Note

Characterization of Corrinoid Compounds from Edible Cyanobacterium Nostochopsis sp.

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*(Received September 26, 2011)*

Summary  Vitamin B12 content of an edible cyanobacterium, Nostochopsis sp., was determined to be 140.6±16.2 μg/100 g dry weight by a microbiological method. To evaluate whether the Nostochopsis cells contain vitamin B12 or inactive corrinoid compounds, corrinoid compounds were purified from the cells and then identified as pseudovitamin B12 (97.4±11.8 μg/100 g dry weight) and vitamin B12 (43.2±6.0 μg/100 g dry weight) on the basis of silica gel 60 TLC bioautograms and LC/ESI-MS/MS chromatograms. Vitamin B12 content was significantly increased in the Nostochopsis cells (254.8±17.6 μg/100 g dry weight) grown in the vitamin B12-supplemented medium.

Key Words  edible cyanobacteria, health foods, Nostochopsis sp., pseudovitamin B12, vitamin B12

Cyanobacteria produce numerous bioactive compounds, most of which have therapeutic properties (1). Substantial amounts of cyanobacterial cells are produced worldwide to meet the high demands of both the food and pharmaceutical industries (1). Several studies (2, 3) have reported that most of the corrinoids found in edible cyanobacteria may not be bioavailable in mammals. Watanabe (4) have also demonstrated that pseudovitamin B12 (adeninilycyanocobamide) inactive for humans is the predominant corrinoid of edible cyanobacteria, which are used as certain health foods for humans.

In northern Thailand, especially along the Nan and Mekong Rivers, an edible cyanobacterium, Nostochopsis sp., which is called “Lon,” occurs in abundance (5). Lon is consumed as a food item containing certain medical ingredients (an antipyretic and so on). Peerapornpisal et al., have reported that an extract of Nostochopsis sp. has various therapeutic activities such as anti-gastric ulcer, anti-inflammatory, anti-oxidant, and anti-hypertensive activities (5).

Although Nostochopsis sp. has been already used as a food item for humans, there is no information on how much vitamin B12 (B12) Nostochopsis cells contain or whether corrinoids found in the cells are B12 or inactive corrinoids. In this study, we have characterized corrinoid compounds from Nostochopsis cells and tried to enrich the cells with B12.

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Experimental Procedures

Materials. B12 was obtained from Sigma (St. Louis, Missouri, USA). Silica gel 60 thin-layer chromatography (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). All other reagents used were of the highest purity commercially available. Nostochopsis sp. was usually grown for 10 d in BG11-N medium (150 mL) without B12 under illumination. The Nostochopsis cells grown in the B12 (5 μg/L)-supplemented medium were used as B12-enriched cells.

Extraction and assay of corrinoids from Nostochopsis cells. Fresh Nostochopsis cells (five different lots) were used. After 2 g of each sample of the cells were suspended in 40 mL of distilled water and homogenized with an ultrasonic disruptor UD-200 (Tomy, Tokyo, Japan). Total corrinoids were extracted with boiling at acidic pH range according to the method described previously (6).

Total corrinoids were assayed by the modified method of an Escherichia coli 215 microbiological method (7). The above extract (10 mL) was partially purified with a Sep-pak® Plus C18 cartridge (Waters Corp., Milford, USA). Two micro-liters of the purified extract and authentic B12 (cyanocobalamin, 10–80 μg/L) were spotted on a filter paper (circle, 6 mm), which was put on 1.5% (w/v) agar containing a basal medium of E. coli 215 and then incubated at 30°C for about 20 h. After the filter papers were removed, the agar plate was sprayed with a methanol solution of 4% (w/v) 2,3,5-triphenyltetrazolium salt and left for 1 h at 30°C. Corrinoids were visualized as red in color indicating E. coli growth. The area of a spot of E. coli 215 growth was...
Corrinoid from *Nostochopsis* sp.

Calculated by the use of ImageJ software. Amounts of B$_{12}$ and pseudo-B$_{12}$ were calculated with the area ratio of their spots after being separated by silica gel TLC and visualized under the same conditions.

**LC/ESI-MS/MS analysis.** *Nostochopsis* cells (300 g wet weight) were suspended in 1 L of distilled water and homogenized with an ultrasonic disruptor UD-200 (Tomy, Tokyo, Japan). Each cell homogenate was supplemented with 50 mL of 0.57 mol/L acetic buffer, pH 4.5, and 0.1 g KCN, and then boiled for 30 min to extract corrinoids. The extraction and partially purified procedures were done under conditions similar to those described above. The purified extract was loaded onto an immunoaffinity column (EASI-EXTRACT® Vitamin B$_{12}$ Immunoaffinity Column (P80) R-Biopharm AG, Darmstadt, Germany) and then corrinoids were purified according to the manufacturer’s recommended protocol.

