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Note

Characterization of a Corrinoid Compound in the Edible (Blue-Green) Alga, Suizenji-nori

Fumio WATANABE,[1,†] Emi MIYAMOTO,[2] Tomoyuki FUJITA,[3]
Yuri TANIOKA,[4] and Yoshihisa NAKANO[4]

[1]*School of Agricultural, Biological and Environmental Sciences, Tottori University, Tottori 680-8553, Japan*
[2]*Department of Health and Nutrition, Nagasaki International University, Sasebo 859-3298, Japan*
[3]*Graduate School of Agriculture, Shinshu University, Kami-ina 399-4598, Japan*
[4]*Graduate School of Life and Environmental Science, Osaka Prefecture University, Sakai 599-8531, Japan*

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The edible blue-green alga (cyanobacterium), Suizenji-nori, contained 143.8 ± 22.4 µg of vitamin B_{12} per 100 g dry weight of the alga (mean ± SE, $n = 4$). A corrinoid compound was purified from the dried Suizenji-nori, and partially characterized. The silica gel 60 TLC and reversed-phase HPLC patterns of the purified corrinoid compound were not identical to those of true vitamin B_{12}, but to those of pseudovitamin B_{12} which is inactive for humans.

Key words: *Aphanothece sacrum*; blue-green alga; pseudovitamin B_{12}; Suizenji-nori; vitamin B_{12}

The usual dietary sources of vitamin B_{12} (B_{12}) are animal food products (meat, milk, eggs and shellfish), but not plant food products.[1] Substantial amounts of B_{12}, however, can be found in various edible algae.[2,3] Our previous studies have demonstrated that true B_{12} was the predominant cobamide of some eukaryotic algae,[4–7] although pseudo-B_{12}, an inactive corrinoid for humans, predominates in the prokaryotic, blue-green alga (cyanobacterium), *spirulina*.[8] It is still unclear whether other edible blue-green algae contain true B_{12} or an inactive corrinoid compound.

Suizenji-nori (*Aphanothece sacrum*) is an edible blue-green alga indigenous to Japan. The dried alga is used as an ordinary food item after being soaked in water as well as a nutritional supplementary food. The nutrition labeling of the algal product shows that the dried algal cells contain substantial amounts of B_{12}. In this study, we purified and characterized a corrinoid compound from suizenji-nori and demonstrate the bioavailability of the algal corrinoids in humans.

After 2 g of the dried suizenji-nori had been suspended in 40 ml of distilled water and homogenized with a UD-200 ultrasonic disruptor (Tomy, Tokyo, Japan), total B_{12} was extracted from the suspension while boiling in the acidic pH range and assayed by the microbiological method with *Lactobacillus delbrueckii* ATCC 7830 as described in the Japanese Standard Tables of Food Composition.[9]

About 600 g of the dried suizenji-nori was added to 40 liters of a 50 mM acetate buffer at pH 4.8. Total B_{12} was extracted from the suspension by boiling with KCN in the acidic pH range;[9] KCN was added to the suspension to a final concentration of 10 mM. The suspension was boiled for 30 min at 98 °C in a draught chamber. The boiled suspension was left for several hours up to 30 °C, and then centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant fraction (about 20 liters) was put into a column (7 × 100 cm) of Amberlite XAD-4 resin (Japan Organo Co., Tokyo, Japan), which had been washed with 10 liters of methanol and then equilibrated with distilled water, at room temperature in the dark. The column was washed with 10 liters of distilled water, and eluted with 10 liters of 80% (v/v) ethanol. The eluate was pooled, evaporated to dryness under reduced pressure, and dissolved in 100 ml of distilled water. The solution was loaded into a column (2.4 × 30 cm) of Cosmosil 140C18-OPN (Nacalai Tesque, Kyoto, Japan), which had been washed with a 75% (v/v) ethanol solution and then equilibrated with distilled water, and eluted with 400 ml of a linear gradient (0–30% v/v) of ethanol. The B_{12}-active fractions were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The solution was further purified by the Cosmosil column (2.4 × 15 cm) chromatography under the same conditions, except for eluting with 200 ml of a linear gradient (0–25% v/v) of ethanol. The B_{12}-active fractions were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was put on to a silica gel 60 TLC sheet (Merck, Darmstadt, Germany) and developed with 2-propanol/

† To whom correspondence should be addressed. Tel/Fax: +81-857-31-5412; E-mail: watanabe@muses.tottori-u.ac.jp

NH_4OH (28%)/water (7:1:2 v/v) as a solvent in the dark at room temperature. The red-colored spot on the TLC sheet was dried, collected, extracted with an 80% (v/v) methanol solution, evaporated to dryness under reduced pressure, and dissolved in 50 µl of distilled water. The solution was further purified by TLC under the same conditions. The concentrated solution was purified by HPLC with Shimadzu (Kyoto, Japan) apparatus (two LC-10ADvp pumps, a DGV-12A degasser, SCL-10Avp system controller, SPD-10Avvp ultraviolet-visible detector, CTO-10Avp column oven, 100-µl sample loop, and C-R6A chromatopac integrator). The sample (50 µl) was loaded into a reversed-phase HPLC column (Wakosil-II 5C18RS, ϕ4.6 × 150 mm; 5-µm particle size; Wako Pure Chemical Industries, Osaka, Japan) which had been equilibrated with a 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40 °C. The flow rate was 1 ml/min. The corrinoid compound was isocratically eluted with the same solution, monitored by measuring the absorbance at 361 nm, and collected in 1-ml fractions. The final red-colored fraction was collected, evaporated to dryness under reduced pressure, dissolved in 20 µl of distilled water, and used as the purified corrinoid compound.

The B_{12} concentration in the edible (blue-green) alga, suizenji-nori, was determined by the microbiological method. The dried alga contained 143.8 ± 22.4 µg of B_{12} per 100 g dry weight of the alga (mean ± SE, n = 4). A similar B_{12} concentration (94 µg) has been shown in the nutrition labeling of the algal product.

To evaluate whether the B_{12} activity detected in the suizenji-nori extract was derived from true B_{12} nor not, a corrinoid compound was purified and partially characterized. The final purified preparation gave a single red-colored spot by silica gel 60 TLC and a single peak by reversed-phase HPLC, indicating that the corrinoid compound had been purified to homogeneity.

The ultraviolet-visible spectrum of the compound purified from the suizenji-nori showed a typical absorption of cobalt-containing corrinoid (Fig. 1); λ_{max} nm (absorbance) was at 548.0 (0.350), 518.0 (0.334), and 360.0 (1.366). The purified corrinoid compound, authentic B_{12}, and cyanocobamides (pseudo-B_{12} and 5-hydroxybenzimidazolyl, and benzimidazolyl cyanocobamides; all kindly provided by Dr. E. Stupperich, Ulm University, Germany) were compared by silica gel 60 TLC and reversed-phase HPLC (Table 1). The R_f values (0.14 and 0.48 in solvents I and II, respectively, by TLC) for the purified compound were identical to those for authentic pseudo-B_{12}, whose retention time (6.7 min by HPLC) was also identical to that of the purified compound. The authentic pseudo-B_{12} (identified by Dr. Stupperich) has been independently analyzed with ^{1}H-NMR spectroscopy by ourselves (unpublished data); the spectrum of the authentic compound was identical to that of the cited reference.[10] The authentic pseudo-B_{12} also gave identical patterns of TLC and HPLC to those of the main *Spirulina* corrinoid compound which has

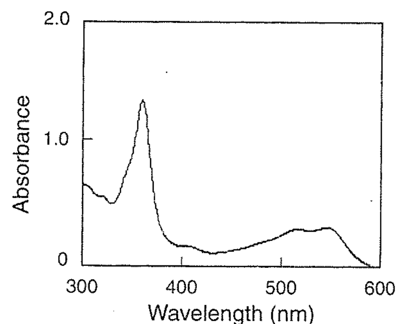

Fig. 1. Ultraviolet-Visible Spectrum of the Purified Compound from the Suizenji-nori.

A portion of the purified preparation was dissolved in 0.1 ml of distilled water. The spectrum was measured with a Shimadzu spectrophotometer (UV-1600) at room temperature, super-micro quartz cuvettes (0.1 ml, d = 1 cm) being used.

Table 1. R_f Values and Retention Times for the Purified Compound from Suizenji-nori, Authentic B_{12}, and Cyanocobamides on TLC and HPLC

Concentrated solutions (2 µl each) of the compound purified from suizenji-nori and cyanocobamides were spotted on silica gel 60 TLC sheets and developed with 1-butanol/2-propanol/water (10:7:10 v/v) and 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) as solvents I and II, respectively, in the dark at room temperature.

In the case of HPLC, diluted solutions (10 µl each) of the purified compound and the cyanocobamides were analyzed in a reversed-phase HPLC column (Wakosil-II 5C18RS) under the same conditions as those described in the text.

