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EFFECT OF GERMINATION ON FREE RADICAL SCAVENGING ACTIVITIES AND ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY OF MELINJO (*Gnetum gnemon* L) SEED PROTEINS

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ABSTRACT

The effect of germination on free radical scavenging activities and Angiotensin I-Converting Enzyme (ACE-I) inhibitory of melinjo (*Gnetum gnemon*) seed proteins was studied to determine its potential use as a nutraceutical ingredient. Investigations into the protein profiles, amino acid composition, free radical scavenging activities and ACE-I-inhibitory of proteins extracted from melinjo seed during germination (0 to 21 days) were carried out. The concentration of amino acids detected in the melinjo seed samples ranged from 0.30 to 12.75 g/100 g protein and the degree of hydrolysis during germination at 21st days was significantly highest than that of either (0-14 days). Furthermore, with all samples a large small-sized peptides degree (<10 kDa) was observed with an increasing degree of hydrolysis value. The free radical scavenging activities were measured using different standard methods, and ACE-I inhibitory activity was determined using in vitro ACE-I inhibitory assay. The time of germination showed a significant effect (p<0.05) on free radical scavenging ($ABTS^{2+}$, DPPH, $O_2^{\bullet}$ and $OH^{\bullet}$) activities and ACE-I inhibitory. The above results indicated that germination time has positively affected ACE-I inhibitory activity and free radical scavenging activities.

Keywords: Melinjo, Protein, Radical Scavenging, ACE–inhibitory, *Gnetum gnemon*, Germination

INTRODUCTION

Bioactive compounds were found in seeds and are known as functional compounds that provide physiological effects, and that can help maintain human health. Some biochemical processes such as germination are considered both simple and economical to improve the nutritive value of seeds by causing desirable changes in the nutrient availability and texture characteristics (**Fernandez et al., 2009**). An extensive breakdown of seed-storage compounds and the synthesis of structural proteins and other cell components take place during the germination. Primary and secondary compounds, many of which are considered beneficial as antioxidants, often change dramatically during the germination. The germination process can make significant changes in the biochemical properties of the seeds due to the activation of enzymatic processes that convert storage macromolecules into nutrients for seed growth. **Sefatie et al., (2013)** reported that the germination process has the potency to produce bioactive peptides directly as a product of the hydrolysis of storage proteins by endogenous proteases in seeds. The protein hydrolyzed is the product of the hydrolysis reaction or the breaking of the peptide bonds in the protein molecule containing a mixture of components of various peptides and free amino acids depending on the degree of hydrolysis, the velocity and degree of hydrolysis by an enzyme influenced by the substrate concentration, enzyme concentration, pH and temperature (**Ramakrishna & Ramakrishna**, **2006**). **Duangmal & Sangsukiam (2015)**, identified and refined small molecular weight peptides (0.5 to 3 kDa) produced at various germination phases of mung beans (*Vigna radiata* (L) Wilczek, sonali b1) by chromatography and ultrafiltration methods. Some researchers had reported the ability of germinated seeds to produce bioactive peptides. **Mamilla & Mishra (2017)** have proved that germination of lentils (*Lens culinaris* Medik cv. Gachsaran) was able to generate bioactive peptide directly and obtained ACE inhibitory peptide extract with activity as much as 84.3%.

Melinjo tree (*Gnetum gnemon* L.) has many benefits because almost all parts of the plant can be utilized as in seed melinjo which can be used as the basic ingredients of making crackers (emping). Indonesian people are generally more familiar with melinjo especially melinjo seeds as vegetables. Melinjo is one of the seeds that have a high protein content of 16-19%, 58% of starch, 16.4% of lipid and 1% of phenolic. Melinjo seed was found two protein major that have activity antioxidants that effectively counteract free radicals (**Siswoyo et al., 2011**). From the potential of melinjo seed protein, yet no further information about protein or peptide ACE-I inhibitor and antioxidant activities during germination of melinjo seeds. Therefore, the experiment was designed to see the effect of germination on the level of ACE-I inhibitor and radical scavenging activities, so that most active stage of the seed is identified for the maximum potential of ACE-I inhibitor and free radical scavenging properties. Furthermore, ACE-I inhibitor and scavenging proteins can be used as a nutraceutical compound.

