Effects of two enzyme extracts of *Aspergillus niger* on green tea aromas

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Abstract Green tea was investigated in terms of its aroma changes induced by two enzyme extracts of *Aspergillus niger*, i.e., crude enzyme extracted from fermentation using tea stalk medium (CETSM) and crude enzyme yielded in potato dextrose medium. The result showed that the former had significant effects on sensory indexes and volatile constituents, with significant increases in toasty and mushroom notes, while the latter had little influence on the aforementioned indexes. In addition, the volatile constituents were significantly affected; in particular, the contents of *cis*-3-hexenol, 1-octen-3-ol, eucalyptol, hexanol, and benzaldehyde increased. Furthermore, gas chromatography–olfactometry (GC–O) analysis showed that an increase in 1-octen-3-ol strengthened the mushroom note. These results indicate that CETSM contains some novel enzymes that can modify the aroma profile of green tea.

This study also provides valuable information and suggestions to use fermented enzymes to modify food aromas.

Keywords Green tea aroma · Enzyme · *Aspergillus niger* · GC–MS · GC–O

Introduction

Tea plant, which is a member of dicotyledonous theaceae plants, has great economic importance globally. According to the Food and Agriculture Organization of the United Nations, it is estimated that more than 5 million tons of tea is produced per annum [1]. The fascination of tea comes from two aspects: its special taste and aroma and its benefits to human health, i.e., antibacterial, antioxidant, anti-aging, anticancer, and eliminating lipid content [2, 3]. Tea is commonly categorized into green, oolong, and black tea depending on the degree of fermentation [1]. Green tea, which does not undergo any fermentation process, is the most important tea product in the world [1].

Tea aroma is the most important character of tea drinks. Various volatile constituents are fundamental for different tea aromas, which can be significantly affected by the relevant processing procedures [4], leading to a diversity of tea products with different aroma profiles. For example, Wang et al. [5] discovered that green tea possessed an aroma dominated by grassy or green odors, while semi-fermented or fermented tea had more fruity or floral notes because of the increased contents of methyl jasmonate and indole. Ramaswamy et al. [6] found that alcohols sharply reduced and aldehydes concomitantly increased during tea fermentation. Gong et al. [7] reported that the concentrations of main aroma constituents, especially methyl ethers of phenolic compounds, increased as the fermentation

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proceeded. Tea volatiles are considered to be generated from carotenoids, lipids, glycosides, and the Maillard reaction [8]. Among these pathways, enzymes play critical roles in catalyzing the generation of most volatiles [1]. In addition to endogenous enzymes in tea leaves, exogenous enzymes such as α -galactosidase, laccase, and polyphenol oxidase have also been demonstrated to improve tea aromas [8, 9]; for instance, the concentrations of *cis*-3-hexenol, hexanol, geraniol, and benzyl alcohol had significant increases in green tea after the treatment of β -glucosidase [9] and the contents of linalool, phenylethyl alcohol, and nerolidol increased after the treatment of α -galactosidase [17]. However, few studies have prepared exogenous enzymes for the diversification of tea aroma profiles.

Aspergillus niger is a fungal that is generally recognized as safety (GRAS) in food processing, which can generate various enzymes, including α -amylase, catalase, cellulase, α -galactosidase, β -glucosidase, glucoamylase, glucose oxidase, β -glucolase, hemicellulase, hesperidinase, lipase, naringinase, pectinase, protease, and tannase [10]. In our recent study, a crude enzyme extract, which was prepared from a fermented broth of *A. niger* growing on tea stalk medium (CETSM), had significant effects on the sensory characteristics of a green tea infusion, while an enzyme extract prepared from the fermented broth of *A. niger* growing in potato dextrose liquid medium (CEPDM) had little effect on the aroma profile. Therefore, this phenomenon prompted us to investigate the effects of the crude enzyme extracts from the fermented broth of *A. niger* on the aroma profile of green tea, in light of changes in sensory characters and volatile constituents. In addition, it prompted us to explore the fermentation on the tea stalk medium (TSM) as a potential process for yielding enzymes to modify and improve tea aromas.

Materials and methods

Tea sample, strain, and medium

Green tea was purchased from Fujian DaMin Development Company (Zhangzhou, Fujian province, China).

Aspergillus niger 41034 was preserved on a potato dextrose medium at 4 °C in Fujian Provincial Key Laboratory of Food Microbiology and Enzyme Engineering, Jimei University (Xiamen, Fujian, China).

Potato dextrose medium (PDM): 200 g of peeled potato in 1 L water were heated to 100 °C and maintained at this temperature for 30 min. The supernatant was collected after filtration through a filter paper. The medium was prepared by adding 20 g of glucose into the supernatant and adjusted to the final volume of 1000 mL with water. Each 30 mL of the medium was poured into a 250 mL

flask, followed by sterilization at 121 °C for 20 min prior to use.

TSM: 5 g of tea stalk and 10 mL of nutrient solution were added to a 250 mL flask, and then, the mixture was sterilized at 121 °C for 20 min. The nutrient solution was composed of (g): ammonium chloride 50, α -lactose 50, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1, KH_2PO_4 1, NaCl 1, and water 1000. The initial pH was 6.0.

