.15 U/g protein). It is significantly (P<0.05) different from others.

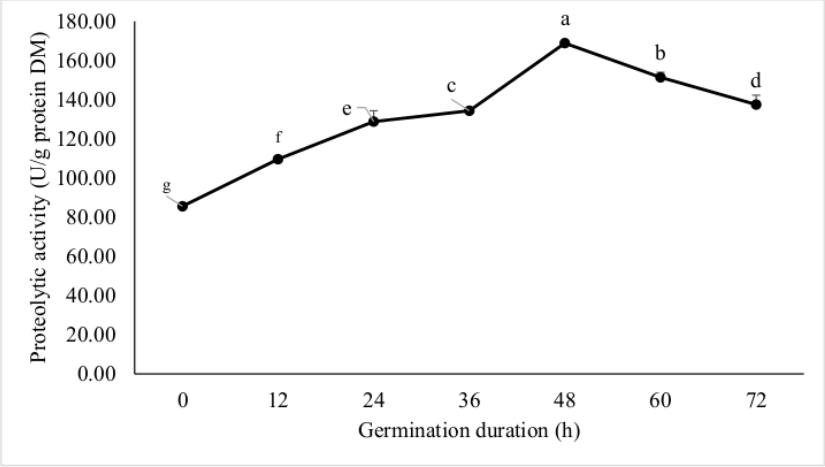

FIGURE 3. Proteolytic activity of germinated Lamtoro Gung flour

The highest proteolytic activity at 48 h germination answers why the soluble protein shows the highest value in that duration. This proves that at 48 h germination, Lamtoro Gung achieves the peak of storage protein hydrolysis. Proteolytic activity increased at the beginning of germination and decreased in the last period. This phenomenon was affected by the types of seeds and the location of the embryonic axis (Ali & Elozeiri 2017; Gepstein & Ilan 1980). A similar trend in the previous study, germination (25 °C; dark condition) of *Phaseolus vulgaris,* showed the increasing protease activity in the first seven days of germination and decreased later

(Gepstein & Ilan 1980). Contrastly, the germination of Indian bean had the maximum proteolytic activity on the 4th day of germination and gradually decreased (Ramakrishna & Rao 2005). However, germinated *Pisum sativum* showed differently. Its proteolytic activity was rapidly enhanced between days 5 - 15 (Basha & Beevers 1975).

DEGREE OF HYDROLYSIS AND PEPTIDE CONCENTRATION

The degree of hydrolysis and peptide concentration can be seen in Figure 4. Germinated Lamtoro Gung for 48 h had the significant (P<0.05) highest DH (23.26±0.15%) (Figure 4). The peptide concentration on 48 h of germination also increased significantly (P<0.05). The highest peptide concentration (70.89±0.26 mg/g DM) was in line with its proteolytic activity (Figure 3) and degree of hydrolysis (Figure 4). Forty-eight h germinated Lamtoro Gung flour shows the highest DH and peptide content. Both of them are closely related to proteolytic activity during germination. The high proteolytic activity triggers hydrolysis and increases its peptide content. Increasing DH and peptide content at 48 h germination explains that storage proteins are hydrolysed and result in simple peptides (Kuo et al. 2004).

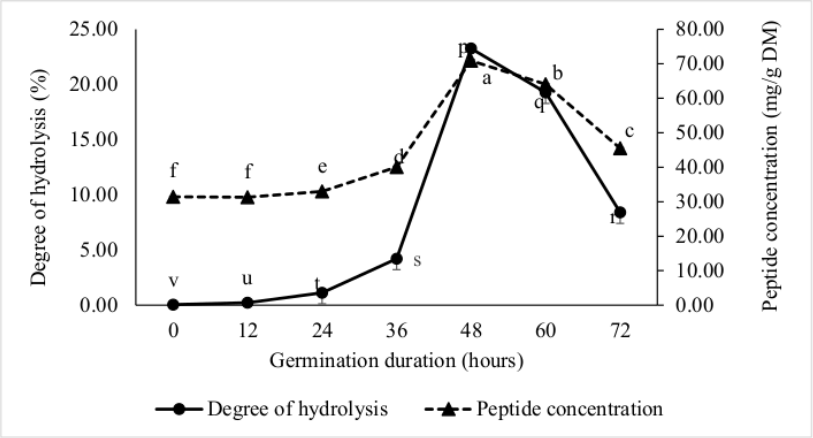

FIGURE 4. Degree of hydrolysis and peptide concentration of germinated Lamtoro Gung flour

