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Inhibitory Effects of (−)-Epigallocatechin Gallate on the Mutation, DNA Strand Cleavage, and DNA Adduct Formation by Heterocyclic Amines

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Green tea is known to be a potential chemopreventive agent against cancer. In this study, we investigated the inhibitory activities of tea extracts, and in particular the polyphenolic component (−)-epigallocatechin gallate (EGCG), against heterocyclic amine-induced genotoxicity. The tea extracts displayed inhibition of 2-hydroxyamino-6-methyldipyrido[1,2-*a*,3′,2′-*d*]imidazole (Glu-P-1(NHOH))-induced mutagenicity. This inhibition can be accounted for by the presence of EGCG in the extracts. The mutagenic effect of Glu-P-1(NHOH), which induces single-strand cleavage in supercoiled circular DNA under neutral conditions, was inhibited by EGCG. Using the *Drosophila* repair test, a test for gross DNA damage, and DNA adduct detection by ^{32}P-postlabeling, we showed that EGCG prevented 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline-induced DNA damage and adduct formation in insect DNA. EGCG was found to accelerate the degradation of Glu-P-1(NHOH) in vitro. This observation suggested that the inhibition by EGCG is associated with an accelerated degradation of metabolically activated heterocyclic amines.

KEYWORDS: Green tea; epigallocatechin gallate; heterocyclic amines; DNA strand break; antimutagenicity; DNA adduct; *Drosophila*

INTRODUCTION

Epidemiological evidence has shown that green tea lowers the risk of cancer (*1, 2*). The antimutagenic and anticarcinogenic activity of EGCG, the main constituent of green tea polyphenols, has been extensively investigated (*3*−*6*). Several mechanisms have been proposed for the effects of EGCG, including modification of signal transduction pathways and apoptosis (*4*), induction of various enzymes (*7*), and the scavenging of reactive oxygen species (*8*). Heterocyclic amines such as Glu-P-1, MeIQx, and Trp-P-2 are formed during the cooking of fish and meat and are potent carcinogens (*9*). In our previous work, we reported that EGCG can suppress the direct acting mutagenic effects of Trp-P-2(NHOH), the metabolically activated form of Trp-P-2, in *Salmonella typhimurium* TA98 (*5*). Bu-Abbas et al. (*10*) and Santana-Rios et al. (*11*) reported that tea extracts decrease the mutagenicity of several heterocyclic amines.

In this study, we investigated the inhibitory mechanism and activities of tea extracts and EGCG toward DNA mutation and strand cleavage by the metabolically activated form of Glu-P-1. Additionally, we investigated whether the effects of EGCG on MeIQx-induced genotoxicity as assayed by the *Drosophila* repair test correlated with observations concerning the in vivo formation of DNA adducts in the larvae.

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MATERIALS AND METHODS

Materials. Green tea was purchased in Okayama. EGCG was a gift from Toyo Hakka Kogyo Co., Ltd. (Okayama, Japan), the purity of which was >96% as determined by high-performance liquid chromatography (HPLC). Glu-P-1 (CAS 67730-11-4) and MeIQx (CAS 77500-04-0) were obtained from Wako Chemicals (Osaka, Japan). Glu-P-1(NHOH) was synthesized from Glu-P-1 according to the literature (*12*). The aforementioned hydroxyamino derivatives were >99.5% pure as determined by HPLC. Tea samples (nos. 1 and 2) were prepared as follows: 110 mL of boiling water was added to green tea leaves (2.3 g), after which the mixture was allowed to stand for 3 min and then filtered to remove the leaves. The tea extracts were evaporated to dryness under reduced pressure. The residues were dissolved in sterile water and subjected to the mutagenicity assays. *S. typhimurium* TA98 was a gift from Dr. Bruce N. Ames of the University of California, Berkeley.

