Analysis of Glycosidically Bound Aroma Precursors in Tea Leaves. 2. Changes in Glycoside Contents and Glycosidase Activities in Tea Leaves during the Black Tea Manufacturing Process#

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Glycosides are known to be precursors of the alcoholic aroma compounds of black tea. They are hydrolyzed by endogenous glycosidases during the manufacturing process. Changes in the amounts of these glycosides during the manufacturing process were investigated by using a capillary gas chromatographic−mass spectrometric analysis after trifluoroacetyl derivatization of the tea glycosidic fractions. Primeverosides were 3-fold more abundant than glucosides in fresh leaves, but they decreased greatly during the manufacturing process, especially during the stage of rolling. After the final stage of fermentation, primeverosides had almost disappeared, whereas glucosides were substantially unchanged. These results show that hydrolysis of the glycosides mainly occurred during the stage of rolling and confirm that primeverosides are the main black tea aroma precursors. This was also supported by the changes in the glycosidase activities in tea leaves. The glycosidase activities remained at a high level during withering but decreased drastically after rolling.

Keywords: *Black tea; aroma formation; primeveroside; glucoside; primeverosidase; glucosidase*

INTRODUCTION

The alcoholic aroma compounds possessing a floral fruity aroma are known to be the main aroma components of black tea. They are mainly present as glycosides in fresh leaves and are released by endogenous glycosidases during the manufacturing process of withering, rolling, and fermentation. Yamanishi et al. (*1*) have investigated the change in flavor constituents during black tea manufacturing and shown that various types of alcohol had increased after withering and that, after fermentation, the aldehyde of (*E*)-2-hexenal had greatly increased, whereas that of (*Z*)-3-hexenol had decreased to less than half its level in withered leaves. These results suggest that the glycosidically bound aroma precursors are present in fresh leaves and that glycosidases play the main role in forming black tea aroma (*2*).

Several glycosides have been isolated as tea aroma precursors in fresh tea leaves and have been identified as β-D-glucopyranosides (Glc), 6-*O*-β-D-xylopyranosyl-β-D-glucopyranosides (β-primeverosides, Prim), 6-*O*-α-L-arabinopyranosyl-β-D-glucopyranosides (β-vicianosides, Vic), and 6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides (β-acuminosides) with aglycons of (*Z*)-3-hexenol, geraniol, linalool, and four linalool oxides (LOs: LO I, *trans*-furanoid; LO II, *cis*-furanoid; LO III, *trans*-pyranoid; LO IV, *trans*-pyranoid), benzyl alcohol, 2-phenylethanol, and methyl salicylate (*3*−*10*). In considering the contribution of these glycosidic aroma precursors to aroma formation in various types of tea, we have recently established a method for the direct qualitative and quantitative determination of the glycosides in tea leaves by a capillary gas chromatographic−mass spectrometric analysis (GC-MS) after trifluoroacetyl (TFA) derivatization of the tea glycosidic fraction. Eleven β-D-glucosides, 10 β-primeverosides, and 1 β-vicianoside were identified and quantified in different fresh leaves for making green tea, oolong tea, and black tea (*11*). However, the change in content of these glycosides during the tea manufacturing process has not been clarified. We report in the present study changes in the amounts of these glycosides during the black tea manufacturing process and changes in the glycosidase activities in tea leaves.

MATERIALS AND METHODS

Reagents and Reference Samples. Analytical reagent grade solvents were used. 4-Nitrophenyl β-D-glucopyranoside (*p*NP-β-D-glucoside), which was used as the substrate of glucosidase in tea leaves, was purchased from Sigma Chemical Co. 4-Nitrophenyl β-D-xylopyranosyl-6-*O*-β-D-glucopyranoside (*p*NP-β-primeveroside) was synthesized according to the method of Sone and Misaki (*12*), and glycosidase [Rohapect D5L (aqueous solution)] was procured from Röhm. All other chemicals were purchased or synthesized in our laboratory as previously described in Part 1 of this study (*11*).

Materials and the Manufacturing Process for Black Tea. Fresh leaves of *Camellia sinensis* var. *sinensis* cv. Benihomare (a hybrid of var. *sinensis* and var. *assamica* for making black tea) were plucked at the National Research Institute of Vegetables, Ornamental Plants and Tea (Kanaya, Shizuoka, Japan) on May 13, 1998.

