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Production and Characterization of Cyanocobalamin-Enriched Lettuce (*Lactuca sativa* L.) Grown Using Hydroponics

Tomohiro Bito,[†] Noriharu Ohishi,[‡] Yuka Hatanaka,[‡] Shigeo Takenaka,[¶] Eiji Nishihara,[†,‡] Yukinori Yabuta,[†,‡] and Fumio Watanabe*[,†,‡]

[†]Division of Applied Bioresources Chemistry, The United Graduate School of Agricultural Sciences, and [‡]School of Agricultural, Biological and Environmental Sciences, Tottori University, Tottori 680-8553, Japan

[¶]Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 598-8531, Japan

ABSTRACT: When lettuces (*Lactuca sativa* L.) grown for 30 days in hydroponic culture were treated with various concentrations of cyanocobalamin for 24 h, its content in their leaves increased significantly from nondetectable to 164.6 ± 74.7 ng/g fresh weight. This finding indicated that consumption of only two or three of these fresh leaves is sufficient to meet the Recommended Dietary Allowance for adults of 2.4 μg/day. Analyses using a cobalamin-dependent *Escherichia coli* 215 bioautogram and LC/ESI-MS/MS demonstrated that the cyanocobalamin absorbed from the nutrient solutions by the leaves did not alter any other compounds such as coenzymes and inactive corrinoids. Gel filtration indicated that most (86%) of the cyanocobalamin in the leaves was recovered in the free cyanocobalamin fractions. These results indicated that cyanocobalamin-enriched lettuce leaves would be an excellent source of free cyanocobalamin, particularly for strict vegetarians or elderly people with food-bound cobalamin malabsorption.

KEYWORDS: *cyanocobalamin, lettuce, nutrient-enriched vegetables, cobalamin deficiency, vitamin B_{12}*

INTRODUCTION

Cobalamin (Cbl) or vitamin B_{12} is synthesized only by certain bacteria[1] and is mainly concentrated in the bodies of higher predatory organisms in the natural food chain. Animal foods such as meat, milk, egg, fish, and shellfish, but not plant foods, are the major dietary sources of Cbl;[2] therefore, strict vegetarians are at a greater risk of developing Cbl deficiency than nonvegetarians.[3] The major symptoms of Cbl deficiency are neuropathy and megaloblastic anemia.[4] It is therefore necessary to identify plant foods containing high Cbl to prevent vegetarians from developing Cbl deficiency. Some plant foods such as edible algae or blue-green algae (cyanobacteria) contain large amounts of Cbl.[5] However, pseudovitamin B_{12} (pseudo Cbl), which is inactive in humans, is reportedly predominant in various edible cyanobacteria.[6]

Mozafar[7] demonstrated that the addition of an organic fertilizer such as cow manure significantly increased the Cbl content of spinach leaves (17.8 ng/g dry weight). Using this value and assuming spinach leaves have a moisture content of 92.4%,[8] the Cbl content is approximately 0.14 μg/100 g fresh weight. Consumption of several hundred grams of fresh spinach would therefore be insufficient to meet the Recommended Dietary Allowance (RDA) of 2.4 μg/day for adult humans.[9] Furthermore, our recent[10] and unpublished works indicate that most organic fertilizers, particularly those made from animal manures, contain considerable amounts of inactive corrinoid compounds. These compounds have also been reported to be present in human feces and account for >98% of the total corrinoid content.[11]

Some researchers have attempted to prepare certain Cbl-enriched vegetables by treating them with a solution containing high levels of Cbl.[12,13] This resulted in a significant increase in the amount of Cbl incorporated into the plants, suggesting that the Cbl-enriched vegetables may be of special benefit to vegetarians. However, the Cbl absorbed by the vegetables was not well characterized in these studies.

A large number of people have low serum Cbl levels that result more commonly from malabsorption of protein-bound Cbl (food-bound Cbl malabsorption) rather than pernicious anemia.[4] Food-bound Cbl malabsorption is found in people with certain gastric dysfunctions, particularly atrophic gastritis with low stomach acid secretion, which prevails in elderly people.[14,15] As the bioavailability of crystalline (free) Cbl is not altered in people with atrophic gastritis,[16] the Institute of Medicine recommended that most of the RDA (2.4 μg/day) should be obtained by consuming foods fortified with Cbl or supplements containing Cbl.[9]

Lettuce (*Lactuca sativa*) is an annual plant, which is most often grown as a leafy vegetable that is easily cultivated, is a popular vegetable, is commonly used in salads, soups, sandwiches, and wraps, and is a good source of β-carotene, potassium, vitamins, and nutrients.[8]

Hydroponic cultivation is an emerging technology that allows better control of water and nutrient supply that improves plant productivity and reduces the use of pesticides.[17] If a sufficient amount of free Cbl can be incorporated into lettuce leaves grown under hydroponic conditions, it would be an excellent source of free Cbl for vegetarians and elderly people.

In this paper, we describe the production and characterization of Cbl-enriched lettuce leaves cultivated using hydroponics.

