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Antioxidant Capacity of Black Tea Obtained Using Tyrosinase and Tannase Treatment

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ABSTRACT: Black tea has a lower antioxidant capacity than other teas, such as green tea, white tea, and oolong tea. Tannase and tyrosinase can be used as a treatment to improve the quality of black tea infusion. Tannase has been reported to be an effective way to enhance antioxidant activity in black tea infusion. Meanwhile, tyrosinase could produce higher theaflavin content than thearubigin. Research about Ready-to-Drink (RTD) black tea preparation with the addition of tannase and tyrosinase to fresh tea leaves before pasteurization has not been reported. This study aimed to find a good combination of tannase (1 mg/ml) and tyrosinase (111; 446; 1785 U/ml) to produce high antioxidant activity of RTD black tea. The results showed that higher tyrosinase concentration decreased the antioxidant activity (DPPH and reducing power), epicatechin (EC), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) content yet increased the theaflavin content and theaflavin (TF)/thearubigin (TR) ratio in the tannase-tyrosinase treated black tea. Still, the highest concentration of tyrosinase (1785 U/ml) in tannase-tyrosinase black tea produces higher antioxidant activities, gallic acid, EC, and EGC content than commercial black tea and tyrosinase without tannase-treated black tea. Thus, the combination of tannase (1 mg/ml) and tyrosinase (1785 U/ml) could be the best treatment to produce high-antioxidant black tea.

Keywords: Tannase; Tyrosinase; Black Tea; Antioxidant.

INTRODUCTION

Tea (*Camellia sinensis* L.) is a drink people consume worldwide. Based on its enzymatic oxidation, tea can be classified into various types. Those are green tea (unfermented), oolong tea (partially fermented), and black tea (fully fermented) (Chaturvedula and Prakash, 2011; Thea *et al*., 2012; Lin *et al*., 2014). Black tea is the most preferred tea among other tea types (Wu and Wei, 2002; Skotnicka *et al*., 2011). Moreover, black tea production and consumption are estimated to grow yearly (Chang, 2015). Due to its complete fermentation process, black tea has a lower antioxidant capacity than green and oolong tea (Carloni *et al*., 2013). Several ways that have been reported to increase antioxidant activity in black tea are the addition of ascorbic acid (Majchrzak *et al*., 2004; Murugesan *et al*., 2020), pectinase, and tannase (Chandini *et al*., 2011; Raghuwanshi *et al*., 2012).

Tannase has been widely used in the food and beverage industries, such as in clarifying wine, beer, and fruit juice (Beniwal *et al*., 2013). Tannase has also been used to enhance the antioxidant activity of tea by catalyzing the hydrolysis of gallate catechin into non-gallate catechin and gallic acid content (Zhang *et al*., 2016; Cao *et al*., 2019). Moreover, applying tannase can produce a higher yield on tea extract (Sanderson and Coggon, 1974) and prevent creaming formation (Lu *et al*., 2009; Kumar *et al*., 2013). Many studies about tannase application to improve the quality of tea have been reported. Previous studies reported the effect of tannase application on the yield and color of fresh tea extract (Sanderson and Coggon, 1974) and green tea's antioxidant profile and sensory attributes (Lu *et al*., 2009; Zhang *et al*., 2016; Cao *et al*., 2019; Xu *et al*., 2019).

Other than that, the quality improvement of black tea was also carried out by adding various exogenous polyphenol oxidase (PPO), such as tyrosinase, lacasse (Zhang and Du, 2015; Verloop *et al*., 2016), peroxidase, bilirubin oxidase, and crude polyphenol oxidase enzymes of tea leaves (Yabuki *et al*., 2017). Tyrosinase is an enzyme that contains copper and has monophenol monooxygenase and diphenol oxidase activity, which play an essential role in the enzymatic oxidation process (Zolghadri *et al*., 2019). The tyrosinase enzyme can produce higher theaflavins compared to laccase enzymes and crude polyphenol oxidase enzymes of tea leaves (Verloop *et al*., 2016). In addition, tyrosinase tends to make non-gallate theaflavins to prevent the formation of more tea creams (Narai-Kanayama *et al*., 2017).

