Food Science and Technology

Ciência e Tecnologia de Alimentos

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Revealing of free radical scavenging and angiotensin I-converting enzyme inhibitor potency of pigmented rice seed protein

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Abstract

e potential source of bioactive protein from pigmented rice seed was investigated by bu ered extracting from nine varieties (Merah-SP, Gogo Niti-2, Merah Wangi, Super Manggis, Lamongan-1, Mota, Ketan Hitam-2, Beureum Taleus, and Aek Sibundong) and non-pigmented rice (IR-64) as a control. e potent contributor to free radical scavenging of extracted proteins was evaluated by analyzing their free amino acid composition. e free radical scavenging and angiotensin-I converting enzyme (ACE-I) inhibitory activity of these proteins were analyzed *in-vitro*. e free radical scavenging activity was analyzed using various standard methods, including radical cation 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonate acid ($ABTS^{•+}$), hydroxyl radical ($OH^{•}$) and defense against radical-mediated DNA damages by hydroxyl. Total free amino acid content ranged between 77.7-181.2 g/100 g protein, and among them, Ketan Hitam-2 displayed the highest abundant antioxidant amino acid grouping (23.23%) and shown signi cantly higher $ABTS^{•+}$ activity (IC_{50}=8.64 µg/mL) and $OH^{•}$ activity (IC_{50}=20.33 µg/mL). Moreover, Ketam Hitam-2 also exhibited notable ACE-I inhibitory activity (IC_{50}=6.20 µg/mL) and protected hydroxyl-induced oxidative damage to DNA. e *in-vitro* systems for free radical scavenging and ACE-I inhibitory were used to acquire the data. e potency of Ketan Hitam-2 seed protein could be utilized as a natural nutraceuticals compound.

Keywords: ACE-I inhibitory; free radical scavenging; pigmented rice; protein.

Practical Application: e pigmented rice seed proteins have a high potential for a human nutraceutical health supplement.

1 Introduction

Rice (*Oryza sativa* L., family: *Poaceae*) is an important cereal crop in the global area. Rice is mainly used as a major staple globally, including in China (143.790 metric tons), India (100.000 metric tons), and Indonesia (38.100 metric tons), according to Global Rice Consumption 2018/2019 (Shahbandeh, 2019). In the Indonesian Center for Rice Research, the germplasm of rice consists of 2095 accession of local rice, 804 accessions of introduction rice, and 270 varieties of superior rice. However, information regarding pigmented rice varieties are inadequate. Farmers identi ed the name brie y based on the colors; black rice, red rice, and black glutinous rice.

Pigmented rice naturally contains higher anthocyanin, protein, and polyphenols than non-pigmented rice (Sati & Singh, 2019). Previous studies have evaluated physicochemical properties (Murdifin et al., 2015), Nuclear Magnetic Resonance (NMR) based on metabolomes information (Wijaya et al., 2018), and morphological variation (Shinta et al., 2014) of pigmented rice in Indonesia. However, further explorations of pigmented rice in Indonesia are necessary to reveal its health bene ts for reducing the risk of human disease. e presence of bioactive compounds in pigmented rice can act be as a scavenger to free radicals caused by oxidative stress leading to cell damage.

e usage of naturally sourced bioactive compounds as an alternative drug is in demand to reduce the dependency on synthetic chemical compounds (Jamshidi-Kia et al., 2018). A plant can produce bioactive peptides as nutrient supplements which have health bene ts as an antioxidant, anti-in ammatory, and antihypertensive agents (Zou et al., 2020). Pigmented rice is well known in possessing high antioxidant capacity in which it can e ectively prevent the unnecessary in ammatory response. e previous report on Lingkang taker and Umling ame of Indian pigmented rice varieties showed antioxidant constituents such as gallic acid, salicylic acid, quercetin, ferulic acid, ca eic acid, apigenin, and anthocyanin, which was proposed to possess a potency in preventing radical-induced related diseases, including hypertension (Samyor et al., 2016).