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MATERIALS AND METHODS

Materials. Cyanocobalamin (CN-Cbl) (crystalline N, average purity of 98.1%) prepared for human food supplementation was purchased from DSM Japan (Tokyo, Japan) and used for hydroponic cultivation. CN-Cbl used as a standard in the bioassay was obtained from Sigma (St. Louis, MO, USA). Pseudo CN-Cbl was prepared in our laboratory as described previously.[18] The Cbl assay medium for *Lactobacillus delbrueckii* subspecies *lactis* (formerly *L. leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan), and silica gel 60 thin-layer chromatography (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). A Shimadzu ultraviolet (UV)−visible spectrophotometer (UV-2550; Kyoto, Japan) was used to measure the turbidity of the *L. delbrueckii* test cultures in the microbiological Cbl assay.

All other reagents were of the highest purity commercially available.

Methods. *Growth Conditions of the Lettuce Leaves.* As shown in Figure 1, the experiments were performed in a glasshouse from June to July 2011(day length, approximately 11 h; temperature range, 18−32 °C) at Tottori University, Japan (35° 52′ N, 134° 17′ E, 3 m above sea level). Seeds of a butter-type head lettuce (*L. sativa* L.) were purchased from Hohoku Seed Co. Ltd. (Tochigi, Japan) and germinated and grown for 24 days in black plastic pots containing sandy soil with an adequate supply of water. Uniform seedlings were then selected, carefully washed, and transferred into 3 L hydroponic lightproof pots (1 plant/pot) containing nutrient solutions (Otsuka House series no. 1 and 2, Otsuka AgriTechno Co., Ltd.) prepared according to the manufacturer's protocol. The nutrient solutions in these pots were continuously aerated using an air pump, covered with styrene foam to protect against light exposure, and renewed every 5 days. The indicated amounts of CN-Cbl were added to the nutrient solution 30 days after transplantation. The plants were left for 24 h under these conditions (not light shielded) and harvested. The edible portions of the plants were weighed, slightly washed with tap water, and lyophilized using a freeze-dryer (DC800, Yamato Scientific Co., Ltd., Tokyo, Japan). The dried leaves were stored at −80 °C until analysis.

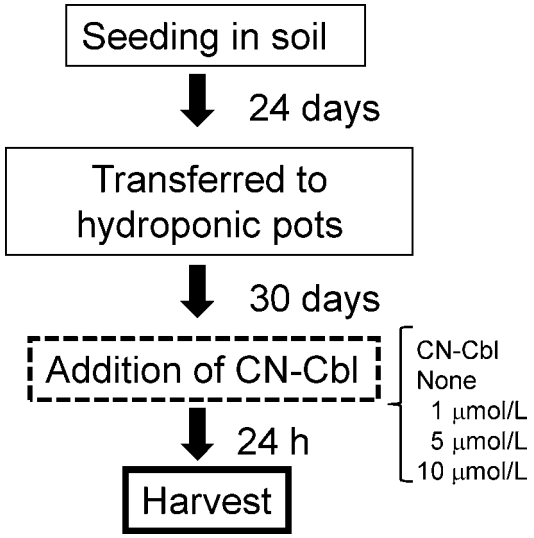

Figure 1. Outline of the preparation of CN-Cbl-enriched lettuces using hydroponic cultivation.

Extraction and Assay of Cbl. Two grams of each dried leaf of the various lettuces grown in the presence or absence of CN-Cbl was used. The total Cbl was extracted by boiling for 30 min in 100 mL of 57 mmol/L acetate buffer (pH 4.5) containing 0.0004% (w/v) KCN to convert the various Cbl compounds with different upper ligands (e.g., Cbl coenzymes) to CN-Cbl. The extraction procedures were performed in a draft chamber (Dalton Co., Tokyo, Japan). The boiled suspensions were centrifuged at 10000*g* for 10 min. A portion of the total Cbl extract was adjusted to pH 11.0 and then placed in an autoclave (MC-23, ALP Co., Ltd., Tokyo, Japan) at 121 °C for 30 min to decompose Cbl in the extract. These extracts were assayed using a microbiological technique based on *L. delbrueckii* ATCC 7830, according to the method described in the Standard Tables of Food Composition in Japan.[19,20] The extract treated at pH 11.0 contained certain compounds, including deoxyribosides and deoxyribonucleotides, that are known as alkali-resistant factors. Because *L. delbrueckii* can utilize both deoxyribosides and deoxyribonucleotides (i.e., alkali-resistant factors) as well as Cbl, the amount of true Cbl was calculated by subtracting the values of the alkali-resistant factor from the values of the total Cbl. The Cbl assay was repeated at least five times, and the data were expressed as the mean ± standard deviation (SD).

For analysis of coenzyme forms of Cbl, the compounds were extracted from 2 g of lyophilized powder of CN-Cbl-enriched lettuce leaves (approximately164.6 ng Cbl/g fresh weight). In total, 100 mL of 80% (v/v) ethanol solution was added to the dried leaves, heated at 98 °C for 30 min under reflux conditions, and cooled to room temperature.[21] The resulting solution was centrifuged at 10000*g* for 10 min, and the supernatant was allowed to evaporate to dryness under reduced pressure and then dissolved in 20 mL of distilled water. This solution was centrifuged at 10000*g* for 10 min to remove insoluble material, and a portion of the supernatant was placed on a Sep-Pak Plus C18 cartridge (Waters Corp., Milford, MA, USA). The cartridge was washed with 10 mL of distilled water and eluted with 2 mL of ethanol. The eluate was used in TLC-bioautogram analysis. All of the procedures were performed in the dark.

