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Characterization of Corrinoid Compounds in the Edible Cyanobacterium *Nostoc flagelliforme* the Hair Vegetable

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ABSTRACT

Vitamin B_{12} contents in the edible cyanobacterium *Nostoc flagelliforme*, also known as hair vegetable, were assayed using a microbiological method. We detected high vitamin B_{12} contents in samples of naturally grown cells (109.2 ± 18.5 µg/100g dry weight) and cultured cells (120.2 ± 53.6 µg/100g dry weight). However, commercially available hair vegetable samples, which comprised fake substitutes and *Nostoc*, had variable contents (4.8 - 101.6 µg/100g dry weight) because concomitant fake items contain very low vitamin B_{12} contents. To evaluate whether natural and cultured *N. flagelliforme* samples contained vitamin B_{12} or pseudovitamin B_{12}, corrinoid compounds were purified and identified as pseudovitamin B_{12} (approximately 72%) and vitamin B_{12} (approximately 28%) using silica gel 60 TLC bioautography and LC/MS. The results suggested that *N. flagelliforme* contains substantial amounts of pseudovitamin B_{12}, which is inactive in humans.

KEYWORDS

Edible Cyanobacteria; Hair Vegetable; *Nostoc flagelliforme*; Pseudovitamin B_{12}; Vitamin B_{12}

1. Introduction

Nostoc flagelliforme is an edible cyanobacterium, which grows naturally in some semidesert regions of China and Mongolia. When dried, the cyanobacterium resembles black hair and hence the name hair vegetable ("Facai" in Chinese), which is one of the most expensive ingredients in Chinese cuisine [1]. At present, fake items and mixtures of pure *N. flagelliforme* with fake substitutes (approximately 90%) are flooding the market [1].

N. flagelliforme contains many nutrients [2], including a novel acidic polysaccharide (nostoflan) that has potent antiviral activity [3]. Takenaka *et al*., [4] demonstrated the oral acute and subacute safety of dried *N. flagelliforme* in rats. Therefore, *N. flagelliforme* is also suitable for pharmaceutical use. Several studies [5,6] have reported that most of the corrinoids found in certain edible cyanobacteria may not be bioavailable in mammals. Watanabe *et al*. [7] also demonstrated that pseudovitamin B_{12} (adeninylcyanocobamide or pseudo B_{12}; **Figure 1**),

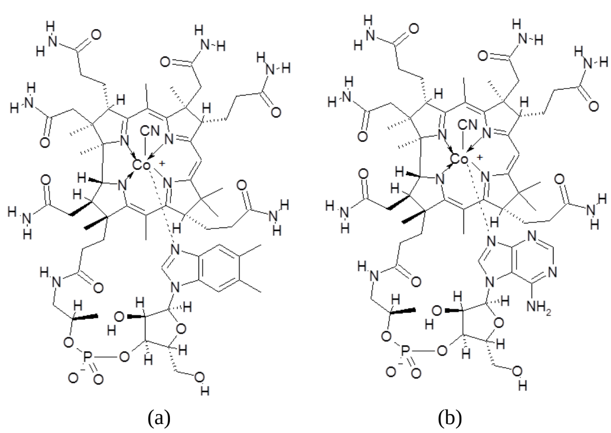

Figure 1. Structures of vitamin B_{12} (B_{12}) and pseudovitamin B_{12} (pseudo B_{12}). (a) B_{12}; (b) pseudo B_{12}.

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which is inactive in humans, is the predominant corrinoid in the edible cyanobacteria used as a health food by humans. *N. flagelliforme*, hair vegetable, is already used as health food, but there is no information on about B_{12} contents in pure *N. flagelliforme* and commercially available hair vegetable, or whether the corrinoids are authentic B_{12} or inactive corrinoids.

In the present study, we characterized corrinoid compounds from *N. flagelliforme* sources, including naturally grown samples, cultured samples, and commercially available hair vegetable samples.

