Inactive Corrinoid-Compound Significantly Decreases in *Spirulina platensis* Grown in a Cobalt-Deficient Medium

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Spirulina platensis NIES-39 was grown under open culture system in the presence or absence of $CoSO_4$ (12 μg/L) and/or vitamin B_{12} (10 μg/L) to confirm whether $CoSO_4$ and/or vitamin B_{12} stimulate or are essential for growth of the algal cells and for accumulation of vitamin B_{12}. The addition of $CoSO_4$ and/or vitamin B_{12} could not affect both cell growth and cell yield of the alga. The amount of corrinoid-compound was increased significantly by the addition of $CoSO_4$ but not by vitamin B_{12}. A C18 reversed-phase HPLC pattern of the *Spirulina* corrinoid-compound increased by the addition of $CoSO_4$ was identical to that of authentic pseudovitamin B_{12}, which is inactive for human. These results indicate that the algal cells grown in the absence of $CoSO_4$ are suitable for use of human health foods because the inactive corrinoid-compound can be reduced significantly.

Keywords: *Vitamin B_{12}; pseudovitamin B_{12}; algal health food; spirulina tablet; intrinsic factor*

INTRODUCTION

A health food fad involves tablets of *Spirulina* sp., a blue-green alga, which has been alleged to have therapeutic properties such as hepatoprotective (*1*), immunological (*2*), and antiviral (*3*). *Spirulina* tablets also contain large amounts of vitamin B_{12} or cyanocobalamin (B_{12}). Herbert and Drivas (*4*), however, have reported that most of B_{12} found in the *Spirulina* tablets are biologically inactive corrinoid-compounds, probably cobinamide-like compounds. The *Spirulina* tablets also contain corrinoid-compounds which can block B_{12}-metabolism (*5*). Several studies (*4, 6, 7*) have showed that the *Spirulina* corrinoid-compounds may not be bioavailable in mammals.

Our previous study (*8*) has demonstrated that pseudo-B_{12} (7-adeninyl cyanocobamide) is the predominant corrinoid of commercially available *Spirulina* tablets (Figure 1). Pseudo-B_{12} appears to be inactive for human pernicious anaemia (*9*) but not to act as a B_{12}-antagonist which can interfere mammalian B_{12}-metabolism (*8*). In supplying the lyophilized algal cells for human health foods, it is necessary to reduce the amount of these inactive corrinoid-compounds and then to fortify biologically active B_{12}.

Here we describe the effect of cobalt required absolutely for synthesis of corrinoid-compounds on the growth of the algal cells under an open culture system. We also analyze an algal corrinoid-compound increased by the addition of cobalt.

MATERIALS AND METHODS

Materials. B_{12} and a reversed-phase high-pressure liquid chromatography (HPLC) column (Wakosil-II 5C18RS, ϕ 4.6 × 150 mm; particle size, 5 μm) were obtained from Wako Pure Chemical Industries (Osaka, Japan). A B_{12} assay medium for *Lactobacillus delbruekii* subsp. *lactis* (formerly *Lactobacillus leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan). Pseudo-B_{12} was kindly provided by Dr. E. Stupperich, Ulm University, Germany. All other reagents used were of the highest purity commercially available.

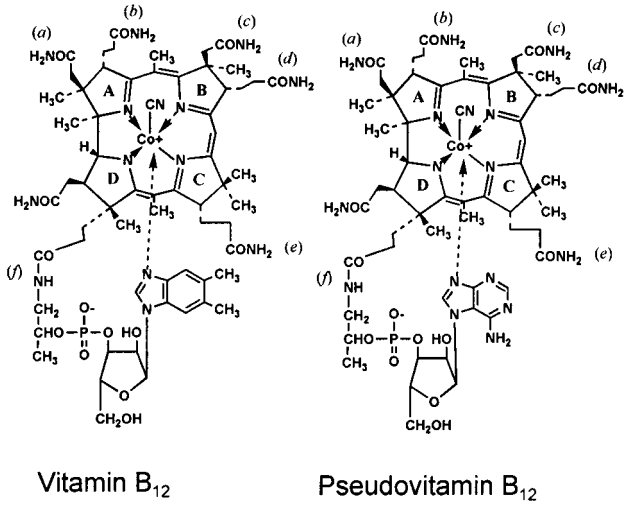

Figure 1. Structure of vitamin B_{12} and pseudovitamin B_{12}.