Stability of CN-Cbl in the Hydroponic Nutrient Solution. CN-Cbl was dissolved in the nutrient solution (pH 5.7) at a final concentration of 5 μmol/L. The solution was continuously aerated using an air pump under the same conditions described above and treated at 25 °C for 24 h (12 h light/dark cycle; photon flux density 25.4 ± 0.3 μmol/m^2/s) in a CLE-303 cultivation chamber (Tomy Seiko Co., Ltd., Tokyo, Japan). CN-Cbl was also dissolved in 100 mmol/L potassium phosphate buffer (pH 6.2), which had a pH value identical to that of the lettuce leaf homogenate, and then treated under the same conditions. The relative amounts of CN-Cbl in the treated solutions were expressed as percentages of a CN-Cbl solution (5 μmol/L) freshly prepared in distilled water.

To determine the stability of aqueous CN-Cbl during 24 h of incubation in a glasshouse, CN-Cbl was dissolved in 100 mmol/L potassium phosphate buffer (pH 6.2) and treated for 24 h in the glasshouse (sunlight; photon flux density 761.7 ± 29.7 μmol/m^2/s). The CN-Cbl solution was covered with aluminum foil to block sunlight exposure, and it represented the solution treated without sunlight exposure.

The UV−visible spectra of authentic CN-Cbl, OH-Cbl, and CN-Cbl solutions (5 μmol/L each) with or without sunlight exposure were measured at room temperature using a Shimadzu spectrophotometer (UV 2550) and then analyzed by HPLC using a JASCO HPLC apparatus (PU-2080 Plus Pump, UV-2070 Plus spectrophotometer, DG-2080-53 degasser, CO-2065 column oven) and CDS ver.5 chromato-data processing system (LAsoft, Ltd. Chiba, Japan). A 20 μL aliquot of each sample was placed on a reversed-phase HPLC column (Wakosil-II 5C18RS, ⌀ 4.6 × 150 mm; particle size = 5 μm) and equilibrated at 40 °C with 20% (v/v) methanol containing 1% (v/v) acetic acid at a flow rate of 1.0 mL/min. The Cbl compounds were isocratically eluted with the same solution and monitored by measuring the absorbance at 361 nm. The retention times of authentic OH-Cbl and CN-Cbl were 2.0 and 7.9 min, respectively.

Bioautography of Cbl Compounds with Cbl-Dependent Escherichia coli 215. Bioautography of Cbl compounds was performed according to the method described by Tanioka et al.[22] After the Cbl extracts were concentrated and partially purified on the Sep-Pak Plus C18 cartridge (Waters Corp.), 2 μL of the extracts and authentic CN-Cbl (10 μg/L) was spotted on a silica gel 60 TLC sheet and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) in the dark at 25 °C. After the TLC sheet was dried, the agar-containing basal medium and precultured *E. coli* 215 were overlaid and then incubated at 37 °C for 12 h. The gel plates were sprayed with a methanol solution

containing 2,3,5-triphenyltetrazolium salt, and the Cbl compounds were visualized as a red color that indicated the growth of *E. coli*.

Identification of Cbl Compounds by LC/ESI-MS/MS. The total Cbl extracts described above (30 mL) were placed in a Sep-Pak Plus C18 cartridge (Waters Corp.) that had been washed with 5 mL of 75% (v/v) ethanol and equilibrated with 5 mL of distilled water. The C18 cartridge was washed with 5 mL of distilled water, and the Cbl compounds were eluted using 2 mL of 75% (v/v) ethanol. The eluate was evaporated to dryness under reduced pressure. The residual fraction was dissolved with 5 mL of distilled water and centrifuged at 10000*g* for 10 min to remove any insoluble material. The supernatant fraction was loaded onto an immunoaffinity column [EASI-EXTRACT Cbl immunoaffinity column (P80) R-Biopharm AG, Darmstadt, Germany], and the Cbl compounds were purified according to the manufacturer's recommended protocol. The purified Cbl compounds and authentic CN-Cbl were dissolved in 50 μL of 0.1% (v/v) acetic acid and filtered using a Nanosep MF centrifuge device (0.4 μm; Pall Corp., Tokyo, Japan) to remove small particles. A 10 μL aliquot of the filtrate was analyzed using an LCMS-IT-TOF system coupled to 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. The Cbl compounds were eluted using 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) at a flow rate of 0.2 mL/min. The electrospray ion (ESI) conditions were determined by injecting authentic CN-Cbl into the MS detector to determine the optimum parameters for detecting parent and daughter ions of CN-Cbl. The ESI-MS was operated in the positive ion mode using argon as the collision gas. The identification of CN-Cbl (m/z 678.291) representing $[M + 2H]^{2+}$ was confirmed by comparison of the observed molecular ions and their retention times.

