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Characterization of Corrinoid Compounds from a Japanese Black Tea (Batabata-cha) Fermented by Bacteria

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A Japanese fermented black tea (Batabata-cha) contained a considerable amount of vitamin B_{12} (456 ± 39 ng per 100 g dry tea leaves and 2.0 ± 0.3 ng per 100 mL of tea drink). A corrinoid compound was partially purified and characterized from the tea leaves. The patterns of the purified compound by the silica gel 60 thin-layer chromatography and C18 reversed phased high-performance liquid chromatography were identical to those of authentic vitamin B_{12}. When 20 week old vitamin B_{12} deficient rats, which excreted substantial amounts (about 250 mg/day) of methylmalonic acid in urine as an index of vitamin B_{12} deficiency, were fed the tea drink (50 mL/day, 1 ng of vitamin B_{12}) for 6 weeks, urinary methylmalonic acid excretion (169 ± 29 mg/day) of the tea drink-supplemented 26 week old rats decreased significantly relative to that (250 ± 32 mg/day) of the deficient rats. The results indicate that the vitamin B_{12} found in the fermented black tea is bioavailable in mammals.

KEYWORDS: Vitamin B_{12}; cobalamin; corrinoid; tea; vitamin B_{12} deficient rat; methylmalonic acid; hepatic vitamin B_{12}

INTRODUCTION

In manufacturing processes of black teas fermented by bacteria, such as Pu'erh tea, tea leaves are heat-treated with steam or roasting and then fermented with certain naturally occurring bacteria (*1*). Thus, they are completely different from the types of self-oxidized black teas (Keemun tea and Darjeeling tea, etc.). These black teas fermented by bacteria, which are found in some Asian countries, may contain various vitamins and/or biofactors synthesized by the concomitant bacteria.

Vitamin B_{12} (B_{12}) is synthesized only in certain bacteria (*2*). Usual dietary sources of B_{12} are known to be animal products but not plant products (*3*). If the fermented black teas contain considerable amounts of B_{12}, the black tea would contribute to human B_{12} needs, especially for vegetarians.

Here, we describe the partial purification and characterization of a corrinoid compound from Japanese fermented black tea (Batabata-cha) leaves and also investigated the effect of feeding the tea drink on the B_{12} status of B_{12} deficient rats.

MATERIALS AND METHODS

Materials. Cyano-B_{12} was obtained from Wako Pure Chemical Industries (Osaka, Japan). A B_{12} assay medium for *Lactobacillus delbrueckii* subsp. *lactis* (formerly *Lactobacillus leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 thin-layer chromatography (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). All other reagents used were of the highest purity commercially available. Japanese fermented black tea (Batabata-cha) leaves and the tea drink were provided by Asahi, Ltd. (Toyama-city, Japan).

A UV-1600 UV−vis spectrophotometer (Shimadzu, Kyoto, Japan) was used for measuring the turbidity of the *L. delbrueckii* test culture in the microbiological method. A fully automated ACS 180 chemiluminescence B_{12} analyzer (Chiron Diagnostics, East Walpole, MA) was used for the B_{12} assay.

Extraction of Corrinoid Compounds for the Determination of B_{12} Content of the Tea Leaves. The dried tea leaves (5 g) were powdered by a food mill and then suspended in 50 mL of 0.25 mol/L acetate buffer, pH 4.8, containing 0.2% (w/v) KCN as cyanation for stabilization. The total corrinoids were extracted from the suspension by boiling for 60 min at 98 °C in the dark. The suspension was centrifuged for 10 min at 5000*g*, and the supernatant was used for the B_{12} assay.

Concentration of the Fermented Black Tea Drink. For the determination of B_{12} in the tea drink, B_{12} was concentrated with a Sep-Pak Vacc 20 cm^3 (5 g) C18 cartridge (Waters Corp., Milford, MA). After the C18 cartridge was washed with 75% ethanol and equilibrated with distilled water, an aliquot (50 mL) of the drink was put on the cartridge. B_{12} was eluted with 50 mL of 25% ethanol, and the eluate was evaporated to dryness under reduced pressure, dissolved in 1.0 mL of distilled water, and used for the B_{12} assay.

Assay of B_{12}. B_{12} was assayed by the microbiological method with *L. delbrueckii* subsp. *lactis* ATCC 7830 and a B_{12} assay medium (Nissui) and by the chemiluminescence B_{12} analyzer with intrinsic factor (IF) as described previously (*4*). The above B_{12} extract and the concentrated drink were directly applied to the chemiluminescence

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analyzer. They were diluted with distilled water to a B_{12} concentration range of 0.01−0.1 μg/L and used as samples for the microbiological method.