Bioactive peptides as antihypertensive agents generally composed of short amino acids structure, which is described by the presence of hydrophobic, aromatic, positive charged, and aliphatic amino acids (Rai et al., 2017). Also, the presence of aromatic amino acids in the C-terminus, positively charged amino acids in the middle,

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and hydrophobic amino acids in the N-terminus of the peptides demonstrated high potential as an ACE inhibitor (Wijesekara et al., 2011). According to (Ahn et al., 2014), a bioactive peptide composed of more than 60% of hydrophobic amino acid could increase the antioxidant activity by transferring electrons from the benzene ring to the radical electrons. However, the anti-oxidative e ects of bioactive compounds in local Indonesian pigmented rice remain unclear. erefore, this study aimed to investigate the antioxidant activity of bioactive peptide of pigmented rice and its role as ACE-inhibitors, which attributed to preventing or delaying hypertension progression.

2 Materials and methods

2.1 Plants collection and reagents

e nine varieties of pigmented rice seeds (Merah-SP, Gogo Niti-2, Merah Wangi, Super Manggis, Lamongan-1, Mota, Ketan Hitam-2, Beureum Taleus, Aek Sibundong) and non-pigmented rice seeds (IR-64) as control were cultivated with conventional plantation measure and their seeds were harvested on August 2019 at the Center of Excellence on Crop Industrial Biotechnology (PUI-PT BioTIn) in Agrotechno Park Research Area, University of Jember, Jember, East Java, Indonesia. e reagents used in this study are: bovine serum albumin (BSA), hydrogen peroxide (H_2O_2), 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$), pyrogallol, thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2-deoxy-D-ribose, ACE Kit (Dojindo Molecular Technology), glutathione (G-SH), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich, Singapore and other supporting chemicals were analytical grade product from Merck Co., USA.

2.2 Amino acid compositions analysis

Amino acid hydrolysis was analyzed by the method of (Szkudzińska et al., 2017). e rice samples were weighing (0.1 g), and HCl (6N, 5 mL) was added. e hydrolysis process is conducted at 110 °C for 22 h. e mixture was cooled at room temperature a er the hydrolysis, then transferred to a measuring ask (50 mL) with aquabidest added up to boundary marker and then ltered using a lter of 0.45 micron. Into the supernatant, alpha aminobutyric acid (50 mM, 0.4 mL) was added as an internal testing standard. A portion of hydrolysate (20 µL) was injected into a thermostat, autosampler, high-pressure binary pump and photodiode array detector (PDA) in the UPLC system (Waters 2475, US). e chromatographic separation was achieved using the AccQ-Tag Ultra C-18 column (2.1 x 100 mm; 1.7 µm). e column heater was set at 55 °C, and the mobile phase ow rate was maintained at 0.7 mL/min. Eluent A was 10% AccQ•Tag Ultra concentrate solvent A, and eluent B was 100% AccQ•Tag Ultra solvent B. e non-linear separation gradient was 0-0.54 min (99.9% A), 5.74 min (90.0% A), 7.74 min (78.8% A), 8.04-8.64 min (40.4% A), 8.73-10 min (99.9% A). A VanGuard™ Waters column (2.1 mm i.d. × 5 mm, 1.7 µm particles) was used as the guard column. One microliter of the sample was injected for analysis. e PDA detector was set at 260 nm to classify the composition of the amino acids, with a sampling rate of 20 points/sec.

2.3 Protein extraction

Rice protein extraction was performed by grounding and homogenizing fresh seed (1 g) with a phosphate bu er (3 mL, 50 mM, pH 6.8). e mixture then centrifugated at 10.000 rpm for 15 min, in which its supernatant was then preserved under liquid nitrogen. e total protein content of the supernatant was measured using the Bradford method (Bonjoch & Tamayo, 2001). Into a portion of the sample (5 µL), aquadest (45 µL) was added prior to Bradford solution addition (950 µL). In order to determine the dissolved protein content, absorbance was recorded at a wavelength of 595 nm, and the result was compared with the BSA standard.

