Occurrence and Characterization of Naturally Occurring and Unnatural Inactive Corrinoid Compounds in Food

（食品に含まれる天然型および非天然型不活性コリノイド化合物の存在と特徴）

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Contents

Chapter I
Introduction

Vitamin B_{12} (B_{12}), also known as cyanocobalamin (CN-B_{12}), is a water-soluble vitamin with the highest molecular weight (1355.4) among vitamins. B_{12} has a cobalt-coordinated nucleotide, which provides 5,6-dimethylbenzimidazole as a base in the lower axial ligand (**Fig. I-1**) [1]. Usually, CN-B_{12}, hydroxocobalamin (OH-B_{12}), and sulfitocobalamin (SO_3^--B_{12}) is readily converted into the B_{12} coenzymes, methylcobalmain (CH_3-B_{12}) and 5′-deoxyadenosylcobalmain (AdoB_{12}). CH_3-B_{12} functions as a coenzyme of methionine synthase (MS) (EC 2.1.1.13) that is involved in methionine biosynthesis [2], and AdoB_{12} is a coenzyme of methylmalonyl-CoA mutase (MCM) (EC 5.4.99.2) involved in branched-chain amino acid and odd-chain fatty acid metabolism in humans [3].

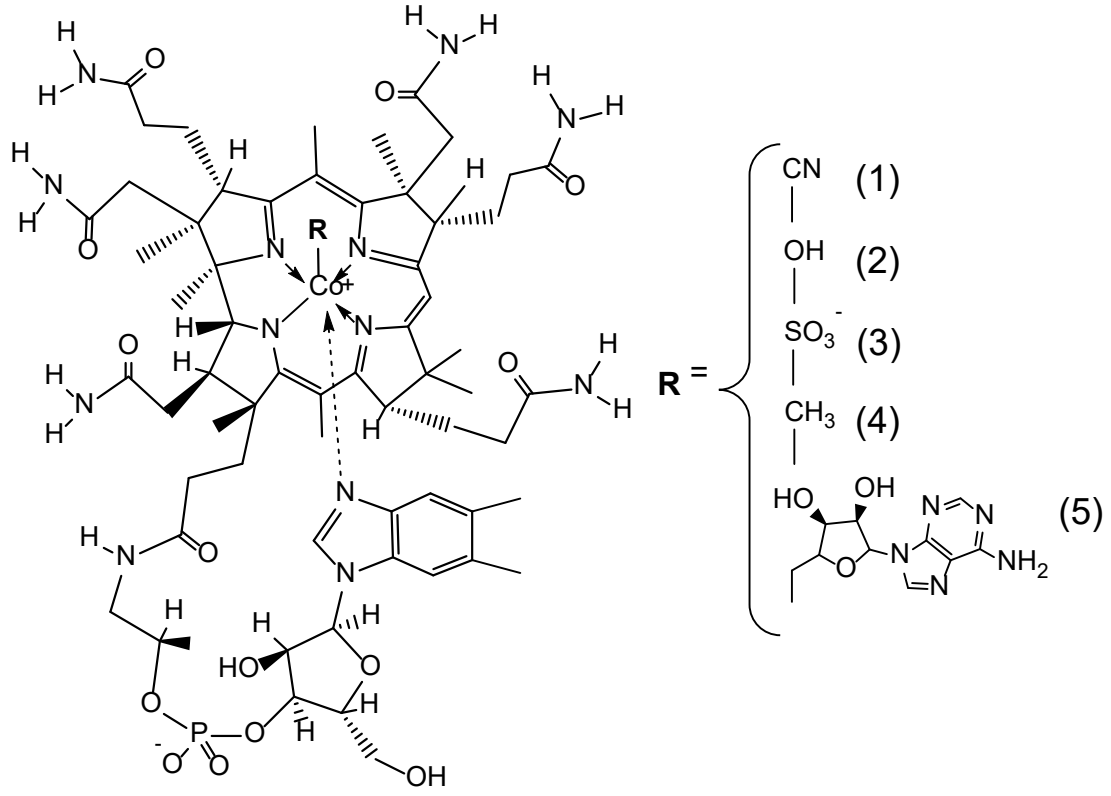

Fig. I-1. Structural formula of vitamin B_{12}. (1) CN-B_{12}, (2) OH-B_{12}, (3) SO_3^--B_{12}, (4) CH_3-B_{12}, and (5) AdoB_{12}, adenosyl B_{12}.

B_{12} deficiency reportedly results in significant increases of methylmalonic acid in urine and homocysteine in serum [4]; which are clinically used as indices of B_{12} deficiency. B_{12} deficiency induces megaloblastic anemia, developmental disorders, growth retardation, and neuropathy [5].

B_{12} is synthesized only by specific archaea and bacteria, but not by animals or plants [6]. It accumulates in animal tissues through microbial interactions in the food chain [7]. For instance, ruminant animals such as cattle and sheep acquire B_{12} from the microbial synthesis in their gastrointestinal tracts [7]. In aquatic environments, Thaumarchaeota and cyanobacteria are the major producers of B_{12} and pseudovitamin B_{12} (pseudoB_{12}), respectively [8,9]. Most phytoplankton obtain their B_{12} through a symbiotic relationship with bacteria, themselves becoming food for larval fish and bivalves. Thus, animal-derived foods such as meat, milk, fish, and shellfish, are considered to be the major dietary sources of B_{12} for humans [10,11]. In contrast, higher plants do not contain biosynthetic pathways for B_{12} and any B_{12}-dependent enzymes. Thus, plant-derived food contains no traces of B_{12}, indicating that vegans (strict vegetarians) are susceptible to B_{12} deficiency. The recommended dietary allowance (RDA) of B_{12} for adults is 2.4 μg/day in the United States and Japan [5].

The intestinal absorption of dietary B_{12} is a complex, multistep process (**Fig. I-2)**. After food is ingested, B_{12} is released from the proteins by the action of HCl present in the stomach and then bound to a salivary B_{12} binding protein, haptocorrin (HC), which is produced by the salivary glands [12]. Subsequently, the HC-B_{12} complex is proteolyzed by pancreatic proteases in the duodenum. Intrinsic factor (IF, gastric B_{12}-binding protein) binds the free-formed B_{12}. The IF-B_{12} complex is subsequently absorbed by enterocytes of the ileum. The IF-B_{12} complex is specifically bound by the receptor involved in gastrointestinal absorption of B_{12} and then internalized into the enterocytes by receptor-mediated endocytosis [13,14]. The IF-B_{12} complex liberated from the receptor is transferred to lysosomes in the enterocytes and then proteases digest the protein component of the complex. Free B_{12} is normally bound

to transcobalamin II (TC II, a B_{12}-binding protein) in blood circulation.

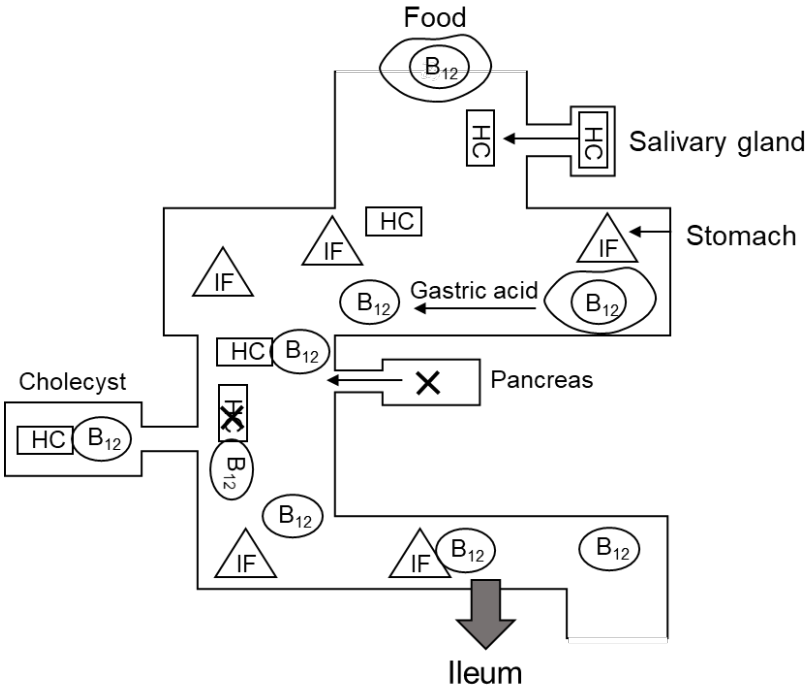

Fig. I-2. Mechanism of gastrointestinal absorption of vitamin B_{12}. HC, haptocorrin; IF, intrinsic factor; X, pancreas.