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Part 1. Qualitative and Quantitative Analyses of Glycosides with Aglycons as Aroma Compounds (*11*).

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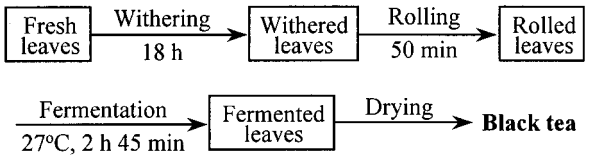

Figure 1. Outline of the manufacturing process for black tea.

Black tea was manufactured from these leaves at the same institute according to the method for making orthodox black tea, as shown in Figure 1. After plucking, the fresh leaves were spread on a net indoors and allowed to naturally wither for 18 h at ambient temperature. After being rolled with conventional equipment for 50 min, the rolled leaves were spread on a tray and allowed to "ferment" for 2.75 h at 27 °C. Finally, the fermented leaves were dried in an oven at 110 °C for 20 min and then at 80 °C for 30 min. The fresh leaves, withered leaves, rolled leaves, and fermented leaves were sampled after each manufacturing stage. Each sample was immediately frozen by adding liquid nitrogen and divided into two parts. One part was lyophilized to dryness for preparing the glycosidic fractions, and the other was stored at −80 °C until needed for preparing the crude enzyme.

Preparation of the Glycosidic Fractions. The glycosidic fractions were prepared on XAD-2 resin from the sample taken at each of the four manufacturing stages according to the method previously described in Part 1 of this study (*11*), using phenyl β-D-glucopyranoside as the internal standard. Three experiments were carried out on each sample.

TFA Derivatization of the Glycosidic Fractions. To each glycosidic extract (8 mg) in a small screw-capped vial were added 25 μL of anhydrous pyridine and 30 μL of *N*-methyl bis-(trifluoroacetamide) (MBTFA) in a nitrogen atmosphere. The resulting mixture was stirred and heated at 60 °C for 50 min and then cooled to room temperature for submission to a GC-MS analysis.

Preparation of the Aroma Concentrate from Black Tea. The aroma concentrate was prepared from black tea by steam distillation under reduced pressure. To 100 g of powdered black tea was added 1 mL of a 1.44 mg/mL solution of ethyl decanoate in diethyl ether (as an internal standard), and then the black tea aroma concentrate was prepared as previously described (*13*). The resulting aroma concentrate of black tea was subjected to GC and GC-MS analyses.

GC and GC-MS Analyses. A Hewlett-Packard 5890 series II gas chromatograph equipped with an FID or with a Hewlett-Packard 5972 series mass selective detector was, respectively, used for GC and GC-MS analyses.

(a) Analyses of TFA Derivatives of the Glycosides. To satisfactorily separate each glycoside studied, we employed two types of columns with different polar stationary phases, a DB-5 [(5% phenyl)-methylpolysiloxane, J&W Scientific] and an HP-50+ [(50% phenyl)-methylpolysiloxane, HP], under the experimental conditions previously described in detail in Part 1 of this study (*11*).

(b) Analyses of the Aroma Concentrates. A DB-Wax (polyethylene glycol, J&W Scientific) fused silica capillary column (60 m × 0.25 mm i.d.) was used to perform the GC and GC-MS analyses on the aroma concentrates. A 0.3-μL aliquot of the aroma concentrate was injected at a 30:1 split ratio into an injector held at 200 °C. The column temperature was programmed at 2 °C/min from 60 °C (held for 4 min) to 200 °C, and the FID temperature was held at 220 °C. Helium at 1 mL/min was used as the carrier gas. Electron impact mass spectra were scanned at 70 eV in an *m*/*z* range from 20 to 400 mass units while the temperature of the ion source was kept at 135 °C.

Preparation of the Crude Enzyme Solution. An acetone powder was prepared from the samples at each of the four manufacturing stages as described by Yano et al. (*2*). Acetone powder (0.8 g) and 0.4 g of Polyclar AT (General Anilin and Film Corp., New York) in 20 mL of a 50 mM sodium citrate buffer solution (pH 5.0) were homogenized twice for 30 s while cooling at 0 °C. After centrifugation at 10000*g* for 20 min at 4 °C, the clear supernatant was collected and used as the crude enzyme solution.