Organism and Culture. *S. platensis* NIES-39 was obtained from Global Environmental Forum (Tsukuba, Japan). The algal cells were aseptically precultured in SOT medium (100 mL) at 25 °C under illumination (40 μmol photon/m^2/s) according to the Forum's instructions. The SOT medium contained the following (per L): 16.8 g of $NaHCO_3$, 0.5 g of K_2HPO_4, 2.5 g of $NaNO_3$, 1.0 g of K_2SO_4, 1.0 g of NaCl, 0.2 g of $MgSO_4$ $7H_2O$, 0.04 g of $CaCl_2$ $2H_2O$, 0.01 g of $FeSO_4$ $7H_2O$, 0.08 g of Na_2EDTA, and 1.0 mL of A5 solution (2.86 g/L of H_3BO_3, 2.5 g/L of $MnSO_4$ $7H_2O$, 0.222 g/L of $ZnSO_4$ $7H_2O$, 0.079 g/L of $CuSO_4$ $5H_2O$, and 0.021 g/L of Na_2MoO_4 $2H_2O$).

In the case of $CoSO_4$ and/or B_{12}-feeding experiments, $CoSO_4$ $7H_2O$ (12 μg/L medium) and/or B_{12} (10 μg/L medium) were added to the SOT medium (25 L). The aseptically precultured *S. platensis* NIES-39 (100 mL) was inoculated into the

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10.1021/jf010733i CCC: $20.00
Published on Web 10/27/2001

experimental medium in a transparent acrylic tank (28 × 44 × 29 cm). The culture was bubbled with air at 25 °C and illuminated at 40 μmol photon/m^2/s for the initial 10 days and then 80 μmol photon/m^2/s for the next 9 days under open culture system. At the indicated times, 2.0 mL of the cell culture was sampled and used to determine the cell growth by measuring absorbance at 750 nm. At stationary growth phase, the cell culture was centrifuged at 3000*g* for 10 min. The precipitated cells were washed twice with distilled water, lyophilized, weighed, and stored at −80 °C until use.

Extraction and Assay of Corrinoid-Compound from *S. platensis* NIES-39 Cells. All procedures are done in the dark. The stored lyophilized cells (about 0.5 g) were added to 100 mL of 0.1 mol/L acetate buffer, pH 4.8, containing 20 mg of KCN, disrupted with sonic treatment (10 kHz, 10 s, three times), and boiled for 30 min at 98 °C. The extraction procedures were done in a Dalton (Tokyo, Japan) draft chamber. The boiled cell suspension was centrifuged at 3000*g* for 10 min. The supernatant was used for the following B_{12} assay.

Spirulina corrinoid-compound was assayed as B_{12} by the microbiological method with *L. delbruekii* ATCC7830 and a B_{12} assay medium (Nissui, Tokyo, Japan) and by the fully automated chemiluminescence B_{12} analyzer ACS 180 (Chiron Diagnostics, East Walpole, MA) as described previously (*10*). The cell extracts were directly applied to the chemiluminescence B_{12} analyzer. They were diluted with distilled water up to B_{12} concentration range of 0.01−0.1 μg/L and used as samples for the microbiological method. The turbidity (%T) of test culture of *L. delbruekii* ATCC7830 grown at 37 °C for 16−21 h was measured at 660 nm with a Shimadzu (Kyoto, Japan) UV−visible spectrophotometer (UV-1600).

HPLC Analysis for *Spirulina* Corrinoid-Compound. The above *Spirulina* extract (1.0 mL) was passed through a C18 cartridge (Sep-Pak Plus C18, Waters Corporation, Milford, MA) which was washed with 5.0 mL of 75% (v/v) ethanol solution and then equilibrated with distilled water. Corrinoid-compound was eluted with 5.0 mL of 25% (v/v) ethanol solution, evaporated to dryness under reduced pressure, and dissolved with 1.0 mL of distilled water. The partially purified *Spirulina* corrinoid-compound (100 μL) was analyzed with a C18 reversed-phase HPLC column (Wakosil-II 5C18RS, φ 4.6 × 150 mm; particle size, 5 μm) using a Shimadzu HPLC apparatus (LC-6A pump, SPD-6A spectrophotometer, CTO-6A column oven, C-R6A Chromatopac). The corrinoid-compound was eluted with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40 °C and monitored by measuring absorbance at 278 nm. The flow rate was 1.0 mL/min. Fractions (1.0 mL) were collected from the HPLC column, evaporated to dryness under reduced pressure, and dissolved in 1.0 mL of distilled water. A small amount of the corrinoid-compound in these fractions was determined by the microbiological B_{12} assay method. In the case of authentic B_{12} and pseudo-B_{12}, concentrated solution (2 μL) of these B_{12}-compounds were analyzed by HPLC under the same conditions. Fractions (1.0 mL) were collected from the HPLC column and monitored by measuring absorbance at 278 nm.

