Study on Black Tea Aroma in Special Reference to the Formation Mechanism of Volatile Carbonyl Compounds

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There is a difference in the process of making tea from fresh leaves of two kinds of tea—Japanese green tea which is made by the successive process of steaming, rolling and drying and black tea, by withering, rolling and fermentation.

It can be considered, therefore, that the specific aromatic substance (the presence of green or black tea) may be composed in the course of the process of tea production.

The author intended to regulate the formation of the aromatic substance and improve the producing method of black tea by studying the formation mechanism of aromatic compounds, especially its principal component, volatile carbonyl compounds, which appear increasingly during the rolling and fermentation process[1),2)].

Aldehydes formation from amino acids during fermentation

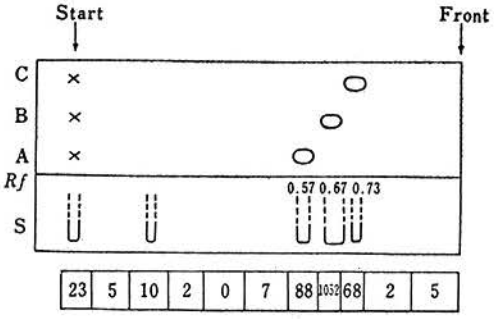
1) Decomposition of phenylalanine-U-^{14}C into phenylacetaldehyde and carbon dioxide[3)]

Fresh tea leaves were fermented at 25°C for an hour and a half in a Petri dish after maceration in a mortar with water diluted with phenylalanine-U-^{14}C which is one of the radioactive amino acids. The carbon dioxide produced from this fermentation was absorbed by potassium hydroxide solution.

The volatile compounds were collected by distilled fermented leaves and dissolved in diethyl ether. After separating both acidic and basic fractions from the ether solution of volatile compounds, 2, 4-dinitrophenylhydrazine reagent was added, and then volatile carbonyl compounds were collected as the precipitate of 2, 4-dinitrophenylhydrazine (2, 4-DNP).

Radioactivity was measured by a gas flow counter, and as the result, 77.5 per cent of the whole radioactivity remained with phenylalanine, 0.2 per cent incorporated into carbon dioxide and 1.8 per cent into volatile carbonyl compounds.

A portion of volatile carbonyl compounds 2, 4-DNP was separated by thin layer chromatography (TLC), and about 85 per cent of the radioactivity of the whole volatile carbonyl compounds were recognized at the



Radio activity (cpm)

Fig. 1. Thin-layer chromatography of 2, 4-DNP separated from radioactive volatile compounds of fermented leaves
S : Sample
A : Authentic acetaldehyde 2, 4-DNP
B : Authentic phenylacetpehyde 2, 4-DNP
C : Authentic benzaldehyde 2, 4-DNP
Developing solvent of benzene-ligroin-ethyl-acetate (6 : 6 : 1)

zone of 0.67 of Rf value which corresponded to that of phenylacetaldehyde 2,4-DNP, as shown in Fig. 1.

To make more detailed analysis, the spots of 0.67 of Rf value were accumulated and recrystallization was repeated with water containing ethanol, and nearly constant values of melting point and specific radioactivity were obtained without any degradation by recrystallization (Table 1).

This means that the crystal of phenylacetaldehyde 2, 4-DNP possesses radioactivity in itself and that a portion of phenylalanine-U-^{14}C added to the material of fresh tea leaves was decomposed to make phenylacetaldehyde and carbon dioxide in the course of fermentation.

Table 1. Specific radioactivity of phenylacetaldehyde 2, 4-DNP

Recrystallization No.	Phenylacetaldehyde 2, 4-DNP	
	mp (°C)	Specific activity (cpm/mg)
1	118.5	750
2	119.0	800
3	119.0	730

2) Formation mechanism of aldehydes derived from amino acids[4]

Crude enzyme solution was prepared from fresh tea leaves[5),6)] and phenylalanine as substrate was added to it with other materials to compose reaction systems. After incubation for two hours at 25°C, phenylacetaldehyde from such system was detected by gas chromatography. The results are shown in Table 2.