A common cause of low serum B_{12} levels in a large number of people is malabsorption of protein-bound B_{12} (food-bound B_{12} malabsorption) rather than pernicious anemia [15]. Food-bound B_{12} malabsorption is found in people with certain gastric dysfunctions, particularly atrophic gastritis with low stomach acid secretion, which is prevalent in elderly people [16,17]. Consequently, vegans and elderly people are at a greater risk of developing B_{12} deficiency [7].

Various bacteria also produce adenyl cobamide, which is known as pseudoB_{12} (**Fig. I-3A**). Although B_{12} contains a corrin ring with a cobalt-coordinated nucleotide containing 5,6-dimethylbenzimidazole as its base (**Fig. I-1**), pseudoB_{12} contains adenine in 5,6-dimethylbenzimidazole [18]. Human feces [19] contain considerable amounts of pseudoB_{12},

and this compound is formed by cyanobacteria [18], *Clostridium cochlearium* [20], *Lactobacillus reuteri* [21], and *Propionibacterium acidipropionici* [22]. PseudoB_{12} is inactive in humans because the affinity for its binding to the IF involved in gastrointestinal absorption of B_{12} is very low [23]. Intestinal bacteria can also synthesize various B_{12} analogs such as 2-methylmercaptoadenyl cobamide (factor S; **Fig. I-3B**) and 5-methoxybenzimidazolyl cobamide (factor IIIm; **Fig. I-3C**), which are reportedly found in foods such as canned escargots [24]. However, it is unclear whether these cobamides inhibit human gastrointestinal absorption of B_{12}.

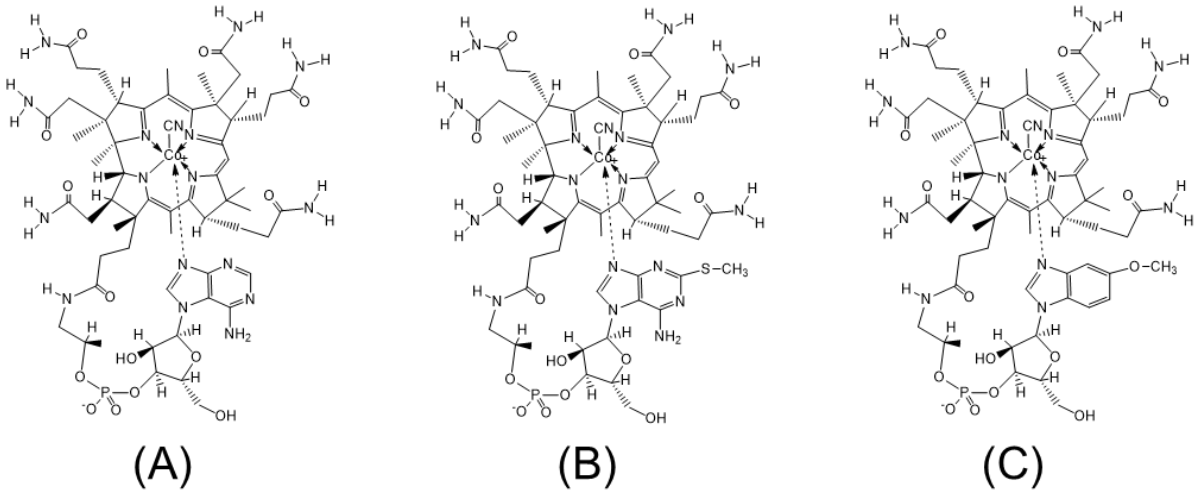

Fig. I-3. The structural formula of vitamin B_{12} analogs. (A) Adenyl cobamide or pseudovitamin B_{12}, (B) 2-methylmercaptoadenyl cobamide or factor S, and (C) 5-methoxybenzimidazolyl cobamide or factor IIIm.

Shellfish are considered to be one of the major B_{12} sources for humans [25]; however, some species of edible snails apparently contain these inactive cobamides [26,27]. Shrimp are popular foods worldwide, but there is no information available on whether they contain these inactive cobamides. In the present study, to determine the nutritional values of B_{12} compounds present in shrimp, the B_{12} compounds of their edible portions (the muscles and innards) were analyzed (**Chapter II**).

Insects have a high nutritive value because they are rich in macronutrients, thus they can contribute to world food security and may replace animal-derived protein (e.g., meat and fish) [28]. Nevertheless, there is a paucity of information on vitamin content in edible insects. To determine the nutritional values of B_{12} found in various edible insects, the B_{12} compounds contained in various, widely distributed edible cricket products were characterized (**Chapter III**).

An unnatural and inactive B_{12} compound, B_{12} [*c*-lactone] formed by treatment with an organochlorine antibacterial agent, was found in some edible mushrooms [29,30]. Moreover, preliminary experiments indicated that B_{12} is completely inactivated by treatment with hypochlorous acid water used to sanitize food products. In **Chapter IV,** the effects of some food additives on the chemical and biological properties of B_{12} under aqueous conditions were evaluated. Furthermore, the likelihood of significant losses of B_{12} in foods treated with these food additives was also assessed.

In this thesis, I described the occurrence and characterized of naturally occurring and unnatural inactive corrinoid compounds in food.

Chapter II

Characterization of vitamin B_{12} compounds in muscles and head innards of edible shrimp

Introduction

Shrimp is one of the most consumed seafood worldwide, and both aquaculture-produced and wild-caught shrimp are used to supply seafood products for human consumption [31]. Shrimp is a good source of several nutrients, such as amino acids, polyunsaturated fats, and minerals [32]; however, the detailed characteristics of B_{12} have not been elucidated in this edible seafood. Although microbiological assays have determined that there is approximately 2 μg of B_{12} per 100 g wet weight in the raw muscle of edible shrimp [33], information on the B_{12} content in the other edible portions the head innards are lacking. Shrimp head innards are used for making shrimp sauces and other products in Asian countries. Moreover, it is unclear whether both shrimp muscles and head innards contain pseudoB_{12}.

In this chapter, I determined the B_{12} contents of the shrimp head innards and muscles in four species of shrimp and characterized B_{12} compounds from both edible portions using liquid chromatography–electrospray ionization/tandem mass spectrometry (LC/ESI−MS/MS).

Materials and Methods

Materials

Authentic B_{12} was obtained from Sigma-Aldrich (St. Louis, MO, USA). B_{12}-*b*-, -*d*-, -*e*-monocarboxylic acids were prepared and their concentrations were determined on the basis of $\varepsilon_{361} = 28.06 \times 10^3\ M^{-1}\ cm^{-1}$ as described previously [34]. The revised designation of these B_{12} acid compounds were used according to Anton et al [35]. *Lactobacillus delbrueckii* subsp.

lactis ATCC 7830 was purchased from American Type Culture Collection (Manassas, VA, USA). The B_{12} assay medium for *L. delbrueckii* subsp. *lactis* ATCC 7830 was obtained from Nissui (Tokyo, Japan). Four types of fresh shrimp [Alaskan pink shrimp *Pandalus eous* (Makarov, 1935), Morotoge shrimp *Pandalopsis japonica* (Balss, 1914), Kuro shrimp *Argis lar* (Owen, 1839), and Togezako shrimp *Argis toyamaensis* (Yokoya, 1933)] were purchased from local markets in Tottori, Japan. Products made from shrimp head innards were purchased from various seafoods markets in Japan.

Extraction and assay of B_{12} in shrimp muscles and head innards

B_{12} was assayed using a microbiological method with *L. delbrueckii* subsp. *lactis* ATCC 7830, which has been adopted in the *Standard Tables of Food Composition in Japan* [10,36]. After the shells were removed from the fresh shrimp, their edible portions (muscles and innards) were separated. Each muscle (2 g) and innard (1 g) was homogenized using a mortar and pestle. Total B_{12} compounds were extracted from each homogenate by boiling with 40 mL of distilled water, 10 mL of 0.57 mol/L acetic acid buffer (pH 4.5), and 0.4 mL of 0.05% (w/v) KCN for 30 min. The extraction procedures were performed in a draft chamber (Dalton Co., Tokyo, Japan). After cooling, 0.6 mL of 10% (w/v) metaphosphoric acid was added to the B_{12} extract and the vessel was filled to 100 mL with distilled water. The prepared extract was filtrated through a Whatman filter paper circle 150 mm in diameter (GE Healthcare UK, Ltd., Bucks, England). The filtrate was then divided into two portions of 25 mL each, the pH of one portion adjusted to 6.0, and the vessel filled to 50 mL with distilled water to use as extract A for the total B_{12} assays. The pH of the remaining portion was adjusted to 11 and autoclaved (MC-23, ALP Co., Ltd., Tokyo, Japan) at 121˚C for 30 min. After cooling, the pH of the solution was readjusted to 6.0, and the vessel was filled to 50 mL with distilled water to use as extract B for determine the alkali-resistant factor.

