Characterization of a Vitamin B_{12} Compound from Unicellular Coccolithophorid Alga (*Pleurochrysis carterae*)

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A unicellular coccolithophorid alga, *Pleurochrysis carterae*, contained 125.4 ± 1.2 μg of vitamin B_{12} per 100 g dry cell weight of the lyophilized algal cells. A vitamin B_{12} compound was purified from the lyophilized algal cells and partially characterized. The silica gel 60 TLC and reversed-phase HPLC patterns of the purified pink-colored compound were identical to those of authentic vitamin B_{12}, but not those of vitamin B_{12} analogues inactive for humans. When 22-week-old B_{12}-deficient rats which excreted substantial amounts of methylmalonic acid (75.5 ± 12.3 mg/day) in urine were fed the *P. carterae* (10 g per kg diet)-supplemented diet for 12 d, urinary methylmalonic acid excretion (as an index of vitamin B_{12} deficiency) of the rats became undetectable and hepatic vitamin B_{12} level of the rats was significantly increased.

Keywords: *Coccolithophorid alga; Lactobacillus delbruekii; intrinsic factor; Pleurochrysis carterae; vitamin B_{12}*

INTRODUCTION

Vitamin B_{12} (B_{12}) is an essential nutrient for animal cells, but not for plant cells (*1*). Some photosynthetic green algae which have no requirement of B_{12} for growth can take up and accumulate exogenous B_{12} (*2*). Although substantial amounts of B_{12} have been found in some edible algae including seaweed, most of the algal B_{12} have been reported to be present as inactive B_{12} analogues (*3*−*6*). Indeed, spirulina (*Spirulina* sp.) tablets available as an algal health food contain substantial amounts of B_{12} analogues inactive for humans (*4, 7*). Several workers have reported that the B_{12} analogues found in edible algae seem to block B_{12}-metabolism in mammals (*8, 9*). Thus, it is still unclear whether the B_{12} found in the algal cells used for human health foods and food supplements is true B_{12} or a harmful B_{12} analogue because there is little information available on the chemical properties of algal B_{12}.

The coccolithophorid alga *Pleurochrysis carterae* is a unicellular marine calcareous phototroph and produces $CaCO_3$ structures (calcified scales around the cells), which are called coccoliths. Takenaka et al. (*10, 11*) have clarified the safety of the lyophilized algal cells for human consumption as a human food supplement. The calcium-rich *P. carterae* cells lyophilized have been already used for a human health food (mainly as a calcium supplement). If the algal cells contain substantial amounts of biologically active B_{12}, the cells would be suitable for use as a human food supplement as a rich source of B_{12} as well as calcium.

Here, we determined B_{12} concentrations of the lyophilized *P. carterae* cells, and also investigated the effects on B_{12} status of feeding the algal cells to B_{12}-deficient rats.

MATERIALS AND METHODS

Materials. Cyano-vitamin B_{12} (CN−B_{12}) was obtained from Sigma (St. Louis, MO). Silica gel 60 thin-layer chromatography (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). A B_{12} assay medium for *Lactobacillus delbruekii* ATCC 7830 was obtained from Nissui (Tokyo, Japan). A reversed-phase HPLC column (Wakosil-II 5C18RS, ϕ 4.6 × 150 mm; particle size, 5 μm) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Culture of *P. carterae* Cells. *P. carterae* cells were cultured for 7 d in a modified Eppley medium (*12*) in raceway ponds (3 m × 1.5 m × 0.1 m) at 22 °C under a continuous illumination (40 μE/m^2/s) as described previously (*10*). The cells were harvested by centrifugation, immediately dehydrated by freeze-drying, and used for experiments.

Assay of Total B_{12}. Total B_{12} was assayed by the microbiological method with *L. delbruekii* ATCC 7830 and a B_{12} assay medium (Nissui), 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 (*13*).

Extraction of Total B_{12} from Lyophilized *P. carterae* Cells. All procedures were done in the dark. One g of the lyophilized cells was added to 100 mL of 0.1 mol/L acetate buffer, pH 4.8.