Glycosidase Assay of the Crude Enzyme. According to the method of Yano et al. (*2*), the activities of glucosidase and primeverosidase in the crude enzymes prepared from the four samples were assayed by using *p*NP-β-D-glucoside or *p*NP-β-primeveroside as the respective substrate. A 200-μL amount of a 25 mM solution of *p*NP-β-D-glucoside (or *p*NP-β-primeveroside) in a 50 mM sodium citrate buffer (pH 5.0) was incubated at 37 °C with 700 μL of the same buffer for 5 min. After the addtion of 100 μL of an appropriately diluted enzyme solution, the reaction mixture was incubated at 37 °C for 15 min. The reaction was stopped by adding 1.4 mL of 0.2 M sodium carbonate. The resulting yellow color was measured at 420 nm by a spectrophotometer, the amount of released *p*-nitrophenol being determined from the resulting values against a calibration curve. One unit of glycosidase activity is defined as the amount of enzyme that hydrolyzed 1 μmol of substrate/min at 37 °C.

RESULTS AND DISCUSSION

Changes in Glycoside Contents during the Black Tea Manufacturing Process. The same methods as those previously described in Part 1 of this study (*11*) were used to prepare the glycosidic fractions on XAD-2 resin from the samples taken at each of the four manufacturing stages. After TFA derivatization and a subsequent GC-MS analysis, the glycosides in each sample were identified by comparing their retention indices and mass spectra with those of authentic synthesized glycosides. The content of each glycoside was determined from the peak area on the total ion chromatogram from GC-MS by making a regression based on the calibration curve equations that had been previously established (*11*).

The glycosides that were identified and their contents in the sample from each manufacturing stage are summarized in Table 1. Fresh leaves contained more disaccharides than glucosides due to the high content of primeverosides. During the withering process, the amounts of both glucosides and primeverosides remained almost the same as those in the fresh leaves. During the stage of rolling, the primeverosides markedly decreased to a level less than one-fourth of that in the withered leaves, whereas the glucoside level was retained. After fermentation, hardly any primeverosides remained, whereas the amount of glucosides was ∼70% of that in the fresh leaves. These results support the hypothesis that primeverosidase is the main glycosidase in tea leaves (*14*, *15*), and primeverosides are considered to be the main black tea aroma precursors.

With respect to the change in amount of each primeveroside, the benzyl and methyl salicylate primeverosides showed a hydrolyzing profile different from that of the (*Z*)-3-hexenyl, 2-phenylethyl, and geranyl primeverosides. The hydrolysis of the former two primeverosides was moderate during the rolling process and lasted for the process of fermentation. However, the hydrolysis of the latter three primeverosides had almost finished after the rolling process. With regard to the primeverosides of linalool and linalool oxides, (*S*)-linalyl primeveroside was observed in the peak of LO I and (3*R*,6*S*)-LO II primeverosides according to the mass spectra. These were also moderately hydrolyzed as in the case of the benzyl and methyl salicylate primeverosides. These results concur with the substrate specificity of the glycosidases in tea leaves toward the aglycon moieties and sugar moieties that has previously been reported by Matsumura et al. (*16*).

Table 1. Changes in the Glycoside Contents of Tea Leaves during the Black Tea Manufacturing Process