2. Materials and Methods

2.1. Materials

Authentic B_{12} 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 were high-grade commercially available reagents. *N. flagelliforme* Born. et Flah. was harvested from Alxa, Inner Mongolia, China, during the summer of 1996. After washing in water, the cyanobacterium was dried in sun and used for the analyses. It was also aseptically cultured in a *Nostoc*-N liquid medium (K_2HPO_4, 40 mg/L; $MgSO_4 \cdot 7H_2O$, 70 mg/L; $Na_2SiO_3 \cdot 7H_2O$ 60 mg/L; $CaCl_2 \cdot 2H_2O$ 36 mg/L; $FeSO_4 \cdot 7H_2O$, 4.8 mg/L, EDTA 2Na, 1 mg/L; H_3BO_3, 2.86 mg/L; $MnCl_2 \cdot 4H_2O$, 1.8 mg/L; $ZnSO_4 \cdot 7H_2O$, 222 μg/L; $Na_2MoO_4 \cdot 2H_2O$, 390 μg/L; $CuSO_4 \cdot 5H_2O$, 80 μg/L; and $Co(NO_3)_2 \cdot 6H_2O$, 50 μg/L; at pH 7.5) at 20 - 25˚C with aeration under illumination (40 μmol/m^2/s). Commercially available hair vegetable samples were purchased from the markets in Japan.

2.2. Samples of *N. flagelliforme*

Samples A-E were naturally grown samples, F-J were cultured samples, and K-N were commercially hair vegetable samples.

2.3. Extraction and Assay of Corrinoids from *N. flagelliforme* Samples

Dried *N. flagelliforme* samples (five different lots of naturally grown and cultured samples and four commercially available hair vegetable samples) were used for the assays. First, 0.5 g of each sample was suspended in 40 mL of distilled water and homogenized with an ultrasonic disruptor UD-200 (Tomy, Tokyo, Japan). Total corrinoids were extracted after boiling at pH 4.8 in the presence of 4.0 × 10^{-4}% KCN and determined using the *Lactobacillus delbrueckii* ATCC 7830 microbiological assay method, according to the method described in the Standard Tables of Food Composition in Japan. *L. delbrueckii* ATCC 7830 can utilize deoxyribosides, deoxyribonucleotides (known as alkali resistant factor), and B_{12}. Thus, accurate B_{12} contents were calculated by subtracting the results for alkali resistant factor from those of total B_{12} [8].

2.4. Bioautography of Corrinoid Compounds Using Vitamin B_{12}-Dependent *Escherichia coli* 215

Bioautography of corrinoid compounds was performed as previously described [9]. B_{12} extracts (20 mL) prepared as mentioned above were partially purified and concentrated using a Sep-Pak Plus® C18 cartridge (Waters Corp., Milfora, USA), which was 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 B_{12} compounds were eluted using 2 mL of 75% (v/v) ethanol. The eluate was evaporated in a centrifugal concentrator (Integrated SpeedVac® System ISS110; Savant Instruments Inv., NY, USA). The residual fraction was dissolved in 5.0 mL of distilled water. Concentrated B_{12} extracts (1 μL) and authentic and pseudo B_{12} (each 50 μg/L) were spotted onto the silica gel 60 TLC sheet and developed in the dark using 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) at room temperature (25˚C). After drying the TLC sheet, it was overlaid with agar containing basal medium and precultured *E. coli* 215, and incubated at 37˚C for 20 h. The gel plate was then sprayed with methanol solution containing 2,3,5-triphenyltetrazolium salt, and B_{12} compounds were visualized as red, indicating *E. coli* growth.

