Antimutagenic Activity of Cacao: Inhibitory Effect of Cacao Liquor Polyphenols on the Mutagenic Action of Heterocyclic Amines

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We investigated the effect of polyphenols derived from cacao liquor on the mutagenic action of heterocyclic amines (HCAs) in vitro and ex vivo. In the Ames test, the cacao liquor polyphenols showed antimutagenic effects in bacteria treated with HCA in the presence of an S-9 mixture; however, they showed less efficacy than quercetin. On the other hand, the cacao liquor polyphenols showed potent antimutagenic activity in bacteria treated with activated forms of HCA, compared with quercetin. We also evaluated the effect of these compounds on enzymatic activation of HCA. They weakly suppressed the production of activated HCA. In the host-mediated assay in mice, a method used to estimate the potential carcinogenicity of chemicals ex vivo, oral administration of the cacao liquor polyphenols, reduced the number of colonies of revertant bacteria recovered from the liver. These data suggest that the cacao liquor polyphenols have an antimutagenic effect not only in vitro, but also ex vivo.

Keywords: *Antimutagenicity; cacao liquor; polyphenol; Ames test; host-mediated assay; heterocyclic amines*

INTRODUCTION

Epidemiological studies have indicated that the frequent intake of substantial amounts of foods derived from plants, such as vegetables and fruits, is associated with a lower incidence of cancer (Kono et al., 1988; You et al., 1989; Hertog et al., 1993). These findings suggested that chemopreventive substances exist in plant-derived foods. Many studies have recently demonstrated the antimutagenic effects of polyphenols present in plant tissues, including fruits and vegetables (Shinohara et al., 1988, 1991; Yamada and Tomita, 1994; Yen and Chen, 1995).

We have been involved in isolation of several polyphenolic substances from cacao liquor, one of the major ingredients of chocolate and cocoa. The polyphenolic compounds in cacao were identified as catechin, epicatechin, clovamide, quercetin, and their glucosides (Sanbongi et al., 1998; Osakabe et al., 1998). Hammerstone et al. (1999) reported that procyanidins also are a major type of polyphenols in cacao liquor.

Many mutagenic heterocyclic amines (HCAs) have been isolated from cooked foods (Sugimura, 1986, 1988; Felton et al., 1986; Wakabayashi et al., 1993). The carcinogenicity of HCA in animal studies has been reported (Ohgaki et al., 1991).

In the present study, we investigated the effects of cacao liquor polyphenols on the mutagenic action of HCA by means of the Ames test in vitro and by means of a host-mediated assay ex vivo.

MATERIALS AND METHODS

Chemicals. Trp-P-2 and MeIQ acetate forms were purchased from Wako Pure Chemical Industries, Ltd., Japan. (−)-Epicatechin was purchased from Sigma Chemicals Co., Ltd., U.S.A. (+)-Catechin and quercetin were purchased from Extrasynthese S.A., France. The S-9 fraction prepared from the liver of rats pretreated with phenobarbital and 5,6-benzoflavone was obtained from Oriental Yeast Co., Ltd., Japan.

Cacao Liquor. Fermented and dried cacao beans imported from Ghana were roasted and ground at Meiji Seika Kaisha Ltd., Japan.

Preparation of Polyphenolic Substances from Cacao Liquor. Cacao liquor polyphenols were prepared from cacao liquor by the following procedure (Figure 1). The cacao liquor was defatted with *n*-hexane and extracted with 80% (v/v) ethanol. The extract (cacao liquor extract, CLE) was applied to a Diaion HP2MG column (25 mm ∅ × 310 mm, Mitsubishi Kasei Co., Ltd., Japan) and washed with 20% (v/v) ethanol containing 0.1% TFA to remove contaminants. The polyphenol fraction (cacao liquor crude polyphenols, CLP) was obtained by elution with 80% (v/v) ethanol containing 0.1% TFA. A small amount of resin (Wako gel C-200) was added to CLP dissolved in methanol. This mixture was stirred under N_2 gas, and chloroform was added to it gradually. After the methanol in the mixture had evaporated completely, the resin which had absorbed the CLP dissolved in chloroform was loaded onto a Wakogel C-200 column (35 mm ∅ × 310 mm, Wako Pure Chemical Industries, Ltd.), and elution was performed stepwise using chloroform followed by a graded series of mixtures of methanol in chloroform, up to 80% (v/v) methanol in chloroform. The chloroform:methanol = 6:4 fraction was

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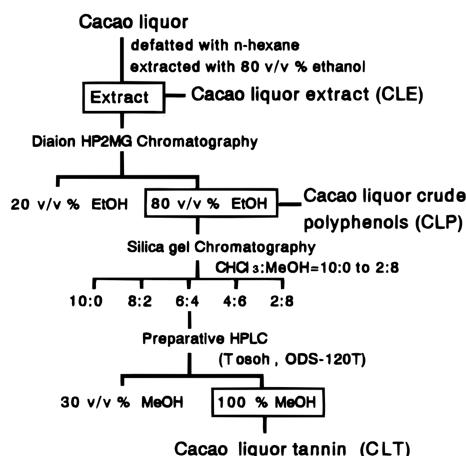

Figure 1. Scheme for preparation of polyphenolic fractions from cacao liquor.