A 2.5 mL of assay mixture was created containing 0.01 mL extract A, B, or standard B_{12} solution (0, 0.5, 1.0, 2.0, 3.0, 4.0, or 5.0 μg/L B_{12}) and 1.25 mL of B_{12} basal medium for the

assay (Nissui) prepared according to the manufacturer's protocol, and 1.24 mL of distilled water. This mixture was placed in polypropylene tubes (13 × 100 mm, Bio-Rad Laboratories Inc., Hercules, CA, USA), vigorously shaken to mix, and autoclaved at 121˚C for 5 min. After *L. delbrueckii* subsp. *lactis* ATCC 7830 were precultured in a B_{12} inoculum broth (Nissui) for 18 h, the bacterial cells were washed several times and diluted with sterile saline. The diluted bacteria solution was aseptically added to each assay mixture and let stand for 16−18 h at 37˚C. To estimate bacterial growth, the turbidity of each assay mixture was measured at 600 nm using the UV-2550 UV–visible spectrophotometer (Shimadzu Corp., Kyoto, Japan).

L. delbrueckii ATCC 7830 can utilize B_{12} as an essential nutrient, but exhibits nucleotides and deoxyribonucleotides (known as alkali resistant factor), as well as B_{12}. Thus, the correct B_{12} contents were calculated by subtracting the values for the alkali-resistant factor from the total B_{12}. The recovery rate of B_{12} from the extraction was calculated to be 105% after adding a known amount of authentic B_{12} in a sample.

Analysis of B_{12} compounds purified from the muscles or head innards of shrimp using LC/ESI–MS/MS

After the muscles (approximately 30 g) or head innards (approximately 2 g) from Alaskan pink shrimp and Kuro shrimp, respectively, were homogenized using a mortar and pestle, the B_{12} compounds were extracted as described above. The B_{12} compounds were partially purified from the extract using Sep-Pak® Vac 20 cc (5 g) C18 cartridges (Waters Corp., Milford, MA, USA) that was washed with 20 mL of 75% (v/v) ethanol and then equilibrated with 20 mL of Milli-Q water (Merck Millipore Corp., Burlington, MA, USA). The extract was filtered through a Whatman filter paper circle 150 mm in a diameter (GE Healthcare UK, Ltd.) and loaded onto the C18 cartridge, which was washed with 25 mL of Milli-Q water (Merck Millipore Corp.). The B_{12} compounds were eluted with 10 mL of 75% (v/v) ethanol. The eluate was evaporated to dryness under reduced pressure using the Integrated

SpeedVac® System ISS110 centrifugal concentrator (Savant Instruments Inc., Holbrook, NY, USA). The residual fraction was dissolved in 1 mL of Milli-Q water and loaded into an EASI-EXTRACT® B_{12} immunoaffinity column (P80; R-Biopharm AG, Darmstadt, Germany), and the B_{12} compounds were purified according to the manufacture's protocol. The purified B_{12} compounds were dissolved in 0.1 mL of Milli-Q water and filtered through a Millex®–LH membrane filter (Merck Millipore Corp.). Aliquots (5 µL) of the filtrate were analyzed using an ACQUITY UPLC H-Class Xevo® G2-S QTof (Waters Corp.). Each purified sample was injected into an InertSustain C18 column (3 µm, 2.1 × 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. The 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, 90–15% solvent B for 11–15 min) at a flow rate of 0.2 mL/min. ESI–MS was operated in the positive ion mode. The identification of authentic B_{12} (m/z 678.2914 representing $[M + 2H]^{2+}$; retention time 9.55 min) was confirmed by comparing the observed molecular ions and their retention times.

To determine the relative content (%) of B_{12}, B_{12}-*d*-monocarboxylic acid, and tentatively identified B_{12}-dicarboxylic acid in the head innards of both shrimp, the absorbance was measured at 361 nm. The relative content (%) of each peak area against total peak area (i.e., the sum of the peak area of B_{12} and B_{12} acid compounds) was calculated. The recovery rate of B_{12} from the extraction and purification was calculated to be 102% after adding a known amount of authentic B_{12} to a sample.

HPLC analysis of B_{12}-b-, -d-, and -e-monocarboxylic acids and unidentified B_{12} compounds found in the head innards of Alaskan pink shrimp

B_{12} compounds were purified from the head innards extract from the Alaskan pink shrimp using the B_{12} immunoaffinity column as described above. The purified compounds were dissolved in 80 µL Milli-Q water (Merck Millipore Corp.), filtered through a Millex®–LH

membrane (Merck Millipore Corp.), and loaded into the Shimadzu HPLC system (SPD-10AV UV-Vis detector, SCL-10A VP system controller, DGU-20A_3 degasser, LC-10Ai liquid chromatograph, and CTO-20A column oven). An aliquot (35 μL) of the purified compounds was loaded into a reversed-phase HPLC column (φ 4.6 × 150 mm; Wakosil®-II 5C18RS; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 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. Authentic B_{12} and B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids (each 1 μg) were analyzed under the same conditions.

Results

B_{12} contents in the muscles and head innards of raw shrimp and commercially available shrimp head innards products

The B_{12} contents of the muscles and head innards of four species of shrimp were determined using a microbiological assay based on *L. delbrueckii* subsp. *lactis* ATCC 7830 (**Table II-1**). The shrimp muscles contained approximately 2.4−4.3 μg B_{12}/100 g wet weight, the values of which were identical to those described in the *Standard Tables of Food Composition in Japan* [33] but approximately eight times greater in the head innards than in the muscles. Shrimp innards products A and B also contained approximately 28.5−30.1 μg B_{12}/100 g wet weight. The muscles, head innards, and whole bodies (except the shells) of the shrimp tested contained approximately 0.2, 0.3, and 0.5 μg B_{12}/ fresh total body weight (g), respectively. These results suggest that shrimp is a source of B_{12} for humans.

Table II-1. B_{12} content in the muscles and innards of raw shrimp and commercially available shrimp head innards products

	B_{12} content				
	Muscles	Innards	Muscles	Innards	Whole body
	(μg/100 g wet weight)		(μg/fresh body)		
Alaskan pink shrimp (*Pandalus eous*)	2.49 ± 0.24	28.24 ± 5.74	0.21 ± 0.04	0.36 ± 0.02	0.57 ± 0.06
Morotoge shrimp (*Pandalopsis japonica*)	2.61 ± 0.29	21.44 ± 4.52	0.18 ± 0.05	0.22 ± 0.06	0.40 ± 0.11
Kuro shrimp (*Argis lar*)	4.12 ± 0.53	33.21 ± 5.70	0.29 ± 0.07	0.41 ± 0.09	0.69 ± 0.16
Togezako shrimp (*Argis toyamaensis*)	4.33 ± 0.72	12.48 ± 4.75	0.30 ± 0.03	0.14 ± 0.07	0.44 ± 0.10
Shrimp head innards product A	-	30.06 ± 4.34	-	-	-
Shrimp head innards product B	-	28.48 ± 6.66	-	-	-

Data are presented as mean ± SD of three independent experiments (n = 3).

LC/ESI–MS/MS analysis in B_{12} compounds of muscles and head innards from Alaskan pink shrimp

To evaluate whether the muscles and head innards of Alaskan pink shrimp contain B_{12} or pseudoB_{12}, corrinoids were purified using an immunoaffinity column and analyzed using LC/ESI−MS/MS. Authentic B_{12} eluted as one ion peak with a retention time of 9.6 min (**Fig. II-1A**). The mass spectrum of authentic B_{12} indicated that a doubly charged ion with an *m/z* of 678.2894 $[M + 2H]^{2+}$ was prominent (**Fig. II-1B**). The exact mass calculated from its formula of $C_{63}H_{88}CoN_{14}O_{14}P$ was 1354.5674 g/mol, and the isotope-distribution data showed that B_{12} was the major doubly charged ion under LC/ESI−MS/MS conditions. The MS/MS spectrum of authentic B_{12} indicated that its singly charged ions at *m/z* 359.1007 and

m/z 997.4782 were attributable to the nucleotide and corrin ring moieties, respectively (**Fig. II-1C**).