Total B_{12} was extracted from the cell suspension by the method of boiling with KCN at acidic pH (*14*); specifically, 30 mg of KCN was added to the cell suspension, which was boiled for 30 min at 98 °C. The extraction procedures were done in a

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Dalton (Tokyo, Japan) draft chamber. The boiled cell suspension was centrifuged at 10000*g* for 10 min. The supernatant was used for the B_{12} assay.

Purification of a B_{12} Compound from Lyophilized *P. carterae* Cells. About 500 g of the lyophilized algal cells was added to 4 L of 0.1 mol/L acetate buffer, pH 4.8. Total B_{12} 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 10000*g* for 10 min and the supernatant was used for the chemiluminescence B_{12} assay. Amberlite XAD-4 resin (1 kg) was washed with 5 L of methanol and then equilibrated with distilled water. The resin was added to the supernatant fraction and stirred for 4 h at room temperature in the dark. The resin suspension was passed through a glass funnel (Buchner type) with a glass filter (type 25G1, Iwaki, Tokyo, Japan) and the resin was washed with 2 L of distilled water. The washed resin was added to 2.5 L of 80% (v/v) methanol solution, and stirred for 3 h at room temperature in the dark. The resin suspension was passed through the glass funnel. B_{12} was determined by the chemiluminescence B_{12} assay method. The eluent containing a B_{12} compound was pooled, evaporated to dryness under reduced pressure, and dissolved in 60 mL of distilled water. Each 20 mL of the solution was put on a column (24 × 70 mm) of Cosmosil 140C18-OPN (Nacalai Tesque) which was washed with 75% (v/v) ethanol solution and then equilibrated with distilled water, and eluted with a linear gradient (0−25% v/v) of ethanol. The fractions containing a B_{12} compound were pooled, evaporated to dryness under reduced pressure, and dissolved with a small amount of distilled water. The concentrated solutions were combined and purified by HPLC using Shimadzu HPLC apparatus (two LC-10ADvp pumps, DGV-12A degasser, SCL-10Avp system controller, SPD-10Avvp ultraviolet−visible detector, CTO-10Avp column oven, 100-μL sample loop, C−R6A chromatopac integrator). The sample (80 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 B_{12} compound was isocratically eluted with the same solution, monitored by measuring absorbance at 361 nm, and collected at 1 mL with a Bio-Rad Laboratories fraction collector (model 2110). The fractions containing a B_{12} compound were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solutions were combined and put on a silica gel 60 TLC sheet and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) as a solvent in the dark at room temperature. A pink-colored spot on the dried TLC sheet was 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 further purified by the HPLC under the same conditions. The final pink-colored fractions were collected, evaporated to dryness under reduced pressure, dissolved in 20 μL of distilled water, and used as a purified B_{12} compound.

Analytical TLC and HPLC. The concentrated solutions (2 μL) of the B_{12} compound purified from the *P. carterae* cells and authentic CN−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. The TLC sheets were dried, and R_f values of the pink-colored spots of these compounds were determined.

In the case of HPLC, the concentrated solutions (2 μL) of the purified B_{12} compound and CN−B_{12} were analyzed with the reversed-phase HPLC column (Wakosil-II 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 Spectra. The spectra were measured with a Shimadzu spectrophotometer (UV-1600) at room temperature. Quartz cuvettes (d = 1 cm) were used. A portion of the purified B_{12} compound was dissolved in 1 mL of distilled water.

Storage Experiments. The lyophilized *P. carterae* cells (10 g) and an oxygen absorber (Sequl AP-100, Nisso, Japan) were transferred into a 25-mL amber glass bottle sealed with a rubber septum and cap. The bottles were wrapped with aluminum sheets and stored at 4 and 25 °C in Sanyo incubators (MIR-152, Tokyo, Japan) for 6 months. The *P. carterae* cells (1 g) were sampled every month and total B_{12} concentration was analyzed by the chemiluminescence B_{12} assay method.