Purification of Corrinoid Compounds from the Fermented Black Tea. About 0.5 kg of the dried tea leaves was powdered by a model MX-X51-H food mill (National, Osaka, Japan), and suspended in 2 L of 0.25 mol/L acetate buffer, pH 4.8. KCN was added to the suspension at the final concentration of 10 mmol/L. Total B_{12} was extracted from the suspension under the same conditions described above. The boiled suspension was centrifuged at 10 000*g* for 10 min. The supernatant was used for purification of corrinoid compounds. Amberlite XAD-4 resin (500 g) washed with 5 L of methanol and equilibrated with distilled water was added to the supernatant fraction and stirred for 3 h at room temperature in the dark. The resin suspension was passed through a glass funnel (Buchner type) with a type 25G1 glass filter (Iwaki, Tokyo, Japan), and the resin was washed with 5 L of distilled water. One liter of 80% methanol solution was added to the washed resin, and the suspension was stirred for 3 h at room temperature in the dark. The resin suspension was passed through the glass funnel. The 80% methanol eluant (about 1 L) containing corrinoid compounds was pooled and evaporated to a final volume of 20 mL under reduced pressure. The solution was put on a 24 mm × 70 mm column of Cosmosil 140C18-OPN (Nacalai Tesque, Kyoto, Japan), which was washed with 75% ethanol solution and equilibrated with distilled water. A corrinoid compound was eluted with 100 mL of a linear gradient (0−25%) of ethanol. The B_{12} active fractions assayed by the microbiological method were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of 70% 2-propanol solution containing 2.8% NH_4OH. The solution was put on a silica gel 60 TLC sheet and developed with 2-propanol/28% NH_4OH/water (7:1:2) as a solvent in the dark at room temperature. The dried TLC sheet was cut into small pieces (0.5 cm × 1.0 cm) with scissors. Corrinoid was extracted from the pieces in 70% 2-propanol solution containing 2.8% NH_4OH several times, and the extract was evaporated to dryness under reduced pressure, dissolved in 1.0 mL of distilled water, and used as samples for the microbiological B_{12} assay. The B_{12} active fractions assayed by the microbiological method were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was further purified by high-performance liquid chromatography (HPLC) using a Shimadzu HPLC apparatus consisting of a LC-6A pump, SPD-6A spectrophotometer, CTO-6A column oven, and C-R6A Chromatopac. The sample (100 μL) was put on a 150 mm × 4.6 mm i.d., 5 μm, Wakosil-II 5C18RS reversed phase HPLC column equilibrated with 20% methanol solution containing 1% acetic acid at 35 °C. The flow rate was 1 mL/min. The corrinoid compound was isocratically eluted with the same solution, monitored by measuring absorbance at 278 nm, and collected in 1 mL fractions. The B_{12} active fractions assayed by the microbiological method were collected, concentrated, and used as a purified corrinoid compound.

Analytical TLC and HPLC of Corrinoid Compound Purified from the Black Tea. The concentrated solution (10 μL) of corrinoid compound purified from the tea leaves and authentic cyano-B_{12} was spotted on the silica gel 60 TLC sheets and developed with solvents I [2-propanol/NH_4OH (28%)/water (7:1:2)] and II [1-butanol/2-propanol/water (10:7:10)] in the dark at room temperature. The TLC sheets were dried, and R_f values of the pink-colored spot of cyano-B_{12} were determined.

The TLC sheets were also cut into small pieces (0.5 cm × 1.0 cm) with scissors. Corrinoids were extracted from the pieces in 80% (v/v) methanol several times, evaporated to dryness under reduced pressure, dissolved in 1.0 mL of distilled water, and used as samples for the microbiological B_{12} assay.

In the case of HPLC, the concentrated solutions (10 μL) of the purified corrinoid compound and authentic cyano-B_{12} were analyzed with the same reversed phase HPLC column as used for purification. The corrinoids were isocratically eluted with 20% methanol solution containing 1% acetic acid at 35 °C and monitored by measuring absorbance at 278 nm. The retention times of corrinoids were determined at a flow rate of 1 mL/min. The eluate from the HPLC column was collected, evaporated to dryness, dissolved in 1.0 mL of distilled water, and used as samples for the microbiological B_{12} assay.