2.5. Liquid Chromatography-Electrospray Ionization/Multistage Mass Spectrometry (LC/ESI-MS/MS) Analysis

Each extract (40 mL) was partially purified and concentrated using a Sep-Pak® Plus C18 cartridge (Waters Corp) as described above. The eluate was evaporated in a centrifugal concentrator (Integrated Speed VacR System ISS110), and the residual fraction was dissolved in 5.0 mL of distilled water. The purified extract was loaded onto an immunoaffinity column [EASI-EXTRACT® Vitamin B_{12} Immunoaffinity Column (P80) R-Biopharm AG, Darmstadt, Germany], and the corrinoids were purified according to the manufacturer's recommended protocol. *Nostoc* corrinoids, authentic pseudo B_{12}, and B_{12} were dissolved in 0.1% (v/v) acetic acid and filtered using a Nanosep MF centrifuge device (0.4 μm, Pall Corp., Tokyo, JAPAN) to separate 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 Inert Sustain 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 using a linear gradient of methanol (15% solvent B for 0 - 5 min, increasing the concentration from 15% to 90% solvent B for 5 - 11 min, and decreasing the concentration from 90% to 15% solvent B for 11 - 15 min). The flow rate was 0.2 mL/min. ESI conditions were determined by injecting authentic pseudo B_{12} or B_{12} into the MS detector to determine the optimum parameters for detecting the parent B_{12} compound and daughter ions. ESI-MS was operated in the positive ion mode. Argon was used as the collision gas. Pseudo B_{12} (*m/z* 672.777) and B_{12} (*m/z* 678.292) as $[M+2H]^{2+}$ were confirmed by comparing the observed molecular ions and the retention times.

2.6. Analytical High Performance Liquid Chromatography (HPLC)

Each immunoaffinity-purified B_{12} fraction (10 µL) was analyzed with a reversed-phase HPLC column (Wakosil-II 5C18RS, 4.6 × 150 mm; 5 µm particle size; Wako Pure Chemical Industries, Osaka, Japan). Corrinoids were isocratically eluted with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40˚C and monitored by measuring the absorbance at 361 nm. The flow rate was 1 mL/min. Retention times of authentic B_{12} and pseudo B_{12} were 8.6 min and 10.7 min, respectively. The relative content ratio of B_{12} and pseudo B_{12} in various *N. flagelliforme* samples was calculated on the basis of peak areas with identical retention times of B_{12} and pseudo B_{12}.

2.7. Evaluation of True and Fake *N. flagelliforme*

Because fake materials generally contain starch [2], commercially available hair vegetable samples were tested using the iodine-starch reaction. The dried samples (0.1 g) were added to 10 mL of distilled water and boiled for 30 min. The treated samples were cooled to a room temperature and centrifuged at 10,000 × *g* for 10 min at 25˚C. Each supernatant solution (0.2 mL) was added to 1.4 mL of distilled water and treated with 0.4 mL of 25% Lugol solution (MP Biomedicals, LLC, Ohio, USA). The solution was allowed to stand for 30 min, and absorbance was measured at 600 nm. Microscopic analysis was performed using a BH-2 type microscope (Olympus Corp., Tokyo, Japan) with a digital camera QV-200 (Casio Computer Co. Ltd, Tokyo, Japan), as previously described [2].

3. Results and Discussion

3.1. Vitamin B_{12} Contents

B_{12} contents were analyzed in various sources of *N. flagelliforme i.e.*, naturally grown samples, cultured samples, and commercially available hair vegetable samples, using the *L. delbrueckii* ATCC 7830 microbiological assay method (**Table 1**). High B_{12} contents were detected in naturally grown cells (109.2 ± 18.5 µg/100g dry weight) and cultured cells (120.2 ± 53.6 µg/100g dry weight). However, commercially available hair vegetable samples had very variable and lower B_{12} contents [45.1 ± 40.6 (range, 4.8 - 101.6) µg/100g dry weight]. B_{12} contents of natural and cultured cells were similar to those of other edible cyanobacteria, *i.e.*, *Spirulina* sp. (127.2 - 244.3 µg/100g dry weight) [10], Suizenji-nori (*Aphanothece sacrum*, 143.8 µg/100g dry weight) [11], and Ishikurage (*Nostoc commune*, 98.8 µg/100g dry weight) [12].