glycoside[a]	content (mg/100 g of dried leaves)			
	fresh leaves	withered leaves	rolled leaves	fermented leaves
(*Z*)-3-hexenyl-Glc	6.6 ± 0.5	5.8 ± 0.4	8.6 ± 0.5	4.8 ± 0.1
benzyl-Glc	23.1 ± 1.1	22.5 ± 0.6	24.1 ± 0.3	15.1 ± 0.3
2-phenylethyl-Glc	1.3 ± 0.0	1.5 ± 0.0	1.7 ± 0.1	1.4 ± 0.0
methyl salicylate-Glc	12.7 ± 1.1	12.2 ± 0.2	12.7 ± 0.2	8.6 ± 0.2
(3*S*,6*S*)-LO I-Glc[b]	1.7 ± 0.2	2.4 ± 0.2	2.6 ± 0.0	2.1 ± 0.3
(3*S*,6*R*)-LO II-Glc[b]	2.6 ± 0.4	2.3 ± 0.0	2.2 ± 0.1	1.8 ± 0.2
(3*S*)-LO III and IV-Glc[b]	4.8 ± 0.9	3.0 ± 0.6	4.3 ± 0.0	2.6 ± 0.2
geranyl-Glc[b]	0.8 ± 0.0	0.7 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
total glucosides	**53.5 ± 4.2**	**50.3 ± 2.0**	**57.2 ± 1.2**	**37.4 ± 1.3**
(*Z*)-3-hexenyl-Prim	6.7 ± 0.8	10.5 ± 0.4	1.4 ± 0.0	0.1 ± 0.0
benzyl-Prim	20.1 ± 1.6	20.9 ± 0.8	12.5 ± 0.1	1.8 ± 0.0
2-phenylethyl-Prim	41.1 ± 4.5	38.2 ± 0.2	2.7 ± 0.0	0.3 ± 0.0
methyl salicylate-Prim[c]	45.2 ± 4.2	40.9 ± 1.0	13.7 ± 0.7	0.8 ± 0.1
LO I and (3*R*,6*S*)-LO II Prim[b,d]	5.3 ± 0.8	4.4 ± 0.1	2.0 ± 0.0	tr
(3*S*,6*R*)-LO II−Prim[b]	13.4 ± 2.7	9.1 ± 0.2	4.1 ± 0.1	tr
LO III and IV-disaccharide[c]	4.4 ± 0.6	4.5 ± 0.2	4.1 ± 0.1	2.4 ± 0.1
geranyl-Prim	47.2 ± 7.0	40.8 ± 1.3	1.4 ± 0.0	1.3 ± 0.0
geranyl-Vic	3.6 ± 0.2	3.4 ± 0.0	2.7 ± 0.2	1.5 ± 0.1
total disaccharides	**187.3 ± 22.5**	**172.6 ± 4.2**	**44.5 ± 1.2**	**8.1 ± 0.3**
total	**240.7 ± 26.7**	**222.9 ± 6.2**	**101.8 ± 2.4**	**45.5 ± 1.6**

[a] Positive identification except for methyl salicylate-Prim and LO III and IV-disaccharides (tentative identification). [b] Quantification is based on the GC-MS analyses in an HP-50+ column or, otherwise, in a DB-5 column. [c] The calibration curve of the corresponding glucoside was used for quantification. [d] Overlapped with (*S*)-linalyl-Prim.

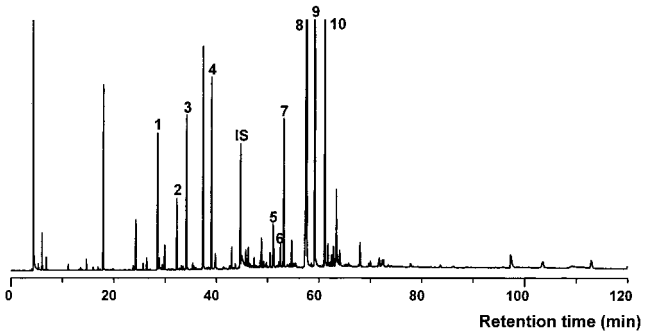

Figure 2. Gas chromatogram of the aroma concentrate of black tea: peak 1, (*Z*)-3-hexenol; peak 2, LO I; peak 3, LO II; peak 4, linalool; peak 5, LO III; peak 6, LO IV; peak 7, methyl salicylate; peak 8, geraniol; peak 9, benzyl alcohol; peak 10, 2-phenylethanol.

We also prepared a free aroma concentrate from the manufactured black tea sample by steam distillation under reduced pressure. Figure 2 shows the gas chromatogram of the black tea aroma concentrate. The alcoholic aroma compounds involved in the hydrolysis of the glycosides are numbered from 1 to 10. It is well-known that these alcohols are present in fresh tea leaves in only small amounts and are increased during the black tea manufacturing processes (*1*). As shown in Figure 2, these alcohols constituted ~74% of the total aroma compounds in black tea. In particular, the high contents of benzyl alcohol, 2-phenylethanol, and geraniol in the black tea aroma concentrate are considered to have been due to the high contents of their corresponding glycosides or to the specific glycosidase activity toward the glycosidic substrates in fresh tea leaves.