Chemical standards and reagents

The internal standard of cyclohexanone and standard chemical series of C_7 – C_{20} alkanes used for the calculation of the linear retention index (RI) were obtained from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA). Forty volatile standards, including pentanal, hexanal, ethyl 2-methylbutyrate, *cis*-3-hexenol, hexanol, 2-heptanone, heptanal, *cis*-2-heptenal, benzaldehyde, 1-octen-3-ol, 6-methyl-5-hepten-2-one, *n*-octanal, *trans*-2,4-heptadienal, hexyl acetate, α -terpinene, *p*-cymene, eucalyptol, *cis*- β -ocimene and *trans*- β -ocimene mixture, 3-methyl-butyl butyrate, *trans*-2-octenal, linalool, *n*-nonanal, *cis*-limonene oxide and *trans*-limonene oxide mixture, *cis*-3-hexenyl butyrate, *cis*-3-hexenyl benzoate, (*trans*-, *cis*-) 2,6-nonadienal, ethyl benzoate, α -terpineol, methyl salicylate, *n*-decanal, β -cyclocitral, neral and geranial mixture, carvone, *cis*-3-hexenyl-hexanoate, *trans*-caryophyllene, α -ionone, *cis*-geranylacetone, and β -ionone, were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA) or Ormah Chemical Inc. Tic Co. (TIC, Hadimkoy, Istanbul), or Penta Manufacturing Co. (PMC, New Jersey, USA). Other chemicals were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Preparation of enzyme extracts

The strain was inoculated onto an agar slant and cultured at 30 °C for 3 days to allow the spores to grow. The spores were collected and transferred into a 0.75% NaCl solution and adjusted to an OD_{600} at 2. The spore concentration was about 1.0×10^8 CFU/mL. The medium was inoculated with 2 mL of the spore suspension. After inoculation, the PDM was cultivated at 30 °C with shaking at 180 rpm for 6 days, and then, the hypha was removed from the fermented broth by centrifugation with a force of 27,000 g for 20 min at 4 °C; the supernatant was further filtrated through a 0.45 μm filter, resulting in the enzyme extract of PDM. In comparison, the inoculated TSM was statically cultured at 30 °C for 4 days, followed by enzyme extraction using 50 mL of 50 mM phosphate buffer (pH = 7) for 1 h at 180 rpm and filtration using a 0.45 μm filter. The two enzyme extracts were precipitated using $(\text{NH}_4)_2\text{SO}_4$ in the saturation range of 10–90%, followed by dissolution in

50 mL of 50 mM phosphate buffer (pH = 7) and dialysis through a membrane with a cutoff of 10 kDa (Sangon Biotech (Shanghai) Co. Ltd. China). As a result, CETSM was obtained in 10 mL with a protein concentration of 1.133 mg/mL, and CEPDM was obtained in 10 mL with a protein concentration of 0.091 mg/mL. Furthermore, CETSM was analyzed to have marked activities of tannase, cellulase, β -glucosidase, and galactosidase. Although CEPDM did not exhibit the activity of cellulase, it was analyzed to contain noticeable amounts of β -glucosidase and galactosidase, as well as a weak activity of tannase. Both of them had very weak volatile chemicals and hardly any smell.

Preparation of tea samples

The original tea sample: 2.0 g of green tea was directly added to a glass bottle with 30 mL of pure water at 40 °C, which will be used as a blank control without the enzyme treatment for sensory and aroma analysis.

The CETSM-treated tea sample: 2.0 g of green tea and 0.5 mL of the enzyme extract solution (CETSM) were added to a glass bottle with 29.5 mL of 40 °C pure water.

The CEPDM-treated tea sample: 2 g of green tea and 0.5 mL of the enzyme extract solution (CEPDM) were added to a glass bottle with 29.5 mL of 40 °C pure water.

Enzyme control: 0.5 mL of CETSM or CEPDM was mixed with 29.5 mL of pure water, which was odorless, and thus, had not submitted to analysis.

After the preparation of respective mixtures, each sample was immediately closed tightly, followed by incubation at 40 °C for 1 h.

Descriptive sensory analysis

Sensory evaluation was conducted according to previous studies [11, 12] and ISO 8589, with minor modifications. In brief, 12 panelists, including 8 women and 4 men aged 25–45 years, were trained to distinguish the aroma characteristics and intensity of a series of standard solutions, which showed green, caramel-like, floral, toasty, woody, and mushroom notes. Formal sensory evaluations were then carried out in a clean room under white illumination at 25 ± 2 °C using a 9-point scoring method, where 1 indicates an unperceived attribute intensity and 9 indicates a very strong attribute intensity. The panelists were asked to randomly rate green, caramel-like, floral, toasty, woody, and mushroom notes, and give a score within 1–9. Mean values were calculated after corrections of effects of session and removal of outliers. A gap of 20 s was allowed between individual odor assessments.

Solid-phase microextraction (SPME) of volatiles

Each sample was added with 10 μ L/L of the internal standard (*IS*) of cyclohexanone. The adsorption of volatiles was operated at 40 °C for 20 min using a DVB/CAR/PDMS (50/30 μ m) SPME fiber (Supelco, Bellefonte, PA, USA) prior to the chemical analysis by gas chromatography coupled with mass spectrometry (GC–MS).

Operation of GC–MS

The analysis of volatile components was performed on a gas chromatograph coupled with a QP 2010 plus mass spectrometer (QP 2010 plus GC-EI/MS). Two different fused silica capillary columns, i.e., 60 m $\times$ 0.32 mm $\times$ 0.25 μ m Rtx-5MS and 60 m $\times$ 0.32 mm $\times$ 0.25 μ m Rtx-Wax (Restek Corporation, Bellefonte, PA, USA), were used to separate and identify the volatiles. Desorption of volatiles was conducted by holding the fiber in the injector at 200 °C for 3 min under the splitless mode. Helium was used as the carrier gas at a flow rate of 3 mL/min. The temperature of the oven with Rtx-5MS was programmed from the initial 50 °C for 5 min, and then increased to 200 °C at a rate of 3 °C/min and held for 1 min. The temperature of the oven with Rtx-Wax was programmed from the initial 30 °C for 5 min, and then increased to 200 °C at a rate of 3 °C/min, and held for 1 min. The temperatures of both the ion source and the interface were set at 200 °C.