The B_{12} compounds purified from the muscles of Alaskan pink shrimp eluted as one ion peak with a retention time of 9.5 min (**Fig. II-2A**), and its mass spectrum primarily comprised a doubly charged ion with *m/z* 678.2894 $[M + 2H]^{2+}$ (**Fig. II-2B**). The MS/MS spectrum of the purified compounds with singly charged ions at *m/z* 359.1007 and *m/z* 997.4782 were identical to those of authentic B_{12} (**Fig. II-2C**). The corrinoid compounds purified from the head innards of Alaskan pink shrimp were eluted as three ion peaks with retention times of 9.4, 9.8, and 10.0 min (**Fig. II-3A**). The MS spectrum of the ion peak with a retention time of 9.4 min showed that the doubly charged ion was formed at *m/z* 678.2894 $[M + 2H]^{2+}$ (**Fig. II-3B**). The MS/MS spectrum of the latter peak was identical to that of authentic B_{12} (**Fig. II-3E**). The MS spectra of the ion peaks with a retention time of 9.8 min (compound A) and 10.0 min (compound B) showed that a major doubly charged ion was formed at *m/z* 678.7849 $[M + 2H]^{2+}$ and 679.2751 $[M + 2H]^{2+}$, respectively (**Fig. II-3C and D**). The MS/MS spectra of compounds A and B with a singly charged ion at *m/z* 359.1007 were identical to that of authentic B_{12}. However, the MS/MS spectra of compounds A and B with singly charged ions at *m/z* 998.4603 and *m/z* 999.4493, respectively, were not identical to that of authentic B_{12} at *m/z* 997.4782 (**Fig. II-3F and G**). These results suggest that the head innards of Alaskan pink shrimp contained B_{12} and two B_{12} compounds A and B, which were B_{12} compounds with different corrin ring moieties that have one and two additional hydrogen atoms, respectively (**Fig. II-2 and -3**). Similar results were obtained for the other shrimp tested.

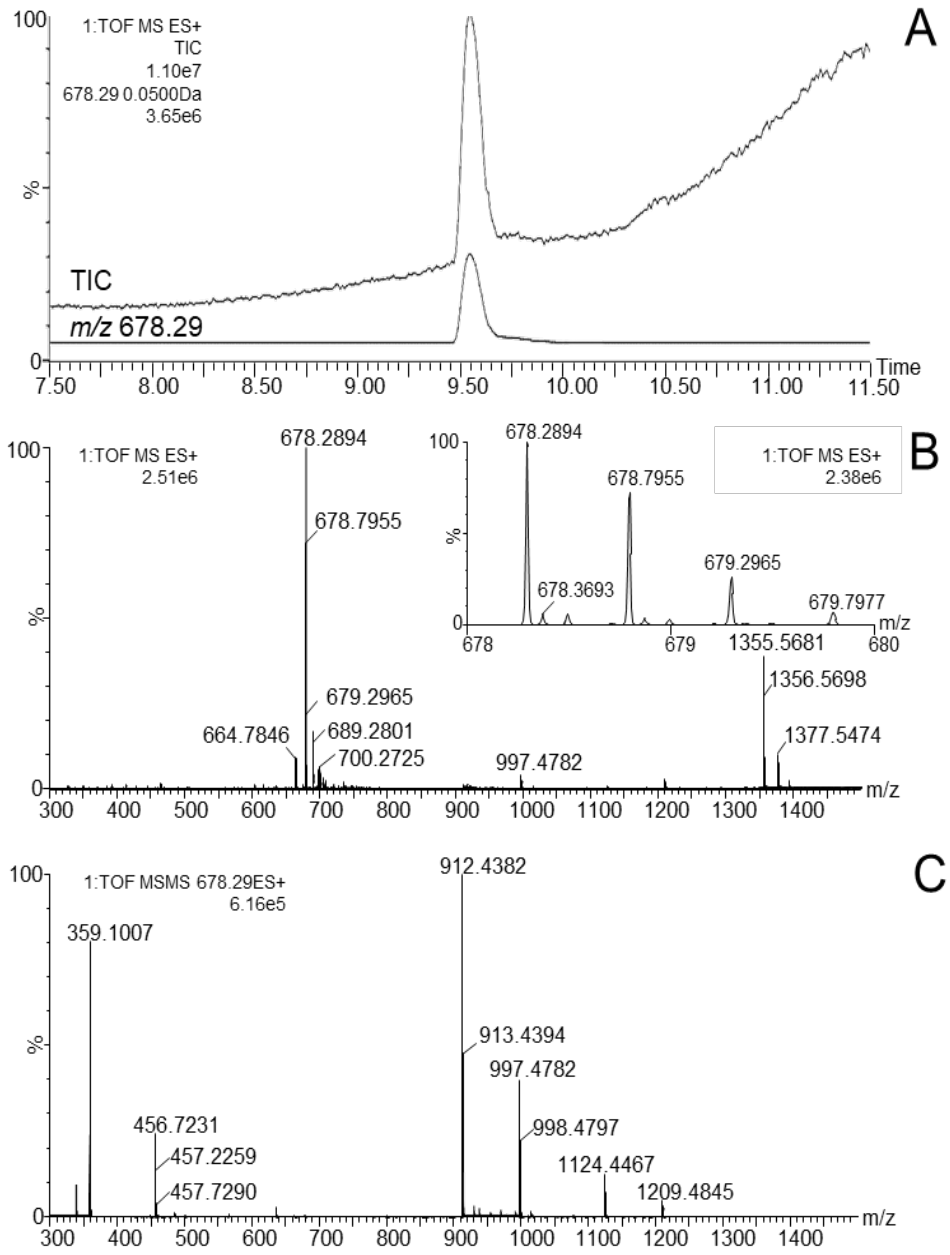

Fig. II-1. LC/ESI−MS/MS chromatograms of authentic B_{12}. (A) Total ion chromatogram (TIC) and mass chromatogram of authentic B_{12} (*m/z* 678.29), (B) MS spectrum of authentic B_{12} (insert magnified spectrum from *m/z* 678 to *m/z* 680), and (C) MS/MS spectrum of the peak of authentic B_{12} at *m/z* 678.2894.

