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

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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

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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 ($n = 2$).

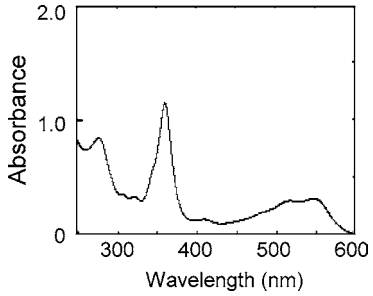

Figure 1. Ultraviolet–visible spectrum of the purified compound from the dried *Aphanizomenon* cells. A portion of the purified preparation was dissolved in 3.0 mL of distilled water. The spectrum was measured with a Shimadzu spectrophotometer (UV-1600) at room temperature, quartz cuvettes (3.0 mL, $d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

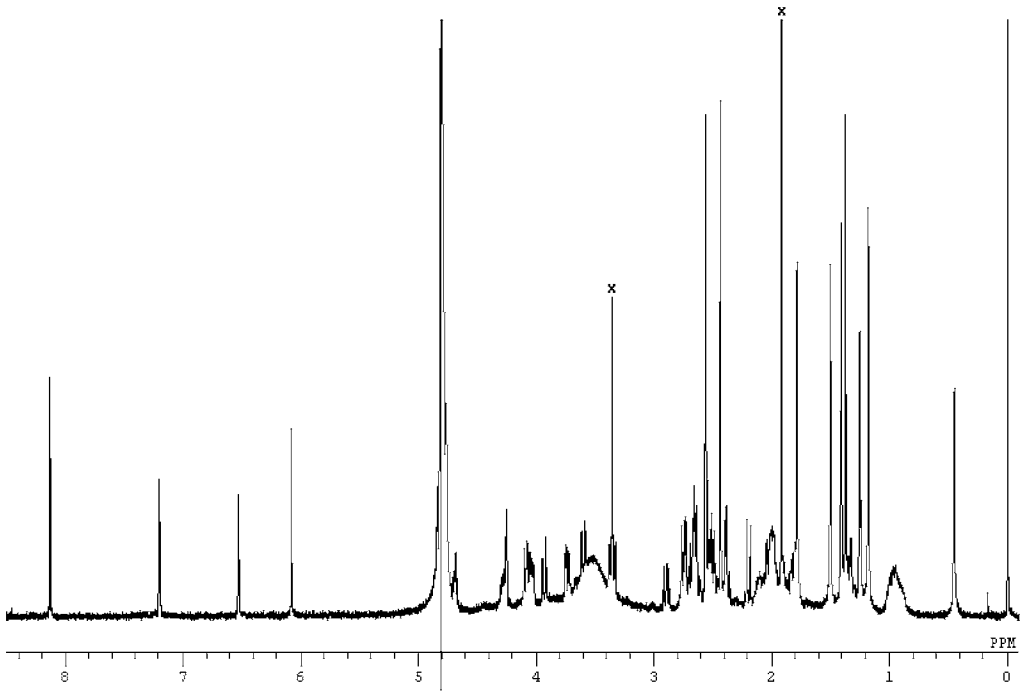

Figure 2. ^{1}H NMR spectrum of the corrinoid purified from dried *Aphanizomenon* cells (500 MHz, D_2O).

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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