The enzymatic oxidation process is influenced by the concentration of oxidizing enzymes present in tea. Narai-Kanayama *et al*. (2017) reported that tyrosinase (313 mU/mL) could produce higher theaflavin content than

thearubigin. Verloop *et al*. (2016) stated that using tyrosinase (5 U/ml) could effectively change the catechins present in tea extracts. However, the effect of tyrosinase concentrations on the quality of black tea has not been reported yet. Moreover, research about tyrosinase application was still limited to improving black tea extract.

Another problem arises when people need to consume a product quickly due to their dense activities. Therefore, ready-to-drink tea (RTD) is one solution to overcome this problem. RTD tea is a form of tea-derivative product that is often consumed in society (Bae *et al*., 2016; Fadlillah *et al*., 2020). The processing of RTD tea usually begins with the leaf processing of tea, followed by the extraction, pasteurization, and packaging process (Dubey *et al*., 2020). The processing of RTD black tea requires a long process due to the processing of dry black tea leaves. The leaves production of black tea needs a longer time than the other tea types due to its withering and enzymatic oxidation process (Owuor and Reeves, 1986). Besides that, the antioxidant content that tends to be low in RTD black tea is another problem.

To the best of our knowledge, research about RTD tea preparation preceded by adding tannase and tyrosinase to fresh tea leaves before pasteurization has not been reported. Raghuwanshi *et al*. (2012) stated that 1000 ppm of tannase could significantly improve the capacity of antioxidants in black tea. Research about tyrosinase were still limited to 1 enzyme concentration (Verloop *et al*., 2016; Narai-Kanayama *et al*., 2017, 2019). The present study aims to find a good combination of tannase (1 mg/ml) and tyrosinase (111; 446; 1785 U/ml) to produce high antioxidants in RTD black tea.

MATERIALS AND METHOD

Materials

Fresh tea leaves (PGL 11 clone) were provided from PT. Pagilaran, Batang, Indonesia. Tannase from *A. niger* (337 U/g solid) was purchased from Xi'an Lyphar Biotech (Xi'an, China). Tyrosinase from mushroom (7,164 U/mg solid), five standards for HPLC ((-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), gallic acid), methanol for HPLC, acetonitrile for HPLC, o-Phosphoric acid, 2,2-Diphenyl-1 picryl-hydrazyl (DPPH), TPTZ, $FeCl_3.6H_2O$, acetic acid, sodium acetate, ascorbic acid, gallic acid, methanol for analysis, ethyl acetate, oxalic acid were bought from Sigma Aldrich (St. Louis, USA), Whatman No.1 filter paper (Maidstone, UK), liquid nitrogen was bought from Samator Gas Industri (Klaten, Indonesia).

Tea leaves preparation

Young shoots of fresh tea leaves Tea buds (p+2) were plucked manually in the morning. After plucking, the leaves were steamed for 10 minutes to inactivate the indigenous enzymes and frozen by pouring liquid nitrogen before storing at -18 ℃.

Preparation of tea leaf extract

Tea leaves were extracted filtrates.

Tannase enzymatic treatment

100 ml filtrate was incubated with 100 mg tannase enzyme using an incubator (Sanyo, Japan) at 37 ℃ for 15 minutes. After incubation, the solution was heated to 90 ℃ for 10 minutes to stop hydrolysis. The tea solution was centrifuged at 27 ℃, 3,000 g, for 10 minutes (Zhang *et al*., 2016; Cao *et al*., 2019; Xu *et al*., 2019). The filtrates were then treated with tyrosinase.