Statistics. Statistical analysis was performed using GraphPad PRISM 3.0 (GraphPad Software, San Diego, CA). One-way ANOVA was used with Tukey's multiple comparison test for the cell yield and amounts of corrinoid-compound determined by both microbiological and chemiluminescence B_{12} assay methods in the algal cells grown in the presence or absence of $CoSO_4$ and/or B_{12}. Differences were considered significant if $P < 0.05$. Values are means ± SD.

RESULTS AND DISCUSSION

Effect of $CoSO_4$ and/or B_{12} on Growth of *S. platensis*. *S. platensis* NIES-39 was grown under open culture system in the presence or absence of $CoSO_4$(12 μg/L) and/or B_{12} (10 μg/L) to confirm whether $CoSO_4$ and/or B_{12} stimulate or are essential for growth of the algal cells (Figure 2). The addition of B_{12} could not affect growth of *S. platensis* NIES-39 during the experimental time course. Although the algal cells were increased slightly at the logarithmic growth phase by the addition of $CoSO_4$, there was no significant difference between the cell growth in the presence or absence of $CoSO_4$. Figure 3 shows effect of the addition of $CoSO_4$ and/or B_{12} on the cell yield of *S. platensis* NIES-39 at the stationary growth phase. The addition of $CoSO_4$ and/or B_{12} could not affect the cell yield (g dry cell weight/L medium) of the alga. The results indicate that both $CoSO_4$ and B_{12} are not essential for growth of *S. platensis* NIES-39 under open culture system.

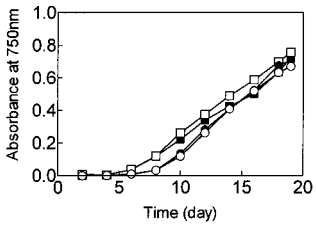

Figure 2. Effect of the addition of $CoSO_4$ and/or B_{12} on the growth of *Spirulina platensis* NIES-39. (○) − $CoSO_4$ − B_{12}; (●) − $CoSO_4$ + B_{12}; (□) + $CoSO_4$ − B_{12}; (■) + $CoSO_4$+ B_{12}. The data are representative of growth pattern in the presence or absence of $CoSO_4$ and/or B_{12} from three independent experiments.

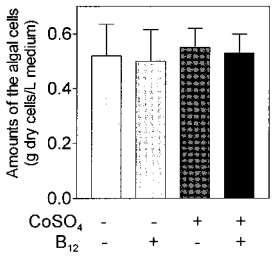

Figure 3. Effect of the addition of $CoSO_4$ and/or B_{12} on the cell yield of *Spirulina platensis* NIES-39. Data represent means ± SD ($n = 3$).

Effect of $CoSO_4$ and/or B_{12} on the Amount of Corrinoid-Compound of *S. platensis*. The amount of corrinoid-compound of the lyophilized algal cells was determined as B_{12} by both microbiological and chemiluminescence B_{12} assay methods (Figure 4). In the corrinoid-compound determination using the microbiological B_{12} assay method, the amount of corrinoid-compound was increased significantly by the addition of $CoSO_4$ but not by B_{12}. The values determined with the microbiological B_{12} assay method were much greater than the values determined with the chemiluminescence B_{12} assay method using mammalian intrinsic factor, the most specific B_{12}-binding protein (*11*). The significant increase in the amount of corrinoid-compound by the addition of $CoSO_4$ could not be found by the chemiluminescence B_{12} assay method. Although in the chemiluminescence B_{12} assay method, the amount of corrinoid-compound was significantly increased only by the addition of B_{12}, the increased amount of corrinoid-compound was less than 1% of B_{12} added to the medium. These results suggest that *S. platensis* NIES-39 does not have the ability to take up and accumulate exogenous B_{12}.

