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Enhancement of Bioactive Components Content and the Antioxidant Activity of Green Tea after Continuous Anaerobic Incubation

J. H. Kim[1], and M. Y. Kim[2*]

ABSTRACT

Although several methods have been developed to make gamma-aminobutyric acid (GABA) accumulate in tea, the active ingredients and health benefits vary widely depending on the manufacturing process of tea products. In this study, the levels of bioactive compounds and antioxidant activity in tea leaves given the continuous anaerobic condition were investigated. GABA and other free amino acids such as alanine, valine, leucine, isoleucine, proline, and asparagine were increased in tea leaves after anaerobic treatment, whereas the contents of organic and fatty acids remained almost constant. Compared with untreated tea, anaerobically incubated tea possesses higher (-)-epigallocatechin and (-)-epigallocatechin gallate contents, and lower IC_{50} values in the 1,1-diphenyl-2-picrylhydrazyl radical scavenging, superoxide radical scavenging, nitric oxide scavenging and xanthine oxidase inhibition assays, which may indicate that continuous anaerobic incubation enhances antioxidant activity. These results suggest that the continuous anaerobic treatment during manufacturing process may provide high quality GABA tea.

Keywords: Gamma-Aminobutyric Acid (GABA), Green tea, Continuous anaerobic incubation, Bioactive component, Antioxidant activity.

INTRODUCTION

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the sympathetic nervous systems (Mody *et al.*, 1994), and plays an important role in the regulation of cardiovascular and cerebrovascular functions (Aoki *et al.*, 2003; Inoue *et al.*, 2003). Owing to these physiological benefits of GABA, there is growing interest in GABA accumulation in functional food research. Various GABA-enriched foods have been characterized: red-mold rice (Wu *et al.*, 2009; Wang *et al.*, 2010), black raspberry juice (Kim *et al.*, 2009), soybean (Aoki *et al.*, 2003), yogurt (Park and Oh, 2007), Korean Kimchi (Cho *et al.*, 2007), brown rice germinated under anoxia (Oh, 2003), wheat germinated by endogenous enzyme (Nagaoka, 2005) and dairy products (Nomura *et al.*, 1998).

GABA-rich green tea (GABA tea) is made by the repeated anaerobic/aerobic sequence fermentation. However, this manufacturing method may cause the loss of other healthy beneficial components. For instance, in one study (Sawai *et al.*, 2001), the amount of GABA increased by the repeating treatment of anaerobic and aerobic incubation. The content of alanine also increased in the first three hours of anaerobic incubation. However, when tea leaves were released

[1] Department of Tourism and Food Service Cuisine, Cheju Tourism College, Jeju 695-900, Republic of Korea.
[2] Faculty of Biotechnology, College of Applied Life Sciences, Jeju National University, Jeju 690-756, Republic of Korea.
[*] Corresponding author; e-mail: jeffmkim@jejunu.ac.kr

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under aerobic condition, alanine was decreased remarkably during one hour of aerobic incubation and its content remained almost constant in the following anaerobic incubation (Sawai *et al.*, 2001). Therefore, there is a need to reduce losses of other bioactive compounds simultaneously with GABA accumulation by employing different treatment strategies. Here, we determined the levels of bioactive compounds such as free amino acids, organic and fatty acids, and polyphenols in tea leaves given the continuous anaerobic conditions. In addition, their antioxidant capacities were evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, superoxide ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$) scavenging, and xanthine oxidase (XO) inhibition assays.

MATERIALS AND METHODS

Fresh young tea leaves (Yabukita cultivar) were collected from Jeju Green tea Agriculture Juridical Person (Jeju green tea agricultural cooperative, Seogwipo-si, Korea). One half of plucked tea leaves were packed into a nitrogen-filled chamber and then continuously incubated at 30ºC for 6 and 12 hours. The other half of tea leaves without anaerobic treatment was used as the control. The tea leaves processed with or without anaerobic treatment were given a roasting process for 5 minutes at 300ºC, followed by cooling and rolling, and then three cycles of hand-rolling for 3 minutes at 250ºC and shaking. After roasting for 90 minutes, a 10 g sample of dried tea leaves was extracted in 1 L of distilled water at 90°C for 10 minutes with moderate agitation. The extracts were isolated by filtering (8 µm; Whatman filter paper, Piscataway, NJ, US), evaporated, lyophilized, and stored in a sealed container at -20°C until use for analysis of amino acids, non-volatile organic acids, fatty acids and phenolic compounds.