Table 1. Polyphenol Content (% w/w) of Fractions Derived from Cacao Liquor

	CLE	CLP	CLT
total polyphenols[a]	27	48	66
simple phenolic fraction[b]	1.2	0.7	0.1
non-tannin flavan fraction[b]	12.1	17.6	10.3
hydrolyzable tannin fraction[b]	3.7	3.2	3.6
condensed tannin fraction[b]	10.0	26.5	52.0
catechin[c]	0.56	0.91	0.09
epicatechin[d]	2.28	2.95	0.24

[a] Total polyphenols were determined by the Prussian blue method using epicatechin as the standard. [b] Each of the samples was fractionated by selective precipitation of polyphenols with cinchonine sulfate and/or formaldehyde (Peri and Pompei). [c,d] Catechin and epicatechin concentrations were measured by HPLC.

obtained, and further purification was done by preparative HPLC. The column (21.5 mm ∅ × 150 mm, Tosoh ODS-120T, Tosoh Co., Ltd., Japan) was washed with 30% (v/v) methanol, and the tannin fraction (cacao liquor tannin, CLT) was obtained by elution with 100% methanol. The polyphenolic fractions were freeze-dried and then used in this study. The total polyphenol content of each fraction was determined by the Prussian blue method (Price and Butler, 1977) using epicatechin as the standard. Each of the samples was fractionated by selective precipitation of polyphenols with cinchonine sulfate and/or formaldehyde (Peri and Pompei, 1971). The polyphenol content of the simple phenolic fraction and that of the hydrolyzable tannin fraction were determined by the Prussian blue method. The polyphenol content of the non-tannin flavan fraction and that of the condensed tannin fraction were obtained by calculation. The epicatechin and catechin concentrations were determined by HPLC according to the method of Kim and Keeney (1983) as shown in Table 1.

Mutagenicity Tests in vitro. *Experiment 1. Ames Test Using HCA with the S-9 Mixture.* The mutagenicity test was carried out by the preincubation method (Yahagi et al., 1975), with slight modification of the Ames method (Ames et al., 1975), using *Salmonella typhimurium* strain TA98 (*his*$^-$). Mixtures were prepared containing various amounts of the test samples in 50 μL of dimethyl sulfoxide (DMSO), 100 μL of Trp-P-2 (0.4 nmol/plate) or MeIQ (0.025 nmol/plate), 300 μL of the S-9 mixture, and 100 μL of the bacterial suspension. The appropriate concentrations of Trp-P-2 and MeIQ were determined in a preliminary study. After preincubation at 37 °C for 20 min, the mixture was added to 2.5 mL of warm soft agar kept at 47 °C, and the soft agar mixture was poured onto a modified Vogel−Bonner medium agar plate (Kada et al., 1984). After incubation at 37 °C for 2 days, the revertant colonies (*his*$^+$) on each plate were counted.

Experiment 2. Ames Test Using Activated HCA. Activated Trp-P-2 and MeIQ were prepared by the methods of Arimoto et al. (1980). HCA was incubated with the S-9 mixture at 37 °C for 20 min; this reaction mixture was added to cold acetone and kept at 4 °C for 20 min. After centrifugation, the supernatant was concentrated in vacuo. The activated HCA was dissolved in DMSO before use. The mutation test was carried out by a method similar to that described above. The appropriate concentration of activated Trp-P-2 was 20 nmol/plate, and that of MeIQ was 0.1 nmol/plate according to the results of a preliminary study. These were dissolved in 0.3 mL of 0.1 M phosphate buffer (pH 7.4) and added instead of the S-9 mixture.

