A Study on Tea Aroma Formation Mechanism: Alcoholic Aroma Precursor Amounts and Glycosidase Activity in Parts of the Tea Plant

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We have shown in molecular basis that alcoholic tea aroma is mainly formed by endogenous enzymatic hydrolysis of glycosidic aroma precursors during manufacturing. Amounts of alcoholic aroma precursor and glycosidase activity in each part of the tea shoot (*Camellia sinensis* var. *sinensis* cv Yabukita and a hybrid of var. *assamica* & var. *sinensis* cv Izumi) were indirectly measured by means of a crude enzyme assay. The aroma precursors were abundant in young leaves and decreased as the leaf aged. Glycosidase activity also decreased as leaves aged, but was high in stems.

Introduction

Tea is classified into nonfermented (green tea), semifermented (oolong tea), and fermented tea (black tea). In particular, floral alcoholic aroma is important in oolong tea and black tea, for quality of the tea is said to mainly depend on the tea aroma.

Monoterpene alcohols (geraniol, linalool, *etc.*) and aromatic alcohols (benzyl alcohol, 2-phenylethanol, methyl salicylate, *etc.*) are major tea aroma constituents and mainly contribute to the floral aroma of oolong and black tea (Yamanishi T., 1989). In the course of our study on aroma formation mechanism of oolong tea, we have isolated some β-primeverosides (6-*O*-β-D-xylopyranosyl-β-D-glucopyranosides) of tea aroma constituents such as linalool, geraniol, 2-phenylethanol, benzyl alcohol, *trans*- and *cis*-linalool 3,6-oxides, and methyl salicylate, *cis*-linalool 3,7-oxide 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside and (*Z*)-3-hexenyl β-D-glucopyranoside as aroma precursors from oolong tea leaves (*C. sinensis* var. *sinensis* cv Shuixian and cv Maoxie) (Guo W. *et al.*, 1994; Guo W. *et al.*, 1993; Moon J. *et al.*, 1994; Moon J. *et al.*, 1994) (Fig. 1). While Kobayashi *et al.* have isolated as aroma precursor glucosides of benzyl alcohol and (*Z*)-3-hexenol from green tea leaves (cv Yabukita) (Kobayashi A. *et al.*, 1994; Yano M. *et al.*, 1991) (Fig. 1). We have also purified a β-primeverosidase from fresh tea leaves (cv Yabukita), which showed high substrate specificity toward each β-primeveroside to hydrolyze them into a disaccharide unit (primeverose) and an aglycone (Guo W. *et al.*,). From the above, most of alcoholic tea aroma constituents have been confirmed to be mainly formed from their β-primeverosides by the action of this β-primeverosidase during oolong tea manufacturing.

Next we were very interested in the distributions of the aroma precursors and the enzymes in tea shoots as a next step to clarify the tea aroma formation mechanism. Here we wish to report the amounts of the alcoholic aroma precursors and glycosidase activity in each part (buds & 1st, 2nd, 3rd, and 4th leaves, stem, and aged leaves) of tea shoot (cvs Yabukita and Izumi) indirectly measured by the crude enzyme assay we have established.

Materials and Methods

Tea leaves

Two kinds of tea leaves (*Camellia sinensis* var. *sinensis* cv Yabukita and a hybrid of var. *assa-*

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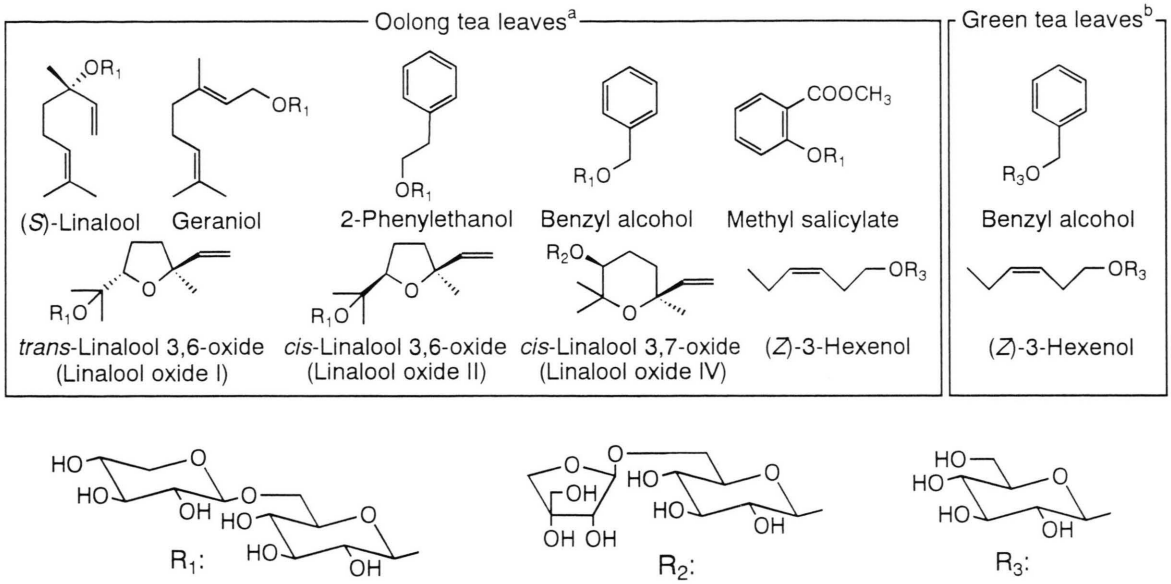