Takeo (*17*) has proposed the concept of the terpene index (TI) to differentiate various cultivars of black tea, which is calculated by the following equation:

$$\mathrm{TI} = (\text{linalool} + \text{linalool oxides})/(\text{linalool} + \text{linalool oxides} + \text{geraniol})$$

Table 2. Changes in the Glycosidase Activities of Tea Leaves during the Black Tea Manufacturing Process

	activity (units/g of fresh leaves)	
	glucosidase	primeverosidase
fresh leaves	0.38	0.46
withered leaves	0.45	0.46
rolled leaves	0.05	0.01
fermented leaves	0.01	0.02

The TI value for Sri Lanka black tea made from var. *assamica* is nearly 1.0 due to the low content of geraniol, and Darjeeling black tea made from var. *sinensis* has a lower TI value because of the high content of geraniol. Benihomare is a hybrid of var. *assamica* and var. *sinensis* that is cultured for making black tea at the National Research Institute of Vegetables, Ornamental Plants and Tea of Japan. The obtained TI value for Benihomare black tea was 0.31. On the other hand, the calculated TI value from the glycosides of linalool, linalool oxides, and geraniol in the dried fresh tea leaves of Benihomare was 0.38. This consistency suggests that the character of black tea aroma is mainly determined by the composition of aglycon moieties in the fresh tea leaves.

These results imply that the hydrolysis of these glycosides plays the main role in the formation of black tea aroma.

Changes in Glycosidase Activities during the Black Tea Manufacturing Process. Ogawa et al. (*14*) and Ijima et al. (*15*) had purified β-primeverosidase from tea leaves and were deeply concerned with the tea aroma formation during oolong tea and black tea manufacturing process. Using *p*NP-glucoside and *p*NP-primeveroside as substrates, we investigated the changes in glucosidase and primeverosidase activities in tea leaves during the black tea manufacturing process.

An acetone powder was prepared as the crude enzyme from the sample taken at each manufacturing stage, and the glucosidase and primeverosidase activities of each sample are shown in Table 2. During the withering

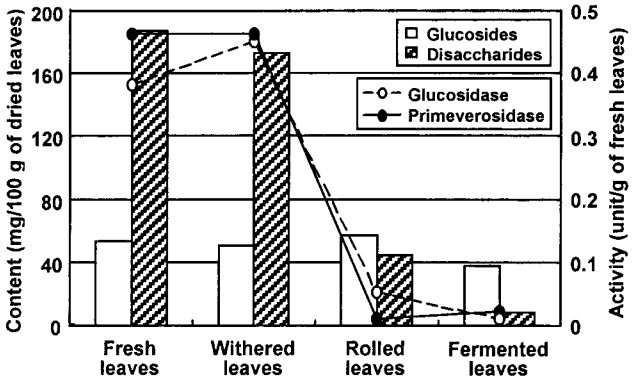

Figure 3. Changes in the glycoside contents and glycosidase activities during the black tea manufacturing process.

process, the primeverosidase activity remained at a level similar to that in the fresh leaves, whereas a small increase was observed in the glucosidase activity. However, the activities of both glucosidase and primeverosidase had decreased greatly after the rolling process. Furthermore, both glycosidases had been almost deactived after fermentation. These results showed the strong likelihood that the glycosides are hydrolyzed during the withering and rolling processes.

Figure 3 summarizes the changes in glycoside amounts and glycosidase activities during the black tea manufacturing process. The decrease in the amounts of glucosides and primeverosides is consistent with the change in activities of the two corresponding glycosidases. Although both glycosidases possessed high activity before and after the withering process, the glycosides were hardly hydrolyzed during natural indoor withering. It seems that the enzymes were also nonactive or that the substrates and glycosidases could not come into contact with each other. During the rolling process, the leaves are mechanically destroyed, enabling the glycosidases to become more active and to have more chance of interacting with the substrates. Because both the glycosidase activities and the amounts of glycosides had decreased greatly after rolling, the formation of the alcoholic aroma from the glycosides occurred mainly during the rolling process.

We are now wondering whether the *p*NP-primeverosidase activity is suitable for determining the total primeverosidase activity, because this enzyme is known to be specific to the aglycon moiety (*15*). It is well-known from experience that the aroma character of black tea is completed by the last stage of fermentation, at which the glycosidase activity is at its lowest, so we should add another factor for the whole aroma formation of black tea.

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