Characterization of Vitamin B_{12} Compounds in Food Ingredients Used in Chinese Cuisine

（中華食材に含まれるビタミン B_{12} 化合物の特徴の解明）

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Contents

Chapter Ⅰ

Introduction

Vitamin B_{12} or cyanocobalamin (B_{12}) is a water-soluble vitamin with the highest molecular weight (1355.4) among vitamins. B_{12} belongs to the "corrinoids", which comprises compounds that contain a corrin ring with a cobalt atom coordinated by the lower ligand (a cobalt coordinated nucleotide with 5, 6-dimethylbenzimidazole as a base moiety) [1]. There are some naturally occurring B_{12}-compounds with different upper ligands (L) (**Fig. 1**). Especially methylcobalamin (MeB_{12}) and adenosylcobalamin ($AdoB_{12}$) function as coenzymes of methionine synthase (EC 2.1.1.13) involved in methionine biosynthesis and methylmalonyl-CoA mutase (EC 5.4.99.2) involved in the amino acid and odd-chain fatty acid metabolisms in mammals, respectively [2, 3].

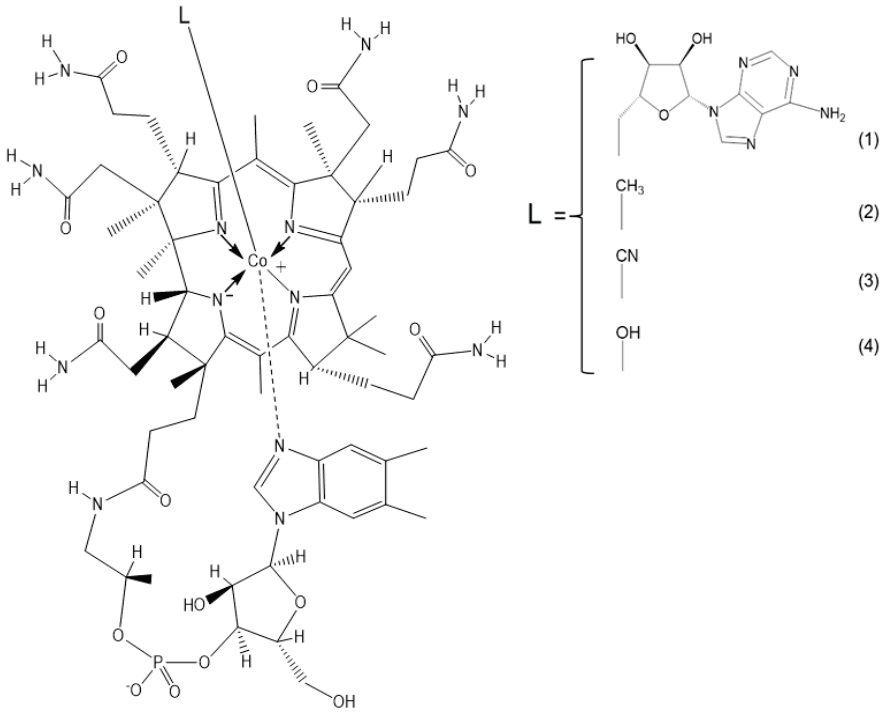


Fig. 1. Structural formula of vitamin B_{12}. (1) 5′-deoxyadenosylcobalamin, $AdoB_{12}$; (2) methylcobalamin, MeB_{12}; (3) hydroxocobalamin, $OH\text{-}B_{12}$; (4) cyanocobalamin or B_{12}, $CN\text{-}B_{12}$.

The intestinal absorption of dietary B_{12} is a complex and multistep process as shown in **Fig. 2**. After food is ingested, B_{12} is released from food in the stomach and then bound to a salivary B_{12} binding protein haptocorrin (HC), which is produced by salivary grands [4, 5]. After proteolysis of HC-B_{12} complex by pancreatic proteases in the duodenum, intrinsic factor (IF, gastric B_{12}-binding protein) binds the released B_{12} in the proximal ileum. The IF-B_{12} complex can enter mucosal cells in the distal ileum by receptor-mediated endocytosis [6, 7]. The IF-B_{12} complex is transferred to lysosomes in enterocytes and then proteases are responsible for digestion of the protein component of the IF-B_{12} complex. Subsequent lysosomal exit of B_{12} involves LMBD1 transporter [8, 9]. Free B_{12} is transported to cellular compartments, synthesized into the coenzyme forms, and exported into portal circulation. B_{12} is normally bound to transcobalamin-II (TC-II, a B_{12}-binding protein) in blood circulation.

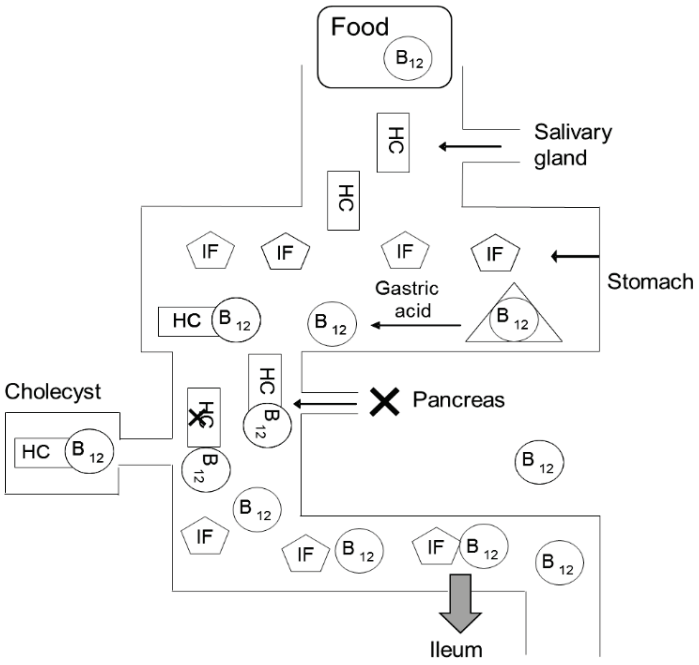


Fig. 2. Gastrointestinal absorption mechanism of B_{12}.

B_{12} is synthesized only by certain bacteria and is concentrated mainly in the bodies of higher predatory organisms in the natural food chain [10, 11]. Animal-derived foods (*i.e.*, meat, milk, egg, fish, and shellfish) are considered to be the major dietary sources of B_{12} [12]. In contrast, higher plants do not contain biosynthetic pathway of B_{12} and any B_{12}-dependent enzymes. Therefore, plant-derived foods contain no or only traces of B_{12}. Thus, strict vegetarians have a greater risk of developing B_{12} deficiency compared with nonvegetarians. The recommended dietary allowance (RDA) of B_{12} for adults is set at 2.4 μg/day in the United States and Japan. The major signs of B_{12} deficiency are megaloblastic anemia and neuropathy [13, 14].

Chinese cuisine has been established from China`s long history and gained a global reputation [15]. Chinese restaurants can be found in the world. However, there is little information on B_{12} content of food ingredients used in Chinese cuisine.

In the present thesis, I characterized B_{12} compounds in various food ingredients used in Chinese cuisine [plant-derived foods (**Chapter II, III** and **IV**) and animal-derived foods (**Chapter V, VI** and **VII**).

Chapter Ⅱ

Characterization of vitamin B_{12} compounds in the edible cyanobacterium *Nostoc flagelliforme* the hair vegetable

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 [16]. At present, fake items and mixtures of pure *N. flagelliforme* with fake substitutes (approximately 90%) are flooding the market [16].

N. flagelliforme contains many nutrients [17], including a novel acidic polysaccharide (nostoflan) that has potent antiviral activity [18]. Takenaka *et al.*, [19] demonstrated the oral acute and subacute safety of dried *N. flagelliforme* in rats. Therefore, *N. flagelliforme* is also suitable for pharmaceutical use. Several studies [20, 21] have reported that most of the corrinoids found in certain edible cyanobacteria may not be bioavailable in mammals. Watanabe *et al.* [22] also demonstrated that pseudo-vitamin B_{12} (adeninylcyanocobamide or pseudo-B_{12}; **Fig. 3**), 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 this chapter, I characterized corrinoid compounds from *N. flagelliforme* sources, including naturally grown samples, cultured samples, and commercially available hair

vegetable samples.

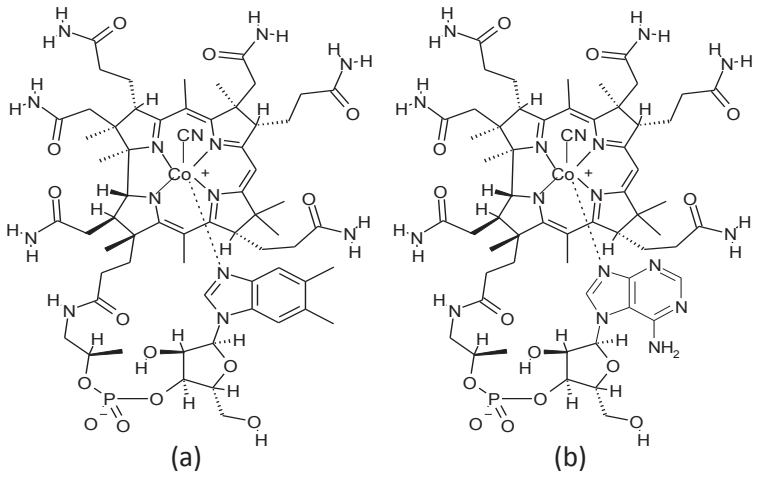


Fig. 3. Structures of vitamin B_{12} and pseudovitamin B_{12}.
(a) B_{12}; (b) pseudo-B_{12}.

Materials and Methods

Materials

Authentic B_{12} was obtained from Sigma (St Louis, MO, USA). A B_{12} assay medium based on *Lactobacillus delbrueckii* subspecies lactis (formerly *L. leichmannii*) ATCC 7830 was obtained from Nissui (Tokyo, Japan). 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* 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.

Samples of N. flagelliforme

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

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 0.0004% (w/v) KCN for 30 min to convert various B_{12} compound with different upper ligands (e.g., coenzyme forms of B_{12}) to CN-B_{12}. The extraction procedures were performed in a draught chamber (Dalton Co., Tokyo, Japan). Then, total B_{12} compounds were determined using the *L. 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} [23].

Bioautogram of corrinoid compounds using B_{12}-dependent Escherichia coli 215

Bioautography of corrinoid compounds was performed as previously described [24].

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.

Identification of B_{12} compounds by LC/ESI-MS/MS

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® B_{12} Immunoaffinity Column (P80) R-Biopharm AG, Darmstadt, Germany], and the corrinoids were purified according to the manufacturer's recommended protocol. The corrinoids of each sample, 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.

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 of various *N. flagelliforme* samples 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}.

Evaluation of true and fake N. flagelliforme

Because fake materials generally contain starch [17], 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 [17].

Results and Discussion

B_{12} contents in N. flagelliforme

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/100 g dry weight) and cultured cells (120.2 ± 53.6 μg/100 g 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/100 g 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/100 g dry weight) [25], Suizenji-nori (*Aphanothece sacrum*, 143.8 μg/100g dry weight) [26], and Ishikurage

(*Nostoc commune*, 98.8 μg/100 g dry weight) [27].

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/100 g 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

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 (**Fig. 4**). 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.

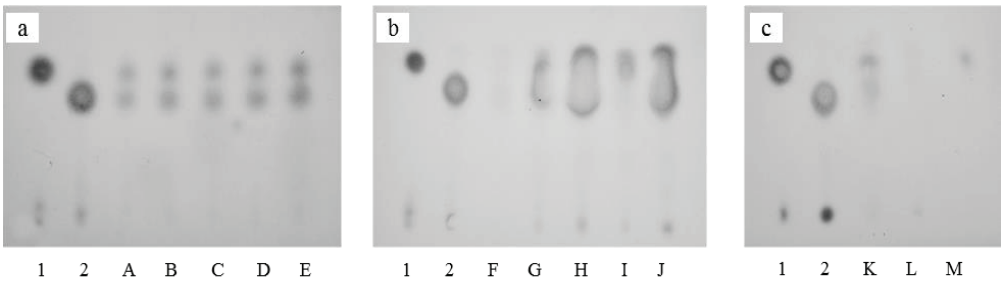


Fig. 4. *Escherichia coli* 215 bioautogram analysis of corrinoids found in various *Nostoc* samples. a) 1, authentic B_{12}; 2, authentic pseudo-B_{12}; A-E, naturally grown samples; b) 1, authentic B_{12}; 2, authentic pseudo-B_{12}; F-J, cultured samples; c) 1, authentic B_{12}; 2, authentic pseudo-B_{12}; K-N, commercially available hair vegetable samples.

LC/ESI-MS/MS analysis

N. flagelliforme extracts were purified using a B_{12} immunoaffinity column and analyzed by LC/ESI-MS/MS (**Fig. 5**). Authentic B_{12} and pseudo-B_{12} were eluted as peaks with retention times of 7.6 and 7.4 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 (**Fig. 5 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 672.7786 $[M + 2H]^{2+}$ was prominent (**Fig. 5 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 (**Fig. 5 C and F**).

Nostoc corrinoids purified from the naturally grown sample were eluted to yield several total ion peaks, indicating the presence of impurities (**Fig. 6 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 7.4 and 7.6 min showed that both pseudo-B_{12} and B_{12} divalent ions were formed at *m/z* 672.7735 (**Fig. 6 B**) and *m/z* 678.2888 (**Fig. 6 D**), respectively. These MS/MS spectra of each compound were identical to those of authentic pseudo-B_{12} (**Fig. 6 C**) and B_{12} (**Fig. 6 E**). Similar results were obtained with cultured cell samples (data not shown).

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 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).

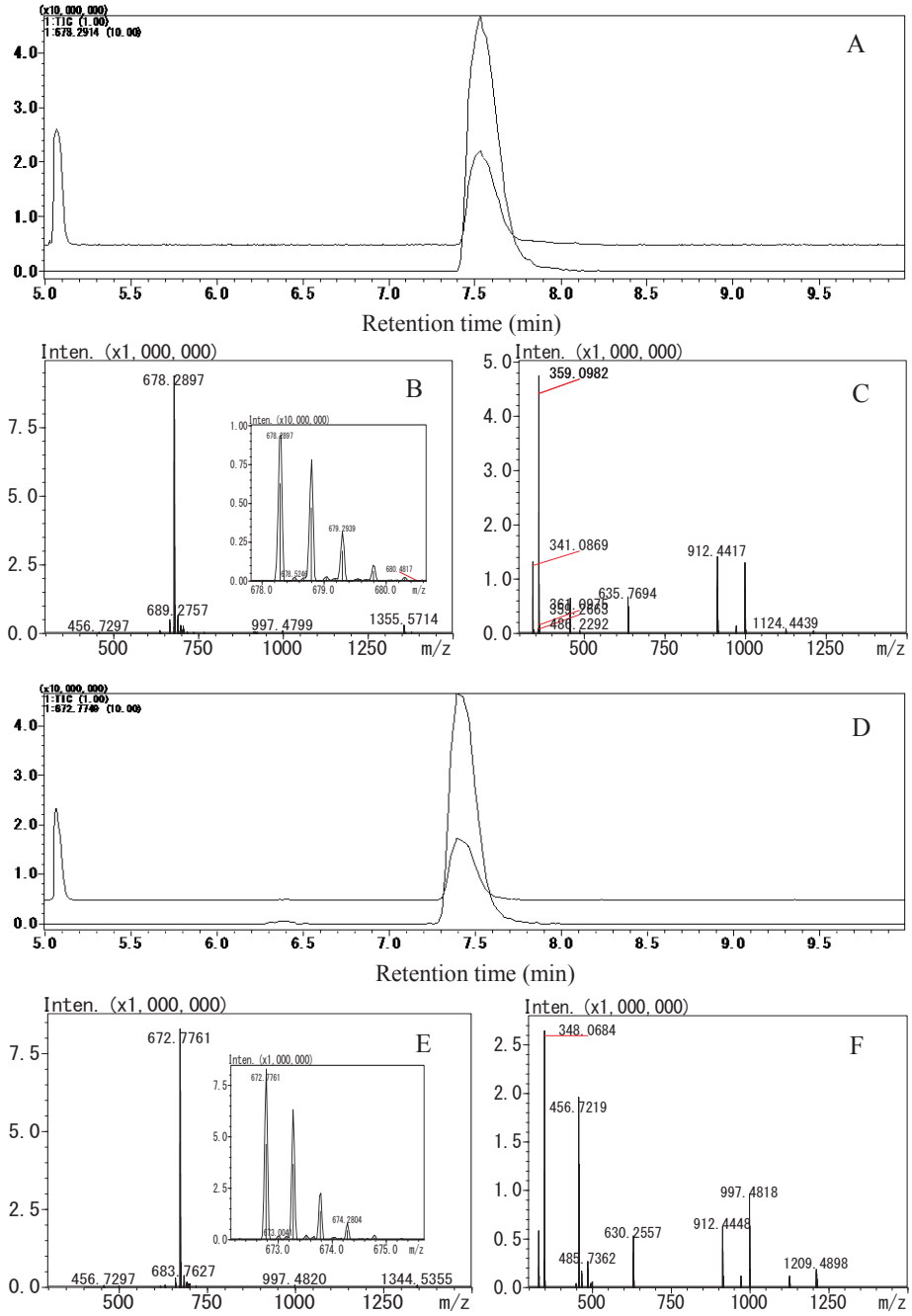


Fig. 5. LC/ESI-MS/MS chromatograms of authentic B_{12} and pseudo-B_{12}. B_{12} and pseudo-B_{12} were analyzed with LCMS-IT-TOF (Shimadzu) as described in the text. The total ion chromatograms (TIC) of authentic B_{12} and pseudo-B_{12} are shown in panels A and D, respectively. The mass spectra of 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.

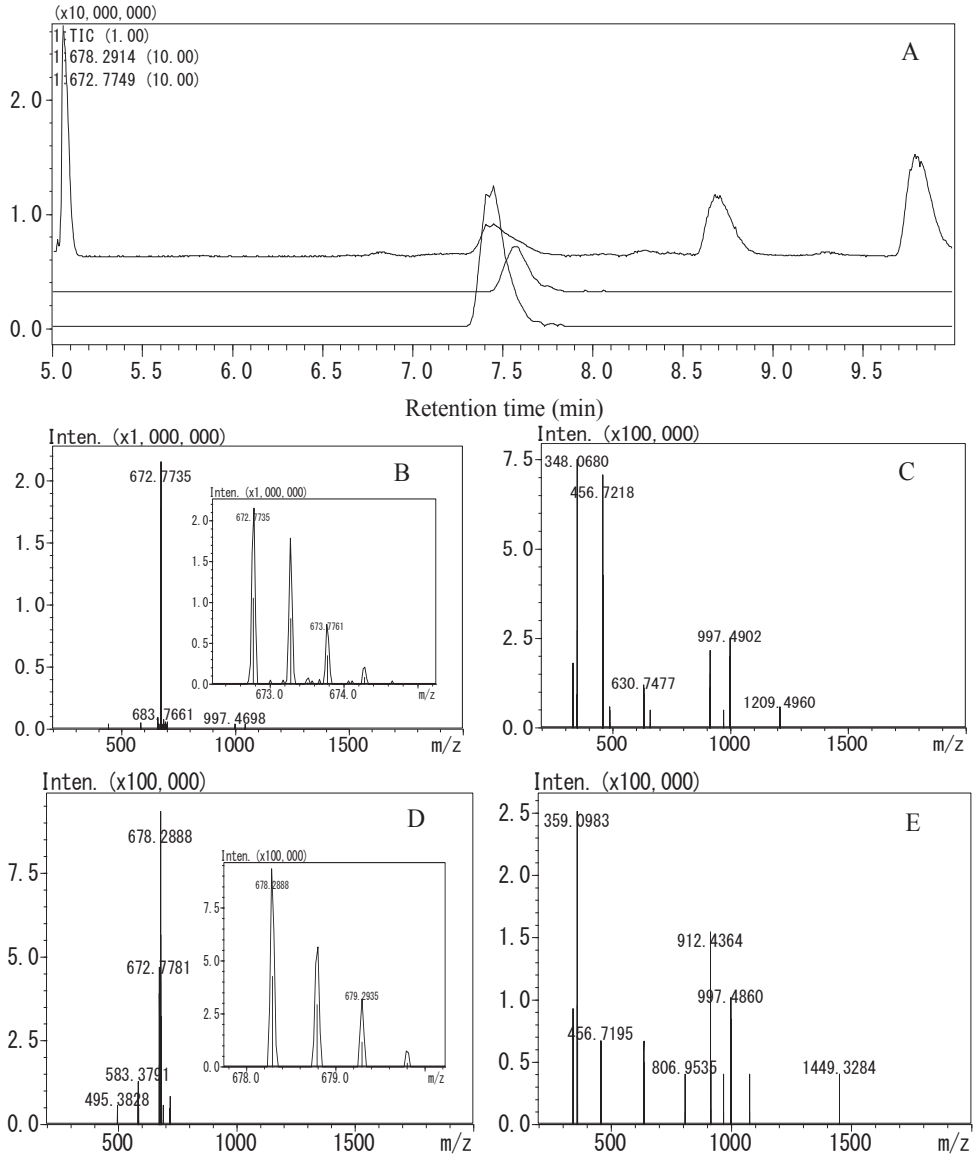


Fig. 6. LC/ESI-MS/MS chromatograms of the purified corrinoids from naturally grown *Nostoc* sample. Total ion chromatograms (TIC) and reconstructed chromatograms for m/z 678.29 (× 10) and 672.77 (× 10) of the Nostoc corrinoids are shown in panel A. The mass spectra of the ion peaks of the Nostoc corrinoids at retention times of 7.4 min and 7.6 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.