Sephadex G-50 Gel Filtration Experiment. Free CN-Cbl was separated from the CN-Cbl-enriched lettuce leaves using an Econo-pack column (1.4 × 10 cm column, Bio-Rad Laboratories, Hercules, CA, USA) of Sephadex G-50 fine (GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, UK) and then assayed. The lettuce leaves (1 g) were extracted in 10 mL of 100 mmol/L potassium phosphate buffer (pH 7.0) at 4 °C using a mortar and pestle and centrifuged at 10000*g* for 10 min at 4 °C to remove the insoluble material. A 1 mL aliquot of the supernatant was applied to a column equilibrated with 100 mmol/L potassium phosphate buffer (pH 7.0). The column was eluted with the same buffer at a flow rate of 1.0 mL/min, and 0.5 mL fractions were collected. Each fraction of the eluate was added to 100 μL of 0.57 mol/L acetate buffer (pH 4.5) and 40 μL of 0.5% (w/v) KCN, vigorously mixed, and boiled in the dark for 30 min. The treated fractions were then centrifuged at 10000*g* for 10 min at 4 °C to remove insoluble material, followed by assay of the CN-Cbl content using the microbiological method. By measuring the absorbance at 280 nm, the macromolecular and free CN-Cbl fractions were estimated using blue dextran and authentic CN-Cbl, respectively.

Statistical Analysis. Differences in the weight and Cbl content of the lettuce leaves placed in the nutrient solutions with or without CN-Cbl for 24 h were analyzed by one-way ANOVA and post hoc analyses using Tukey's multicomparison test. Experimental data on the stability of CN-Cbl in aqueous solution were also analyzed using the same tests. These analyses were performed using GraphPad Prism for Windows version 5.03 (GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as the mean and SD, with statistical significance defined as $p < 0.05$.

RESULTS AND DISCUSSION

Stability of CN-Cbl in Hydroponic Nutrient Solution. To evaluate the stability of CN-Cbl in a hydroponic nutrient solution, CN-Cbl was dissolved in the nutrient solution (pH 5.9) and treated at 25 °C for 24 h with or without 12 h of light exposure (room fluorescent lighting; photon flux density = 25.4 ± 0.3 μmol/m^2/s). No significant decrease in CN-Cbl concentration was observed in either solution treated with or without light exposure (Figure 2). The nutrient solutions were hardly exposed to light because the hydroponic lightproof pots were covered with styrene foam.

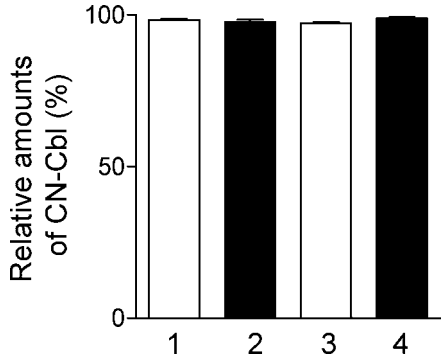

Figure 2. Stability of CN-Cbl in the hydroponic nutrient solution. CN-Cbl was dissolved in the nutrient solution at a final concentration of 5 μmol/L. The solution was continuously aerated and treated with (1) or without (2) light exposure (12 h light/dark cycle) at 25 °C for 24 h. CN-Cbl was also dissolved in 100 mmol/L potassium phosphate buffer (pH 6.2), which had a pH value identical to that of the lettuce leaf homogenate, and was treated with (3) or without (4) light exposure (12 h light/dark cycle) under the same conditions. The CN-Cbl solution was covered with aluminum foil to block light exposure and represented the solution treated without light exposure. The CN-Cbl content of these samples (20 μL each) was analyzed using C18 reversed-phase HPLC. The relative amounts of CN-Cbl in the treated solutions were expressed as percentages of the 5 μmol/L CN-Cbl solution freshly prepared in distilled water. The data represent typical HPLC elution patterns from three independent experiments.

When CN-Cbl was also dissolved in 100 mmol/L potassium phosphate buffer (pH 6.2), which had a pH value identical to that of the lettuce leaf homogenate, and treated under the same conditions, CN-Cbl was found to be stable in aqueous solution under the conditions. These results indicated that the procedures for preparation of CN-Cbl-supplemented nutrient solution and subsequent transplantation of lettuce plants could be performed under fluorescent light.

However, when the CN-Cbl solutions (pH 6.2) were treated for 24 h in the glasshouse (sunlight; photon flux density = 761.7 ± 29.7 μmol/m^2/s), the color of these CN-Cbl solutions slightly changed to that of the solution containing authentic CN-Cbl treated without sunlight exposure. However, the UV−visible spectrum of the sunlight-exposed CN-Cbl (Figure 3A-4) was not identical to that of authentic CN-Cbl (absorption maxima at 361 and 550 nm) (Figure 3A-1) but was similar to that of authentic OH-Cbl (absorption maxima at 351 and 525 nm) (Figure 3A-2). During reversed-phase HPLC, the sunlight-treated CN-Cbl was eluted as two peaks [main (90.8 ± 0.4%) and minor (4.7 ± 0.7%) with retention times of 2.0 and 7.9 min, respectively]. These retention times were identical to those of authentic OH-Cbl and CN-Cbl, respectively (Figure 3B-1,-2,-4). The UV−visible spectrum and retention time of the CN-Cbl solution treated without sunlight exposure remained unchanged (Figure 3A-3,B-3). These results indicated that CN-Cbl was converted to OH-Cbl with CN^- of the corrin ring being replaced by H_2O owing to sunlight exposure. Ahmad et al.[23] demonstrated that CN-Cbl was readily photolyzed in an aqueous solution to produce OH-Cbl and that light-induced photolysis of CN-Cbl occurred in a pH-dependent manner (i.e., a fast decrease at pH 4.5−6.5 and constant rate at pH 6.5−8.5).