Mutagenicity Assays, Detection of DNA Strand Breaks, and HPLC Analysis. The preincubation method (*13*) was employed in the *Salmonella* mutagenicity assays (*14*). The assays were performed without the addition of S9 mix. Modifying effects on the mutagenicity were examined by the procedure described earlier (*15*). The mutagenic activity (%) was obtained as follows: [(His$^+$ revertants in the presence of inhibitor) − (spontaneous revertants)] × 100/[(His$^+$ revertants in the absence of inhibitor) − (spontaneous revertants)]. The amount of Glu-P-1(NHOH) used in the reaction mixture was 0.5 nmol, which gave 783 ± 132 His$^+$ revertants from *S. typhimurium* TA98 per plate in the absence of inhibitor without metabolic activation. In the assay,

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duplicate plates were used for individual dose points. In all experiments, we observed that the background lawns of bacteria on the plates were not impaired, unless otherwise stated.

The quantitative analysis of EGCG in a tea sample was performed with HPLC on a Inertsil ODS, reversed phase column (4.6 mm × 250 mm, GL Sciences Inc., Tokyo, Japan). The elution was performed with methanol−water−acetic acid (20:75:5) at a flow rate of 0.7 mL/min.

Single-strand breaks in DNA were assayed using phage PM2 replicative form DNA (RF I) (*16*). Reactions were carried out by incubating the superhelical DNA of PM2 (0.05 mg/mL) in 0.1 M sodium phosphate (pH 7.4) and 1 mM Glu-P-1(NHOH) in the presence or absence of EGCG. The treated DNA sample was analyzed by agarose gel electrophoresis (0.7% gels in tris−borate−ethylenediaminetetraacetic acid buffer). Electrophoresis was performed for 3 h, and the gels were subsequently stained with ethidium bromide. Single-strand breaks result in the formation of the relaxed circular form (RF II), which migrates more slowly in the gel than the RF I form.

The *Drosophila* strain sc z^1 $w^{+(TE)}$ mei-9^a mei-41^{D5}/-C(1)DX, y f, which consists of DNA repair deficient males and DNA repair proficient females, was a gift from Dr. H. Ryo (Osaka University) and Dr. K. Fujikawa (Kinki University) (*17*). The protocols used for the in vivo DNA repair test are described in our previous paper (*18*). Briefly, third instar larvae were fed a diet containing 150 ppm MeIQx (1 mg/6.5 g diet/bottle) for 6 h, with or without EGCG (0−4.5%; 0, 100, 200, or 300 mg/6.5 g diet). A proportion of these larvae was grown on the normal diet to form adult flies. The male/female ratio was counted as an indication of the damaged DNA lesions in the somatic cells. A lower male/female ratio reflects an increased amount of DNA damage. For the DNA analysis, the treated larvae were immediately frozen in liquid nitrogen until use. The amount of heterocyclic amine−DNA adduct in the DNA of treated larvae was determined by the modified adduct−intensification analysis in the ^{32}P-postlabeling method (*19*). Briefly, DNA was isolated by phenol−chloroform extraction from larvae. The extracted DNA was digested with micrococcal nuclease and spleen phosphodiesterase (Worthington, Freehold, NJ), and the digest was then labeled with ^{32}P by use of T4 polynucleotide kinase (Takara, Kyoto, Japan) and [γ-^{32}P]ATP (ICN Biochemicals, Irvine, CA). The labeled nucleotide mixture was further digested with nuclease P1 (Yamasa, Choshi, Japan) and phosphodiesterase I (Worthington). The digest was subjected to thin-layer chromatography (TLC) on polyethyleneimine cellulose (Polygram, Cel 300 PEI, Machery-Nagel, Duren, Germany), and the plate was developed as described (*18*). The adduct level was indicated by the relative adduct labeling using a Bio-Imaging Analyzer (BAS 2000, Fuji Photo Film, Tokyo).