Amino acid quantification was carried out using the EZ:faast^{TM} kit (Phenomenex, CA) for clean-up and derivatization of free amino acids. Analyses were performed according to Kugler *et al.*, (2006) using a gas chromatography (GC) device equipped with a Flame Ionization Detector (FID) (Hewlett Packard; HP, 5890 Series II). Aliquots of the derivatized amino acids (2 µL) were injected at 250°C into a Zebron column (ZB – AAA, 10 m and 0.25 mm in diameter) programmed from 110-320°C at 32 °C min^{-1}. Flame ionization detection was carried out at 320°C with a split ratio of 1:15.

Measurement of non-volatile organic and fatty acids was performed by an HP5890 GC-FID instrument equipped with an SP-2340 column (30 m×0.32 mm i.d.×0.2 µm film thickness) according to the method of Court and Hendel (1978). Three microliters of the solution were injected at 230°C in split mode. The oven temperature program was: 10 minutes at 50°C, from 160 to 230°C at 2°C min^{-1}, then 60 min at 230°C. Helium (1.4 bar) and nitrogen (0.8 bar) were used as carrier and auxiliary gases, respectively.

Phenolic compounds were determined using a modification of the procedure described previously (Yang *et al.*, 2007). The powdered tea leaves (0.5 g) were extracted with 40 mL boiling water at 90°C for 30 minutes while shaking. The extracts were diluted with HPLC grade water and passed through a 0.45 µm syringe filter (Sigma). A 20 µL aliquot of filtered sample was injected into a C-18 symmetry (5 µm, 3.9 mm×150 mm) column of the Waters HPLC system equipped with a 626 pump, a 486 UV detector plus autosampler (Waters, Milford, MA), and eluted with a linear gradient starting at a proportion of 90:10 (v:v) of 5% AcOH/H_2O/0.1% AcOH/MeOH for 10 minutes and then changing to 80:20 in 20 minutes and to 90:10 (v:v) in 15 minutes at a flow rate of 1 mL min^{-1}. The column was operated at 40°C, and the eluent was monitored using a UV detector at the wavelength of 230 nm. Standard solutions of theobromine (TB), caffeine (Caff), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (GCG) and (-)-

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epicatechin-3-gallate (ECG) dissolved in acetonitrile solution were prepared. The phenolics were identified by comparing their retention times and UV spectra with those of authentic standards stored in a data processor. The content of each phenolic compound was calculated from the integrated peak area of the sample and the corresponding standard.

DPPH radical scavenging activity was measured by the modified method described previously (Cavin *et al.*, 1998). Briefly, 100 μL of each test solution with various concentrations was prepared in 96-well plates and equal volume of 0.4 mM DPPH in methanol was added in each well. The solution was kept in the dark for 10 minutes at room temperature and absorbance of the solution was measured at 517 nm using an ELISA reader (EL340, Bio-Tek).

Measurement of $O_2^{\bullet-}$ scavenging activity of the test extracts was based on the methods described previously (Chang *et al.*, 1996; Furuno *et al.*, 2002). Briefly, test extracts were added to the reaction mixture containing 50 mM sodium carbonate (Na_2CO_3) buffer, 3 mM xanthine, 3 mM ethylenediamine tetraacetic acid, 0.5 mM nitroblue tetrazolium and 0.15% bovine serum albumin solution. After incubation at 25°C for 25 minutes, the reaction was started by the addition of XO (0.25 units mL^{-1}). Absorbance was read at 560 nm after 20 minutes.

The XO inhibitory activity was assayed based on the procedure reported previously (Nguyen *et al.*, 2004). The assay mixture, consisting 50 μL of test solutions, 35 μL of 200 mM phosphate buffer (pH 7.5), 30 μL of XO (0.05 units mL^{-1} in 200 mM phosphate buffer), was prepared immediately before use. After preincubation at 25°C for 15 minutes, the reaction was initiated by the addition of 60 μL of 0.5 mM xanthine. The assay mixture was incubated at 25°C for 30 minutes and then the absorption increments at 290 nm were determined.

$NO^{\bullet}$ generated from sodium nitroprusside (SNP) was measured according to the method of Green *et al.* (1982). Briefly, the reaction mixture (200 mL) containing 10 mM SNP in phosphate-buffered saline (pH 7.0), with or without the test extract at different concentrations, was incubated at 25°C for 3 hours. The incubation mixture was then reacted with an equal amount of Greiss reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylene diamine dihydrochloride in 2.5% polyphosphoric acid) for 5 minutes. The absorbance of assay mixture at 540 nm was measured using a spectrophotometer (DU640i, Beckman).

For antioxidant activity assays, the concentration of the extract giving 50% inhibition (IC_{50}) was determined from a dose response curve. IC_{50} represented the concentration of the extract that inhibited 50% of radicals.

