

Table 1. Vitamin B₁₂ Contents^a of the *Chlorella* Tablets Commercially Available for Human Health Food

Chlorella tablet	Vitamin B ₁₂ contents (μg/100 g dry weight)		
	claim on bottle	microbiological assay	chemiluminescence assay
A	20–50	241.0 ± 6.9	200.9 ± 4.1
B	60–100	285.7 ± 2.3	211.6 ± 2.9
C	50–150	201.3 ± 19.9	208.2 ± 4.5

^aAll values obtained represent mean ± SEM (n = 3).

pure hog intrinsic factor (IF–CM method). The cell extracts were directly applied to the IF–CM analyzer as described by Watanabe et al. (11). They were diluted with distilled water up to a B₁₂ concentration range of 10–100 ng/L and used as samples for the microbiological method. The turbidity (%T) of the test culture of *L. leichmannii* ATCC7830 grown at 37 °C for 16–21 h was measured at 600 nm with the UV-1600 UV-vis spectrophotometer according to the manufacturer's recommended method.

Purification of a Corrinoid Compound from *Chlorella* Tablets. The *Chlorella* tablets (B tablets shown in Table 1) were used in the following purification experiments. The amount of corrinoid compound in the *Chlorella* tablets used for the purification experiments was determined to be 223 μg and 205 μg of B₁₂ per 100 g dry weight by the microbiological and IF–CM methods, respectively. *Chlorella* tablets (400 g) were powdered by the use of the food mill and added to 4 L of 0.25 mol/L acetate buffer, pH 4.8, containing 0.2% (w/v) KCN. Total corrinoid was extracted under the same conditions described above. Amberlite XAD-4 (1 kg) resin (after washing with 10 L of methanol and then equilibration with distilled water) was added to the extract and stirred for 3 h at room temperature in the dark. The resin suspension was passed through a glass funnel with filtered disk (type 25G1, Iwaki, Tokyo, Japan), and the resin was washed with 5 L of distilled water. The washed resin was added to 2 L of an 80% aqueous methanol and stirred for 3 h at room temperature in the dark. The resin suspension was passed through the glass funnel. The eluate containing corrinoids was pooled, and evaporated to the final volume of 30 mL under reduced pressure. The solution was put on a 70 mm × 28 mm column of Cosmosil 140C18-OPN, which was washed with ethanol and then equilibrated with distilled water. Corrinoid was eluted with 100 mL of a linear gradient of 0–25% (v/v) ethanol, and fractionated. B₁₂ activity was assayed in these fractions (1 mL) by the microbiological method. The B₁₂-active fractions were pooled, and evaporated under reduced pressure. The concentrated solution was streaked on 20 cm × 20 cm silica gel 60 TLC sheets, which were developed with 2-propanol/NH₄OH (28%)/distilled water (7:1:2 v/v). The TLC sheets were dried and cut into small pieces (5 mm) by scissors. Each TLC piece was added to 5 mL of an 80% aqueous methanol and left for 24 h at 4 °C in the dark to extract corrinoids. Each soluble fraction was evaporated to dryness under reduced pressure, and dissolved in 1 mL of distilled water. The B₁₂-active fractions were pooled, evaporated to dryness under reduced pressure, and dissolved in 1 mL of distilled water. The concentrated solution was purified by HPLC using a Shimadzu HPLC apparatus (LC-6A pump, SPD-6A spectrophotometer, CTO-6A column oven, C-R6A chromatopac). The sample (100 μL) was put on a Wakosil-II 5C18RS reversed-phase HPLC column equilibrated with a 20% aqueous methanol solution containing 1% acetic acid at 35 °C. The flow rate was 1 mL/min. The red-colored fraction eluted from the HPLC column was collected, evaporated to dryness under reduced pressure, dissolved in 0.5 mL of distilled water, and used as a purified corrinoid compound. The corrinoid compound was purified from total amounts of 3.2 kg of the *Chlorella* tablets.

Analytical TLC and HPLC. The concentrated solutions (2 μL) of the purified *Chlorella* corrinoid compound and authentic B₁₂ were spotted on the silica gel 60 TLC sheets, which were developed with 1-butanol/2-propanol/distilled water (10:7:10 v/v) as solvent I, or with 2-propanol/NH₄OH (28%)/distilled water (7:1:2 v/v) as solvent II.

In the case of HPLC analysis, the Shimadzu HPLC apparatus was used. The solutions (20 μL) of the purified *Chlorella* corrinoid

compound and authentic B₁₂ were injected onto the reversed-phase HPLC column equilibrated with a 20% aqueous methanol solution containing 1% acetic acid at 35 °C. The corrinoids were eluted with the same mobile phase and monitored by measuring the absorbance at 278 nm. The flow rate was 1 mL/min.