Animals and Diets. B_{12}-deficient rats were prepared by feeding with a B_{12}-deprived diet [400 g (per kg of diet) of soybean protein (Fuji Oil Ltd., Osaka, Japan), 443 g of glucose anhydrous (Nacalai Tesque, Ltd., Kyoto, Japan), 100 g of soybean oil (Nacalai), 50 g of salt mixture, 5 g of *dl*-methionine (Nacalai), 5 g of B_{12}-free vitamin mixture, and 2 g of choline chloride (Nacalai)] as described previously (*15*).

Male weanling Wistar rats (4 weeks old, 50.0 ± 5.0 g), born to 14-week-old parents fed on the B_{12}-deficient diet for 8 weeks, were used. Parent rats aged 6 weeks were obtained from KIWA Laboratory Animals Co. Ltd. (Wakayama, Japan). The 4-week-old weanling rats were housed at 24 °C in a room with a 12-h light:dark cycle. They were given the B_{12}-sufficient and -deficient diets under pair meal feeding for 18 weeks. All experimental procedures involving laboratory animals were approved by the Animal Care and Use Community of Osaka Prefecture University.

Feeding Experiments of Lyophilized *P. carterae* Cells. Effects of feeding the lyophilized *P. carterae* cells on growth and urinary methylmalonic acid (MMA) levels in the B_{12}-deficient rats were studied. Cellulose powder (10 g; Nacalai) was added to the original B_{12}-deprived diet and used as the B_{12}-deficient diet. The CN−B_{12}-supplemented diet was identical to the B_{12}-deficient diet, except that 10 μg per kg diet was included. A 10-g aliquot of the lyophilized *P. carterae* cells (containing 1.05 μg/g dry weight) was added to the original B_{12}-deprived diet instead of cellulose powder, and used as *P. carterae*-supplemented diet. The B_{12}-deficient 22-week-old rats were used and given free access to the two experimental diets and tap water for 12 d.

Urinary Methylmalonic Acid Assay. The urine of the CN−B_{12}- and *P. carterae*-supplemented rats were sampled for 24 h in individual metabolic cages at the indicated times during the experiments. Urinary MMA was assayed by HPLC as described previously (*16*).

Extraction and Assay of B_{12} from Rat Liver. After food was withheld from rats overnight, rats were killed by decapitation under diethyl ether anesthesia. Livers were washed with a chilled 0.9% (w/v) NaCl solution, weighed, and stored at −80 °C until analysis. A portion (1 g) of the liver was cut into small pieces with a razor blade, homogenized in 10 mL of 0.9% (w/v) NaCl solution, and centrifuged at 3000*g* for 30 min at 4 °C. The supernatant was used as a liver homogenate. The extraction mixture (9.9 mL) contained 10 mmol/L acetate buffer, pH 4.8, 25 μg of KCN, and the live homogenate (0.1 mL). The mixture was autoclaved for 10 min at 120 °C. Metaphosphoric acid (10%; 0.1 mL) was added to the mixture, and the mixture was centrifuged at 3000*g* for 10 min. The supernatant was used as a sample for the microbiological B_{12} assay.

Statistics. Statistical analysis was performed using GB-STAT$_{TM}$5.4 (Dynamic Microsystems, Inc., Silver Spring, MD). Two-way ANOVA was used with Tukey/Kramer procedure for the lyophilized *P. carterae*-feeding experiment in the B_{12}-deficient rats. The Mann−Whitney *U*-test was used for hepatic B_{12} concentrations of the rats. Differences were considered significant if $P < 0.05$. Values are means ± SD.