Qualitative and quantitative analyses of volatiles

Most of the volatiles (40 of 44) were identified by matching their MS spectra and Kovats Retention Indices (RI) on the Rtx-5MS column to those of the standards. Other volatiles (4 of 44) that currently lack standards were tentatively identified by matching their MS spectra to those in Mass Spectral Library (NIST08, NIST08s, FFNSC1.3) and their RIs to those reported in relevant references. The identified volatiles were quantitatively analyzed according to their respective calibration curves by the selective ion monitoring (SIM) mode with the aid of the *IS* (i.e., cyclohexanone) to correct the internal errors. The concentrations of other volatiles were estimated using the calibration curve of the *IS* by the total ion chromatogram (TIC) mode.

GC–O analysis

An Agilent 6890 GC with an FID detector (Agilent Technologies, Palo Alto, CA, USA) coupled with an olfactory detection port Gerstel ODP-2 (Gerstel AG Enterprise, Mülheim an der Ruhr, Germany) was used to analyze the aroma intensities of the volatiles. The GC was

Table 1 Sensory evaluation of the green tea after CETSM and CEPDM treatments

	Green	Caramel-like	Floral	Toasty	Woody	Mushroom
Original tea	7.4 ± 1.1 ^a	6.5 ± 1.1 ^a	4.5 ± 1.4 ^a	2.3 ± 0.8 ^b	3.7 ± 1.1 ^a	1.9 ± 0.8 ^b
CETSM	2.4 ± 0.5 ^b	2.5 ± 0.9 ^b	4.0 ± 0.8 ^a	5.8 ± 0.8 ^a	2.3 ± 1.0 ^c	6.1 ± 1.6 ^a
CEPDM	7.1 ± 0.8 ^a	6.5 ± 0.8 ^a	4.1 ± 1.5 ^a	2.8 ± 0.6 ^b	3.0 ± 1.1 ^b	1.9 ± 0.3 ^b

Columns with different superscripts (i.e., a, b, c) have significantly statistical differences ($P < 0.05$)

fitted with an HP-Innowax column (60 m × 0.25 mm × 0.25 μm, Agilent, Palo Alto, CA, USA). The oven temperature was programmed as follows: an initial temperature of 40 °C was kept for 1 min and then increased to 220 °C at a speed of 4 °C/min. High-purity nitrogen was used as the carrier gas at a flow rate of 1 mL/min. The temperature of the injector port was 250 °C, and the SPME was injected into the GC-FID/O system in the splitless mode. GC–O was performed by three panelists. The aroma character of each eluted volatile compound was evaluated by sniffing, and its aroma intensity was rated on a five-point scale (1 = extremely weak, 2 = clear but not intense odor, 3 = medium odor, 4 = intense odor, 5 = extremely strong) [13]. The identification of volatiles was conducted by matching their RI values to those of the standards.

Statistical analysis

All experiments were repeated three times to calculate the mean values of the chemical concentrations. The data from sensory evaluation were analyzed by reliability and validity analysis using the SPSS-IBM 19.0 software. The mean values were calculated after a correction for session, panelist effects, and removal of outliers (one of the data). Principal components analysis (PCA) was conducted using the method of biplot visualization (SPSS-IBM 19.0 software), which was used to show the relationships between and among detailed flavor attributes, as well as flavor attributes. The PCA was under varimax orthogonal rotation. The significant analysis of concentrations of volatile components was performed by the software SPSS 19.0 and Microsoft Excel 2013 (SPSS-IBM 19.0 software). Hierarchical clustering was performed through OmicsOffice[®] built in TIBCO[®] Spotfire[®] using the hierarchical average linkage method with correlation. Data were normalized using a linear method. The similarity metric was measured with the Pearson distance method.

Results and discussion

Effects of enzymatic treatments on the sensory index of green tea

The original aroma of the untreated original green tea was dominated by green and caramel-like notes, as well as

weak toasty, woody, and mushroom notes, which was consistent with previous studies in which the green note represented the typical aroma of green tea [14].

Compared to the original aroma profile of the green tea, the CEPDM-treated tea did not have significant differences ($P > 0.05$) in green, caramel-like, floral, toasty, and mushroom notes; in contrast, the CETSM-treated tea showed significant decreases in the aroma intensities of green (from 7.4 to 2.4) and caramel-like (from 6.5 to 2.5) notes and significant increases in the toasty (2.3–5.8) and mushroom (1.9–6.1) notes ($P < 0.05$) (Table 1). In comparison to the CEPDM-treated tea, the CETSM-treated tea had significantly weaker green and caramel-like notes and significantly stronger toasty and mushroom notes ($P < 0.05$). The aromas were further compared by PCA, which is commonly used to extract interpretable and statistical reliable information from data. As a result, two PCs (i.e., PC1 and PC2) that contributed more than 80% to the multiple variables were extracted, among which PC1 contributed 62.2% and PC2 contributed 19.3% of the total variances (Fig. 1). Component matrix and scores of the coefficient matrix indicated that PC1 contributed to the most parts of the green, caramel-like, toasty, and mushroom notes, and PC2 contributed to the floral and woody notes. The PCA scatter plots showed that the CEPDM-

Fig. 1 PCA scatter plot of the note (A) and scatter diagram (B) of the tea samples