Our previous study (*10*) has indicated that except for foods containing substantial amounts of inactive corrinoid and/or B_{12}-substitutive compounds (probably deoxyribosides or deoxynucleotides), the observed correlation coefficient between the microbiological and chemiluminescence B_{12} assay methods is excellent. It

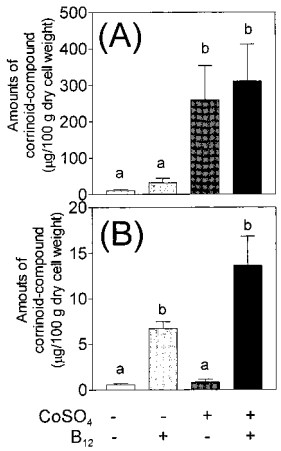

Figure 4. Effect of the addition of $CoSO_4$ and/or B_{12} on the amount of corrinoid-compound in *Spirulina platensis* NIES-39. *Spirulina* corrinoid-compound was assayed as B_{12} by the microbiological B_{12} assay (A) and chemiluminescence B_{12} assay (B) methods. Group means with different letters are significantly different ($p < 0.05$). Data represent means ± SD ($n = 3$).

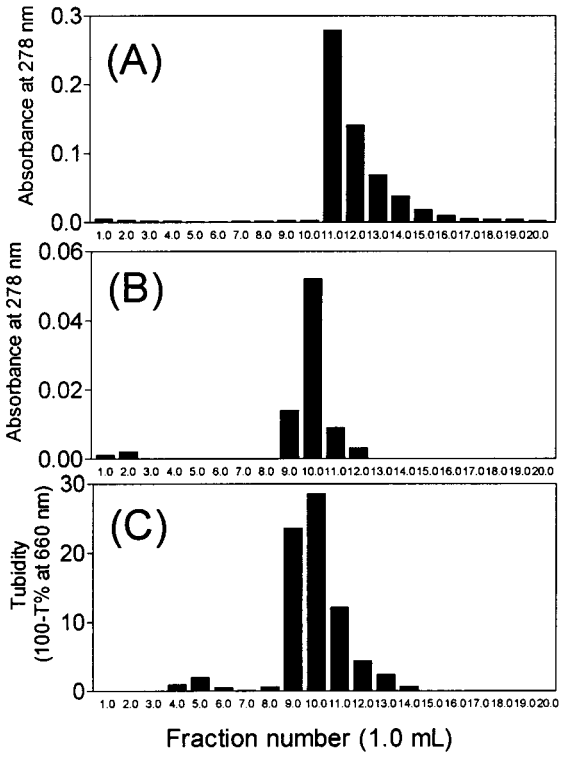

Figure 5. HPLC pattern of the corrinoid-compound found in *Spirulina platensis* NIES-39 grown in the $CoSO_4$-supplemented medium: (A) authentic B_{12}; (B) authentic pseudo-B_{12}; and (C) corrinoid-compound found in the algal cells grown in the $CoSO_4$-supplemented medium. The data are representative of HPLC patterns of the *Spirulina* corrinoid-compound from three independent experiments.

suggests that the significant increase in the amount of corrinoid-compound by the addition of $CoSO_4$ is due to formation of corrinoid-compound inactive for binding of the intrinsic factor.

The amounts of corrinoid-compound (314.90 ± 77.60 and 1.19 ± 0.56 μg/100 g of dry weight by the microbiological and chemiluminescence B_{12} assay methods, respectively) in *S. platensis* NIES-39 grown in the $CoSO_4$-supplemented medium under open culture system were similar to those (127.2−244.3 μg and 6.2−17.4 μg/100 g of dry, respectively) in commercially available *Spirulina* tablets as described previously (*8*).