	TLC (R_f value)		HPLC (retention time, min)
	Solvent I	Solvent II	
Purified compound	0.14	0.48	6.7
Vitamin B_{12}	0.24	0.61	7.9
Benzimidazolyl cyanocobamide	0.18	0.57	6.3
5-Hydroxybenzimidazolyl cyanocobamide	0.20	0.49	6.1
Pseudovitamin B_{12}	0.14	0.48	6.7

been identified as pseudo-B_{12}.[8] The TLC and HPLC patterns of the compound purified from suizenji-nori, pseudo-B_{12} purified from *Spirulina* tablets, and authentic pseudo-B_{12} are shown in Fig. 2. These results indicate that the red-colored compound purified from suizenji-nori was not true B_{12}, but pseudo-B_{12} that is inactive for humans. No further detailed information on the purified compound is available because only a small amount of the purified sample was obtained (for the NMR study).

These results indicate that suizenji-nori is not suitable for use as a B_{12} source, especially for vegetarians.

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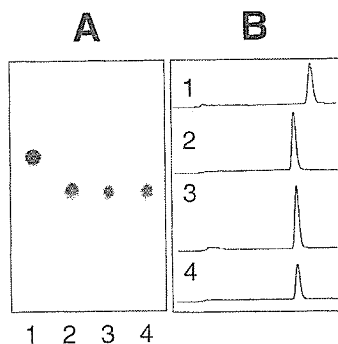

Fig. 2. TLC and HPLC Patterns of the Compound Purified from the Suizenji-nori, Pseudo-B_{12} Purified from *Spirulina* Tablets, and Authentic Pseudo-B_{12}.

In the TLC analysis (A), concentrated solutions (2 µl) of authentic B_{12} (1), authentic pseudo-B_{12} (2), pseudo-B_{12} purified from *Spirulina* tablets (3), and the purified compound from suizenji-nori (4) were spotted on silica gel 60 TLC sheets and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) in the dark at room temperature. In the case of HPLC (B), diluted solutions (10 µl) of authentic B_{12} (1), authentic pseudo-B_{12} (2), pseudo-B_{12} purified from *Spirulina* tablets (3), and the purified compound from suizenji-nori (4) were analyzed in a reversed-phase HPLC column (Wakosil-II 5C18RS) under the same conditions as those described in the text.

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Purification and Characterization of a Corrinoid-Compound in an Edible Cyanobacterium *Aphanizomenon flos-aquae* as a Nutritional Supplementary Food

EMI MIYAMOTO,*,† YURI TANIOKA,‡ TOMOYUKI NAKAO,‡ FLORIN BARLA,‡ HIROSHI INUI,‡ TOMOYUKI FUJITA,§ FUMIO WATANABE,‖ AND YOSHIHISA NAKANO‡

Department of Health and Nutrition, Nagasaki International University, Sasebo 859-3298, Japan, Department of Applied Biological Chemistry, Osaka Prefecture University, Sakai 599-5831, Japan, Graduate School of Agriculture, Shinsyu University, Kami-ina 399-4598, Japan, and School of Agricultural, Biological and Environmental Sciences, Tottori University, Tottori 680-8553, Japan

The vitamin B_{12} concentration of the dried cells of *Aphanizomenon flos-aquae* was determined by both microbiological method with *Lactobacillus delbrueckeii* ATCC7830 and chemiluminescence method with intrinsic factor. The *Aphanizomenon* cells contained 616.3 ± 30.3 μg ($n = 4$) of vitamin B_{12} per 100 g of the dried cells by the microbiological method. The values determined with the chemiluminescence method, however, were only about 5.3% of the values determined by the microbiological method. A corrinoid-compound was purified from the dried cells and characterized. The purified corrinoid-compound was identified as pseudovitamin B_{12} (an inactive corrinoid-compound for humans) by silica gel 60 TLC, C18 reversed-phase HPLC, ultraviolet–visible spectroscopy, and ^{1}H NMR spectroscopy. The results suggest that the *Aphanizomenon* cells are not suitable for use as a vitamin B_{12} source, especially in vegans.

KEYWORDS: *Aphanizomenon flos-aquae*; cyanobacteria; nutritional supplementary food; pseudovitamin B_{12}; vitamin B_{12}

INTRODUCTION

Strict vegetarians (vegans) have a greater risk of developing vitamin B_{12} deficiency relative to non-vegetarians because natural food sources of vitamin B_{12} are not plant food products, but animal food products (*1*). They must consume vitamin B_{12}-fortified foods or vitamin B_{12}-containing dietary supplements to prevent vitamin B_{12} deficiency. Plant foods, edible algae, and/or blue-green algae (cyanobacteria), however, contain substantial amounts of B_{12} (*2, 3*). Our previous studies have demonstrated that true vitamin B_{12} is the predominant cobamide of many species of eukaryotic algae (*4–7*), although pseudovitamin B_{12}, an inactive corrinoid for humans, predominated in a cyanobacterium *Spirulina* (*8*). Substantial amounts of cyanobacteria, *Spirulina* (3000 t/year), *Nostoc* (600 t/year), and *Aphanizomenon* (500 t/year), are produced worldwide to meet the high demands of both food and pharmaceutical industries (*9*). It is still unclear whether the other edible cyanobacteria, which are used as nutritional supplementary foods, contain true vitamin B_{12} or the inactive corrinoid-compound.

Aphanizomenon flos-aquae, a fresh water cyanobacterium, grow naturally in Upper Klamath Lake, OR. The bacterial cells contain various nutrients (polyunsaturated fatty acids, protein, carotenoids, vitamins, minerals, and so on) and also have therapeutic effects (*9–13*). Kay (*13*) has described that the bacterial cells contain some corrinoid-compounds that can be utilized as vitamin B_{12} in humans.

Thus, the dried *Aphanizomenon* cells (commercially available in a capsule form) are used as a vitamin B_{12}-rich nutritional supplementary food. The bacterial cells can contribute to human vitamin B_{12} needs, especially for vegans. There is, however, little information available on chemical properties of the corrinoid-compound in the *Aphanizomenon* cells.

In the present paper, we determine vitamin B_{12} concentration of the dried *Aphanizomenon* cells, which are used as a nutritional supplementary food by both microbiological method with *Lactobacillus delbrueckii* ATCC7830 and chemiluminescence method with intrinsic factor. We also describe the purification and characterization of corrinoid-compound from the bacterial cells to clarify whether the bacterial corrinoid-compound is true vitamin B_{12} or not.

MATERIALS AND METHODS

Materials. Vitamin B_{12} (cyanocobalamin) was obtained from Sigma (St. Louis, MO). Silica gel 60 TLC aluminum sheets were obtained

* Author to whom correspondence should be addressed [telephone/fax +81-956-20-5530; e-mail miyamo@niu.ac.jp].
† Nagasaki International University.
‡ Osaka Prefecture University.
§ Shinsyu University.
‖ Tottori University.

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Table 1. Vitamin B_{12} Concentrations of the Three Microalgae Commercially Available for Human Nutritional Supplementary Food (or Health Food)

	vitamin B_{12} concentration (μg/100 g of dry weight)			
	claim on bottle[a]	microbiological assay	chemiluminescence assay	refs
Chlorella	20–150	201.3–285.7	200.9–211.6	7
Spirulina	100–250	127.2–244.3	6.2–17.4	8
Aphanizomenon	800	616.3 ± 30.3[b]	32.3[c]	this study

[a] Determined by microbiological assay. [b] Values obtained represent mean ± SEM ($n = 4$). [c] Values obtained represent mean values ($n = 2$).

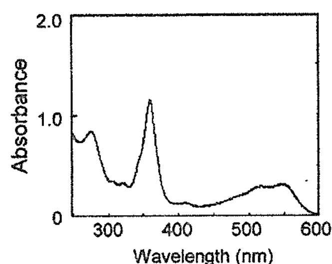

Figure 1. Ultraviolet–visible spectrum of the purified compound from the dried *Aphanizomenon* cells. A portion of the purified preparation was dissolved in 3.0 mL of distilled water. The spectrum was measured with a Shimadzu spectrophotometer (UV-1600) at room temperature, quartz cuvettes (3.0 mL, $d = 1$ cm) being used.

Table 2. R_f Values and Retention Times of the Purified Corrinoid-Compound from the Dried *Aphanizomenon* Cells, Authentic Vitamin B_{12}, and Pseudovitamin B_{12} on TLC and HPLC[a]

	TLC (R_f values)		
	solvent I	solvent II	reversed-phase HPLC (retention time, min)
purified compound	0.10	0.46	6.4
vitamin B_{12}	0.12	0.58	8.7
pseudovitamin B_{12}	0.10	0.46	6.4

[a] Concentrated solutions (2 μL) of the compound purified from the dried cells, vitamin B_{12} (cyanocobalamin), and pseudovitamin B_{12} were spotted on silica gel 60 TLC sheets and developed with 1-butanol/2-propanol/water (10:7:10 v/v) and 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) as solvents I and II, respectively, in the dark at room temperature. In the care of HPLC, concentrated solutions (2 μL) of the purified compound from the dried cells, vitamin B_{12} (cyanocobalamin), and pseudovitamin B_{12} were analyzed in a reversed-phase HPLC column (Wakosil-II 5C18RS) under the same conditions described in the text.

from Merck (Darmstadt, Germany). A B_{12} assay medium for *Lactobacillus delbrueckii* (formerly *Lactobacillus leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan). Pseudovitamin B_{12} was kindly provided by Dr. E. Stupperich, Ulm University, Germany. A reversed-phase high-performance liquid chromatography (HPLC) column (Wakosil-II 5C18RS, ϕ4.6 × 150 mm; particle size, 5 μm) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The dried *Aphanizomenon* cells as a nutritional supplementary food were purchased from market in Japan.