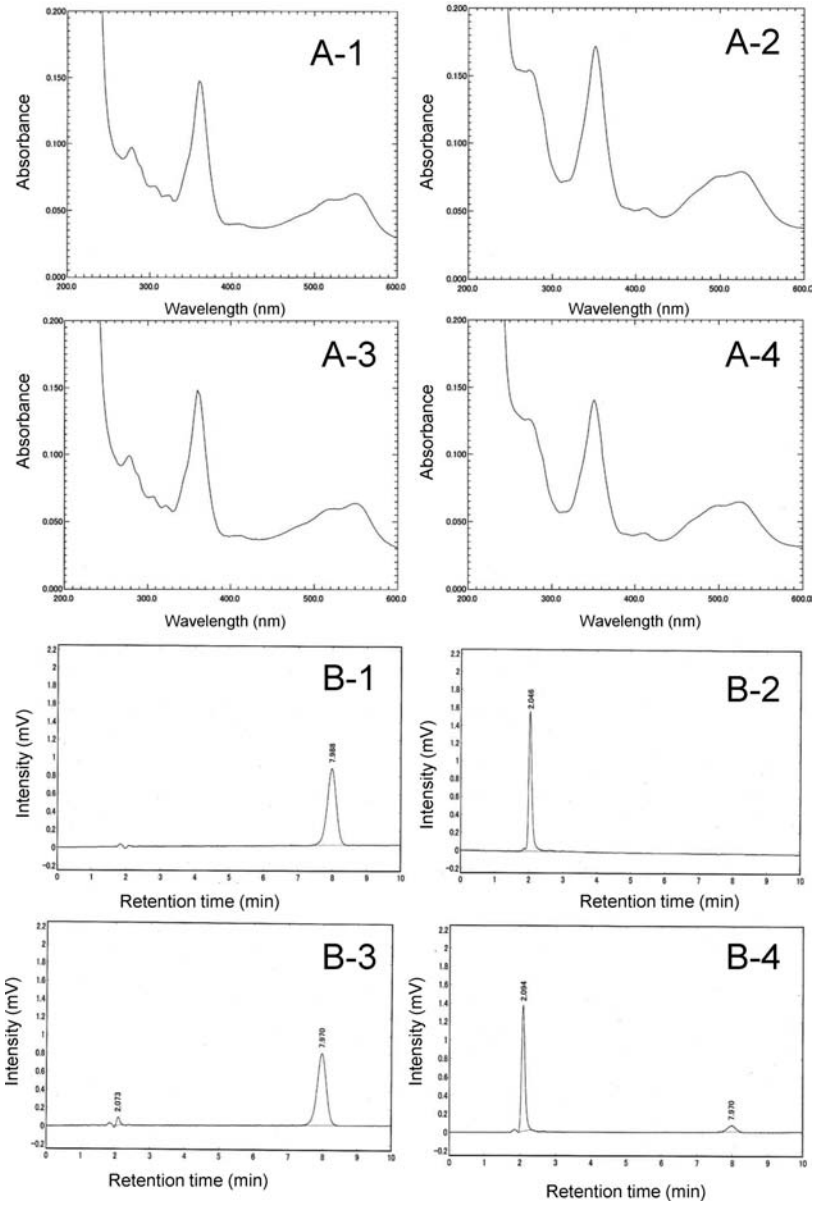

Figure 3. Photolysis of CN-Cbl in aqueous solution under glasshouse conditions. CN-Cbl was dissolved in 100 mmol/L potassium phosphate buffer (pH 6.2), which had a pH value identical to that of the lettuce leaf homogenate, and was treated with or without sunlight exposure under glasshouse conditions. The CN-Cbl solution was covered with aluminum foil to block sunlight exposure and used as the solution treated without sunlight exposure. The UV−visible spectra of authentic CN-Cbl (A-1), OH-Cbl (A-2), and CN-Cbl solutions treated with (A-4) or without (A-3) sunlight exposure (5 μmol/L each) were measured using a Shimadzu spectrophotometer. The authentic CN-Cbl (B-1), OH-Cbl (B-2), and CN-Cbl solutions treated with (B-4) or without (B-3) sunlight exposure (20 μL each) were analyzed by HPLC and monitored by measuring the absorbance at 361 nm. The data represent typical ultraviolet−visible spectra and HPLC elution patterns from three independent experiments.

It has also been reported that OH-Cbl is readily converted to coenzyme forms of Cbl and is therefore more effective in Cbl deficiency than CN-Cbl.[24]

These results indicated that this CN-Cbl reagent would be useful for the preparation of CN-Cbl-enriched lettuce leaves cultivated using hydroponics.

