The Formation of Aldehydes from Amino Acids by Tea Leaves Extracts

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Received July 3, 1969

In the reaction system containing amino acid, tea leaves extract and (–)-epicatechin, some amino acids such as glycine, alanine, valine, leucine, isoleucine, methionine and phenylalanine produced formaldehyde, acetaldehyde, isobutyraldehyde, isovaleraldehyde, 2-methylbutanal, methional and phenylacetaldehyde, respectively. The production of these aldehydes was regarded to proceed as Strecker degradation. On the production of phenylacetaldehyde it was revealed in the tea leaves extract-phenol-phenylalanine system that: 1) di-phenol was the most effective co-factor in comparison with mono- and tri-phenols; 2) the optimum concentration of (–)-epicatechin was $5 \times 10^{-4}M$ and the production was depressed at the concentration more than $5 \times 10^{-4}M$; 3) the production decreased by diluting tea leaves extract.

Several carbonyl compounds of black tea were supposed to be formed from amino acids by Strecker degradation.¹⁻³⁾ In the previous paper we reported that phenylalanine degraded into phenylacetaldehyde and carbon dioxide in the process of tea fermentation. The objectives of the present study were to reveal the production of the corresponding aldehydes from various amino acids by tea leaves extract and to study the formation mechanism of aldehydes using phenylalanine as the substrate.

MATERIALS AND METHODS

Preparation of tea leaves extract. The young tea leaves, field-grown in autumn of variety Benifuji were homogenated by the method reported previously⁴⁾ and

stored in $(NH_4)_2SO_4$ solution, 42 g $(NH_4)_2SO_4$ per 100 ml buffer solution, until winter when it was used. The homogenate was dialysed against 0.2 M McIlvaine buffer (pH 7.0), containing 1% sodium ascorbate, for 48 hr and filtered. The resultant residue was suspended in 0.2 M McIlvaine buffer (pH 7.0), containing 5% Tween-80⁴⁾ and 1% sodium ascorbate, extracted for 2 hr, and then centrifuged for 5 min at $12,000 \times g$. The supernatant was applied to Sephadex G-25 column, 2×15 cm. Using 0.2 M McIlvaine buffer as the eluting buffer, fractions containing protein were collected. The protein fractions were precipitated with $(NH_4)_2SO_4$, 21 g per 100 ml buffer solution, collected by centrifugation for 15 min at $10,000 \times g$ and the supernatant was again applied to Sephadex G-25 column. The resultant protein fractions, having activity of polyphenol oxidase, were used as tea leaves extract to the following experiments.

Incubation methods. Incubation was carried out in 50 ml flask containing 6 to 9 ml of phosphate buffer solution for 2 hr at 25°C unless otherwise stated. Phosphate buffer solution (pH 7.0) was prepared from M/15 KH_2PO_4 and M/15 $NaHPO_4$. The composition of the reaction mixture was shown in each table and figure.

1) M. A. Bokuchava and V. R. Popov, *Doklady Akad. Nauk S.S.S.R.*, **99**, 145 (1954) [*C.A.*, **49**, 3439c (1955)].

2) T. Nakabayashi, *Nippon Nogeikagaku Kaishi*, **32**, 941 (1958).

3) R. Saijō and T. Takeo, *Agr. Biol. Chem.*, **34**, 222 (1970).

4) T. Takeo, *ibid.*, **29**, 558 (1965).

Quantitative analysis of phenylacetaldehyde by gas chromatography. Volatile compounds were extracted from the reaction mixture with diethylether. The extract was dehydrated with Na_2SO_4 and concentrated. All of the resultant concentrate was applied to gas chromatography, the condition of which was the same as described previously³⁾ except 1/30 sensitivity of flame ionization detector. The retention time of phenylacetaldehyde was 29.5 min*. From the peak area its quantity was calculated by the use of the calibration curve shown in Fig. 1.

FIG. 1. Calibration Curves of Phenylacetaldehyde.

Column: Carbowax 6000, Temp.: 150°C, He flow rate: 30 ml/min. Detector: FID, Sens.: 1/30, Chart speed: 1 cm/min.

Measurement of total carbonyl contents by titration. Total carbonyl compounds were extracted with diethylether from the reaction mixture, and then reacted with 5 ml of 1 N NaHSO_3 for 70 min at 5°C to form carbonyl compounds- NaHSO_3 complex. After residual NaHSO_3 was titrated with 1 N I_2 (indicator, 1% starch solution), excess I_2 was also titrated with 0.1 N NaHSO_3 and precisely with N/100 I_2 . The carbonyl compounds- NaHSO_3 complex was decomposed by adding NaHCO_3 and stand for 10 min at room temperature. Resultant SO_2 liberated was titrated with N/100 I_2 . The titration value was proportional to original carbonyl compounds.