Table 2 Identification and calibration equations of the volatiles

No.	Chemical	Standard curves	R^2	Range	CF	Retention index				CI	Identification	References
						RI 1	RI 2	RI 3	RI 4			
1	Pentanal	$Y = 0.00418X - 0.00127$	0.997	4.0–128.0	343.6	703	701	974	968	44 81	Std MS	Ho et al. [8]
2	Hexanal	$Y = 0.01096X + 0.00289$	0.998	25.0–160.0	72.2	803	802	1074	1073	44 56 82	Std MS	Zhang et al. [17]
3	Ethyl 2-methylbutyrate	$Y = 0.06082X + 0.00320$	0.996	0.1–64.0	15.6	846	849	1052	1051	57 102	Std MS	Tschiggerl et al. [20]
4	cis-3-Hexenol	$Y = 0.69237X - 0.09658$	0.998	0.2–32.0	1.7	852	855	1381	1381	41 67	Std MS	Ho et al. [8]
5	Hexanol	$Y = 0.00479X - 0.00143$	1.000	0.4–64.0	297.6	866	869	1359	1365	56 43 69	Std MS	Ho et al. [8]
6	2-Heptanone	$Y = 0.03304X + 0.00075$	0.997	0.5–32.0	29.6	889	898	1233	1172	43 114 58	Std MS	Zhang et al. [17]
7	Heptanal	$Y = 0.03524X - 0.01615$	0.999	1.0–16.0	52.4	901	904	1180	1187	41 70	Std MS	Kim et al. [18]
8	cis-2-Heptenal	$Y = 0.01430X - 0.00645$	0.992	0.43–3.45	127.4	955	954	1319	1319	41 83 112	Std MS	Wang et al. [5]
9	Benzaldehyde	$Y = 0.00373X - 0.00168$	0.999	0.25–16.0	487.8	959	966	1509	1514	106 77 51	Std MS	Zhang et al. [17]
10	1-Octen-3-ol	$Y = 0.02958X - 0.00154$	0.999	1.0–32.4	35.7	979	952	1456	1458	57 72 43	Std MS	Du et al. [19]
11	5-Hepten-2-one, 6-methyl-	$Y = 0.02149X - 0.00137$	0.998	0.1–64.0	49.7	987	989	1333	1337	43 69 108	Std MS	Kim et al. [18]
12	n-Octanal	$Y = 0.03766X - 0.02471$	0.998	1.0–32.0	37.2	1003	1008	1286	1276	93 41	Std MS	Zhang et al. [17]
13	trans-2,4-Heptadienal	$Y = 0.05064X + 0.00038$	0.999	0.25–32.0	19.6	1013	1013	1484	1488	81 39 111	Std MS	Zhang et al. [17]
14	Hexyl acetate	$Y = 0.38218X - 0.02945$	0.998	0.05–3.2	2.8	1014	1014	1269	1261	43 84	Std MS	Zhang et al. (2015)
15	α -Terpinene	$Y = 0.04353X - 0.00321$	0.999	0.1–16.0	25.2	1016	1017	1162	1172	93 41 136	Std MS	Alasalvar et al. [21]
16	p-Cymene	$Y = 0.84675X - 0.23700$	0.999	0.5–16.0	1.6	1024	1028	1259	1278	119 134	Std MS	Zhang et al. [17]
17	Eucalyptol	$Y = 0.04232X - 0.02134$	0.999	10.0–320.0	26.1	1030	1039	1202	1183	43 154	Std MS	Cheng et al. [23]
18	cis- β -Ocimene	$Y = 0.15508X - 0.02105$	0.993	0.16–2.5	7.5	1038	1038	1230	1241	93 79	Std MS	Zhang et al. [17]
19	trans- β -Ocimene	$Y = 0.40427X + 0.00226$	0.892	1.0–32.0	2.5	1048	1048	1247	1263	93 79 136	Std MS	Zhang et al. [17]
20	3-Methyl-butyl butyrate	$Y = 0.45475X - 0.06299$	0.998	0.16–2.56	2.6	1056	1060	1261	1133	71 43	Std MS	Ferrari et al. [22]
21	trans-2-Octenal	$Y = 0.05321X + 0.21231$	0.998	0.16–10.24	20.1	1058	1060	1420	1411	121 136	Std MS	Du et al. [19]
22	3,5-Octadien-2-one	–	–	–	–	1071	1072	1513	1493	93 41 136	MS	Kraujalytė et al. [3]
23	Undecene, 4-methyl-	–	–	–	–	1101	–	–	–	71 43 95	MS	Kraujalytė et al. [3]
24	Linalool	$Y = 0.02416X - 0.00658$	0.992	2.0–64.0	56.9	1102	1103	1553	1552	71 93 136	Std MS	Zhang et al. [17]
25	Nonane, 2,6-dimethyl-	–	–	–	–	1094	–	–	–	43 71 113	MS	–
26	n-Nonanal	$Y = 0.12830X + 0.00269$	1.000	0.2–6.4	7.6	1105	1107	1388	1385	41 57 114	Std MS	Ho et al. [8]
27	Dodecane, 4-methyl-	–	–	–	–	1107	1327	–	–	93 79 136	MS	–
28	cis-Limonene oxide	$Y = 0.08208X + 0.01395$	0.997	0.2–6.4	10.4	1108	1107	1436	1433	43 67 108	Std MS	Kraujalytė et al. [3]
29	trans-Limonene oxide	$Y = 0.00816X - 0.00065$	0.999	0.2–6.4	133.2	1139	1138	1447	1441	43 67 108	Std MS	Kraujalytė et al. [3]
30	cis-3-Hexenyl benzoate	$Y = 0.09213X + 0.06521$	0.999	0.1–6.4	9.1	1145	1151	1619	1619	67 43 82	Std MS	–
31	Trans/cis-2,6-Nonadienal	$Y = 0.10981X + 0.03871$	1.000	0.05–3.2	8.4	1154	1155	–	–	41 69	Std MS	Du et al. [19]
32	Hethyl benzoate	$Y = 0.17867X + 0.00303$	1.000	0.05–3.2	5.5	1172	1170	1657	1661	105 150 71	Std MS	Cheng et al., 2013
33	α -Terpineol	$Y = 0.00374X - 0.00131$	0.996	0.4–6.4	411.5	1192	1199	1697	1694	59 93 136	Std MS	Du et al. [19]
34	Methyl salicylate	$Y = 0.03919X + 0.00446$	0.999	0.2–6.4	22.9	1195	1197	1759	1779	120 152 39	Std MS	Zhang et al. [17]
35	n-Decanal	$Y = 0.39084X - 0.02062$	0.999	0.05–3.2	2.7	1206	1413	1496	1499	43 57 112	Std MS	Zhang et al. [17]