2.4 ABTS•+ radical scavenging activity assay

e free radical scavenging activity was performed based on the $ABTS^{\bullet+}$ activity, using the method Karami et al., (2019). e radical cations were prepared by mixing ABTS solution (7 mM, 1 mL) with potassium persulfate (2.45 mM, 1 mL) followed by incubation for 12-16 h in the dark place. e ABTS working solution was diluted with phosphate saline bu er (0.2 M, pH 7) to produce an absorbance of 0.700-0.750 at the wavelength of 734 nm. e photometric assay was conducted on ABTS solution (950 µL) and protein extracts (20 µg), in which the absorbance was recorded at a wavelength of 734 nm. e antioxidant activity of the tested samples was calculated by using the following Formula 1:

$$/\%(\,)\ ABTS^{\bullet 2} : \left(/Ac\ 4\ As(\,)/Ac\right)\ x\ 100\% \quad (1)$$

where is Ac = absorbance control, and As = absorbance sample.

2.5 Hydroxyl radical scavenging activity assay

Hydroxyl radical scavenging activity was evaluated through the analytical protocol described by (Hazra et al., 2008). Hydroxyl radical was generated by mixing 2-deoxyribose (28 mM, 50 µL), $FeCl_3$ (10 mM, 10 µL), EDTA (1 mM, 100 µL), H_2O_2 (1 mM, 10 µL), ascorbic acid (1 mM, 100 µL), and protein sample (20 µg). e mixture was then diluted until the nal volume of 1 mL with a phosphate bu er (pH 7.4) and was incubated at 37 °C for 1 h. Into the mixture, TBA (1%, 500 µL) and TCA (2,8%, 500 µL) were added. e mixture was incubated for 30 min at 80 °C, and its absorbance at a wavelength of 532 nm was recorded. Percentage inhibition was assessed by comparing the sample absorbance with blank solution sample by using the following Formula 2:

$$/\%(\,)\ Hydroxyl : \left(/Ab\ 4\ As(\,)/Ab\right)\ x\ 100\% \quad (2)$$

where is Ab = absorbance blank and As = absorbance sample.

2.6 Angiotensin I-Converting Enzyme (ACE-I) inhibitor activity assay

e activity of ACE-I inhibitor was performed using the ACE Kit (Dojindo Molecular Technology). Crude protein sample (20 µg) was loaded into a sample well followed by the addition of substrat buffer and enzyme working solution. The mixture was than incubated for 1 h at 37 °C, which was then an indicator working solution (200 µL) applied. The mixture was then re-incubated for 10 min at room temperature, followed by recording the absorbance at wavelength of 450 nm under a microplate reader system (ZN-320, Zenix). Blank 1 (positive control) was made without a sample solution, and blank 2 was made without a sample solution and enzyme working solution. ACE inhibition was evaluated by the following Formula 3:

$$\left(\left(Ab_1 - As\right) / \left(Ab_1 - Ab_2\right)\right) x\ 100\% \qquad (3)$$

where is Ab_1= absorbance blank 1, Ab_2 = Absorbance blank 2, and As = absorbance sample and the result was compared with the captopril as reference drug.

2.7 Protective DNA assay

Protective DNA was analyzed using the method described by (Siswoyo et al., 2011). The *p*BT7 plasmid from the collection of Nutraceutical and Pharmaceutical Laboratory, Center for Development of Advanced Science and Technology, University of Jember, Jember, East Java, Indonesia was used. The DNA plasmid (2.5 µg) was treated by a Fenton's reagent (80 mM $FeCl_3$, 30 mM H_2O_2, and 50 mM ascorbic acid) until the final volume of 1 mL and was incubated at 37 °C for 15 min. The protein samples were then added and made the final volume up to 20 µL with ddH_2O. The mixture was then incubated at 37 °C for 15 min. The mixture then running to 1.5% gel electrophoresis and was visualized through the gel documentation system (Major Science, USA).

2.8 Statistical analysis

All values are expressed as the mean of three replicates ± standard deviations (SD). Each data was analyzed statistically using analysis of variance (ANOVA). Duncan's multiple range tests with a significance level of $p<0.05$ were followed. The SPSS® 20.0 Package (IBM Cooperation, Chicago, USA) was used throughout.

3 Results

3.1 Variation shapes and color morphology of rice seed

The rice seeds of each variety were hulled to determine the variation of shape and color morphologies. The results showed that rice seed of each variety has variation in the shape and color. Visually, the shape could be determined as slender, medium, and round. Whereas, the color of the seeds varies from brown to black (Figure 1).