3.2. *E. coli* 215 Bioautography Analysis

Corrinoids found in all *Nostoc* samples were analyzed using the *E. coli* 215 bioautogram after separation by silica gel 60 TLC (**Figure 2**). Corrinoids found in all *Nostoc* samples and the commercially available hair vegetable sample K were separated to yield two spots, the *R*f values of which were identical to those of authentic pseudo B_{12} and B_{12}, respectively. No or faint spots were obtained with commercially available hair vegetable samples L-N because of their lower B_{12} contents.

3.3. LC/ESI-MS/MS Analysis

N. flagelliforme extracts were purified using a B_{12} immunoaffinity column and analyzed by LC/ESI-MS/MS (**Figure 3**). Authentic B_{12} and pseudo B_{12} were eluted as

Table 1. Vitamin B_{12} contents of various sources of *Nostoc flagelliforme* (naturally grown and cultured samples and commercially available hair vegetable samples).

Vitamin B_{12} content (µg/100g dry weight)					
Naturally grown cells		Culture cells		Commercially available hair vegetable	
A	92.6	F	58.4	K	101.6
B	109.4	G	109.7	L	35.4
C	89.5	H	198.4	M	38.5
D	133.2	I	90.6	N	4.8
E	120.5	J	144.1		
Mean ± SD	109.2 ± 18.5	Mean ± SD	120.2 ± 53.6	Mean ± SD	45.1 ± 40.6

[*]Total corrinoids were extracted from 0.5 g of each sample by boiling at pH 4.8 in the presence of KCN and determined using the *Lactobacillus delbrueckii* ATCC 7830 microbiological assay method. Because *L. delbrueckii* ATCC 7830 can utilize deoxyribosides, deoxyribonucleotides (known as alkali resistant factor), and B_{12}, B_{12} values were corrected by subtracting the results for alkali resistant factor from those for total B_{12}. B_{12} was assayed in triplicate for each sample, and the data is presented as mean values.

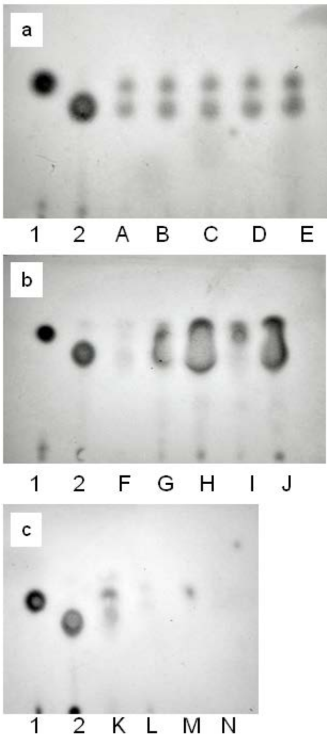

Figure 2. ***Escherichia coli*** **215 bioautogram analysis of corrinoids found in various** ***Nostoc flagelliforme*** **samples. (a) 1, authentic B_{12}; 2, authentic pseudo B_{12}; A-E, naturally grown samples; (b) 1, authentic B_{12}; 2, authentic pseudo B_{12}; F-J, cultured samples; (c) 1, authentic B_{12}; 2, authentic pseudo B_{12}; K-N, commercially available hair vegetable samples. One microliter of concentrated cell extracts and authentic B_{12} and pseudo B_{12} (each 50 µg/L), were spotted onto a silica gel 60 TLC sheet and developed in the dark using 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) at 25˚C. After drying the TLC sheet, it was overlaid with agar medium containing pre-cultured** ***E. coli*** **215 and incubated at 37°C for 20 h. B_{12} compounds on the gel were visualized as red spots using 2,3,5-triphenyltetrazolium salt. The data are representative of typical bioautograms from three independent experiments.**