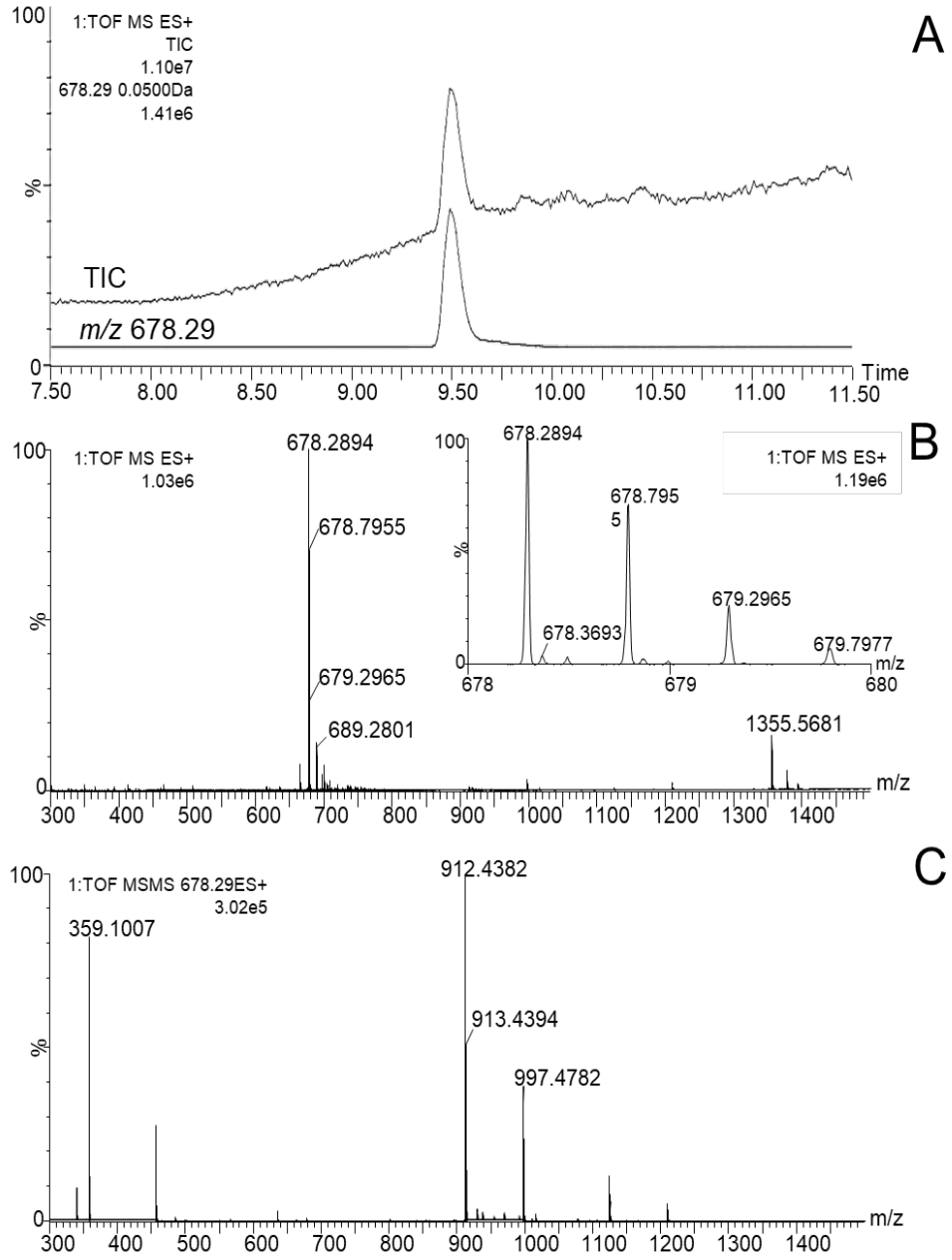

Fig. II-2. LC/ESI−MS/MS chromatograms of B_{12} compounds purified from Alaskan pink shrimp muscles. (A) Total ion chromatogram (TIC) and mass chromatogram of the B_{12} compounds (*m/z* 678.29), (B) MS spectrum of the B_{12} compounds at 9.49 min (insert magnified spectrum from *m/z* 678 to *m/z* 680), and (C) MS/MS spectrum of the peak of the B_{12} compounds at *m/z* 678.2894.

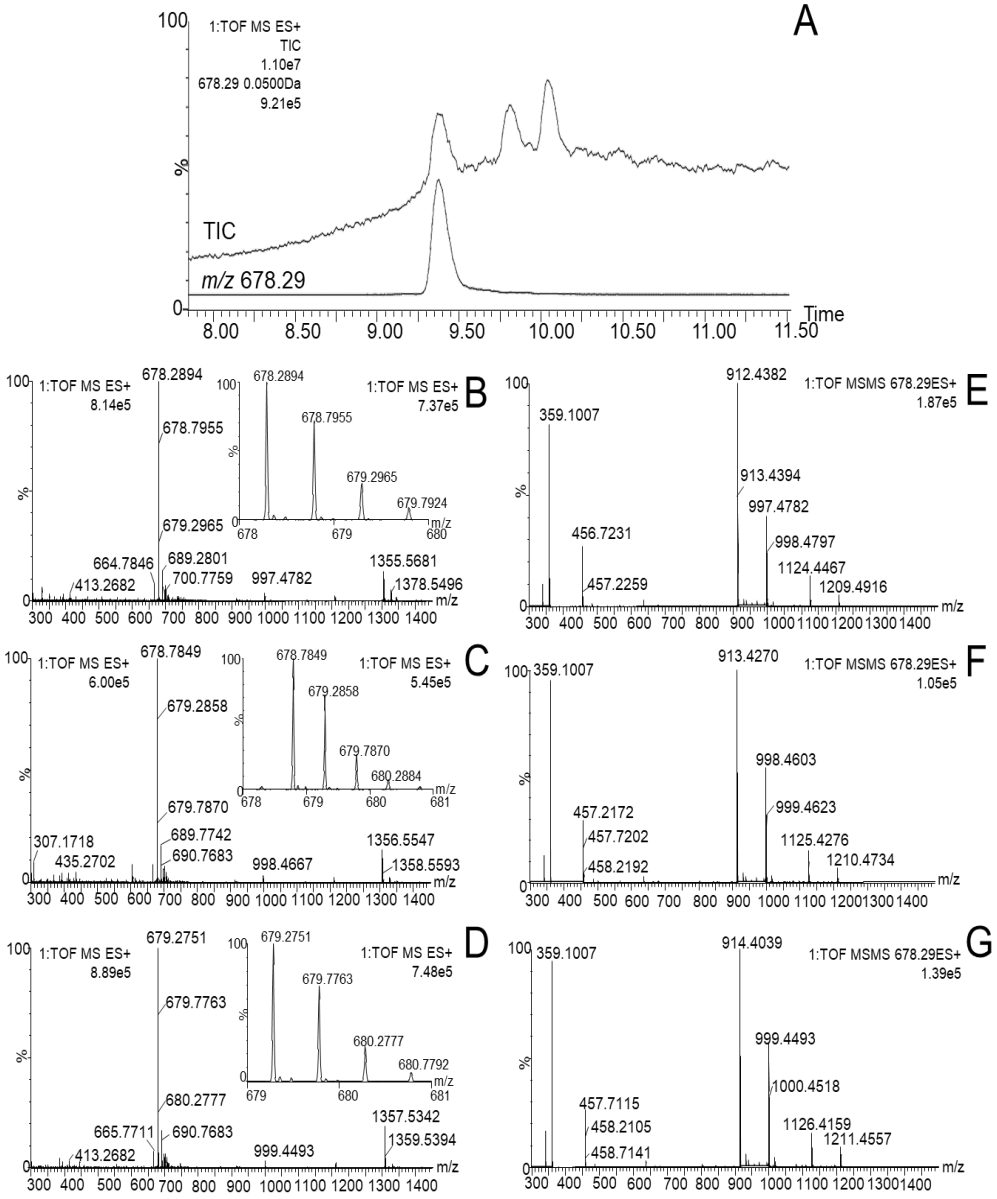

Fig. II-3. LC/ESI−MS/MS chromatograms of B_{12} compounds purified from Alaskan pink shrimp head innards. (A) Total ion chromatogram (TIC) and mass chromatogram of the B_{12} compounds (*m/z* 678.29). (B)–(D) MS spectra of the B_{12} compounds with retention times of 9.49 min (the magnified spectrum from *m/z* 678 to *m/z* 680 is shown as an insert in B), 9.81 min (the magnified spectrum from *m/z* 678 to *m/z* 681 is shown as an insert in C), and 10.04 min (the magnified spectrum from *m/z* 679 to *m/z* 681 is shown as an insert in D), respectively. (E)–(G) MS/MS spectra of the peaks of the c B_{12} compounds with retention times of 9.49 min (*m/z* 678.2894), 9.81 min (*m/z* 678.7849), and 10.04 min (*m/z* 679.2751), respectively.

HPLC analysis of B_{12}-b-, -d-, and -e-monocarboxylic acids and unidentified B_{12} compounds from in the head innards of Alaskan pink shrimp

The chemical structure of B_{12} and the molecular masses of the unidentified B_{12} compounds were used to predict their identity. Compound A was hypothesized to be B_{12}-monocarboxylic acid while compound B was predicted to be B_{12}-dicarboxylic acid (**Fig. II-4**). Immunoaffinity chromatography was used to purify corrinoids from head innards extract obtained from Alaskan pink shrimp. This was followed by analysis using a C18 reversed-phase HPLC column. HPLC studies showed that B_{12}, unidentified compounds A and B eluted as three separate peaks with retention times of 9.8, 16.2, and 18.3 min, respectively (**Fig. II-5A**). HPLC analysis of known forms of B_{12} under the same conditions showed that authentic B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids eluted as single peaks with retention times of 9.7, 13.5, 16.2, and 20.0 min, respectively (**Fig. II-5B, C, D, and E**). The retention time of B_{12}-*d*-monocarboxylic acid was identical to that of compound A (retention time of 16.2 min). When B_{12}-*d*-monocarboxylic acid was analyzed using LC/ESI−MS/MS, it eluted as an ion peak with a retention time of 9.6 min (**Fig. II-6A**). This was similar to be the retention time observed for compound A (retention time of 9.8 min) (**Fig. II-3A**). Thus, MS and MS/MS spectra of B_{12}-*d*-monocarboxylic acid (**Fig. II-6B and C**) were identical to that of compound A (**Fig. II-3C and F**).

However, the identity of compound B could not be established as the preparation methods for B_{12}-*bd*-, -*be*-, and -*de*-dicarboxylic acids have not been reported. At present, compound B can be hypothetically identified as one of these B_{12}-dicarboxylic acids.