Tyrosinase enzymatic treatment

100 mL of tannase-treated filtrate was added with 100 mL of tyrosinase enzyme (111; 446; and 1,785 U/ml) and incubated at 25 ℃ for 20 minutes. The reaction mixture was added with 100 µL of saturated ascorbic acid to stop the reaction. The mixture was then centrifuged (10,000 g, 20 ℃, 5 minutes) to obtain black tea and stored at 4 ℃ for further analysis (Verloop *et al*., 2016; Narai-Kanayama *et al*., 2019).

Antioxidant Activity

DPPH Radical Scavenging Activity Determination

Radical scavenging activity was determined using the assay according to Zaiter *et al*. (2016) method with slight modification. 1 mL of 0.2 M DPPH solution was added to 1 mL of tea extract. The mixture was incubated in the dark for 30 minutes. After incubation, the absorbance was measured at a wavelength of 517 nm. The radical scavenging activity was expressed as mg Gallic Acid Equivalent (GAE)/g fresh tea leaves (DW). Radical scavenging activity was determined using the following equation:

$$\text{Radical scavenging activity (\%)} = \frac{(\text{A blank} - \text{A sample})}{\text{A blank}} \times 100\%$$

Reducing Power Determination

Reducing power was determined by following the method from Benzi and Strain (1996). FRAP reagent was prepared by mixing 300 mM acetic buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ (10:1:1, v/v/v). The reagent was incubated at 37 ℃ for 5 minutes before use. 10 µL tea extract was added to 30 µL H_2O and 300 µL FRAP reagent. The mixture was incubated in the dark for 4 minutes. The absorbance was measured at a wavelength of 593 nm. Reducing power was expressed as mg Ascorbic Acid Equivalent (AAE)/g fresh tea leaves (DW).

Table 1. Antioxidant activity of black tea treated with different treatments.

Treatment	Tyrosinase Activity (U)	Radical scavenging activity (mg GAE/g fresh tea leaves (db))	Reducing power (mg AAE/g fresh tea leaves (db))
Tannase-Tyrosinase	223.2	67.66±4.64^{c}	275.42±16.74^{d}
	892.85	56.80±2.11^{b}	214.28±6.36^{c}
	3571.4	52.28±1.05^{ab}	161.79±6.15^{b}
Tyrosinase	3571.4	52.05±2.26^{ab}	135.89±17.08^{a}
Commercial Black Tea		49.94±2.80^{a}	115.50±10.98^{a}

Note: Different superscript in the same column means significant differences ($p < 0.05$).

Theaflavin and Thearubigin Determination

The spectrophotometric method was used to analyze theaflavin and thearubigin based on a procedure by Ullah (1986). 20 ml tea extract was added into 6 ml 2.5% sodium bicarbonate and 20 ml ethyl acetate. The solution was shaken for 1 minute. 10 ml of the top part of the solution containing theaflavin was added with 15 ml methanol. The mixture was shaken for 1 minute. The optical density was measured at a wavelength of 380 nm and recorded as E1. 1 ml tea extract was added with 1 ml saturated oxalic acid, 8 ml distilled water, 15 ml methanol, and shaken for 1 minute. The optical density was measured at a wavelength of 380 nm and recorded as E2. Theaflavin and thearubigin were determined using the following equation:

$$TF\ (\%) = 2.25 \times E1$$

$$TR\ (\%) = 7.06 \times (4E2 - E1)$$

TF: Theaflavin
TR: Thearubigin

Individual Catechin and Gallic Acid Determination

Individual catechin and gallic acid were analyzed using high-performance liquid chromatography (HPLC) (Martono and Martono, 2012). Tea extract was filtered through a nylon filter membrane (0.45 µm). The filtered samples were injected into the HPLC system. HPLC system was equipped with a PDA detector (Shimadzu, Japan) and C18 column Shimadzu (SHIM-PACK GISH, 150 × 4.6 mm; 5 µm). HPLC conditions were controlled as follows: oven temperature at 40 ℃, the flow rate at 1 ml/min, and the wavelength at 210 nm for 35 min. The mobile phase in this assay was the mixture of 0.1% ortho-Phosphoric acid, water, acetonitrile, and methanol (14:7:3:1, v/v/v/v). The quantification was determined by comparing the peak area of samples with catechin (EGCG, ECG, EC, EGC) and gallic acid standards.

