Agric. Biol. Chem., 53 (9), 2307~2311, 1989

Antibacterial Substances in Japanese Green Tea Extract against *Streptococcus mutans*, a Cariogenic Bacterium

Senji SAKANAKA, Mujo KIM,* Makoto TANIGUCHI and Takehiko YAMAMOTO

Central Research Laboratories of Taiyo Kagaku, Co., Ltd., 1-3, Takaramachi, Yokkaichi-shi, Mie 510, Japan
**Faculty of Science, Osaka City University, Sugimoto, Sumiyoshi-ku, Osaka 558, Japan*

Received February 7, 1989

An extract of Japanese green tea, one of the most popular drinks in Japan, was an inhibitor of the growth of *Streptococcus mutans*, a bacterium responsible for causing dental caries. The analysis of the extract revealed that the main antibacterial components of the extract were several polyphenolic compounds, especially gallocatechin (GC), epigallocatechin (EGC), and epigallocatechin gallate (EGCg). GC was the most active component and its minimum inhibitory concentration against the bacterium was around 250 μg per ml.

Recent studies on Japanese green tea have shown that the extract shows antimutagenic[1] and antihepatotoxic effects[2] capillary blood congestion resistant activity,[3] antitumor activity,[4] and hypolipemic[5,6] and antioxidative effects.[7~9]

A green tea extract is usually tasted after every meal as a custom in Japan. A traditional saying that drinking green tea makes the mouth clean led us to this work, and it was found that several polyphenols in the tea extract inhibited the growth of *Streptococcus mutans*, a cariogenic bacterium. This paper describes the isolation of the active polyphenolic compounds from the tea extract and their modes of bacterial growth inhibition.

Materials and Methods

Tea leaves. Tea leaves were purchased from a tea dealer at Suizawa in Mie Prefecture.

Microorganisms examined. The cariogenic bacteria examined in this study were *Streptococcus mutans* MT8148 (serotype c), *S. mutans* IFO 13955 (serotype c), and *S. sobrinus* 6715DP (serotype g).

To culture the bacteria, brain heart infusion (BHI, Kyokuto Seiyaku Co., Tokyo and DIFCO Laboratories, Detroit) and sensitive meat extract broth (Eiken Chemical Co., Tokyo) were used.

Before testing, the bacteria were cultured in the liquid sensitive meat extract broth at 37 °C overnight. For the paper disc test, about a 0.6-ml sample of the precultured bacterial cell broth was taken and smeared uniformly on an agar plate (140 mm × 100 mm) made of 60 ml of sensitive meat extract. Paper discs (Toyo Roshi Co., 8 mm dia. and about 1.5 mm thick) containing 3 mg or 1 mg of partitioned fractions were placed on the seeded plates. The incubation was done at 37°C for 48 hr, and the growth inhibitory zones were visually compared.

For measurement of the minimum inhibitory concentration (MIC), each polyphenol isolated was examined against *S. mutans* by two-fold serial dilution as follows: 0.1 ml of the compound solution to be tested was added to 9.9 ml of sterile media containing 1.5% agar at around 60°C in a petri dish. This mixture was mixed thoroughly and solidified. One platinum loop of the bacterial suspension precultured at 37°C overnight was inoculated on the agar medium, and incubated at 37°C for 48 hr. The minimum inhibitory concentration was estimated by visually comparing the bacterial growth.

Isolation and identification of the antibacterial components of Japanese green tea [*Camellia sinensis* (L.) O. Kuntze]. The isolation of the antibacterial components was monitored by the anti-growth activity against *S. mutants*. One kg of pulverized Japanese green tea was suspended in 10 l of methanol and kept at room temperature for two days. After filtration the extract was evaporated to dryness *in vacuo*. The residue (298 g) was dis-

solved in water (6 l), and successively partitioned with equal volumes of hexane, chloroform, and ethyl acetate.

The anti-growth activity was observed in the ethyl acetate fraction (96 g), and a portion (7.5 g) of this fraction was chromatographed on a silica gel column [500 g (BW-300, Fuji-Davison Co.), 5 cm × 60 cm]. The column, which had adsorbed the fraction, was washed with a mixture of chloroform and methanol 20 : 1, 5 l and then eluted with a similar mixture but in the ratio of 10 : 1, 5 l. The eluates were separated into three fractions which were confirmed by thin-layer chromatography (Polygram SIL G/UV_{254}, Macherey-Nagel, Germany). The solvent used was methanol–chloroform, 10 : 3, and the compounds developed were detected by spraying a mixture of sulfuric acid and vanillin.