Table 2. Relative content ratio of vitamin B_{12} and pseudovitamin 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.

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 [17]. 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 **Fig. 7**, 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 pseudo-B_{12}, which is inactive in humans [22] 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} [28], which functions as a coenzyme of methionine synthase to catalyze the synthesis of methionine from homocysteine and N^5-methyltetrahydrofolate [29]. 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.

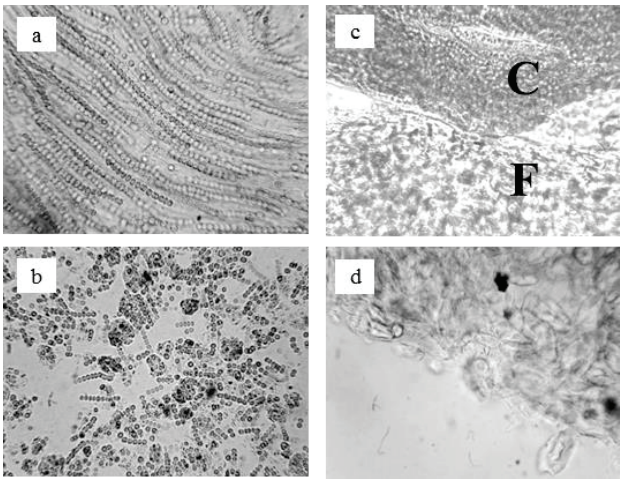


Fig. 7. Microscopic analysis of various *N. flagelliforme* samples.
(a) Naturally grown samples; (b) cultured samples, (c) commercially available hair vegetable samples K-M (C, cells; and F, fake item), and (d) commercially available hair vegetable sample N. The results represent typical microscopic data from various *N. flagelliforme* samples**.**

Summary

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

Chapter III

Characterization of vitamin B_{12} compounds in cultured and dried lion's mane mushroom (*Hericium erinaceus*) fruiting bodies

Introduction

The edible medicinal mushroom *Hericium erinaceus* is well-known as the lion's mane mushroom and is also called "yamabushitake" and "houtou" in Japan and China, respectively. The fruiting body of *H. erinaceus* is commercially cultivated and has attracted increasing attention in pharmaceutical and functional food industries because of its health promotion and disease alleviation effects on human health [30, 31]. Thus, pills containing the dried fruiting body of *H. erinaceus* are manufactured and used as a health food.

Animal-derived foods (*i.e.*, meat, milk, egg, fish, and shellfish), but not plant-derived foods, are considered to be the major dietary sources of B_{12} [12]. Thus, strict vegetarians have a greater risk of developing B_{12} deficiency compared with nonvegetarians [32]. The major symptoms of B_{12} deficiency are neuropathy and megaloblastic anemia [13, 14].Thus, we need to identify edible mushrooms that contain high levels of B_{12} to prevent vegetarians from developing B_{12} deficiency. Previously, Watanabe *et al.* demonstrated that black trumpet (*Craterellus cornucopioides*) and golden chanterelle (*Cantharellus cibarius*) mushrooms contained considerable levels (1.09 - 2.65 μg/100 g dry weight) of B_{12}, whereas porcini mushrooms (*Boletus* spp.), parasol mushrooms (*Macrolepiota procera*), oyster mushrooms (*Pleurotus ostreatus*), and black morels (*Morchella conica*) had zero or trace levels (0.01 - 0.09 μg/100 g dry weight) of B_{12} [33]. If the fruiting body of *H. erinaceus* contains large amount of B_{12}, it would be a

good source of B_{12} in vegetarians. However, there is little information available on the B_{12} content of *H. erinaceus* fruiting bodies and particularly whether this mushroom contains B_{12} or an inactive pseudo-B_{12}.

In **Chapter III**, I analyzed the B_{12} contents of various dried *H. erinaceus* fruiting body samples and characterized the B_{12} compounds present in this mushroom.

Materials and Methods

Materials

B_{12} was obtained from Sigma (St Louis, MO). A B_{12} assay medium based on *L. delbrueckii* ATCC 7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 TLC aluminum sheets were obtained from Merck (Darmstadt, Germany). The dried lion's mane mushroom samples were purchased in Japan.

Extraction and assay of B_{12} in edible mushroom

Each *H. erinaceus* fruiting body (approximately 10 g) was homogenized using a mixer (TML160; Tescom & Co. Ltd., Tokyo, Japan). A portion (5 g) of the homogenate was used as the test sample. B_{12} was assayed using a microbiological technique based on *L. delbrueckii* ATCC 7830, according to the method described in **Chapter II**.

In the case of B_{12} assay using B_{12}-dependent *E. coli* 215, each B_{12} compound was spotted onto a filter paper (circle, 6 mm), which was placed on 1.5% (w/v) agar containing a basal medium of *E. coli* 215 and incubated at 30 °C for approximately 20 h [24]. The gel plate was sprayed with a methanol solution containing 2,3,5-triphenyltetrazolium salt to enable visualization of the B_{12} compounds, indicating *E. coli* growth.

Identification of mushroom B_{12} compounds by LC/ESI-MS/MS

B_{12} compounds were extracted under the same conditions described above-mentioned method. Aliquots (approximately 200 mL) of the supernatant were placed in Sep-Pak® Vac 20 cc (5 g) C18 cartridges (Waters Corp.), which had been washed with 75% (v/v) ethanol and equilibrated with distilled water. The C18 cartridges were washed with 30 mL of distilled water, and B_{12} compounds were eluted using 30 mL of 75% (v/v) ethanol. The remaining supernatant was treated in a similar manner. The combined eluates were evaporated to dryness under reduced pressure and the residual fraction was dissolved in 5 mL of distilled water and centrifuged at centrifuged at 10,000 × *g* for 10 min to remove any insoluble material. The supernatant fraction was loaded onto an immunoaffinity column (EASI-EXTRACT B_{12} Immunoaffinity Column (P80) R-Biopharm AG, Darmstadt, Germany), and B_{12} compounds were purified according to the manufacturer's protocol.

The purified mushroom B_{12} compounds and authentic 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 remove small particles. We analyzed an aliquot (2 μL) of the filtrate using an LC/MS-IT-TOF system coupled to an Ultra-Fast LC system (Shimadzu, Kyoto, Japan). Each purified sample 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. B_{12} 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). The flow rate was 0.2 mL/min. The ESI conditions were determined by injecting authentic B_{12} into the MS detector to identify the optimum parameters for detecting the parent and daughter ions of the B_{12} compound. ESI-

MS was operated in the positive ion mode using argon as the collision gas. The identification of B_{12} (*m/z* 678.2914) representing $[M + 2H]^{2+}$ was confirmed by comparing the observed molecular ions and their retention times.

Preparation of B_{12}[c-lactone]

B_{12}[*c*-lactone] was synthesized according to the method of Pathare *et al* [34]. In brief, B_{12} (cyanocobalamin, 0.5 g), was dissolved in 150 mL of distilled water, and 7.5 mL of acetic acid was added. Sodium iodide (50 mg) and N-chlorosuccinimide (90 mg) were then added, and the reaction mixture was stirred at 25 °C for 16 h in the dark. After the reaction mixture was desalted using Sep-Pak® Vac 20 cc (5 g) C18 cartridges, the desalted solution was evaporated to dryness under reduced pressure and then dissolved in a small amount of distilled water. The solution was further purified using a reversed-phase HPLC column (Wakosil-II 5C18RS, 4.6 × 150 mm; 5-μm particle size; Wako Pure Chemical Industries, Osaka, Japan). B_{12}[*c*-lactone] was isocratically eluted with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40 °C and monitored by measuring the absorbance at 280 nm. The flow rate was 1 mL/min. A main peak with a retention time of 12.2 min was recovered and treated as B_{12}[*c*-lactone] in the experiments. B_{12}[*c*-lactone] concentration was determined using the molar extinction coefficient ($\varepsilon = 2.63 \times 104$) at an absorbance of 359 nm as described previously [34].

Analytical TLC and HPLC

Concentrated solutions of prepared B_{12}[*c*-lactone] and authentic B_{12} were spotted onto the silica gel 60 TLC sheet and developed at 25 °C in the dark using 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) and 1-butanol/2-propanol/water (10:7:10 v/v)

as solvents I and II, respectively. After drying the TLC sheet, the R_f values of the red spots were measured. In HPLC analysis, prepared B_{12}[*c*-lactone], authentic B_{12}, or a reaction mixture was analyzed using 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. The retention times of authentic B_{12} and prepared B_{12}[*c*-lactone] were 8.4 and 12.2 min, respectively.

Conversion of B_{12} to B_{12}[c-lactone] by Chloramine-T

The reaction mixture contained 10 μmol/L B_{12} (cyanocobalamin), 10 μmol/L chloramine-T, and 100 mmol/L citrate buffer (pH range 3.0 - 6.0), 2-(N-morpholino) ethane sulfonic acid buffer (pH range 5.0 - 8.0), 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid buffer (pH range 7.0 - 9.0), or borate buffer (pH range 8.0 - 10.0). After leaving the reaction mixture for 24 h at 25 °C in the dark, each mixture was immediately analyzed using the reversed-phase HPLC system, as described above. The relative concentrations of B_{12} and B_{12}[*c*-lactone] were calculated based on the peak areas with the identical retention times of B_{12} and B_{12}[*c*-lactone], respectively. B_{12}[*c*-lactone] formation was represented as the percentage of B_{12}[*c*-lactone] versus the sum of B_{12} and B_{12}[*c*-lactone].

Results and Discussion

B_{12} content in dried lion's mane mushroom

The B_{12} contents were determined in seven samples of dried lion's mane mushroom

(*H. erinaceus*) fruiting body using a microbiological assay method based on *L. delbrueckii* ATCC 7830 (**Table 3**). Trace levels (0.04 - 0.36 μg/100 g dry weight) of B_{12} were found in most samples (A, B, D, E, and F), and samples C and G contained 0.56 and 1.04 μg/100 g dry weight of B_{12}, respectively. As these cultured and dried lion's mane mushroom fruiting body samples were obtained from different manufacturers as food items, precise strains of the tested lion's mane mushrooms, culture conditions, and postharvest processing methods (probably heat-drying and then storage at an ambient temperature) of their fruiting bodies are not available. Thus, I have little information on why such differences in B_{12} contents occurred among these dried fruiting bodies. *L. delbrueckii* ATCC 7830 can utilize deoxyribosides and deoxyribonucleotides (known as alkali-resistant factors) as well as B_{12}. High levels (0.51 - 4.03 μg as B_{12} equivalent/100 g dry weight) of the alkali-resistant factor were found in all mushroom fruiting body samples.

Table. 3. Vitamin B_{12} contents of various types of dried lion's mane mushroom fruiting bodies.

Sample	Vitamin B_{12} content (μg/100 g dry weight)
A	0.04
B	0.36
C	0.56
D	0.10
E	0.27
F	0.20
G	1.04
Mean ± SD	0.37 ± 0.34

LC/ESI-MS/MS analysis

Corrinoids were purified from the extracts of dried lion's mane mushroom fruiting bodies using an immunoaffinity column and identified by LC/ESI-MS/MS (**Fig. 8**). Authentic B_{12} was eluted as a peak with a retention time of 7.6 min (**Fig. 8 A**). The mass spectrum of authentic B_{12} mainly contained a doubly charged ion with an *m/z* of 678.2883 $[M + 2H]^{2+}$ (**Fig. 8 A** and **B**). Its exact mass was calculated from its formula ($C_{63}H_{88}CoN_{14}O_{14}P$, 1354.5674) and the isotope distribution data showed that B_{12} mainly existed as a doubly charged ion under LC/ESI-MS conditions. The MS/MS spectrum of B_{12} indicated that a monovalent ion with an *m/z* of 359.0984 predominated, which was mainly attributable to the nucleotide moiety of B_{12} (**Fig. 8 C**). The compounds purified from dried lion's mane mushroom sample G were eluted as several ion peaks, which indicated that impurities were still present. The mass spectrum of the main peak had a retention time of 7.6 min in the purified sample (**Fig. 8 D**), where the B_{12} doubly charged ions had an *m/z* value of 678.2873 (**Fig. 8 E**). The MS/MS spectrum of the compound was identical to that of B_{12} (**Fig. 8 F**). These results indicated that dried lion's mane mushroom sample G contained B_{12} but not inactive corrinoid compounds. Similar LC/ESI-MS/MS chromatograms were obtained for the samples B, D, and F. No spectrum was obtained for mushroom sample A because it only had a trace level of B_{12}.

However, the compounds purified from mushroom sample C were eluted as two ion peaks in the chromatogram of *m/z* values of 678.2914 (**Fig. 9 A**). The mass spectrum of the minor ion peak had a retention time of 7.5 min in the purified compounds, where the B_{12} doubly charged ions had *m/z* values of 678.2877 (**Fig. 9 B**). The MS/MS spectrum of the compound was identical to that of B_{12} (**Fig. 9 C**). The mass spectrum of the major ion peak had a retention time of 7.7 min in the purified compounds (**Fig. 9 A**). The mass

spectrum of the major peak had a doubly charged ion with an *m/z* of 677.7748 $[M + 2H]^{2+}$ (**Fig. 9 D**). The MS/MS spectrum of the major peak indicated that a monovalent ion with an *m/z* of 359.0974 (the nucleotide moiety of B_{12}) predominated (**Fig. 9 E**). The identical spectra were also obtained with sample E. These results indicated that dried lion's mane mushroom samples C and E contained another B_{12} compound as well as B_{12}.

Occurrence of the unnatural corrinoid B_{12}[c-lactone] in commercially available dried lion's mane mushroom fruiting bodies

Chloramine-T, an antimicrobial agent, has widespread uses in medical, dental, veterinary, food processing, and agricultural fields because of its low cytotoxicity [35]. Bonnett *et al.* reported that chloramine-T is a source of electrophilic chlorine and it reacts with B_{12} to form B_{12}[*c*-lactone], which contains no halogen, at pH 4.0 (**Fig. 10**) [36**]**. Thus, B_{12}[*c*-lactone] was synthesized according to the method of Pathare *et al*. and then analyzed using the silica gel 60TLC and reversed-phase HPLC (**Table 4**) [34]. Although B_{12} and B_{12}[*c*-lactone] showed similar R_f values on TLC, B_{12}[*c*-lactone] was eluted as a single peak with different retention time of B_{12} during HPLC.

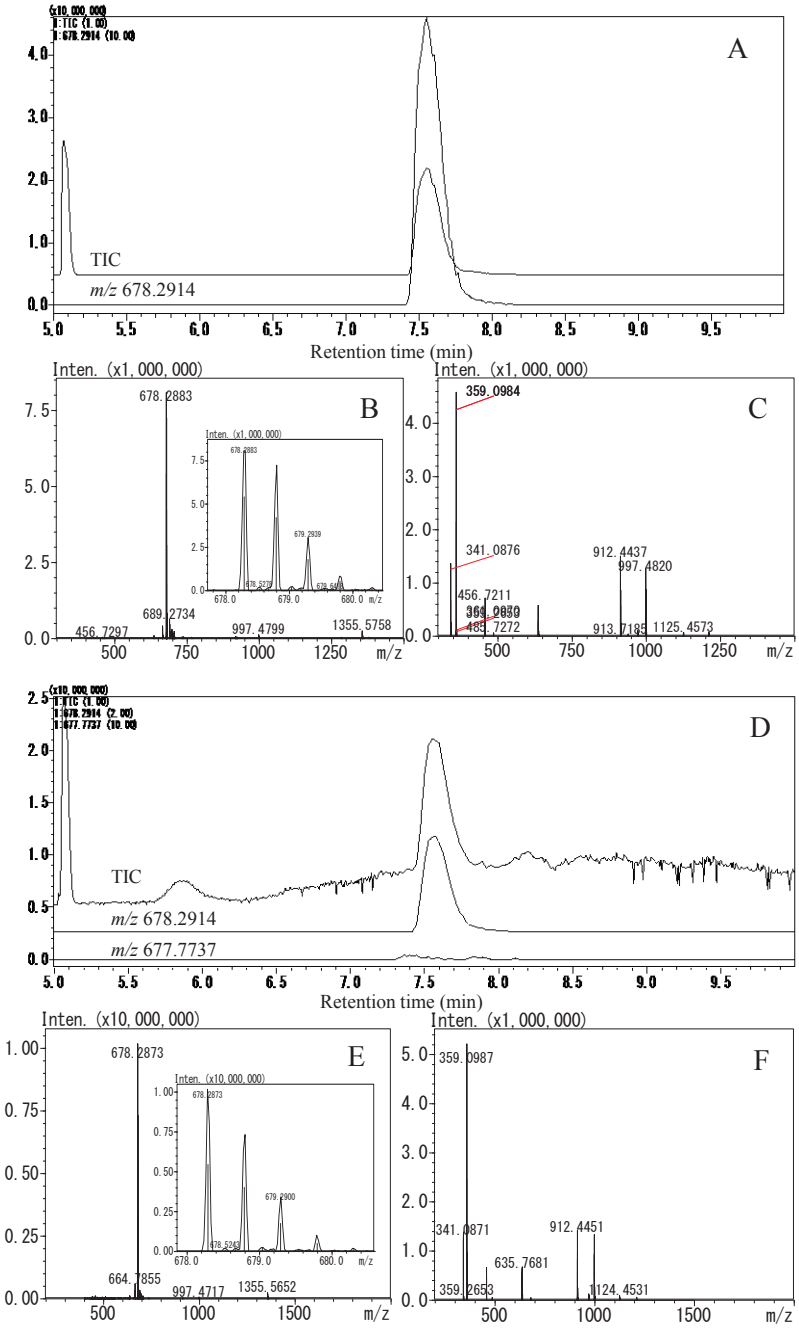


Fig. 8. LC/ESI-MS/MS chromatograms of authentic B_{12} and the B_{12} compounds purified from the lion's mane mushroom sample G. Panels A and D show the TICs and the chromatograms (*m/z* 678.2914) of authentic B_{12} and the purified B_{12} compounds from lion's mane mushroom sample G, respectively. The mass spectra of authentic B_{12} and the B_{12} compounds purified from mushroom sample G at 7.6 min are shown in panels B and E, respectively (the magnified spectrum range from *m/z* 678 to *m/z* 680 is shown as an insert in each panel). The MS/MS spectra for *m/z* 678.2883 peak of authentic B_{12} and the B_{12} compounds purified from the mushroom sample G are shown in panels C and F, respectively.