Table 2 continued

No.	Chemical	Standard curves	R^2	Range	CF	Retention index				CI	Identification	References
						RI 1	RI 2	RI 3	RI 4			
36	β -Cyclocitral	$Y = 0.20631X + 0.09871$	0.999	0.8–12.8	5.3	1222	1223	1607	1609	137 152	Std MS	Zhang et al. [17]
37	Neral	$Y = 0.01557X - 0.00166$	0.997	0.09–2.94	71.9	1243	1242	1674	1667	41 69	Std MS	Zhang et al. [17]
38	Carvone	$Y = 0.01210X - 0.00056$	0.999	0.05–3.2	86.7	1246	1250	1718	1731	82 54 150	Std MS	Zhang et al. [17]
39	Geranial	$Y = 0.02075X - 0.00092$	0.999	0.05–3.45	50.4	1272	1273	1697	1686	69 41	Std MS	Zhang et al. [17]
40	<i>cis</i> -3-Hexenyl-hexanoate	$Y = 0.47030X + 0.05057$	0.999	1.0–64.0	1.9	1383	1380	1655	1651	82 67 43	Std MS	Zhang et al. [17]
41	<i>trans</i> -Caryophyllene	$Y = 0.40826X - 0.05463$	0.996	1.0–32.0	2.8	1423	1426	1581	1583	69 204	Std MS	Lasekan et al. [24]
42	α -Ionone	$Y = 0.09651X - 0.2811$	0.997	0.18–2.94	10.2	1431	1429	1841	1840	171 43	Std MS	Ho et al. [8]
43	<i>cis</i> -Geranylacetone	$Y = 0.11878X - 0.00387$	0.998	0.5–16.0	8.7	1455	1453	1852	1858	43 69 151	Std MS	Ho et al. [8]
44	β -Ionone	$Y = 0.08615X - 0.01096$	0.998	0.1–12.4	13.3	1490	1478	1929	1945	177 43	Std MS	Ho et al. [8]

RI 1 was obtained by GC–MS analysis using an Rtx-5MS column. RI 2 was reported in the literature or NIST Chemistry Web Book (<http://webbook.nist.gov/chemistry>), which was using a column similar to Rtx-5MS (the column stationary phase is 5% diphenyl and 95% dimethyl polysiloxane, equivalent to United States Pharmacopoeia G27). RI 3 was obtained by GC–MS analysis using an Rtx-wax column. RI 4 was reported in the literature or NIST Chemistry Web Book (<http://webbook.nist.gov/chemistry>) using a column similar to Rtx-wax (the column stationary phase is polyethylene glycol, equivalent to United States Pharmacopoeia G16). Std in reference indicates that the identification has been confirmed by matching a standard. All of the equations of the calibration curves of ASCs were calculated in the SIM mode. Y is the ratio of the peak area of the ASC to that of the internal standard (IS), and X is the ratio of the concentration of the ASC to that of the IS. CF represents correction factors using this formula: $CF = (As/Ms)/(Ar/Mr)$. As represents the corresponding quantitative ion (SIM mode) area of the IS; Ar represents the corresponding quantitative ion (SIM mode) area of the ASC, Ms represents the concentration of IS, Mr represents the concentration of the ASC