MATERIAL AND METHODS

Samples and Germination Process

Germination was carried out following the procedure of **Kiran et al. (1991)**. Melinjo seeds were treated to warm stratification to stimulate and accelerate the germination of the seed, the seed skins were discarded, and then the seeds were sterilized with 70% ethanol then washed with aquadest. After drying for 2 h, the seeds were wrapped with a damp cloth filled with chaff and incubated for 3 weeks at 40^{o}C. Every 3 days a once the seeds were sprayed using distilled water during the germination period. Seeds were harvested at 0 (ungerminated), 3rd, 7rd, 14th and 21st germination days (Figure 1). Germinated seeds were frozen at -20^{o}C for 12 h to stop the germination.

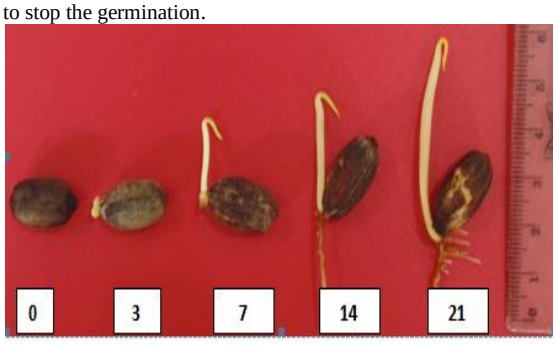

Figure 1 *Gnetum gnemon* seed during germination (days)

Extraction sample

Protein extraction of melinjo seed was done by grounding and homogenized 1 g of fresh seed with Tris-HCl buffer (3 mL, 50 mM, pH 6.8) then centrifuged 10.000 rpm for 15 min, and the supernatant was stored at liquid nitrogen. The supernatant was analyzed for total protein content using the Bradford method (**Bonjoch &Tamayo, 2001**). As much as 5 µL of seed extracts solution were added with 45µL aquadest and then introduced with 950 µL of Bradford solution. Absorbance was measured at a wavelength of 595 nm, and the result was compared with bovine serum albumin (BSA) standard to determine the dissolved protein content.

Degree of Hydrolysis

The determination of degree of hydrolysis (DH) values were using trinitrobenzenesulphonic acid (TNBS) reaction (**Alder-Nissen, 1979**), and the measurement was adapted from **Thiansilakul et al.** (**2007**). The amount of α-amino acids were determined in the sample of the original of the protein extract after acid hydrolysis with 6 M HCl (100ºC, 24 h) and soluble α-amino acid estimated in the hydrolytic solution at the beginning and the end of the reaction. As much as 125 µL of protein seed extract solution was mixed with phosphate buffer (2.0 mL, 200 mM, pH 8.2) and TNBS solution (1.0 mL, 0.1%) and was incubated (50ºC, 30 min) in a water bath. The reaction was stopped by adding Na_2SO_3 (2.0 mL, 0.1M) followed by cooling to room temperature for 15 min. Absorbance was quantified using a spectrophotometer at a wavelength of 420 nm, and the results were compared with L-leucine standard to quantification the dissolved α-amino acid. The degree of hydrolysis was calculated as follows: Degree of hydrolysis (%) = [(At-Ao)/Amax-Ao)] x100, where At is the amount of α-amino acid released at time t; Ao is the amount of free amino acid in the original protein extract; Amax is total amino acid in the original protein extract obtained after acid hydrolysis.