To study the effect of EGCG on degradation, a solution of Glu-P-1(NHOH) with or without EGCG was analyzed by HPLC. The reaction was initiated by mixing an aqueous solution of Glu-P-1(NHOH) (50 μM) with EGCG (50 μM) in sodium phosphate buffer (0.1 M, pH 6.0) at 21 °C. At 0 and 60 min, a sample (20 μL) was taken and subjected to HPLC to quantify the amount of Glu-P-1(NHOH). Given that the automatic degradation of Glu-P-1(NHOH) occurs more rapidly at higher pH values and that the half-life of Glu-P-1(NHOH) is about 60 min at pH 7.4 (*20*), we examined the effects on degradation at pH 6.0, rather than at pH 7.4. The HPLC conditions were the same as those described above for the quantitative analysis of EGCG.

RESULTS AND DISCUSSION

The tea extracts displayed inhibition of Glu-P-1(NHOH)-induced mutagenicity (**Figure 1A**). EGCG also inhibited the mutagenicity of Glu-P-1(NHOH) (**Figure 1B**). With 10 nmol of EGCG, the mutagenicity of Glu-P-1(NHOH) dropped to 40%. The survival fractions of *S. typhimurium* treated with Glu-P-1(NHOH) plus 10 nmol of EGCG did not decrease as compared to the original. This indicated that the inhibition of Glu-P-1(NHOH)-induced mutagenicity by EGCG was not due to the bacteria being killed.

We performed a quantitative analysis of EGCG in the tea sample (no. 2) using HPLC. The peak fraction at 13.7 min shown in **Figure 2** was identified as EGCG by cochromatography with an authentic specimen. The amount of EGCG in this tea sample was thus estimated at 160 nmol/mL. The amount of tea sample (no. 2) needed for 50% inhibition of Glu-P-

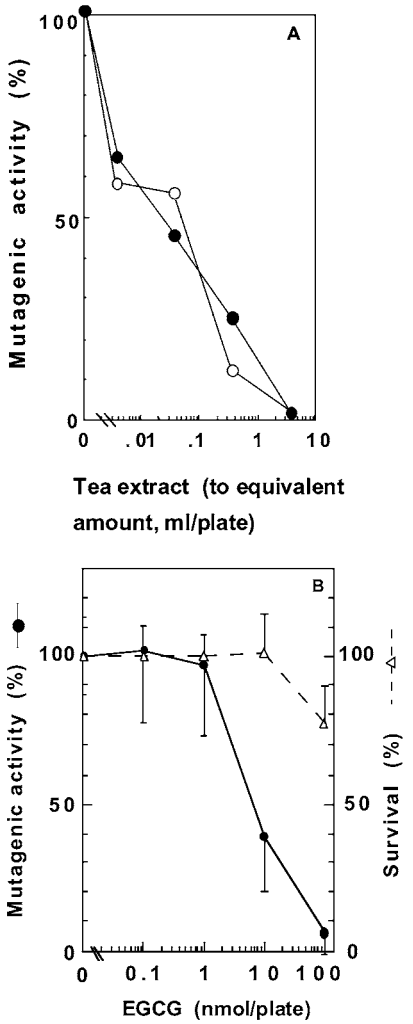

Figure 1. (**A**) Effects of tea samples on the mutagenicity of Glu-P-1(NHOH); sample 1 (●) and sample 2 (○). (**B**) Effects of EGCG on the mutagenicity of Glu-P-1(NHOH) (●) and bacterial survival (△) following treatment. *S. typhimurium* TA98 was used. The data are averages of three independent experiments, and the bar attached to each datum point indicates the standard deviation.

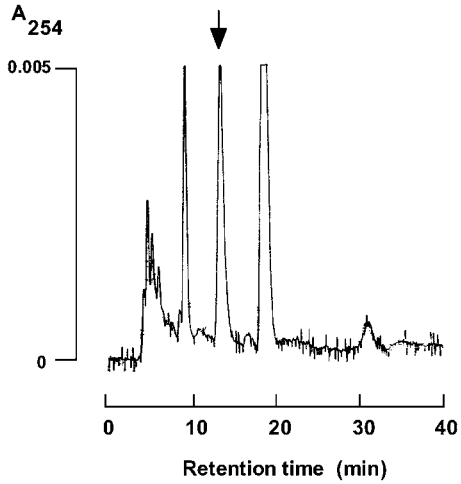

Figure 2. Quantitative analysis of EGCG in tea sample no. 2 by HPLC. The arrow indicates the peak for EGCG.