Table 3 Concentration of the volatiles

No.	Volatile	Concentration (µg/L)			Cluster
		Original	CETSM-treated	CEPDM-treated	
Terpenes					
15	α-Terpinene	4.5 ± 0.7a	ndb	ndb	1
18	cis-β-Ocimene	0.4 ± 0.2a	0.5 ± 0.3a	0.4 ± 0.1a	4
19	trans-β-Ocimene	2.2 ± 0.4b	3.2 ± 0.6a	1.8 ± 0.5c	4
41	trans-Caryophyllene	2.4 ± 0.7a	ndb	ndb	1
	Subtotal	9.4	3.8	2.1	
Alcohols					
4	cis-3-Hexenol	ndb	13.5 ± 1.9a	ndb	5
5	Hexanol	ndb	29.7 ± 7.6a	ndb	5
10	1-Octen-3-ol	3.0 ± 1.0b	10.0 ± 0.7a	3.5 ± 0.2b	4
17	Eucalyptol	ndb	155.2 ± 29.6a	ndb	5
24	Linalool	10.5 ± 11.2a	5.2 ± 0.4c	7.3 ± 5.8b	4
33	α-Terpineol	13.0 ± 7.6b	13.8 ± 8.3b	28.2 ± 35.4a	4
	Subtotal	26.5	227.4	38.9	
Aldehydes					
1	Pentanal	26.3 ± 7.2a	23.4 ± 3.3a	14.2 ± 4.5b	4
2	Hexanal	139.1 ± 27.4a	134.4 ± 24.4a	124.5 ± 8.9a	4
7	Heptanal	3.7 ± 0.3a	4.1 ± 0.3a	3.7 ± 0.4a	4
8	cis-2-Heptenal	1.4 ± 0.4b	1.9 ± 0.2	1.0 ± 0.4b	4
9	Benzaldehyde	ndb	12.8 ± 1.1	ndb	5
12	n-Octanal	5.9 ± 0.2a	4.9 ± 3.2b	6.3 ± 0.6a	4
13	trans-2,4-Heptadienal	13.7 ± 1.4c	18.8 ± 2.8a	16.8 ± 1.7b	4
21	trans-2-Octenal	6.7 ± 0.8b	10.4 ± 0.2	7.0 ± 0.4b	4
26	n-Nonanal	1.3 ± 0.1a	1.5 ± 0.0a	2.1 ± 0.9a	4
31	Trans/cis-2,6-Nonadienal	0.6 ± 0.1a	0.6 ± 0.0a	0.7 ± 0.1a	4
35	n-Decanal	2.7 ± 0.3a	2.7 ± 0.1a	2.4 ± 0.1b	4
36	β-Cyclocitral	2.8 ± 0.2b	3.0 ± 0.1a	2.8 ± 0.2b	4
37	Neral	0.7 ± 0.0a	ndb	0.6 ± 0.2a	3
39	Geranial	2.4 ± 0.6b	4.5 ± 0.3a	1.7 ± 0.2c	4
	Subtotal	207.5	222.8	183.6	
Ketones					
6	2-Heptanone	13.1 ± 4.2a	16.1 ± 2.8a	15.4 ± 2.1a	4
11	5-Hepten-2-one, 6-methyl-	35.4 ± 4.0b	41.6 ± 1.6a	35.6 ± 2.9b	4
22	3,5-Octadien-2-one	3.7 ± 0.3a	4.7 ± 0.1a	4.4 ± 0.4a	4
38	Carvone	1.1 ± 0.1a	ndb	0.9 ± 0.3a	3
42	α-Ionone	1.4 ± 0.0a	1.3 ± 0.1a	1.3 ± 0.1a	4
43	cis-Geranylacetone	7.0 ± 1.4a	7.0 ± 1.0a	7.5 ± 1.3a	4
44	β-Ionone	6.2 ± 0.8b	7.2 ± 0.1a	6.2 ± 0.7b	4
	Subtotal	67.8	77.9	71.3	
Esters					
3	Ethyl 2-methylbutyrate	16.3 ± 4.1a	15.8 ± 1.2a	16.7 ± 1.5a	4
14	Hexyl acetate	ndb	2.7 ± 0.8a	3.0 ± 0.1a	2
20	3-Methyl-butyl butyrate	0.4 ± 0.0b	0.8 ± 0.2a	0.4 ± 0.0b	4
30	cis-3-Hexenyl butyrate	1.3 ± 0.1a	1.3 ± 0.2a	1.2 ± 0.2a	4

Table 3 continued

No.	Volatile	Concentration ($\mu\text{g/L}$)			Cluster
		Original	CETSM-treated	CEPDM-treated	
32	<i>cis</i> -3-Hexenyl benzoate	$1.3 \pm 0.7a$	$1.0 \pm 0.2b$	$1.2 \pm 0.2a$	4
34	Methyl salicylate	$2.3 \pm 0.7b$	$5.5 \pm 2.6a$	$2.6 \pm 0.4b$	4
40	<i>cis</i> -3-Hexenyl-hexanoate	ndb	ndb	$12.2 \pm 1.6a$	2
	Subtotal	21.6	27.1	37.4	
	Miscellaneous				
23	Undecene, 4-methyl	ndb	$4.1 \pm 0.9a$	ndb	5
25	Nonane, 2,6-dimethyl	ndb	$0.9 \pm 0.3a$	ndb	5
16	<i>p</i> -Cymene	$2.9 \pm 0.7b$	$3.6 \pm 0.4a$	$3.5 \pm 0.6a$	4
27	Dodecane, 4-methyl-	ndb	$2.3 \pm 0.5a$	ndb	5
28	<i>cis</i> -Limonene oxide	$1.2 \pm 0.0a$	ndb	$1.1 \pm 0.0a$	3
29	<i>trans</i> -Limonene oxide	$1.6 \pm 0.5a$	ndb	$1.6 \pm 0.4a$	3
	Subtotal	5.7	10.9	6.2	
	Total	338.5	569.8	339.6	

0 means the concentration was $<0.3 \mu\text{g/L}$ or not detected. Rows with different letters a, b, c indicate significant statistical differences ($p < 0.05$)

treated tea possessed a very similar aroma to that of the original green tea, while the CETSM-treated tea displayed a distinct aroma from those of the two counterparts, indicating that CETSM had significantly changed the original aroma profile of the green tea. Xu et al. [15] reported that the herbaceous note of green tea decreased after the treatment of a proteolytic enzyme from *Aspergillus oryzae*. Chen et al. [16] reported that tea infusions had a stronger floral note after 42 days of fermentation. Nevertheless, previous studies did not reveal the changes in other aroma notes, e.g., caramel-like, toasty, and mushroom notes after enzymatic treatments, although they reported that enzymatic treatments could decrease the green note of green tea [15]. In this context, it was worthy of studying the effect of CETSM on the modification of the original and generation of novel aromas of tea.