Gel Electrophoresis

Analysis of protein pattern was done by denaturing gel electrophoresis (SDS-PAGE) method performed according to the **Laemmli** method (**1970**). A total of 10 µg protein samples taken from the extracted sample plus 10 µL buffer loading (95% buffer loading plus 5% *β*-mercaptoethanol) were denatured in boiling water for 3 min. The prepared protein samples were then loaded into gel well and electrophoresed for 2 to 4 h. 10 µL of markers were also added to simplify marking of sample protein molecular weight. After removal from the plate sequence, the protein band staining was performed by immersing the electrophoresis gel in a 10% Coomassie brilliant blue solution for 24 h. The excess color was then removed by soaking the gel in destaining solution (50 mL of aquadest, 40 mL of methanol, 10 mL of glacial acetic acid) until the gel becomes clear forming revealing clear bands parted from each other. The bands on the electrophoresis gel were documented.

Determination of Amino Acid

Amino acid hydrolysis was carried out by weighing 0.1 g each of the five samples and added 5 mL of 6N HCl. The hydrolysis process is carried out at 110ºC for 22 h. After the hydrolysis, the mixture was cooled at room temperature then transferred to a 50 mL measuring flask with aquabidest added up to boundary marker and then filtered using a 0.45 µm filter. It was then added with 0.4 mL 50 mM alpha aminobutyric acid (AABA) as an internal testing standard. As much as 20 µL of hydrolysate was injected into the UPLC system (Waters 2475, US) by using AccQ. Tag Ultra C18 µm (2.1 x 100 mm) column with Photodiode Array (PDA) as a detector at 260 nm to identify the amino acid compositions.

Determination of ACE-I Inhibitory Activity

The sample (10 mg/1 mL) was dissolved in demineralized water and analyzed by an ACE-I inhibition assay. The ACE-I inhibitory activity was measured in-vitro as described by **Li et al**. (**2005**). For each test, 20 µL of sample solution of ACE-I Inhibitor with 50 µL of 5 mM Hippuryl-His-Leu (HHL) in sodium borate buffer (100 mM, pH 8,3) containing 300 mM NaCl was pre-incubated at 37ºC for 5 min. The reaction was started by the addition of ACE solution (10 µL, 100 mU/mL), and was incubated at same temperature for 30 min. The reaction was terminated by the addition of HCl (100 µL, 1 M) and then added of sodium borate buffer to a volume of 0.5 mL. Hippuric acid (HA) released from HHL by ACE-I was observed by addition of quinoline and benzene sulfonyl chloride. Finally, the absorbance was quantified by spectrophotometer at wavelength of 228 nm and the results were compared with HA standard. The results are displayed as a mean of triplicate measurements. The IC_{50} value was defined as the peptide concentration that inhibits the 50% of ACE-I activity.

2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Ability

DPPH antioxidant activity was carried out according to the method proposed by **Galvez et al**. (**2005**). About 0.5 mM DPPH solution was dissolved in methanol. A volume of 100 µL supernatant obtained from the sample extract was taken and then added 100 µL methanol and 800 µL of 50 mM DPPH. The solution was then incubated for 20 min and measured its absorbance at 517 nm wavelength.

2,2'-azino-bis (3-ethyl benzothiazole-6-sulfonic acid) (ABTS) Radical Scavenging Ability

The antioxidant activity was assayed by $ABTS^{2+}$ method **Re et al**. (**1999**). The ABTS was generated by the reaction of an ABTS aqueous solution (7 mmol/L) with potassium persulfate (2.45 mmol/L). Before the assay, ABTS was incubated in the dark for 16 h, and the absorbance was 0.7 (734 nm). The assay contained 950 µL of ABTS solution in sodium phosphate buffer saline and the protein extracts in various concentrations. The absorbance was measured at 734 nm.

