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Inactivation of *Polyphenol oxidase* with Microwave and Its Influence on Total Polyphenol Content and Antioxidant Activity of Cocoa Beans (*Theobroma Cacao L.*)

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Abstract:
Cocoa beans contain natural antioxidants of polyphenols but its degradation occurs during processing by polyphenol oxidase (PPO) activity. This study aimed to investigate PPO inactivation using the microwave and to evaluate its influence on total polyphenol content (TPC) and antioxidant activity (AA) in cocoa beans. Physicochemical characterization (color, moisture content, temperature, and pH), TPC, AA, PPO activity, and functional groups (FTIR) of samples were analyzed. The results showed that the use microwave could inactivate PPO, generate TPC and AA higher than control. This indicates that the use of the microwave is more effective and can be used as an alternative treatment method to improve polyphenol cocoa beans.

Keywords*: cocoa beans, microwave and polyphenol oxidase*

1. Introduction

Cocoa is a source of food and beverages consumed in the world. In addition to its unique taste and aroma, cocoa is rich in compounds called polyphenols, in particular procyanidins and flavan-3-ols. There are three groups of polyphenols able to be identified including catechins (37%), anthocyanins (4%) and proanthocyanidins (about 58%) (Kyi et al. 2005). Cocoa beans contain polyphenol compounds as much as 12 – 18% in dry beans (Ioannou et al. 2012). It has been reported that total polyphenols range from 120 – 180 g/kg defatted unfermented cocoa powder (Misnawi, Nazamid, and Jamilah 2002). Compared to the study of Cieślik et al. (2006), the result of Misnawi et al. (2002) will be equivalent to 6 kiwi fruits, 15 apples, 22 pink grapes, 10 tomatoes, 10 carrots, 11 onions and 8 broccoli.

In recent years, many researchers are interested in cocoa polyphenol compounds because it is potential as antioxidants beneficial for health (Misnawi et al., 2002, Wollgast and Anklam 2000). (Lee et al. 2003) reported the total antioxidant capacities of cocoa > red wine > green tea > black tea. Therefore, it is necesarry to develop the new cocoa beans processing to produce products that are rich in polyphenol as a natural antioxidant. However, degradation of polyphenol occurs during the fresh cocoa beans process to be products. The reduction is caused by the fermentation and drying process (Kyi et al., 2005; Wollgast and Anklam 2000). It is likely caused by oxidized cocoa polyphenol compounds for the enzymatic reaction supported by the presence of oxygen and moisture content of the beans still around 40 until 10% (Jinap 1994). The oxidation induced by polyphenol oxidase (PPO) needs to be inhibited early using blanching treatment (Menon et al., 2015; Tomas-Barberán et al., 2007).

Blanching with the principle of heat transfer by convection through the heat source to material causes lower penetration rate than a microwave. It was confirmed by Mudgett et al. (1989) that a conventional heating rate is 10 – 20 times slower than microwave. It is

contrast with a microwave with the principle of heat generation by the material it self. This causes microwave energy 10 times faster than conventional heating (Supriyanto et al., 2014a).

The microwave is quite effectively used mainly for large scale production because it is easy to use, save energy and processing time by 70% due to the faster rate penetration, and reduce the loss of nutritional value, especially unstable compounds to heat such as polyphenols, carotenoids and other secondary metabolites (Chávez-Reyes et al., 2013; Hayat et al., 2010; Yoshida et al., 1995)**.** Some researchers have done inactivation of polyphenol oxidase on loquat fruit (Chávez-Reyes et al. 2013) and kiwi juice (Benlloch-Tinoco et al. 2013). There is limited information concerning on cocoa bean, so in this study, we report the use of microwave energy to inactivate PPO in the fresh cocoa bean. Then, the effect of this treatment on the phenolic and antioxidant activity was investigated.

2. Material and Methods

2.1. Samples

Forastero cocoa were collected in August 2015 from plantations of cocoa farmers in the Gunung Kidul, Yogyakarta, Indonesia. They were stored for a week at room temperature and opened by a wood splitter, and then cocoa beans were taken after that pulp was removed. Finally, the fresh cocoa beans were sorted based on visual appearance to impurities, defects, diseases, and pests. Hexanes, methanol and gallic acids were supplied from Merck Milipore (Germany), acetone was supplied from Mallinckrodt (USA), DPPH and follin-ciocalteu were supplied from Sigma-Aldrich (Germany), all other chemicals and solvents were analytical grade.