This result agrees with Noviyanti et al. (2020), who found an increasing degree of hydrolysis of *melinjo* during 21 days of germination. Lichtenfeld et al. (1979) also found that on the 3rd day of field beans germination, the peak of proteolytic activity was observed, and the storage protein was modified. The increase in peptide concentration is similar to Gulewicz et al. (2008) and Urbano et al. (2005). Germination *Pisum sativum* for 2, 4, and 6 days caused hydrolysis of storage proteins that released peptides and free amino acids. It was observed by reducing the density of polypeptide bands from the storage proteins and lower MW peptides obtained with the increasing germination periods (Urbano et al. 2005).

ACE INHIBITORY ACTIVITY

Lamtoro Gung ACE-I activity increase during a particular germination period. Germinated Lamtoro Gung at 48 h had the significant (P<0.05) highest ACE-I activity (70.62±0.53%) (Figure 5). Significant differences (P<0.05) were noted between and within seeds after and before germination (25.42±0.65%). In addition, the 48 h germinated Lamtoro Gung flour approached the inhibitory activity of captopril (90.06±0.39%).

There was three-point that positively correlated with ACE-I activity. First is the proteolytic activity, the degree of hydrolysis, and the last is the peptide concentration. Maximum DH at 48 h germination confirms the highest proteolytic activity and results in high content of peptides. The high content of peptides clarifies that small peptides were formed a lot. The small peptides can quickly enter the narrow, deep active site of

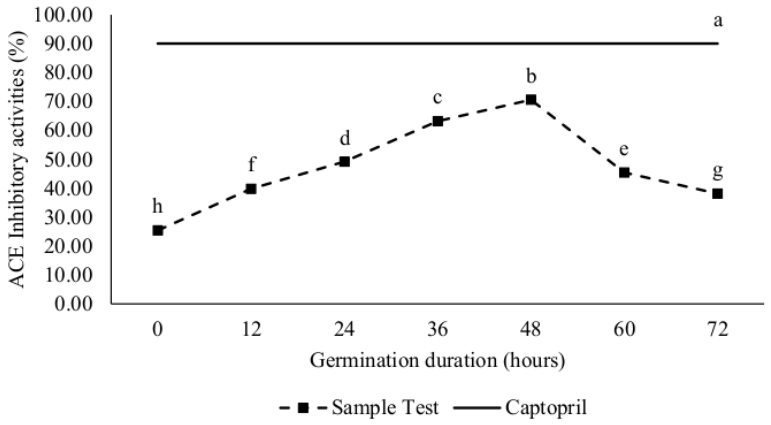

FIGURE 5. ACE-I activity of germinated Lamtoro Gung flour

ACE and inhibit ACE's activity (Fan, Liao & Wu 2018). This result follows the germination of lentils (Bamdad et al. 2009) and black soybean (Mamilla & Mishra 2017). Bamdad et al. (2009) reported that germinated lentils (20 °C; dark condition) had a sharp increment of 80% in ACE-I activity at 72 h germination. Mamilla and Mishra (2017) also found the increase of ACE-I during soybean germination (40 °C; five days; dark condition) to about 83.5%.

EFFECT OF BLANCHING ON ACE-I ACTIVITY

The blanching process applied to the highest ACE-I germinated Lamtoro Gung (48 h germinated) aims to evaluate the effect of cooking on its ACE-I activity. Sprouts are usually freshly consumed, water blanched for a short time, steam blanched, or soaked in the boiling water for a short time. The 48 h germinated Lamtoro Gung has the highest ACE-I activity (70.62±0.53%). It was then blanched to evaluate the effect of blanching on its ACE-I activity.