For further purification of the active components, recycling HPLC was done on a JAI-LC-908 HPLC (Japan Analytical Industry Co., Tokyo, JAPAN) equipped with a JAI RI- and JAI UV-detector, operating at 280 nm. A prepacked PVA HP-GPC-column (JAIGEL GS-320, 50 cm × 2 cm i.d.) was used. Methanol was used as the eluting solvent at a flow rate of 3 ml per min.

The active compounds isolated were identified by analyzing them on a GC mass spectrum (JMS-DX303, JEOL, Tokyo, JAPAN) and NMR (GSX-400, JEOL, Tokyo, JAPAN).

Results

Preparation of crude tea polyphenolic compounds

The methanol extract of "Japanese green tea" inhibited the growth of cariogenic bacterium, *Streptococcus mutans*. The methanol extract was partitioned with several solvents separating it into four fractions as described above. The ethyl acetate fraction showed the antibacterial activity, but other fractions were not active.

The ethyl acetate fraction was further partitioned on a silica gel column. After the sample was put on, the column was washed with methanol–chloroform (20 : 1) and then the absorbed substance was eluted with methanol–chloroform (10 : 1). Three peak fractions were obtained and they all inhibited the growth of cariogenic bacteria.

To isolate the active compound, each fraction obtained above was recycled on HPLC using a PVA column as a gel-permeation type column and methanol as the solvent. Fraction 1 gave rise to (+)-catechin and (−)-epicatechin on recycling twice and fraction 2, (+)-gallocatechin, (−)-epigallocatechin, and (−)-epicatechin gallate on recycling three times (Fig. 1). Fraction 3 gave (−)-epigallocatechin gallate. Each compound isolated was identified on the basis of spectral evidence (UV, MS, ^{1}H-NMR, ^{1}H–^{1}H-2D NMR, and ^{13}C-NMR). Their structures are summarized in Fig. 2.

Epigallocatechin gallate, for example, was identified based on the following evidence. The purified sample gave the molecular ion peak at m/z: 459 $(M+H)^+$ on the FAB mass spectrum. In the ^{1}H-NMR spectrum in CD_3OD, two meta coupled doublets [d, $J_{6,8}=2.2$] at δ 6.00

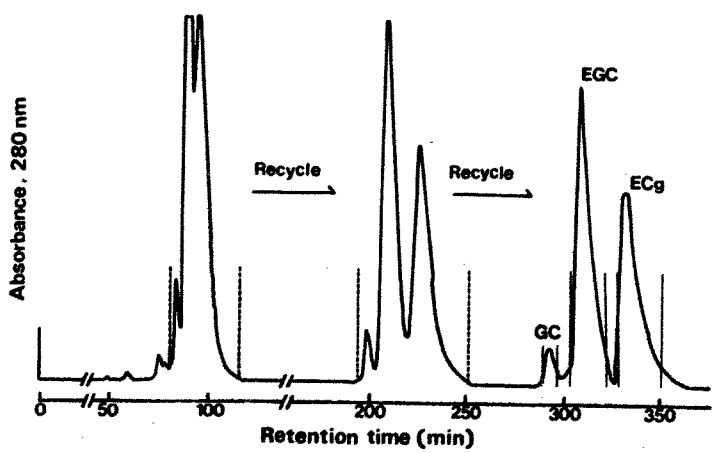

Fig. 1. Isolation of Polyphenols by Recycling HPLC.

Column JAIGEL GS-320 (50 cm × 2 cm i.d.); eluent, MeOH; flow rate, 3 ml/min; detector, UV at 280 nm; injection amount, 180 mg of fraction 2 eluted from silica gel column. GC, EGC, and ECg were isolated respectively.

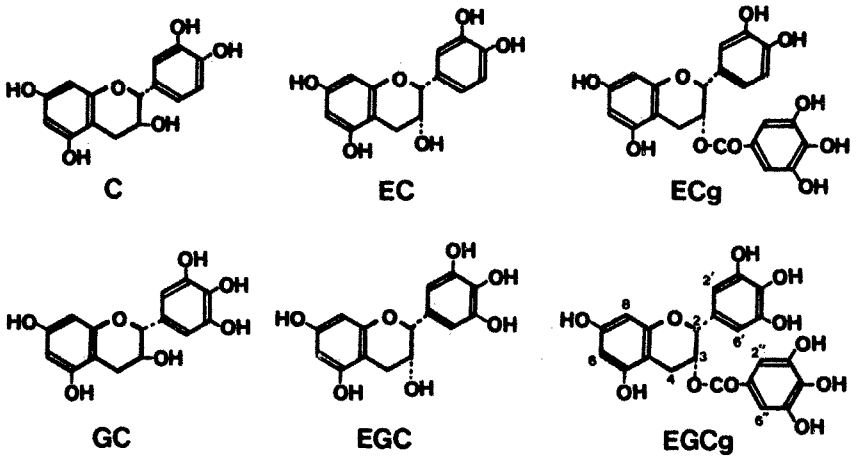

Fig. 2. Structures of Isolated Polyphenols.