HPLC Analysis of *Spirulina* Corrinoid-Compound Increased by the Addition of $CoSO_4$. To clarify the corrinoid-compound increased by the addition of $CoSO_4$, an extract of the algal cells grown in the $CoSO_4$-supplemented medium was analyzed by a C18 reversed-phase HPLC. The corrinoid-compound in the eluate from the HPLC column was determined by the microbiological B_{12} assay method. The elution pattern of the corrinoid-compound was identical to that of authentic pseudo-B_{12} but not to B_{12}; the identical elution pattern was also obtained in the algal cells grown in the $CoSO_4$ and B_{12}-supplemented medium (data not shown). These results strongly suggest that the *Spirulina* corrinoid-compound increased by the addition of $CoSO_4$ is pseudo-B_{12}, coinciding our previous study that pseudo-B_{12} is the predominant corrinoid of commercially available *Spirulina* tablets (*8*). Pseudo-B_{12} appears to be inactive for human pernicious anaemia (*9*) but not act as a B_{12}-antagonist (*8*).

Although most of corrinoid-compounds found in the *Spirulina* tablets have been reported to be biologically inactive corrinoid-compounds (*4*), some of which can block B_{12}-metabolism (*5*), there is little information available on chemical properties of these corrinoid-compounds. The results presented here indicate that the formation of these corrinoid-compounds including pseudo-B_{12} can be reduced significantly in the *Spirulina* cells grown in the absence of $CoSO_4$. If the *Spirulina* cells grown in the absence of $CoSO_4$ are lyophilized and fortified with a crystalline B_{12}, they would be suitable for use of human health foods.

ACKNOWLEDGMENT

This study was supported by a fund from The Salt Science Research Foundation, No. 0035, (F.W.).

LITERATURE CITED

(1) Vadiraja, B. B.; Gaikwad, N. W.; Madyastha, K. M. Hepatoprotective effect of C-phycocyanin: protection for carbon tetrachloride and R−(+)-pulegone-mediated hepatotoxicity in rats. *Biochem. Biophys. Res. Commun.* **1998**, *249*, 428−431.

(2) Yang, H. N.; Lee, E. H.; Kim, H. M. *Spirulina platensis* inhibits anaphylactic reaction. *Life Sci.* **1997**, *61*, 1237−1244.

(3) Hayashi, K.; Hayashi, T.; Kojima, I. A natural sulfated polysaccharide, calcium spirulan, isolated from *Spirulina plantesis*: in vitro and ex vivo evaluation of anti-herpes simplex virus and anti-human immunodeficiency virus activities. *AIDS Res. Hum. Retroviruses* **1996**, *12*, 1463−1471.

(4) Herbert, V.; Drivas, G. Spirulina and vitamin B_{12}. *JAMA* **1982**, *248*, 3096−3097.

(5) Herbert, V. Vitamin B_{12}: plant sources, requirements, and assay. *Am. J. Clin. Nutr.* **1988**, *48*, 852−858.

(6) Dagnelie, P. C.; van Staveren, W. A.; van den Berg, H. Vitamin B_{12} from algae appears not to be bioavailable. *Am. J. Clin. Nutr.* **1991**, *53*, 695−697.

(7) van den Berg, H.; Dagnelie, P. C.; van Staveren, W. A. Vitamin B_{12} and seaweed. *Lancet* **1988**, *1*, 242−243.

(8) Watanabe, F.; Katsura, H.; Takenaka, S.; Fujita, T.; Abe, K.; Tamura, Y.; Nakatsuka, T.; Nakano, Y. Pseudovitamin B_{12} is the predominant cobamide of an algal health food, spiriulina tablets. *J. Agric. Food Chem.* **1999**, *47*, 4736−4741.

(9) Schneider, Z. Biosynthesis of vitamin B_{12}. In *Comprehensive B_{12}*; Schneider, Z., Stroiñski, A., Eds.; Walter de Gruyter: Berlin, 1987; pp 93−110.
(10) Watanabe, F.; Takenaka, S.; Abe, K.; Tamura, Y.; Nakano, Y. Comparison of a microbiological assay and fully automated chemiluminescent system for the determination of vitamin B_{12} in foods. *J. Agric. Food Chem*. **1998**, *46*, 1433−1436.
(11) Kolhouse, J. F.; Allen, R. H. Absorption, plasma transport, and cellular retention of cobalamin analogues in the rabbit. *J. Clin. Invest*. **1977**, *60*, 1381−1392.

Received for review June 4, 2001. Revised manuscript received September 6, 2001. Accepted September 7, 2001.

JF010733I