2.2. Microwave Treatments

100 and 200 g of fresh cocoa beans were heated by microwave (Samsung ME-731K) at 600 W. The heating is done by the 3 times intervals (60, 120 and 180 s). The microwave energy intensity (E) was calculated as follows using Equation (1). Each of the E was repeated 3 times (Moreno et al. 2003).

$$E\left[KJ/g\right] = \left[{}^{(W \times s)}/_{g}\right] / 1000 \qquad (1)$$

"E" shows the microwave energy intensity, "*W*" is the microwave oven power (watt), "*s*" denote the treatment time (s), "*g*" is the sample weight (g) and 1000 J represents a conversion factor.

2.3. Analysis of Physicochemical Parameters

Cocoa beans that have been microwave treated was analyzed of moisture content by thermogravimetric methods (AOAC, 1997) and pH was determined using ((.::Detail SNI::. n.d.). The percentage of weight change (before and after microwave treated) were measured using an analytical balance. The temperature of cocoa beans on the end microwave process was measured by thermocouple (type K). The color of cocoa bean was determined using Chromameter (Minolta CR 200, Osaka Japan), referring to color space CIE L*a*b. The determination was based on measuring the specific color parameters (L is brightness, a is redness, b is yellowness). Data of color per samples were repeated by five readings. Each of the treatment measured 3 times (Supriyanto et al., 2014a).

2.4. Determination of polyphenol oxidase (PPO) Activity

Polyphenol oxidase was isolated using the isolation Worthington Enzyme Manual sample. Fresh cocoa beans were blended to a powder and weigh as much as 10 g then added 20 ml with aquabidest. The mixture was homogenized using a stirrer at 4 °C for 10 minutes and then filtered through the paper of Whatman # 1 using a vacuum pump. The filtrate (crude polyphenol oxidase) was accommodated on the bottle falcon then characterized by the method of (Putra, Wartini, and Anggreni 2012)) 0.1 ml of crude PPO was added with 2.6 ml of buffer Na-acetate-acetic acid (pH 5.0) and 0.3 ml of 0.5 M catechol substrate at 25 °C. The mixture was recorded using a spectrophotometer at 420 nm. Absorbance readings was done for 3 minutes with an interval of 1 minute. One unit (U) activity of the enzyme polyphenol oxidase is equal to change in absorbance of 0.001 per minute. Furthermore, U unit per volume (ml) of enzyme used in the test was converted into Unit per gram of fresh weight (FW) of cocoa beans. Determination is repeated 3 times.

2.5. Extraction of total polyphenol content (TPC) Cocoa Bean

The dried beans were deshelled, milled, and defatted by Soxhlet extraction using hexane. The milled cocoa samples were extracted using 100 ml acetone : water (80 : 20 v/v) under a sonicator at 4 °C for 30 minutes then filtered using a vacuum pump, and the acetone was removed using rotary evaporator at 30 °C (Noor-Soffalina et al. 2009)

2.6. Determination of total polyphenol Content (TPC)

1 ml extract sample was dissolved in 10 ml of 80% acetone then reacted with 0.5 ml of Folin-Ciocalteu reagent for 2 – 3 minutes. 1 ml of 15% sodium carbonate (15 g dissolved in distilled water) was added to stabilize the color formation. The resulting mixture was shaken and left for 2 h at room temperature in the dark to formate of a blue color. The samples were measured at 765 nm. The results of total polyphenols were expressed in milligrams of gallic acid equivalent g^{-1} DS and obtained from standard curve equation of gallic acid solution with a concentration range of 1 – 100 mg L^{-1} (Noor-Soffalina et al. 2009).

2.7. Determination of DPPH Radical Scavenging Activity

Aqueous extract at different concentration (0 – 40 mL/L), solution was prepared by adding 1 ml extract, and 2 mL of 0.1 mM DPPH solution in methanol. The mixture was vortex then stored for 30 minutes at room temperature in a dark room. The samples were

measured 517 nm and the same treatment was done for control using acetone. The results were expressed by IC_{50} and percent of radical scavenging activity (RSA) DPPH was calculated using the equation (2) as follows: % RSA DPPH (Burda and Oleszek 2001)

$$[(absorbance\ of\ control - absorbance\ of\ sample)\ /\ absorbance\ of\ control\ ] \times 100\ \% \qquad (2)$$

2.8. Measurement of FTIR

1 mg of dried samples were crushed in 200 mg potassium bromide and pressed with a thin discs or pellets. The mixtures were analyzed using FTIR instrument (PERKIN ELMER 100). The spectra obtained was matched with an index of literature.