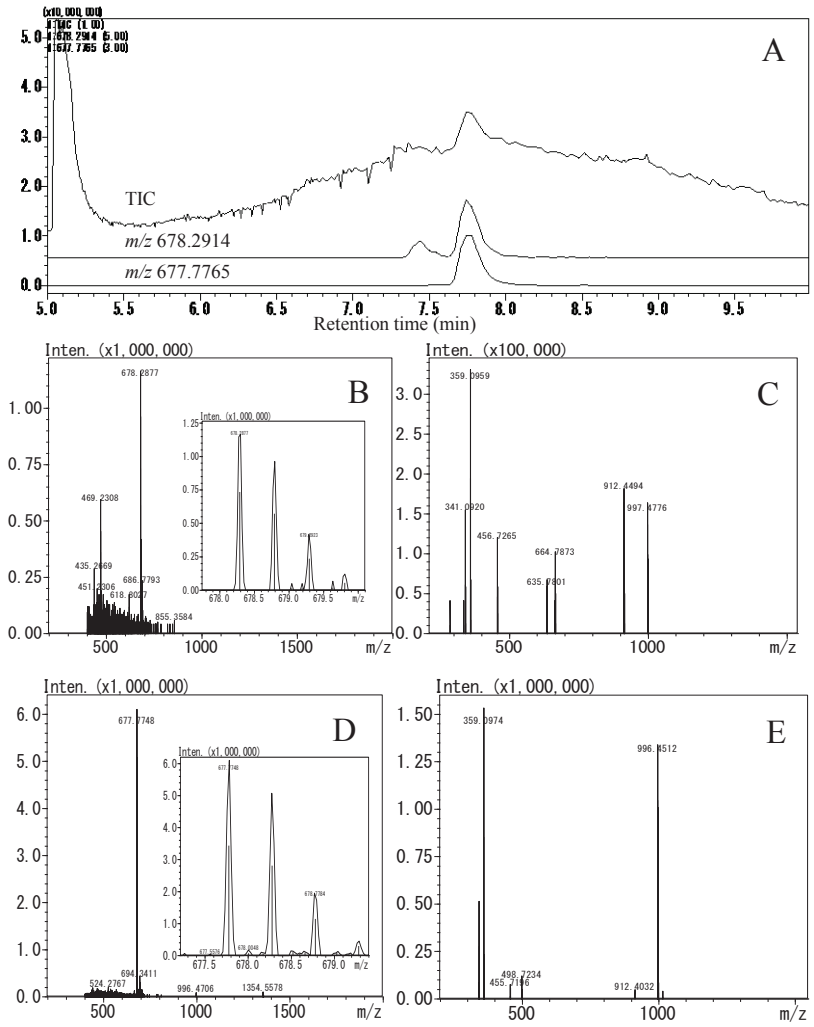
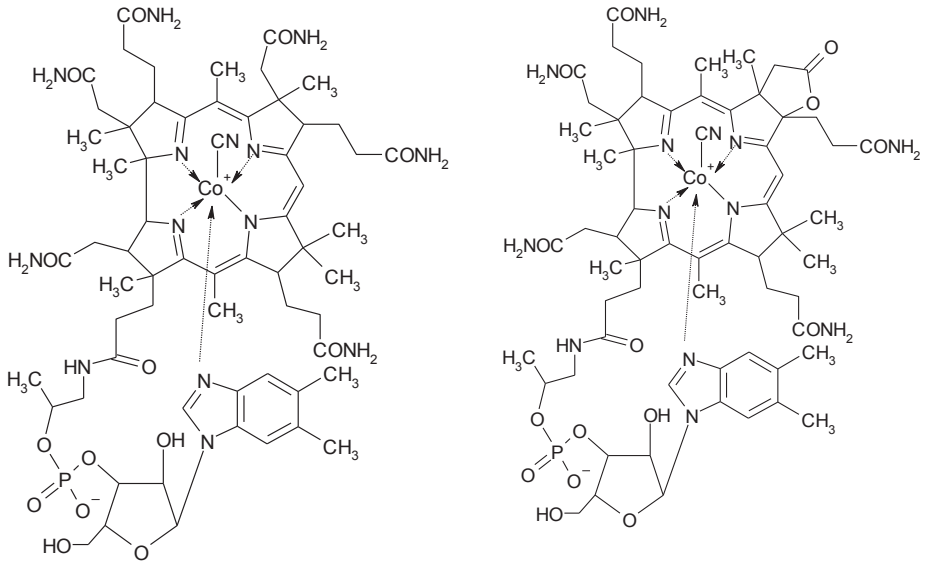


Fig. 9. LC/ESI-MS/MS chromatograms of the B_{12} compounds purified from the lion's mane mushroom sample C. Panel A shows the TICs and the chromatograms of *m/z* 678.2914 and *m/z* 677.7765 in the purified B_{12} compounds from mushroom sample C. The mass spectra of the *m/z* 678.2914 (at 7.5 min) and *m/z* 677.7765 (at 7.7 min) peaks of the B_{12} compounds purified from mushroom sample are shown in panels B and D (the magnified spectrum range from *m/z* 678 to *m/z* 680 is shown as an insert in each panel), respectively. The MS/MS spectra for the *m/z* 678.2877 and *m/z* 677.7748 peaks of the B_{12} compounds purified from the mushroom sample C are shown in panels C and E, respectively.



A B

Fig. 10. Structures of vitamin B_{12} and vitamin B_{12}[*c*-lactone]. (A) B_{12} and (B) B_{12}[*c*-lactone]

Table 4. R_f values and retention times of authentic B_{12} and prepared B_{12}[*c*-lactone] during TLC and HPLC.

	silica gel 60 TLC (R_f)		reversed-phase HPLC
compound	solvent I	solvent II	(min)
authentic B_{12}	0.57	0.21	8.4
B_{12}[*c*-lactone]	0.58	0.29	12.2

To identify the mushroom B_{12} compound with a doubly charged ion with an *m/z* of 677.7748 $[M + 2H]^{2+}$, B_{12}[*c*-lactone] was prepared and analyzed by LC/ESI-MS/MS (**Fig. 11**). The prepared B_{12}[*c*-lactone] was eluted as a peak with a retention time of 7.7 min (**Fig. 10 A**). The mass spectrum of B_{12}[*c*-lactone] mainly had a doubly charged ion with an *m/z* of 677.7758 $[M + 2H]^{2+}$ (**Fig. 11 B and C**). Its exact mass was calculated from

its formula ($C_{63}H_{87}CoN_{14}O_{14}P$, 1353.5674). The MS/MS spectra of B_{12}[*c*-lactone] indicated that a monovalent ion with an *m/z* of 359.0993 predominated (the nucleotide moiety of B_{12}) (**Fig. 11 C**). These data were identical to those of the unidentified B_{12} compound found in lion' mane mushroom, which indicated that some dried lion's mane mushroom contained an unnatural B_{12} compound, B_{12}[*c*-lactone]. I have no information on precise strains of the tested lion's mane mushrooms and their culture conditions and postharvest processes because the tested fruiting body samples were obtained as food items. Therefore, the reasons for such differences in B_{12} contents and occurrence of B_{12}[*c*-lactone] among the individual dried fruiting bodies is unclear. Previously, I demonstrated that the dried fruiting bodies of dried shiitake mushrooms contained higher amounts (5.61 μg/100 g dry weight) of B_{12} than those of the cultured lion's mane mushrooms, also B_{12}[*c*-lactone] was detected in these dried mushroom fruiting bodies [33].

Biosynthesis of B_{12} in edible mushroom

It is known that mushrooms neither have the ability to synthesize B_{12} *de novo* nor contain any B_{12}-dependent enzymes. Thus, mushroom B_{12} was probably derived from B_{12} sources outside the mushrooms, probably sawdust substrate (but no evidence of any B_{12}-containing substrates), or it was derived from B_{12} synthesized by bacteria living on the surfaces of the mushroom fruiting body. It is unclear at the present time whether such B_{12}-synthesizing bacteria can grow symbiotically with the mushroom during growth of its fruiting body or they are contaminated during preparation and storage of the dried mushroom fruiting body. However, occurrence of B_{12}[*c*-lactone] suggests that the antimicrobial agent chloramine-T was used during postharvest processing of the mushroom fruiting body.

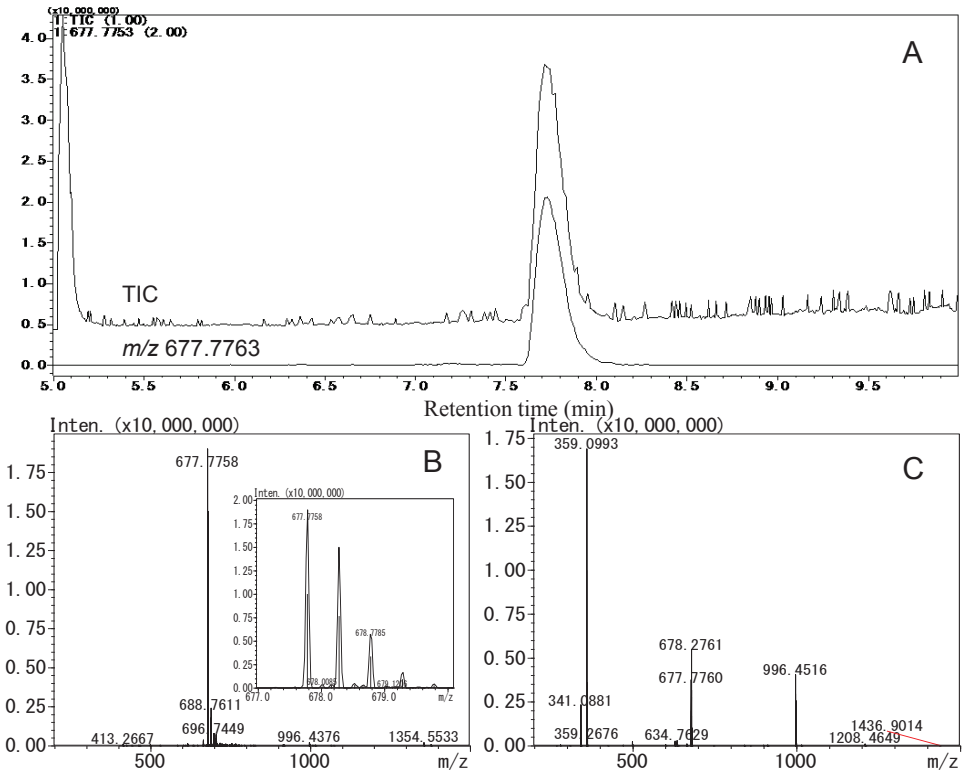


Fig. 11. LC/ESI−MS/MS chromatograms of prepared B_{12}[*c*-lactone]. Panel A shows the TICs and the chromatogram (*m/z* 677.7753) of prepared B_{12}[*c*-lactone]. The mass spectrum of prepared B_{12}[*c*-lactone] at 7.7 min is shown in panel B (the magnified spectrum range from *m/z* 678 to *m/z* 680 is shown as an insert in each panel). The MS/MS spectra for the *m/z* 677.7758 peak of prepared B_{12}[*c*-lactone] is shown in panel C.

Conversion of B_{12} to B_{12}[c-lactone] by chloramine-T

Fig. 12 shows the effects of varying pH on the conversion of B_{12} to B_{12}[*c*-lactone]. The formation of B_{12}[*c*-lactone] was highest at pH 3.0 in the experimental conditions, but considerable (approximately 40 - 50%) B_{12}[*c*-lactone] was also produced at neutral pH values (pH 6.0 - 7.0). These results indicate that B_{12}[*c*-lactone] is simple to be produced by incubating B_{12} with chloramine-T at a varying pH values (3.0 - 7.0).

I investigated whether B_{12}[*c*-lactone] exhibited any B_{12} activity in two B_{12}-dependent bacteria, which are used in microbiological B_{12} assays. B_{12}[*c*-lactone] was

completely inactive in *E. coli* 215 (**Fig. 13 A**) and *L. delbrueckii* ATCC 7830 (**Fig. 13 B**). These results suggest that the lower B_{12} contents of dried lion' mane mushroom samples were due to the presence of B_{12}[*c*-lactone]. Stabler *et al.* demonstrated that hydroxo (OH)-B_{12}[*c*-lactone] binds very weakly to the most specific B_{12}-binding protein, intrinsic factor [37]. When OH-B_{12}[*c*-lactone] was injected into a rat blood vessel using an osmotic pomp, the indices of B_{12} deficiency (the serum methylmalonic acid and homocysteine levels) significantly increased because methylmalonyl-CoA mutase and methionine synthase were strongly inhibited by the addition of OH-B_{12}[*c*-lactone].

This study indicates that the use of the antimicrobial agent chloramine-T in food processing and agricultural fields readily promotes the formation of B_{12}[*c*-lactone] in food materials. Because nutritional values of foods treated with chloramine-T are reduced significantly, it would be necessary to make a detailed survey on the occurrence of B_{12}[*c*-lactone] in various foods.

The consumption of approximately 240 g of dried mushroom (even dried lion's mane mushroom sample G; 1.04 μg/100g dry weight) could provide the recommended dietary allowance for adults (2.4 μg/day), although they would not be able to ingest such large amounts of this dried mushroom daily [13]. Thus, dried lion's mane mushroom is not suitable for use as a B_{12} source by vegetarians because of its low B_{12} contents and the occasional presence of B_{12}[*c*-lactone] which is inactive in humans.

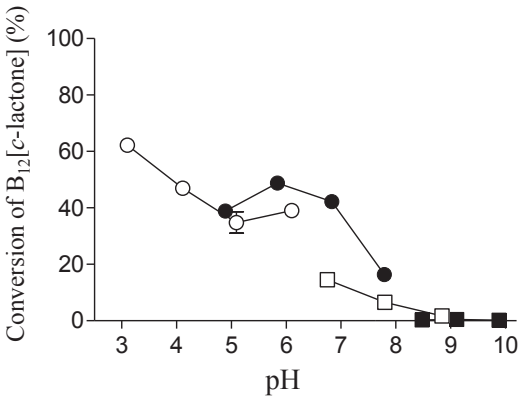


Fig. 12. Effects of varying pH on the conversion of B_{12} to B_{12}[*c*-lactone]. B_{12}[*c*-lactone] formation is represented as the percentage of B_{12}[*c*-lactone] versus the sum of B_{12} and B_{12}[*c*-lactone]. Citrate buffer (○), 2-(N-morpholino) ethane sulfonic acid buffer (●), 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid buffer (□), or borate buffer (■). The values represent the mean ± SD from three independent experiments.

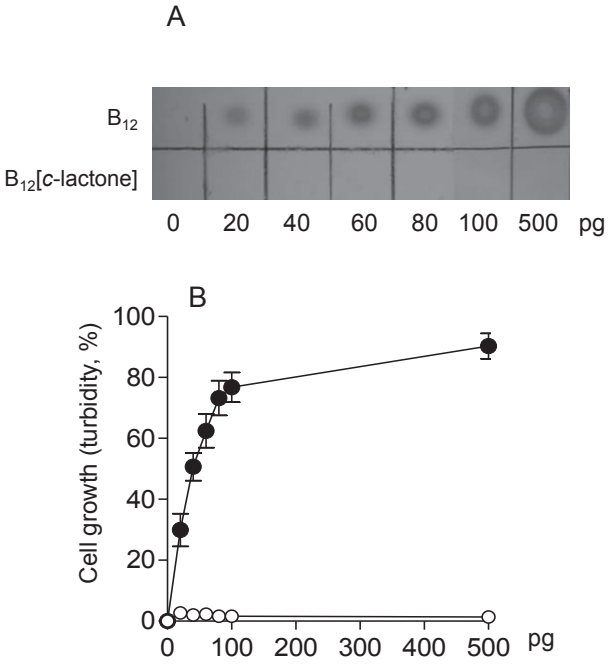


Fig. 13. Effects of B_{12}[*c*-lactone] on two B_{12}-dependent bacteria used in microbiological B_{12} assay methods. (A) *E. coli* 215 and (B) *L. delbrueckii* ATCC 7830: Authentic B_{12} (○) and prepared B_{12}[*c*-lactone] (●). The values represent the mean ± SD from three independent experiments.

Summary

I determined the B_{12} content of the edible medicinal mushroom *H. erinaceus,* lion's mane mushroom fruiting body, using a microbiological assay based on *L. delbrueckii* ATCC 7830. Trace levels (0.04 - 0.36 μg/100 g dry weight) of B_{12} were found in most of the dried mushroom samples, and two samples contained slightly higher levels (0.56 and 1.04 μg/100 g dry weight, respectively) of B_{12}. We purified the corrinoid compounds from the extracts of dried lion's mane mushroom fruiting bodies using an immunoaffinity column and identified them as B_{12} or B_{12}[*c*-lactone] (or both) based on LC/ESI-MS/MS chromatograms. This is the first report on an unnatural corrinoid, B_{12}[*c*-lactone], occurring in foods. B_{12}[*c*-lactone] was simple to produce during incubation of authentic B_{12} and chloramine-T, an antimicrobial agent, at varying pH values (3.0 - 7.0) and was completely inactive in the B_{12}-dependent bacteria that are generally used in B_{12} bioassays.

Chapter IV

Characterization of vitamin B_{12} compounds in Chinese black tea leaves

Introduction

Tea is the second highest consumed nonalcoholic beverage worldwide, and an important dietary source of flavonoid compounds [38]. Although these tea polyphenols possess therapeutic properties, including anti-cardiovascular and anti-cancer effects *in vitro* and *in vivo*, epidemiological and clinical studies suggest an association with moderately reducing the risk of chronic diseases [39]. Chinese tea is generally divided into at least three categories on the basis of different production methods: nonfermented (green tea), semi-fermented (oolong tea), and fully fermented (black tea) [40].Among these, only black tea leaves are withered, rolled ,and fermented with bacteria [40]. Biologically active compounds containing anti-oxidative [41], anti-mutagenic [42], and anti-hypertriacylglycerolemia [43] properties have been observed in black tea leaves. Kittaka-katsura *et al.* [44] demonstrated that the Japanese black tea leaf Batabata-cha contains approximately 0.5 mg of B_{12} per 100 g of dried tea leaves, which is bioavailable in mammals. In addition, they determined that B_{12} content of two types of Chinese black tea leaves [45] is, similar to that of Batabata-cha. However, B_{12} content in various types of Chinese black tea leaves and the identification of B_{12} compounds in Chinese black tea leaves as "true" B_{12} or inactive corrinoid compounds in humans remain to be established.

In **Chapter IV**, I described the characterization of B_{12} compounds from various Chinese fermented black tea leaves using TLC-*E. coli* 215 bioautography and LC/ESI-MS/MS.

Materials and Methods

Materials

Authentic B_{12} was obtained from Sigma (St. Louis, Missouri, USA). Silica gel 60 TLC aluminum sheets were purchased from Merck (Darmstadt, Germany). All other reagents were high-grade and commercially available. Various types of Chinese black tea leaves (Pu'er, Ryubao, Fu, and Brick) were purchased from local markets in Japan (**Fig. 14**).

Extraction and assay of B_{12} from Chinese black tea leaf samples

Each sample (5 g) of dried Chinese black tea leaves was homogenized in a mixer (TML160; Tescom & Co., Ltd., Tokyo, Japan). A portion (2 g) of the homogenate was used as the test sample. B_{12} compounds of samples were extracted as described in **Chapter II**.

Tea from 3 g Ryubao leaves (sample H) with the highest B_{12} content among those tested was extracted for 5 min with 150 mL of boiling water. After cooling down to 40 ºC, the extract was used as a tea drink, and B_{12} was extracted from 50 mL of liquid using the above-mentioned method.

Bioautography of corrinoid compounds using B_{12}-dependent E. coli 215

B_{12} extract (50 mL) prepared as described above was partially purified and concentrated using Sep-Pak Plus® C18 cartridge (Waters Corp., Milford, USA). Bioautography of the B_{12} compounds was done according to the method of Tanioka *et al*. [24].