Extraction of Corrinoid-Compound from the Dried *Aphanizomenon* Cells. One gram of the dried cells was added to 10 mL of 0.1 mol/L acetate buffer, pH 4.8. Corrinoid-compound was extracted from the cell suspension by the method of boiling with KCN at acidic pH; specifically 0.05% (w/v) of KCN was added to the cell suspension, which was boiled for 30 min at 98 °C in the dark. The extraction procedures were done in a Dalton (Tokyo, Japan) draft chamber. The boiled cell suspension was centrifuged at 10 000*g* for 10 min. The supernatant was used for vitamin B_{12} assay.

Assay of Total Vitamin B_{12}. The bacterial corrinoid-compound was assayed as vitamin B_{12} by the microbiological method with *L. delbrueckii* subsp. *lactis* ATCC 7830 and by the fully automated chemiluminescence B_{12} analyzer ACS 180 (Chiron Diagnostics, East Walpole, MA) according to the manufacturer's instructions as described previously (*14*). The extracts were diluted with distilled water up to a vitamin B_{12} concentration range of 10–100 ng/L and used as samples for the microbiological method. The turbidity (*%T*) of the test culture of *L. delbrueckii* ATCC7830 grown at 37 °C for 16–21 h was measured at 660 nm with the UV-1600 UV–visible spectrophotometer according to the manufacturer's recommended method.

Purification of a Corrinoid-Compound from the Dried *Aphanizomenon* Cells. About 550 g of the dried *Aphanizomenon* cells was added to 5.5 L of 0.1 mol/L acetate buffer, pH 4.8. Corrinoid-compound was extracted from the suspension by boiling with KCN at acidic pH; KCN was added to the suspension at the final concentration of 10 mmol/L. The suspension was boiled for 30 min at 98 °C in the dark. The extraction procedures were done in the Dalton draught chamber. The boiled suspension was centrifuged at 10 000*g* for 10 min. Corrinoid-compound remaining in the precipitate fraction was re-extracted under the same conditions. The combined supernatant fractions (about 8 L) were put on a column (5 × 100 cm) of Amberlite XAD-4 resin (Japan Organo Co., Tokyo, Japan), which had been washed with 5 L of methanol and then equilibrated with distilled water. The column was washed with 5 L of distilled water and then eluted with 5 L of 80% (v/v) methanol solution in the dark. The eluate containing a corrinoid-compound was evaporated to dryness under reduced pressure, and dissolved in 60 mL of distilled water. Each 20 mL of the concentrated solution was put on a column (24 × 180 mm) of Cosmosil 140C18-OPN (Nacalai Tesque, Kyoto, Japan), which had been washed with 75% (v/v) ethanol solution and then equilibrated with distilled water. The column was eluted with a stepwise gradient [0%, 10%, 20%, 30%, and 80% (v/v)] of ethanol. The 10% (v/v) ethanol fraction containing a corrinoid-compound was evaporated to dryness under reduced pressure, and dissolved with a small amount of distilled water. The concentrated solution was put on a silica gel 60 TLC sheet (Merck, Darmstadt, Germany) and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) as a solvent in the dark at room temperature. Red-colored spots on the dried TLC sheet were collected, extracted with 80% (v/v) methanol solution, evaporated to dryness under reduced pressure, and dissolved in 50 μL of distilled water. The concentrated solution was put on a silica gel 60 TLC sheet and developed with 1-butanol/2-propanol/water (10:7:10 v/v) as a solvent in the dark at room temperature. Red-colored spots on the dried TLC sheet were collected, extracted with 80% (v/v) methanol solution, evaporated to dryness under reduced pressure, and dissolved in 100 μL of distilled water. The concentrated solution was purified by HPLC using Shimadzu HPLC apparatus (two LC-10ADvp pumps, DGV-12A degasser, SCL-10Avp system controller, SPD-10Avp ultraviolet–visible detector, CTO-10Avp column oven, 100 μL sample loop, C-R6A Chromatopac integrator). The sample (50 μL) was put on a reversed-phase HPLC column (Wakosil-II 5C18RS) equilibrated with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40 °C. The flow rate was 1 mL/min. The corrinoid-compound was isocratically eluted with the same solution and monitored by measuring absorbance at 361 nm. The fractions (1 mL) were collected from the reverse phase HPLC column with a Bio-Rad Laboratories fraction collector (model 2110). The final red-colored fractions were collected, evaporated to dryness under reduced pressure, dissolved in 100 μL of distilled water, and used as a purified corrinoid-compound.

Analytical TLC and HPLC. The concentrated solutions (2 μL) of the corrinoid-compound purified from the *Aphanizomenon* cells, vitamin B_{12} (cyanocobalamin), and pseudovitamin B_{12} were spotted on the silica gel 60 TLC sheets and developed with 1-butanol/2-propanol/water (10:7:10 v/v), solvent I, and 2-propanol/NH_4OH (28%)/water (7:1:2 v/v), solvent II, in the dark at room temperature. After TLC sheets were dried, R_f values of the red-colored spots of these corrinoid-compounds were determined.

In the case of HPLC, the concentrated solutions (2 μL) of the purified corrinoid-compound, vitamin B_{12} (cyanocobalamin), and pseudovitamin B_{12} were analyzed with the reversed-phase HPLC column (Wakosil-II

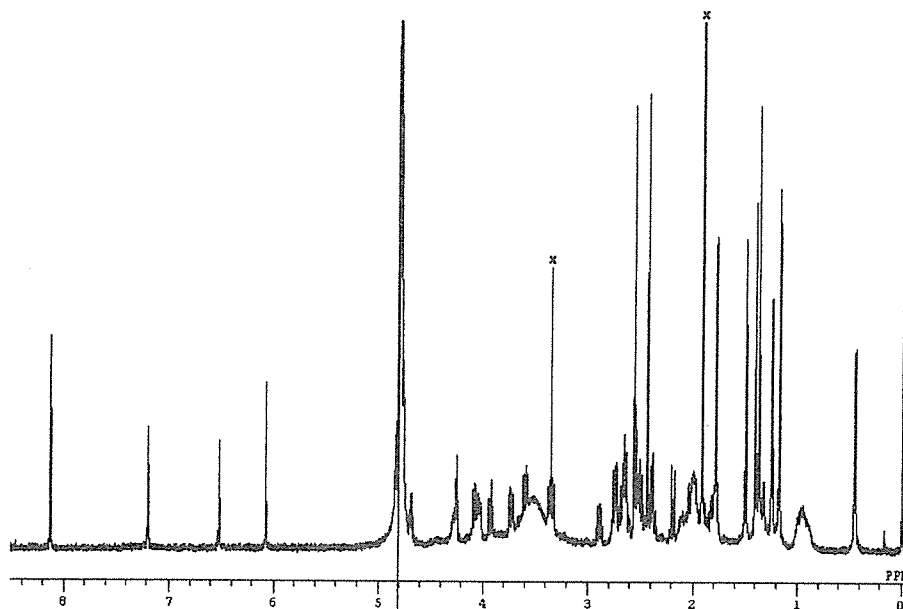

Figure 2. ^{1}H NMR spectrum of the corrinoid purified from dried *Aphanizomenon* cells (500 MHz, D_2O).

5C18RS). They were isocratically eluted with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40 °C, and monitored by measuring absorbance at 361 nm. The flow rate was 1 mL/min.

Ultraviolet–Visible Spectrum. The spectrum was measured with a Shimadzu spectrophotometer (UV-1600) at room temperature. Quartz cuvettes (d = 1 cm) were used. A portion of the purified corrinoid-compound was dissolved in 3 mL of distilled water.

^{1}H NMR Spectrum. ^{1}H NMR spectrum was obtained in D_2O with a JEOL JNM α-500 spectrometer. Chemical shifts are given on a δ (ppm) scale with 3-(trimethylsilyl)propionic acid-d_4 sodium salt (TSP) as an internal standard. ^{1}H NMR spectral data of the purified corrinoid-compound: δ_H 8.13 (B2, s), 7.20 (B8, s), 6.53 (R1, d, J = 3.4 Hz), 6.08 (C10, s), 4.69 (R3, dt, J = 4.3, 8.5 Hz), 4.29 (Pr2, m), 4.26 (R2, t-like, J = 3.7 Hz), 4.08 (C3, m), 4.08 (C19, m), 4.03 (R4, m), 3.93 (R5a, dd, J = 2.1, 13.1 Hz), 3.74 (R5b, dd, J = 3.7, 13.1 Hz), 3.60 (Pr1a, br d, J = 14.3 Hz), 3.37 (C8, m), 3.35 (C13, m), 2.89 (Pr1b, dd, J = 10.1, 14.3 Hz), 2.56 (C53, s), 2.44 (C35, s), 1.79 (C25, s), 1.50 (C47, s), 1.41 (C54, s), 1.37 (C36, s), 1.25 (Pr3, d, J = 6.1 Hz), 1.18 (C46, s), 0.45 (C20, s). The assignment of these signals was carried out in comparison with those of authentic vitamin B_{12}.