peaks with retention times of 7.55 and 7.42 min, respectively. Mass spectrum of authentic B_{12} indicated that a doubly-charged ion with an *m/z* of 678.2897 $[M+2H]^{2+}$ was prominent (**Figures 3(a)** and **(b)**). The exact mass calculated from its formula ($C_{63}H_{88}CoN_{14}O_{14}P$) was 1354.5674, and the isotope distribution data showed that B_{12} was the major divalent ion under the LC/ESI-MS conditions. For authentic pseudo B_{12} with an exact mass of 1343.5375 ($C_{59}H_{83}CoN_{17}O_{14}P$), a doubly-charged ion with an *m/z* of 672.77861 $[M+2H]^{2+}$ was prominent (**Figures 3(d)** and **(e)**). The MS/MS spectra of B_{12} and pseudo B_{12} indicated that the dominant ions at *m/z* 359.0982 and *m/z* 348.0684, respectively, were attributable to the nucleotide moiety of each corrinoid compound (**Figures 3(c)** and **(f)**). *Nostoc* corrinoids purified from the naturally grown sample E were eluted to yield several total ion peaks, indicating the presence of impurities (**Figure 4(a)**). Ion peaks at *m/z* 672.77 and *m/z* 678.29 for pseudo B_{12} and B_{12}, respectively, were also detected, and their retention times were identical to those of authentic pseudo B_{12} and B_{12}. The mass spectra at the retention times of 7.42 and 7.55 min showed that both pseudo B_{12} and B_{12} divalent ions were formed at *m/z* 672.7735 (**Figure 4(b)**) and *m/z* 678.2888 (**Figure 4(d)**), respectively. There spective MS/MS spectra of each compound were identical to those of authentic pseudo B_{12} (**Figure 4(c)**) and B_{12} (**Figure 4(e)**). Similar results were obtained with other naturally grown and cultured cell samples (data not shown).

3.4. Relative Content Ratios of B_{12} and Pseudo B_{12} in Various *Nostoc* Samples

Table 2 summarizes the relative contents ratios of B_{12} and pseudo B_{12} in various *Nostoc* samples. The ratios of B_{12} (approximately 28%) and pseudo B_{12} (approximately 72%) are shown for naturally grown (A-E) and cultured (G and H) samples and for commercially available hair vegetable samples (K-M). Pseudo B_{12} was the predominant corrinoid in cultured samples F and J. In contrast, sample I contained approximately 76% of B_{12}. The variable ratios of B_{12} and pseudo B_{12} in the cultured samples

Table 2. **Relative content ratio of B_{12} and Pseudo B_{12} contents in various sources of** ***Nostoc flagelliforme*** **(naturally grown and cultured samples and commercially available hair vegetable samples).**

Naturally grown cells			Cultured cells			Commercially available hair vegetable		
	B_{12} (%)	Pseudo B_{12}(%)		B_{12} (%)	Pseudo B_{12}(%)		B_{12} (%)	Pseudo B_{12}(%)
A	28.0	72.0	F	6.2	93.8	K	20.7	79.3
B	29.3	70.7	G	22.7	77.3	L	29.9	70.1
C	28.5	71.5	H	22.4	77.6	M	25.5	74.5
D	25.1	74.9	I	76.2	23.8	N	nd	nd*
E	26.2	73.8	J	11.9	88.1			
Mean ± SD	27.4 1.7	72.6 1.7	Mean ± SD	27.9 27.9	72.1 27.9	Mean ± SD	25.4 4.6	4.6 4.6

*nd: not detected. Each B_{12} fraction (10 µL) was purified using a B_{12} immunoaffinity column and analyzed with a reversed-phase HPLC column (Wakosil-II 5C18RS, 4.6 × 150 mm; 5 µm particle size). B_{12} compounds were isocratically eluted with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40°C and monitored by measuring the absorbance at 361 nm. The relative content ratio of B_{12} and pseudo B_{12} of each sample was calculated on the basis of peak areas with identical retention times of B_{12} and pseudo B_{12}.Total corrinoids were extracted from 0.5 g of each sample by boiling at.