Effects of enzyme treatments on volatile compounds of green tea

A total of 44 volatiles were detected in the tea samples (Table 2), among which 40 were identified based on matching their MS and RI values with those of the standards using the Rtx-5MS and Rtx-wax columns. Other 4 volatiles were tentatively identified according to the MS library searching and RI value matching with those reported in previous references [3, 5, 8, 17–24]. These 44 volatile compounds included 4 terpenes, 6 alcohols, 14 aldehydes, 7 ketones, 7 esters, and 6 miscellaneous. The original green tea, CETSM-treated tea, and CEPDM-treated tea had 35, 37, and 35 volatile components being detected, respectively (Table 3). All these samples had

similarity, in a large extent, in their volatile constituents that mainly consisted of linalool ($5.2\text{--}10.5 \mu\text{g/L}$), α -terpineneol ($13.0\text{--}28.2 \mu\text{g/L}$), 2-heptanone ($13.1\text{--}16.1 \mu\text{g/L}$), *trans*-2,4-heptadienal ($13.7\text{--}18.8 \mu\text{g/L}$), pentanal ($14.2\text{--}26.3 \mu\text{g/L}$), ethyl 2-methylbutyrate ($15.8\text{--}16.7 \mu\text{g/L}$), 6-methyl-5-hepten-2-one ($35.4\text{--}41.6 \mu\text{g/L}$), and hexanal ($124.5\text{--}139.1 \mu\text{g/L}$). These results were consistent with a previous report that hexanal, linalool, etc., were the major volatiles of green tea [25]. However, these three samples contained many other volatiles existing in delicate differences in their concentrations, which have also affected the aroma profiles that will be explored by the multivariate statistics and described below.

The hierarchical clustering (Fig. 2) was conducted to find the interconnectivity and closeness of individual volatiles, which revealed the similarities of the individual volatiles and their contributions to the aromas in the 3 samples. After normalization, each volatile concentration was represented by a color in a heat map, where the maximum (0.43), average (0.21), and minimum (0.00) values (relative contents) were represented by red, green, and blue, respectively. As a result, the volatiles were classified into 5 clusters according to their concentration changes (Table 3; Fig. 2). The volatiles, including α -terpinene and *trans*-caryophyllene (*trans*-) in cluster 1, were only detected in the original green tea (Table 3; Fig. 2). The volatiles in cluster 2 included hexyl acetate and *cis*-3-hexenyl-hexanoate, whose concentrations significantly increased after the enzymatic treatments of CETSM and CEPDM (Table 3; Fig. 2). All volatiles in cluster 3 (i.e., *cis*-limonene oxide, *trans*-limonene oxide, neral, and carvone) vanished after the CETSM treatment, while their

Fig. 2 Hierarchical clustering of volatile compounds

concentrations had no significant differences after the CEPDM treatment. In comparison, most of the volatiles in cluster 4 (i.e., pentanal, hexanal, 2-heptanone, ethyl 2-methylbutyrate, heptanal, *cis*-2-heptenal, 1-octen-3-ol, 6-methyl-5-hepten-2-one, *trans*-2,4-heptadienal, *n*-octanal, *p*-cymene, *cis*- β -ocimene, *trans*- β -ocimene, 3-methyl-butyl butyrate, *trans*-2-octenal, 3,5-octadien-2-one, linalool, *n*-nonanal, *cis*-3-hexenyl *cis*-hexanoate, (*trans*-, *cis*-) 2,6-nonadienal, *cis*-3-hexenyl benzoate, α -terpineol, methyl salicylate, *n*-decanal, β -cyclocitral, geranial, α -ionone, *cis*-geranylacetone, and β -ionone) had no significant statistical differences ($P > 0.05$) in their concentrations in all tea samples. Cluster 5 had 7 volatiles, including *cis*-3-hexenol, hexanol, benzaldehyde, eucalyptol, 4-methyl-undecene, 2,6-dimethyl-nonane, and 4-methyl-dodecane, which were only detected in the CETSM-treated tea. This result also

indicated that the enzyme(s) in the CETSM had released or generated those novel flavors via the enzymatic reactions from the original sample.

Based on the hierarchical clustering analysis, it is obvious that the CETSM-treated tea had significantly different volatile compositions from those of original green tea and the CEPDM-treated tea (Table 3). For example, there were decreased contents of the chemicals in cluster 1 (α -terpinene and *trans*-caryophyllene) and cluster 3 (*cis*-limonene oxide, *trans*-limonene oxide, neral, and carvone) and increased contents in cluster 2 (hexyl acetate and *cis*-3-hexenyl-hexanoate) and cluster 5 (*cis*-3-hexenol, hexanol, benzaldehyde, eucalyptol, 4-methyl-undecene, 2,6-dimethyl-nonane, and 4-methyl-dodecane) (Fig. 2). Among these volatiles, α -terpinene and *trans*-caryophyllene were reported to be transformed into alcohols through enzymatic

oxidations [1, 26]; the concentrations of *cis*-3-hexenol, hexanol, geraniol, and benzyl alcohol had significant increases in green tea after a β -glucosidase treatment [9]; and the contents of linalool, phenylethyl alcohol, and nerolidol were reported to be increased after an α -galactosidase treatment [17]. Besides, some researchers have shown that α -linolenic acid, linoleic acid, oleic acid, and palmitoleic acid are precursors of short-chain aroma compounds, such as 2-hexanal, 2-hexanol, 3-hexanol, and 1-octen-3-ol [27]. Particularly, 1-octen-3-ol, which had a typical odor of mushroom, may be generated from the linoleic acid degradation [28]. Although the CETSM-treated tea has shown some volatile changes similar to that in previous studies, the CETSM treatment also exhibited some novel influences on tea flavors, e.g., the increased contents of 1-octen-3-ol, hexanol, and eucalyptol (Fig. 2), which have been not reported yet. These results can also be attributed to the activities of glycosidase in CETSM, such as β -glucosidase and galactolase, which have

been reported to release alcohol volatiles from glycosides precursors [9, 17]. Eucalyptol belongs to an alcohol in structure, and thus, it is reasonable to conclude that the glycosidases in CETSM transform the aroma precursors to generate eucalyptol.