All analyses were carried out at least three times and the data are presented as an average of three independent determinations. One-way non-parametric ANOVA, Kruskal–Wallis tests were used to compare the difference between control and treated groups. The Mann-Whitney test was used for comparing two independent samples and values were set as significant when $P < 0.05$ (SPSS for Windows, 12.0, SPSS Inc. Chicago, IL, USA). After multiple comparisons, the means in the following tables were followed with different small letter "*a-c*" based on their values and statistical differences.

RESULTS AND DISCUSSION

The amino acid contents of green tea leaves processed with or without continuous anaerobic treatment are shown in Table 1. Significant increases (9- and 12-folds, respectively) of GABA in tea leaves incubated anaerobically for 6 and 12 h were observed in comparison with the untreated control ($P < 0.05$) (Table 1). This result is in good agreement with the previous studies indicating that the repeated anaerobic/aerobic sequence treatment of tea leaves produced a green tea with elevated GABA levels (Sawai *et al.*, 2001; Wang *et*

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Table 1. Contents of amino acid (mg 100 g^{-1}) in green tea after continuous anaerobic treatment.

	Untreated control	Nitrogen 6 h	Nitrogen 12 h
Alanine	9.8 ± 3.50[a]	19.1 ± 2.84[a]	38.8 ± 5.18[b]
Valine	10.9 ± 3.33	15.2 ± 2.03	16.4 ± 3.75
Leucine	3.6 ± 1.02	7.5 ± 2.18	8.9 ± 3.10
Isoleucine	27.6 ± 4.86	59.4 ± 7.73	61.7 ± 6.31
Proline	9.5 ± 2.81[a]	40.4 ± 8.93[b]	62.3 ± 8.35[b]
Asparatic acid	11.1 ± 4.92	10.5 ± 5.76	8.9 ± 6.04
Asparagine	29.7 ± 8.30	57.8 ± 13.58	65.4 ± 5.20
GABA	25.8 ± 4.62[a]	228.7 ± 6.78[b]	312.7 ± 5.23[c]

[a~c] Values with different superscripts in a row are significantly different (P< 0.05).

al., 2006). However, while the content of GABA increased continuously in their study, the content of alanine remained constant during the repeating treatment of anaerobic and aerobic incubation (Sawai *et al*., 2001). In contrast, our results showed the accumulation of alanine in the continuous anaerobic process; the alanine content of tea treated with nitrogen for 12 hours was 4-fold higher than that of untreated control (Table 1). Alanine supports the metabolism of glucose, a simple carbohydrate which is used by the body for energy production, and contributes to immunopotentiation (Kakuda, 2002). Benefits of proline include the healthy maintenance of connective tissue and repairing damage to tissue, skin and muscle (Shetty, 2004). It also acts as a free radical scavenger and further contributes to a healthy immune system (Shetty, 2004). In the present study, the contents of valine, leuicine, isoleucine, proline and asparagine also increased, indicating that green tea given the continuous anaerobic condition not only contains an increased amount of GABA but also higher bioactive amino acids (Table 1).

Table 2 shows the changes of organic and fatty acid compositions of green tea under continuous anaerobic treatment. The organic and fatty acid contents of tea were not affected by anaerobic treatments in our study, which is consistent with a previous finding that there is no difference in contents of free fatty acids between GABA and green teas (Wang *et al*., 2006).

Polyphenols are generally considered to be

Table 2. Contents of organic and fatty acids in green tea after continuous anaerobic treatment. [a]

	Untreated control	Nitrogen 6 h	Nitrogen 12 h
Organic acids			
Oxalic acid	5.1 ± 0.47[*]	5.8 ± 0.97	5.4 ± 0.56
Malonic acid	0.1 ± 0.004	0.1 ± 0.004	0.1 ± 0.007
Succinic acid	0.2 ± 0.08	0.3 ± 0.01	0.2 ± 0.04
Malic acid	1.3 ± 0.16	1.6 ± 0.12	1.3 ± 0.42
Citric acid	4.9 ± 0.26	4.4 ± 0.33	4.2 ± 0.35
Fatty acids			
Palmitic acid	6.0 ± 0.25	6.3 ± 0.43	6.5 ± 0.46
Stearic acid	0.3 ± 0.03	0.3 ± 0.03	0.4 ± 0.06
Oleic acid	1.1 ± 0.21	1.2 ± 0.12	1.1 ± 0.17
Linoleic acid	5.7 ± 0.33	5.8 ± 0.45	5.4 ± 0.16
Linolenic acid	20.8 ± 0.57	21.4 ± 0.85	22.1 ± 1.31

[a] mg g^{-1} on dry weight basis.