Cbl Content of Lettuce Leaves 24 h after the Plants Were Placed in Nutrient Solutions Containing Various Concentrations of CN-Cbl. Lettuces grown for 30 days in hydroponic culture were treated with the indicated amounts of CN-Cbl for 24 h and then harvested (Figure 1). The Cbl concentration of the lettuce leaves significantly increased CN-Cbl concentration up to 5 μmol/L (Table 1). No Cbl was detected in lettuce leaves placed in a solution without CN-Cbl. Although fresh weights (per plant stock) of lettuces appear to increase depending on CN-Cbl concentrations, there is no significant difference in weights of the treated lettuces, except for control (no addition of CN-Cbl) versus 10 μmol/L CN-Cbl-supplemented lettuces. These results indicated that although lettuce plants do not require Cbl for growth, they can take up and concentrate Cbl in their leaves. The Cbl content of lettuce leaves may be controlled, to some extent, by changing the CN-Cbl concentration in the nutrient solution. Because lettuce leaves treated with 1 μmol/L CN-Cbl contained 1.3 ± 0.6 μg of Cbl per total fresh weight (g) of plant stock, consumption of two stocks of these plants would supply the RDA for adults (2.4 μg/day). For lettuce leaves treated with 5 μmol/L CN-Cbl (6.8 ± 3.4 μg of Cbl per plant

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Table 1. Cbl Content of Leaves of Lettuce Plants Placed for 24 h in Nutrient Solutions Supplemented with Different Concentrations of CN-Cbl[a]

	fresh wt of plant stock* (g)	Cbl content	
		μg/total fresh wt of plant stock	ng/g fresh wt
none	28.8 ± 3.8a	ND	ND
addition of CN-Cbl			
1.0 μmol/L	36.1 ± 8.5ab	1.3 ± 0.6a	38.6 ± 22.5a
5.0 μmol/L	40.3 ± 3.1ab	6.8 ± 3.4b	164.6 ± 74.7b
10.0 μmol/L	46.7 ± 3.2b	7.2 ± 0.8b	154.9 ± 26.7b

[a]The edible portions (leaves)* of the treated lettuces were weighed and lyophilized, followed by extraction of Cbl and assay using the microbiological method. Values are expressed as the mean ± SD (n = 3). The letters define statistically significant differences ($p < 0.05$). ND, undetectable.

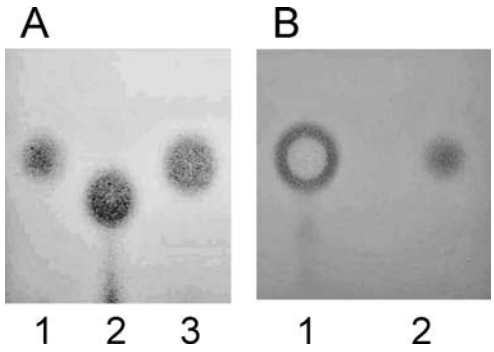

Figure 4. Cbl-dependent *E. coli* 215 bioautogram after silica gel 60 TLC of Cbl extracts of enriched leaves. The Cbl compounds were extracted from the leaves in the presence (A) or absence (B) of KCN, separated on silica gel 60 TLC, and visualized using Cbl-dependent *E. coli* 215. Lanes: (A) 1, authentic CN-Cbl; 2, authentic pseudo CN-Cbl; 3, lettuce leaf extract; (B) 1, authentic CN-Cbl; 2, lettuce leaf extract. The data represent a typical bioautogram from three independent experiments.

stock), consumption of only two or three leaves would be sufficient to meet the RDA.

Our data showed that lettuce plants could take up only 0.03% of the CN-Cbl contained in the 1 and 5 μmol/L solutions and that the CN-Cbl uptake ratio was reduced to 0.02% in the 10 μmol/L CN-Cbl solution. These results indicated that the CN-Cbl-supplemented nutrient solution was reused several times during hydroponic cultivation.

Characterization of CN-Cbl Absorbed by Lettuce Leaves. To evaluate whether CN-Cbl absorbed by the leaves was converted to other corrinoid compounds (i.e., pseudo Cbl, OH-Cbl, coenzyme forms of Cbl, and others), Cbl in the leaves was extracted with or without KCN treatment that converts various Cbl compounds with different upper ligands (e.g., OH-Cbl and Cbl coenzymes) to CN-Cbl and then analyzed using a Cbl-dependent *E. coli* 215 bioautogram after separation on silica gel 60 TLC. The KCN-treated extract of Cbl-enriched leaves (approximately 164.6 ng Cbl/g fresh weight) produced a single clear spot with an R_f value identical to that of the authentic CN-Cbl but not that of pseudo Cbl, an inactive form in humans (Figure 4A). An extract treated without KCN produced a single clear spot with a R_f value (0.63) identical to that of authentic CN-Cbl (Figure 4B) but not those of other