Investigation of the aroma changes by GC–O analysis

A total of 26 volatiles were found to have aroma-impact activities in the samples by the GC–O analysis (Table 4). Among these chemicals, hexanal, hexanol, heptanal, nonanal, octanal, and β -cyclocitral showed typical grassy notes; carvone showed a minty note, eucalyptol showed a strong camphor note, *p*-cymene showed a strong oily note, and α -ionone and β -ionone exhibited a typical woody note (Table 4), which are all consistent with previous references [1, 12, 19, 21, 24, 29–35]. Furthermore, hexanal, decanal, and β -ionone showed the strongest intensity, which was

Table 4 GC–O analysis of the odor intensities

No.	Volatiles	Odor characteristic	Flavor intensity			Odor references
			Original	CETSM	CEPDM	
1	Pentanal	Apple, aldehydes	5	5	4	Joshi et al. [32]
2	Hexanal	Green, grassy, aldehydes	5	5	5	Yang et al. [1]
3	Hexanol	Green	0	1	0	Takei et al. [35]
4	2-Heptanone	Woody, fruity	2	2	2	Joshi et al. [32]
5	Heptanal	Fatty, green	5	4	4	Joshi et al. [32]
6	<i>cis</i> -2-Heptenal	Green, tomato	4	4	3	Alasalvar et al. [21]
7	Benzaldehyde	Sweet, almond	3	5	3	Fanaro et al. [31]
8	1-Octen-3-ol	Mushroom	1	5	1	Lasekan et al. [24]
9	<i>trans</i> -2,4-Heptadienal	Nut, oily, aldehydes	4	4	4	Joshi et al. [32]
10	<i>n</i> -Octanal	Aldehydes, green	5	5	5	Márquez et al. [29]
11	Hexyl acetate	Fruity, sweet	0	1	1	Ma et al. (2013)
12	<i>p</i> -Cymene	Oily, sweet	1	1	1	Alasalvar et al. [21]
13	Eucalyptol	Camphor, pungent	0	4	0	Lasekan et al. [24]
14	<i>trans</i> - β -Ocimene	Pungent, burnt	0	2	0	Fanaro et al. [31]
15	Linalool	Floral, fruity	3	1	3	Joshi et al. [32]
16	<i>n</i> -Nonanal	Green, citrus	3	3	5	Qin et al. 2011
17	<i>cis</i> -Limonene oxide	Sweet, floral	1	1	1	Lasekan et al. [24]
18	<i>trans</i> -Limonene oxide	Sweet, floral	1	1	1	Lasekan et al. [24]
19	α -Terpineol	Oily, resinous	2	1	1	Lv et al. [34]
20	Methyl salicylate	Sweet	1	2	1	Qin et al. [12]
21	<i>n</i> -Decanal	Floral, fruity, aldehydes	5	5	5	Lv et al. [34]
22	β -Cyclocitral	Green, minty, fruity	1	1	1	Lv et al. [34]
23	Neral	Lemon, minty	3	1	3	Kowalski et al. [30]
24	Carvone	Minty	3	1	3	Lasekan et al. [24]
25	Geraniol	Lemon, minty	2	1	2	Fanaro et al. [31]
26	β -Ionone	Woody	4	4	4	Lv et al. [34]

Note: Odor characteristics were evaluated by GC–O and corresponding references. Flavor intensity: 5: very strong, 4: strong, 3: medium, 2: lower, 1: detected

consistent with the previous report these compounds were major aroma contributors of green tea according to Ho et al. [8].

It has been reported that 1-octen-3-ol has a typical mushroom note [24], eucalyptol possesses a camphor and pungent odor [24], and benzaldehyde has a sweet note [31]. After the CETSM treatment, the odor intensity of 1-octen-3-ol increased from 1 to 5 (Table 4), giving an acceptable explanation to the increased mushroom note (from 1.9 to 6.1), as shown in the sensory evaluation (Table 2). In addition, benzaldehyde showed an odor intensity increase from 3 to 5 (Table 4), which was linked to the decreased green note (from 7.4 to 2.4), as illustrated in Table 1. In particular, the note intensity of eucalyptol increased from 0 to 4 (Table 4), which might cover, or counteract, the green and caramel-like notes, and thus, indirectly decreased the green note (from 7.4 to 2.4) and caramel-like note (from 6.5 to 2.5) (Table 1). These results indicated that the volatile changes in terms of their concentration as well as aroma intensities, induced by the CETSM treatment, were the cause of changes in sensory descriptions.

In summary, sensory evaluation, GC–MS, and GC–O analysis revealed that, compared to the control without the enzyme extract treatment, CEPDM had little effect on the volatile constituents and aroma profile, while CETSM significantly changed the aroma intensities and volatile components of green tea. The CETSM treatment resulted in lower intensities of green and caramel-like notes and higher intensities of toasty and mushroom notes. In addition, the CETSM treatment significantly changed the volatile constituents, especially increasing the contents of 1-octen-3-ol, benzaldehyde, hexanol, and eucalyptol. GC–O analysis demonstrated that the increased contents of benzaldehyde and eucalyptol were the major reason for the decrease in green and caramel-like notes, while the increase in 1-octen-3-ol was the major cause for the increased mushroom note. These results indicated that CETSM contains some enzymes responsible for the changes in the green tea aroma profiles. Although we have not isolated and purified those interesting flavoring enzymes yet, the relevant experiments have been designed and scheduled for the purpose of revealing the mechanisms of flavor changes due to the enzymatic reactions.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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