RESULTS AND DISCUSSION

Vitamin B_{12} Concentration of the Dried *Aphanizomenon* Cells. Total vitamin B_{12} concentration of the dried *Aphanizomenon* cells was determined by both microbiological and chemiluminescence methods. Using the microbiological assay, the *Aphanizomenon* cells contained significantly higher amounts of vitamin B_{12} (616.3 ± 30.3 μg, n = 4) relative to the other edible microalgae (*Chlorella* and *Spirulina*) previously characterized (*8, 14*) (**Table 1**). The values determined with the microbiological assay were, however, 20.5-fold greater than the values determined with the chemiluminescence assay in the *Aphanizomenon* cells. The similar result has been obtained in the *Spirulina* cells that contain pseudovitamin B_{12} (*8*). In the *Chlorella* cells containing true vitamin B_{12}, the values determined with the microbiological assay are similar to the values determined by the chemiluminescence assay (*14*).

Purification and Characterization of a Corrinoid-Compound from the Dried *Aphanizomenon* Cells. To evaluate whether the vitamin B_{12} activity detected in the *Aphanizomenon* cells by the microbiological assay method is derived from true vitamin B_{12} or not, a corrinoid-compound was purified and characterized.

The final purified preparation gave a single red-colored spot on the silica gel 60 TLC and a single peak by the C18 reversed-phase HPLC, indicating that the corrinoid-compound was purified to homogeneity. The ultraviolet–visible spectrum of the purified corrinoid-compound showed a typical absorption spectrum of cobalt-containing corrinoid (**Figure 1**); λ_{max} nm (absorbance) values were at 548.0 (0.317), 518.0 (0.296), 360.0 (1.153), and 277.5 (0.846).

The purified corrinoid-compound, authentic vitamin B_{12} (cyanocobalamin), and pseudovitamin B_{12} were analyzed by the silica gel 60 TLC and reversed-phase HPLC (**Table 2**). The R_f values (0.10 and 0.46 in solvents I and II, respectively, TLC) for the purified compound were identical to the values for authentic pseudovitamin B_{12}, whose retention time (6.4 min) by HPLC was also identical to that of the purified compound.

In the ^{1}H NMR spectrum of the corrinoid purified from the dried *Aphanizomenon* cells (**Figure 2**), the typical signals due to corrin skeleton and adenyl and ribose moieties were observed (see Material and Methods). These spectral data were identical to those of pseudovitamin B_{12} isolated from *Spirulina* tablet (*7*).

These results indicate that the red-colored compound purified from the dried *Aphanizomenon* cells is not true vitamin B_{12}, but pseudovitamin B_{12} inactive for humans.

Although only one corrinoid-compound (true vitamin B_{12}) has been purified from the *Chlorella* cells, the *Spirulina* cells contain two corrinoid-compounds (main, pseudovitamin B_{12}; and minor, true vitamin B_{12}). Our unpublished work demonstrated that the true vitamin B_{12} found in the *Spirulina* cells was derived from some vitamin B_{12}-synthesizing bacteria concomitant with the *Spirulina* cells grown under the open culture system. *Eschricha coli* 215-bioautography of the *Aphanizomenon* extract indicated that the bacterial cells contained substantial amounts of pseudovitamin B_{12} alone (data not shown). Peudovitamin B_{12} has been reported to reveal moderate affinity to the intrinsic

factor (most specific vitamin B_{12}-binding protein) (*15*) used in the chemiluminescence vitamin B_{12} assay method. These observations suggest that these cyanobacteria have the ability to synthesize pseudovitamin B_{12} *de novo*.

Some preclinical studies suggest that the *Aphanizomenon* cells have therapeutic properties such as macrophage-activation (*16*), antioxidant (*17*), immunological (*18*), and anti-inflammatory (*12, 17*) activities. Although the taking of the *Aphanizomenon* cells may give some health promotion effects for humans, the results presented here strongly suggest that the bacterial cells are not suitable for use as a vitamin B_{12} source, especially in vegans.

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原　著

カツオ魚肉のビタミン B_{12} 含量と各種加熱調理が魚肉ビタミン B_{12} 含量に及ぼす影響

[1]高知女子大学生活科学部*，[2]高知女子大学大学院人間生活学研究科*，[3]大阪府立大学大学院生命環境科学研究科
[4]長崎国際大学健康管理学部，[5]鳥取大学農学部

西岡　道子[1]，彼末　富貴[1]，谷岡　由梨[2,3]，宮本　恵美[1,4]，渡辺　文雄[1,2,5]

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Characterization of Vitamin B_{12} in Skipjack Meats and Loss of the Vitamin from the Fish Meats by Various Cooking Conditions

Michiko Nishioka[1], Fuki Kanosue[1], Yuri Tanioka[2,3], Emi Miyamoto[1,4], Fumio Watanabe[1,2,5]

[1]Faculty of Human Life and Environmental Science, and [2]Graduate School of Human Life, Kochi Women's University, Japan
[3]Graduate School of Life and Environmental Science, Osaka Prefecture University, Sakai 599-8531, Japan
[4]Department of Health and Nutrition, Nagasaki International University, Sasebo 859-3298, Japan
[5]School of Agricultural, Biological and Environmental Sciences, Faculty of Agriculture, Tottori University, 680-8553, Japan

Vitamin B_{12} contents of skipjack meats were assayed by the microbiological method authorized in the Standard Tables of Food Composition in Japan (5th Edition). The dark muscles contained substantial amounts (158.5±16.3 μg/100 g) of vitamin B_{12} relative to the ordinary muscles (dorsal portion 9.9±0.6 μg/100 g ; ventral portion 8.4±0.6 μg/100 g). A corrinoid compound was purified from the dark muscles and characterized, then it was confirmed that the dark muscles contain true vitamin B_{12}. Various heat-cooking treatments (boil, steam, sauté, fly, and microwave) did not give significant losses (2.3~14.8%) of vitamin B_{12} from the treated fish meats. The results indicate that skipjack meats (especially dark meat) are excellent vitamin B_{12} sources.
Key words: Skipjack, fish meats, dark muscle, vitamin B_{12}, heat-cooking

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緒　言

日本は世界有数の魚介類の消費国であり，魚介類から24.6%ものタンパク質を摂取しており，畜肉類の16.6%を大きく上回っている[1]．また，魚介類はその他の栄養素（脂質，ビタミン，ミネラル）のよい供給源ともなっており[1]，魚介類が日本人にとって重要な食品であることを示している．

ビタミン B_{12} (B_{12}) は主に動物性食品に含まれており，一部の藻類や微生物が関与する発酵食品（糸引き納豆[2]，テンペ[3]，後発酵茶[4)5]）を除き植物性食品にはほとんど含まれていない[6]．米国では，畜肉や牛乳が B_{12} のよい供給源であるが[7]，我が国では魚介類が主要な供給源となっており[8]，栄養学的に重要である．しかし，畜肉[9]や牛乳[10]に比べ，魚肉の B_{12} 含量や化学的性質，ならびに加熱調理による B_{12} の損失についての知見は非常に少ない．そこで，比較的大型で血合肉も食する赤身魚としてカツオに着目し，魚肉各部位の B_{12} 含量ならびに血合肉中に含まれるコリノイド化合物の同定を行った．また，カツオは，生で刺身やタタキとして食する場合が多いが，ま

*〒780-8515　高知市永国寺町 5-15

ぐろなどの赤身魚のモデルとして各種加熱調理が魚肉 B_{12} 含量に及ぼす影響を検討した．

実験方法

1．試料

カツオ（春獲り，生，6 匹，重量 1.5 ～ 2.0 kg）は，高知県で水揚げされたものを高知市内の市場で購入し実験に用いた．カツオの重量を測定後，内臓を除き 3 枚におろした．その後，5 匹の魚肉を血合肉と普通肉に分け，普通肉はさらに背肉と腹肉に分け，それぞれの重量を測定した．カツオ魚肉の各部位の一部は分析に供するまで－20℃の冷凍庫で保存した．

残りのカツオ魚肉は，普通肉と血合肉を分けずにフードプロセッサーで破砕・均質化した後，50 g を正確に秤量し，魚肉パテ（縦 5 x 横 5 x 高さ 2 cm）を調製した．この魚肉パテを用いて直ちに各種加熱調理の実験を行った．

ブリ，サワラ，ゴマサバの魚肉（切り身）は，高知市内の市場で購入した．

2．ビタミン B_{12} の抽出・定量法

カツオ魚肉に含まれる B_{12} の定量は，五訂日本食品標準成分表で採用されている分析マニュアル[11]に準じて *Lactobacillus delbrueckii* subsp. *lactis*（旧名 *L. leichimannii*）ATCC7830 を用いた微生物学的定量法で行った．なお，定量に用いる培地は，日水製薬株式会社製のライヒマニ保存用培地，ライヒマニ接種用培地，ライヒマニ用 B_{12} 定量基礎培地を用いた．