UV-Vis Spectrum. The spectrum was measured with a Shimadzu UV-1600 spectrophotometer at room temperature in 1-cm quartz cuvettes. The purified corrinoid compound from *Chlorella* tablets was dissolved in 1 mL of methanol. The UV-Vis spectrum of the compound (3.2 μmol/L) had λ_{max} (absorbance) at 550 (0.032), 518 (0.029), 361 (0.0952), and 278 (0.0564) nm.

¹H NMR Spectrum. The purified corrinoid compound was dissolved in D₂O, and the ¹H NMR spectrum was measured with a JEOL JNM α-500 spectrometer. Chemical shifts are given as δ (ppm) with [2,2,3,3-d₄] sodium 3-(trimethylsilyl)propanoate as an internal standard. ¹H NMR spectral data of the corrinoid: δ 7.28 (1H, s, B4), 7.09 (1H, s, B2), 6.51 (1H, s, B7), 6.35 (1H, d, J = 3.1 Hz, R1), 6.09 (1H, s, C10), 4.73 (1H, m, R3), 4.30 (1H, m, Pr2), 4.28 (1H, t, J = 3.3 Hz, R2), 4.18 (1H, m, C3), 4.04–4.12 (2H, m, C19 and R4), 3.92 (1H, dd, J = 1.9, 13.2 Hz, R5), 3.75 (1H, dd, J = 4.0, 13.2 Hz, R5'), 3.61 (1H, d-like, J = 15.3 Hz, Pr1), 3.42 (1H, dd, J = 4.3, 11.3 Hz, C8), 3.34 (1H, d, J = 10.4 Hz, C13), 2.96 (1H, dd, J = 9.2, 14.3 Hz, Pr1'), 2.76 (2H, m, C56), 2.67 (1H, m, C18), 2.63 (1H, t, m, C55'), 2.57 (3H, s, C53), 2.54 (3H, s, C35), 2.42 (1H, d, J = 13.7 Hz, C26), 2.39 (1H, d, J = 13.7 Hz, C26'), 2.26 (6H, s, B10, B11), 1.87 (3H, s, C25), 1.45 (3H, s, C47), 1.40 (3H, s, C54), 1.39 (3H, s, C36), 1.25 (3H, d, J = 6.4 Hz, Pr3), 1.19 (3H, s, C46), 0.45 (3H, s, C20).

RESULTS AND DISCUSSION

Amount of Corrinoid Compounds Determined with the Microbiological and IF–CM Methods in *Chlorella* Tablets. Amounts of B₁₂ were assayed in three brands of the commercially available *Chlorella* tablets by both the *L. leichmannii* microbiological method and the IF–CM method. As shown in Table 1, the values determined by the microbiological method were similar to the values determined by using the IF–CM method in the three brands of the *Chlorella* tablets (A–C). These results were significantly different from the findings that the values determined with the microbiological method are ~9-fold greater than the values determined with the IF–CM method in the *Spirulina* tablets (4).

Purification and Characterization of the Corrinoid Compound from *Chlorella* Tablets. To evaluate whether the corrinoid compound found in the *Chlorella* tablets is B₁₂ or not, a corrinoid compound was purified and characterized.

The final purified preparation was pale red, and it gave a single peak on the reversed-phase HPLC. The UV-vis spectrum of the purified compound showed a typical absorption spectrum of cobalt-containing corrinoid (data not shown). The R_F values (0.22 and 0.54, in solvents I and II, respectively) for the purified compound on silica gel 60 TLC were identical to the values for authentic B₁₂, whose retention time (17.5 min) by the reversed-phase HPLC was also identical to that of the purified compound.

In the ¹H NMR spectrum of the corrinoid purified from the *Chlorella* tablets (Figure 1), two methyl groups [δ 2.26 (6H, s, B10, B11)] and three protons [δ 7.28 (1H, s, B4), δ 7.09 (1H, s, B2), and δ 6.51 (1H, s, B7)] due to the 5,6-dimethylbenzimidazolyl moiety were present. B₁₂ (5,6-dimethylbenzimidazolyl cyanocobamide) and inactive corrinoid compounds [5-hydroxybenzimidazolyl cyanocobamide (factor III), benzimidazolyl cyanocobamide, 7-adenyl cyanocobamide (pseudovitamin B₁₂), etc.] can be distinguished from one another on the basis of the structure of the base moiety. One anomeric proton [δ 6.35 (1H, d, J = 3.1 Hz, R1)] and five protons (δ 4.73, 4.28, 4.1, 3.92, 3.37) of ribose were observed in the spectrum. Eight