3.2 Variation and ratio of antioxidant amino acid to total amino acid

The seventeen amino acid compositions of each rice were analyzed by UPLC. The data were analyzed by the coefficient variance (CV) value. The CV value is used to determine the percentage of data variability, and the high CV values indicate a wide variety of data. The results showed that the CV value of each amino acid compositions of nine rice is between 19.91-59.52% (Table 1). It was indicated that there is a high variation of amino acid concentrations among rice varieties.

It was known that arginine, lysine, tyrosine, histidine, cysteine, and methionine are the amino acids that correlate with antioxidant activity. The ratio of antioxidant amino acid group to a total amino acid of each variety was analyzed. The results showed the highest ratio of antioxidant amino acid to total amino acids found in Ketan Hitam-2, followed by Beureum Taleus, 23.23%, and 23.17% (Table 1). It was indicated that Ketan Hitam-2 and Beureum Taleus rice seed has a high potency of antioxidant.

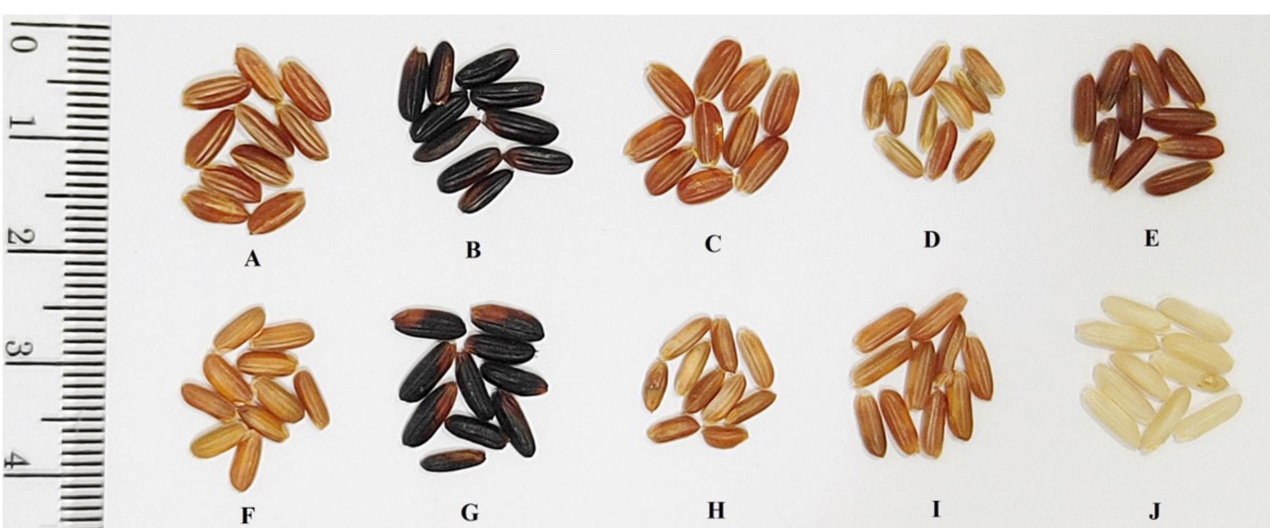

Figure 1. Variation shape and color morphology of pigmented (A-I) and non-pigmented (J) rice seeds. (A) Merah-SP; (B) Gogo Niti-2; (C) Merah Wangi; (D) Super Manggis; (E) Lamongan; (F) Mota; (G) Ketan Hitam-2; (H) Beureum Taleus; (I) Aek Sibundong; (J) IR-64.

Table 1. The amino acid composition of rice seed protein.