2.9. Statistical Analysis

Results of tables and figures are presented as mean ± standard deviation, all of the analyses were done in triplicate. All data obtained were tabulated and analyzed using variance analysis (ANOVA). The differences of the result would be tested using Duncan Multiple Range Test (DMRT)

3. Results and Discussions

3.1. The effect of Microwave treatment on PPO Activity and TPC

The results showed that the TPC value increased with an increase of the microwave energy (Table 1). The highest TPC value occurred by combination of 100 g cocoa beans, 180 s and 600 W microwave power (E= 1.08 KJ/g) of the other E value. The means of the highest TPC was 101.97 mg GAE/g defatted sample (DS). It is also exhibited that the E value increased from 0.18 up to 1.08 kJ/g, but PPO activity was decreased up to inactive.

The polyphenol content is closely related to polyphenol oxidase activity (Kyi et al. 2005; Menon et al. 2015). Several previous studies have reported that the value of E is used to disable the PPO like on strawberry (E = 0.15 kJ/g, at 400 W for 150 s) (Moreno et al. 2000), on hawthorn puree with (E = 0.54 kJ/g, at 900 W for 120 s), and Mamey fruit (E = 0.51 kJ/g, 300 g at 937 W for 165 s) (Palma-Orozco et al., 2012). Microwave energy intensity turns beneficial especially when the processes are scaled up (Chávez-Reyes et al. 2013; Palma-Orozco et al. 2012). Compared to the control, the means of TPC and activity of PPO were 72.63 mg/g DS and 445.9 U/g FW respectively. TPC value was higher than control beginning from E = 0.72 KJ/g.

The difference results of the control and microwave showed the microwave energy can inactivate PPO, so it can have improved the polyphenol compounds in cocoa beans. This is consistent with previous findings of (Gulati et al. 2003) who reported that the TPC of fresh tea shoots (cover glass container treatment) increased more than control by using the E=14 KJ/g (1500 g at 945 W for 240 s).(Chávez-Reyes et al. 2013) also explained on loquat fruit with optimal condition value E = 0.44 kJ/g (225 g, at 478 W for 210 s). The TPC on broccoli, spinach, green beans and pepper were increased by combination of 100 g at 1000 W (Turkmen, Sari, and Velioglu 2005). The use of microwave has a positive and negative effect on the TPC, depending on the difference of optimum condition and morphological characteristics of each of the commodities. The negative effects have been studied on fresh cabbage (Jaiswal and Abu-Ghannam, 2013) and on dried sour cherry (Wojdyło et al., 2014) when the TPC decrease were 33.3 – 36.3% and 44 – 49% respectively.

PPO activity was affected by temperature and moisture content (Table 1), showing that the cocoa beans temperature increased with increase the E. The temperature was means ranging from 49.0 to 76.4 °C. Lee et al. (1991) explained that the optimum temperature of PPO in cocoa beans was 45 °C and relatively stable at 50 – 70 °C. The above result agreed with Putra et al. (2012) who confirmed the optimum condition of PPO at 53.43 °C. However, the temperature increase over optimal temperature decreases enzyme activity, because the rate of enzyme damage is over the rate of enzyme activity (Lee et al., 1991)

The opposite results happened to the moisture content, where the moisture content decreased due to the heat by oscillation from the water and polar molecules of cocoa beans with the microwave emission (Chandrasekaran et al., 2013). PPO activity decreased slowly with the moisture content reduction until 10% (Rohan 1963). Furthermore, the decrease of moisture content would affect bean weight (Mechlouch et al., 2015), where the percentage of weight loss occurred with increase of E (Table 1). Ruiz-Ojeda and Peñas 2013) also explained that the weight loss increased by processing time and also microwave power level. However, water distribution in cellular structure and variation in individual samples greatly affect the transformation of microwave energy (Wojdyło et al. 2014). In addition, pH affects PPO activity (Biehl et al., 1977), Putra et al. (2012) reported that the optimum pH was 5.42 for PPO. In our study, the pH of cocoa beans was between 6.73 up to 7.26. On the other hand, the pH in controls was 6.59.