Statistical analysis

All data were analyzed by one-way ANOVA using SPSS 16.0 (IBM SPSS Statistics, IBM Corp, Somers, NY, USA). Duncan's multiple range test (DMRT) was used to evaluate the significant differences (p<0.05).

RESULT AND DISCUSSION

Antioxidant activity

The antioxidant activity (radical scavenging and reducing power) of black tea with different treatments can be seen in Table 1. The higher concentration of tyrosinase could significantly ($p < 0.05$) decrease the radical scavenging activity and reducing power. The highest tyrosinase concentration (1,785 U/ml) obtained the lowest radical scavenging activity among tannase-tyrosinase-treated black tea. The results aligned with previous studies that both radical scavenging and reducing power in black tea were lowered as long as the oxidation process (Chen *et al*., 2018). It happens because theaflavin and thearubigin that formed from enzymatic oxidation had a lower amount of hydroxyl group in their structure compared to catechin (Engelhardt, 2010). Hydroxyl groups in phenolic compounds were reported to play a role in free radical scavenging activity and reducing power (Nanjo *et al*., 1999; Takeuchi *et al*., 2007; Huang *et al*., 2019). Table 1 also showed that the antioxidant activity of the highest tyrosinase concentration among tannase-tyrosinase treated black tea was still higher than commercial RTD black tea. Thus, the highest tyrosinase activity without tannase treatment was used as a comparison to clarify the effect of tannase application to improve the antioxidant activities.

Tannase application in tyrosinase-treated black tea generally gave significantly higher antioxidant activity than in tyrosinase-treated black tea without tannase treatment and commercial black tea (Table 1). In agreement with the previous study, tannase could enhance green and black tea's antioxidant activity. Tannase plays an essential role in the hydrolysis process of gallated catechin to non-gallated catechin and gallic acid (Raghuwanshi *et al*., 2012; Zhang *et al*., 2016; Cao *et al*., 2019; Xu *et al*., 2019). Thus, gallic acid might be the reason for the increase in antioxidant activity.

Table 2. Catechin and gallic acid content of black tea treated with different treatments

Treatment	Tyrosinase Activity (U)	GA	EGC	ECG	EC	EGCG
Tannase-Tyrosinase	223.2	66.86	97.05	9.03	24.99	10.05
	892.85	57.98	37.68	9.21	13.07	9.70
	3571.4	63.58	35.16	9.12	10.20	9.83
Tyrosinase	3571.4	8.42	13.73	9.36	9.33	9.60
Fresh Tea Leaves		7.26	34.15	9.33	12.67	10.40
Commercial Black Tea		8.30	11.98	9.10	9.07	9.80

Note: (GA): gallic acid, (EGC): epigallocatechin, (ECG): epicatechin gallate, (EC): epicatechin, (EGCG): epigallocatechin gallate

Table 3. Theaflavin and thearubigin content of black tea treated with different treatments.

Treatment	Tyrosinase Activity (U)	Theaflavin (%)	Thearubigin (%)	TF/TR Ratio
Tannase-Tyrosinase	223.2	1.25 ± 0.05^{a}	2.04 ± 0.79^{a}	0.61
	892.85	2.51 ± 0.09^{b}	2.43 ± 1.03^{a}	1.03
	3571.4	5.25 ± 0.12^{d}	2.59 ± 0.43^{a}	2.03
Tyrosinase	3571.4	4.89 ± 0.09^{c}	2.74 ± 0.29^{a}	1.78
Commercial Black Tea		1.30 ± 0.01^{a}	21.46 ± 0.21^{b}	0.06

Note: Different superscript in the same column means significant differences ($p<0.05$).