Experiment 3. Ames Test with Metabolic Activation of HCA. The suppressive effect of the test samples on the mutagenic action of Trp-P-2 and MeIQ upon metabolic activation was examined by the method of Osawa et al. (1991). Mixtures were prepared containing HCA (10 μg/mL), S-9 fraction (2 mg of protein/mL), 2.7 mg/mL NADPH, various amounts of the test samples in DMSO, and 1/15 M phosphate buffer (pH 7.4), and these were incubated at 37 °C for 20 min. Thereafter, cold acetonitrile was added to each mixture, and the resulting mixtures were kept at 4 °C for 20 min. The mixture was centrifuged, and the concentration of nonactivated HCA in the supernatant was determined by HPLC analysis. HPLC analysis of Trp-P-2 was performed by the method of Minamoto and Kanazawa (1995) using a high-performance liquid chromatograph equipped with a UV detector and an ECD connected in series, under the following conditions: column, Develosil ODS-5 (4.6 mm ∅ × 250 mm, Nomura Chemical Co., Ltd., Japan); column temperature, 40 °C; mobile phase, 20 mM KH_2PO_4 (pH 4.6)/acetonitrile (80:20, v/v) containing 0.1 mM EDTA·2Na; flow rate, 1.0 mL/min; detector, UV 267 nm and ECD +300 mV.

HPLC analysis of MeIQ was performed by the method of Alexander et al. (1989). The concentration of nonactivated MeIQ in the supernatant was determined by HPLC analysis, using an HPLC equipped with a UV detector, under the following conditions: column, Develosil ODS-5 (4.6 mm ∅ × 250 mm, Nomura Chemical Co., Ltd.); column temperature, 40 °C; mobile phase, linear gradient of methanol in water from 10% to 45% containing 0.1% diethylamine/acetic acid buffer (pH 5.0); flow rate, 1.0 mL/min; detector, UV 267 nm.

The changes in the levels of nonactivated Trp-P-2 and MeIQ were calculated on the basis of the measured peak areas.

Mutagenicity Test ex Vivo. Intrasanguinous Host-Mediated Assay. This experiment was carried out by the method of Anri et al. (1977) with slight modification. Female BALB/c mice, with body weight in the range of 20−22 g, were purchased from Clea Japan Co. Ltd., Japan. The mice were orally administered 500 mg of CLE/kg of body weight. CLE was easy to suspend in distilled water, as compared to CLP and CLT. The mice in the "no treatment" group and those in the control group received an equivalent amount of distilled water. After 1 h, all mice were treated intravenously with 0.1 mL of a concentrated overnight culture (5×10^{10} colony forming units (cfu)/mL) of *S. typhimurium* strain TA98. Immediately after receiving this injection, the mice were administered Trp-P-2 orally at a dose of 1.5 mg/kg body weight. The mice in the no treatment group received an equivalent amount of 10 mM sodium acetate buffer (pH 4.2). One hour later, the mice were sacrificed, and their livers were removed. The liver was homogenized in a 10-fold volume of 50 mM Tris−HCl buffer (pH 7.4), using a Poter−Elvehjem glass Teflon homogenizer. After gentle centrifugation (100*g*, 10 min, 4 °C) to remove cell debris, the supernatant was collected and centrifuged (2500*g*, 20 min, 4 °C) to recover the bacteria. The bacterial pellet was resuspended in 1 mL of Tris−HCl buffer, 0.1 mL of this bacterial suspension was added to 2.5 mL of top agar containing 0.05 mM histidine, 0.05 mM biotin, and 25 μg/mL ampicillin, and the mixture was poured onto a Vogel−Bonner agar plate (Maron and Ames, 1983). All plates were incubated for 2 days. The revertant colonies on each plate were counted, and the mean value for five replicate plates per liver was determined. The total bacterial count for each liver was determined by spreading 0.1 mL of appropriately diluted samples onto nutrient agar plates containing 5 μg/mL ampicillin.

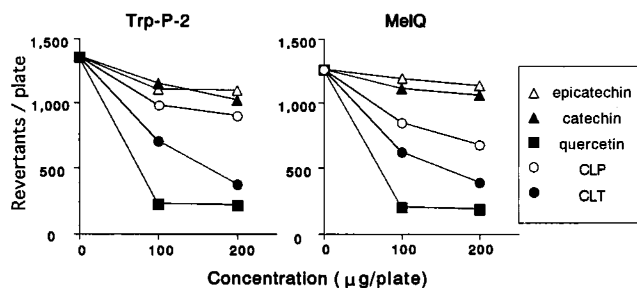

Figure 2. Effects of polyphenolic fractions derived from cacao liquor on mutagenicity induced by Trp-P-2 (0.4 nmol/plate) and MeIQ (0.025 nmol/plate) in *S. typhimurium* TA98 with the S-9 mixture. Each point represents the mean of two independent experiments.