Phenylacetaldehyde was recognized after incubation in the No. 2 reaction system which contained phenylalanine, crude enzyme solution and (−)-spicatechin and the addition of hydrogen peroxide to this No. 2 system (this becomes No. 4 system) resulted in accelerating the formation of phenylacetaldehyde.

Consequently, it is assumed that phenylacetaldehyde was formed by the reaction of phenylalanine and orthoquinone, which is composed of oxidation of (−)-epicatechin. This was ascertained in the Nos. 9 and 10 reaction

Table 2. Production of phenylacetaldehyde in various reaction systems

No.	Reaction system Composition	Phenylacetaldehyde $\times 10^{-2}$ μmole
1	Phe+TLE	0
2	Phe+TLE+(−)EC	1.9
3	Phe+TLE+H_2O_2	0
4	Phe+TLE+(−)EC+H_2O_2	3.3
5	Phe+TLE+(−)EC+Mn^{2+}	2.1
6	Phe+HRP+(−)EC+H_2O_2	8.3
7	Phe+HRP+(−)EC+Mn^{2+}	4.5
8	Phe+TLE (100°C, 10 min)+(−)EC	0
9	Phe+β-Naphthoquinone	8.2
10	Phe+Sodium β-Naphthoquinone 4-sulfonate	10.2
11	Phe+TLE+α-Ketoglutaric acid+Pyridoxal phosphate	0
12	Phe+TLE+Pyridoxal phosphate+Mn^{2+}+p-Cresol	0
		(Benzaldehyde *ca.* 30.8$\times 10^{-2}$ μmole)

Reaction systems 1∼10: 6 ml total volume (M/15 phosphate buffer, pH 7.0), 25°C, 2 hr. Phe; 300 μmoles, TLE; 1 ml of tea leaves extract, (−)EC; 3 μmoles (−)-epicatechin, H_2O_2 or Mn^{2+}; 0.3 μmole, HRP; 30μg of horseradish peroxidase, quinones; 3 μmoles.

Reaction systems 11[6)]: 9 ml total volume (M/15 Tris-HCl buffer, pH 8.5), 37°C, 3 hr. Phe; 90 μmoles, TLE; 1 ml, α-Ketoglutaric acid; 45 μmoles, Pyridaxal phosphate; 3 μmoles.

Reaction system 12: 9 ml total volume (M/15 phosphate buffer, pH 7.0), 25°C, 2 hr. Phe; 90 μmoles, TLE; 1 ml, Pyridoxal phosphate; 30 μmoles, Mn^{2+}; 0.3 μmole, p-Cresol; 0.09 μmole.

systems.

Subsequently, the effects of various phenols (mono-, di-, and tri-phenols) in the production of phenylacetaldehyde in the reaction system of phenylalanine combined with crude enzyme solution and phenol were examined (Table 3). As the result, mono-phenols were not effective in the production of phenylacetaldehyde while di-phenols were most effective and tri-phenols had a little effect.

The production of phenylacetaldehyde was rather depressed in the reaction mixture of (−)-epicatechin and (−)-epigallocatechin gallate than that of (−)-epicatechin only. Therefore, orthoquinone, the derivative of di-phenol, could be effective in the decarboxylation and deamination of phenylalanine but, orthoquinone, the derivative of tri-phenol, might be inhibitive to the reaction.