Figure 6 shows the effect of blanching on germinated Lamtoro Gung ACE-I activity. The longer the cooking time, the lower the sample's ACE-I activity. For example, blanching for 2 s has no significant (P<0.05) difference

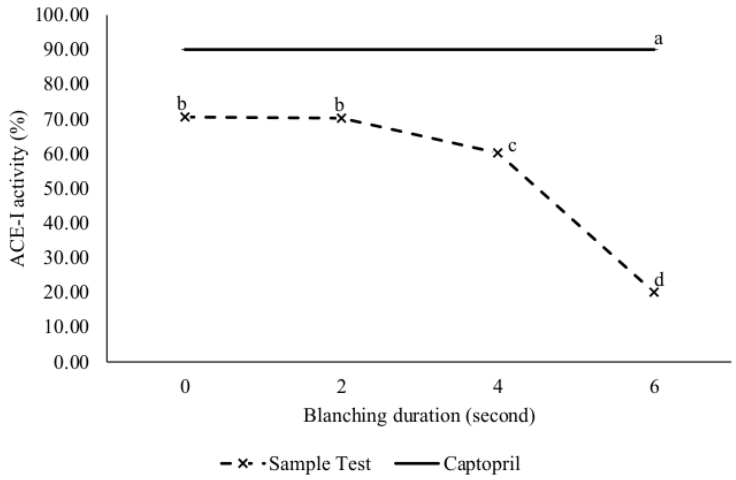

FIGURE 6. ACE-I activity of the 48 h germinated Lamtoro Gung during blanching

compared with the fresh model (70.62±0.53% inhibition); it had 70.29±0.08% inhibition. However, prolonged blanching time showed a negative impact on ACE-I activity. For example, blanching germinated Lamtoro Gung for 4 and 6 s significantly (P<0.05) decreased the ACE-I activity, they are 60.25±0.16% (14.66% reduction) and 20.08±0.24% (71.55% reduction), respectively.

Based on the results, it was found that the ACE-I activity of germinated Lamtoro Gung can be held during the blanching process for 2 s. The reduction of ACE-I activity during blanching was due to 2 possibilities. Those are thermal destruction and peptide leaching (Fellows 2000; Fitriani et al. 2021b; Xiao et al. 2017). Peptides' primary characteristic is labile at high environmental temperatures. Since germinated Lamtoro Gung has many hydrophilic group amino acids (Table 1), this amino acid type is polar and easy to leach to water as a cooking medium. Zhang, Pechan and Chang (2018) also reported that the ACE-I activity of black soybean and black turtle seed decreased during cooking. ACE-I from bovine casein-derived peptides also decreased during heating at 110 and 120 °C for 2 h, about 6.55% and 14.14%, respectively, compared to unheated ones (Wu et al. 2014). In contrast, Escudero, Mora and Toldrá (2014) found that ACE-I peptides from dry-cured ham remained active and had good resistance during heat treatment. ACE-I peptides derived from tuna cooking juice showed good stability at temperatures (20-100 °C) for 2 h (Hwang 2010). Similarly, ACE-I peptides from Koro Kratok tempeh increased after boiling (Pertiwi, Yustinus & Retno 2019). This finding shows that ACE-I peptides from germinated Lamtoro Gung are heat-labile and recommended to consume in fresh form.

Conclusion

Germination of Lamtoro Gung involves reactivated proteases in the peas to hydrolyse the storage protein. Higher proteolytic activity increases the hydrolysis of protein and high molecular peptides to form smaller peptides and increase the soluble protein. This result is confirmed by the degree of hydrolysis and peptide concentration. The low molecular weight peptides act as an ACE-I through their ability to inhibit ACE activity. Forty-eight h germinated Lamtoro Gung had the highest proteolytic activity (168.79 U/g protein DM), DH (23.26%), and ACE-I activity (70.62%). Forty-eight hours of germination of Lamtoro Gung resulted in the highest ACE-I activity. Blanching of germinated Lamtoro Gung for 2 s could hold the ACE-I activity, but the longer duration decreased it.

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