Hydroxyl Radical Scavenging

Hydroxyl radical scavenging activity was analyzed as described by **Halliwell et al**. (**1987**). As much as 150 µL of protein extract was introduced with 50 µL of 28 mM 2-deoxy-D-ribose (in 20 mM potassium phosphate buffer, pH 7.4), 100 µL of 1 mM Ethylenediaminetetraacetic acid (EDTA), 10 µL of 10 mM Ferric chloride solution ($FeCl_3$), 10 µL of 1 mM Hydrogen peroxide (H_2O_2), 100 µL of 1 mM ascorbic acid and incubated at 37ºC for 1 h. After incubated, the solution was then added with 500 µL of 1% tertiary butyl alcohol (TBA) and 500 µL of 2.8% trichloroacetic acid (TCA) then reincubated for 30 min at a temperature of 80ºC. The absorbance was measured at 532 nm using a spectrophotometer.

Superoxide radical scavenging

Superoxide anion radicals were analyzed by **Tang et al**. (**2010**), 200 µL sample was added into 1.7 mL of 50 mM Tris-HCl pH 8.2 than incubate for 10 min, followed by addition of 100 µL of 10 mM pyrogallol (in 10 mM HCl). Measured slop for 4 min at wavelength 320 nm.

Statistical analysis

The result was expressed as mean values and standard deviation (±SD). The analysis of variance was performed using one-way analysis of variance (ANOVA) and differences between the means of samples were analyzed by Duncan's test at a significance level at $p < 0.05$.

RESULTS AND DISCUSSION

In this study, the content of total protein of melinjo seeds was found about 16.8%. According to **Bhat & Yahya (2014),** the protein content of melinjo ranged from 16 to 19%. Furthermore, the percentage value of total protein extracted can be obtained about 80-90% of the total content of protein. Protein extracted from germinated melinjo seed for different periods of the time were subjected to SDS-PAGE and analyzed degree of hydrolysis (DH). An appreciable increase in DH was observed from 0 to 21 days as shown in Figure 2B. The rate of DH of protein for interval time at 0, 3, 7, 14 and 21 days during the germination period was significantly increased from 0.01 to 25.31%. Protein profiles from different periods of germination by SDS-PAGE yielded two major polypeptides bands with a molecular weight ranging from 100 to 10 kDa, the most prominent being 37 and 12 kDa polypeptides (Figure 2A). During germination, the 37 kDa polypeptide was degraded at 3rd days and the gradual disappearance at 7th days. Several new polypeptide bands formed in a range of 15 to 10 kDa started appearing on the 7th day and further intensified with the progress of the germination.

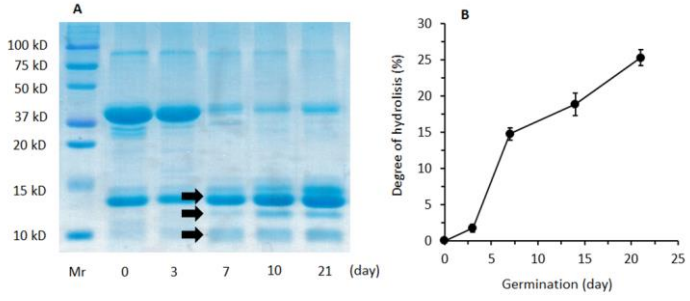

Figure 2 Effect of germination on protein profile of germinated melinjo seed. A. SDS-PAGE profile of germinated melinjo seed protein; B. Degree of hydrolysis of melinjo seed protein during germination. Arrows indicate a new band which is a polypeptide fragment resulting from the germination process.

The change in free amino acid content in the germinated melinjo seeds was shown in Table 1. The free amino acid in melinjo seed at 0 day samples, concentrations of Glu and Arg were the highest, whereas Met and Cys concentrations were relatively low. The variability in Arg Glu and Pro concentrations of the samples during the germination period was relatively very high, as exemplified by the CV= 2.07, 1.37 and 1.19%. Also, the concentrations of Tyr, Leu, Asp, Phe and Ser among the samples were moderately high, as shown by the CV values (in a range 0.42-0.61%).