* As the retention time of benzaldehyde was 18.6 min, these aldehydes could be separated clearly.

2,4-Dinitrophenylhydrazones and thin-layer chromatography of volatile carbonyl compounds. The preparation of 2,4-dinitrophenylhydrazones (2,4-DNPH) of carbonyl compounds and thin-layer chromatography were done according to the methods described previously.³⁾ After the development of thin layer chromatography main 2,4-DNPH was scraped off with a spatula, eluted by acetone and recrystallized from ethanol-water.

Spectrometry. Absorption spectra were determined in water solution by a Beckman DB spectrophotometer.

Chemicals. (+)-Catechin, (–)-epicatechin, (–)-epigallocatechin, (–)-epigallocatechin gallate were supplied by Dr. M. Nakagawa.⁵⁾ Those were isolated from tea and other plants in pure state. Other chemicals were the highest grade commercially available.

RESULTS

Reaction systems in which phenylacetaldehyde was produced from phenylalanine by tea leaves extract

As shown in Table I, phenylacetaldehyde was produced, when (–)-epicatechin was added to the reaction mixture of phenylalanine and tea leaves extract (No. 2). Since hydrogen peroxide and Mn^{2+} showed the stimulating effect on the production of phenylacetaldehyde (No. 4, 5), it was revealed as shown in No. 6, 7 that these factors activated the peroxidase system in tea leaves extract. Reaction systems of No. 9, 10 showed that phenylacetaldehyde was formed by the reaction between phenylalanine and *o*-quinone. Therefore, the mechanism of phenylacetaldehyde production was considered to be the reaction between phenylalanine and *o*-quinone derived from (–)-epicatechin by polyphenol oxidase or peroxidase. When pyridoxal phosphate, Mn^{2+} and mono-phenol were added in tea leaves extract and phenylalanine (No. 12), benzaldehyde was produced

5) M. Nakagawa and H. Torii, *Agr. Biol. Chem.*, **28**, 160 (1964).

6) M. H. Yu, D. K. Salunkhe and L. E. Olson, *Plant & Cell Physiol.*, **9**, 633 (1968).

TABLE I. THE PRODUCTION OF PHENYLACETALDEHYDE IN VARIOUS REACTION SYSTEMS

No.	Reaction system	Phenylacetaldehyde $\times 10^{-2}$ μ mole
	Composition	
1	Phe + TLE	0
2	Phe + TLE + (–)EC	1.9
3	Phe + TLE + H ₂ O ₂	0
4	Phe + TLE + (–)EC + H ₂ O ₂	3.3
5	Phe + TLE + (–)EC + Mn ²⁺	2.1
6	Phe + HRP + (–)EC + H ₂ O 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⁺ + <i>p</i> -Cresol	0

(Benzaldehyde *ca.* 30.8
 $\times 10^{-2}$ μ mole)

Reaction systems 1~10: 6 ml total volume (M/15 phosphate buffer, pH 7.0), 25°C, 2 hr. Phe; 300 μ moles, TLE; 1 ml of tea leaves extract, (–)EC; 3 μ moles (–)-epicatechin, H₂O₂ or Mn²⁺; 0.3 μ mole, HRP; 30 μ g of horseradish peroxidase, quinones; 3 μ moles.

Reaction systems 11⁹⁾: 9 ml total volume (M/15 Tris-HCl buffer, pH 8.5), 37°C, 3 hr. Phe; 90 μ moles, TLE; 1 ml, α -Ketoglutaric acid; 45 μ moles, Pyridoxal phosphate; 3 μ moles.

Reaction system 12: 9 ml total volume (M/15 phosphate buffer, pH 7.0), 25°C, 2 hr. Phe; 90 μ moles, TLE; 1 ml, Pyridoxal phosphate; 30 μ moles, Mn²⁺; 0.3 μ mole, *p*-Cresol; 0.09 μ mole.

in higher yield as reported by J. M. Hill and P. J. G. Mann.⁷⁾

Carbonyl compounds produced from various amino acids by tea leaves extract

Examinations using various amino acids were attempted in the reaction system, containing amino acid, tea leaves extract and (–)-epicatechin. These results were summarized in Table II. It was revealed that glycine, alanine, valine, leucine, isoleucine, methionine and phenylalanine produced the corresponding aldehydes; formaldehyde, acetaldehyde, isobutyraldehyde, isovaleraldehyde, 2-methylbutanal, methional and phenylacetaldehyde, respectively. Glutamic acid, tyrosine and tryptophan were also presumed to produce the

corresponding carbonyl compounds, however the products were not determined in this study. Although it was presumed that the value of determined carbonyl contents did not always represent the contents of the corresponding carbonyl compounds derived from the amino acid, methionine, isoleucine, valine and leucine might produced larger amounts of carbonyl compounds in the investigated amino acids.