Levels of Individual Catechin and Gallic Acid

Table 2 shows a decrease in catechin levels, especially in EGC, EC, and EGCG, due to higher tyrosinase enzyme concentration. Tyrosinase was reported to be effective in oxidizing EC, EGC, and EGCG to produce theaflavins and thearubigins (Verloop *et al*., 2016). Compared to tyrosinase-treated black tea without tannase treatment, a combination of tannase and tyrosinase could increase catechin oxidation. Tyrosinase with the highest activity treated black tea without tannase treatment only showed 59%, 26%, 7% oxidation for EGC, EC, and EGCG, respectively. Meanwhile, the application of the highest tyrosinase concentration (1785 U/ml) in tyrosinase-tannase-treated black tea resulted in a decrease of 63%, 59%, 2% of EGC, EC, and EGCG, respectively, compared to the lowest tyrosinase concentration (111 U/ml). Therefore, combining tannase and tyrosinase could increase the oxidation of catechin.

An increase of gallic acid, EGC, and EC content also were observed in this research to clarify the effect of tannase application on increasing antioxidant activity. Tannase-tyrosinase-treated black tea showed higher gallic acid, EGC, and EC content than tyrosinase-treated black tea without tannase treatment and commercial black tea (Table 2). According to previous studies, tannase plays an important role in the hydrolysis of EGCG and ECG into EGC, EC, and gallic acid by cleaving ester bonds (Hong *et al*., 2013; Li *et al*., 2017). Besides that, catechin and gallic acid content in the tyrosinase-treated black tea without tannase treatment had a similar amount with commercial black tea. It means 20 minutes of oxidation with tyrosinase was equal to 2 hours of oxidation in the black tea oxidation process.

Theaflavin and thearubigin content

Theaflavin and thearubigin content significantly increased ($p<0.05$) as the tyrosinase concentration increased (Table 3). It also was found that the theaflavin content was higher than thearubigin content. The oxidation using PPO could obtain higher theaflavin content, while peroxidase (POD) was in charge of higher thearubigin formation (Verloop *et al*., 2016; Zhu *et al*., 2020). Due to the higher theaflavin content was found in the present research, increasing tyrosinase activity increased TF/TR ratio (Table 3). TF/TR ratio can be used to determine tea quality, which can be categorized as good quality (up to 0.04), better quality (0.04-0.08), and best quality (more than 0.08) (Borse and Rao, 2012). Therefore, all tyrosinase treatments could result black tea with the best quality. The highest tyrosinase activity gave the highest TF/TR ratio that indicates this activity was the best activity to transform tannase-treated green tea into black tea. Higher theaflavin content was known to be good for health benefits. (Imran *et al*., 2018) reported that theaflavin based diet could minimize lipid oxidation better than thearubigin based diet. Similar research also stated that lipid peroxidation inhibition by theaflavin was more effective than thearubigin (Yoshino *et al*., 1994). Another study reported by Halder *et al*. (Halder *et al*., 2006) showed that theaflavin had more protective effects than thearubigin as antimutagenic and anticlastogenic.

From Table 3, it also could be seen that tyrosinase with the highest activity and tannase treated black tea brought

in higher theaflavin content rather than tyrosinase alone. It indicated that the combination of tannase and tyrosinase had more substrates to be oxidized. On the other hand, it gave different content of theaflavin and thearubigin to commercial black tea. The commercial black tea gave higher content in thearubigin and lower theaflavin content. Therefore, the application of tannase and tyrosinase can be used instead of conventional enzymatic oxidation in producing "best" quality black tea.

CONCLUSION

As the tyrosinase transformed green tea into black tea, the antioxidant capacities were decreased. Thus, tannase treatment was added to improve the antioxidant capacity of black tea. Combining Tyrosinase and tannase resulted in sample with the highest with the highest antioxidant activity, TF and TR content, galic acid content, and cathecin level. Results show that this treatment can be used as an alternative method for conventional fermentation to produce black tea. This treatment could obtain black tea with the best category, higher antioxidant activity, and similar catechin levels compared to commercial black tea.

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