凍結したカツオ魚肉各部位を解凍後，フードプロセッサーにより破砕・均質化した．この均質化した魚肉各部位 2 g を正確に秤量し，定法によりシアン化カリウムを含む酢酸緩衝液（pH4.5）中で B_{12} を加熱抽出した．

また，本定量菌は，B_{12} 以外にデオキシリボースやデオキシリボヌクレオチドにも B_{12} 活性を示すため，分析マニュアル[11]に記載された以下の方法で補正した．

上記 B_{12} 抽出液の一部に 1 mol/*l* 水酸化ナトリウム溶液を加え，pH を 11 ～ 12 に調整後，オートクレーブ（121℃，30 分）処理を行い，B_{12} を分解させた．その後，1 mol/*l* 塩酸溶液で pH を 6 に調整・定容し，この溶液をアルカリ耐性因子画分として B_{12} 抽出液と同様に微生物学的定量法でみかけの B_{12} 含量を測定した．

B_{12} 抽出液画分に検出された B_{12} 含量からアルカリ耐性因子画分に検出されたみかけの B_{12} 含量を差し引き，試料中の正味の B_{12} 含量を算出した．

3．カツオ血合肉からのコリノイド化合物の単離方法

カツオ血合肉（約 600 g）をフードプロセッサーで破砕・均質化した後，0.5 g シアン化カリウムを含む 10 mmol/*l* 酢酸緩衝液（pH4.5）4 *l* を加えドラフト内で 30 分間加熱抽出した．室温まで冷却後，8000 g，10 分間の遠心分離を行い，上澄み画分を抽出液とした．

あらかじめエタノールで洗浄後，蒸留水で平衡化させたアンバーライト XAD4 樹脂（約 1 kg）をガラスカラム（5.0 x 50 cm）に充填した．上記抽出液を当該カラムにかけコリノイド化合物を樹脂に吸着させた．蒸留水 1 *l* でカラムを洗浄後，80%（v/v）エタノール 2 *l* で樹脂に吸着したコリノイド化合物を溶出させた．溶出液は，エバポレーターで乾固した後，30 ml の蒸留水に溶解させた．この時生じた不溶性画分は，遠心分離により除去した．

あらかじめエタノールで洗浄後，蒸留水で平衡化させたコスモシル 140C18-OPN 樹脂をガラスカラム（2.4 x 10 cm）に充填した．上記溶出液を本カラムにかけコリノイド化合物を樹脂に吸着させた．蒸留水 100 ml でカラムを洗浄後，10%（v/v）エタノール 100 ml と 20%（v/v）エタノール 100 ml を用いて樹脂に吸着したコリノイド化合物を溶出させた．溶出された赤色画分を回収し，エバポレーターで乾固した後，少量の蒸留水に溶解させた．この時生じた不溶性画分は，遠心分離により除去した．

この赤色溶液をシリカゲル 60 の薄層クロマトグラフィー（TLC）用アルミニウムプレートに負荷し，展開溶媒 2-プロパノール / アンモニア水 / 蒸留水（7/2/1）を用いて室温・暗黒下で展開させた．展開した TLC プレートを風乾後，赤色スポットをハサミで切り取り，80%（v/v）エタノール 20 ml を加え 4℃で一晩放置することで赤色化合物を再抽出した．抽出液は減圧下，35℃にて遠心エバポレーターで乾固した後，100 μl の蒸留水に溶解させた．

上記赤色溶液 100 μl を高速液体クロマトグラフィー（HPLC）［カラム，Wakosil-II 5C18RS（φ 4.6 x 150 mm）；移動相，1%（v/v）酢酸を含む 20%（v/v）メタノール溶液；カラム温度，35℃；流速，1.0 ml/min；検出波長，278 nm］で分離後，溶出液を 1.0 ml ずつフラクションコレクターで分画した．赤色画分を減圧下 35℃にて遠心エバポレーターで乾固させた後，50 μl の蒸留水に溶解させ，精製標品として実験に用いた．

4．コリノイド化合物の同定方法

カツオ血合肉より精製したコリノイド化合物溶液ならびに標準の B_{12} 溶液（20 μmol/*l*）をシリカゲル 60 の TLC アルミニウムプレートに負荷し，展開溶媒 I［1-ブタノール /2-プロパノール / 蒸留水（10/7/10）］ならびに展開溶媒 II［2-プロパノール / アンモニア水 / 蒸留水（7/2/1）］を用いて室温・暗黒下で展開させた．展開した TLC プレートは風乾後，各赤色スポットの R_f 値を測定した．

また，カツオ血合肉より精製したコリノイド化合物溶液ならびに標準の B_{12} 溶液（20 μmol/*l*）5 μl をそれぞれ HPLC［カラム，Wakosil-II 5C18RS（φ4.6 x 150 mm）；移動相，1%（v/v）酢酸を含む 20%（v/v）メタノール溶液；カラム温度，35℃；流速，1.0 ml/min；検出波長，278 nm］で分析し，各画分の保持時間を測定した．

5．調理条件

上記の方法で調製したカツオ魚肉パテを用いて以下の条件下で加熱調理した．

ゆでる(Boil)は魚肉パテ1個を水道水200 ml中で6分間加熱した．焼く(Sauté)は魚肉パテをホットプレート(180℃)で7分30秒加熱した．揚げる(Fly)は，魚肉パテ1個を食用油200 ml(180℃)中で1分30秒間加熱した．蒸す(Steam)は，魚肉パテ1個を蒸し器で4分間加熱した．電子レンジ加熱(Microwave)は，魚肉パテを耐熱性ラップで包み電子レンジで1分間加熱した．

各種条件下で加熱調理した後，魚肉パテの重量と煮汁がある場合は煮汁の容量を測定した．加熱調理前後の魚肉パテそれぞれ2 gを正確に秤量し，上述した方法でB_{12}を抽出・定量した．同じ加熱調理実験を3回行った．また，調理前後で魚肉パテ中の水分含量が変化する可能性があるため，調理後の魚肉パテ1個の重量も測定し，調理後の魚肉パテ1個に含まれるB_{12}含量を求め調理前の魚肉パテ1個に含まれるB_{12}含量に対するパーセントで示した．

結果および考察

1. カツオ魚肉各部位のビタミンB_{12}含量

五訂日本食品標準成分表で採用されている*L. delbrueckii subsp. lactis* ATCC7830を用いたB_{12}定量法でカツオ魚肉(普通肉背部，普通肉腹部，血合肉)に含まれるB_{12}含量を測定した(表1)．可食部100 gあたりのB_{12}含量は，普通肉の背部と腹部でほぼ同レベルであり，五訂日本食品標準成分表[6]のカツオ(春獲り，生)および(秋獲り，生)のB_{12}含量(それぞれ8.4 μgと8.6 μg)とよく一致していた．B_{12}が魚肉の血合肉に高濃度で存在することは古くから知られているが[12]，カツオの血合肉(平均値158.5 μg/100 g)は，他の魚の血合肉(ブリ，平均値48.3 μg/100 g；サワラ，平均値54.8 μg/100 g；ゴマサバ，平均値47.2 μg/100 g)と比べてもB_{12}を高濃度含有していた．また，カツオ魚肉全体に含まれる全B_{12}含量の約74%が血合肉に含まれていることが明らかとなった．

なお，本定量菌は，B_{12}以外にデオキシリボースやデオキシリボヌクレオチド(アルカリ耐性因子)にもB_{12}活性を示すため，アルカリ耐性因子量を補正したが，カツオ血合肉には，B_{12}含量の0.6%以下のアルカリ耐性因子量

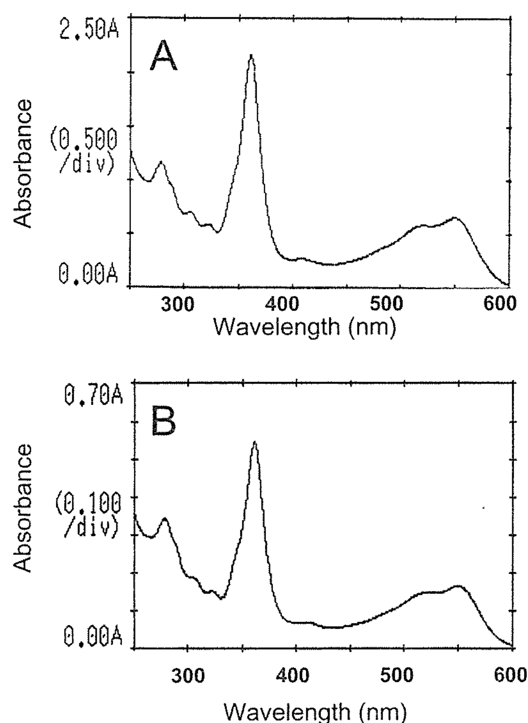

図1. Ultraviolet-visible spectrum of the purified corrinoid compound from skipjack dark muscle. (A) Authentic B_{12} (cyanocobalamin) solution (0.1 mmol/*l*). (B) A portion of the purified compound was dissolved in 0.1 ml of distilled water. These spectra were obtained with a Shimadzu spectrophotometer (UV-16000) at room temperature (25 ℃). Super micro-quartz cuvettes (0.1 ml, *d* = 1 cm) were used.