In general, microwave was able to improve polyphenol in cocoa beans because it can suppress the oxidation because of the PPO enzyme. During the treatment, the level of free flavonols increased as reported by (Stewart et al. 2000). It also explained that the power of microwave causes the cells breakdown in the skin. Thus, the large percentage of phenolic compounds are bound to cellular structures and release bound phytochemicals from the matrix to make them more accessible in extraction (Boateng et al. 2008; Dewanto, Wu, and Liu 2002; Wojdyło et al. 2014).

Weight (g)	Time (s)	Energy Intensity (KJ/g)	TPC (mg GAE/g DS)	PPO activity (U/g FW)	Temperature of bean (^oC)	% Moisture content	% weight loss	pH
100	180	1.08	101.97 ± 6.21^b	0 ± 0^c	76.4 ± 0.4^b	23.35 ± 0.91^d	21.71 ± 0.27^f	7.26 ± 0.00^d
100	120	0.72	96.54 ± 8.14^b	6.33 ± 2.58^c	71.1 ± 1.4^c	28.37 ± 1.35^b	14.03 ± 0.65^e	7.17 ± 0.08^d
200	180	0.54	71.43 ± 0.32^a	13.79 ± 6.97^c	70.5 ± 1.2^d	32.31 ± 0.33^b	9.56 ± 0.50^d	7.06 ± 0.06^c
200	120	0.36	72.85 ± 7.64^a	29.82 ± 2.54^c	65.3 ± 4.3^d	32.47 ± 1.96^b	5.51 ± 0.23^c	7.01 ± 0.02^c
200	60	0.18	62.44 ± 3.74^a	195.1 ± 14.84^b	49.0 ± 2.0^e	33.97 ± 2.83^b	2.85 ± 0.40^b	6.73 ± 0.09^b
control			72.63 ± 0.85^a	445.9 ± 37.36^a	25.4 ± 1.3^a	55.07 ± 1.97^a	0^a	6.59 ± 0.03^a

Table 1: Effect of microwave treatment on PPO activity and TPC of cocoa bean

3.2. The Effect of Microwave Treatment on Color

The degradation of polyphenols due to PPO activity can also be seen by a color change from purple to brown. The purple is a marker of the polyphenols that changes into a bright purple and even brown. The color change of food can be estimated indirectly by measuring colors instead of chemical analysis because it is simpler and faster (Maskan 2001). In the microwave treatment showed that, the higher E, the lower L (*lightness*), a (*Redness*), and b (*yellowness*). These findings were in agreement with (Jaiswal and Abu-Ghannam 2013) who explained that the opposite effect occurs in the Microwave processing when the processing time and power level increased along with the decreased of chroma value. Similar results were also reported by (Ruiz-Ojeda and Peñas 2013) on green beans. The decrease L, a, b caused the color was darker than that in control (Table 2). This indicated polyphenols oxidation by PPO activity caused a color change from purple to brown. The purple is the combination of the results of L, a, b where dark violet showed the higher polyphenol compounds than bright purple. This is consistent with previous findings of Barampama and Simard (1995) who reported that dry beans that have darker seed coats have relatively higher total phenolics than lighter seed coats

Weight (g)	Time (s)	Energy Intensity (KJ/g)	Color		
			L	a	b
100	180	1.08	34.72 ± 2.75^a	7.60 ± 0.93	-1.21 ± 0.32^c
100	120	0.72	35.87 ± 1.63^{ab}	7.65 ± 0.70	-1.20 ± 0.10^c
200	180	0.54	36.93 ± 0.60^{ab}	7.63 ± 0.41	-0.80 ± 0.27^c
200	120	0.36	38.02 ± 1.01^{bc}	7.97 ± 0.61	-0.19 ± 1.23^{bc}
200	60	0.18	39.77 ± 1.37^c	8.00 ± 0.49	0.94 ± 0.94^{ab}
		control	44.62 ± 0.23^d	6.89 ± 0.25	1.08 ± 0.25^a

Table 2: Effect of microwave treatment on color of cocoa bean

3.3. The effect of Microwave Treatment on Antioxidant Activity

Antioxidant activity was represented in percentage of radical scavenging activity (RSA) DPPH from equation (2). Our results showed that the %RSA DPPH increases with the increases of E. The highest %RSA DPPH (E= 1.08 KJ/g) was means of 74.44%. In addition, the amount of antioxidant activity was characterized by IC_{50}, where the higher % RSA, the lower of the IC_{50}. The IC_{50} value was concentration of the sample solution needed for scavenging of 50% DPPH radicals. It did not represent the amount of the antioxidant content of the material, but only classified the level of antioxidant capacity. The smaller of the IC_{50} value, the more effective the compound as a radical scavenger (Molyneux, 2004).