Amino acid*	Variety										Mean	SE	CV (%)
	A	B	C	D	E	F	G	H	I	J			
L-Arginine**	9.34	14.22	9.72	12.61	11.76	16.60	10.36	9.96	7.48	10.56	11.26	0.83	23.44
L-Aspartic acid	7.56	10.96	8.61	10.24	9.35	15.72	7.73	7.89	5.90	8.56	9.25	0.85	29.02
L-Cysteine**	0.40	0.67	0.49	0.63	0.53	0.80	0.65	0.55	0.40	0.57	0.57	0.04	21.85
L-Glutamic acid	16.18	24.43	19.02	23.02	20.71	33.32	16.38	16.89	12.40	18.58	20.09	1.84	28.96
L-Histidine**	2.89	4.57	3.38	4.10	3.52	5.20	3.23	3.47	2.58	3.60	3.65	0.25	21.34
L-Lysine**	3.75	4.55	5.30	5.32	4.33	6.71	3.77	3.95	2.86	4.64	4.52	0.34	23.71
L-Serine	6.37	9.61	7.55	8.82	7.58	10.45	6.45	7.26	5.41	7.72	7.72	0.49	19.91
L-Threonine	4.19	6.73	5.07	5.96	5.81	7.40	4.64	4.71	3.64	5.30	5.35	0.36	21.56
L-Alanine	5.53	8.23	6.61	7.70	6.72	10.33	5.76	5.82	4.29	6.42	6.74	0.53	24.93
L-Phenylalanine	5.76	9.64	6.13	8.90	7.90	12.40	6.62	6.84	5.13	7.10	7.64	0.69	28.40
L-Tyrosine**	4.23	6.73	4.06	6.62	5.77	8.42	4.98	5.70	3.51	4.75	5.48	0.47	27.14
Glycine	5.45	8.22	6.29	7.34	6.47	8.90	5.68	5.79	4.42	6.42	6.50	0.42	20.57
L-Isoleucine	4.59	6.89	5.16	6.46	5.73	8.07	4.67	4.64	3.52	5.19	5.49	0.42	24.20
L-Leucine	9.19	14.19	10.36	13.20	11.91	16.55	9.44	9.19	7.08	10.47	11.16	0.89	25.25
L-Methionine**	0.01	0.86	0.78	0.51	0.68	0.70	0.96	0.62	0.37	0.02	0.55	0.10	59.52
L-Valine	6.68	10.07	7.63	9.22	8.41	11.52	6.97	6.60	5.09	7.57	7.98	0.60	23.60
L-Proline	4.69	6.96	5.42	6.44	5.72	8.13	4.80	4.80	3.62	5.39	5.60	0.41	23.12
TAA (g/100 g Protein)	96.81	147.53	111.58	137.09	122.9	181.22	103.09	104.68	77.7	112.86	119.55	9.29	24.58
TAAAnt (g/100 g Protein)	20.62	31.60	23.73	29.79	26.59	38.43	23.95	24.25	17.20	24.14	26.03	1.89	23.01
TAAAnt /TAA (%)	21.30	21.42	21.27	21.73	21.64	21.21	23.23	23.17	22.14	21.39	21.85	0.24	3.49

A: Merah-SP; B: Gogo Niti-2; C: Merah Wangi; D: Super Manggis; E: Lamongan-1; F: Mota; G: Ketan Hitam-2; H: Beureum Taleus; I: Aek Sibundong; J: IR-64; SE: Standard Error; CV: Coefficient of Variance; TAA: Total Amino Acid, TAAAnt: Total Amino Acid Antioxidant.
*g/100 g protein; **Antioxidant amino acid grouping.

3.3 Free radical scavenging activity of pigmented rice seed protein

Free radical scavenging activity was analyzed by ABTS•+ and OH• assay. The results showed that the IC_{50} value of all pigmented rice seed protein was significantly lower than non-pigmented rice (control). The lowest IC_{50} value of $ABTS^{•+}$ scavenging activity was shown in the seed protein of Ketan Hitam-2 (8.64 µg/mL), while the IC_{50} value of G-SH as positive control is 0.22 µg/mL (Table 2). It was indicated that the seed protein of Ketan Hitam-2 has the capability to scavenge radical.