RESULTS AND DISCUSSION

B_{12} Concentration of Lyophilized *P. carterae* Cells. Total B_{12} concentration of the lyophilized *P. carterae* cells was determined by the chemiluminescence

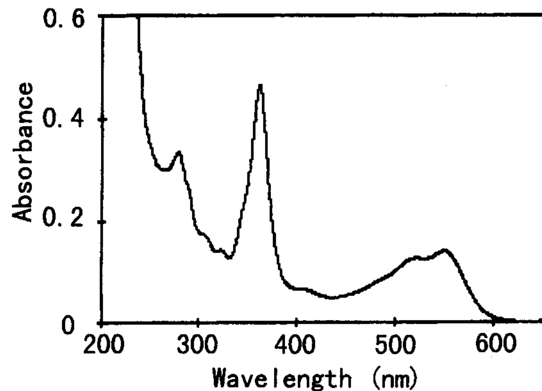

Figure 1. Ultraviolet–visible spectrum of the purified B_{12} compound from lyophilized *P. carterae* cells. A portion of the final purified preparation was dissolved in distilled water. Detailed procedures are described in the text.

B_{12} analyzer with hog intrinsic factor (IF), the most specific B_{12}-binding protein. The algal cells contained 125.4 ± 1.2 μg of B_{12} per 100 g dry weight of the algal cells; the identical values were also obtained by *L. delbruekii* ATCC7830 bioassay.

Dried laver sheet (nori) appears to be most widely eaten among the edible seaweed types. Our previous study indicated that the edible purple laver (*Porphyra yezoensis*) used as nori contains a substantial amount of biologically active B_{12} (*17*). The B_{12} concentration was about 2.4-fold greater in the lyophilized *P. carterae* cells than in the purple laver.

Purification and Characterization of a B_{12} Compound from Lyophilized *P. carterae* Cells. B_{12} compound was purified and partially characterized from the lyophilized algal cells. The final purified preparation gave a single pink-colored spot by the silica gel 60 TLC and a single peak by the reversed-phase HPLC, indicating that the B_{12} compound was purified to homogeneity.

The ultraviolet–visible spectrum of the B_{12} compound purified from the algal cells showed a typical absorption of cobalt-containing corrinoid (Figure 1); λmax/nm (absorbance) were at 551.0 (0.141), 522.0 (0.126), 361.5 (0.466), and 278.5 (0.335). The purified B_{12} compound and authentic CN–B_{12} were analyzed by the silica gel 60 TLC and reversed-phase HPLC. The R_f values (0.25 and 0.61 in solvents I and II, respectively, by the TLC) of the purified B_{12} compound were identical to the values of authentic CN–B_{12}, of which the retention time (8.5 min by the HPLC) was also identical to that of the purified B_{12} compound. These results indicate that the pink-colored compound purified from the algal cells is true B_{12}, but not B_{12} analogues inactive for mammals.

Loss of B_{12} in Lyophilized *P. carterae* Cells during Storage. Loss of B_{12} during storage of the lyophilized *P. carterae* cells was determined (Figure 2). There was no loss of total B_{12} during 6-month storage of the cells at 4 and 25 °C. The results indicate that the B_{12} compound is stable in the lyophilized algal cells.

Feeding of Lyophilized *P. carterae* Cells in B_{12}-Deficient Rats. To clarify whether the B_{12} compound found in the lyophilized *P. carterae* cells is absorbed in the mammalian intestine and accumulated in the liver, feeding experiments of the *P. carterae*-supplemented diet to the 22-week-old B_{12}-deficient rats were conducted.

The body weights of the 22-week-old rats fed with the B_{12}-deficent diet were ~18.1% of those of the B_{12}-sufficient rats under pair meal feeding. The 22-week-old B_{12}-deficient rats excreted 75.5 ± 12.3 mg of MMA per day in urine (as an index of B_{12} deficiency). Severely B_{12}-deficient (14 weeks old) rats have been reported to excrete about 214.3 μmol (about 25.3 mg) MMA per day in urine (*15*). These results indicated that the 22-week-old rats fed with the B_{12}-deficient diet developed a severe B_{12} deficiency.