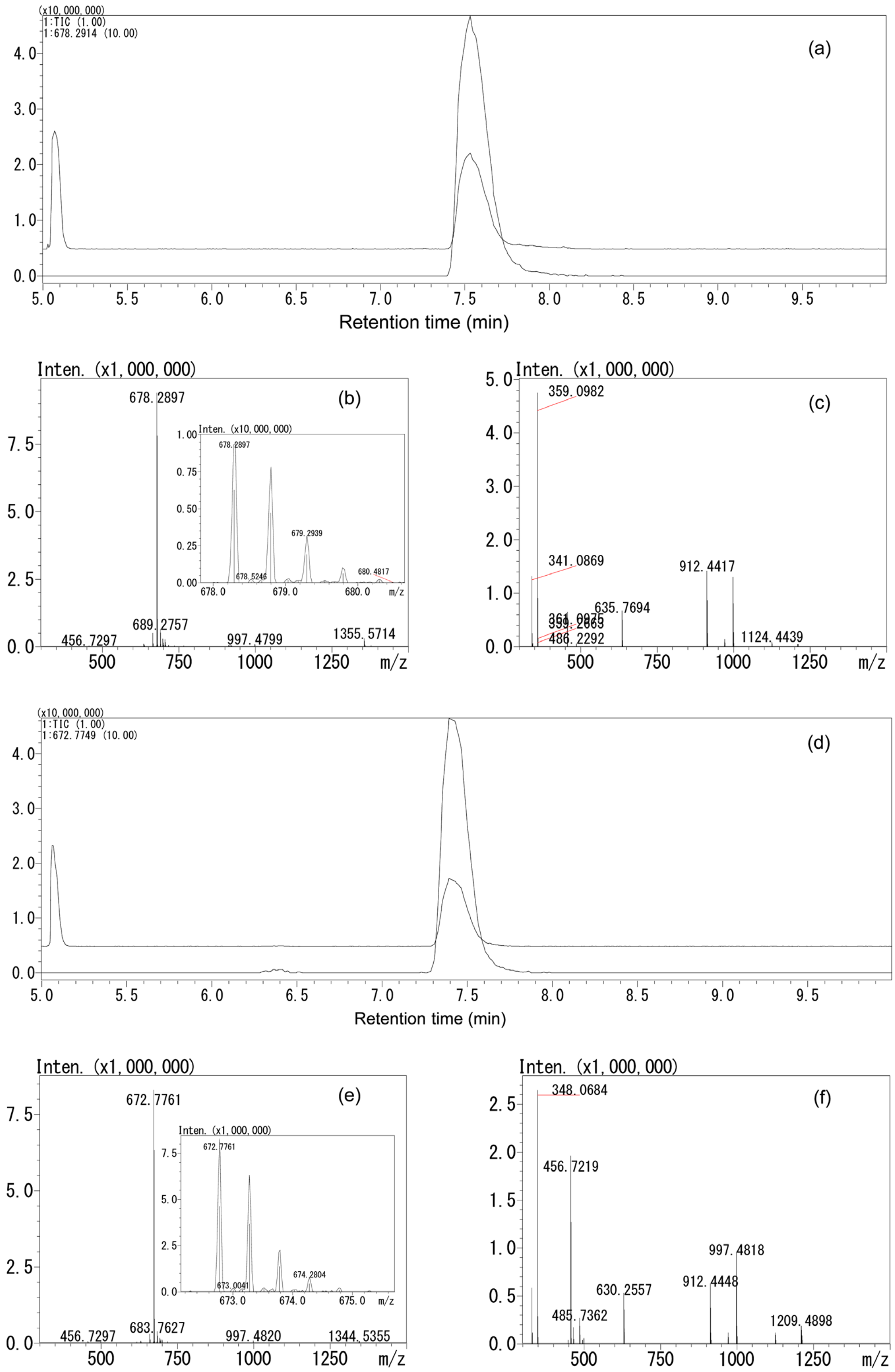

Figure 3. **Liquid chromatography-electrospray ionization/multistage Mass spectrometry (LC/ESI-MS/MS) of authentic B_{12} and pseudo-B_{12}. B_{12} and pseudo B_{12} were analyzed with LCMS-IT-TOF (Shimadzu) as described in the text. The total ion chromatograms (TIC) of authentic B_{12} and pseudo B_{12} are shown in panels (a) and (d), respectively. The mass spectra of each ion peak from B_{12} and pseudo B_{12} are shown in panels (b) and (e), respectively. The magnified mass spectra from *m/z* 678 to 680 in B_{12} and from *m/z* 672 to 675 in pseudo B_{12} are shown as inserts. The MS/MS spectra of the peaks of B_{12} and pseudo B_{12} are shown in panels (c) and (f), respectively.**