Table I. Minimum Inhibitory Concentrations (MIC) of Tea Polyphenols Isolated for Cariogenic Bacteria

Test compounds[3]	MIC (μg/ml)					
	S. mutans MT8148		*S. mutans* IFO 13955		*S. sobrinus* 6715DP	
	a[1]	b[2]	a	b	a	b
C	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
EC	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
GC	250	250	250	250	250	250
EGC	500	250	500	250	500	250
ECg	>1,000	1,000	>1,000	>1,000	>1,000	>1,000
EGCg	1,000	500	1,000	500	1,000	500

[1] BHI agar medium.
[2] Sensitive meat extract agar medium.
[3] Test compounds: C, (+)-catechin; EC, (−)-epicatechin; GC, (+)-gallocatechin; EGC, (−)-epigallocatechin; ECg, (−)-epicatechin gallate; EGCg, (−)-epigallocatechin gallate.

and 6.03 which are due to C-6 and C-8 protons were observed. C-2′ and C-6′ (C-2″ and C-6″, also) proton signals, were observed in the form of singlets at δ 6.59 and 6.19, respectively. The signals at aliphatic regions were characteristic of flavanols and three typical signals were visible at δ 2.91 for two protons at C-4 [dd, $J_{gem}=17$, $J_{3,4}=2.5$ Hz] δ 5.04 and 5.52 each integrating for a single proton assignable to the C-2 and C-3 protons. The weak coupling between these two protons is an indication of the *cis* nature of these protons. The compound isolated from fraction 3 was thus identified as epigallocatechin gallate.

The structures of the other five polyphenolic compounds were identified similarly.

Minimum inhibitory concentration of isolated polyphenolic compounds

The minimum inhibitory concentrations (MIC) of the isolated polyphenolic compounds were estimated as shown in Table I. GC and EGC completely inhibited the growth of three strains of cariogenic bacteria in 250 and 250 or 500 μg/ml, respectively. The MIC of EGCg whose amount in the green tea extract was comparatively large, was between 500 and 1,000 μg/ml. Their growth inhibitory effects were doubled or more when examined using sensitive meat extract medium.

Minimum time for the bactericidal action

To measure the minimum time for the bac-

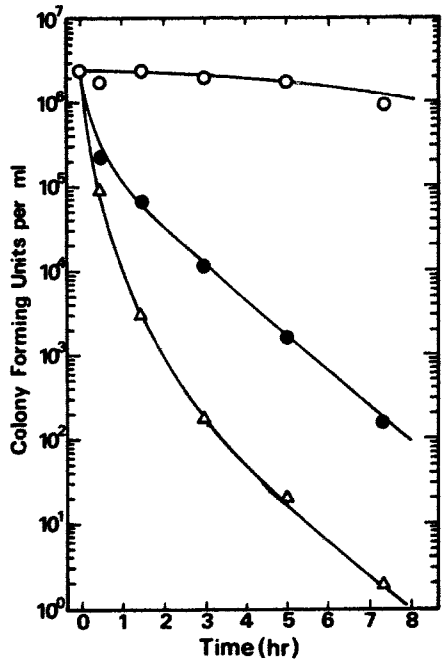

Fig. 3. Relationship between Bacterial Cells Surviving and Period of Exposure to Tea Polyphenols.

S. mutans MT8148 precultured in BHI broth at 37°C overnight were harvested and washed with sterile phosphate-buffered saline solution (pH 7.0), followed by centrifugation. A 0.1-ml sample of the washed cell suspension (about $10^6 \sim 10^7$ cells/ml) was added to 4.9 ml of phosphate-buffered saline solution (pH 7.0) containing the test compounds and the mixture was incubated for the time indicated at 37°C. Samples (0.1 ml) of the incubation mixture were then diluted properly with a phosphate-buffered saline solution, and a certain portion of the diluent was inoculated in BHI agar medium, then incubated at 37°C for 48 hr to count the colonies on the plate. Symbols: ○, control; ●, ethyl acetate fraction (1,000 μg/ml); △, EGCg (1,000 μg/ml).

tericidal action of the above polyphenolic compounds, the cells of *S. mutans* MT8148 were suspended in a solution of ethyl acetate fraction or EGCg for certain periods. The colony forming units (CFU) of the bacterial cells thus treated were then measured by cultivation at 37°C for 48 hr. Figure 3 shows a sharp decrease of CFU with time of incubation with the compounds. By incubation of the cell suspension for 30 min with ethyl acetate fraction or EGCg of concentration of 1,000 μg/ml, the CFU of *S. mutans* was reduced to about one tenth of the initial amount. After eight hrs of incubation with the ethyl acetate fraction, the bacterial CFU decreased to 10^2 CFU/ml, and in the case of EGCg, it became negligible.