Table 1 Amino Acid Composition (g/100 g protein) of *Gnetum gnemon* seeds during germination

Amino Acid	Germination Period (day)					CV (%)
	0	3	7	14	21	
Histidine (His)*	1.62	1.53	1.70	1.65	1.50	0.01
Threonine (Thr)	3.66	2.92	3.08	2.92	3.18	0.07
Proline (Pro)	5.11	3.35	2.40	2.22	2.34	1.19
Tyrosine (Tyr)*	5.42	4.55	4.53	4.66	4.25	0.15
Leucine (Leu)	5.90	4.32	4.91	4.28	3.98	0.46
Aspartatic Acid (Asp)	7.15	5.89	5.49	5.36	5.15	0.51
Lysine (Lys)*	4.15	4.30	4.44	4.11	4.24	0.01
Glycine (Gly)	4.41	3.47	3.12	2.91	2.89	0.32
Arginine (Arg)*	8.86	7.24	5.68	5.22	5.10	2.07
Alanine (Ala)	4.02	3.54	3.14	3.21	3.14	0.11
Valine (Val)	4.69	3.82	3.77	3.42	3.29	0.24
Isoleucine (lle)	3.12	2.65	2.88	2.63	2.52	0.05
Phenylalanine (Phe)	3.78	2.06	2.19	2.22	2.19	0.42
Glutamic Acid (Glu)	12.75	11.74	10.32	10.31	9.48	1.37
Serine (Ser)	5.35	4.86	3.75	3.49	3.42	0.61
Methionine (Met)*	0.92	0.86	0.81	0.82	0.88	0.00
Cysteine (Cys)*	0.65	0.44	0.47	0.40	0.46	0.01
TOTAL AA (g/100 g protein)	81.56	67.54	62.68	59.83	58.01	

Legend: *antioxidant amino acid grouping ; CV = coefficient of variation.

Table 2 shows the total amino acid (TAA), total antioxidant amino acid grouping (TAntAA) and ratios in different germination period of the melinjo seeds. The amount of amino acid composition in germinated melinjo seeds was presented as TAA. The different compositions of amino acids in the germinated melinjo seeds (0-21 days) reflected the different amounts of TAA. The ungerminated period (0 days) contain more TAA than did either (3-21 days), in a range of 14.02–23.55 g/100 g protein. The amount of TAA in the samples was several dominated by the composition of Lys, Arg and Tyr are similar to the antioxidant amino acid group reported by **Xu et al**. (**2017**). The amount of those antioxidant amino acids is presented as TAntAA. Similar to the TAA value, ungerminated seeds contain higher TAntAA than do either, in a range of 2.70–5.19 g/100 g protein. This is also represented in the ratio of TAntAA/TAA, in which ungerminated (0 days) has a ratio of 0.265, a lower value than the either (3-14 days) and the germinated melinjo seeds at 21st days with its high TAntAA/TAA ratio about 0.283, had more potent antioxidant activity than either. The result of the germination process, melinjo seeds undergo considerable metabolic changes in their storage proteins. Germination is a natural biological process of all superior plants by the seed comes out its latency stage, once the minimal environmental conditions needed for its growth and development, such as humidity, temperature, nutrients (**Sangronis et al., 2007**).

Table 2 Average amino acid groupings of *Gnetum gnemon* seed during germination and some ratios

Groups	Germination Period (day)					CV (%)
	0	3	7	14	21	
TAA (g/100 g protein)	81.56	67.54	62.68	59.83	58.01	0.14
TAntAA (g/100 g protein)	21.62	18.92	17.63	16.86	16.43	0.11
TAntAA/TAA (ratio)	0.265	0.280	0.281	0.282	0.283	0.03

Legend: TAA=Total Amino Acid; TantAA=Total Amino Acid Antioxidant; CV = coefficient of variation.

