Characterization and bioavailability of Vitamin B\textsubscript{12} compound from a Japanese piled-black tea, Batabata-Cha.

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Summary

A Japanese piled-black tea, Batabata-cha, contained considerable amounts of vitamin B\textsubscript{12} (B\textsubscript{12}) [1.2 \textmu g/100 g dry tea leaves, 2 ng/100 mL tea leaf extract (used as a tea beverage)]. A B\textsubscript{12} compound was partially purified from the tea leaves and characterized. The silica gel 60 TLC and reversed-phase HPLC patterns of the partially purified B\textsubscript{12} compound were identical to those of authentic CN-B\textsubscript{12}, but not those of B\textsubscript{12} analogues inactive for humans.

When 20-week-old B\textsubscript{12}-deficient rats which excreted substantial amounts of methylmalonic acid (250 mg/day) in urine (as an index of B\textsubscript{12} deficiency) were fed the tea leaf extract (50 mL/day; 1 ng of B\textsubscript{12}) for 6 weeks, urinary methylmalonic acid excretion of the tea leaf extract-supplemented rats decreased significantly relative to authentic CN-B\textsubscript{12}-supplemented rats. The hepatic B\textsubscript{12} content was about twofold greater in the tea leaf extract-supplemented rats than in the CN-B\textsubscript{12}-supplemented rats. The results indicate that the Japanese piled-black tea, Batabata-cha, contains considerable amounts of B\textsubscript{12} bioavailable in mammals.

Keywords

piled-black tea, batabata-cha, vitamin B\textsubscript{12}, vitamin B\textsubscript{12}-deficiency, urinary methylmalonate excretion

Introduction

Piled-black teas have been heat-treated by steam or roast, piled, and fermented by certain bacteria. Various piled-black teas are found only in Asian countries. The piled-black teas may contain some vitamins and/or biofactors synthesized by the concomitant bacteria.

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

Here we described the partially purification and characterization of a B\textsubscript{12} compound from a Japanese piled-black tea, Batabata-cha, and also investigated the effects on B\textsubscript{12} status of feeding the black tea extract used as a tea beverage to B\textsubscript{12}-deficient rats.

Materials and Methods

Extraction of B\textsubscript{12}. The dried tea leaves and extract (used as a tea beverage) of Batabata-cha were kindly provided by Asahi co. (Toyama-prefecture, Japan). The tea leaves were powdered by the use of a food mill. Total B\textsubscript{12} was extracted from the tea
leave powder and extract by boiling with KCN at acidic pH as described previously.

**Assay of B$_{12}$.** Total B$_{12}$ was assayed by the microbiological method with *Lactobacillus leichmannii* ATCC7830 and a B$_{12}$ assay medium (Nissui, Tokyo, Japan) according to the manufacturer's instructions as described previously.

**Partially Purification of a B$_{12}$ compound from the tea leaves.** About 400 g of Batabata-cha leaves were powdered by the use of the food mill and added to 4 L of 0.25 mol/L acetate buffer, pH 4.8, containing 0.2% (w/v) KCN. The suspension was boiled for 60 min and then centrifuged for 10 min at 5000 r.p.m. The supernatant was used as a B$_{12}$ extract for the following experiment. About 1 kg of Amberlite XAD-4 (Organo Co. Tokyo, Japan) resin, after had washed with 10 L of methanol and then equilibrated with distilled water, was added to the B$_{12}$ extract and stirred for 3 h at room temperature in the dark. A B$_{12}$ compound was eluted with 1 L of 80% (w/v) methanol and concentrated under reduced pressure. The concentrated solution was put on a column (28×70mm) of COSMOSIL 140C18-OPN, which was washed with ethanol and then equilibrated with distilled water. A B$_{12}$ compound was eluted with 100 mL of a linear gradient of 0-25%(v/v) ethanol. The B$_{12}$-active fractions were pooled, concentrated under reduced pressure, and used as a partially purified B$_{12}$ compound.

**Analytical TLC and HPLC.** The partially purified B$_{12}$ compound and authentic CN-B$_{12}$ was put on silica gel-60 TLC sheets, which were developed with solvent I; 2-propanol/NH$_4$OH (28%)/distilled water (7:1:2 v/v), or with solvent II; 1-butanol/2-propanol/distilled water (10:7:10 v/v). The TLC sheets were dried and cut into small pieces (5 mm) by scissors. The B$_{12}$ compounds were extracted from each TLC piece with 80% (v/v) methanol, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. B$_{12}$ was assayed in these fractions by the microbiological method.

The partially purified B$_{12}$ compound was also analyzed by HPLC using a Shimadzu HPLC apparatus (CLC-6A pump, SPD-6A spectrophotometer, CTO-6A column oven, C-R6A chromatopac). The partially purified B$_{12}$ compound and authentic CN-B$_{12}$ were put on a reversed-phase HPLC column (Wakosil- II 5C18RS, φ 4.6×150mm; particle size = 5 μm) equilibrated with a 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 35°C. The flow rate was 1 mL/min. The B$_{12}$ compounds were isocratically eluted and collected at 1 mL. These fractions were evaporated to dryness and dissolved in a small amount of distilled water. B$_{12}$ was assayed in these fractions by the microbiological method.