The microwave treatment (Table 3) showed that the lowest IC_{50} values were 0.93 mL/L. The results of this study also showed that the antioxidant activity of cocoa beans was higher than synthetic antioxidants such as BHT and ascorbic acid, where % RSA DPPH and IC_{50} values of BHT (20.85% and 20.46 mg/g) and ascorbic acid (18.64% and 13.90 mg/g).

In polyphenol molecular, OH functional group is the most responsible antioxidant activity acting as a donor of H^+ (Supriyanto et al. 2010b). That phenomenon might be described by FTIR (Fig.1), it is seen that the spectra of functional groups was in the range of wave numbers between 4000-400 cm^{-1}. The polyphenols represented the stretch O-H group was detected in the range of wave numbers 3200-3500 cm^{-1}. In the microwave treatment, the number of OH groups in the polyphenols molecules is higher than the control. It is shown from the smaller measured transmittance value (0.333%) than that in control (0.474%). This indicates that the OH functional group on microwave treatment compared to controls, does not oxidize. It also proved that the use of microwave is more effective to improve the polyphenols in cocoa beans.

Weight (g)	Time (s)	Energy Intensity (KJ/g)	IC_{50} (mL/L)	% RSA
100	180	1.08	2.12 ± 0.93^a	74.44 ± 1.18^b
100	120	0.72	2.64 ± 0.81^{ab}	74.61 ± 1.47^b
200	180	0.54	5.38 ± 0.20^c	52.01 ± 1.44^a
200	120	0.36	5.05 ± 0.24^c	56.75 ± 1.14^a
200	60	0.18	7.74 ± 0.51^d	43.68 ± 3.18^a
		control	3.39 ± 0.13^{ab}	55.36 ± 9.41^a

Table 3: Effect of microwave treatment on antioxidant activity of cocoa bean

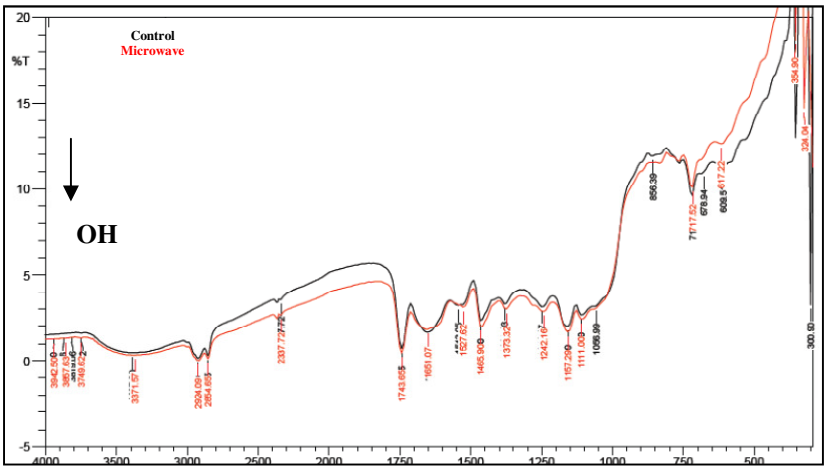

Figure 1: Comparison FTIR spectra of cocoa beans on microwave and control

4. Conclusion

PPO activity greatly affects the total polyphenols in cocoa beans. Our study results show that the value of E 1.08 KJ/g (100 g at 600 W for 180s) was able to inactivate PPO and generate TPC of 101.97 mg GAE/g DS as well as produce antioxidant activity of 74.44% with IC_{50} values of 2.12 mL/L. Compared to microwave, the TPC in control was 72.63 mg GAE/g DS and the antioxidant activity was 55.36% (RSA) and 3.39 mL/L (IC_{50}).The antioxidant activity in cocoa beans with the microwave treatments produced a higher antioxidant activity than synthetic antioxidant (BHT and ascorbic acid). Thus, microwave is able to improve polyphenol cocoa beans as a natural antioxidant

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