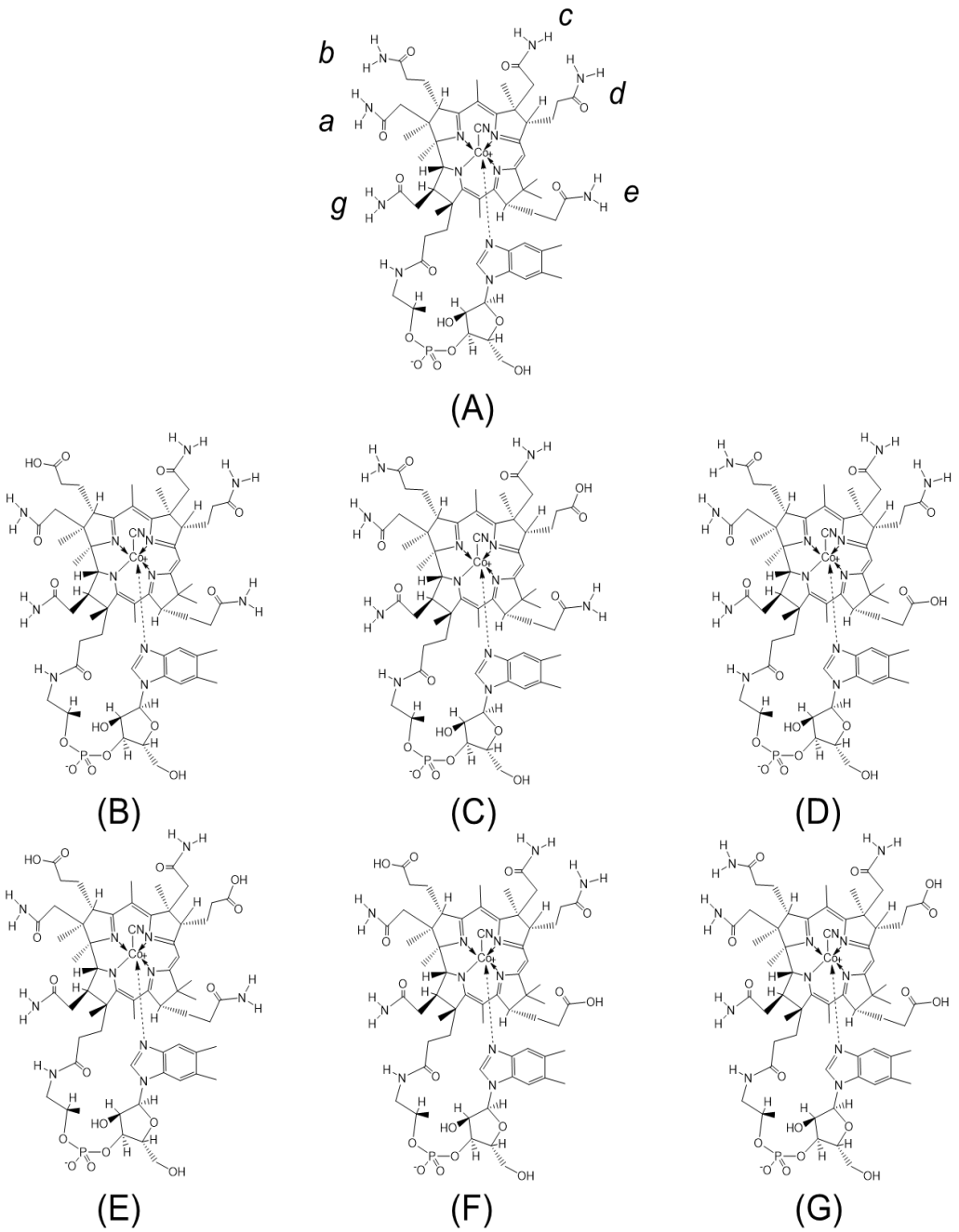

Fig. II-4. Chemical structures of B_{12}-mono and -dicarboxylic acids. (A) CN-B_{12}, (B) B_{12}-*b*-monocarboxylic acid, (C) B_{12}-*d*-monocarboxylic acid, (D) B_{12}-*e*-monocarboxylic acid, (E) B_{12}-*bd*-dicarboxylic acid, (F) B_{12}-*be*-dicarboxylic acid, and (G) B_{12}-*de*-dicarboxylic acid.

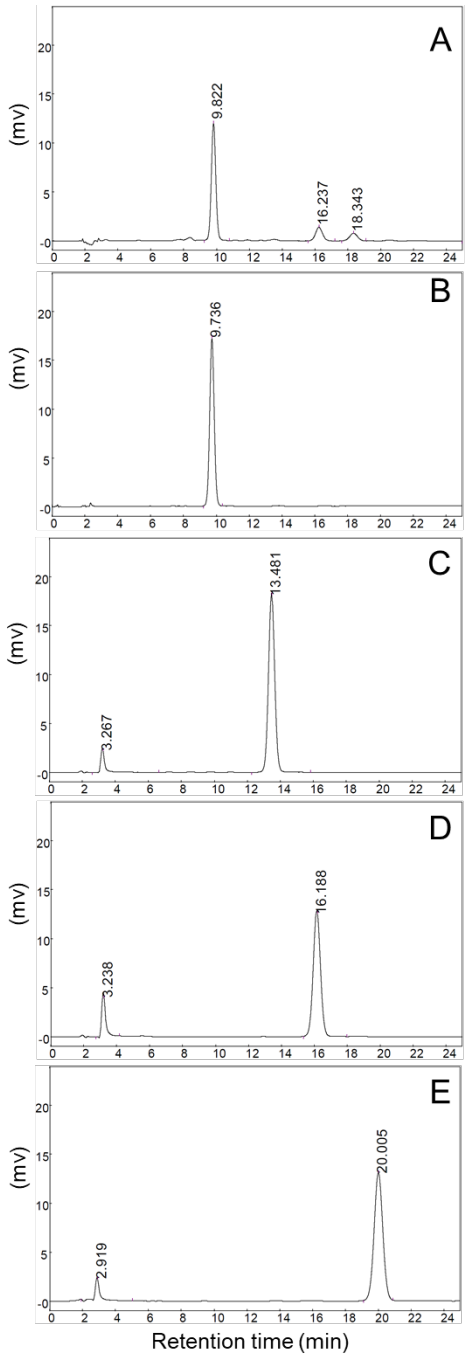

Fig. II-5. HPLC chromatograms of B_{12} compounds present in in head innards extract of Alaskan pink shrimp and authentic B_{12}-monocarboxylic acids. (A) Head innards extract, (B) CN-B_{12}, (C) B_{12}-*b*-monocarboxylic acid, (D) B_{12}-*d*-monocarboxylic acid, and (E) B_{12}-*e*-monocarboxylic acid.