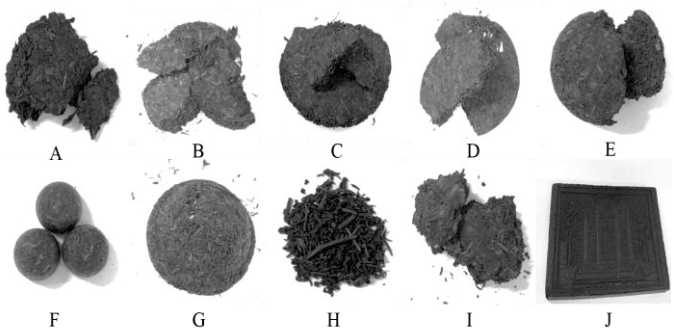


Fig. 14. Types of Chinese black tea leaves. Pu'er tea (samples A-G), Ryubao tea (sample H), Fu tea (sample I), and Brick tea (sample J) leaves were used in this study.

Identification of B_{12} compounds by LC/ESI-MS/MS

Sample H (10 g) containing high levels of B_{12} was suspended in 500 mL of distilled water and homogenized in a mixer (TML 160). The homogenate was added to 50 mL of 0.57 mol/L acetic buffer (pH 4.5) with 0.05 g KCN and boiled for 30 min to extract B_{12} compounds. Extraction procedures were performed in a draught chamber (Dalton Co., Tokyo, Japan). The boiled suspension was centrifuged at 5,000 × *g* for 10 min. An aliquot (approximately 200 mL) of the supernatant was placed in Sep-pak Vac 20cc (5 g) C18 cartridges (Waters Corp.) prewashed with 75% (v/v) ethanol and equilibrated with distilled water. The C18 cartridges were washed with 30 mL of distilled water and B_{12} compounds were eluted using 30 mL of 75% (v/v) ethanol. The remaining supernatant was treated in the same manner. Combined eluates were evaporated to dryness under reduced pressure, and the residual fraction was dissolved in 5 mL of distilled water and centrifuged at 10,000 × *g* for 10 min to remove any insoluble material. The supernatant fraction was loaded onto EASI-EXTRACT B_{12} Immunoaffinity Column (P80) [R-Biopharm AG, Darmstadt, Germany], and corrinoids were purified according to the manufacturer's protocol. B_{12} compounds, pseudo-B_{12}, and B_{12} were dissolved in 0.1%

(v/v) acetic acid and filtered using a Nanosep MF centrifual device (0.4 μm, Pall Corp., Tokyo, Japan) to separate small particles. Aliquots (2 μL) of filtrate were analyzed using 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 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, followed by decreasing the concentration from 90% to 15% solvent B for 11 - 15 min) at a flow rate of 0.2 mL/min. ESI conditions were determined by injecting pseudo-B_{12} or B_{12} into the MS detector to ascertain the optimum parameters for detecting the parent B_{12} compound and daughter ions. ESI-MS was operated in the positive ion mode with argon as 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 retention times molecular ions and retention times.

Results and Discussion

B_{12} contents in black tea leaves

B_{12} levels were assayed in 10 Chinese black tea leaves that are commercially available worldwide using the microbiological B_{12} assay method based on *L. delbrueckii* ATCC 7830 (**Table 5**). Traces (0.25 - 0.69 μg/100 g dry weight) of the corrected B_{12} were observed in Pu`er, Fu, and Brick tea leaves. However, Ryubao tea leaves (sample H) contained the highest B_{12} content (1.37 μg/100 g dry weight), which is similar to that previously reported [45]. To further clarify whether Ryubao tea leaves generally contain

high levels of B_{12}, we determined the B_{12} content of the other Ryubao leaf samples. As shown in **Table 6**, the corrected B_{12} content of various Ryubao tea leaves varied (0.06 - 1.37 μg/100 g dry weight), and their mean value was calculated as approximately 0.69 μg of B_{12}, which is only slightly higher than that for Pu`er tea leaves (approximately 0.49 μg/100 g dry weight). High levels (0.61 - 2.02 μg B_{12} equivalent/100 g dry weight) of the alkali-resistant factor were detected in all tested Chinese black tea leaves.

Table 5. Vitamin B_{12} content of various types of Chinese black tea leaves.

	Vitamin B_{12} content (μg/100 g dry weight)		
	Apparent B_{12}	Alkali-resistant factor	Corrected B_{12}
Pu`er tea leaves			
Sample A	1.94	1.32	0.62
Sample B	1.75	1.50	0.25
Sample C	2.55	1.86	0.69
Sample D	1.53	1.05	0.48
Sample E	2.35	1.77	0.58
Sample F	1.52	1.07	0.45
Sample G	2.42	2.02	0.40
Ryubao tea leaves			
Sample H	2.94	1.57	1.37
Fu tea leaves			
Sample I	1.87	1.30	0.57
Brick tea leaves			
Sample J	0.74	0.61	0.13
Mean ± SD	1.96 ± 0.63	1.41 ± 0.43	0.55 ± 0.33

E. coli 215 Bioautography Analysis

B_{12} compounds identified in Chinese black tea leaf samples A-J were analyzed using the *E. coli* 215 bioautogram after separation using silica gel 60 TLC (**Fig. 15**). The Ryubao tea leaf extract (sample H) produced a single, clear spot with an R_f value identical

to that of authentic B_{12}. Indistinct spots with the R_f value identical to that of authentic B_{12} were detected in samples C, D, E, G, and I. The remaining samples showed no spot because of their lower B_{12} contents.

Table 6. Vitamin B_{12} content of various types of Ryubao tea leaves.

	Vitamin B_{12} content (μg/100 g dry weight)		
	Apparent B_{12}	Alkali-resistant factor	Corrected B_{12}
Sample H	2.94	1.57	1.37
Sample K	1.57	1.23	0.34
Sample L	2.89	1.90	0.99
Sample M	1.68	1.62	0.06
Mean ± SD	2.27 ± 0.75	1.58 ± 0.27	0.69 ± 0.60

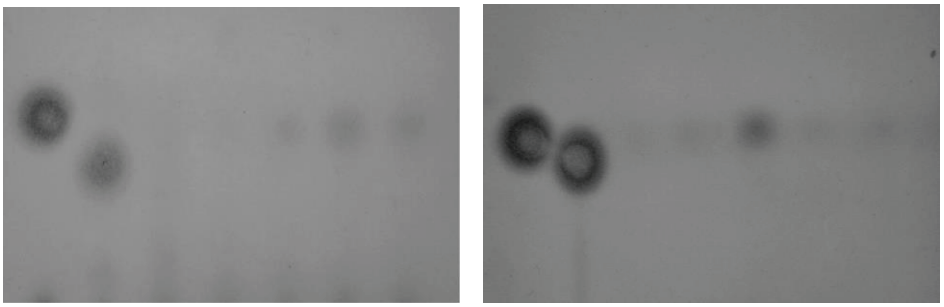


Fig. 15. *E. coli* 215 bioautogram analysis of B_{12} compounds detected in various black tea leaf samples. Authentic B_{12} (1), pseudo-B_{12} (2), and concentrated extracts of various black tea leaf samples A-J. Typical bioautograms from three independent experiments are presented.

LC/ESI-MS/MS analysis

To more precisely identify the corrinoid compounds present in Chinese black tea leaves, corrinoids were purified from the Ryubao tea leaf extract (sample H) containing high B_{12} content and identified using LC/ESI-MS/MS (**Fig. 16**). Authentic B_{12} was eluted as a peak with a retention time of 7.5 min. The mass spectrum of authentic B_{12} primarily comprised a doubly charged ion with *m/z* 678.2910 $[M + 2H]^{2+}$ (**Fig. 16 A and B**). MS/MS spectra revealed a predominant monovalent ion with *m/z* 359.0994, which was largely attributable to the nucleotide moiety of B_{12} (**Fig. 16 C**). The corrinoid purified from the Ryubao tea leaf sample H was eluted as several ion peaks, indicating the presence of impurities. The mass spectrum of the main peak with *m/z* 687.2914 had a retention time of 7.5 min in the purified sample (**Fig. 16 D and E**). The MS/MS spectrum of the purified compound with a monovalent ion with *m/z* 359.0960 was identical to that of authentic B_{12} (**Fig. 16 C and F**). These results indicate that the Ryubao tea leaf sample H contained authentic B_{12} but not pseudo-B_{12} which is inactive in humans.

B_{12} content in the tea drink prepared from the Ryubao tea leaf sample H was 0.8 ng/100mL of black tea. Therefore, consumption of approximately 300 L of this tea would provide the recommended dietary allowance for adults (2.4 μg/day) [13], although ingestion of such large quantities of tea on a daily basis is not recommended. Notably, Kittaka-Katsura *et al.* [44] demonstrated that administration of the Japanese black tea drink (B_{12} content, approximately 2.0 ng/100mL) considerably improves B_{12} status in B_{12}-deficient rats. Considering these earlier observations and our present findings, we propose that Ryubao tea leaves containing significantly levels of B_{12} can be utilized as a source of B_{12} for vegetarians.

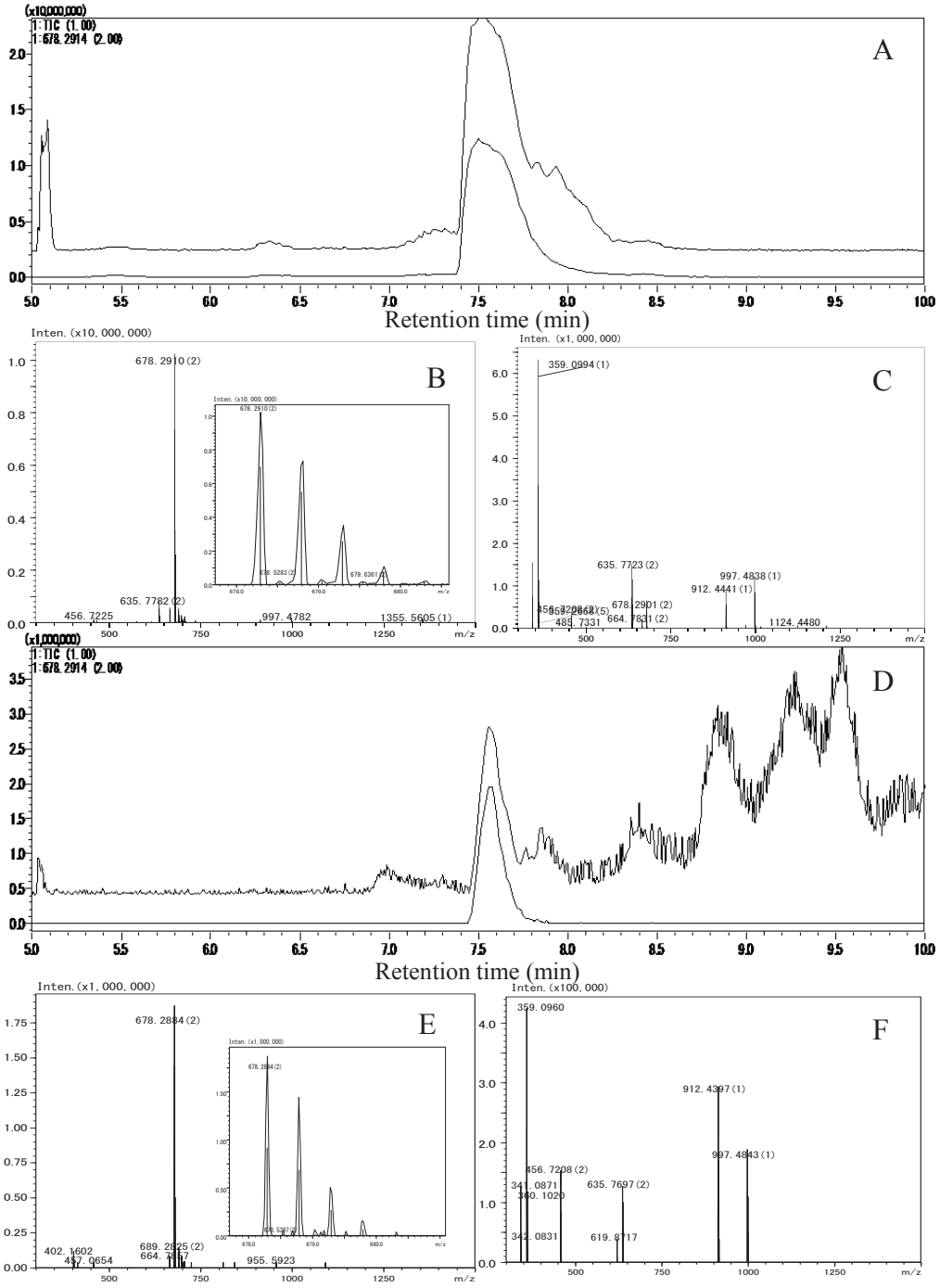


Fig. 16. LC/ESI-MS/MS chromatograms of authentic B_{12} and the B_{12} compounds purified from black tea leaf sample H. B_{12} compounds were analyzed using the LC/MS-IT-TOF system (Shimadzu). Panels A and D show total ion chromatograms and those (*m/z* 678.2914) of authentic B_{12} and B_{12} compounds purified from sample H. Mass spectra of authentic B_{12} and purified B_{12} compounds at 7.5 min are shown in panels B and E, respectively (magnified spectrum range from *m/z* 678 to *m/z* 680 is shown as an insert in each panel). MS/MS spectra for the peak of authentic B_{12} at *m/z* 678.2910 and that of purified B_{12} compounds at *m/z* 678.2884 are shown in panels C and F, respectively.

Summary

I determined B_{12} content of Chinese black tea leaves using a microbiological assay based on *L. delbrueckii* ATCC 7830. Trace levels (0.25 - 0.69 μg/100 g dry weight) of B_{12} were detected in Pu'er, Fu, and Brick tea leaves. However, B_{12} content (0.06 - 1.37 μg/100 g dry weight) of Ryubao tea leaves significantly varied. To determine whether Chinese black tea leaves contain B_{12} or other corrinoid compounds that are inactive in humans, corrinoid compounds were purified from Ryubao tea by an immunoaffinity column chromatography and B_{12} was identified by LC/ESI-MS/MS. B_{12} content in the tea drink prepared from Ryubao tea leaves was very low (0.8 ng/100 mL). Our results indicate that Chinese black tea is usually not a good source of B_{12}, although Ryubao tea leaves with the highest B_{12} content may be utilized as a source of this vitamin for vegetarians.

Chapter V

Characterization of vitamin B_{12} compounds in Chinese boiled and dried oyster and oyster sauce

Introduction

Shellfish that siphon large quantities of B_{12}-synthesizing bacteria from seawater and/or freshwater are excellent sources of B_{12} [46]. The previous studies indicated that B_{12} levels were significantly higher in popular edible bivalves (approximately 60 μg/100 g wet weight) than in edible snails (approximately 20 μg/100 g wet weight) [47]. The corrinoid compounds purified from most edible bivalves (clams, oysters, mussels etc.) have been identified as "true" B_{12} using NMR spectroscopy [48]. Boiled and dried oyster is a popular food ingredient in Chinese cuisine. In addition, the broth made with boiled oyster is further concentrated to form oyster sauce. However, there is no information on B_{12} content of boiled and dried oyster and oyster sauce and on whether these Chinese oyster products contain "true" B_{12} or inactive corrinoid compounds.

In **Chapter V**, I described determination of the B_{12} contents of boiled and dried oyster and oyster sauce and characterization of their B_{12} compounds to evaluate whether they are good sources of B_{12}.

Materials and Methods

Materials

B_{12} was obtained from Sigma (St Louis, Missouri, USA). A B_{12} assay medium based on *L. delbrueckii* ATCC 7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60

TLC aluminum sheets were obtained from Merck (Darmstadt, Germany). Boiled and dried oysters were purchased from local markets in Japan and China. Various types of oyster sauces were purchased in Japan.

Extraction and assay of B_{12} in boiled and dried oyster and oyster sauce

Boiled and dried oyster (5g) was homogenized in a mixer (TML160; Tescom & Co., Ltd., Tokyo, Japan). A portion (2.0 g) of the oyster homogenate and oyster sauce was used as the test sample. B_{12} compounds were extracted from the samples as described in **Chapter II**.

Bioautography of corrinoid compounds using B_{12}-dependent E. coli 215

B_{12} extract (50 mL) prepared as described above was partially purified and concentrated using Sep-Pak Plus® C18 cartridge (Waters Corp., Milford, USA). Bioautography of the B_{12} compounds was done according to the method of Tanioka *et al*. [24].

Preparation of boiled and dried oyster

Raw oysters (approximately 10 bodies) were boiled for 3 min in 2.5 g/L NaCl solution. When the temperature of the treated samples was below 40 ºC, oysters and broth were separated. The boiled oysters were dried for 2 - 3 days. B_{12} was extracted for dried oysters and broth using the above-mentioned method.

Determination of water content of boiled and dried oyster

The water contents of various types of boiled and dried oysters were determined

using a moisture analyzer (MOC-120H Shimadzu Co., Japan) according the manufacturer's instructions.

Identification of B_{12} compounds by LC/ESI-MS/MS

B_{12} compounds were purified under the same conditions described above. The purified samples, authentic B_{12}, pseudo-B_{12} were dissolved in 0.1% (v/v) acetic acid and filtered with a Nanosep MF centrifuge device (0.4 μm, Pall Corp., Tokyo, Japan) to remove small particles. Aliquots (2 μL) of filtrate were analyzed using LC/MS-IT-TOF coupled with an Ultra-Fast LC system (Shimadzu, Kyoto, Japan) as described in **Chapter II**. The identities of pseudo B_{12} (*m/z* 672.7749) and B_{12} (*m/z* 678.2914) as $[M + 2H]^{2+}$ were confirmed by comparing the observed molecular ions and retention times.

Results and Discussion

B_{12} content in dried oyster and oyster sauce

B_{12} was assayed in various boiled and dried oysters and oyster sauces that are commercially available in China using a microbiological method based on *L. delbrueckii* ATCC 7830 (**Table 7**). Raw oysters and boiled and dried oyster from China and Japan contained similar B_{12} content (approximately 15.5 - 22.2 μg/100 g wet weight). However, B_{12} contents (approximately 20.4 - 28 μg) of 100 g dry weight of boiled and dried oyster samples were approximately 25% or less of B_{12} content of raw oysters (approximately 133.8 μg/100 g dry weight). The identical results were obtained in the prepared oyster samples. The broth prepared by boiling of oysters contained small amount of B_{12} (0.6 μg/100 mL) (**Table 7**). By calculating the B_{12} content of raw and boiled and dried oysters,

approximately 33% of B_{12} found in raw oysters were recovered in boiled and dried oyster products. Such significant loss of B_{12} may be due to heat denaturation of B_{12}. In previous studies indicated that B_{12} content of round herring meats decreases by up to approximately 62% by grilling, boiling, frying, steaming, or microwaving [11, 49]. As shown in **Table 8**, commercially available oyster sauces contained small amount (0.4 - 3.5 μg/100 g wet weight) of B_{12}.

Table 7. Vitamin B_{12} content in in various boiled and dried oysters.

	Vitamin B_{12} content	
	(μg/100 g wet weight)	(μg/100 g dry weight)
Raw oyster (Japan)		
Sample A	21.2 ± 2.1	127.9 ± 12.7
Sample B	23.4 ± 1.0	141.1 ± 6.0
Sample C	21.9 ± 0.9	132.1 ± 5.4
Mean ± SD	22.2 ± 1.1	133.8 ± 6.7
Commercially available samples		
Boiled and dried oyster (China)		
Sample A	18.3 ± 1.8	23.5 ± 2.3
Sample B	16.5 ± 2.7	21.8 ± 3.5
Sample C	20.9 ± 1.8	27.3 ± 2.4
Sample D	33.2 ± 4.1	44.1 ± 5.4
Sample E	18.6 ± 2.9	23.3 ± 3.6
Mean ± SD	21.5 ± 2.7	28.0 ± 3.5
Boiled and dried oyster (Japan)		
Sample A	15.5 ± 3.8	28.6 ± 7.0
Sample B	17.9 ± 3.2	20.4 ± 3.6
Prepared samples		
Boiled and dried oyster (Japan)		
Sample A	19.9 ± 1.5	28.4 ± 2.1
Sample B	21.1 ± 2.3	30.1 ± 3.3
Sample C	16.9 ± 1.1	24.1 ± 1.6
Mean ± SD	19.3 ± 2.2	27.7 ± 3.1
Broth (Japan)		
Sample A	0.7 ± 0.3 (μg/100 mL)	
Sample B	0.4 ± 0.2 (μg/100 mL)	
Sample C	0.6 ± 0.3 (μg/100 mL)	
Mean ± SD	0.6 ± 0.1 (μg/100 mL)	

Table 8. Vitamin B_{12} content in the various oyster sauces.