Effect of various phenols on the producibility of phenylacetaldehyde

Table III showed that mono-phenols were no effective on the production of phenylacetaldehyde and that tri-phenols were a little and that di-phenols were most effective, especially (–)-epicatechin. In the reaction mixture of (–)-epicatechin and (–)-epigallocatechin gallate, which was one of the formation systems

⁷⁾ J. M. Hill and P. J. G. Mann, *Biochem. J.*, **99**, 454 (1966).

TABLE II. CARBONYL COMPOUNDS PRODUCED FROM VARIOUS AMINO ACIDS BY TEA LEAVES EXTRACT

Amino acid	Main carbonyl compound 2,4-DNPH			Produced total carbonyl content (N/100 I ₂ ml)
	<i>R_f</i> (TLC) ^{a)}	mp °C	Identification	
Gly	0.50	163	Formaldehyde	0.23
Ala	0.54	167	Acetaldehyde	0.15
Val	0.77	180	Isobutyraldehyde	0.45
Leu	0.88	121	Isovaleraldehyde	0.33
Ileu	0.87	126	2-Methylbutanal	0.46
Met	0.57	128	Methional	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 7.0), contained 500 μ moles of amino acids, 3 μ moles of (—)-epicatechin and 1 ml of tea leaves extract.

^{a)} Thin-layer chromatography with developing solvent of benzene-ligroin-ethylacetate (6:6:1).

TABLE III. EFFECT OF VARIOUS PHENOLS ON THE PRODUCTION OF PHENYLACETALDEHYDE

Phenol	Phenylacetaldehyde (relative quantity)
<i>p</i> -Coumaric acid	0
<i>p</i> -Cresol	0
(+)-Catechin	31
Catechol	57
(—)-Epicatechin	100
(—)-Epigallocatechin	10
(—)-Epigallocatechin gallate	10
Pyrogallol	trace
(—)-Epicatechin+(—)-Epigallocatechin gallate	17

Reaction mixture, of 6 ml total volume (M/15 phosphate buffer, pH 7.0), contained 300 μ moles of phenylalanine, 1 ml of tea leaves extract and 3 μ moles of phenol; where mono-phenol was used, 0.3 μ mole of H₂O₂ was added in above mixture. Reaction was carried out at 25°C for 2 hr.

of theaflavin⁸⁾ (a reddish orange pigment in black tea infusion), the production of phenylacetaldehyde was depressed. Therefore, it was revealed that *o*-di-phenols, especially (—)-epicatechin, reacted as the effective co-factor on the production system of phenylacetaldehyde

from phenylalanine and that the effectiveness of (—)-epicatechin was inhibited by the co-existence of tri-phenol.

Effects of the concentrations of phenols and quinone on the phenylacetaldehyde production

As shown in Table IV, the most suitable concentration of (—)-epicatechin for the phenyl-

8) Y. Takino and H. Imagawa, *Agr. Biol. Chem.*, **28**, 255 (1964).

TABLE IV. EFFECTS OF THE CONCENTRATIONS OF PHENOLS AND QUINONES ON THE PHENYLACETALDEHYDE PRODUCTION

Phenol or quinone	Concentration (M)	5×10^{-6}	5×10^{-5}	5×10^{-4}	5×10^{-3}	5×10^{-2}
(-)-Epicatechin		0.3	5.7	8.1	2.1	—
Catechol		trace	0.7	4.6	19.2	15.0
Sodium β -naphthoquinone 4-sulfonate		0	trace	10.2	125.0	217.0

The figures represent the produced phenylacetaldehyde ($\times 10^{-2}$ μ mole).

Reaction mixture, of 6 ml total volume (M/15 phosphate buffer, pH 7.0), contained 300 μ moles of phenylalanine, 1 ml of tea leaves extract and phenols or quinone.