On the other hand, the aldehydes derived from various amino acids were examined in the aldehyde production system which is composed of amino acid, crude enzyme solution and (−)-epicatechin. The results are listed in Table 4 and show that methanal, ethanal, 2-methylpropanal, 3-methylbutanal, 2-methyl-

Table 3. Effect of various phenols on the production of phenylacetaldehyde

	Phenol	Phenylacetaldehyde (relative quantity)
Mono	*p*-Coumaric acid	0
	p-Cresol	0
Di	(+)-Catechin	31
	Catechol	57
	(−)-Epicatechin	100
Tri	(−)-Epigallocatechin	10
	(−)-Epigallocatechin gallate	10
	Pyrogallol	trace
Di+Tri	(−)-Epicatechin+(−)-Epigallocatechin gallate	17

Reaction mixture, of 6 ml total volume (M/15 phosphate buffer, pH 7.0), contained 300 μmoles of phenylalanine, 1 ml of tea leaves extract and 3 μmoles of phenol; where mono-phenol was used, 0.3 μmole of H_2O_2 was added in above mixture. Reaction was carried out at 25°C for 2 hours

Table 4. Carbonyl compounds produced from various amino acids by tea leaves extract

Amino acid	Main carbonyl compound 2, 4-DNP			Produced total carbonyl content (N/100 I_2 ml)
	Rf(TLC)*	mp °C	Identification	
Gly	0.50	163	Methanal	0.23
Ala	0.54	167	Ethanal	0.15
Val	0.77	180	2-methylpropanal	0.45
Leu	0.88	121	3-methylbutanal	0.33
Ileu	0.87	126	2-methylbutanal	0.46
Met	0.57	128	3-methylthiopropanal	0.65
Phe	0.69	119	Phenylacetaldehyde	0.25
Glu	—			0.06
Tyr	—			0.13
Try	—			0.27

Reaction mixture, of 9 ml total volume (M/15 phosphate buffer, pH 0.7), contained 500 μmoles of amino acids, 3 μmoles of (−)-epicatechin 1 ml of tea leaves extract

* Thin-layer chromatography with developing solvent of benzene-ligroin-ethylacetate (6 : 6 : 1)

butanal, 3-methylthiopropanal were produced from glycine, alanine, valine, leucine, isoleucine and methionine respectively, losing one C from each origin.

From these results, it might be concluded that aldehydes are formed from amino acids by Strecker degradation, as shown in Fig. 2. That is, the oxidation of polyphenol is proceeded by polyphenol oxidase (or by peroxidase) at first, and subsequently the quinone produced reacts with amino acid to be followed by nonenzymatic decarboxylation and deamination reaction, and then aldehydes appeared finally.

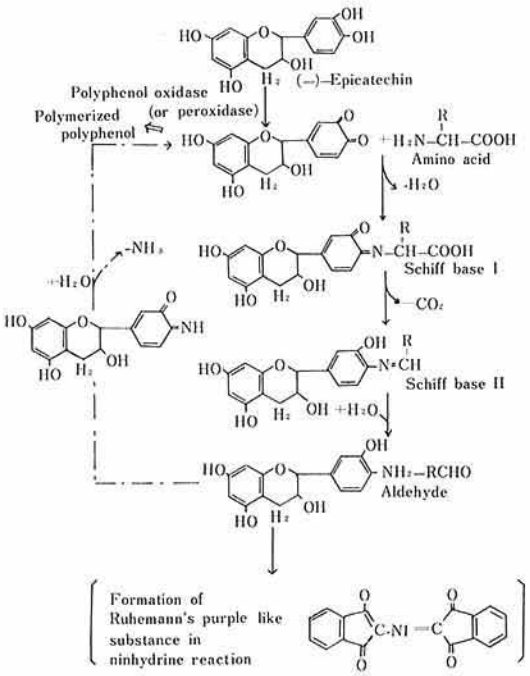


Fig. 2. Formation mechanism of aldehyde from amino acid during fermentation (presumed figure)

Formation of aldehydes from fatty acids during fermentation[7]

1) Changes in fatty acids during fermentation

Fatty acids were extracted by solvent from tea leaves at various stages of fermentation, then the extract was separated into several fractions and, after esterification, the methyl ester was analyzed by gas chromatography.