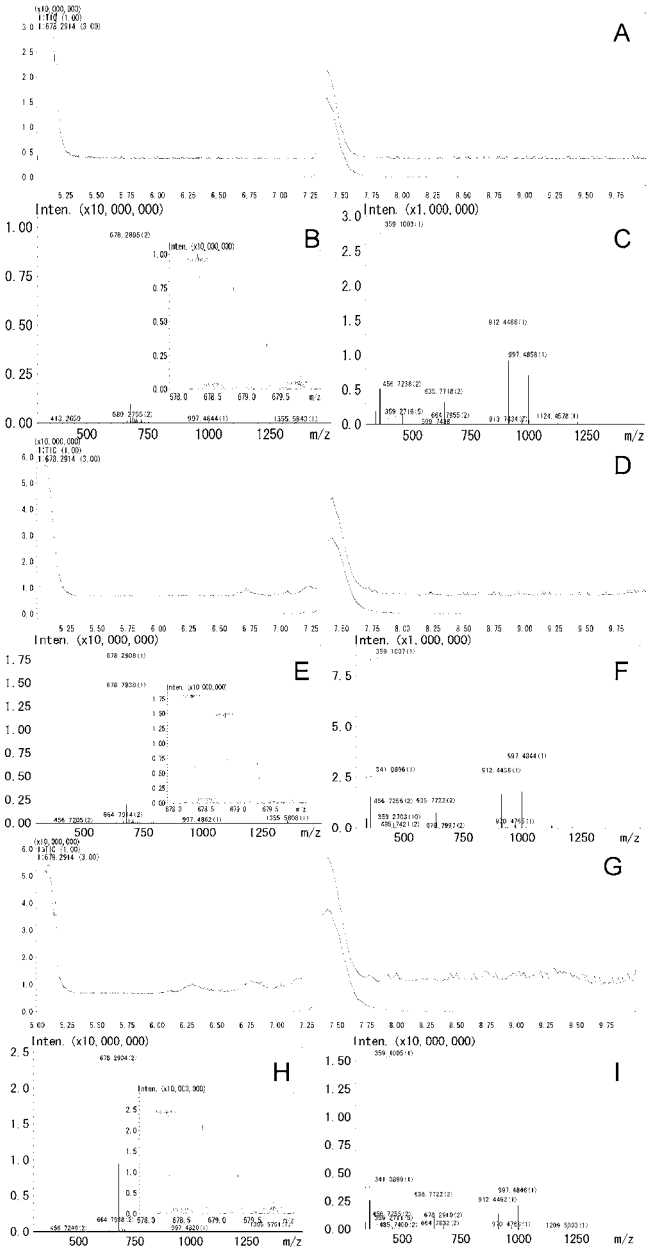

Figure 5. LC/ESI-MS/MS chromatograms of authentic CN-Cbl and leaf extracts treated with or without KCN. CN-Cbl was analyzed using an LCMS-IT-TOF system (Shimadzu) as described under Materials and Methods. The total ion chromatogram (TIC) of authentic CN-Cbl is shown in panel A. Panels D and G show the TICs and reconstructed chromatograms of the purified Cbl compounds (m/z 678.2914) from the leaf extracts with and without KCN, respectively. The mass spectra of authentic CN-Cbl and Cbl compounds purified from the extracts treated with and without KCN at 7.30 min are shown in panels B, E, and H, respectively (the magnified spectrum ranging from m/z 678 to 680 is shown as an inset in each panel). The MS/MS spectra for the m/z 678.29 peak of authentic CN-Cbl and Cbl compounds purified from the extracts treated with and without KCN are shown in panels C, F, and I, respectively.

Cbl compounds [R_f values: OH-Cbl (0.05), 5′-deoxyadenosylcobalamin (0.53), and methylcobalamin (0.67)].

These Cbl extracts treated with or without KCN were further purified using a Cbl immunoaffinity column and analyzed by LC/ESI-MS/MS. As shown in Figure 5, the mass spectra of authentic CN-Cbl had a major divalent ion (precursor ions)

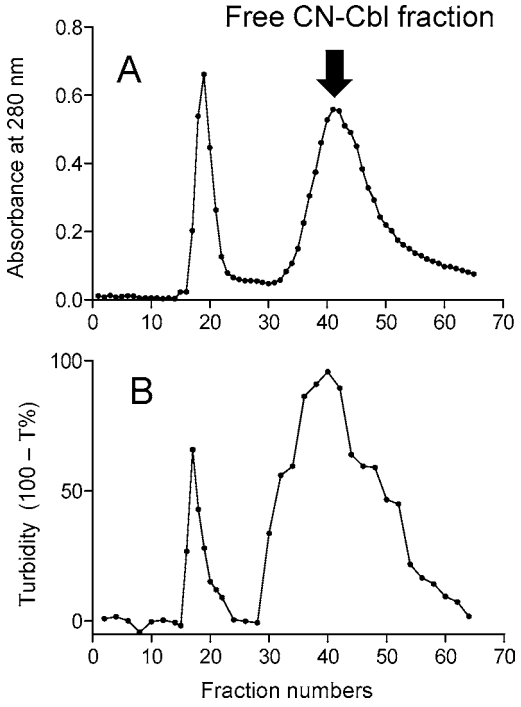

Figure 6. Elution patterns of Cbl-active compounds in leaves analyzed using Sephadex G-50 gel filtration: authentic blue dextran and CN-Cbl mixture (A) and CN-Cbl-enriched lettuce leaves (B). The macromolecular and free CN-Cbl fractions were estimated by measuring the absorbance at 280 nm of blue dextran and authentic CN-Cbl, respectively. The Cbl content in the Cbl-enriched lettuce leaves was assayed using the microbiological method. The data represent a typical elution pattern of the Cbl-active compound of lettuce leaves from four independent experiments.