Oyster sauce	Vitamin B_{12} content (μg/100 g wet weight)
Sample A	3.5 ± 0.8
Sample B	0.4 ± 0.1
Sample C	2.8 ± 0.8
Sample D	3.5 ± 0.7
Sample E	1.3 ± 0.2
Mean ± SD	2.3 ± 1.4

E. coli 215 Bioautography Analysis

B_{12} compounds found in various oyster products were analyzed using the *E. coli* 215 bioautogram after separation using silica gel 60 TLC (**Fig. 17**). All the samples produced a single and clear spot with an R_f value identical to that of authentic B_{12} but not to that of pseudo-B_{12} (inactive in humans).

Identification of corrinoid compounds using LC/ESI-MS/MS analysis

To evaluate whether boiled and dried oyster products contain "ture" B_{12}, each B_{12} extract was purified using a B_{12} immunoaffinity column and analyzed by LC/ESI-MS/MS. Authentic pseudo-B_{12} and B_{12} were eluted as peaks with retention times of 7.3 and 7.5 min, respectively **(Fig. 18 A** and **D**). The mass spectrum of authentic pseudo-B_{12} indicated that a doubly charged ion with an *m/z* of 672.7785 $[M + 2H]^{2+}$ was prominent (**Fig. 18 B**). The exact mass calculated from its formula ($C_{59}H_{83}CoN_{17}O_{14}P$) was 1343.5375 and the isotope distribution data showed that pseudo-B_{12} was the major doubly charged ion under the LC/ESI-MS conditions used in my analyses. For authentic B_{12}, which has an exact mass of 1354.5674 ($C_{63}H_{88}CoN_{14}O_{14}P$), a doubly charged ion with an *m/z* of

678.2943 [M + 2H] $^{2+}$ was prominent (**Fig. 18 E**). The MS/MS spectra of authentic pseudo-B_{12} and B_{12} indicated that their dominant ions at *m/z* 348.0688 and *m/z* 359.0986, respectively, were attributable to the nucleotide moiety of each corrinoid compound (**Fig. 18 C** and **F**).

The corrinoids purified from Japanese boiled and dried oysters from Japan were eluted as only an ion peak with *m/z* 678.2914 at a retention time of 7.5 min. The mass spectrum showed that a doubly charged ion was formed at *m/z* 678.2893 (**Fig. 19 A and B**). The MS/MS spectrum of the compound was identical to that of B_{12} (**Fig. 19 C**). These results indicate that B_{12} is the predominant corrinoid compound in dried oysters from Japan.

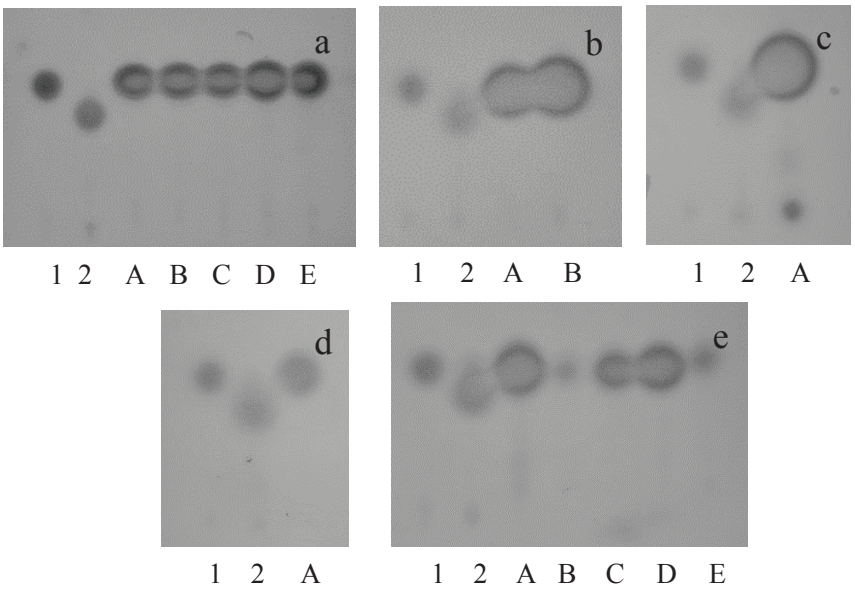


Fig. 17. *E. coli* 215 bioautogram analysis of corrinoids found in various boiled and dried oysters and oyster sauces. a) 1, authentic B_{12}; 2, pseudo-B_{12}; commercially available boiled and dried oysters from China A, B, C, D and E; b) 1, authentic B_{12}; 2, pseudo-B_{12}; commercially available boiled and dried oysters from Japan A and B; c) 1, authentic B_{12}; 2, pseudo-B_{12}; prepared boiled and dried oyster A; d) 1, authentic B_{12}; 2, pseudo-B_{12}; broth A; e) 1, authentic B_{12}; 2, pseudo-B_{12}; oyster sauce A, B, C, D and E.

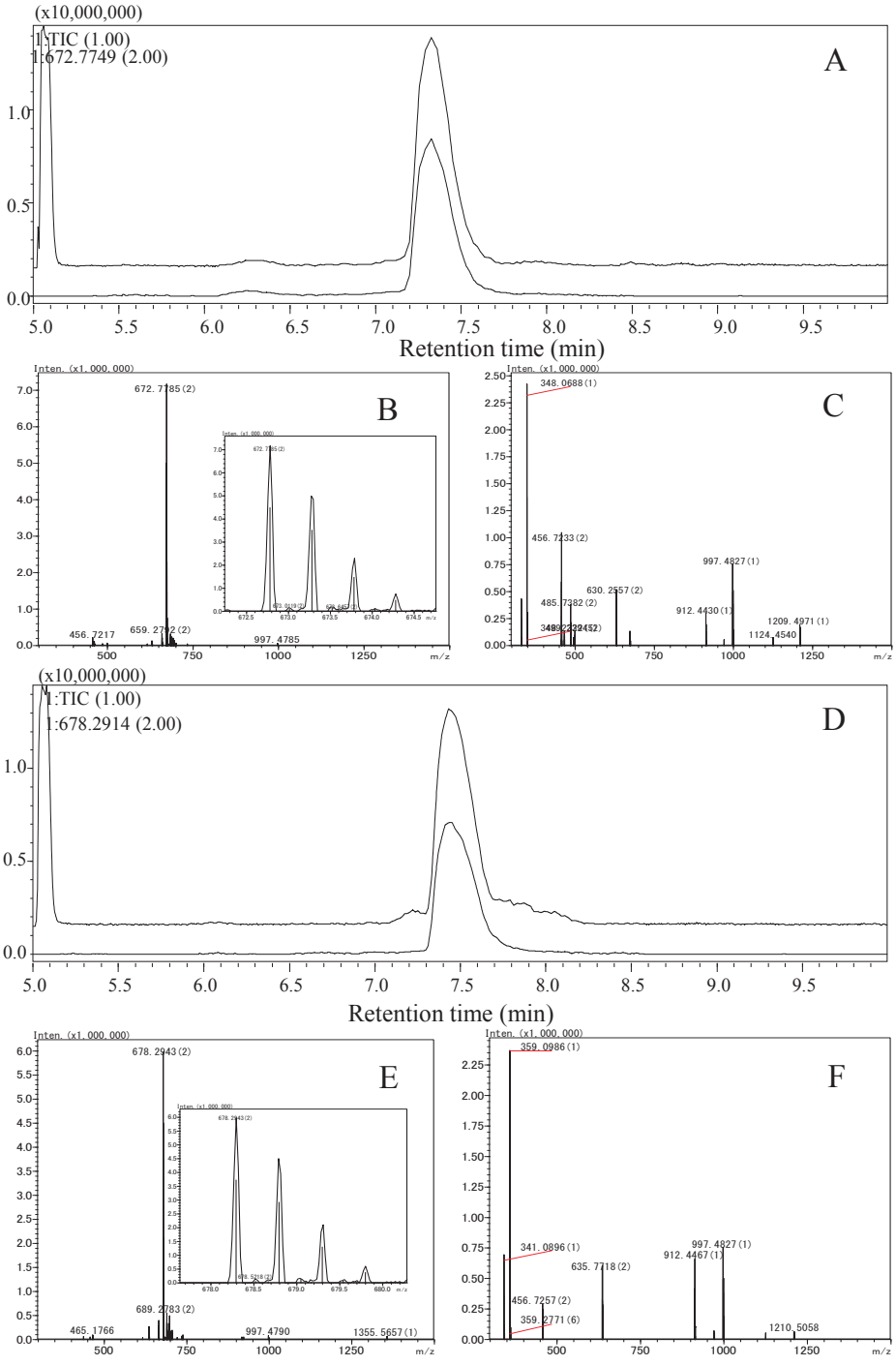


Fig. 18. LC/ESI-MS/MS chromatograms of authentic pseudo-B_{12} and B_{12}. Pseudo-B_{12} and B_{12} were analyzed with LC/MS-IT-TOF as described in the text. The TIC of authentic pseudo-B_{12} and B_{12} are shown in panels A and D, respectively. The mass spectra of the ion peaks from pseudo-B_{12} and B_{12} are shown in panels B and E, respectively. The magnified mass spectra from *m/z* 672 to 675 in pseudo-B_{12} and from *m/z* 678 to 680 in B_{12} are shown as inserts. The MS/MS spectra of the peaks of pseudo-B_{12} and B_{12} are shown in panels C and F, respectively.

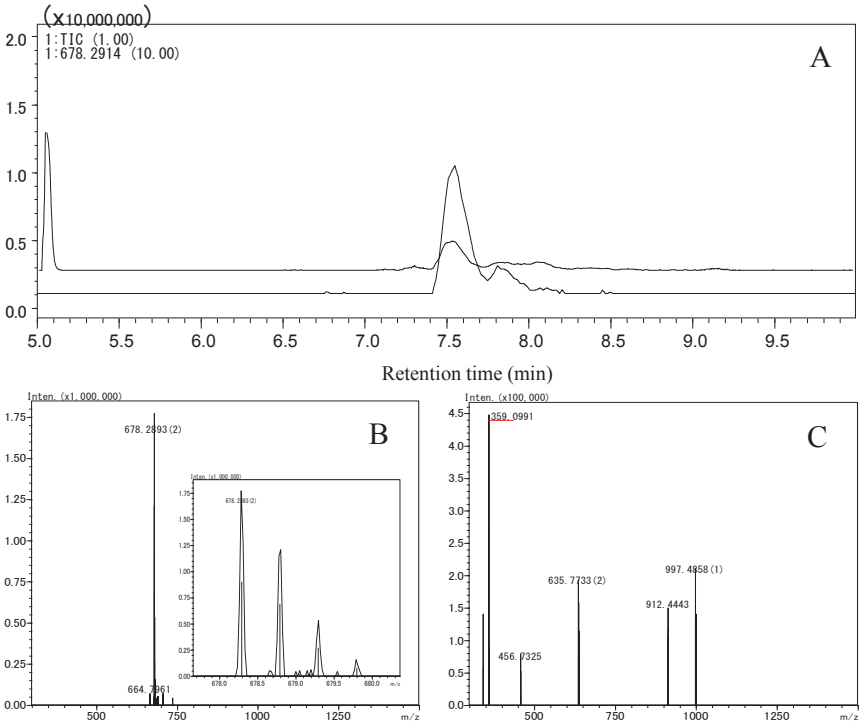


Fig. 19. LC/ESI-MS/MS chromatograms of boiled and dried oysters from Japan. TIC and ion chromatograms for *m/z* 678.2914(×10) of B_{12} compounds purified from dried oysters are shown in panels A. The mass spectra of the ion peaks of B_{12} compounds at retention times of 7.4 min are shown in panel B (the magnified mass spectrum from *m/z* 678 to 680 is shown as an insert). The MS/MS spectra for the peak of B_{12} compounds at *m/z* 678.2893 is shown in panels C.

However, the compounds purified from Chinese boiled and dried oyster were eluted as several total ion peaks, indicating that impurities remained. The ion peaks of *m/z* 672.7749 and *m/z* 678.2914 due to pseudo-B_{12} and B_{12}, respectively, were also found (**Fig. 20 A**). Their retention times of 7.3 and 7.5 min, respectively, were similar to those of authentic pseudo-B_{12} and B_{12} respectively. The mass spectra of the materials eluting at retention times of 7.3 and 7.5 min showed doubly charged ions at *m/z* 672.7790 (**Fig. 20 B**) and *m/z* 678.2919 (**Fig. 20 D**), respectively. The MS/MS spectra of these compounds were identical to those of pseudo-B_{12} (**Fig. 20 C**) and B_{12} (**Fig. 20 E**). These results indicate that pseudo-B_{12} is found in Chinese boiled and dried oyster. Although the

Chinese boiled and dried oyster contained only trace of pseudo-B_{12}, they would be a good source of B_{12} for Chinese people.

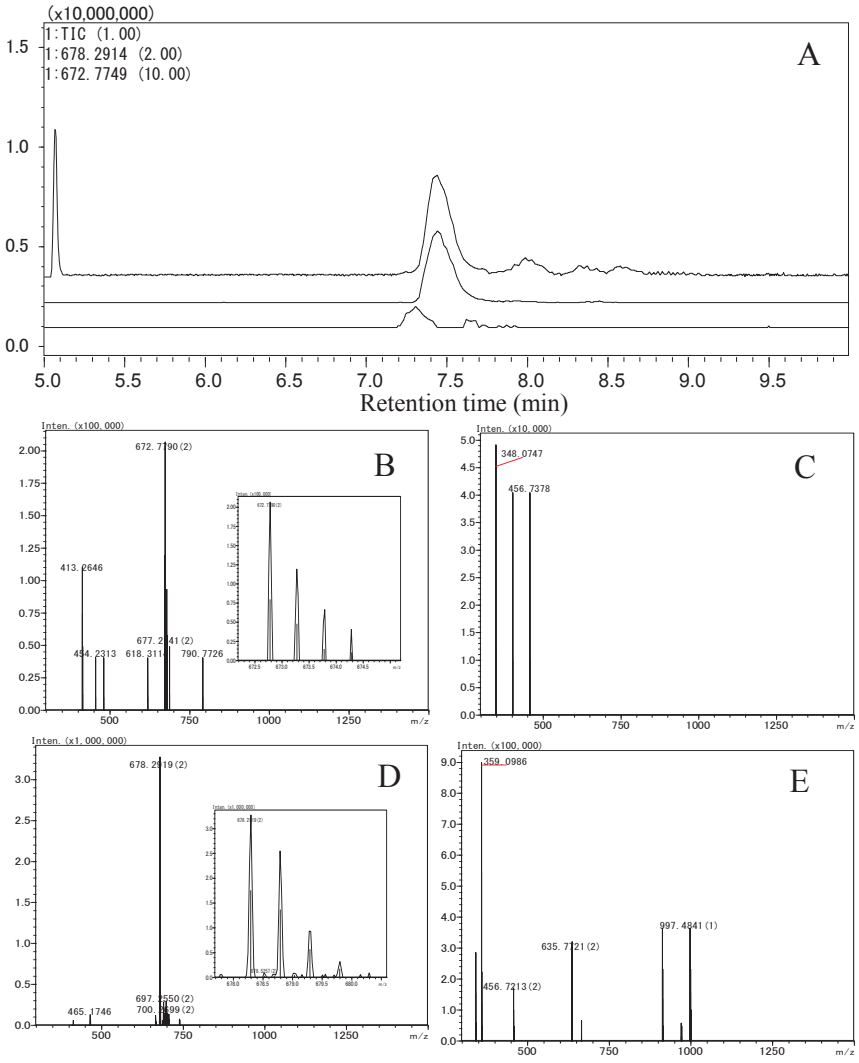


Fig. 20. LC/ESI-MS/MS chromatograms of B_{12} compounds purified from boiled and dried oysters from China. TIC and ion chromatograms for *m/z* 678.2914(× 20) and 672.7749 (× 20) of B_{12} compounds are shown in panel A. The mass spectra of the ion peaks of B_{12} compounds at retention times of 7.3 min 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 spectrum from *m/z* 678 to 680 is shown as an insert), respectively. The MS/MS spectra for the peaks of B_{12} compounds at *m/z* 672.7764 and at *m/z* 678.2856 are shown in panels C and E, respectively.

Boiled and dried oysters as a B_{12} source

The consumption of approximately 3 - 4 boiled and dried oyster whole body could provide the recommended dietary allowance of B_{12} for adults (2.4 μg/day) [13]. However, oyster sauce products contained only trace of B_{12}. These observations indicate that Chinese boiled and dried oyster are a good or practical source of B_{12}.

Summary

The boiled and dried oyster products contained a high B_{12} (approximately 20.4 - 28 μg/100 g dry weight). By calculating B_{12} content of raw oysters and oyster products, approximately 33% of B_{12} found in raw oysters were recovered in boiled and dried oyster products. B_{12} content of oyster sauce products was low (2.3 ± 1.4 μg/100 g wet weight). *E. coli* 215-bioatuography analysis suggested that all commercially available oyster products contained "true" B_{12}. However, LC/ESI-MS/MS analysis indicated that trace of pseudo-B_{12} was detected in Chinese boiled and dried oyster products. The consumption of 3 - 4 boiled and dried oysters could provide the recommended dietary allowance of B_{12} for adults (2.4 μg/day). These observations indicate that boiled and dried oyster products are good sources of B_{12}.

Chapter VI

Characterization of vitamin B_{12} compounds in edible sea snails, ivory shell *Babylonia japonica* and turban shell *Turdo Batillus cornutus*

Introduction

The Japanese obtain most (approximately 84%) of their daily B_{12} intake from fish and shellfish [50]. Shellfish siphon large quantities of B_{12}-synthesizing bacteria from seawater and/or freshwater and are excellent sources of B_{12} (>10 μg/100 g wet weight) [22, 46]. However, these B_{12}-synthesizing bacteria can also synthesize other corrinoids that contain a different base moiety in the lower ligand of the molecule [25].

Previous studies indicated that B_{12} levels were significantly higher in edible bivalves (approximately 60 μg/100 g wet weight) than in edible snails (approximately 20 μg/100 g wet weight) [47]. The corrinoid compounds purified from most edible bivalves (clams, oysters, mussels etc.) have been identified as "true" B_{12} [48]. In the edible sea snail abalone, B_{12} and pseudo-B_{12} (an inactive corrinoid) were observed to be the major and minor corrinoid compounds, respectively [51]. However, there is little information available on the B_{12} compounds of other edible sea snails. Among the edible sea snails, ivory shell *Babylonia japonica* and turban shell *Turdo Batillus cornutus* are the most popular food items in Japan and China. Their meat and viscera are edible because high levels of B_{12} accumulate in the viscera of shellfish [47]. If these popular snails contain a large amount of "true" B_{12}, they would be good sources of B_{12} in humans.

In this chapter, I identified B_{12} compounds from the edible portions (meat and viscera) of *B. japonica* and *T. cornutus* using the *L. delbrueckii* ATCC 7830

microbiological assay method and LC/ESI-MS/MS.

Materials and Methods

Materials

B_{12} was obtained from Sigma (St Louis, Missouri, USA). A B_{12} assay medium based on *L. delbrueckii* ATCC 7830 was obtained from Nissui (Tokyo, Japan). Raw *B. japonica* and *T. cornutus* were purchased from local markets in Tottori prefecture, Japan.

Extraction and assay of B_{12} from edible snails

After the shells were removed from *B. japonica* and *T. cornutus*, their edible portions (meat and viscera) were sampled. Each sample was homogenized using a mixer (TML160; Tescom & Co., Ltd., Tokyo, Japan). An aliquot (2.0 g) of the homogenate was used as the sample for the assay. B_{12} compounds of samples were extracted as described in **Chapter II**.