As shown by the results in Table 5, the free fatty acid fraction was not much in quantity and its change in quality was obscure. As for the neutral fat fraction, lauric acid, myristic acid, oleic acid, linoleic acid and linolenic acid decreased, especially the decreases of C_{18} unsaturated fatty acids in the latter three acids were conspicuous.

2) Decomposition of linoleic acid-U-^{14}C *and linolenic acid*-U-^{14}C *into hexanal and trans-2-hexenal*

New tea stems were immersed in the buffer

Table 5. Changes in fatty acids in each fraction during tea fermentation

Fatty acid*	Free fatty fraction			Neutral fat fraction			Phospholipid fraction		
	Fermentation time (hr)			Fermentation time (hr)			Fermentation time (hr)		
	0	1	2	0	1	2	0	1	2
Lauric	—	—	—	0.7	0.2	—	1.1	0.7	0.4
Myristic	0.2	0.1	0.1	2.0	1.5	0.7	5.3	1.8	2.4
Stearic	—	—	—	—	—	—	—	1.7	2.4
Oleic	—	—	—	4.9	3.8	1.1	1.6	1.8	2.4
Linoleic	0.2	0.3	0.8	4.2	1.4	0.6	10.2	14.6	11.7
Linolenic	0.2	0.2	0.2	22.6	10.2	4.2	27.2	27.0	18.3

Each value is the average peak area (cm^2) of two gas chromatograms obtained under the following conditions: Column packing, 20% poly-ethylene glycol adipate on Chromosorb W (60–80 mesh); Column temperature, 180°C; He flow rate, 30 ml/min; attenuation range of detector, 5×10^2. Under these conditions 1 cm^2 of peak area corresponded to 6.2 μg of lauric acid

* Fatty acid was identified by comparing its log retention time with that of the authentic fatty acid ester

solution (pH 7.0) which is a dispersion of fatty acids (linoleic acid-U-^{14}C or linolenic acid-U-^{14}C). After absorption of the buffer solution, the tea leaves were macerated in a mortar and then fermented at 25°C for two hours.

The fermented materials were treated in the same way as the radioactive phenylalanine experiment that is, preparation of volatile carbonyl compounds, separation by TLC and measurement of radioactivity were carried out.

The results are shown in Fig. 3 (fermented with linoleic acid-U-^{14}C) and in Fig. 4 (fermented with linolenic acid -U-^{14}C). And the maximum radioactivity exists near F (hexanal 2, 4-DNP) in Fig. 3 and near E (*trans*-2-hexenal 2, 4-DNP) in Fig. 4.

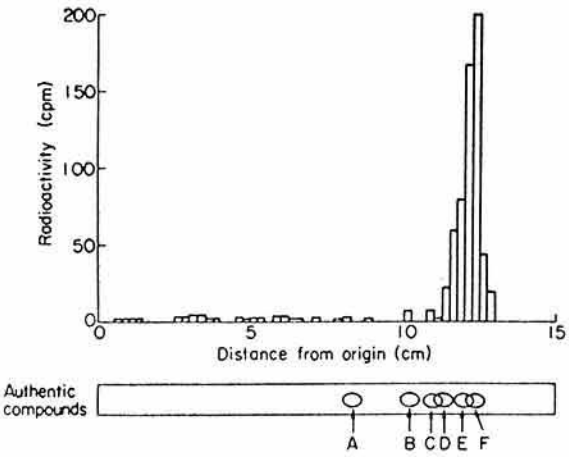


Fig. 3. Thin-layer chromatography of 2, 4-DNP derivatives from the carbonyle fraction of tea leaves fermented with radioactive linolenic acid. A, acetaldehyde 2.4-DNP; B, propionaldehyde 2.4-DNP; C, butyraldehyde 2.4-DNP; D, valeraldehyde 2.4-DNP; E, trans-2-hexanal 2.4-DNP; F, hexanal 2.4-DNP