Animals and Experimental Diets. Fifteen male Wister rats (20 weeks old), born to 14 week old parents fed on a B_{12} deficient diet for 8 weeks, were used. The B_{12} deficient diet fed to the parents contained (g/kg diet): 400 soyabean protein (Fuji Oil Ltd, Osaka, Japan), 438 anhydrous glucose (Nacalai Tesque Ltd., Kyoto, Japan), 100 soyabean oil (Nacalai Tesque Ltd.), 50 salt mixture, 5 dl-methionine (Nacalai Tesque Ltd.), and 5 B_{12}-free vitamin mixture and 2 choline chloride (Nacalai Tesque Ltd.), as described previously (*5*). The 3 week old weanling rats were housed in individual metabolism cages at 24 °C in a room with a 12 h light−dark cycle. They were given free access to 16 g/day of the B_{12} deficient diet and distilled water for 17 weeks. In the feeding experiments, the 20 week old B_{12} deficient rats (four rats/group) were given free access to 16 g/day of the B_{12} deficient diet and 50 mL of either distilled water, authentic cyano-B_{12} solution (1 ng of B_{12} per 50 mL), or the tea drink (1 ng of B_{12} per 50 mL) for 6 weeks. All experimental procedures involving laboratory animals were approved by the Animal Care and Use Committee of Osaka Prefecture University.

Urinary Methylmalonic Acid Assay. The urine of the B_{12} deficient, cyano-B_{12}-supplemented, and tea drink-supplemented rats was sampled for 24 h in individual methabolic cages at weeks 0, 1, 2, and 6 during the experiments. Urinary methylmalonic acid was assayed by HPLC as described previously (*6*).

Extraction of B_{12} from Rat Liver. After food was withheld from the 26 week old rats overnight, the rats were killed by decapitation under diethyl ether anesthesia. Livers were washed with a chilled 9 g/L NaCl solution, weighed, and stored at −80 °C until analyzed. A portion (1 g) of the liver was cut into small pieces using a razor blade and homogenized in 10 times its volume of 10 mmol/L acetate buffer, pH 4.8. B_{12} was extracted from the liver homogenate by boiling with KCN at acidic pH as described above and assayed by the microbiological assay.

Statistics. Statistical analysis was performed using GB-STAT5.4 (Dynamic Microsystems, Inc., Silver Spring, MD). One way and two way repeated measure analysis of variance (ANOVA) were used for assay of hepatic B_{12} and urinary methylmalonic acid in the animal feeding test, respectively. When ANOVA results were significant, a posthoc two-tailed Student's *t*-test also was performed and considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

B_{12} Content Determination. The B_{12} contents were determined by two methods, the IF-chemiluminescence and the microbiological methods. The B_{12} contents of the dry tea leaves were 456 ± 39 ng and 368 ± 56 ng per 100 g dry weight by the IF-chemiluminescence method and the microbiological method, respectively. In the case of B_{12}, contents of the tea drink used for the feeding experiments were 2.0 ± 0.3 ng and 2.0 ± 0.8 ng per 100 mL by those two methods, respectively. Although B_{12} contents were considerably lower in the black tea drink than in cow's milk (0.3 μg/100 g) (*7*), which greatly contributes to the B_{12} intake of U.S. adult women (*8*), this is the first report on the occurrence of B_{12} in tea leaves and their drinks.

Characterization of the Corrinoid Compound from the Black Tea. A corrinoid compound was purified from the tea leaves. The purified corrinoid compound and authentic cyano-B_{12} were analyzed by TLC and HPLC. The R_f values for the purified compound were 0.64 and 0.22 on silica gel 60 TLC in solvents I and II, respectively. These values were identical to those for authentic cyano-B_{12}. The retention time of authentic B_{12} by reversed phase HPLC was 9.4 min; it was also identical to that of the purified compound. These results strongly suggest that the compound purified from the fermented tea leaves is true B_{12} but not corrinoid compounds inactive for humans. UV−vis spectroscopy and NMR spectroscopy could not be determined because a substantial amount of the purified compound was not obtained.