Identification of sea snail B_{12} compounds by LC/ESI-MS/MS

Each B_{12} extract (40 mL) was partially purified and concentrated using a Sep-Pak® Plus C18 cartridge (Waters Corp.), as described previously. The eluate was evaporated to dryness under reduced pressure, and then was dissolved in 3 mL distilled water and centrifuged at 10,000 × *g* for 10 min to remove insoluble material. The supernatant fraction was loaded onto an immunoaffinity column [EASI-EXTRACT® B_{12} Immunoaffinity Column (P80) R-Biopharm AG, Darmstadt, Germany], and were purified according to the manufacturer's protocol. The purified vitamin B_{12} compounds were

dissolved in 0.1% (v/v) acetic acid and filtered through a Nanosep MF centrifuge device (0.4 μm; Pall Corp., Tokyo, Japan) to remove small particles. An aliquot (2 μL) of the filtrate was analyzed using LC/MS-IT-TOF coupled with an Ultra-Fast LC system. Each purified corrinoid was injected into an Inert-Sustain column (3 μm, 2.0 × 100 mm; GL Science, Tokyo, Japan) equilibrated with 85% solvent A [0.1% (v/v) acetic acid] and 15% solvent B (100% methanol) at 40 ˚C. Corrinoids 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). The flow rate was 0.2 mL/min. ESI conditions were determined by injecting the corrinoids into the MS detector, thereby identifying the optimum parameters for detecting parent and daughter ions of B_{12} compounds. The ESI-MS system was operated in the positive ion mode, and argon was used as the collision gas. The identities of pseudo-B_{12} (*m/z* 672.7749) and B_{12} (*m/z* 678.2914) as $[M + 2H]^{2+}$ were confirmed by comparing the observed molecular ions and retention times.

Results and Discussion

B_{12} content of edible sea snails

I analyzed the B_{12} content of the edible sea snails ivory shell *B. japonica* and turban shell *T. cornutus*, which are commonly consumed in Japan, using the *L. delbrueckii* ATCC 7830 microbiological assay method (**Table 9**). The viscera of *B. japonica* contained a substantial amount of B_{12} (approximately 92.8 μg/100 g wet weight); 3.4 times greater than that of the meat (approximately 27.2 μg/100 g wet weight). The meat and viscera of *T. cornutus* contained significantly lower amounts of B_{12} (approximately 3.0 and 15.1 μg/100 g wet weight, respectively). These results indicated that high levels of B_{12}

accumulate in the viscera of these edible sea snails. The B_{12} content (approximately 4.7 μg) per whole body of *B. japonica* was 2.8 times greater than that of *T. cornutus*. The B_{12} contents determined in our analysis are significantly higher than those (4.3 and 1.3 μg of B_{12} per 100 g of edible portion (without shell and viscera) of ivory shell and turban shell, respectively) described in the Standard Tables of Food Composition in Japan 2010 [52].

Table 9. Vitamin B_{12} contents of edible sea snails.

	Amount of vitamin B_{12} compounds			
	Muscle (μg/100 g wet weight)	Viscera	Whole body (μg)	Body weight (g)
Ivory shell				
Babylonia japonica (*n*=5)	27.2 ± 9.1	92.8 ± 25.8	4.7 ± 0.7	11.0 ± 0.9
Turban shell				
TurdoBatillus cornutus (*n*=5)	3.0 ± 1.5	15.1 ± 8.3	1.7 ± 0.6	20.1 ± 4.0

The B_{12} content of foods were determined using the *L. delbrueckii* ATCC 7830 bioassay method. Our previous studies showed that the observed correlation rate between the values determined by the *L. delbrueckii* ATCC 7830 bioassay and intrinsic factor (the most specific B_{12}-binding protein)-based chemiluminescence method is excellent, except for foods containing substantial amounts of pseudo-B_{12} [53]. These results indicated that *L. delbrueckii* ATCC 7830 utilizes pseudo-B_{12} as well as B_{12}. Thus, pseudo-B_{12} found in the edible portions of turban shells was determined as B_{12} using the *L. delbrueckii* ATCC 7830 bioassay.

The differences in content and B_{12} compounds between these edible sea snails would be dependent on their dietary habitats, because *B. japonica* and *T. cornutus* are carnivorous and herbivorous sea snails, respectively. The B_{12} is synthesized only by certain bacteria and is concentrated mainly in the bodies of higher predators in the natural

food chain. The usual dietary sources of B_{12} are animal-derived products but not plant-derived products [22]. The B_{12} content of tengusa *Gelidium pacificum Okamura* and wakame *Undaria pinnatifida*, the foods of *T. cornutus*, are very low (0.2 - 0.5 μg/100 g dry weight) [52]. Yamada *et al*. [54] demonstrated that wakame predominantly contained certain vitamin B_{12} analogues. Moreover, various blue-green algae (cyanobacteria) contain substantial amount of pseudo-B_{12} [22].

Identification of corrinoid compounds from edible sea snails using LC/ESI-MS/MS analysis

Edible snail extracts were purified using a B_{12} immunoaffinity column and then analyzed using LC/ESI-MS/MS. Authentic pseudo-B_{12} and B_{12} were eluted as peaks with retention times of 7.4 and 7.5 min, respectively (**Fig. 21 A and D**). The mass spectrum of authentic pseudo-B_{12} indicated that a doubly-charged ion with an *m/z* of 672.7769 $[M + 2H]^{2+}$ was prominent (**Fig. 21 B**). The exact mass calculated from its formula ($C_{59}H_{83}CoN_{17}O_{14}P$) was 1343.5375 and the isotope distribution data showed that pseudo B_{12} was the major doubly-charged ion under the LC/ESI-MS conditions used in our analyses. For authentic B_{12}, which has an exact mass of 1354.5674 ($C_{63}H_{88}CoN_{14}O_{14}P$), a doubly charged ion with an *m/z* of 678.2883 $[M + 2H]^{2+}$ was prominent (**Fig. 21 E**). The MS/MS spectra of authentic pseudo-B_{12} and B_{12} indicated that their dominant ions at *m/z* 348.0695 and *m/z* 359.0984, respectively, were attributable to the nucleotide moiety of each corrinoid compound (**Fig. 21 C and F**).

The corrinoids purified from the meat of *B. japonica* were eluted as an ion peak with *m/z* 678.2914 at a retention time of 7.5 min. The mass spectrum showed that a doubly charged ion was formed at *m/z* 678.2928 (**Fig. 22 A and B**). The MS/MS spectrum of the

compound was identical to that of B_{12} (**Fig. 22 C**). Identical spectral data were obtained for the corrinoids purified from *B. japonica* viscera (**Fig. 22 D, E, and F**). These results indicate that B_{12} is the predominant corrinoid compound in *B. japonica*. The compounds purified from *T. cornutus* meat eluted as several total ion peaks, indicating that impurities remained. The ion peaks of *m/z* 672.7749 and *m/z* 678.2914 due to pseudo-B_{12} and B_{12}, respectively, were also found (**Fig. 23 A**). Their retention times of 7.3 and 7.4 min, respectively, were similar to those of authentic pseudo-B_{12} (retention time of 7.4 min) and B_{12} (retention time of 7.5 min). Such slight differences in retention times may be due to the existence of impurities in the purified compounds. The mass spectra of the materials eluting at retention times of 7.3 and 7.4 min showed doubly charged ions at *m/z* 672.7764 (**Fig. 23 B**) and *m/z* 678.2856 (**Fig. 23 D**), respectively. The MS/MS spectra of these compounds were identical to those of pseudo-B_{12} (**Fig. 23 C**) and B_{12} (**Fig. 23 E**). Similar results were obtained with the visceral sample, but no B_{12} was detected (**Fig. 23 F, G, and H**). These results indicate that pseudo-B_{12} is the predominant corrinoid compound in *T. cornutus*. Similar results were reported in abalone [47]. This result indicated that *T. cornutus* would not be a suitable source of B_{12}.

The consumption of one whole body (meat and viscera, approximately 11 g) of *B. japonica*, which contains a considerably high B_{12} level (approximately 4.7 μg), could supply the entire recommended dietary allowance for an adult (2.4 μg/day) [13]. Our unpublished studies indicated that the edible portions (without shell and viscera) of other carnivorous sea snails, e.g., whelks *Buccinum striatissium* and *Neptunea intersculpta*, also contain considerable amounts of B_{12} [10.13 ± 3.33 (n = 5) and 28.72 ± 5.48 (n = 5) μg/100 g, respectively]. The results presented here indicate that these edible carnivorous sea snails would be excellent sources of B_{12} for humans.

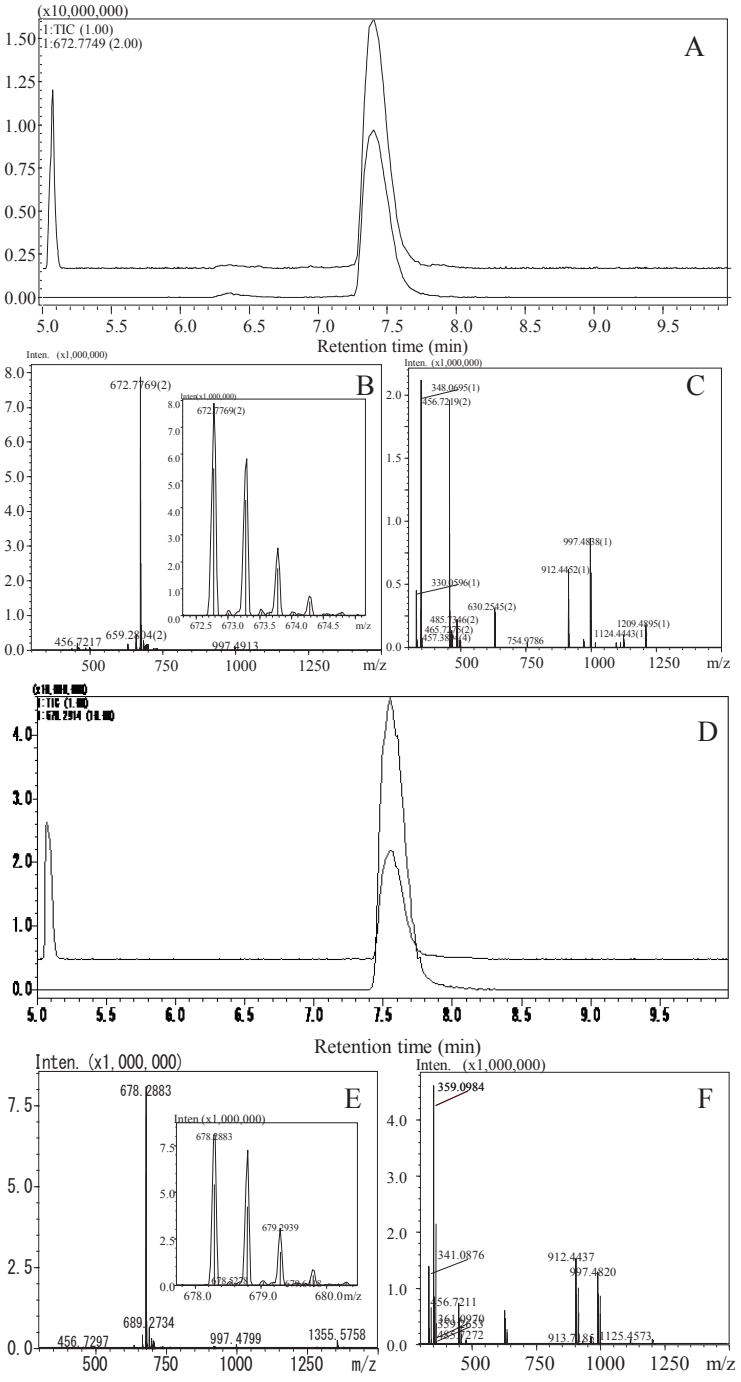


Fig. 21. LC/ESI-MS/MS chromatograms of authentic pseudo-B_{12} and B_{12}. Pseudo-B_{12} and B_{12} were analyzed with LCMS-IT-TOF as described in the text. The TIC of authentic pseudo-B_{12} and B_{12} are shown in panels A and D, respectively. The mass spectra of the ion peaks from pseudo-B_{12} and B_{12} are shown in panels B and E, respectively. The magnified mass spectra from *m/z* 672 to 675 in pseudo-B_{12} and from *m/z* 678 to 680 in B_{12} are shown as inserts. The MS/MS spectra of the peaks of pseudo-B_{12} and B_{12} are shown in panel C and F, respectively.

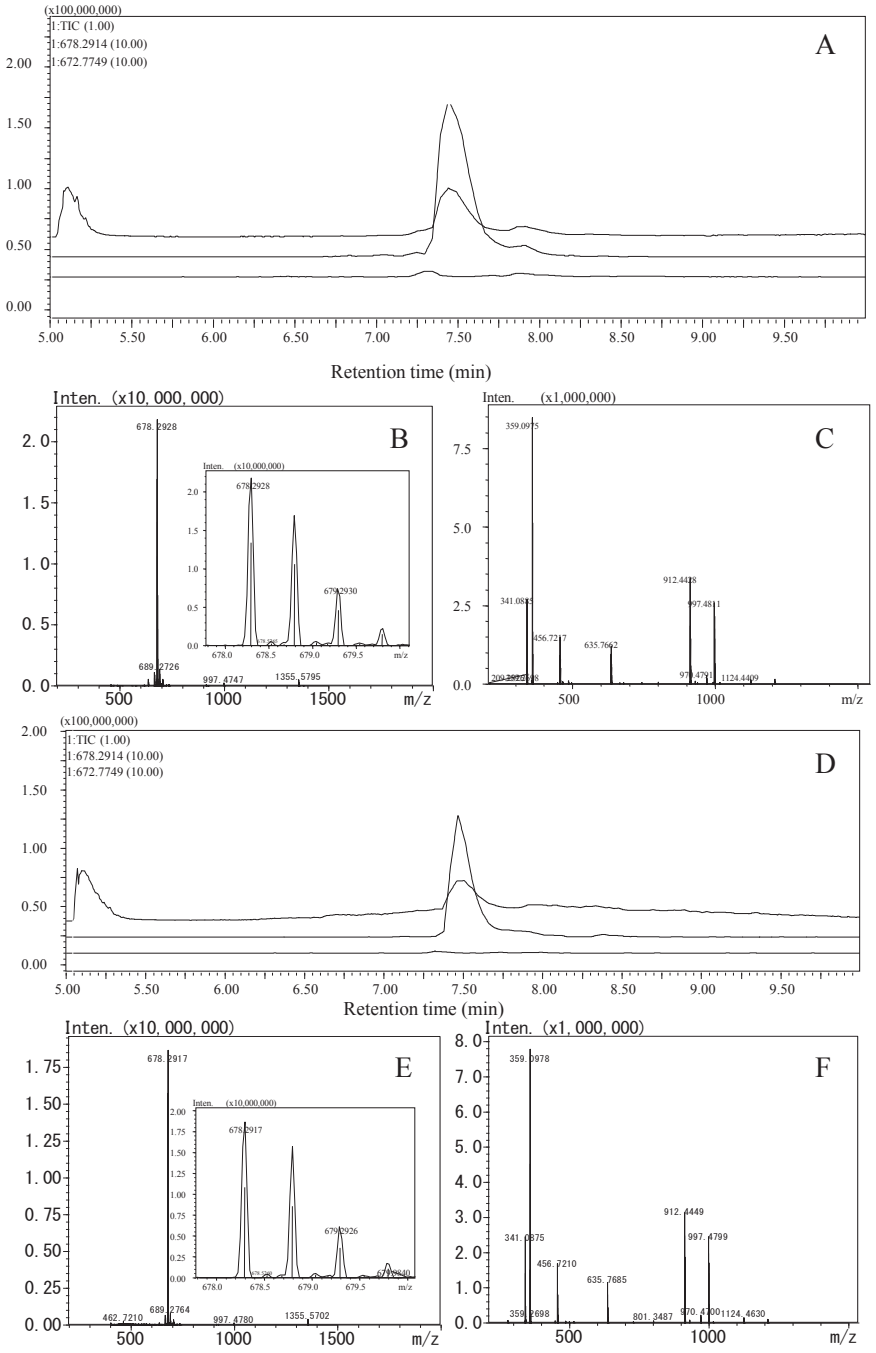


Fig. 22. LC/ESI-MS/MS chromatograms of B_{12} compounds purified from the meat and viscera of *B. japonica*. TIC and ion chromatograms for *m/z* 678.2914 (×10) and 672.7749 (×10) of B_{12} compounds purified from the meat and viscera of *B. japonica* are shown in panels A and D, respectively. The mass spectra of the ion peaks of the meat and visceral B_{12} compounds at retention times of 7.5 min are shown in panel B and panel E, respectively. The MS/MS spectra for the peaks of the muscle and visceral B_{12} compounds at *m/z* 678.2928 and at *m/z* 678.2917 are shown in panels C and F, respectively.

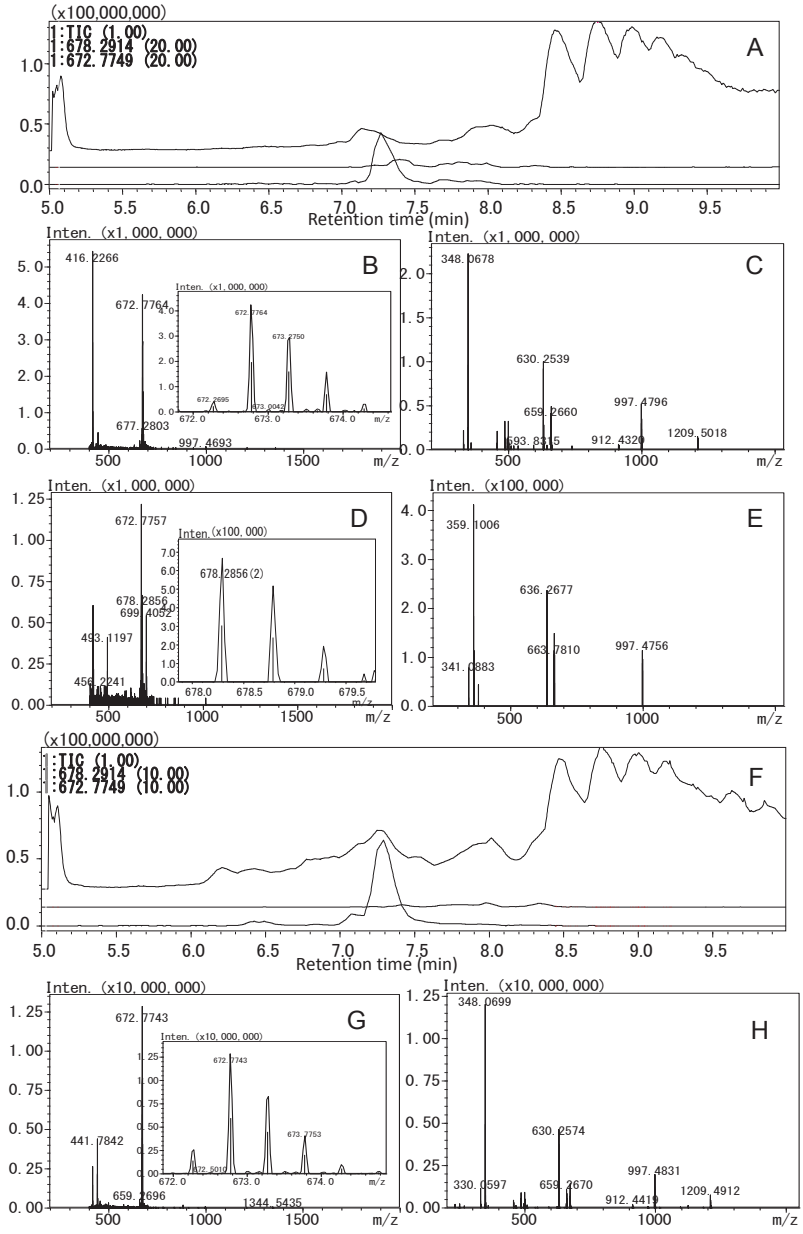


Fig. 23. LC/ESI−MS/MS chromatograms of B_{12} compounds purified from the meat and viscera of *T. cornutus*. TIC and ion chromatograms for *m/z* 678.2914 (×20) and 672.7749 (×20) of B_{12} compounds purified from the meat and viscera of *T. cornutus* are shown in panel A. The mass spectra of the ion peaks of the meat B_{12} compounds at retention times of 7.3 min and 7.4 min are shown in panel B and panel C, respectively. The MS/MS spectra for the peaks of the muscle B_{12} compounds at *m/z* 672.7764 and at *m/z* 678.2856 are shown in panels C and E, respectively. TIC and ion chromatograms for *m/z* 678.29 14 (×10) and 672.7749 (×10) of B_{12} compounds purified from the viscera of *T. cornutus* are shown in panel F. The mass spectrum of the ion peak of the visceral B_{12} compounds at a retention time of 7.3 min is shown in panel G. The MS/MS spectrum for the peak of the visceral B_{12} compound at *m/z* 672.7743 is shown in panel H.