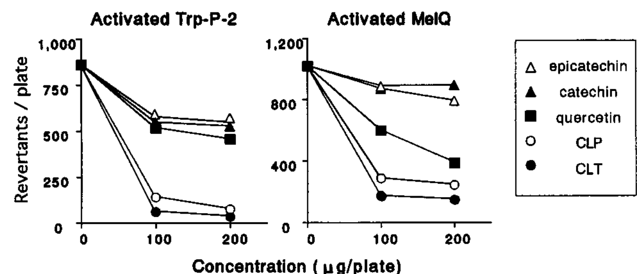

Figure 3. Effects of polyphenolic fractions derived from cacao liquor on mutagenicity induced by activated Trp-P-2 (20 nmol/plate) and activated MeIQ (0.1 nmol/plate) in *S. typhimurium* TA98. Each point represents the mean of two independent experiments.

Statistical Analysis. Data are expressed as the mean plus or minus the standard deviation. Analyses were performed using SPSS statistical software. When ANOVA showed $p < 0.05$, the data were further analyzed using Scheffe's multiple-range test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Mutagenicity Tests in Vitro. *Experiment 1. Ames Test of HCA with the S-9 Mixture.* The effects of CLP, CLT, epicatechin, catechin, and quercetin on the mutagenic action of Trp-P-2 and that of MeIQ in the presence of the S-9 mixture were evaluated. As shown in Figure 2, quercetin markedly decreased the incidence of mutations induced by Trp-P-2 and MeIQ. CLT and CLP were also effective to decrease the number of revertant colonies, and epicatechin and catechin showed slight effects. These chemicals did not affect bacterial growth at the dosage used.

Experiment 2. Ames Test Using Activated HCA. The effectiveness of the test samples in suppressing the mutagenic action of activated Trp-P-2 and MeIQ was evaluated. As shown in Figure 3, CLT and CLP were markedly effective to reduce the incidence of mutations induced by activated HCA. Quercetin showed a weak effect in this experiment as compared with its effect in the presence of the S-9 mixture described below. The antimutagenicity of EC and CA was also weak in the case of this method, similar to experiment 1.

Experiment 3. Ames Test with Metabolic Activation of HCA. The suppressive effect of the test samples on the mutagenic action of Trp-P-2 and MeIQ upon metabolic activation was evaluated. The 50% inhibitory concentration of each of the test samples as determined by monitoring the production of activated HCA is shown in Table 2. Quercetin markedly suppressed the metabolic activation of HCA. Its efficacy was more than 25 times that of CLT and more than 96 times that of CLP. Epicatechin and catechin did not show any suppression in this experiment at concentrations up to 1.0 mg/mL.

Table 2. Effects of Polyphenols in Cacao Liquor on Metabolic Activation of Trp-P-2 and MeIQ

	IC_{50} (μg/mL)[a]	
	Trp-P-2	MeIQ
CLP[b]	499.6	2.28
CLT[c]	332.3	2.95
quercetin	5.2	0.24

[a] IC_{50} = 50% concentration on HCA activated form production. [b] CLP = cacao liquor crude polyphenols. [c] CLT = cacao liquor tannnin.

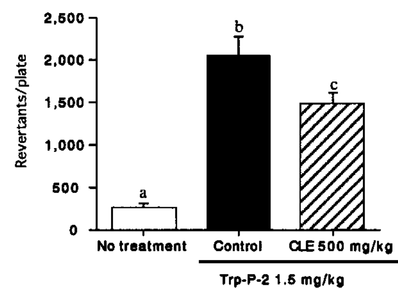

Figure 4. Effect of CLE on mutagenicity induced by Trp-P-2 in *S. typhimurium* TA98 ex vivo. Each value represents the mean and standard deviation ($n = 8$). Means without a common letter were significantly different ($p < 0.01$).

Mutagenicity Test ex Vivo. The effect of CLE on the mutagenic action of Trp-P-2 was evaluated using a host-mediated assay. The number of bacteria recovered from the liver varied from 2.9×10^7 to 1.1×10^8 cfu/mL. As shown in Figure 4, the control group treated with Trp-P-2 showed a markedly increased number of revertant colonies compared with the no treatment group. The CLE pretreatment group showed a significantly decreased number of revertant colonies compared with the control group.

DISCUSSION

The effects of cacao liquor polyphenols on the mutagenic action of HCA were evaluated.