with an m/z value of 678.2895 $[M + 2H]^{2+}$ in the LC/ESI-MS conditions employed (Figure 5A,B). MS/MS spectra analysis indicated that the ion with an m/z value of 359.1003 was attributable to the nucleotide moiety of authentic CN-Cbl, whereas the ion with an m/z value of 997.4858 was attributable to a corrin ring moiety that also formed in authentic CN-Cbl (Figure 5C). As shown in the LC/ESI-MS/MS chromatograms of the KCN-treated extract, Cbl compounds were eluted at a single ion peak with a retention time of 7.3 min (m/z 678.2914) (Figure 5D). The retention time of the ion peak with an m/z value of 678.2914 was identical to that of authentic CN-Cbl. The MS and MS/MS spectra of the ion peak were also identical to those of authentic CN-Cbl (Figure 5E,F). Moreover, LC/ESI-MS/MS chromatograms of the Cbl extract without KCN were identical to those of authentic CN-Cbl (Figure 5G–I). These results indicated that the CN-Cbl absorbed by lettuce leaves was not changed to any other corrinoids, although CN-Cbl was readily converted to OH-Cbl in an aqueous solution under the same conditions (Figure 3A-4,B-4). These findings suggested that lettuce plant tissues have the ability to block photolysis of aqueous CN-Cbl from sunlight exposure. These results and the fact that higher plants have neither the ability to synthesize Cbl de novo nor an absolute requirement for the vitamin to promote growth also indicate that the accumulation of CN-Cbl by leaves has no effect on the physiological functions of lettuce plants.

To evaluate whether CN-Cbl-enriched lettuce leaves contained "free CN-Cbl" or "protein-bound CN-Cbl," homogenates of selected leaves were analyzed using Sephadex G-50 gel filtration. The results demonstrated that the majority of CN-Cbl present in the leaves (86%) was recovered in the free CN-Cbl fractions (Figure 6B). These results suggest that CN-Cbl-enriched lettuce leaves are an excellent source of free CN-Cbl.

Table 2 shows a comparison of Cbl contents in CN-Cbl-enriched lettuce and other vegetables treated with solutions containing high concentrations of CN-Cbl. Mozafar and Oertli[12] reported that uptake of CN-Cbl by soybean leaves did not reach saturation even at an extremely high concentration of Cbl (3.2 mmol/L) in the nutrient solution. A previous study also showed that when soybean seedlings were placed in solution containing 10 μmol/L CN-Cbl for 24 h, the leaves contained a significantly higher level of Cbl (9.8 μg/g fresh weight). That study also showed that Japanese radish sprouts (kaiware daikon) had significant increases in Cbl content (1.3 μg/g fresh weight) after their seeds were soaked for 6 h in a solution containing 200 μg/mL (147.6 μmol/L) CN-Cbl.[13] In the present study, we demonstrated that the Cbl content in lettuce leaves (approximately 164.6 ng/g fresh weight) reached saturation at a CN-Cbl concentration of 5 μmol/L. Because vegetables are generally a good source of dietary fibers, vitamins, and minerals,[8] consumption of a large amount of vegetables enriched with a low level of CN-Cbl would be better for human health than consumption of a small amount of a vegetables with a high level of CN-Cbl. Consumption of 62.2 g of CN-Cbl-enriched lettuce leaves

Table 2. Comparison of the Cbl Content in Various Cbl-Enriched Plants Following Treatment with CN-Cbl-Supplemented Solutions[a]

		Cbl content			
		solution	plants		
plant	method	μmol/L	μg/g fresh wt	ng/g fresh wt	ref
soybean leaves	hydroponic culture for 24 h	10.0	9.8a	(0.2 g)[b]	12
radish sprout	seed soaking for 6 h	18.4	0.2a	(12.0 g)	13
		147.6	1.3a	(1.9 g)	
lettuce leaves	hydroponic culture for 24 h	1.0	38.6b	(62.2 g)	this study
		5.0	164.6b	(14.6 g)	
		10.0	154.9b	(15.5 g)	

[a]The mean Cbl values of soybean leaves and radish sprouts were calculated from the cited references. [b]The amount of each enriched fresh plant that provides the adult RDA for Cbl of 2.4 μg/day is shown in parentheses.

(approximately 38.6 ng/g fresh weight) grown in a low CN-Cbl (1 μmol/L)-supplemented nutrient solution supplies the RDA for Cbl of 2.4 μg/day. In addition, these leaves provide the daily intake of other nutrients, particularly folic acid and B_6, which are essential for normal homocysteine metabolism and for preventing disorders such as atherosclerosis and neuropathy.[25] The use of low levels of CN-Cbl in nutrient solutions would also lower the production costs of the enriched plants. Indeed, the cost of CN-Cbl for the preparation of 1 stock of Cbl-enriched lettuce leaves grown in the low CN-Cbl (1 μmol/L)-supplemented nutrient solution was calculated to be only approximately ¥ 6 (U.S. $0.06) under the experimental conditions used. If the nutrient solution is reused several times, this cost would be reduced significantly. Therefore, the cost for adding CN-Cbl would not a major factor in the preparation of CN-Cbl-enriched plants.

On the basis of the fact that approximately 30% of people older than 50 years are estimated to have atrophic gastritis with low acid secretion in the stomach and decreased bioavailability of Cbl from food (food-bound Cbl malabsorption),[14,15] the Institute of Medicine recommended that the majority of the RDA should be obtained from foods fortified with Cbl or supplements containing Cbl.[9] The results of this study indicate that CN-Cbl-enriched lettuce leaves would be an excellent source of free CN-Cbl, particularly for vegetarians and elderly people.

AUTHOR INFORMATION

Corresponding Author

*Phone/fax: +81 857 31 5412. E-mail: watanabe@muses.tottori-u.ac.jp .

Notes

The authors declare no competing financial interest.

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