The IC_{50} value of $ABTS^{•+}$ and $OH^{\cdot}$ scavenging of seed protein of Ketan Hitam-2 variant is significantly less than other pigmented rices. The IC_{50} rate of $OH^{\cdot}$ scavenging of the seed protein of Aek Sibundong variant has the same significance as the seed protein of Ketan Hitam-2 variant. The low IC_{50} value of $ABTS^{•+}$ and $OH^{\cdot}$ scavenging in seed protein of Ketan Hitam-2 and Aek Sibundong indicates a potential antioxidant agent as cellular protection against oxidative stress (Table 2). Also, the oxidative DNA damage protection against hydroxyl radicals was evaluated by a fenton's reagent. Results suggested that the *p*BT7 plasmid incubation of in fenton's reagent for 15 min resulted in supercoiled (SC) cleavage to create the open circular (OC) form (Figure 2), indicating that single-strand DNA breaks were created by the hydroxyl radical formed by the fenton reaction. As shown in Figure 2, extra protein does not always respond to DNA damage. However, the addition of Ketan Hitam-2 seed protein may decrease SC to OC conversion. Its ability is almost the same as shown by G-SH as the positive control.

Table 2. The free radical scavenging activities of pigmented rice seed protein.

Variety	Free Radical (*IC_{50}, µg/mL)	
	$ABTS^{•+}$ (µg/mL)	Hydroxyl (µg/mL)
Merah-SP	45.66 ± 0.93^{c}	25.30 ± 0.53^{d}
Gogo Niti-2	47.34 ± 1.15^{c}	33.08 ± 0.26^{a}
Merah Wangi	44.63 ± 1.09^{c}	23.43 ± 0.15^{f}
Super Manggis	43.99 ± 0.24^{c}	22.11 ± 0.17^{g}
Lamongan-1	61.62 ± 1.02^{b}	24.44 ± 0.15^{e}
Mota	43.61 ± 0.34^{c}	30.30 ± 0.19^{b}
Ketan Hitam-2	8.64 ± 0.68^{e}	20.33 ± 0.23^{i}
Beureum Taleus	36.87 ± 0.35^{d}	20.90 ± 0.18^{h}
Aek Sibundong	63.66 ± 1.94^{b}	20.35 ± 0.17^{i}
IR-64	72.53 ± 1.67^{a}	29.47 ± 0.23^{c}
G-SH	0.22 ± 0.08^{f}	8.24 ± 0.12^{j}

*IC_{50} value (µg/mL) is the effective concentration of antioxidants to inhibit 50% radical ABTS and hydroxyl. Glutathione (G-SH) was used as a positive control. All values are expressed as the mean of three replicates ± standard deviations (SD), the Duncan's multiple range tests with a significance level of $p<0.05$ were followed. The different letters in the column are represented significantly different.

3.4 Angiotensin I-Converting Enzyme (ACE-I) inhibitory activity

This study also investigated each pigmented rice seed protein's potency to inhibited the activity of ACE-I. The crude protein of each variety has been analyzed *in-vitro* using the ACE kit. The IC_{50} value of the Ketan Hitam-2 seed protein is significantly less than other seed proteins of pigmented rice

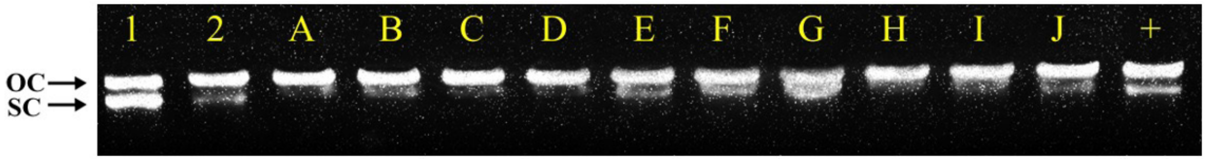

Figure 2. Inhibitory effect of pigmented rice seed protein on DNA damage treat by hydroxyl radicals. DNA damage was introduced by mixing the fenton reagent into the plasmid DNA for 0 and 15 minutes (lanes 1 and 2, respectively). Seed protein of Merah SP, Gogo Niti 2, Merah Wangi, Super Manggis, Lamongan, Mota, Ketan Hitam-2, Beureum Taleus, Aek Sibundong, and IR-64 (lanes A-J respectively) and Gluthatione as a positive control (lane +). OC: Open Circular; SC: Supercoiled.

Table 3. The ACE-I inhibitor activities of pigmented rice seed protein.