The *Nostochopsis* corrinoids, authentic pseudo-B$_{12}$, and B$_{12}$ were dissolved in 0.1% (v/v) acetic acid and filtrated with a Nanosep MF centrifuge device (0.4 μm, Pall Corp., Tokyo, Japan) to remove small particles. We analyzed an aliquot (2 μL) of the filtrate using a LCMS-IT-TOF coupled with an Ultra-Fast LC system (Shimadzu, Kyoto, Japan). Each purified corrinoid was injected into an InertSustain column (3 μm, 2.0×100 mm, GL Science, Tokyo, Japan) and equilibrated with 85% solvent A [0.1% (v/v) acetic acid] and 15% solvent B (100% methanol) at 40˚C. Corrinoid compounds were eluted with a linear gradient of methanol (15% solvent B for 0–5 min, 15–90% solvent B for 5–11 min, and 90–15% solvent B for 11–15 min). The flow rate was 0.2 mL/min. ESI conditions were determined by injection of authentic pseudo-B$_{12}$ or B$_{12}$ to the MS detector to achieve optimum parameters to detect B$_{12}$ compound parent and daughter ions. The ESI-MS was operated in positive ion mode. Argon was used for collisions.
as the collision gas. The identification of pseudo-B\textsubscript{12} (m/z 672.777) and B\textsubscript{12} (m/z 678.292) representing [M+2H]\textsuperscript{2+} was confirmed by comparison of the observed molecular ions and the retention times.

**Results and Discussion**

Amounts of corrinoids were determined in the *Nostochopsis* cells by the *E. coli* 215 microbiological method. Total corrinoid content of the cells was estimated to be 140.6±12.6 μg/100 g dry weight; the values are similar with the values of other edible cyanobacteria. *Spirulina* sp. (127.2–244.3 μg) (4), *Suizenji-nori* (*Aphanotoce sacrum*, 143.8 μg) (8), Ishikurage (*Nostoc commune*, 98.8 μg) (9). Cyanobacteria have the ability to synthesize corrinoids (10), which function as a coenzyme of methionine synthase (11).

The corrinoids found in the *Nostochopsis* cells were analyzed with the *E. coli* 215 bioautogram after being separated by silica gel 60 TLC (Fig. 1). The *Nostochopsis* corrinoids were given as two (main and minor) spots, whose Rf values were identical to those of authentic pseudo-B\textsubscript{12} and B\textsubscript{12}, respectively (Fig. 1, lane 3). The *Nostochopsis* cells were purified by a B\textsubscript{12} immunoaffinity column and then analyzed by LC/ESI-MS/MS (Fig. 2).

Authentic B\textsubscript{12} and pseudo-B\textsubscript{12} were eluted as a peak (Fig. 2A and D); its exact mass calculated from its formula (C\textsubscript{57}H\textsubscript{89}CoN\textsubscript{17}O\textsubscript{4}P) is 1354.5674 and isootope distribution data supported that B\textsubscript{12} formed predominantly the divalent ion under the LC/ESI-MS conditions. If the mass spectrum of authentic B\textsubscript{12} indicated that a divalent ion of m/z 678.2937 [M+2H]\textsuperscript{2+} was major (Fig. 2A and D); its exact mass calculated from its formula (C\textsubscript{57}H\textsubscript{89}CoN\textsubscript{17}O\textsubscript{4}P) is 1354.5674 and isootope distribution data supported that B\textsubscript{12} formed predominantly the divalent ion under the LC/ESI-MS conditions. In the case of authentic pseudo-B\textsubscript{12} whose exact mass is 1343.5375 (C\textsubscript{56}H\textsubscript{88}CoN\textsubscript{21}O\textsubscript{4}P), a divalent ion of m/z 672.7787 [M+2H]\textsuperscript{2+} was major (Fig. 2B and E). MS/MS spectra of B\textsubscript{12} and pseudo-B\textsubscript{12} indicated that the ions of m/z 359.0981 and 348.0698, respectively, due to the nucleotide moiety of each corrinoid compound, were predominantly formed (Fig. 2G and H). The purified *Nostochopsis* corrinoids were eluted as several total ion peaks, indicating impurities still existed (Fig. 2C).

The ion peaks of pseudo-B\textsubscript{12} and B\textsubscript{12} showed both pseudo-B\textsubscript{12} and B\textsubscript{12} divalent ion peaks, indicating impurities still existed (Fig. 2C). A hundred grams (dry weight) of the B\textsubscript{12}-supplemented cells contained both pseudo-B\textsubscript{12} (58.6±11.4 μg) and B\textsubscript{12} (254.8±17.6 μg), indicating that B\textsubscript{12} was enriched significantly in the cells grown in the B\textsubscript{12}-supplemented medium (Fig. 1, lane 6). These results indicated that about 2.2% of the B\textsubscript{12} added to the medium was taken up by the *Nostochopsis* cells.

Even if humans take pseudo-B\textsubscript{12} from the *Nostochopsis* cells, pseudo-B\textsubscript{12} would not be absorbed in the intestine because pseudo-B\textsubscript{12} has been reported to reveal low affinity to the intrinsic factor (B\textsubscript{12}-binding protein) involved in mammalian intestinal absorption of B\textsubscript{12} (13, 14). When trascobalamin II-pseudo-B\textsubscript{12} or -authentic B\textsubscript{12} complex is intravenously injected in mammals, urinary excretion is slightly greater in pseudo-B\textsubscript{12} than in B\textsubscript{12} (15). The adenosyl coenzyme form of pseudo-B\textsubscript{12} has a 1,000-fold higher Km value for adenosyl-B\textsubscript{12} dependent mammalian methylmalonil-CoA mutase than does adenosyl-B\textsubscript{12} (16). These observations indicated that even if a certain amount of pseudo-B\textsubscript{12} is absorbed in the intestines, pseudo-B\textsubscript{12} would not be disturbed in B\textsubscript{12}-related cellular metabolisms.

Consumption of only 1 g of the dried *Nostochopsis* cells (or 100 g of fresh cells) can supply the recommended dietary allowance for adults (2.4 μg/d) (17).

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