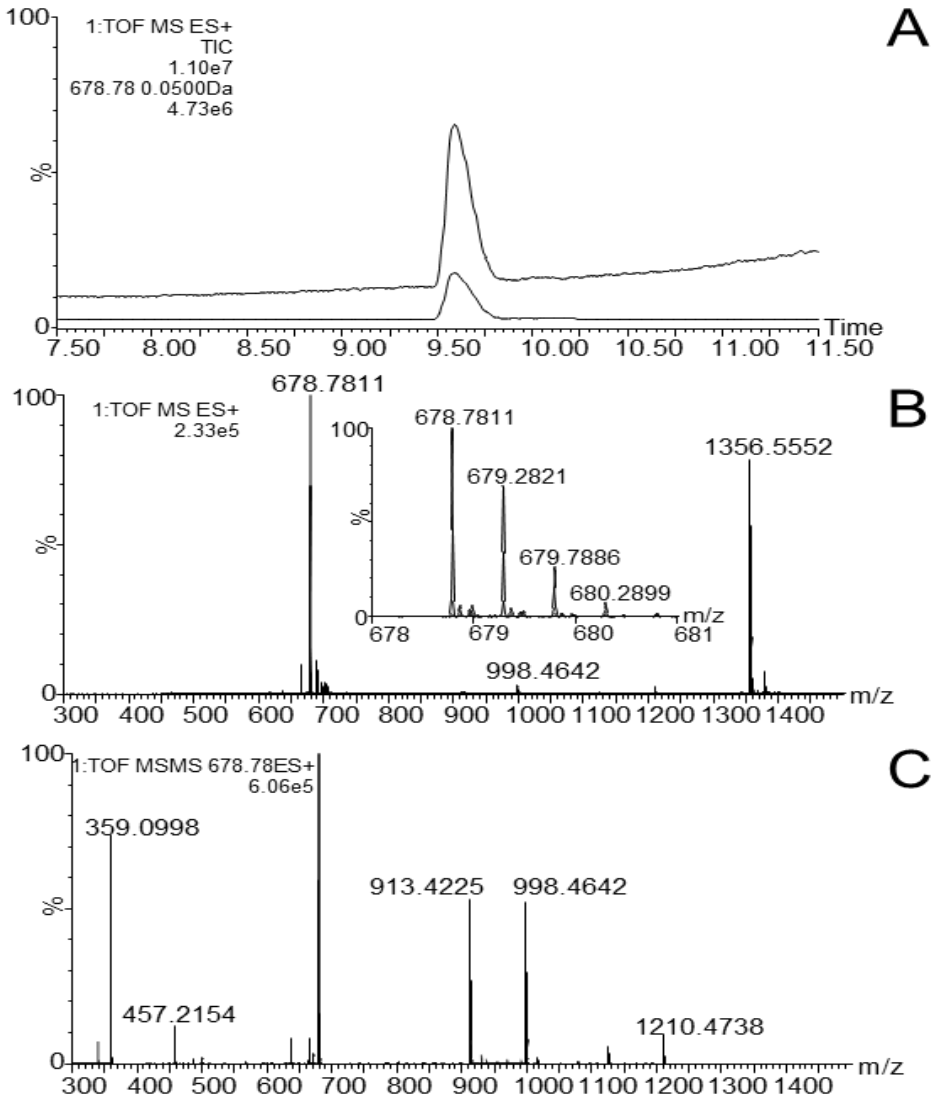

Fig. II-6. LC/ESI−MS/MS chromatograms of B_{12}-*d*-monocarboxylic acid. (A) Total ion chromatogram (TIC) and mass chromatogram of B_{12}-*d*-monocarboxylic acid (*m/z* 678.78), (B) MS spectrum of B_{12}-*d*-monocarboxylic acid (insert magnified spectrum from *m/z* 678 to *m/z* 681), and (C) MS/MS spectrum of the peak of B_{12}-*d*-monocarboxylic acid at *m/z* 678.7811.

Relative content (%) of B_{12} compounds found in the head innards of shrimp

To determine the relative B_{12} content (%), B_{12}-*d*-monocarboxylic acid and B_{12}-dicarboxylic acid in the shrimp head innards of Alaskan pink shrimp and Kuro shrimp were selected because of their high B_{12} contents compared with those of the other shrimp tested.

The relative B_{12} content (%) of each peak area against the total peak area (i.e., the sum of the peak area of B_{12} and unidentified compounds) was calculated by measuring the absorbance at 361 nm (**Table II-2**). Approximately 65% of the B_{12} compounds identified in the head innards of Alaskan pink shrimp and 26% in the case of Kuro shrimp were derived from B_{12}-monocarboxylic and -dicarboxylic acids. The total B_{12} content in shrimp head innards products A and B contained approximately 22% these B_{12} acid compounds.

Table II-2. Relative contents (%) of B_{12} compounds in selected shrimp head innards and their products

	Relative content (%)		
	B_{12}	B_{12}-*d*-monocarboxylic acid	tentative B_{12}-dicarboxylic acid
Alaskan pink shrimp (*Pandalus eous*)	34.6 ± 0.1	24.6 ± 0.2	40.8 ± 0.3
Kuro shrimp (*Argis lar*)	73.9 ± 0.3	11.0 ± 0.2	15.0 ± 0.1
Shrimp innards product A	82.3 ± 0.6	11.5 ± 0.2	6.1 ± 0.4
Shrimp innards product B	78.2 ± 1.3	13.9 ± 0.2	7.9 ± 1.1

Data are presented as mean ± SEM of triplicates.

B_{12} activity assay of B_{12}-d-monocarboxylic acid towards L. delbrueckii ATCC 7830

The ability of B_{12}-*d*-monocarboxylic acid to show B_{12} activity was studied using the standard microbiological assay with *L. delbrueckii* subsp. *lactis* ATCC 7830. In particular, the effect of various doses of B_{12} and B_{12}-*d*-monocarboxylic acid (0.01–0.05 pmol) on the growth of the lactic acid bacterium was studied (**Fig. II-7**). At a concentration of 0.05 pmol, the activity shown by B_{12}-*d*-monocarboxylic acid was only 6% of that displayed by the authentic B_{12}. These results suggest that B_{12}-*d*-monocarboxylic acid hardly functions as B_{12} in this bacterium.

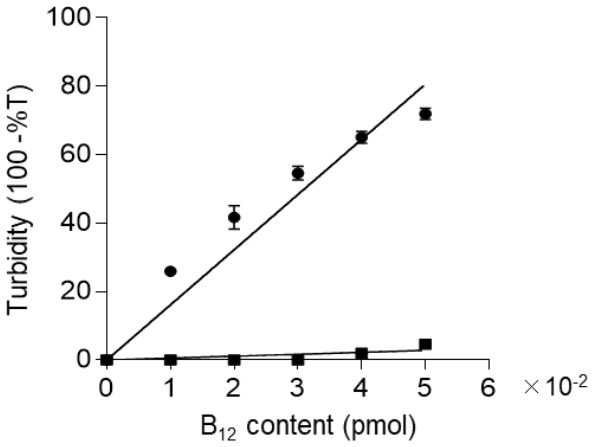

Fig. II-7. Effect of authentic B_{12} and B_{12}-*d*-monocarboxylic acid on growth of *L. delbrueckii* subsp. *lactis* ATCC 7830. The growth of the bacterium was expressed as turbidity as a function of dose. The experiment was performed in triplicate and results are given as mean ± SEM. B_{12} (●) and B_{12}-*d*-monocarboxylic acid (■).

Discussion

Although shrimp is one of the most consumed seafoods worldwide and is a good source of several nutrients [32], the detailed characteristics of B_{12} have not been elucidated in this seafood. Thus, I determined the B_{12} content in both the muscles and head innards of various shrimp species using a microbiological assay based on *L. delbrueckii* subsp. *lactis* ATCC7830. As shown in **Table II-1**, approximately 2–4 μg B_{12}/100 g wet weight was detected in shrimp muscles. The shrimp head innards contained significantly higher levels of B_{12} (approximately 12−33 μg/100 g wet weight). Consumption of approximately 8−14 shrimp muscles, 6−7 shrimp head innards, and 3−6 whole shrimp bodies could supply the RDA of B_{12} for adults (2.4 μg/day) [5], which suggests that shrimp is a suitable source of B_{12} for humans.

The purified B_{12} compounds from the extracts of shrimp muscles and head innards were identified using LC/ESI–MS/MS. The shrimp muscle extracts contained only B_{12} while the

extracts obtained from shrimp head innards contained three corrinoid compounds. These head innards extracts contained large amount of B_{12} and smaller amounts of two unidentified B_{12} compounds denoted as compound A and compound B. The HPLC and LC/ESI–MS/MS analyses identified compound A as B_{12}-*d*-monocarboxylic acid (**Fig. II-5 and -6**). The identity of compound B could not be established completely and it was hypothetically identified as one of the B_{12}-dicarboxylic acids because of the presence of additional masses of two hydrogen atoms for B_{12} compounds having different corrin ring moieties. The content of the B_{12}-mono and -dicarboxylic acids varied from 18% to 65% of the total B_{12} content in shrimp head innards and their products (**Table II-2**), which suggested that these B_{12} acid compound levels were dependent on the species samples.

IF plays an important role in the gastrointestinal absorption of B_{12} in humans. This protein is known to have high specific binding for B_{12}. However, it lacks any significant affinity towards B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids or B_{12}-*bde*-tricarboxylic acid [37]. The low affinity of this IF protein for B_{12} -mono and -tricarboxylic acids explains the poor absorption of these compounds following their oral administration in rabbits [37]. Saido et al. [38] reported that oral and intravenous administration of B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids did not show any improvement in the B_{12} levels of B_{12}-deficient rats. These studies strongly suggest that both B_{12}-*d*-monocarboxylic acid and B_{12}-dicarboxylic acids found in shrimp head innards might not be absorbed by the human intestine. However, shrimp head innards can be still considered a source of B_{12} for humans owing to their contents of B_{12}-mono and -dicarboxylic acids (approximately 18–65% of the total B_{12} content).

Anton et al. [35] reported the formation of B_{12}-mono and -dicarboxylic acids on mild acid hydrolysis of B_{12}. These B_{12} acids are derived from the propionamide side chains *b*, *d*, and *e*, which are more susceptible to hydrolysis than the acetamide side chains *a*, *c*, and *g* [39] (**Fig. II-4**). The presence of these B_{12} acid compounds in shrimp head innards is quite unusual. It is possible that trace elements are absorbed from sea water and accumulate in shrimp head innards [32], and might react with B_{12} to produce these acid compounds.

However, no detailed mechanism has been reported so far to explain this hypothesis.