Reaction was carried out at 25°C for 2 hr.

acetaldehyde production in the tea leaves extract-phenol-phenylalanine system was 5×10^{-4} M and that of catechol, 5×10^{-3} M. At higher concentration of di-phenols the phenylacetaldehyde production was depressed. The optimum concentration of tea polyphenols on the reaction of tea leaves polyphenol oxidase was about 3 to 5×10^{-3} M. Therefore, the optimum concentration of (-)-epicatechin in the tea leaves extract-phenol-phenylalanine system was one tenth of that of (-)-epicatechin in polyphenol oxidase-polyphenol system. These results showed that the phenylacetaldehyde production system was accelerated by the low concentration of quinone derived from (-)-epicatechin and that the high concentration of quinone reacted as inhibitor on the system. In the case of quinone-phenylalanine system the production was nearly proportional to the concentration of quinone under 5×10^{-3} M.

Effect of concentration of enzyme on the phenylacetaldehyde production and (-)-epicatechin oxidation

The production of phenylacetaldehyde decreased by diluting tea leaves extract (Fig. 2). Moreover, it was found that the phenylacetaldehyde production proceeded for 3 or 4 hr though the formation of oxidized (-)-epicatechin, which was determined by the absorption at 398 m μ ,⁹⁾ almost stopped within

FIG. 2. Effect of Concentration of Enzyme on the Phenylacetaldehyde Production and (-)-Epicatechin Oxidation.

Reaction mixture, of 6 ml total volume, contained 300 μ moles Phe., 3 μ mole (-)-epicatechin and tea leaves extract (○—○ and ●—●; 1 ml, □—□ and ■—■; 1/2 ml, △—△ and ▲—▲; 1/4 ml). Real line; phenylacetaldehyde, broken line; oxidized (-)-epicatechin.

1 hr after the start of reaction. From the previous results, it was thought that the pro-

⁹⁾ M. Nakagawa and H. Torii, *Agr. Biol. Chem.*, **29**, 278 (1965).

duction of phenylacetaldehyde from phenylalanine was effected by oxidative enzyme in tea leaves extract. However, it was presumed that this reaction was not catalysed directly by enzymic system and might be stimulated by small amount of quinone which was produced in the reaction mixture by enzymic oxidation of di-phenols, and that the reaction continued until quinone produced was consumed by the reaction between quinone and amino acid.

Visible-light absorption spectra of the phenylacetaldehyde production system

As shown in Fig. 3, the tea leaves extract-

FIG. 3. Visible-light Absorption Spectra of Two Reaction Mixtures.

Real line; 300 μ moles Phe + 3 μ moles (—)-epicatechin + 1 ml tea leaves extract, 6 ml total volume.

Broken line; 3 μ mole (—)-epicatechin + 1 ml tea leaves extract, 6 ml total volume.

Measurement was done at one fourth concentration of original reaction mixture of 2 hr.

phenol-phenylalanine system gave the absorption maximum at 428 $m\mu$ which was different from that of the polymerized substance formed by (—)-epicatechin alone.

DISCUSSION

The reaction that the aldehyde is produced

from amino acid by tea leaves extract is regarded as Strecker degradation from the following reasons: 1) the ratio of radioactive phenylacetaldehyde to carbon dioxide produced from phenylalanine- $U^{14}C$ on the process of black tea fermentation is about 8:1; 2) in the tea leaves extract-(—)-epicatechin-phenylalanine system it is supposed that the first step is the formation of *o*-quinone from (—)-epicatechin and then *o*-quinone reacts with phenylalanine; 3) in the same system the colored substance which is different from polymerized (—)-epicatechin is also formed.

In the tea leaves extract-phenol-amino acid system the effectiveness of (—)-epicatechin is inhibited by the coexistence of triphenol. This indicates that one of the $>CO$ radicals of visinal tri-quinone reacts as the inhibitory factor. The optimum concentration of (—)-epicatechin on the tea leaves extract-phenol-amino acid system was $5 \times 10^{-4}M$, and the higher concentration of that inhibited the production of carbonyl compounds. This optimum concentration was about one tenth of that on the polyphenol oxidase-polyphenol system. In the system containing higher concentration of *o*-quinone derived from (—)-epicatechin it is thought that most of *o*-quinone changes to polymerized product and the production of carbonyl compound is depressed, since the polymerization rate of *o*-quinone is much higher than the production rate of carbonyl compound from *o*-quinone and amino acid. Therefore, these two reaction may be competitive each other.

The production does not depend on the enzyme which is characteristic to aldehyde production, because the reaction system does not show the substrate specificity for amino acids (Table II). Therefore, it is considered concerning with the production of aldehyde from amino acid in black tea fermentation that the first step of the reaction is the formation of quinone from catechin by oxidative

enzyme in tea leaves and then the quinone reacts with amino acid to produce carbonyl compound at the relatively low concentration of quinone.

Acknowledgement. The authors wish to express their gratitude to Dr. I. Uritani, University of Nagoya, for his valuable suggestions.