Fig. 1. Glycosidic aroma precursors isolated from tea leaves. [a] *Camellia sinensis* var *sinensis* cvs Shuixian and Maoxie; [b] cv Yabukita.

mica & var. *sinensis* cv Izumi) were plucked at the National Research Institute of Vegetables, Ornamental Plants and Tea, Kanaya, Shizuoka, and Shizuoka University, Japan, in June and July 1993.

Tea leaves to measure amounts of aroma precursors were steamed just after plucking and then stored under ice-cooling. Both steamed and fresh tea shoots were separated into each part (buds & 1st, 2nd, 3rd, and 4th leaves, stem, and aged leaves) stored at −20 °C before use. Aged leaves were plucked in June 1993 and March 1994. Aged leaves which are tough and deep-green colored and have been at the plant more than a year, are not used for tea manufacturing.

Table I. Dry weights of acetone powder prepared from 10 g of each part of tea shoot.

Part of plant	Yabukita variety	Izumi variety
Bud & 1st leaf[b]	1.72[a]	1.53
2nd Leaf[b]	1.84	1.51
3rd Leaf[b]	1.75	1.66
4th Leaf[b]	2.07	1.45
Stem[b]	1.73	1.62
Aged leaf[c]	2.63	2.72
Aged leaf[d]	3.23	3.72

[a] g/10 g fresh weight.
[b] Plucked in June 1993.
[c] Yabukita was plucked in July 1993, and Izumi was plucked in June 1993.
[d] Plucked in March 1994.

Preparation of acetone powder (crude enzyme)

Fresh tea leaves were finely chopped, crushed by a homogenizer ['Physcotron' (Niti-on Medical & Physical Instruments MFG. CO., LTD.)] in dry ice-acetone, and then filtered under *in vacuo*. Chilled acetone (−20 °C) was added to wash the residue until the filtrate became nearly colorless. The residue was transferred into a desiccator and sucked by an aspirator pump until the acetone was completely removed. The dried powder (acetone powder) was stored at −20 °C before use. Yields of the acetone powder from each part are shown in Table I. The glycosidase activity of the acetone powder was found to remain >80% even after one year when stored at −20 °C.

Measurement of alcoholic aroma precursor amounts

Each steamed tea leaf sample (30 g) was homogenized in 150 ml of 50 mM citrate buffer (pH 6.0), filtered through clothes, and washed with 75 ml of methylene chloride to remove volatile compo-

Table II. Main aroma constituents liberated from a crude aroma precursor solution from 4th leaves (Izumi) by enzymatic hydrolysis with the acetone powder[a].

Peak No.	Compound	1st Exp.	2nd Exp.	3rd Exp.	Average	SD[b]
1	(*Z*)-3-Hexenol	43[c]	39	31	38	5.0
2	*trans*-Linalool 3,6-oxide	7.9	8.4	7.6	8.0	0.33
3	*cis*-Linalool 3,6-oxide	13	14	12	13	0.82
4	Linalool	61	59	52	57	3.9
5	Methyl salicylate	22	24	19	22	2.1
6	Nerol	5.0	4.8	4.8	4.9	0.094
7	Geraniol	130	120	120	120	4.7
8	Benzyl alcohol	22	30	19	24	4.0
9	2-Phenylethanol	43	53	36	44	7.0
	Total	350	350	300	330	28

[a] Prepared from fresh tea leaves (cv Yabukita).
[b] Standard deviation.
[c] μg/fresh weight 30 g [calculated from peak area comparing with that of 25 μg of internal standard (ethyl decanoate)].