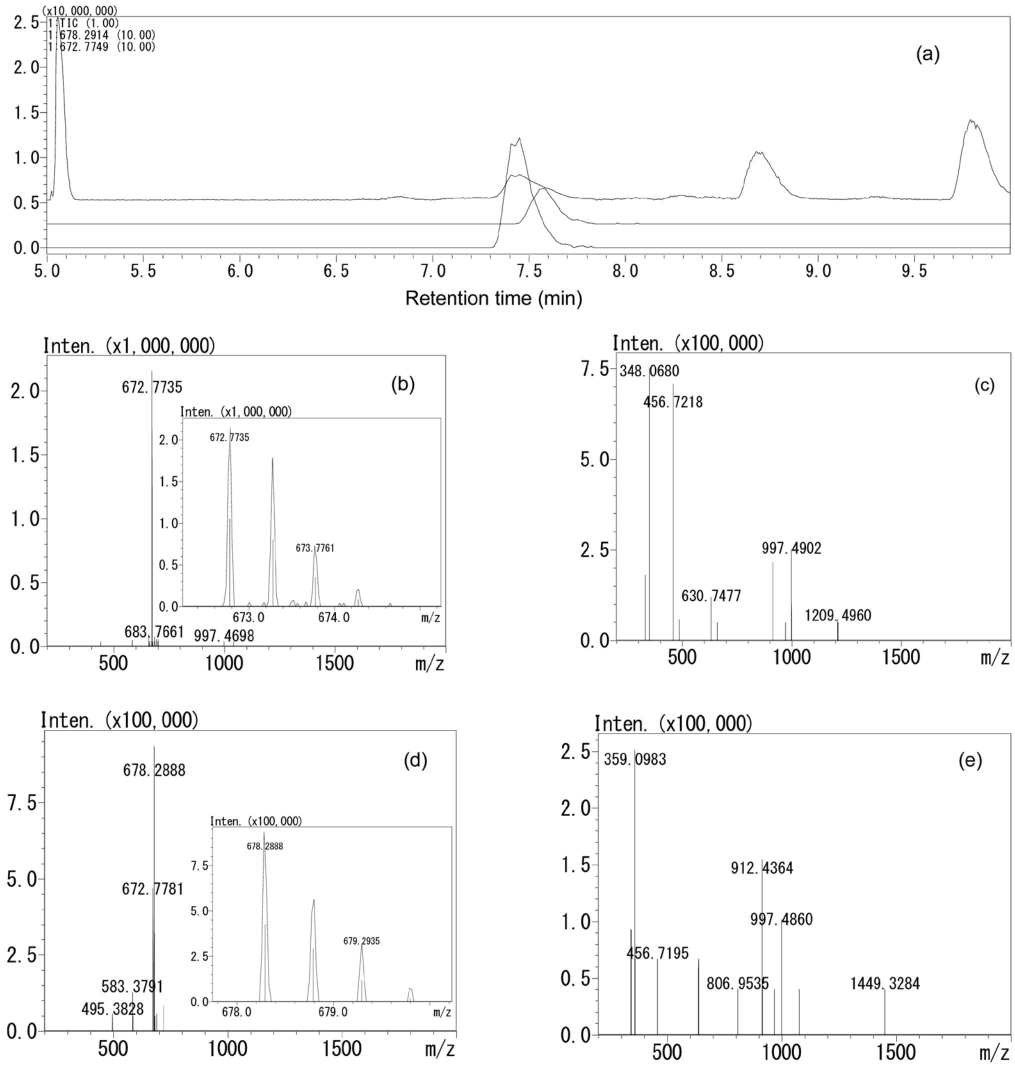

Figure 4. Liquid chromatography-electrospray ionization/multistage mass spectrometry (LC/ESI-MS/MS) of the purified corrinoids from naturally grown *Nostoc* sample (sample E). Total ion chromatograms (TIC) and reconstructed chromatograms for *m/z* 678.29 (×10) and 672.77 (×10) of the *Nostoc* corrinoids are shown in panel (a). The mass spectra of the ion peaks of the *Nostoc* corrinoids at retention times of 7.2 min and 7.4 min are shown in panel (b) (the magnified mass spectrum from *m/z* 672 to 675 is shown as an insert) and panel (d) (the magnified mass spectra from *m/z* 678 to 680 are shown as an insert), respectively. The MS/MS spectra for the peaks of the *Nostoc* corrinoids at *m/z* 672.7735 and at *m/z* 678.2888 are shown in panels (c) and (e), respectively.

may have been due to differences in the culture conditions, but we have no detailed information on the key factor that affected B_{12} and pseudo B_{12} ratios. These results indicate that most Nostoc samples and commercially hair vegetable samples contained pseudo B_{12} (major) and B_{12} (minor).

3.5. Evaluation of True and Fake *N. flagelliforme*

Because hair vegetable is one of the most expensive ingredients in Chinese cuisine, certain fake items represent a large proportion of the commercially available hair vegetable [2]. No color change was observed in all cultured samples, whereas all commercially available hair vegetable samples (K-N) exhibited significant staining by the iodine-starch method (optical densities of 0.29, 0.65, 0.34, and 1.19, respectively, at 600 nm). As shown in **Figure 5**, microscopic analysis indicated that although naturally grown