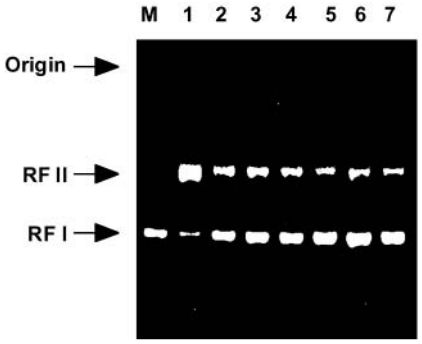

Figure 3. Effects of EGCG on DNA strand cleavage by Glu-P-1(NHOH). Lane M, marker (PM2 DNA RF I); lane 1, Glu-P-1(NHOH) only; lane 2, Glu-P-1(NHOH) + EGCG (0.2 mM); lane 3, Glu-P-1(NHOH) + EGCG (2 mM); lane 4, Glu-P-1(NHOH) + EGCG (20 mM); lane 5, Glu-P-1(NHOH) + EGCG (200 mM); lane 6, without Glu-P-1(NHOH) or EGCG; lane 7, EGCG (200 mM).

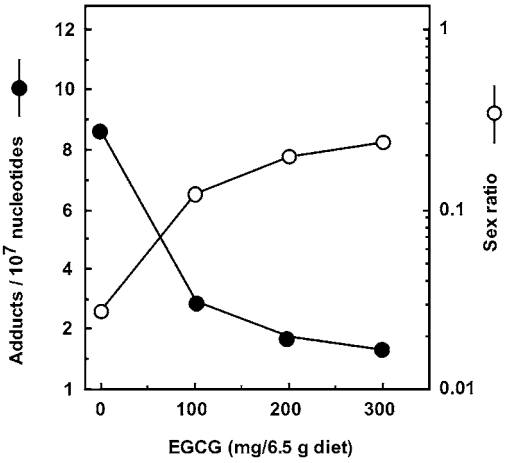

Figure 4. Inhibitory effects of EGCG on MeIQx-induced adduct formation and DNA damage. The amount of adducts (●) and sex ratios (○) shown represents the means of two experiments.

1(NHOH)-induced mutagenicity (I_{50}) was about 0.05 mL. The amount of EGCG in this 0.05 mL of tea was 8 nmol, and the I_{50} value for EGCG was approximately 6 nmol (**Figure 1B**). This confirmed that EGCG in this tea sample could account for the inhibitory effect of the tea extract against Glu-P-1(NHOH)-induced mutagenicity.

Glu-P-1(NHOH) produced single-strand cleavage in supercoiled circular DNA (PM2) under neutral conditions (**Figure 3**, lane 1). The cleavage was inhibited by EGCG in a dose-dependent manner (lanes 2−5). In our earlier study (*16*), Trp-P-2(NHOH) was found to produce single-strand cuts in a supercoiled DNA (ϕX174 RFI), probably through the production of active oxygen radicals as a result of the spontaneous degradation of Trp-P-2(NHOH). It is known that EGCG can scavenge free radicals, thereby inhibiting the H_2O_2-induced oxidative damage of DNA (*8*). It is possible that EGCG might scavenge oxygen radicals produced from Glu-P-1(NHOH), thus preventing the strand cleavage of PM2 DNA.