HCAs are mutagens isolated from cooked foods and are classified into two groups, the IQ [2-amino-3-methylimidazo[4,5-*f*]quinoline] type and the non-IQ type (Wakabayashi et al., 1993). In this study, we selected one typical HCA from each group, MeIQ and Trp-P-2. Metabolic activation is required for these compounds to induce mutations in *S. typhimurium* TA98. The amino moiety of these HCA is *N*-hydroxylated by cytochrome P-450, and the resulting N-hydroxyarylamines are further activated by enzymatic esterification, O-acylation, O-acetylation, O-sulfonylation, and O-phosphorylation, resulting in compounds capable of forming covalent bonds with DNA and proteins (Kato and Yamazoe, 1987).

In the present study, the antimutagenicity of cacao liquor polyphenols and their identified components was examined in bacteria treated with HCA in the presence/absence of the S-9 mixture. In terms of efficacy, these compounds could be ranked in the following order: quercetin > CLT > CLP ≫ EC = CA (Figure 2). The results obtained for three flavans, EC, CA, and quercetin, confirmed the findings in a previous report (Edenharder et al., 1993).

It is known that the antimutagenic mechanism of action of quercetin involves suppression of the metabolic activation of HCA in the liver (Alldrick et al., 1986). We evaluated its antimutagenic effect using an activated form of HCA, and quercetin showed slight inhibition of revertant colony formation. In contrast, cacao liquor polyphenol fractions were shown to have potent antimutagenic effects by this method (Figure 3). It was suggested that the antimutagenic mechanism of action of quercetin differs from that of cacao liquor polyphenols. We investigated the effects of these chemicals on the metabolic activation of HCA. Quercetin showed a potent inhibitory effect on enzymatic metabolism of HCA as determined by HPLC analysis. Compared to CLT and CLP, its efficacy was about 25 and 96 times stronger, respectively (Table 2). These results suggest that the antimutagenic mechanism of action of cacao liquor polyphenols involves not only the suppression of HCA activation. CLP and CLT each contain various types of polyphenols. It has been reported that EC, CA, and their conjugated forms such as procyanidin are the main components (Sanbongi et al., 1998; Osakabe et al., 1998; Hammerstone et al., 1999). Procyanidins are classified as condensed tannins, and condensed tannins are a major component of CLT as shown in Table 1.

Okuda et al. reported that the tannin geraniin showed a marked inhibitory effect on the mutagenic action of Trp-P-2 as compared with its components, corilagin and elladic acid (Okuda et al., 1984). They suggested that the difference in efficacy may be related to their affinity for the mutagen. In general, tannins have a strong affinity for proteins, as indicated by the reactive affinity for methylene blue and the relative astringency values observed. The reactive affinity for methylene blue and the relative astringency of tannins are considered to be correlated with the efficacy in suppression of mutagenesis in the case of heterocyclic amines. In this study, CLP showed less activity than CLT. The difference between them is thought to be a reflection of the content of condensed tannins, such as procyanidins. We conclude that one of the mechanisms of action of cacao liquor polyphenols in suppressing the mutagenic action of HCA might involve adsorption of HCA by certain components, especially procyanidins.

In addition, the effect of cacao liquor polyphenols on the mutagenic action of Trp-P-2 was evaluated using a host-mediated assay. The host-mediated assay has been extensively used in evaluation of antimutagenic agents ex vivo (Allderick et al., 1989, 1995). It has been reported that Trp-P-2 is absorbed, distributed to the liver, and metabolically activated by cytochrome P-450. The activated Trp-P-2 induces mutations in bacteria present in this organ. In this study, the number of revertant colonies of bacteria recovered from the liver increased in the control group upon treatment with Trp-P-2. In contrast, CLE treatment resulted in suppression of this change (Figure 4). These findings suggest several possibilities: (1) cacao liquor polyphenols inhibit the metabolic activation of Trp-P-2 in the liver; (2) cacao liquor polyphenols have a direct effect on activated Trp-P-2, diminishing its mutagenicity; (3) cacao liquor polyphenols induce an anticarcinogenic enzyme; (4) cacao liquor polyphenols inhibit the absorption of orally administered Trp-P-2 in the gastrointestinal tract.

In conclusion, we have confirmed the antimutagenic activity of polyphenols derived from cacao liquor as demonstrated by tests examining their effect on the mutagenic action of HCA in vitro and ex vivo. Their antimutagenic mechanism of action remains obscure; however, the results of this study suggest that the mechanism involves not only inhibition of HCA activation. To fully define its mechanism of action, further studies are required.

ABBREVIATIONS USED

HCAs, heterocyclic amines; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; MeIQ, 2-amino-3,4-dimethyl-3*H*-imidazo[4,5-*f*]quinoline; EC, epicatechin; CA, catechin; CLE, cacao liquor extract; CLP, cacao liquor crude polyphenols; CLT, cacao liquor tannin.

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