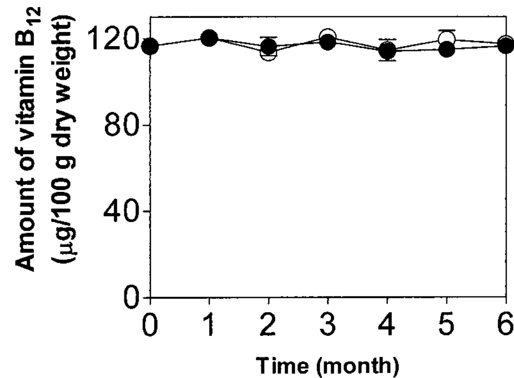

Figure 2. Loss of B_{12} in lyophilized algal cells during storage. The lyophilized algal cells were stored at 4 °C (●) and 25 °C (○) for the indicated times.

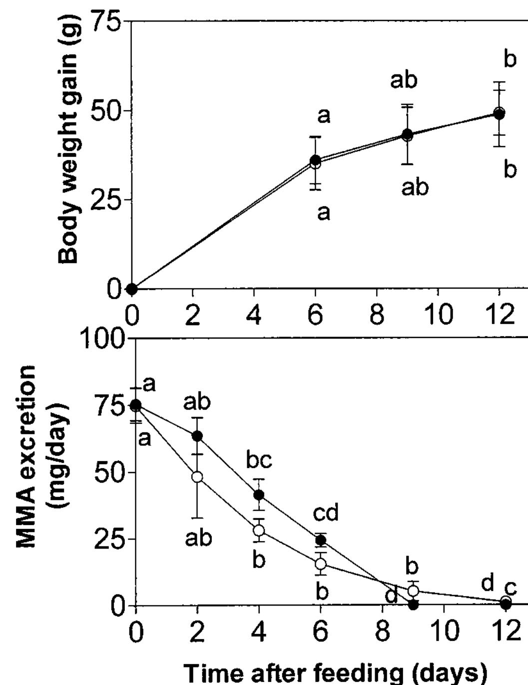

Figure 3. Effects of feeding *P. carterae*-supplemented diet on the body weight and urinary MMA excretion of the 22-week-old B_{12}-deficient rats. The 22-week-old B_{12}-deficient rats were fed CN–B_{12}-supplemented (●) and *P. carterae*-supplemented (○) diets for 12 d. Group means with different letters on the same line are significantly different ($P < 0.05$). Data represent means ± SD ($n = 4$).

In the rats fed the CN–B_{12}- and the *P. carterae*-supplemented diets, the MMA excretion became undetectable at 11 d after the feeding (Figure 3). There was no significant difference in body weight among the rats fed the two experimental diets for 12 d.

Total B_{12} levels were assayed in the livers of the rats fed the CN–B_{12} and *P. carterae*-supplemented diets for 12 d (Table 1). The hepatic total B_{12} levels of the CN–B_{12}- and *P. carterae*-supplemented rats were about 5.5- and 5.8-fold greater, respectively, than the 22-week-old B_{12}-deficient rats. The increased total B_{12} level in the

Table 1. Hepatic B_{12} Concentrations of the B_{12}-Deficient Rats Fed the CN−B_{12} and *P. Carterae*-Supplemented Diets[a]

rat type	B_{12} concentration (ng/g wet liver weight)
B_{12}-deficient rats	18.2 ± 1.5^{a}
CN−B_{12}-supplemented rats	99.3 ± 1.2^{b}
P. carterae-supplemented rats	105.1 ± 7.0^{b}

[a] The mean values within a column with different superscript letters are significantly different ($n = 4$ rats/group), $P < 0.05$.

P. carterae-supplemented rats was not significantly different from that in the CN−B_{12}-supplemented rats.

The results presented here indicate that the lyophilized *P. carterae* cells contain substantial amounts of biologically active true B_{12}, and that feeding of the lyophilized algal cells significantly improves B_{12} status in the B_{12}-deficient rats. Although our results might imply that the B_{12} compounds from the lyophilized algal cells are also active in humans, bioavailability of the algal B_{12} against humans remains to be determined in detail because rat metabolism is not necessarily similar to human metabolism.

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