nents. The aqueous layer was subjected to vacuum evaporation to remove residual methylene chloride. As enzymatic reactions were considered to be possibly inhibited by catechins, Polyclar AT (6 g) purchased from Wako Pure Chemical (Osaka) was added into the aqueous solution to adsorb catechins. This crude aroma precursor solution (fresh weight 30 g equivalent) was reacted at 37 °C for 24 h with the acetone powder (crude enzyme, fresh weight 0.5 g equivalent) prepared from cv Yabukita. Liberated aroma was extracted by simultaneous distillation and extraction (SDE) method using methylene chloride as an extraction solvent, dried with anhydrous Na_2SO_4, and concentrated. Into the aroma extract was added 5 μl of ethyl acetate solution containing ethyl decanoate (25 μg) as an internal standard. The sample solution was transferred into a sample tube for microanalysis, concentrated by a stream of nitrogen, and then analyzed by GC and GC-MS. Liberated aroma amounts were calculated from their peak areas comparing with that of the internal standard. The figure does not represent real amounts, but is a sufficient parameter to indicate the aroma precursor amounts in each part.

In order to confirm the reproducibility of the above experiments, a sample (4th leaves of cv Izumi) was subjected to measurement in triplicates (Table II). From the results, the experimental error was within 10 percents.

Measurement of glycosidase activity

To determine the optimum reaction time for the aroma analysis, the hydrolysis reaction was carried out for 30 min and 12 h, respectively. Both results showed no significant difference in the aroma composition and contents. Therefore, the reaction time was set for 1 h. The acetone powder (fresh weight 0.4 g equivalent) prepared from each part of the tea shoot reacted with a crude aroma precursor mixture (fresh weight 20 g equivalent) prepared from steamed tea leaves (cv Benihomare, a hybrid of var. *assamica* & var. *sinensis*) in 50 mM citrate buffer (pH 6.0) at 30 °C for 1 h. Liberated aroma was extracted with ethyl ether. The aroma extract was transferred into a sample bottle and concentrated under reduced pressure in a desiccator. Into the concentrate was added 2 μl of the internal standard solution of ethyl decanoate (10 μg). The sample solution was passed through a column (*ca.* 3 ml) of anhydrous Na_2SO_4 to dry, concentrated by a stream of nitrogen, and analyzed by GC and GC-MS. Total liberated aroma amounts, reflecting glycosidase activity of each part of the tea shoot, were calculated from their peak areas based on that of the an internal standard in the same manner as shown above.

In order to confirm the reproducibility of the above experiments, a sample (buds & 1st leaves of cv Yabukita) was subjected to measurement in triplicates. Aroma precursor solution prepared from cv Maoxie was used for these experiments.

Table III. Main aroma constituents liberated from glycosidic aroma precursorsa by the enzymatic hydrolysis with a acetone powder prepared from buds & 1st leaves (Yabukita).

Peak No.	Compound	1st Exp.	2nd Exp.	3rd Exp.	Average[b]	SD[c]
1	*trans*-Linalool 3,6-oxide	5.1[d]	4.9	4.6	4.9	0.21
2	*cis*-Linalool 3,6-oxide	6.4	5.9	5.8	6.0	0.26
3	Linalool	65	63	57	62	3.4
4	Geraniol	49	50	41	47	4.0
5	Benzyl alcohol	2.3	2.5	2.3	2.4	0.094
6	2-Phenylethanol	16	16	15	16	0.47
	Total	140	140	130	140	8.5

[a] Prepared from cv Maoxie (fresh weight 20 g eq.).
[b] Average.
[c] Standard deviation.
[d] μg/acetone powder (68.8 mg: fresh weight 0.4 g eq.).

The results showed in Table III. From the results, the experimental error was within 15 percents.

Analytical conditions (GC and GC-MS)

(a) *GC*: A Hitachi 163 gas chromatograph with FID equipped with a TC-WAX capillary column (0.25 mm i.d. ×30 m) was used. The GC conditions were as follows: carrier gas, N_2 (1 ml/min); split ratio, 100:1; temp. program, holding at 60 °C for 10 min and then raising to 200 °C at 3 °C/min; injector temp., 250 °C.

(b) *GC-MS*: A JEOL JMS-DX 302 mass spectrometer with a JEOL JMA-DA 5000 mass data system linked with a Hewlett-Packard 5890A gas chromatograph equipped with a PEG-20M capillary column (0.25 mm i.d. ×50 m) was used. The GC-MS conditions were as follows: carrier gas, He (1 ml/min); split ratio, 65 : 1; temp. program, 60 °C to 220 °C at 3 °C/min; injector temp., 150 °C; ionization voltage, 70 eV.