The ^{32}P-postlabeling of *Drosophila* DNA treated with MeIQx in the presence or absence of EGCG yielded a spot in the TLC assay. In the MeIQx minus control, no spot was observed at the position of the MeIQx adduct (data not shown). The amount of MeIQx−DNA adduct was 8.6 residues/10^7 nucleotides in the absence of EGCG, which decreased to 1.3 residues/10^7 nucleotides in the presence of 4.5% EGCG. The decrease in DNA adduct formation followed a EGCG dose-dependent pattern (**Figure 4**). At the same time, the sex ratio was restored from 0.03 to 0.24. This indicates that EGCG may prevent MeIQx-induced adduct formation and DNA damage.

In an effort to elucidate the antimutagenic mechanism(s) of EGCG, we examined the possibility that EGCG affects the stability of Glu-P-1(NHOH) and analyzed an equimolar mixture of Glu-P-1(NHOH) and EGCG by HPLC. The peak at retention time 8.7 min, which corresponded to 1 nmol of Glu-P-1(NHOH), decreased after incubation with EGCG for 60 min at 21 °C (**Figure 5A**,**B**). The amount of Glu-P-1(NHOH) after 60 min dropped by 28% as compared to the original (**Figure 5A**,**B**). As shown in **Figure 5C**,**D**, the level of Glu-P-1(NHOH), in the absence of EGCG, did not change during the 60 min incubation period. These results indicate that EGCG stimulated the degradation of Glu-P-1(NHOH). The accelerated degradation of Glu-P-1(NHOH) could be one of the mechanisms by which EGCG acts on inhibiting the genotoxicity of heterocyclic amines.

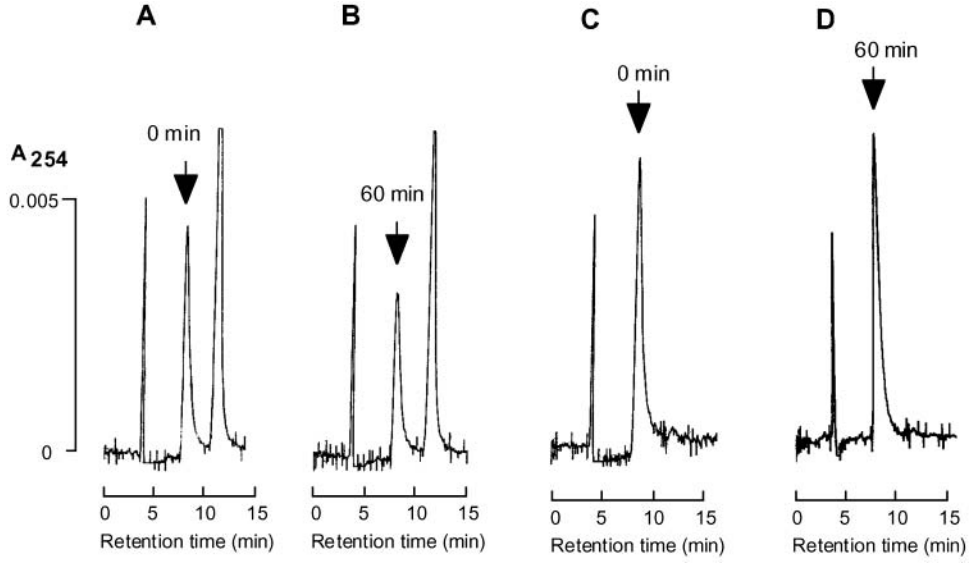

Figure 5. Time course of HPLC profile of Glu-P-1(NHOH) in the presence (**A**,**B**) or absence (**C**,**D**) of EGCG in sodium phosphate buffer (pH 6.0) at 21 °C. The arrow marks the Glu-P-1(NHOH) peak. The peak at retention time 12.2 min corresponded to EGCG (**A**,**B**).

ABBREVIATIONS USED

EGCG, (−)-epigallocatechin gallate; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline; Glu-P-1(NHOH), 2-hydroxyamino-6-methyldipyrido[1,2-*a*,3′,2′-*d*]imidazole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2(NHOH), 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole.

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