Discussion

Recent investigations are focusing on the nutritional effects of tea on the human body. However, detailed examinations of its biological or physiological effects have not yet been reported. This paper suggests that drinking green tea extract is an effective prevention of dental caries to a considerable extent. The chemical investigation of the tea extract as indicated by the anti-microbial test led to isolation of several polyphenolic compounds and they were found to inhibit the growth of *S. mutans*, a cariogenic bacterium responsible for tooth decay.

The inhibitory activity of (+)-gallocatechin and (−)-epigallocatechin were stronger than (+)-catechin and (−)-epicatechin. Also (−)-epigallocatechin gallate was more active than (−)-epicatechin gallate. These facts indicate that the presence of the three hydroxy moieties at 3′, 4′, and 5′ on the B ring in the catechin and epicatechin molecules strengthen the inhibitory activity.

The inhibitory activity of the phenolic compounds varied depending on the kind of medium. The minimum inhibitory concentrations (MIC) were from 250 to 1,000 μg per ml. This MIC is much higher than that of various antibiotics. However, it is noteworthy that a cup of green tea extract (100 ml) usually contains 50 to 100 mg of the polyphenols. This concentration of the tea polyphenols is higher than those used in the experiments in this paper. This study also indicates that only a five to ten minutes exposure of the cariogenic bacteria to the tea polyphenols resulted in a great reduction of colony forming units.

A clinical test of the effect of drinking green tea extract on dental caries was once reported by Onisi *et al.*, whose experiment was conducted at primary schools over a year. Incidences of dental caries among children who took a cup of tea immediately after lunch were found to be significantly lower.[11] This

result suggested that green tea contained certain effective substances for the prevention of dental caries. They concluded that the green tea extract was more effective than fluoride compounds.[12] However, they did not indicate the responsible substances in the tea extract. It is now known that the amount of fluoride in the tea extract is so negligible that the fluoride is hardly thought to be the effective substance in the tea extract responsible for the prevention of dental caries. Our paper seems to clearly explain the effects of polyphenols in green tea.

Also, our recent study revealed that Japanese tea extract strongly inhibits the formation of dextran and levan from sucrose by the cariogenic bacteria; these results will be published elsewhere. This also supports the idea that green tea polyphenols can inhibit the growth of *S. mutans* and prevent the cause of caries.

References

1) T. Kada, K. Kaneko, S. Matsuzaki and Y. Hara, *Mutation Res.*, **150**, 127 (1985).
2) H. Hikino, Y. Kiso, T. Hatano, T. Yoshida and T. Okuda, *J. Ethnopharmacol.*, **14**, 19 (1985).
3) D. N. Das, *Ann. Biochem. Exp. Med.*, **23**, 219 (1963).
4) I. Oguni, K. Nasu, S. Yamamoto and T. Nomura, *Agric. Biol. Chem.*, **52**, 1879 (1988).
5) Y. Fukuo, Y. Kobayashi, Y. Nakazawa, H. Inaba, T. Shibuya, H. Ootsuka, K. Hada, N. Oouchi, K. Iijima, A. Terashi, K. Oohashi, M. Kawamorita, T. Tsushima, K. Seta and J. Atarashi, *Domyaku Koka* (in Japanese), **10**, 981 (1982).
6) K. Muramatsu, M. Fukuyo and Y. Hara, *J. Nutr. Sci. Vitaminol.*, **32**, 613 (1986).
7) T. Matsuzaki and Y. Hara, *Nippon Nōgeikagaku Kaishi*, **59**, 129 (1985).
8) T. Okuda, Y. Kimura, T. Yoshida, T. Hatano, H. Okuda and S. Arichi, *Chem. Pharm. Bull.*, **31**, 1625 (1983).
9) Y. Kimura, H. Okuda, K. Mori, T. Okuda and S. Arichi, *Nippon Eiyō Shokuryō Gakkaishi*, **37**, 223 (1984).
10) S. Maeda and M. Nakagawa, *Chagyo Kenkyu Hokoku* (in Japanese), No. 45, 85 (1977).
11) M. Onisi, N. Shimura, C. Nakamura and M. Sato, *J. Dent. Hlth.*, **31**, 13 (1981).
12) M. Onisi, F. Ozaki, F. Yoshino and Y. Murakami, *J. Dent. Hlth.*, **31**, 158 (1981).