and cultured *N. flagelliforme* possessed a bead-like morphology, no such morphology was found in the commercially available hair vegetable sample N (fake item only). The remaining hair vegetable samples K-M contained both *Nostoc* and fake substitutes. These microscopic data coincided with the results of iodine-starch reaction. Our results indicated that because naturally grown *N. flagelliforme* contain substantial amounts of

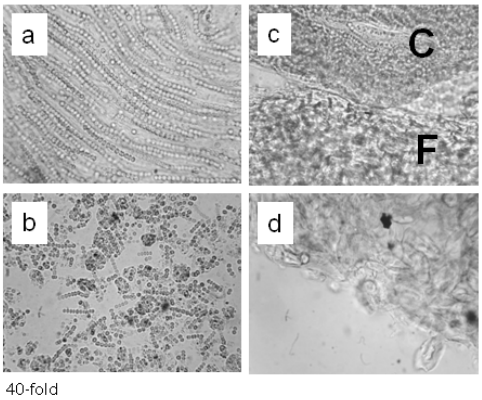

Figure 5. Microscopic analysis of various *Nostoc flagelliforme* samples. (a) Naturally grown samples; (b) cultured samples, (c) commercially available hair vegetable samples K-M (C, cells; and F, fake item), and (d) commercially available hair vegetable sample *N*. The results represent typical microscopic data from various *N. flagelliforme* samples.

pseudo B_{12}, which is inactive in humans [7] and because the fake items have very low B_{12} contents, commercially available hair vegetable is not suitable for use of B_{12} source, regardless of the presence of the fake items.

Cyanobacteria have the ability to synthesize pseudo B_{12} [13], which functions as a coenzyme of methionine synthase to catalyze the synthesis of methionine from homocysteine and N^5-methyltetrahydrofolate [14]. In the present study, the cultured *Nostoc* sample I predominantly contained B_{12} but not pseudo B_{12} (Table 2), suggesting that *N. flagelliforme* may synthesize both B_{12} and pseudo B_{12} *de novo*. Further biochemical and genetic studies are required to elucidate the detailed physiological functions of each corrinoid in this terrestrial cyanobacterium.

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