表1. Vitamin B_{12} contents of skipjack meats. Total B_{12} was extracted by boiling at acidic pH and assayed by the microbiological method with *Lactobacillus delbrueckii* sub. *lactis* ATCC 7830 according to the method described in the Japanese Standard Tables of Food Composition. Since *L. delbrueckii* ATCC 7830 can utilize both deoxyribosides and deoxyribonucleotides (known as an alkali-resistant factor) as well as B_{12}, the amount of true B_{12} was calculated by subtracting the values of the alkali-resistant factor from the values of total B_{12}.

	Vitamin B_{12} contents		Weight
	(μg/100 g)	(μg/total muscle tissue)	(g)
Ordinary meat			
Dorsal portion	9.9 ± 0.6	38.0 ± 0.6	390.2 ± 20.1
Ventral portion	8.4 ± 0.6	29.3 ± 1.5	346.7 ± 12.9
Dark meat	158.5 ± 16.3	190.3 ± 28.2	120.7 ± 13.2

Mean ± SE (n = 5)

が検出されたに留まり，普通肉背部および腹部においては検出限界以下であった．

2. カツオ血合肉に含まれるコリノイド化合物の単離・同定

五訂日本食品標準成分表で採用されている B_{12} 定量菌 *L. delbrueckii* subsp. *lactis* ATCC7830 は B_{12} 以外のコリノイドにも感応するため，本定量法で測定した結果，B_{12} が多量に含まれていると評価されても，ヒトに生理活性を有しないシュード B_{12} である場合が淡水産の藻などにおいて報告されている[13]．そこで，カツオ血合肉中に多量に検出される B_{12} が真の B_{12} であるかどうかを確認するために，カツオ血合肉からコリノイド化合物を単離・同定した．カツオ血合肉抽出液から各種クロマトグラフィーを用いて HPLC で単一のピークになるまで赤色化合物を精製した．カツオ血合肉より精製した赤色化合物の紫外・可視吸収スペクトル分析を行った結果（図1），コリノイド化合物特有の吸収スペクトルを示した．シリカゲル 60TLC 分析と逆相 HPLC 分析において，カツオ血合肉より精製したコリノイド化合物と標準の B_{12} の挙動が完全に一致したことから（表2），カツオ血合肉には真の B_{12} が多量に含まれていることが明らかとなった．

3. カツオ魚肉のビタミン B_{12} 含量に及ぼす各種加熱調理の影響

カツオ魚肉パテを用いて各種加熱調理による魚肉 B_{12} の調理損失について検討した結果を表3に示す．焼く（Sauté）・蒸す（Steam）・揚げる（Fly）では，カツオ魚肉 B_{12} の残存率は 94.5 ～ 97.7% と調理損失はほとんどなかった。また，ゆでる（Boil）において煮汁中への溶出は 3.4% 程度に留まり，よい残存率を示した．電子レンジ加熱（Microwave）による調理損失が最も大きかった．

B_{12} の供給源である畜肉や牛乳の調理損失について，焼く・ゆでる・揚げるなどの加熱調理による B_{12} の残存率は牛肉各部位で 61 ～ 88%，豚肉各部位で 76 ～ 90% と報告されている[14]．また，牛乳中の B_{12} は加熱調理により顕著に減少し，電子レンジ3分加熱および直火30分の加熱で約 50% の B_{12} が消失する[10]．加熱調理の条件が異なるため単純に残存率を比較して評価することはできないが，畜肉や牛乳に比べ，カツオ魚肉中の B_{12} の調理損失は少ないと考えられる．

以上の結果からカツオ魚肉（特に血合肉）は，調理方法に関係なく B_{12} のよい供給源となりえることが明らかとなった．

結　論

1. 可食部 100 g あたりの B_{12} 含量は，普通肉の背部（9.9 ± 0.6 μg）と腹部（8.4 ± 0.6 μg）でほぼ同レベルとなり，五訂日本食品標準成分表の値とよく一致していた．
2. 血合肉には普通肉に比べ多量（158.5 ± 16.3 μg）の B_{12} が含まれており，カツオ魚肉全体に含まれる B_{12} の約 74% が血合肉に含まれていた．
3. カツオ血合肉抽出液から各種クロマトグラフィーを用

表2. R_f values and retention times of the purified compound and authentic B_{12} on silica gel 60 TLC plate and reversed-phase HPLC. Solvent I, 1-butanol/2-propanol/water (10: 7: 10); Solvent II, 2-propanol/25% NH_4OH /water (7: 1: 2) Mobile phase for HPLC, 20% (v/v) methanol solution containing 1% (v/v) acetic acid

	TLC (R_f)		HPLC (min)
	Solvent I	Solvent II	
Purified compound	0.25	0.63	9.5
Authentic B_{12}	0.25	0.63	9.5

表3. Loss of vitamin B_{12} from the skipjack meat putty treated under various cooking conditions.

Cooking conditions	Relative contents (%) of remaining vitamin B_{12}*
None	100
Boil	91.6 ± 3.5
Steam	94.5 ± 3.9
Sauté	97.7 ± 0.9
Fly	95.8 ± 2.4
Microwave	85.2 ± 7.1

*Percent against vitamin B_{12} content of the meat without cooking

いてコリノイド化合物を精製・同定した結果，カツオ血合肉に含まれるコリノイド化合物は真のB_{12}であった．

4. ゆでる・焼く・蒸す・揚げる・電子レンジ加熱によるカツオ魚肉のB_{12}残存率は85.2～97.7%であった．

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（平成18.1.6受付）

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市販ふりかけおよび茶漬けの素のビタミンB_{12}含量

西岡道子[1]，彼末富貴[1]，谷岡由梨[2]，宮本恵美[1]，渡辺文雄[1,2]

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Vitamin B_{12} contents of commercially available seasoned powders for sprinkling over boiled rice and for boiled rice with tea.

Michiko NISHIOKA[1], Fuki KANOSUE[1], Yuri TANIOKA[2], Emi MIYAMOTO[1], Fumio WATANABE[1,2]

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要　旨

市販されているふりかけおよび茶漬けの素に含まれるビタミンB_{12}含量を五訂日本食品標準成分表に準じた*Lactobacillus delbrueckii* subsp. *lactis* ATCC7830を用いた微生物学的定量法で検討した。かつお，小魚，のり，たまごを原料に含むふりかけのB_{12}含量は，比較的高値を示したが，その他のふりかけのB_{12}含量は，低値であった（平均3.84 μg/100g)。一方，茶漬けの素のB_{12}含量は，平均2.25 μg/100gであった。

Abstract

Vitamin B_{12} contents of commercially available seasoned powders for sprinkling over boiled rice and for boiled rice with tea were assayed by the microbiological method authorized in the standard tables of food composition in Japan (5th Edition). These seasoned powders (100 g) for sprinkling over boiled rice and for boiled rice with tea contained 3.84μg and 2.25μg of vitamin B_{12} (mean values), respectively.

キーワード：ふりかけ，茶漬け，調味料，ビタミンB_{12}

Key words : seasoned powder for sprinkling over boiled rice, seasoned powder for boiled rice with tea, seasonings, vitamin B_{12}

1 高知女子大学生活科学部健康栄養学科 Department of Health Science, Faculty of Human Life and Environmental Science, Kochi Women's University

2 高知女子大学大学院人間生活学研究科 Graduate School of Human Life, Kochi Women's University

1．緒　　言

日本は世界有数の水産食品の消費国であり，魚介類や藻類は栄養素（タンパク質，脂質，ビタミン，ミネラル）のよい供給源ともなっており[1]，日本人にとって重要な食品である。

これらの栄養素のうち，ビタミンB_{12}（B_{12}）は一部の藻類（あおのりやあまのり）[2~5]を除き，植物性食品には含まれておらず，主に動物性食品から摂取している。米国では，畜肉や牛乳がB_{12}のよい供給源であるが[6]，我が国では魚介類や藻類が主要な供給源となっており[7]，栄養学的に重要である。

しかし，B_{12}のよい供給源である魚介類や藻類を用いた加工食品の中で，日本人が日常的に飯と共に食するふりかけと茶漬けの素については，五訂日本食品標準成分表に記載されていない。そこで，これらふりかけおよび茶漬けの素に着目し市販品中のB_{12}含量を測定し，数値を定めると共に，B_{12}のよい供給源になりえるかどうかを検討した。

2．実験方法

(1) 試　　料

市販のふりかけおよび茶漬けの素は，高知市内の市場で購入して実験に用いた。

(2) ビタミンB_{12}の抽出・定量法

B_{12}の定量は，五訂日本食品標準成分表で採用されている分析マニュアル[8]に準じて*Lactobacillus delbrueckii* subsp. *lactis*（旧名*L. leichimannii*）ATCC7830を用いた微生物学的定量法で行った。なお，定量に用いる培地は，日水製薬株式会社製のライヒマニ保存用培地，ライヒマニ接種用培地，ライヒマニ用B_{12}定量基礎培地を用いた。