Summary

I characterized and quantified B_{12} compounds in popular edible snails *Babylonia japonica* and *Turdo Batillus cornutus* using a microbiological assay based on *L. delbrueckii* ATCC 7830. The meat and viscera of *B. japonica* contained 27.2 ± 9.1 and 92.8 ± 25.8 μg of B_{12} per 100 g, respectively. However, the meat and viscera of *T. cornutus* contained extremely low amounts of B_{12} (3.0 ± 1.5 and 15.1 ± 8.3 μg of B_{12} per 100 g, respectively). I identified the B_{12} compounds from the edible portions (meat and viscera) of *B. japonica* and *T. cornutus* using LC/ESI-MS/MS. I found that *B. japonica* contained substantial amounts of true B_{12}, while pseudo-B_{12} was the predominant corrinoid in *T. cornutus*. These results indicate that the meat and viscera of *B. japonica* are excellent sources of B_{12} for humans.

Chapter VII

Characterization of vitamin B_{12} compounds in century egg (pidan)

Introduction

A large number of people that have low serum B_{12} levels result more commonly from malabsorption of protein-bound B_{12} (food-bound B_{12} malabsorption) rather than pernicious anemia [55]. Food-bound B_{12} malabsorption is found in people with certain gastric dysfunctions, particularly atrophic gastritis with low stomach acid secretion, which prevails in elderly people [56, 57]. As the bioavailability of crystalline (free) B_{12} is not altered in people with atrophic gastritis [58], the Institute of Medicine recommended that most of the RDA (2.4 μg/day) should be obtained by consuming foods fortified with B_{12} or supplements containing B_{12} [13].

Animal foods (*i.e.*, milk, egg and fish) are considered to be the major dietary sources of B_{12}. B_{12} content in the whole egg is about 0.9 - 1.4 μg/100 g [59, 60], and most of the B_{12} is located in the egg yolk [61]. The B_{12} intake from egg is generally large, because it is a popular food item [13]. Bioavailability of B_{12} from scrambled egg yolks, scrambled whole eggs, boiled eggs, and fried eggs (1.1 - 1.4 μg B_{12} per 100 g) averaged 8.2%, 3.7%, 8.9%, and 9.2%, respectively [60]. The B_{12} of eggs is poorly absorbed relative to other animal food products [62].

Century egg (“Pidan” in Chinese) is an alkaline-fermented ethnic food in China. Century eggs were traditionally made by preserving chicken or duck eggs in a mixture of salt, lime and ash, then wrapping in rice husks for several weeks. During this treatment, pH of the egg raises and components of egg are significant changed. During this process, egg yolk becomes a dark green with a creamy consistency, while egg white becomes as

amber jelly (**Fig. 24**). As B_{12} is unstable under alkaline conditions, there is the possibility that such alkali treatment results in a significant loss of B_{12} in eggs. However, little information is available on B_{12} content of century egg. If century egg contains substantial amounts of free B_{12}, it would be excellent source of free B_{12} for elderly persons with food-bound B_{12} malabsorption.

Chapter VII, I described determination of the B_{12} contents of century eggs and characterization of their B_{12} compounds to evaluate whether they are good sources of B_{12}.

Fig. 24. Picture of century egg.

Materials and Methods

Materials

B_{12} was obtained from Sigma (St Louis, Missouri, USA). A B_{12} assay medium based on *L. delbrueckii* ATCC 7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 TLC aluminum sheets were obtained from Merck (Darmstadt, Germany). Various century eggs were purchased from local markets in Japan.

Extraction and assay of B_{12}

After the egg shells were removed from century eggs, egg white and egg yolk were

separated. Each sample was homogenized using a mortar and pestle. An aliquot (4.0 g) of each homogenate was used as the sample for the B_{12} assay. B_{12} compounds were extracted and determined as described in **Chapter II.**

Determination of pH value

The pH values of century egg yolk and white were determined using a pH electrode (0040-10D Horiba, Ltd. Japan) according the manufacturer's instructions.

Bioautography of B_{12} compounds with B_{12}-dependent E. coli 215

B_{12} extract (50 mL) prepared as described above was partially purified and concentrated using Sep-Pak Plus® C18 cartridge (Waters Corp., Milford, USA). Bioautography of the B_{12} compounds was done according to the method of Tanioka *et al*. [24].

Identification of B_{12} compounds by LC/ESI-MS/MS

B_{12} compounds of each sample were purified under the same conditions described above. The purified extract was loaded onto an immunoaffinity column [EASI-EXTRACT® B_{12} Immunoaffinity Column (P80) R-Biopharm AG, Darmstadt, Germany], and the corrinoids were purified according to the manufacturer's protocol. The corrinoids of each sample and authentic B_{12} were dissolved in 0.1% (v/v) acetic acid and filtered with a Nanosep MF centrifuge device (0.4 μm, Pall Corp., Tokyo, Japan) to remove small particles. Aliquots (2 μL) of filtrate were analyzed using LC/MS-IT-TOF coupled with an Ultra-Fast LC system (Shimadzu, Kyoto, Japan) as described in **Chapter II.** The identity of B_{12} (*m/z* 678.2914) as $[M + 2H]^{2+}$ were confirmed by comparing the observed

molecular ions and retention times molecular ions.

Sephadex G-50 gel filtration experiment

The free B_{12} was separated from the egg yolk and white of century egg using a column (1.4 × 10 cm, econo-pack column, BioRad) of Sephadex G-50 fine (GE Healthcare U.K.) and then assayed. An aliquot (2.5 g) of egg white and egg yolk was added 100 mmol/L potassium phosphate buffer (10 mL) and then homogenized using a mortar and pestle. Each homogenate was centrifuged at 10,000 × *g* for 10 min at 25 ^{O}C to remove insoluble materials. An aliquot (1.0 mL) of the supernatant was applied on to the Sephadex G-50 column, which had been 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. The eluate from the column was fractionated at 0.5 mL. A thousand microliter of 0.57 mol/L acetate buffer, pH 4.5 and 40 μL of 0.5% (w/v) KCN were added to each fraction, mixed vigorously, and left overnight at 4 °C in the dark. B_{12} was assayed by the microbiological method. The macromolecular and free B_{12} fractions were estimated with blue dextran and authentic B_{12}, respectively, by measuring the absorbance at 280 nm.

In vitro gastric digestion of century egg and boiled chicken egg

The *in vitro* gastric digestion of egg was done using a method simulating the human digestion system [63]. Century egg and boiled chicken egg (each egg yolk and white) were sampled. An aliquot (5 g) of each sample was homogenized with distilled water (5 mL) using mortar and pestle. The homogenate was mixed with 3 mL of 0.01% (w/v) pepsin solution and 29 mL of 0.06 mol/L HCl and incubated at 37 °C in the dark for 1.5

h *in vitro* (gastric digestion). The digestive reaction was stopped by adding 5 mL of 1 mol/L $NaHCO_3$ and incubated in the ice for 5 min. The mixture was centrifuged at 10,000 × *g* for 10 min and the supernatant was used a sample of free B_{12} assay as described above.

Results and Discussion

B_{12} content of century egg (pidan)

The B_{12} contents of various century eggs (samples A, B, C, and D; n=3) were analyzed using *L. delbrueckii* ATCC 7830 microbiological assay method (**Table 10**). The yolks and whites (each 100 g weight) of century eggs contained approximately 1.9 ± 0.59 μg of B_{12} and 0.83 ± 0.20 μg of B_{12}, respectively. The B_{12} content of whole century eggs (per 100 g weight) was calculated to be approximately 1.41 ± 0.39 μg of B_{12}. Although there is no information on the B_{12} content of fresh duck egg, considerable amount (3.0 μg per 100 g) of B_{12} is reportedly found only in the yolk fraction of fresh chicken egg but not in the white fraction [52]. The pH values of century egg yolk and white were 8.6 ± 0.4 and 8.7 ± 0.5, respectively. These results suggested that B_{12} found in the egg yolk was partly transferred to the egg white by the denaturation of egg proteins during the process.

Identification of corrinoid compounds using the E. coli 215 bioautography

The B_{12} compounds found in century eggs were analyzed using an *E. coli* 215 bioautogram after separation by silica gel 60TLC (**Fig. 25**). Each yolk sample of century eggs produced a single clear spot with an R_f value that was identical to that of authentic B_{12} (**Fig. 25 a**), The identical result were also obtain in the egg white of samples (**Fig. 25 b**). These results indicate that egg yolks and whites of century eggs contained B_{12} but not

pseudo-B_{12}, which is biologically inactive in humans.

Table 10. B_{12} content in various century egg.

	Vitamin B_{12} content			Egg weight	pH	
	egg yolk	egg white	whole egg		egg yolk	egg white
	(μg/100 g wet weight)			g		
Sample A (n=3)	2.34 ± 0.21	0.60 ± 0.29	1.63 ± 0.24	54.60	8.84 ± 0.12	8.95 ± 0.10
Sample B (n=3)	2.47 ± 0.26	1.24 ± 0.09	1.84 ± 0.10	48.50	8.10 ± 0.53	8.04 ± 0.56
Sample C (n=3)	1.46 ± 0.20	0.84 ± 0.20	1.19 ± 0.12	56.40	9.00 ± 0.20	9.21 ± 0.30
Sample D (n=3)	1.33 ± 0.26	0.64 ± 0.06	0.99 ± 0.12	48.70	8.49 ± 0.10	8.75 ± 0.09
Mean ± SD	1.90 ± 0.59	0.83 ± 0.29	1.41 ± 0.39	52.05 ± 4.05	8.61 ± 0.40	8.74 ± 0.50

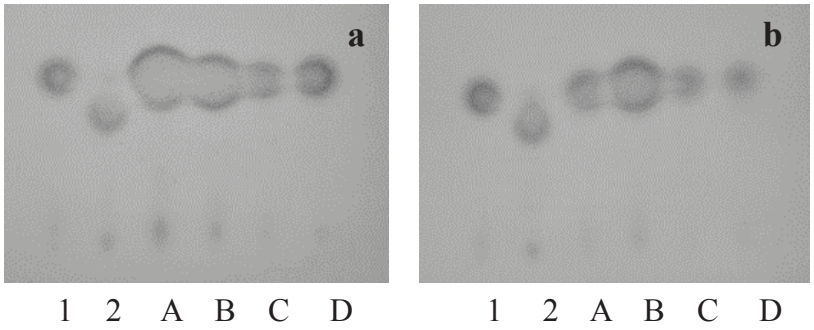


Fig. 25. *E. coli* 215 bioautogram analysis of corrinoids found in century eggs.
a) 1, authentic B_{12}; 2, pseudo-B_{12}; A-D, egg yolk of century egg samples. b) 1, authentic B_{12}; 2, pseudo-B_{12}; A-D, egg white of century egg samples.

LC/ESI-MS/MS Analysis

To precisely identify the corrinoid compounds present in century eggs, corrinoids were purified from the egg yolk and identified using LC/ESI-MS/MS (**Fig. 26**). Authentic B_{12} was eluted as a peak with a retention time of 7.5 min. The mass spectrum of authentic B_{12} primarily comprised a doubly charged ion with *m/z* 678.2943 $[M + 2H]^{2+}$ (**Fig. 26 A and B**). MS/MS spectra revealed a predominant monovalent ion with *m/z* 359.0986,

which was largely attributable to the nucleotide moiety of B_{12} (**Fig. 26 C**). The corrinoid purified from the egg yolk of century egg was eluted as one ion peak. The mass spectrum of the main peak with *m/z* 687.2902 had a retention time of 7.5 min in the purified sample (**Fig. 26 D and E**). The MS/MS spectrum of the purified compound with a monovalent ion with *m/z* 359.0984 was identical to that of authentic B_{12} (**Fig. 26 C and F**). The egg white of century egg also obtained the identical results. These results indicate that the egg yolk and whites of century egg contained authentic B_{12} but not pseudo-B_{12}, which is inactive in humans.

Free B_{12} analysis

To evaluate whether egg yolk and white of century egg contained "free B_{12}" or "protein-bound B_{12}", the homogenates of selected samples were analyzed using Sephadex G-50 gel filtration. As shown in **Fig. 27**, B_{12} found in egg yolk and white was recovered only in the protein-bound B_{12} fractions. These results indicate that both yolk and white of century egg contained protein bound-B_{12}, but not free B_{12}.

In vitro gastric digestion of century egg and boiled chicken egg

The release of free B_{12} from ingested food during gastric digestion is the first step for the absorption of B_{12}. To evaluate whether free B_{12} is formed from protein-bound B_{12} during gastric digestion of century egg, the egg yolk and white were treated by the *in vitro* gastric digestion and free B_{12} was separated Sephadex G-50 gel filtration and assayed. Boiled egg was treated with the identical *in vitro* digestion as a control (**Fig. 28**). Although 52.67 ± 9.07% of the B_{12} found in the egg yolk of century egg was recovered in the free B_{12} fractions, no free B_{12} was detected in the egg white. While 10.33 ± 2.08% of B_{12}

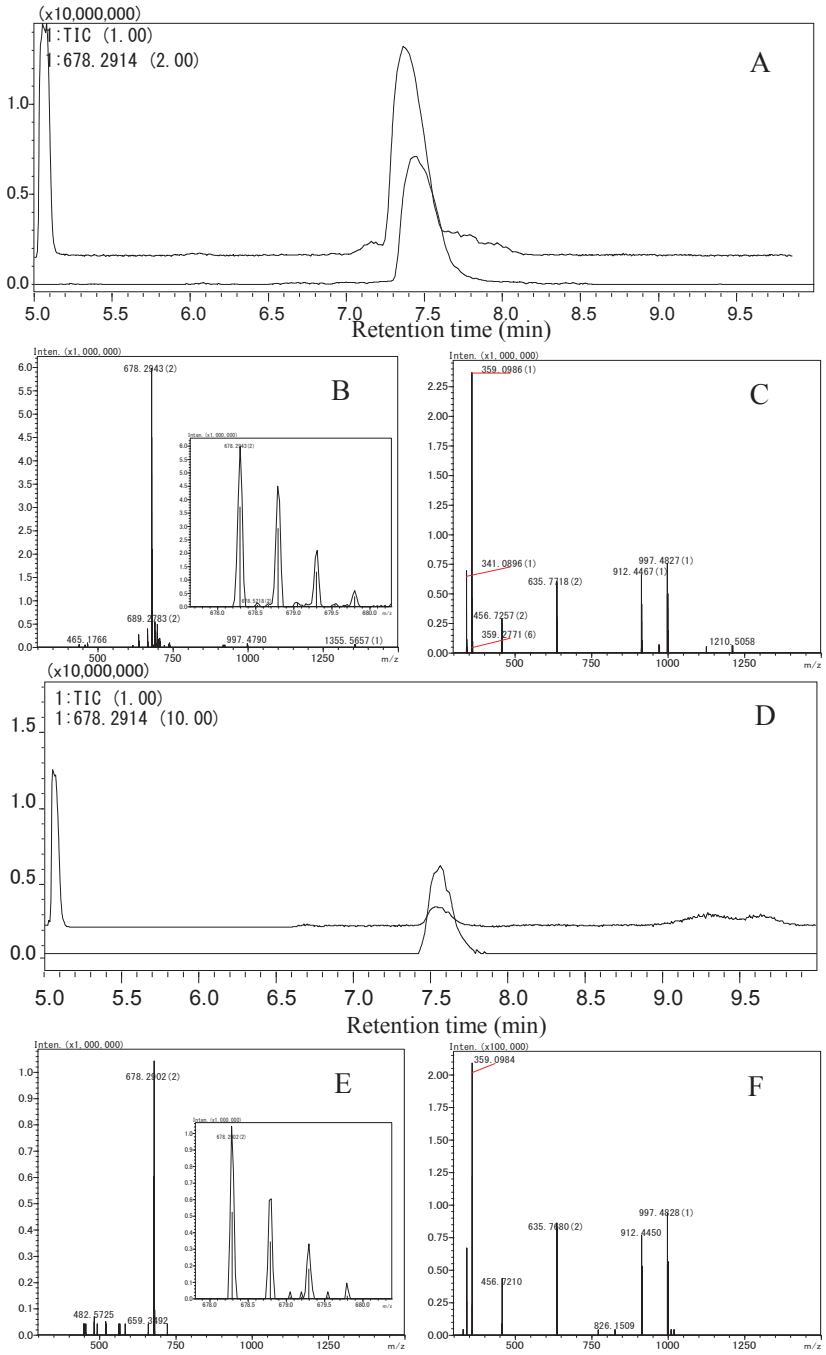


Fig. 26. LC/ESI-MS/MS chromatograms of authentic B_{12} and the B_{12} compounds purified from egg yolk of century eggs. B_{12} compounds were analyzed using the LC/MS-IT-TOF system. Panels A and D show total ion chromatograms and those (*m/z* 678.2943) of authentic B_{12} and B_{12} compounds purified from egg yolk. Mass spectra of authentic B_{12} and purified B_{12} compounds at 7.5 min are shown in panels B and E, respectively (magnified spectrum range from *m/z* 678 to *m/z* 680 is shown as an insert in each panel). MS/MS spectra for the peak of authentic B_{12} at *m/z* 678.2943 and that of purified B_{12} compounds at *m/z* 678.2902 are shown in panels C and F, respectively.

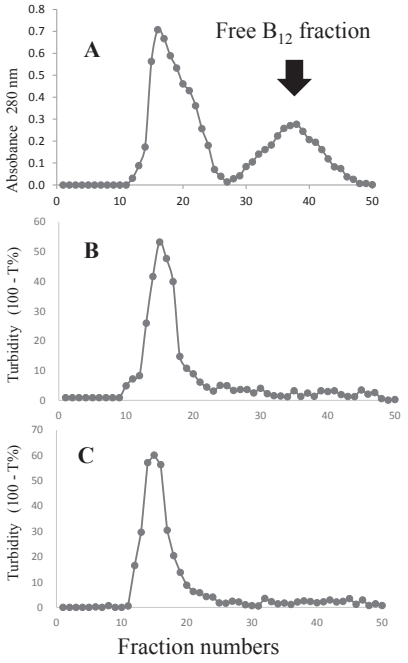


Fig. 27. Elution patterns of B_{12} compounds using Sephadex G-50 gel filtration of century egg yolk and white: (A) authentic blue dextran and B_{12} mixture, (B) egg yolk and (C) egg white.