Variety	ACE-I inhibitor (*IC_{50}, µg/mL)
Merah-SP	15.75 ± 0.04^{f}
Gogo Niti-2	7.66 ± 0.63^{e}
Merah Wangi	6.39 ± 0.06^{c}
Super Manggis	6.76 ± 0.05^{d}
Lamongan-1	6.31 ± 0.02^{c}
Mota	7.05 ± 0.02^{e}
Ketan Hitam-2	6.20 ± 0.05^{b}
Beureum Taleus	17.30 ± 0.11^{g}
Aek Sibundong	26.60 ± 0.14^{i}
IR-64	21.63 ± 0.02^{h}
Captopril	2.08 ± 0.20^{a}

*IC_{50} value (µg/mL) is the effective concentration of antioxidants to inhibit 50% ACE-I activity. Captopril was used as a positive control of ACE-I. All values are expressed as the mean of three replicates ± standard deviations (SD), the Duncan's multiple range tests with a significance level of $p<0.05$ were followed. The different letters in the column are represented significantly different.

(Table 3). This result demonstrated that the seed protein of Ketan Hitam-2 has the highest capability to inhibit the ACE-I enzyme than other pigmented rice, therefore this variant was selected as an antihypertensive compound. Nevertheless, the activity still three folds higher than the control (IC_{50} value of captopril is 2.08 µg/mL).

4 Discussion

Previous reports showed rice to contain relatively low amounts of proteins within the range of 4-10% (Balindong et al., 2018). This protein content considerably varies among different varieties and cultivar depending on climatic, adaptive, and growth conditions as well as a level of maturity at harvest (Dabi & Khanna, 2018). Compared to non-pigmented rice, pigmented rice varieties tendentially have higher protein content with a well-balanced composition of amino acids (Samyor et al., 2017). The amount of amino acid in each variety of pigmented rice is very different in which CV value are ranging from 19.91% to 59.52% (Table 1) and could represent different potencies. The amino acid profiling in this current study (Table 1) showed higher abundant of L-tyrosine and L-methionine amino acids in pigmented rice. Previous research reported tyrosine and methionine have positive effects on antioxidant capacity due to its structures. Tyrosine have capability to act as hydrogen transferor, while methionine tends to oxidize methionine sulphoxide (Karami et al., 2019). In addition, the amino acid component was previously reported to have high correlation with radical scavenging capacity (Pérez et al., 2007). In the current study, high of L-methionine reported in pigmented rice protein could play a role as an antioxidant. According to Xu et al. (2017), there are several amino acid which were classified as antioxidant group, these includes L-arginin, L-lysine, L-tyrosine, L-histidine, L-cysteine, and L-methione. As a result, the abundances of TAAAnt/TAA in pigmented rice could contribute to the antioxidant peptides activity.

The scavenging of ABTS $^{\bullet+}$ and $OH^{\bullet}$ radicals were carried out to confirm antioxidant potential in each rice seed protein. The results showed that the IC_{50} value in $ABTS^{\bullet+}$ radical and $OH^{\bullet}$ radical of Ketan Hitam-2 is the lowest; it is mean that Ketan Hitam-2 protein has the best antioxidant capabilities (Table 2). Several previous studies documented a good association between the percentage of hydrophobic amino acid and the $OH^{\bullet}$ radical activity (Ajibola et al., 2011; Zou et al., 2016). Based on data shown in Table 1, the variety with high percentage of hydrophobic amino acid are Gogo Niti-2, Super Manggis and Ketan Hitam-2 with percentage value of 81.37%, 80.47% and 80.35%, respectively. This suggested the high hydroxyl scavenger activity in Ketan Hitam-2 seed protein was related to its high content of hydrophobic amino acids. The mechanism of action may be that antioxidant peptides can enter smoothly through hydrophobic interactions with membrane lipid bilayers by their hydrophobicity, where they can exercise significant scavenging capacity (Pouzo et al., 2016). Bioactive peptides with antioxidant IC_{50} values under 500 µg/mL suggested to possess a significant potency as nutraceutical resources (Supriyadi et al., 2019; Xu et al., 2017).