ふりかけおよび茶漬けの素５～10ｇ程度をフードプロセッサーで粉砕後，分析試料2ｇを正確に秤量し，常法によりシアン化カリウムを含む酢酸緩衝液（pH 4.5）中でB_{12}を加熱抽出した。

また，本定量菌は，B_{12}以外にデオキシリボースやデオキシリボヌクレオチドにもB_{12}活性を示すため，分析マニュアル[8]に記載された以下の方法で補正した。

上記B_{12}抽出液の一部に１mol/L水酸化ナトリウム溶液を加え，pHを11～12に調整後，オートクレーブ（121℃，30分）処理を行い，B_{12}の分解を行った。その後，１mol/L塩酸溶液でpHを６に調整・定容し，この溶液をアルカリ耐性因子画分としてB_{12}抽出液と同様に微生物学的定量法でB_{12}含量を測定した。

B_{12}抽出液画分に検出されたB_{12}含量からアルカリ耐性因子画分に検出されたB_{12}含量を差し引き，試料中の正味のB_{12}含量を算出した。

3．結果および考察

五訂日本食品標準成分表で採用されている*L. delbrueckii* subsp. *lactis* ATCC7830を用いたB_{12}定量法で市販ふりかけ中のB_{12}含量を測定した（Table １）。その結果，かつおや小魚ふりかけ中のB_{12}含量は，2.39～10.88μg/100gと比較的高く，次いでのりやたまごのふりかけ（1.32～6.50μg/100g）であった。一方，さけ，たらこ，その他のふりかけのB_{12}含量は，低値を示した。使いきりタイプの小包装では，内容物の重量が２ｇ程度であったので，一食あたりの摂取含量の目安として併記した。今回測定した26種の市販ふりかけ中のB_{12}含量の平均値は3.84μg/100gであり，一食あたり約0.1μgが摂取できると考えられる。

一方，市販茶漬けの素中のB_{12}含量において，主要な素材の違いがB_{12}含量へ及ぼす影響は，ふりかけほど大きくなかった（Table ２）。内容物の重量が８ｇ程度のものが多かったので，一食あたりの摂取含量の目安として併記した。茶漬けの素中のB_{12}含量の平均値は，2.25μg/100gとなり，一食あたり約0.18μgのB_{12}が摂取できると推定される。

以上の結果から飯にかけて食するふりかけや茶漬けの素の中には比較的高いB_{12}含量を示すものもあったが，推定される一食あたりのB_{12}摂取量は日本人の食事摂取基準（2005年版）[9]の成人男女の推奨量（2.4μg）の1/10程度であった。

Table 1　市販ふりかけに含まれるビタミンB_{12}含量

種類	ビタミンB_{12}含量 (μg/100g)	(μg/2g)
かつお，小魚など		
A	9.14	0.18
B	2.39	0.05
C	9.02	0.18
D	7.04	0.14
E	10.88	0.22
F	8.59	0.17
G	8.95	0.18
H	7.67	0.15
のり，たまご		
I	3.55	0.07
J	3.00	0.06
K	2.40	0.05
L	1.32	0.03
M	6.50	0.13
たらこ（めんたいこを含む）		
N	4.59	0.09
O	0.44	0.01
P	1.15	0.02
さけ		
Q	0.23	Trace*
R	0.48	0.01
S	2.26	0.05
その他		
T	3.14	0.06
U	0.20	Trace
V	2.87	0.06
W	1.30	0.03
X	0.35	Trace
Y	1.60	0.03
Z	0.80	0.02
平均**	3.84±0.67	0.09±0.01

*0.01μg以下の含量は，Traceとして示した。
**平均値±SEM

Table 2　市販茶漬けの素に含まれるビタミンB_{12}含量

種類	ビタミンB_{12}含量 (μg/100g)	(μg/2g)
A	3.05	0.24
B	1.80	0.14
C	2.25	0.18
D	4.60	0.37
E	5.25	0.42
F	1.15	0.09
G	1.50	0.12
H	2.00	0.16
I	0.90	0.07
J	0.25	0.02
K	1.95	0.16
平均*	2.25±0.46	0.18±0.04

*平均値±SEM

4．結　　論

1．ふりかけのB_{12}含量は，平均3.84μg/100gであり，一食あたり約0.1μgが摂取できると評価された。

2．茶漬けの素のB_{12}含量は，平均2.25μg/100gとなり，一食あたり約0.18μgのB_{12}が摂取できると評価された。

謝　　辞

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Low folate status increases chromosomal damage by X-ray irradiation

KAORI ENDOH[1,2], MASAHIRO MURAKAMI[1], RISA ARAKI[3], CHIZUKO MARUYAMA[3] & KEIZO UMEGAKI[2]

[1]*Department of Domestic Science, Kyoritsu Women's University, Chiyoda-ku, Tokyo,* [2]*Division of Food Analysis for Health Research, National Institute of Health and Nutrition, Shinjuku-ku, Tokyo, and* [3]*Department of Food and Nutrition, Japan Women's University, Bunkyo-ku, Tokyo, Japan*

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Abstract

Purpose: To examine how folate status influences chromosomal damage following X-ray irradiation.

Material and methods: In an animal study, mice were fed either a low, basal, or high folic acid diet (0, 2, or 40 mg/kg diet, respectively) for 4 weeks, and then given total body irradiation (TBI) at 0.5 Gy. In a human study, subjects were supplemented with folic acid (800 μg/day) for 2 weeks and their peripheral blood was irradiated at 0.5 Gy *in vitro*. Chromosomal damage was determined by micronucleus assay.

Results: In an animal study, TBI-induced chromosomal damage was higher and folate concentration was lower in the bone marrow of the low folic acid group compared to the other two diet groups. The chromosomal damage and folate concentration were comparable between the basal and high folic acid groups. TBI administered to mice decreased folate in the plasma, erythrocyte and bone marrow. In a human study, supplementation with folic acid increased plasma folate, but did not influence either plasma homocysteine or X-ray-induced chromosomal damage in lymphocytes.

Conclusion: Low folate status increases susceptibility to X-ray-induced chromosomal damage, but excessive folic acid supplementation under normal conditions yields no further protection due to folate saturation in the target tissue.

Keywords: *Folate, chromosomal damage, irradiation, bone marrow, diet*

Introduction

Irradiation produces oxygen radicals and thereby induces oxidative damage to biomolecules such as lipids, proteins, and DNA (Riley 1994). Oxidative damage can be controlled by antioxidant defence systems such as antioxidants and antioxidant enzymes. As endogenous antioxidants do not have the capacity to prevent oxidative damage completely (Halliwell 1994), exogenous antioxidants from food have been gaining much attention for the prevention of oxidative damage. We have examined the effects of antioxidant vitamins and oxidative damage in DNA and lipids, especially in the bone marrow of mice and rats given total body irradiation (TBI) (Umegaki et al. 1995, 1999a, 2001, Umegaki & Ichikawa 1994, Yoshimura et al. 2002). In those studies, we showed that bone marrow is particularly susceptible to radiation-induced oxidative damage, which was accompanied by a marked decrease in antioxidants in the bone marrow. We have also examined the preventive effect of dietary antioxidants such as vitamin C, vitamin E and beta-carotene against radiation-induced DNA damage in bone marrow, and found that few antioxidant vitamins prevent radiation-induced DNA damage (Sugisawa et al. 2002, Umegaki et al. 1994a, 1994b, 1997).

Various epidemiological studies strongly suggest that a low folate status increases the risk of cancer (Choi & Mason 2000, Duthie et al. 2002, Kim 1999). In mouse studies, it has been reported that mice with a low folate status showed an increase in DNA damage in the bone marrow after treatment with caffeine and sodium arsenate (MacGregor et al. 1990, McDorman et al. 2002). The mechanism of enhanced DNA damage with a low folate status could be related to DNA synthesis and/or repair based on one-carbon metabolism by folate (Choi &

Correspondence: Keizo Umegaki, Division of Food analysis for Health Research, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo, 162-8636 Japan. Tel: +81 3 3203 8063. Fax: +81 3 3205 6549. E-mail: umegaki@nih.go.jp

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Mason 2000). On the other hand, it has been reported that the radical scavenging capacity of folate is equivalent to that of vitamin C (Stocker et al. 2003). If folate acts as a radical scavenger in the body, folate could prevent radiation-induced DNA damage in two ways: by the modification of DNA synthesis and/or repair through one-carbon metabolism, and as a radical scavenger that traps oxygen radicals produced by irradiation. Several *in vitro* studies have shown that folate affects chromosomal stability (Duthie et al. 2002, Fenech 2001). Low folate status and gamma-ray irradiation increase chromosomal damage *in vitro* (Beetstra et al. 2005, Courtemanche et al. 2004). To our knowledge, however, only a few reports have been published that have examined these topics *in vivo*.