Feeding Test of the Tea Drink with the B_{12} Status of B_{12} Deficient Rats. To evaluate whether the B_{12} found in the tea

Table 1. Effects of Feeding the Fermented Black Tea Drink on Urinary Methylmalonic Acid of B_{12} Deficient Rats[a]

	urinary methylmalonic acid (mg/day)		
groups	control	CN-B_{12} supplement	tea drink supplement
week 0	246 ± 51^{a}	253 ± 41^{a}	251 ± 35^{a}
week 1	246 ± 32^{a}	200 ± 25^{a}	181 ± 66^{a}
week 2	251 ± 104^{a}	218 ± 35^{a}	151 ± 50^{b}
week 6	250 ± 32^{a}	201 ± 28^{a}	169 ± 29^{b}

[a] The 20 week old B_{12} deficient rats (four rats/group) were given free access to 50 mL of either distilled water, the cyano-B_{12} solution (1 ng/50 mL), or the tea drink (1 ng of B_{12}/50 mL) per day. [ab]The mean values with different superscript letters are significantly different; $P < 0.05$.

Table 2. Hepatic B_{12} Contents of the 26 Week Old B_{12} Deficient Rats Fed the CN-B_{12} Solution and Tea Drink[a]

groups	B_{12} contents (pg/g wet tissue)
control	746 ± 97^{a}
CN-B_{12} supplement	768 ± 129^{a}
tea drink supplement	1473 ± 252^{b}

[a] The 20 week old B_{12} deficient rats (four rats/group) were given free access to 50 mL of either distilled water, the cyano-B_{12} solution (1 ng/50 mL), or the tea drink (1 ng of B_{12}/50 mL) per day. [ab]The mean values within a column with different superscript letters are significantly different; $P < 0.01$.

leaves is absorbed in the mammalian intestine and accumulated in the liver, feeding experiments of the tea drink to 20 week old B_{12} deficient rats were conducted. There was no significant difference in the intakes of the diet and drink (water, cyano-B_{12} solution, or the tea drink) among the rat groups during the experimental time course. When the 20 week old B_{12} deficient rats, which excreted substantial amounts of methylmalonic acid (about 250 mg/day) in urine (as an index of B_{12} deficiency), were given the tea drink (1 ng of B_{12} per day) for 6 weeks, urinary methylmalonic acid excretion of the tea drink-supplemented 26 week old rats decreased significantly relative to the B_{12} deficient (control) rats, but that of the cyano-B_{12}-supplemented rats did not (**Table 1**).

Although the rate of growth (61.6 ± 18.2 g) of the B_{12} deficient rats given the tea drink had a tendency to be greater than that of the control (28.2 ± 7.4 g) and cyano-B_{12}-supplemented (20.6 ± 15.8 g) rats during the experiment, there was no significant difference in body weight among the rats fed the three experimental drinks after 6 weeks.

The hepatic B_{12} contents were about 2-fold greater in the tea drink-supplemented rats than in both control and cyano-B_{12}-supplemented rats (**Table 2**); there was no significant difference in the hepatic B_{12} contents between control and cyano-B_{12}-supplemented rats. Although the methylmalonic aciduria of the B_{12} deficient rats could not be completely recovered by the 6 week feeding of the tea drink, the significant increase in the hepatic B_{12} content of the tea drink-supplemented rats indicated that the feeding of the tea drink considerably improved B_{12} statue in the B_{12} deficient rats.

Our previous study (*9*) has indicated that urinary levels of methylmalonic acid became undetectable in the B_{12} deficinet rats fed a cyano-B_{12} (about 100 ng/day)-supplemented diet for 10 days. In this study, however, the cyano-B_{12} (1 ng/day)-supplemented 26 week old rats did not show both significant recovery of methylmalonic aciduria and increase in hepatic B_{12} content. Even the 26 week old B_{12} deficient rats given the tea drink did not completely recover from methymalonic aciduria. The results may be due to a lesser B_{12} content (1 ng/day) of the administered authentic B_{12} and tea drink. We did not use any concentrated or purified compound from the tea leaves because of evaluation for bioavailability of B_{12} found in the tea drink commercially available for humans.

Our preliminary experiments indicated that considerable amounts of the coenzyme B_{12} (adenosylcobaramin and methylcobalamin) were found in the tea leaves and that the B_{12} found in the tea drink existed as a free form (without binding to a macromolecular compound). These results suggest that the B_{12} found in the tea drink would be assimilated more easily in the B_{12} deficinet rats than authentic cyano-B_{12}.

The results presented here indicate that the B_{12} found in the Japanese black tea (Batabata-cha) fermented by bacteria is bioavailable in mammals. Although only 1−2 L of the fermented tea drink (20−40 ng of B_{12}) is not sufficient to satisfy the recommended dietary allowance (2.4 μg/day) for human adults, intakes of various B_{12}-containing plant foods [purple and green lavers (*10*), Chlorella tablets (*11*), and the fermented tea extract] would contribute to prevention of B_{12} deficiency for vegetarians.

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