Free Radical Scavenging and ACE-I inhibitory Activities

The results obtained showed that during the germination process the seeds of melinjo with the long period of germination have antioxidant activity in IC_{50} that tends to decrease in $ABTS^{2+}$, DPPH, and $O_2^{\bullet}$, but not with $OH^{\bullet}$ which increases after the 14th days germination period, as listed in Table 3. According to **Donkor et al**. (**2007**) that higher antioxidant activity as shown in low IC_{50} is likely to be a contribution to each phytochemical compound and as a result of metabolic activity. The germination process can make significant changes in the biochemical properties of the seeds due to the activation of enzymatic processes that convert protein storage into bioactive peptides. The protein hydrolyzed is the product of the hydrolysis reaction or the breaking of the peptide bonds in the protein molecule containing a mixture of components of various peptides and free amino acids depending on the degree of hydrolysis. **Chen et al**. (**2017**) reported that the degree hydrolysis of protein storage high related with increased of antioxidant capacity on the germination seed and they have observed antioxidant activity in hydrolyzed proteins (small size peptide) due to the exposure of amino acids, resulting in high free radical scavenging activity, reducing power, inhibition of lipid oxidation and metal chelation capacity. The variety of peptides or free amino acid seed *Gnetum gnemon* was higher than the rest of the seeds and comparable to germinate, this can be attributed to the differential activity of hydrolases and oxidase during germination and differences in seed matrices control in antioxidant capacity.

Table 3 The IC_{50} value of Antioxidant and ACE-I Inhibitor Activities[#]

Antioxidant Activity	Germination Period (day)				
	0	3	7	14	21
DPPH (mg/mL)	2.63 ± 1.05^{a}	2.38 ± 0.38^{ab}	1.85 ± 0.87^{ab}	1.65 ± 0.56^{ab}	1.30 ± 0.30^{bc}
$ABTS^{2+}$ (mg/mL)	0.62 ± 0.12^{a}	0.32 ± 0.15^{ab}	0.19 ± 0.06^{ab}	0.013 ± 0.002^{ab}	0.006 ± 0.001^{bc}
$O_2^{\bullet}$ (mg/mL)	9.12 ± 0.12^{a}	3.17 ± 0.17^{b}	2.17 ± 0.10^{bc}	2.77 ± 0.23^{cd}	1.32 ± 0.33^{d}
$OH^{\bullet}$ (mg/mL)	1.91 ± 0.13^{d}	1.30 ± 0.14^{bc}	1.05 ± 0.19^{ab}	0.73 ± 0.14^{a}	1.58 ± 0.13^{c}
ACE-I (µg/mL)	13.23 ± 0.92^{d}	9.65 ± 0.57^{cd}	8.69 ± 0.31^{c}	7.02 ± 0.42^{b}	1.14 ± 0.55^{a}

Legend: [#]The values are means of followed by standard deviation and Duncan's test at $p < 0.05$, where the same letter in a column is not significantly different.

Angiotensin-I converting enzyme assay was performed to evaluate ACE-I inhibitory activity by bioactive proteins produced during the germination process (**Magana et al., 2007**). The results obtained for the inhibition of ACE-I (IC_{50}) at the start of germination were 13.23 µg/mL at the beginning of germination and improved to 21st days by 1.14 µg /mL. This suggests that with decreasing IC_{50} ACE-Inhibitors, there is an increase in the formation of bioactive proteins during the germination process and reaches an optimal value on the 21st days of the germination process. This suggests that relatively fewer specific peptides are produced during the germination process and undergo further cleavage into smaller peptides and more bioactive peptides during the 21st days germination process. Further protein during the longer germination process results in smaller peptides and fewer bioactive peptides. *Gnetum gnemon* germination process may change the pattern of proteolysis which then form and deactivate proteins with anti-ACE-I activity (**Meisel, 1997**).

CONCLUSION

During the process of germination melinjo seed (*Gnetum gnemon*) a decrease in the value of amino acid due to a process of protein storage hydrolysis which resulted in small-sized peptides with strongly inhibit of the ACE-I and free radical scavenging activities. The results indicated that germination time has positively affected on free radical scavenging activities and ACE-I inhibitory activity.

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