In this study, we fed mice various folic acid diets, and then gave them TBI to examine changes in folate concentration and the modification of chromosomal damage in bone marrow. We also examined radiation-induced chromosomal damage in a human study, where folic acid was given as a dietary supplement to healthy volunteers for 2 weeks and their peripheral blood was sampled before and after the study to determine radiation-induced chromosomal damage in lymphocytes *in vitro*. Micronucleus (MN) assays in mice (Hayashi et al. 1994) and human lymphocytes (Fenech 2000) are able to detect DNA damage better than the measurement of 8-hydroxydeoxyguanosine (Kobus et al. 1993), a well-known biomarker of oxidative DNA damage, and X-ray-induced damage is mainly related to oxidative damage (Riley 1994). Therefore, in this study we used the MN assay to evaluate X-ray-induced DNA damage.

Materials and methods

Materials

Fetal bovine serum, Roswell Park Memorial Institute (RPMI) 1640 medium, antibiotic solution, phytohemagglutinin, Hanks' Balanced Salt Solution, and L-glutamine were purchased from GIBCO (Grand Island, NY, USA). Cytochalasin B and trypsin were obtained from Sigma (St Louis, MO, USA). Giemsa's Solution and 2-thiobarbituric acid were from Merck (Darmstadt, Germany). Ficoll-Paque was from Amersham Bioscience (Uppsala, Sweden). Folinic acid calcium salt pentahydrate was from Fluka (Buchs, Switzerland). *Lactobacillus rhamnosus* (American Type Culture Collection (ATCC) number 27773) was from the ATCC (Rockville, MD, USA). Folic Acid Casei Medium was obtained from Becton Dickinson (Sparks, MD, USA). Rat serum was purchased from Nippon Biotest Laboratories Inc. (Tokyo, Japan). Other chemicals were obtained from Wako Pure Chemical Ltd. (Osaka, Japan).

Animals and diets

Four-week-old male ICR mice (Japan Clea, Tokyo, Japan) were individually housed in polypropylene cages in a room with a constant temperature of 23 ± 1°C and a 12-h light-dark cycle. The mice were fed either low, basal or high folic acid diets (folic acid; 0, 2, 40 mg/kg diet, respectively), which were prepared based on the AIN-93G formula (Reeves 1997). The composition was as follows: cornstarch 397.486 g/kg diet, vitamin-free casein (>85% protein) 200 g/kg diet, dextrinized cornstarch (90–94% tetrasaccharides) 132 g/kg diet, sucrose 100 g/kg diet, soybean oil (no additives) 70 g/kg diet, fiber 50 g/kg diet, mineral mix (AIN-93G-MX) 35 g/kg diet, folic acid-free vitamin mix (AIN-93-VX) 10 g/kg diet, L-cystine 3 g/kg diet, choline bitartrate (41.1% choline) 2.5 g/kg diet, *tert*-butylhydroquinone 14 mg/kg diet. Mice were divided into six groups (six mice per group), given *ad libitum* access to food and tap water, and weighed once every 3 days throughout the study. After a 4-week feeding period, half of the mice in each diet group received TBI with X-rays by a soft X-ray unit (OM-150-RS; Ohmic, Tokyo, Japan) at a dose rate of 0.5 Gy/min (140kV, 9mA). The beam was filtered through Cu (0.1 mm) and Al (0.2 mm). For the analysis of chromosomal damage in bone marrow, peripheral blood was taken from the tail after an overnight fast at 44 h post TBI. The mice were anesthetized with sodium pentobarbital and blood was taken from the large abdominal vein with a heparinized syringe. Part of the blood was collected in a capillary tube and centrifuged at 10,000*g* for 10 min for hematocrit measurement and the rest of the blood was immediately centrifuged (RS-20IV; TOMY SEIKO Co., LTD, Tokyo, Japan) at 1,500*g* for 15 min at 4°C to prepare plasma. The liver was immediately removed, frozen, and stored at −80°C until analyses. The bone marrow cells were prepared from the femurs and tibiae according to the method reported previously (Umegaki & Ichikawa 1994). For the analysis of folate, the blood was immediately mixed with 0.5% ascorbic acid and the bone marrow cell samples were similarly mixed with 0.5% ascorbic acid sodium salt for the analysis of folate. The plasma was immediately mixed with 5 volumes of 6% metaphosphoric acid for the analysis of vitamin C. In an *in vitro* study, mice plasma was prepared and irradiated with X-ray at a dose of 3Gy to examine changes in the concentration of folate and vitamin C in the sample. All procedures were in accordance with the National Institute of Health and Nutrition guidelines for the care and use of laboratory animals.

Human study

The subjects were 22 male healthy volunteers in their 20s (22.7 $\pm$ 2.0 years old). They were randomly divided into a placebo ($n = 10$) or folic acid group ($n = 12$) and their diets were supplemented with folic acid 800 μg/day (Nature Made Folic acid, Otsuka Pharmaceuticals, Tokyo, Japan) or a placebo (Lactose tablets) for 2 weeks. They consumed folic acid supplements three times a day just after each meal. During the study, the consumption of the supplement was recorded and the subjects were instructed to maintain their habitual diet and physical activity. Before and after the supplementation period, their peripheral blood was collected after an overnight fast. Chromosomal damage in the lymphocytes and the plasma concentration of folate were determined. The Japan Women's University Human Ethics Committee approved this study, and written informed consent was obtained from the subjects.

Analytical methods

Folate was analysed as follows: The liver and bone marrow cells samples were homogenized (Polytron PT3100; Kinematica, Lucerne, Switzerland) or sonicated (Ultra Sonic processor; Seiko Sonics & Materials, Tokyo, Japan) with 9 volumes of folate extraction buffer (50 mM phosphate buffer, 0.5% ascorbic acid, final pH 6.1). These samples were autoclaved (KS-323; Tomy Seiko Co., LTD, Tokyo, Japan) at 121°C for 30 min, cooled in an ice-water bath, and centrifuged at 2000 *g* for 15 min. The supernatant (150 μl) was incubated with 100 μl rat serum conjugase and 2.79 ml folate extraction buffer at 37°C for 6 h. The whole blood samples were incubated at 37°C for 30 min to hydrolyze pteroylpolyglutamates with endogenous conjugase. The plasma and conjugase-treated liver, bone marrow, and whole blood samples were diluted with 0.5% ascorbic acid sodium salt, if necessary (O'Leary & Sheehy 2001). The folate concentration in the samples was analysed by microbiological assay using 96-well microplate and glycerol-cryoprotected *Lactobacillus rhamnosus* methods (Horne 1997). Erythrocyte folate concentration was calculated using the following formula: [whole blood folate − {plasma folate $\times$ (1-hematocrit)}]/hematocrit (Bills et al. 1992).

In the mouse study, chromosomal damage in the bone marrow cells was evaluated with the micronucleus assay using peripheral blood according to the method reported previously (Umegaki et al. 1994c). Briefly, reticulocytes with and without micronuclei (MN) among over 1000 cells were counted, and chromosomal damage was expressed in terms of the number of cells containing MN per 1000 cells by fluorescence microscope (Olympas BHS, Tokyo, Japan). In the human study, chromosomal damage to lymphocytes was assessed by the cytokinesis-block micronucleus (CBMN) assay according to the method of Fenech (2000). Briefly, peripheral whole blood from each human subject was divided into two parts (0.5 ml each) in culture tubes. One sample was not irradiated and the other was irradiated with X-rays at 0.5 Gy. The two samples from each subject were each added to 4.5 ml of RPMI-1640 culture medium supplemented with 1% L-glutamine, 10% fetal bovine serum and 1% antibiotics. Culture was immediately initiated by adding phytohemagglutinin. Cytochalasin B (final concentration 6.6 μg/ml) was added at 44 h to induce binucleated cells. The cells were harvested at 72 h and slides were prepared using a cytocentrifuge (Cytospin-3; Shandon Southern Products, Cheshire, UK). At least 500 binucleated cells with preserved cytoplasma were scored for each culture using a microscope. Percentages of lymphocytes with MN in the binucleated cells were calculated from each culture.

Vitamin C was analysed by high pressure liquid chromatography (HPLC) with an electrochemical detector (Shiseido, Tokyo, Japan) according to the method reported previously (Umegaki et al. 1999b). Plasma homocysteine concentration was determined by HPLC with a fluorescence detector (Shimadzu, Kyoto, Japan) by the method of Frick et al. (2003). Protein was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) and Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Statistical methods

Data are presented as the mean $\pm$ standard deviation (SD) for individual groups. Statistical analyses of the data were performed with analysis of variance followed by a post hoc test of Fisher's Protected Least Significant Difference. Correlation analysis between TBI-induced chromosomal damage and folate concentration in various tissues was performed with Pearson's correlation coefficient. These statistical analyses were performed with Stat View 5.0 (Abacus Concepts, Inc., Berkeley, CA, USA). Values of $p < 0.05$ were considered significant.

Results

Animal study

The final body weight and relative liver weight did not differ among the groups (Table I). Relative spleen weight was lower in the TBI groups, but no difference was found among the three diet groups. The proportion of micronucleated (MNed) reticulocytes in the peripheral blood, an indicator of