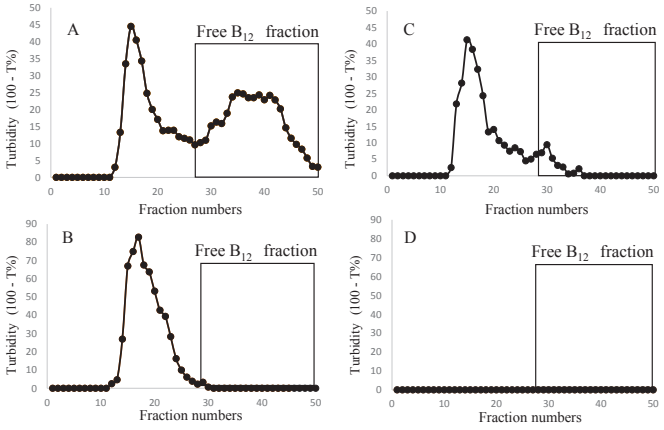


Fig. 28. Elution patterns of B_{12} compounds using Sephadex G-50 gel filtration of the pepsin-treated century egg and boiled chicken egg: (A) century egg yolk, (B) century egg white, (C) boiled chicken egg yolk, (D) boiled chicken egg white.

found in the boiled chicken egg yolk was recovered in free B_{12} fraction and no B_{12} was detected in the egg white; the result coincided with the previous results that bioavailability of B_{12} from boiled egg is very low (8.9%) [60]. It is the first report that century egg yolk is easier to digest than the usually cooked egg yolk and that approximately 52% of B_{12} found in the century egg yolk were formed as free B_{12} during gastric digestion. Considering the *in vitro* digestion rate (52%), the consumption of five century egg yolks could supply the entire RDA for an adult (2.4 μg/day) [13]. These result present in this chapter indicated that century egg is a good source of B_{12}.

Summary

I determined the B_{12} content of commercially available century eggs and characterized their B_{12} compounds in detail. The yolk and white of century eggs (each 100 g basis) contained 1.9 ± 0.6 and 0.8 ± 0.3 μg of B_{12}, respectively. B_{12} compounds purified from egg yolk and white were identified as B_{12} using LC/ESI-MS/MS. The B_{12} that existed in yolk or white of century eggs was completely recovered in macromolecular fractions, but not in free B_{12} fractions during Sephadex G-50 gel filtration. Although B_{12} was completely bound to protein in century egg yolk and white, approximately 52% of B_{12} found in the egg yolk were formed as free B_{12} during *in vitro* gastric digestion and no free B_{12} was detected in the egg white. These results indicated that century egg is a good source of B_{12}.

Chapter VIII

Conclusion

B_{12} is synthesized only by certain bacteria and is then concentrated mainly in the bodies of higher predatory organisms in the natural food chain [11]. Therefore, animal-derived foods are considered to be the major dietary sources of B_{12}. In contrast, higher plants do not contain biosynthetic pathways for B_{12} and B_{12}-dependent enzymes. Thus, plant-derived foods contain no or only traces of B_{12}. Vegetarians have a great risk of developing B_{12} deficiency. Most Chinese vegetarians are Buddhists and Chinese people who are not vegetarians tend to eat vegetarian food in order to prevent illness and disease in the future [64]. Thus, I need to identify plant-derived foods that contain high levels of B_{12} to prevent vegetarians from developing B_{12} deficiency. Chinese cuisine is one of the world`s greatest cuisines and has become popular internationally. The ingredients used in Chinese cuisine are based on natural and agricultural products. However, there is little information on B_{12} content of food ingredients used in Chinese cuisine. In the present thesis, I characterized B_{12} compounds in various food ingredients used in China.

In **Chapter II**, B_{12} contents were analyzed in various sources of hair vegetable (*N. flagelliforme*). As shown in **Table 1**, high B_{12} contents were detected in naturally grown cells (109.2 ± 18.5 μg/100 g dry weight) and cultured cells (120.2 ± 53.6 μg/100 g dry weight). However, commercially available samples had very variable and lower B_{12} contents [45.1 ± 40.6 (range, 4.8 - 101.6) μg/100 g dry weight]. LC/ESI-MS/MS analysis indicated that pseudo-B_{12} was the predominant corrinoid in hair vegetable (*N. flagelliforme*) (**Fig. 3 and Table 2**). These results were similar to those of other edible cyanobacteria, *Spirulina sp.*, Suizenji-nori (*Aphanothece sacrum*), and Ishikurage

(*Nostoc commune*) [25, 26, 27]. Cyanobacteria have the ability to synthesize pseudo-B_{12} [63], which functions as a coenzyme of methionine synthase to catalyze the synthesis of methionine from homocysteine and N^5-methyltetrahydrofolate [29]. Microscopic analysis indicated that although naturally grown and cultured hair vegetable possessed a bead-like morphology, no such morphology was found in the commercially available samples (fake item only) (**Fig. 7**). The fake items have very low B_{12} contents. These results indicated that hair vegetable (*N. flagelliforme*) contains substantial amount of pseudo-B_{12} inactive in humans and it is not suitable for use of B_{12} source, regardless of the presence of the fake items.

The wild edible mushroom fruiting bodies that are consumed by European vegetarians, black trumpet (*Craterellus cornucopioides*) and golden chanterelle (*Cantharellus cibarius*) mushrooms, contained considerable levels (1.09 - 2.65 μg/100 g dry weight) of B_{12} [33]. In this study, I determined B_{12} content in Lion's mane mushroom fruiting bodies (*H. erinaceus*) (**Table 3**). Low B_{12} was detected in the mushroom fruiting bodies (0.37 ± 0.34 μg/100 g dry weight). To determine whether this mushroom fruiting body contained B_{12} or other corrinoid compounds that are inactive in humans, the mushroom corrinoid compounds were identified using B_{12}-dependent *E. coli* 215 bioautogram and LC/ESI-MS/MS chromatograms. The dried mushroom fruiting bodies rarely contained an unnatural B_{12}[*c*-lactone] that is inactive in humans. Thus, dried lion's mane mushroom is not suitable for use as a B_{12} source because of its low B_{12} contents and the occasional presence of B_{12}[*c*-lactone] which is inactive in humans.

In **Chapter IV**, determination and characterization of Chinese black tea leaves were described. As shown in **Table 5**, Traces (0.25 - 0.69 μg/100 g dry weight) of B_{12} were observed in Pu`er, Fu, and Brick tea leaves. Although, Ryubao tea leaves (sample H)

contained the highest B_{12} content (1.37 μg/100 g dry weight).The other Ryubao leaf samples (approximately 0.69 μg of B_{12}) was only slightly higher than that for Pu`er tea leaves (approximately 0.49 μg/100 g dry weight). B_{12} content in the tea drink prepared from the Ryubao tea leaf sample H was 0.8 ng/100 mL of black tea.

Therefore, consumption of approximately 300 L of this tea would provide the recommended dietary allowance for adults (2.4 μg/day) [13], although ingestion of such large quantities of tea on a daily basis is not recommended.

As summarized in **Table 11**, the plant-derived foods tested in this study are not suitable for use of B_{12} source.

Table 11. Vitamin B_{12} content of plant-derived foods.

	B_{12} content (μg/100 g dry weight)	Corrinoid compounds
Hair vegetable (*N. flagelliforme*)	109.2 ± 18.5	B_{12} (27%), Pseudo-B_{12} (73%)
Lion' mane mushroom (*H. erinaceus*)	0.4 ± 0.3	B_{12}, B_{12} [*c*-lactone]
Black tea leaves	0.6 ± 0.3	B_{12}

Animal-derived foods (*i.e.*, meat, milk, egg, fish, and shellfish) are considered to be the major dietary sources of B_{12}. In **Chapter V-VII**, I described characterization of B_{12} compounds in animal-derived foods used in Chinese cuisine. In **Chapter V**, I determined B_{12} content in boiled and dried oyster products and oyster sauce. The dried oyster products from China and Japan contained a considerably high B_{12} level (approximately 20.4 - 28 μg/ 100 g dry weight) (**Table 7**).

This study showed significant loss (67%) of B_{12} during the preparation of boiled and dried oyster. However, boiled and dried oyster products contained substantial amount of B_{12} and 3 - 4 of them could provide the recommended dietary allowance of B_{12} for adults

(2.4 μg/day). These observations indicate that boiled and dried oyster products is a good source of B_{12}. B_{12} contents of oyster sauce products were low (2.3 μg/ 100 g wet weight).

In **Chapter VI**, characterization of B_{12} compounds in edible sea snails (*B. japonica* and *T. cornutus*) were described. *B. japonica* contained a high "true B_{12}" (approximately 27.2 μg/100 g muscle) (**Table 9**). However, *T. cornutus* has low B_{12} content (approximately 3.0 μg/100 g muscle) and pseudo-B_{12} was the predominant corrinoid in *T. cornutus.* The results indicate that *B. japonica* would be an excellent source of B_{12}.

I determined the B_{12} content of commercially available century eggs and characterized their B_{12} compounds in **Chapter VII**. The yolk and white of century eggs (each 100 g basis) contained 1.9 ± 0.6 and 0.8 ± 0.3 μg of B_{12}, respectively (**Table 10**). The B_{12} (approximately 52%) found in the century egg yolk were formed as free B_{12} during gastric digestion. These results indicated that century egg is a good source of B_{12}.

Table 12. Vitamin B_{12} content in animal-derived foods.

	B_{12} content (μg/100 g wet weight)	Corrinoid compounds
Oyster		
Raw oyster (Japan)	22.2 ± 1.1	B_{12}
Boiled and dried oyster (China)	21.5 ± 2.7	B_{12}, Pseudo B_{12} (trace)
Oyster sauce	2.3 ± 1.4	B_{12}
Edible snails		
Babylonia japonica	27.2 ± 9.1	B_{12}
Turdo Batillus cornutus	3.0 ± 1.5	B_{12}, Pseudo B_{12}
Century egg		
Egg yolk	1.9 ± 0.6	B_{12}
Egg white	0.8 ± 0.3	B_{12}

B_{12} content of various animal-derived foods tested in this study summarized in **Table 12**. Boiled and dried oyster products and sea snail *B. japonica* contain high B_{12} levels (approximately 21.5 - 27.2 μg/100 g wet weight) and would be excellent sources of B_{12}.

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Summary

Vitamin B_{12} (B_{12}) is synthesized only by certain bacteria and is concentrated mainly in the bodies of higher predatory organisms in the natural food chain. Animal-derived foods (*i.e.*, meat, milk, egg, fish, and shellfish) are considered to be the major dietary sources of B_{12}. Chinese cuisine is one of the world's greatest cuisines and has become popular internationally. However, there is little information on B_{12} content of food ingredients used in Chinese cuisine. In the present thesis, I characterized B_{12} compounds in various food ingredients used in Chinese cuisine. As vegetables, fruits, and nuts do not contain B_{12}, an edible cyanobacterium *Nostoc flagelliforme* (known as hair vegetable), an edible mushroom *Hericium erinaceus*, and black tea leaves (fermented by certain bacteria) were selected as plant-derived foods. Boiled and dried oyster products, edible sea snails (*Babylonia japonica* and *Turdo Batillus cornutus*), and century eggs were used as animal-derived foods. B_{12} was extracted from the food samples and assayed using a microbiological assay method with *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830. The B_{12} compounds were identified using *Escherichia coli* 215 bioautography and liquid chromatography-electrospray ionization/tandem mass spectrometry (LC/ESI-MS/MS).

Naturally grown and cultured hair vegetable (*N. flagelliforme*) samples contained high B_{12} (109.2 - 120.2 μg/100 g dry weight). However, commercially available samples showed variable B_{12} contents (4.8 - 101.6 μg/100 g dry weight) because they comprised *Nostoc* cells and fake substitutes, which contain very low B_{12} content. Corrinoid compounds from *N. flagelliforme* samples were identified as pseudo-B_{12} (approximately 72%) and B_{12} (approximately 28%) using *E. coli* 215 bioautography and LC/ESI-MS/MS. The results suggested that hair vegetable *N. flagelliforme* contained substantial amounts of pseudo-B_{12}, which is inactive in humans.

B_{12} compounds in the edible medicinal mushroom *Hericium erinaceus,* lion's mane mushroom fruiting bodies were characterized. Trace levels (0.04 - 0.36 μg/100 g dry weight) of B_{12} were found in most of the dried mushroom samples, and a few samples contained slightly higher B_{12} levels (approximately 1.04 μg/100 g dry weight). Corrinoid compounds were purified from these samples and identified as B_{12} or B_{12}[*c*-lactone] (or both) using LC/ESI-MS/MS chromatograms. B_{12}[*c*-lactone] was simply produced during incubation of authentic B_{12} and chloramine-T, an antimicrobial agent, at varying pH values (3.0 - 7.0) and was completely inactive in the B_{12}-dependent bacteria that are generally used in B_{12} bioassay.

I characterized B_{12} compounds of Chinese black tea leaves. Trace levels (0.25 - 0.69 μg/100 g dry weight) of B_{12} were detected in Pu'er, Fu, and Brick tea leaves. However, B_{12} content (0.06 - 1.37 μg/100 g dry weight) of Ryubao tea leaves significantly varied. B_{12} compounds were purified from Ryubao tea leaves and identified as B_{12} by LC/ESI-MS/MS. B_{12} content in the tea drink prepared from Ryubao tea leaves was very low (0.8 ng/100 mL). These results indicated that Chinese black tea was not suitable for use of B_{12} source.

The boiled and dried oyster products contained a high B_{12} (approximately 20.4 - 28 μg/100 g dry weight). Approximately 33% of B_{12} found in raw oysters were recovered in boiled and dried oysters. B_{12} content of oyster sauce products was low (2.3 ± 1.4 μg/100 g wet weight). LC/ESI-MS/MS analysis indicated that B_{12} was the predominant corrinoid in Chinese boiled and dried oysters. The consumption of 3 - 4 boiled and dried oysters could provide the recommended dietary allowance of B_{12} for adults (2.4 μg/day). These observations indicate that boiled and dried oyster products are a good source of B_{12}.

I characterized and quantified B_{12} compounds in popular edible snails *Babylonia*

japonica and *Turdo Batillus cornutus*. The meat and viscera of *B. japonica* contained 27.2 ± 9.1 and 92.8 ± 25.8 μg of B_{12} per 100 g, respectively. However, the meat and viscera of *T. cornutus* contained extremely low amounts of B_{12} (3.0 ± 1.5 and 15.1 ± 8.3 μg of B_{12} per 100 g, respectively). LC/ESI-MS/MS analysis showed that *B. japonica* contained substantial amounts of true B_{12}, while pseudo-B_{12} was the predominant corrinoid in *T. cornutus*. These results indicate that the meat and viscera of *B. japonica* are excellent sources of B_{12} for humans.

B_{12} compounds of century eggs were characterized. The yolk and white of century eggs (each 100 g basis) contained 1.9 ± 0.6 and 0.8 ± 0.3 μg of B_{12}, respectively. B_{12} compounds were purified from egg yolk and white and identified as B_{12} using LC/ESI-MS/MS. B_{12} found in the egg yolk and white existed in the protein-bound form. However, approximately 52% of B_{12} found in the egg yolk were formed as free B_{12} during *in vitro* gastric digestion and no free B_{12} was detected in the egg white. These results indicated that century egg is a good source of B_{12}.

摘要

ビタミン B_{12}(B_{12})は一部の細菌で生合成され、食物連鎖により動物組織に蓄積されるため、動物性食品(畜肉、牛乳、鶏卵、魚介類)が B_{12} の良い供給源である。従って、植物性食品には B_{12} が含有されておらず、厳格な菜食主義者は B_{12} 欠乏症を呈しやすい。現在、中華食材の B_{12} 含量や B_{12} 化合物の特性についての情報は極めて少ない。そこで、本研究では、多種類の中華食材に含まれる B_{12} 化合物の特性を検討した。分析試料は日本と中国で市販されている中華食材を使用し、植物性と動物性食品に分けて研究を行った。野菜などの植物性食品には B_{12} は含まれていないが、藍藻の髪菜やキノコのヤマブシタケ及び微生物が関与する中国黒茶葉を植物性食品の試料とした。動物性食品としては煮干しガキ、バイ貝、サザエ、ピータンに着目した。B_{12} の分析は、日本食品標準成分表に準じて *Lactobacillus delbrueckii* subspecies *lactis* ATCC 7830 を用いた微生物学的定量法で行った。また、*Escherichia coli* 215 によるバイオオートグラムと Liquid chromatography-electrospray ionization/tandem mass spectrometry (LC/ESI-MS/MS)を用いて各中華食品に含まれる B_{12} 化合物を同定した。

髪菜の B_{12} 含量は天然品と人工培養品においてほぼ同量で乾燥重量 100 g あたり約 120 μg であったが、市販品は 45 μg 程度と低かった。さらに、LC/ESI-MS/MS を用いて詳細に分析した結果、髪菜には B_{12} とシュード B_{12} の両方が約 3 対 7 の割合で存在することが明らかとなった。また、髪菜の市販品には本物と偽物の混合物か、完全な偽装品である場合が多かった。髪菜の主要なコリノイド化合物はヒトで生理的不活性なシュード B_{12} であり、B_{12} の供給源にはならないことが明らかになった。

また、食用キノコであるヤマブシタケに含まれるB_{12}化合物について検討した。B_{12}含量は乾燥キノコ100 gあたり約0.04-1.04 μgと低値であった。さらに

LC/ESI-MS/MSで分析を行った結果、一部の乾燥ヤマブシタケにはB_{12}とB_{12}[*c*-lactone]が検出された。B_{12}[*c*-lactone]は生体内で不活性であり、B_{12}と抗菌剤クロラミン-Tとの反応で容易に生成されることを明らかにした。従って、乾燥ヤマブシタケで検出されたB_{12}[*c*-lactone]は、加工段階に用いられた塩素系消毒剤とヤマブシタケ中のB_{12}の反応で生成されたと推察した。

次に発酵食品である中国黒茶のB_{12}含量とB_{12}化合物を検討した。中国産の十種類の黒茶葉(プーアル、六堡茶、磚茶と茯茶)の中で、とくに六堡茶のB_{12}含量(1.37 μg/100 g)が高かった。その六堡茶に含まれていたB_{12}化合物をLC/ESI-MS/MSを用いて分析した結果、B_{12}のみが検出された。しかし、黒茶抽出物100 mLあたり0.8 ngのB_{12}含量と極めて低値であるため、B_{12}の供給源にならないことが明らかとなった。

また、中国で一般的に食されている煮干しガキとオイスターソースに含まれるB_{12}化合物について分析した。煮干しガキ(中国産と日本産)には多量のB_{12}(15.5-21.5 μg/100 g)が含まれていたが、生ガキのB_{12}含量と比較すると約75%減少していた。このB_{12}含量の減少の理由を調べるため、生ガキを用いて煮干しガキを作成した結果、カキの加熱調理により約67%のB_{12}が損失した。また、煮汁を濃縮したオイスターソースのB_{12}含量は低値(2.3±1.4 μg/100 g)を示した。LC/ESI-MS/MS分析結果から、中国産の干しガキにシュードB_{12}が極微量検出されたが、煮干しガキを3-4個摂取することでB_{12}の推奨量(2.4 μg/日)を満たすことができ、B_{12}の良い供給源になることが示唆された。

次に中華料理でも利用される食用巻貝のバイ貝とサザエのB_{12}化合物について検討した。バイ貝の筋肉部および内臓部のB_{12}含量は、サザエの各部位に比べ6-9倍高い含量を示した。また、サザエの筋肉部および内臓部にはシュードB_{12}が多量に検出されたが、バイ貝のいずれの部位ではシュードB_{12}は検出されず、すべて

B_{12}であった。サザエに比べ、バイ貝に多量のB_{12}が含有されている理由として、バイ貝は肉食性であり、サザエは藻食性であり、食性の違いが関係していると考えられた。

最後にピータンのB_{12}化合物を検討した。卵黄のB_{12}含量は100 gあたり約1.90 μgであったが、卵白にも0.83 μg程度のB_{12}が検出された。B_{12}化合物をLC/ESI-MS/MSで詳細に分析した結果、卵黄と卵白にはB_{12}のみが検出された。また、ピータンのタンパク質が変性していることから遊離型B_{12}の存在が示唆されたが、卵黄と卵白の両方で遊離型B_{12}は検出されず、B_{12}はタンパク質と結合した状態で存在することが明らかとなった。人工消化試験においてピータンの卵黄から遊離型B_{12}が52.67±9.07%生成したが、卵白から遊離型B_{12}は生成しなかった。一方、ゆで卵の人工消化試験では、卵黄から遊離型B_{12}が約10%生成したにとどまった。これらの結果より、ピータンの卵黄は消化性が良く、B_{12}の良い供給源になることが明らかとなった。

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