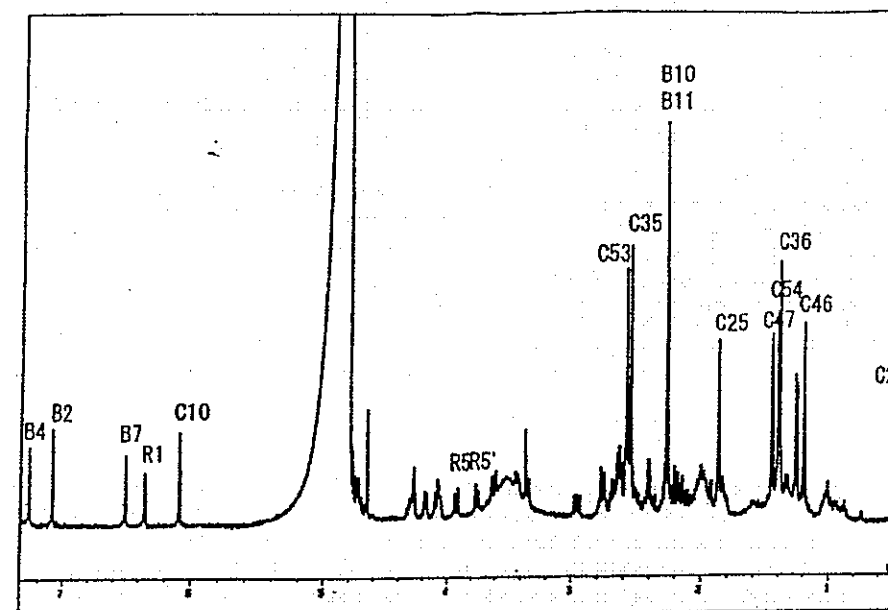


Figure 1. ¹H NMR spectrum of the corrinoid purified from *Chlorella* tablets (500 MHz, D₂O).

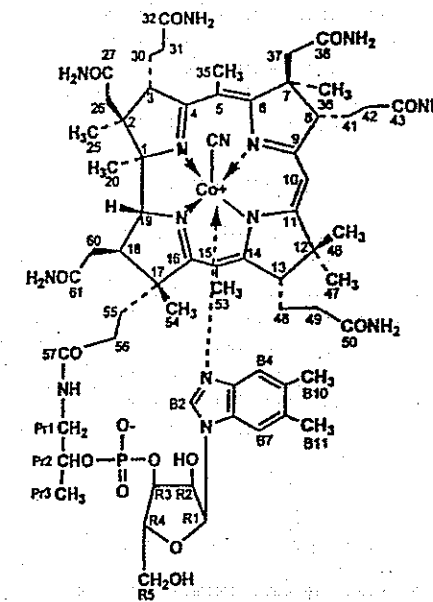


Figure 2. Structure of the corrinoid purified from *Chlorella* tablets.

singlet methyl signals of the corrin skeleton at δ 2.57, 2.54, 1.87, 1.45, 1.40, 1.39, 1.19, and 0.45, and one doublet methyl signal at δ 1.25 (3H, d, J = 6.4 Hz, Pr3) on the propyl moiety were also observed in the high-field region. These spectral data of the corrinoid compound were identical to those of authentic B₁₂ cited in the literature (12). These results indicate that the corrinoid compound purified from the *Chlorella* tablets is B₁₂ (Figure 2), but not corrinoid compound inactive for humans. Although two corrinoid compounds [major (83%) and minor (17%)] have been purified from *Spirulina* tablets and identified as pseudo-B₁₂ and B₁₂, respectively (4), only one corrinoid compound (identified as B₁₂) was purified from the *Chlorella* tablets using the similar purification procedures. Our previous study (11) has indicated that, except for foods containing substantial amounts of inactive corrinoids, the observed cor-

relation coefficient between the microbiological and IF–CM methods in foods is excellent. These observations also support the results in Table 1.

Some *Chlorella* species grown under open culture system are used for preparation of the *Chlorella* tablets. Green-algae *Chlorella* species are known to be unable to synthesize B₁₂. Watanabe et al. (13) have reported that *Chlorella vulgaris* can take up and accumulate exogenous B₁₂. These observations may imply that the *Chlorella* species used for the *Chlorella* tablets have the ability to take up and accumulate exogenous B₁₂, which may be derived from B₁₂-synthesizing bacteria concomitantly occurring in the open culture system. The results presented here indicate that *Chlorella* tablets contain substantial amounts of B₁₂ so that they may be suitable for use as a B₁₂ source, especially in vegetarians, but bioavailability of the green-algal B₁₂ to humans remains to be determined in detail.