Studies have shown the presence of natural antioxidants as nutraceutical ingredients was able to reduce chronic diseases such as mutagenesis, cancer, and DNA damage (Gupta et al., 2013; Tan et al., 2018). Evaluation of the protective role of rice protein against hydroxyl radicals caused by oxidative DNA damage was performed which in the case of plasmid, the OC DNA conformation resulted in a single-strand break in hydroxyl radical-exposed SC plasmid DNA due to the Fenton reaction. The study indicated incubation of *p*BT7 in 15 min Fenton's reagent resulted in SC cleavage and created OC form (Figure 2, lane 2). This suggested that the Fenton reaction-formed hydroxyl radical which induced DNA damage in single-strand. Nevertheless, adding of Ketan Hitam-2 seed protein to the Fenton reagent mixture clearly reduced the SC converting to OC DNA significantly (Figure 2). This can be caused by the high antioxidant amino acid group contents, L-tyrosine and L-methionine amino acids. The activities of antioxidants contribute to fenton reaction inhibition, protecting SC DNA from radical-induced hydroxyl breaks in the strands. Hydroxyl radicals are highly reactive and toward biological molecules, such as DNA, proteins, and lipids, which can cause chemical cation changes (Dizdaroglu

& Jaruga, 2012). Hydroxyl radicals may react with sugar units through base groups or hydrogen abstraction. DNA damage occurred as an unpleasant oxygen-metabolism by product and other environmental insults. Most of the DNA damage that is regularly repaired and removed may not be adequately repaired, accumulating inside cells, causing genetic damage (Chatgilialoglu & O'Neill, 2001). Previous study has reported that increased antioxidant capacity could be responsible for reducing cell damage (Samsampour et al., 2018).

Apart from free radical scavenging study, the activity of ACE-I inhibitor of each rice protein was also analysed. ACE-I inhibitors related to decreasing the blood pressure (Arora et al., 2014) in which ACE-I enzyme is responsible for the development of angiotensin II from angiotensin I. Angiotensin II is a vasoconstrictor causing blood pressure to increase. ACE-I inhibitors prevent renal disease development by decreasing intraglomerular pressure induced by angiotensin II (Tutor & Chichioco-Hernandez, 2017). Captopril was selected as the standard ACE inhibitor drug and was proved to be effective in inhibiting ACE activity at relatively lower concentrations than sample extracts. This was confirmed from the IC_{50} values (Table 3), where captopril showed a significant low inhibitory concentration compared to the other samples. Several studies stated that some ACE inhibitory peptides were directly isolated from food materials without *in vitro* proteolysis (Maruyama & Suzuki, 1982; Shin et al., 2001). Li et al. (2004) reviewed that the ACE-inhibitory peptide activities were ranged from 0.20 to 246.7 mg/mL on with the IC_{50} values. Most food-protein-derived antihypertensive peptides with high ACE-inhibitory activity were purified to have a relatively low molecular weight (Pihlanto-Leppälä et al., 2000). The current study showed inhibitor activity Ketan Hitam-2 was the highest indicated by its lowest IC_{50} value and this indicated Ketan Hitam-2 seed protein has the best potency to inhibit the activity of ACE-I. As well as antioxidant properties, it has been reported that ACE-I inhibitor peptides are also known to have high hydrophobic amino acids (López-Barrios et al., 2014). Another study has published that ACE-I inhibitor peptides have high hydrophobicity, aromatics, and branched side chains amino acids (Rai et al., 2017).

In this study, the analysed were carried out on crude protein of pigmented rice seed. The high activity reported were the activity of crude protein which was isolated from pigmented rice seeds. Thus, it is a premature conclusion if consuming pigmented rice directly could reduce the blood pressure.

5 Conclusion

In conclusion, the present study successfully explored and demonstrated bioactive protein in pigmented rice seed related to their potential protective effect against free radicals and ACE activity-induced by oxidative stress. Seed protein of Ketan Hitam-2 showed a higher potency as a protection agent with high content of the antioxidant amino acid group, scavenge of ABTS •+ and OH• radicals, protect DNA damage, and increase the activity of ACE-I. These results provide information related to nutraceutical sources in the prevention of cellular disease including hypertension.

Conflict of interest

The authors reported no potential conflict of interest.

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