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the most important compounds of green tea, with the catechins being the most important polyphenols. The primary catechins in green tea are EC, ECG, EGC and EGCG (Ahmad and Mukhtar, 1999). In addition, TB, Caff, theophylline, and phenolic acids, such as gallic acid, are also present as minor constituents of green tea (Ahmad and Mukhtar, 1999). Table 3 shows the contents of purine alkaloids and catechins present in anaerobically treated tea leaves. The amounts of TB did not change during the anaerobic treatment, whereas Caff in anaerobically treated tea leaves was a little higher than that in untreated control. The amount of catechins in all samples showed the order: EGC> EGCG> GC> ECG> EC> C> GCG (Table 3). Yen and Chen (1996) reported that EGC is the more prevalent flavonol in tea extracts, while other findings suggested that EGCG is the most abundant catechin in green tea and GABA tea extracts (Wang *et al.*, 1992; Wang *et al.*, 2006). Inconsistencies in study findings regarding the composition of catechin in tea extract may be due to the different contents of catechins in different varieties and to the cultivation conditions of teas.

As shown in Table 3, the contents of EGC and EGCG in anaerobically treated tea leaves were significantly higher than those of untreated control ($P< 0.05$). This result is in contrast with that of previous study showing that both green tea and GABA tea have similar amounts of most phenolic compounds (Wang *et al.*, 2006). These authors reported that EGCG and EC were lower in GABA tea than green tea, and both teas had a similar antioxidant activity (Wang *et al.*, 2006). The only difference in processing steps between the previous study and ours is that the tea leaves were repeatedly treated under both anaerobic and aerobic incubations in previous studies, while tea underwent continuous anaerobic treatment in the present study. This suggested that the contents of bioactive compounds in GABA tea can be modulated by manufacturing process (Tables 1 and 3). EGCG, one of the major polyphenol components of green tea, was reported to inhibit low density lipoprotein oxidation *in vitro* by scavenging oxygen radicals and chelating metal ions which act as catalysts of lipid oxidation (Terao *et al.*, 1994). In addition, EGCG possess many cytoprotective properties such as inhibition of pro-inflammatory cytokines and inhibition of growth factors inducing neovascularization (Kaszkin *et al.*, 2004). To investigate the possible relationship between the antioxidant capacity and the contents of these phenolics in the literatures, we next examined antioxidant activities of

Table 3. Contents of purine alkaloids and catechins in green tea after continuous anaerobic treatment.

Compound [a]	Untreated control	Nitrogen	
		6 h	12 h
TB	1.6 ± 0.14 [b]	1.5 ± 0.18	1.7 ± 0.12
Caff	47.5 ± 2.01	49.2 ± 1.54	60.6 ± 1.34
GC	63.7 ± 0.87	64.0 ± 1.83	64.7 ± 3.15
EGC	110.4 ± 4.67^a	231.2 ± 11.32^b	264.6 ± 11.75^b
C	10.2 ± 0.34	11.9 ± 0.62	12.0 ± 0.88
EC	14.5 ± 0.92	14.4 ± 1.92	14.6 ± 1.23
EGCG	94.6 ± 9.46^a	193.5 ± 8.50^b	244.6 ± 6.74^b
GCG	8.2 ± 0.35	8.6 ± 0.34	8.5 ± 0.29
ECG	16.1 ± 0.70	15.9 ± 0.94	16.1 ± 0.73

[a] TB: Theobromine; Caff: Caffeine; GC: Gallocatechin; EGC: (-)-epicatechin-3-gallate; EGC: Epigallocatechin; C: Catechin; EC: Epicatechin; EGCG: Epigallocatechingallate; GCG: Gallocatechin gallate, ECG: Epicatechin gallate.

[b] mg g^{-1} on dry weight basis.

[a~b] Values with different superscripts in a row are significantly different ($P< 0.05$).

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the tea extracts obtained from continuous anaerobic incubation.

Scavenging activity for DPPH radical has been widely used to evaluate the antioxidant activity of natural products obtained from plant sources. As shown in Table 4, anaerobically incubated tea leaves possess significantly higher DPPH radical scavenging activity than that of untreated green tea (P< 0.05). The tea leaves treated with nitrogen gas for 12 h were found to be more effective (IC_{50} 6.6 μg mL^{-1}) than the standard positive controls *L*-ascorbic acid (24.7 μg mL^{-1}), quercertin (83.5 μg mL^{-1}) and curcumin (51.3 μg mL^{-1}).