Results and Discussion

We have shown in molecular basis that alcoholic tea aroma like geraniol, linalool, *etc.* of oolong tea is mainly liberated by enzymatic hydrolysis of glycosidic aroma precursors (β-primeverosides) with an endogenous β-primeverosidase during manufacturing (Guo W., *et al.*, 1994; Guo W., *et al.*, 1993; Guo W., *et al.*, ; Moon J., *et al.*, 1994; Moon J., *et al.*, 1994). In order to clarify the distributions of the aroma precursors and the enzymes in tea shoots, we indirectly measured the amounts of the alcoholic aroma precursors and glycosidase activity in each part (buds & 1st, 2nd, 3rd, and 4th leaves,

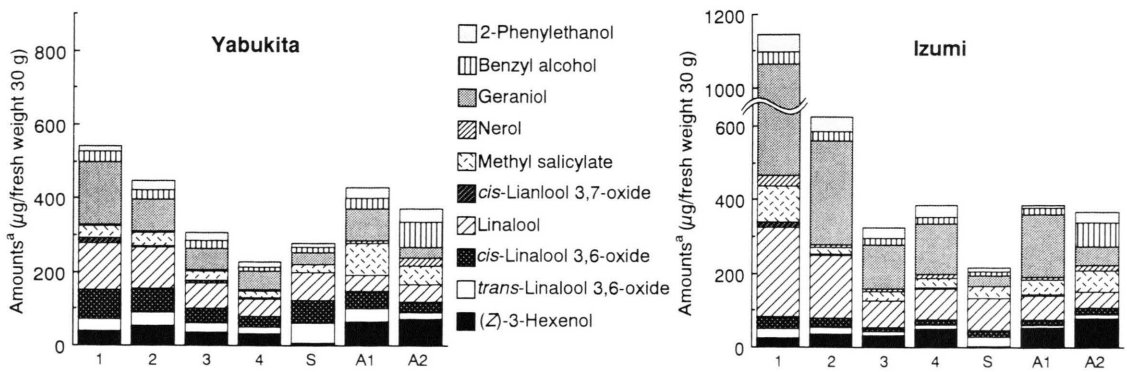

Fig. 2. Aroma precursors in each part of tea shoot. 1, buds & 1st leaf; 2, 2nd leaf; 3 3rd leaf; 4, 4th leaf; A1, aged leaf plucked in June 1993; A2, aged leaf plucked in March 1994; S, stem. [a] Amounts of tea aroma generated by the hydrolysis with the crude enzyme (from cv Yabukita) from the crude aroma precursor solution prepared from each part of tea shoot (details in the text). The amounts are considered to roughly reflect the aroma precursor amounts of each sample.

stem, and aged leaves) of tea shoot (cvs Yabukita and Izumi).

To measure amounts of aroma precursors in each part of a tea shoot, a crude aroma precursor solution prepared from each steamed sample of cvs Yabukita and Izumi plucked in June, was reacted at 37 °C for 24 h with a crude enzyme prepared from cv Yabukita. Liberated aroma was extracted by SDE method and analyzed by GC and GC-MS. The results of the aroma precursor measurements are shown in Fig. 2. Liberated aroma amounts were considered to roughly reflect the aroma precursor amounts.

Total aroma precursor amounts of cv Izumi having *Camellia assamica* gene are nearly twice of those of cv Yabukita for Japanese green tea. Aroma precursor amounts of linalool and geraniol in both cultivars were a lot in younger leaves such as buds & 1st and 2nd leaves, especially extremely abundant in those of cv Izumi, and decreased as leaf aged in both cultivars. Those of *trans*- and *cis*-linalool 3,6-oxides (linalool oxides I and II) were contained considerably more in each part of cv Yabukita than in that of cv Izumi, and quite interestingly abundant in stem of cv Yabukita, although much more geraniol and linalool were liberated from cv Izumi. Aroma precursor amounts of methyl salicylate were relatively abundant in aged leaves of cv Yabukita. A larger amounts of (*Z*)-3-hexenol were liberated from aged leaves of both cultivars than young leaves. Aroma precursor amounts of benzyl alcohol were a lot in aged leaves of both cultivars plucked in March and much more than those in aged leaves plucked in June.