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Purification and Characterization of a Corrinoid Compound from *Chlorella* Tablets as an Algal Health Food

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Vitamin B₁₂ content of an algal health food, *Chlorella* tablets (*Chlorella* sp.), was determined by both *Lactobacillus leichmannii* ATCC 7830 microbiological and intrinsic factor-chemiluminescence methods. The values of 200.9–211.6 μg/100 g dry weight determined by the chemiluminescence method were similar to the values (201.3–285.7 μg/100 g dry weight) determined by the microbiological method. A corrinoid compound was purified to homogeneity from the *Chlorella* tablets and characterized. The purified corrinoid compound was identified as vitamin B₁₂, on the basis of silica gel 60 TLC, C18 reversed-phase HPLC, ¹H NMR spectroscopy, and UV-Vis spectroscopy.

KEYWORDS: Vitamin B₁₂; algal health food; *Chlorella* tablets; intrinsic factor; *Lactobacillus leichmannii*

INTRODUCTION

Several studies (1–3) have reported that most of the vitamin B₁₂ or cyanocobalamin (B₁₂) found in edible algae may not be bioavailable in mammals. Watanabe et al. (4) have also demonstrated that pseudo-B₁₂ (7-adenyl cyanocobamide), a corrinoid inactive for humans, is the predominant corrinoid of commercially available *Spirulina* (*Spirulina* sp., blue-green algae) tablets, an algal health food for humans. Although Rauma et al. (5) have indicated that some seaweeds (*Chlorella* and nori), when substantially consumed by strict vegetarians, can supply adequate amounts of bioavailable B₁₂, Dagnelie (6) and Davis (7) have contradicted these results. Recently, a corrinoid compound has been purified from a dried purple laver (nori) and identified as B₁₂ (8). Feeding of the dried purple laver to B₁₂-deficient rats has indicated that the algal B₁₂ is bioavailable in rats (9). Thus, the bioavailability in humans of the B₁₂ found in edible algae is still unclear.

B₁₂-Enriched *Chlorella* (green algae) cells have been prepared and used as food for rotifer, which are in turn supplied as a food for fry in fish farming, growth of which was significantly increased by feeding of the *Chlorella* cells (10). *Chlorella* (*Chlorella* sp.) tablets have been used already for a human health food. There is, however, little information on the amount of B₁₂ contained in the commercially available *Chlorella* tablets and whether the *Chlorella* corrinoid compound is B₁₂ or an inactive corrinoid. In this study, we have purified and character-

ized a corrinoid compound from the *Chlorella* tablets and discuss the bioavailability of the compound in mammals.

MATERIALS AND METHODS

Materials. B₁₂ and a 150 mm × 4.6 mm i.d., 5 μm, Wakosil-II 5C18RS reversed-phase high-performance liquid chromatography (HPLC) column were obtained from Wako Pure Chemical Industries (Osaka, Japan). A B₁₂ assay medium for *Lactobacillus leichmannii* was obtained from Nissui (Tokyo, Japan). Silica gel 60 thin-layer chromatography (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). Amberlite XAD-4 was obtained from Japan Organo Co. (Tokyo, Japan). Cosmosil 140C18-OPN was obtained from Nacalai Tesque Inc. (Kyoto, Japan). All other reagents used were of the highest purity commercially available. The *Chlorella* tablets tested were purchased from a local market in Kochi-City, Japan.

A Shimadzu (Kyoto, Japan) UV-1600 UV-vis spectrophotometer was used for measuring the turbidity of *L. leichmannii* test culture in the microbiological method, and the absorbance of B₁₂ and the corrinoid compound purified from the *Chlorella* tablets. A fully automated ACS 180 chemiluminescence B₁₂ analyzer (Chiron Diagnostics, East Walpole, MA) was used for B₁₂ assay.

Extraction of Corrinoid Compounds in *Chlorella* Tablets. *Chlorella* tablets (three different manufacturer's products, n = 3) were used. A 1-g portion of each *Chlorella* tablet was powdered by the use of a MX-X51 food mill (National, Osaka, Japan) and added to 50 mL of 0.25 mol/L acetate buffer, pH 4.8, containing 0.2% (w/v) KCN. Total corrinoid compounds were extracted from the suspension by boiling the suspension for 60 min in a Dalton draft chamber (Tokyo, Japan). The boiled suspension was centrifuged for 10 min at 5000 rpm. The clear supernatant was obtained and used for the B₁₂ assay described below.

Assay of B₁₂. *Chlorella* corrinoid compound was assayed as B₁₂ by the microbiological method with *L. leichmannii* ATCC7830 and by the fully automated ACS 180 chemiluminescence B₁₂ analyzer using

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