Reactive oxygen species, including $O_2^{\bullet -}$, $NO^{\bullet}$, hydrogen peroxide, hydroxyl radical and peroxinitrite, play an important role in oxidative stress related to the pathogenesis of various diseases such as inflammation, heart disease, diabetes, gout and cancer (Slater, 1984). In the present work, a significantly higher antioxidant activity (IC_{50} 5.0-7.2 μg mL^{-1}) was found in anaerobically treated tea extracts compared with that of untreated tea extracts (12.0 μg mL^{-1}), which exhibited $O_2^{\bullet -}$ scavenging activity using the NBT dye reduction assay (P< 0.05) (Table 4). We also evaluated the effect of the extract on the XO activity by the metabolic conversion of xanthine to uric acid. Anaerobically treated tea extracts (IC_{50} 258.5-265.1 μg mL^{-1}) showed a statistically higher XO inhibitory activity than that of untreated tea extracts (IC_{50} 344.2 μg mL^{-1}) (P< 0.05) (Table 4). The same trend was observed for the level of $NO^{\bullet}$ scavenging effects in tested tea leaves. The IC_{50} values of tea extracts anaerobically treated with nitrogen gas for 6 and 12 hours were 374.1 and 380.2 μg mL^{-1}, respectively, which were statistically lower than that of untreated control (493.2 μg mL^{-1}) (P< 0.05) (Table 4).

Table 4. IC_{50} values of antioxidant activities of green tea after continuous anaerobic treatment.

		IC_{50} (μg Ml^{-1})			
		DPPH scavenging assay	$O_2^{\bullet -}$ scavenging assay	XO inhibition assay	$NO^{\bullet}$ scavenging assay
Untreated control		13.0 ± 0.20[a]	12.0 ± 1.78[a]	344.2 ± 9.06[a]	493.2 ± 11.01[a]
Nitrogen	6 h	8.5 ± 0.55[b]	7.2 ± 0.79[b]	265.1 ± 2.96[b]	380.2 ± 4.02[b]
	12 h	6.6 ± 0.62[c]	5.0 ± 0.74[c]	258.5 ± 1.31[c]	374.1 ± 1.74[b]

[a~c] Values with different superscripts in a column are significantly different (P< 0.05).

CONCLUSIONS

Overall, the continuous anaerobic incubation enhances antioxidant activity of GABA tea, possibly because of the correlation between antioxidant activity and the contents of EGC and EGCG (Tables 3 and 4), which is inconsistent with the one obtained in the previous report (Wang *et al.*, 2006). Collectively, our results indicate that the continuous anaerobic fermentation may be a suitable procedure for the production of GABA tea as a healthy beverage, which could be protective against free radicals-mediated damages. Because this study was based on green tea cultivar Yabukita, different cultivars of green tea should be further analyzed to improve the significance and precision of experimental results obtained.

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افزایش میزان مواد تشکیل‌دهنده زیست‌فعال و فعالیت آنتی‌اکسیدانی در چای سبز پس از انکوباسیون غیرهوازی پیوسته

ج. ه. کیم و م. ی. کیم

چکیده

گرچه روش‌های متعددی برای افزایش تجمع اسید گاما-آمینوبوتیریک (گابا) در چای توسعه یافته-اند، اجزای تشکیل‌دهنده فعال و فواید آنها برای سلامتی بسته به فرایند تولید چای متفاوتند. در این مطالعه، میزان ترکیبات زیست‌فعال و فعالیت آنتی‌اکسیدانی برگ‌های چای در شرایط تیمار پیوسته غیرهوازی مورد مطالعه قرار گرفت. مقادیر گابا و دیگر آمینواسیدهای آزاد مانند آلانین، والین، لئوسین، ایزولئوسین، پرولین و آسپاراجین در برگ‌های چای پس از تیمار غیرهوازی افزایش یافتند در حالی که میزان اسیدهای آلی و چرب تقریبا ثابت باقی ماند. در مقایسه با چای تیمار نشده، چای تیمارشده غیرهوازی در آزمون‌های جذب رادیکال 1,1-diphenyl-2-picrylhydrazyl، جذب رادیکال سوپراکسید، جذب اسید نیتریک و منع زانتین اکسیداز، مقادیر (-)-epigallocatechin و (-)-epigallocatechin gallate بیشتر و IC50 پایین‌تری داشت که این امر می‌تواند نشان دهنده بهبود فعالیت آنتی‌اکسیدانی در نتیجه انکوباسیون غیرهوازی پیوسته باشد. این نتایج نشان می‌دهند که تیمار غیرهوازی پیوسته در حین فرایند تولید ممکن است به تولید چای گابا با کیفیت بالا بیانجامد.

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