**Animals and diets.** Fifteen male weanling Wister rats (20-week-old), born to 14-week-old parents fed on a B$_{12}$-deficient diet for 8 weeks, were used. The B$_{12}$-deficient diet contained (g/kg): 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), 5 B$_{12}$-free vitamin mixture and 2 choline chloride (Nacalai Tesque Ltd), as described previously. The 3-week-old weanling rats were housed individual metabolism cages at 24°C in a room with a 12 h light-dark cycle. They were given free access to the B$_{12}$-deficient diet and tap water for 17 weeks. All experimental procedures involving laboratory animals were approved by the Animal Care and Use Committee of Osaka Prefecture University.

**Feeding the tea leave extract in B$_{12}$-deficient rats.** The effects of feeding the tea leave extract on urinary methylmalonic acid level in the B$_{12}$-deficient rats were studied.
The 20-week-old B$_{12}$-deficient rats (4 rats/group) were given free access to 50 mL of water, authentic CN-B$_{12}$ (1 ng/50 mL) solution, and tea leaf extract (1 ng of B$_{12}$/50 mL).

**Urinary methylmalonic acid assay.** The urine of the B$_{12}$-deficient, CN-B$_{12}$-supplemented, and tea leaf extract-supplemented rats was sampled for 24 h in individual metabolism cages at day 0, 1, 3, 7, 12, 15, 42 during the experiments. Urinary methylmalonic acid was assayed by HPLC as described previously.$^{3}$

**Extraction of B$_{12}$ from rat liver.** After food was withheld from rats overnight, the rats were killed by decapitation under diethyl ether anesthesia. Liver were washed with a chilled 9 g NaCl/L 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 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.$^{4}$

**Results and Discussion**

A Japanese piled-black tea, Batabata-cha, is available at Toyama-prefecture, Japan. Batabata-cha contained 1.2 $\mu$g of B$_{12}$ per 100 g dry tea leaves and 2 ng of B$_{12}$ per 100 mL tea extract used as a tea beverage.

To clarify whether the B$_{12}$ found in the tea leaves is true B$_{12}$ or inactive B$_{12}$ analogues, a B$_{12}$ compound was partially purified from the tea leaves and characterized. The $R_f$ values (0.22 and 0.54 in solvent I and II, respectively, on silica gel 60 TLC) of B$_{12}$ activity of the partially purified compound were identical to the values of that of authentic CN-B$_{12}$ (Fig. 1), of which the retention time (17.5 min by the reversed-phase HPLC) was also identical to that of the partially purified compound (Fig. 2). These results strongly suggest that the B$_{12}$ compound partially purified from the tea leaves is true B$_{12}$, but not B$_{12}$ analogues inactive for humans.

To evaluate whether the B$_{12}$ compound found in the tea leaves is absorbed in the mammalian intestine and accumulated in the liver, feeding experiments of the tea leaf extract to the 20-week-old B$_{12}$-deficient rats were conducted. When the 20-week-old B$_{12}$-deficient rats which excreted substantial amounts of methylmalonic acid (250 mg/day) in urine (as an index of B$_{12}$ deficiency) were given the tea leaf extract (50 ml/day, 1 ng of B$_{12}$) for 6 weeks, urinary methylmalonic acid excretion of the tea leaf extract-supplemented rats decreased significantly relative to both B$_{12}$-deficient (control)}
and CN-B₁₂-supplemented rats (Fig. 3).

The hepatic B₁₂ content was about twofold greater in the tea leaf extract-supplemented rats than in both control and CN-B₁₂-supplemented rats (Table 1); there is no significant difference in the hepatic B₁₂ contents between control and CN-B₁₂-supplemented rats. The fact that the B₁₂ compound found in the tea leaf extract showed a better bioavailability in the B₁₂-deficient rats relative to authentic CN-B₁₂ may imply that most of B₁₂ found in the tea leaves are existed as B₁₂ coenzymes.

![Fig. 3 Effect of feeding the Batabata-cha extract on urinary MMA (an index of vitamin B₁₂ deficiency) of vitamin B₁₂-deficient rats.](image)

**Table 1** Hepatic vitamin B₁₂ contents of the vitamin B₁₂-deficient rats fed the CN-B₁₂ and Batabata-cha extract

<table>
<thead>
<tr>
<th>Groups</th>
<th>vitamin B₁₂ contents (pg/g wet tissue)</th>
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<tbody>
<tr>
<td>Control</td>
<td>746±97</td>
</tr>
<tr>
<td>CN-B₁₂-supplemented</td>
<td>768±129 *</td>
</tr>
<tr>
<td>Batabata-cha extract</td>
<td>1473±252 *</td>
</tr>
<tr>
<td>Batabata-cha extract</td>
<td>1473±252 *</td>
</tr>
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*The mean values within a column are significantly different, P< 0.01.

Although the methylmalonic acidemia in the B₁₂-deficient rats could not be completely recovered by the 6-week-feeding of the tea leaf extract, the significant increase in the hepatic B₁₂ content of the tea leaf extract-supplemented rats indicate that the feeding of the tea leaf extract considerably improved B₁₂ status in the B₁₂-deficient rats.

These results presented here indicate that the Japanese piled-black tea, Batabata-cha, contains considerable amounts of B₁₂ bioavailable in mammals.

**References**