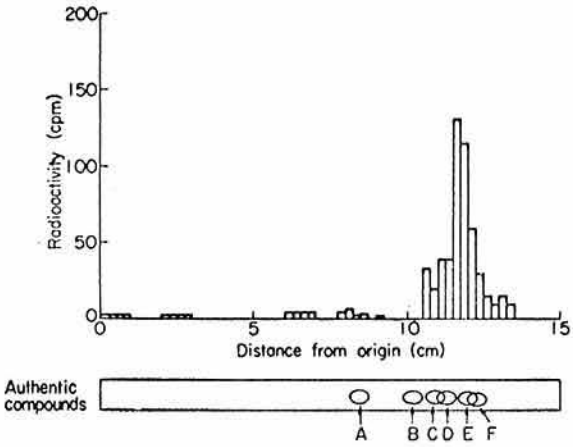


Fig. 4. Thin-layer chromatography of 2, 4-DNP from the carbonyl fraction of tea shoots fermented with radioactive linolenic acid. A, B, C, D, E and F are the same as compounds mentioned in Fig. 2

Portions of maximum radioactivity were gathered from chromatograph and recrystallizations were repeated with water containing ethanol. In Table 6, the values of melting point and specific radioactivity appear to be nearly constant in both cases; therefore, it was ascertained that radioactivities reside in each crystal.

From these results, it was proved that the linoleic acid-U-^{14}C absorbed by tea leaves decomposed into hexanal and also linolenic acid-U-^{14}C into *trans*-2-hexenal respectively during fermentation.

Furthermore, in another experiment, non-radioactive hexanal or *trans*-2-hexenal was absorbed by tea leaves and analyzed by gas chromatography after fermentation, and then it was noted that hexanoic acid or *trans*-2-hexenoic acid increased.

Table 6. Specific radioactivity of hexanal and trans-2-hexenal 2, 4-DNPH derivatives

Recrystallization No.	Hexanal 2, 4-DNP		Trans-2-Hexenal 2, 4-DNP	
	mp (°C)	sp. activity (cpm/mg)	mp (°C)	sp. activity (cpm/mg)
1	106.0	82	137.0	28
2	107.0	77	136.0	25
3	106.0	72	137.0	29
4	106.0	70	137.0	32

Therefore, hexanoic acid and *trans*-2-hexenoic acid might have been derived from hexanal and *trans*-2-hexenal respectively by oxidation during fermentation.

The mechanism of oxidation and decomposition of linoleic acid or linolenic acid are shown in Fig. 5.

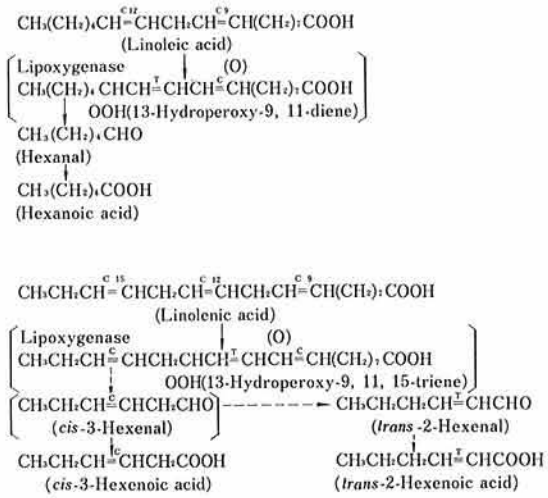


Fig. 5. Oxidation and decomposition process of linoleic acid and linolenic acid during fermention (presumed figure)

It is also assumed that the first oxidation reaction of linoleic acid or linolenic acid is an enzymatic reaction caused by lipoxygenase because there was no increase of hexanal and *trans*-2-hexanal in green tea production.

Though the results on the formation mechanism of volatile carbonyl compounds have been described, studies on aliphatic alcohols and terpens which are also important aromatic elements of black tea are now under way.

References

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