Although aroma precursor amounts of either terpene alcohols or aromatic alcohols decreased in order of buds & 1st leaves, 2nd, 3rd, and 4th leaves in both cvs Yabukita and Izumi, those of (*Z*)-3-hexenol were quite different. This may be explained by the different biosynthetic pathways of the aroma precursors. For example, it has been shown that terpene alcohols of tea aroma are biosynthesized through a mevalonate pathway (Takeo T., 1981). (*Z*)-3-Hexenol has been shown to be liberated in tea leaves through the following two pathways. Hatanaka has clarified that (*Z*)-3-hexenal is formed from oxidative degradation of linolenic acid, and converted to (*Z*)-3-hexenol by alcohol dehydrogenase (Hatanaka A. and Harada T., 1973). On the other hand, Kobayashi isolated and identified (*Z*)-3-hexenyl β-D-glucopyranoside as an aroma precursor from tea leaves (cv Yabukita) and has proposed that (*Z*)-3-hexenol biosynthesized through above pathway is accumulated as its glucoside in tea leaves, and liberated by an enzymatic hydrolysis during tea processing (Yano M. *et al.*, 1990).

To measure glycosidase activity, which is also responsible for the aroma formation from glycosidic alcoholic aroma precursors, an acetone powder prepared from each part of fresh tea leaves (buds & 1st leaves, 2nd, 3rd, and 4th leaves, stem, and aged leaves of cvs Yabukita and Izumi, respectively) was reacted at 30 °C for 1 h with a crude aroma precursor solution prepared from cv Beni-

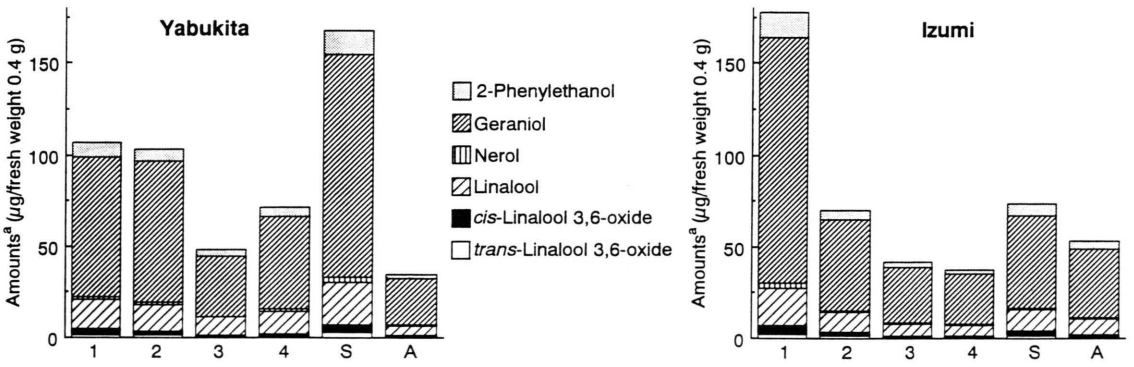

Fig. 3. Glycosidase activity in each part of tea shoot. 1, buds & 1st leaf; 2, 2nd leaf; 3, 3rd leaf; 4, 4th leaf; A, aged leaf; S stem. [a] Amounts of tea aroma liberated from a crude aroma precursor solution (prepared from cv Benihomare) by hydrolysis with the crude enzyme prepared from each part of tea shoot (details in the text). The amounts are considered to roughly reflect the glycosidase activity of each sample.

homare, a cultivar for black tea. Liberated aroma was extracted with ether, dried, concentrated, and analyzed by GC and GC-MS. Fig. 3 shows the results. Kinds of liberated aroma are much less than those in the experiments to measure aroma precursor amounts. Because a crude aroma precursor solution prepared from cv Benihomare was used in this experiment. Benihomare is a cultivar selected by breeding to manufacture black tea in Japan. Liberated aroma amounts in this experiments were considered to roughly reflect the glycosidase activity of each part.

Glycosidase activities of both cvs Yabukita and Izumi were high in young leaves such as buds & 1st leaves and 2nd leaves, and decreased as leaf aged, but were exceptionally high in stem of cv Yabukita. The glycosidases are suggested to be transferred from mature leaves to young one through a sieve tube in the stem. Recently big molecules like proteins have been reported to be possibly transported through a sieve tube of plants (Pernollet J.-C. *et al.*, 1993). The glycosidases of aged leaves plucked in June, July, and March showed low activity. The glycosidases were suggested to be produced in developed and/or aged leaves and transported to very young leaves such as buds, 1st and 2nd leaves.

On the basis of the foregoing, buds & 1st leaves were found to contain large amounts of aroma precursors and high glycosidase activity, indicating that high quality tea are reasonably made from young tea leaves from aroma formation mechanistic points of view. We also confirmed in molecular basis that a tea cultivar (Izumi) having *C. assamica* gene contains much more amounts of aroma precursors and higher glycosidase activity in very young leaves than cv Yabukita (*C. sinensis* var. *sinensis*) for Japanese green tea manufacturing.

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