Euglena gracilis Z, a B_{12}-dependent alga, has been widely utilized in microbiological assays for B_{12}. Supplementation of growth culture medium with B_{12}-*d*-monocarboxylic acid increased the growth of *Euglena* to similar levels to those observed in authentic B_{12}-supplemented culture medium [34]. *L. delbrueckii* subsp. *lactis* ATCC 7830 is a bacterium commonly used to determine the B_{12} content of food. As shown in **Fig. II-7**, B_{12}-*d*-monocarboxylic acid did not show any significant effect on the growth of this bacterium. There are no reports on the effect of B_{12}-dicarboxylic acids on the growth of *L. delbrueckii* subsp. *lactis* ATCC 7830. Since the structure of B_{12} changed more in the case of B_{12}-dicarboxylic acid compared to B_{12}-*d*-monocarboxylic acid, it is possible that B_{12}-dicarboxylic acids are inactive in this bacterium. The possible inactivity of B_{12}-*d*-monocarboxylic and B_{12}-dicarboxylic acids in *L. delbrueckii* subsp. *lactis* ATCC 7830 suggests that these compounds might not affect the B_{12} content of shrimp head innards.

Summary

I determined the B_{12} content in both the muscles and head innards of various shrimp species using a microbiological assay based on *L. delbrueckii* subsp. *lactis* ATCC7830. A considerable amount (approximately 2–4 μg B_{12}/100 g wet weight) was detected in shrimp muscles. The shrimp head innards contained significantly higher levels of B_{12} (approximately 12–33 μg/100 g wet weight). Commercially available shrimp head innards products contained approximately 30 μg B_{12}/100 g wet weight. The muscle extract contained only one corrinoid compound, which was identified as B_{12} using LC/ESI–MS/MS, whereas the shrimp head innards contained three corrinoid compounds, which included large amounts of B_{12} and two smaller amounts of B_{12}-*d*-monocarboxylic acid and tentatively identified B_{12}-dicarboxylic acids.

Chapter III
Characterization of vitamin B_{12} compounds in edible insects

Introduction

Traditionally, edible insects have only been consumed in specific parts of the world. However, nowadays, they are considered to have the potential to contribute to global food security [28]. Although around 2,000 different insects are known to be eaten by humans, only a limited number of edible insect products are commercially available [28,40]. In particular, lepidopteran (butterflies and moths), coleopteran (beetles and grubs), and orthopteran (crickets and grasshoppers) food products are widely available in certain areas [28]. Although investigations have shown edible insects to be rich in macronutrients such as protein and fats, data on their vitamin and mineral content is currently limited [40,41]. Some studies have described the B_{12} content of edible insect products [28,41–43], however, little is known about the inactive corrinoid compounds, such as pseudoB_{12}, contained in these products. Therefore, to more precisely evaluate the nutritional value of edible insects, I quantified the B_{12} content of six different insect products using a bioassay. Furthermore, in the cricket products that are most widely circulated as food, B_{12} compounds were identified using LC/ESI–MS/MS.

Materials and Methods

Materials

B_{12} was purchased from Sigma-Aldrich (St Louis, MO, USA). B_{12} assay and inoculum media for *L. delbrueckii* subspecies *lactis* ATCC 7830 were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). *L. delbrueckii* ATCC 7830 was obtained from ATCC (Manassas, VA, USA). All of the edible insects used, i.e., giant water bugs, bee larvae, grasshoppers, weaver ants, diving beetles, crickets, and cricket products were commercially

available products obtained from markets in Japan or imported from overseas. The scientific names of the six insects tested, except for the crickets (*Acheta domesticus* and *Gryllus bimaculatus*), could not be assigned because the food description was not sufficiently detailed. Raw or frozen samples were first lyophilized before being used in experiments.

Extraction and assay of B_{12} in edible insect products

The whole bodies of the edible insects (2 g) were homogenized using a mortar and pestle. B_{12} compounds were extracted from the homogenized or powdered products (2 g) as described in **Chapter II**. Recovery of B_{12} from the extract was 98% by determining a B_{12} amount from a sample added to a known amount of B_{12}.

Cricket-feeding experiments

Newly emerged house crickets (*A. domesticus*) (4–6 mm in size) were obtained from a pet food vendor in Japan. Twenty-five crickets were housed in a plastic container [27 (length) × 15 (width) × 17 (height) cm], given free access to cricket food diets A or B (described below) and tap water, and maintained at 25˚C for 23 days. Cricket food A, which was also purchased from a pet food vendor in Japan, was comprised of wheat bran (30%), soy bean (27%), fish powder (12%), corn (8%), concentrated whey (7%), rice bran (7%), desugared sugar cane (5%), garlic powder (1%), calcium carbonate (1%), glucose (1%), and bifidobacteria (1%). Cricket food B was dog food consisting of proteins (> 25%), fats (> 25%), crude fibers (< 5%), ash (8.5%), and water (< 10%). The specific ingredients of cricket food B included cereals (including corn, flour, bran, corn gluten feed, and corn gluten meal), meats (including beef meat meal, pork meat meal, chicken meal, and chicken liver powder), vegetables (including beet pulp, carrot, pumpkin, and spinach), yeast extract, roasted aroma, lactic acid bacteria, minerals, vitamins, and antioxidants (mixed tocopherol and herb extract). The B_{12} content of both cricket food A and B was determined using the bioassay described above. After the cricket feeding period, adult crickets were collected and stored at -20˚C

until further use. The B_{12} compounds from the cricket foods and crickets were extracted and determined the B_{12} content as described above.

Identification of B_{12} compounds in edible cricket products by LC/ESI–MS/MS

B_{12} extracts were prepared from five commercially available edible cricket products (each 3 g) using the extraction method described above. Extracts were filtrated with a Whatman filter paper circle 150 mm in diameter (GE Healthcare UK, Ltd.) and then partially purified using C18 cartridges (Sep-Pak® Vac 20 cc, Waters Corp.). The cartridges were treated with 75% (v/v) ethanol and Milli-Q water (each at 20 mL) before use. An aliquot (20 mL) of each filtrate was loaded onto the C18 cartridge and washed with 20 mL of Milli-Q water. Corrinoids were eluted with 10 mL of 75% (v/v) ethanol. Each elution was evaporated to dryness in the SpeedVac® centrifugal concentrator (System ISS110, Savant Instruments Inc.). The residue was dissolved in 1 mL of Milli-Q water and B_{12} compounds were purified with an immunoaffinity column (EASI-EXTRACT® B_{12}, R-Biopharm AG). The purified compound was dissolved in 0.15 mL of Milli-Q water, filtered with a membrane filter (Merck Millipore Corp.), and identified by UPLC–MS/MS (Xevo® G2-S QTof, Waters Corp.). Each filtrate (5 μL) was loaded into an InertSustain C18 column (3 μm, 2.1 × 100 mm; GL Science) and isocratically eluted with a 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40˚C. The flow rate was 0.2 mL/min. The identities of pseudoB_{12} (*m/z* 672.7769 and retention time 4.6 min), authentic B_{12} (*m/z* 678.2894 and retention time 5.4 min), and factor S (*m/z* 695.7720) were determined by comparing the retention times and observed molecular ions.

To quantify the relative content (%) of pseudoB_{12}, B_{12}, and factor S in the edible cricket products, each peak area was compared with the sum of all peak areas by determining the absorbance at 361 nm.

Identification of B_{12} compounds in cricket foods and adult crickets by HPLC

In the cricket foods and adult crickets grown in my laboratory, corrinoid compounds were analyzed using the following HPLC method. Corrinoid compounds were first purified from crickets and cricket foods using the B_{12} immunoaffinity column. The purified corrinoids were dissolved in 200 μL of Milli-Q water, treated with a membrane (Millex®–LH, Merck Millipore Corp.), and analyzed using the HPLC system (detector, SPD-10AV UV–Vis; system controller, SCL-10A VP; degasser, DGU-20A_3; liquid chromatograph pump, LC-10Ai; and column oven, CTO-20A; Shimadzu Corp.). The purified sample (50 μL) was injected into Wakosil®-II 5C18RS (4.6 × 150 mm) HPLC column (FUJIFILM Wako Pure Chemical Corp.) at 40˚C and eluted with a 20% (v/v) methanol containing 1% (v/v) acetic acid at a flow rate of 1 mL/min. Corrinoids were detected at 361 nm wavelength. Authentic B_{12} and pseudoB_{12}, and cricket and food samples, were analyzed under the same conditions.

Statistical analysis

Differences in the B_{12} content of cricket foods and adult crickets grown in the laboratory were evaluated by Student's *t*-test. Analyses were conducted in GraphPad Prism 3 for Windows version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). All data are presented as the means ± SEM. Differences were considered statistically significant when $p < 0.05$.

Results

B_{12} content of edible insect products

The B_{12} content of the six types of commercially available edible insect products was shown in **Table III-1**. Diving beetle and cricket products contained relatively high amounts of B_{12} (approximately 89.5 and 65.8 μg/100 g dry weight, respectively). In comparison, the B_{12} content of the other four edible insect products, namely giant water bug, bee larva, grasshopper, and weaver ant products, were relatively low (approximately 1.1–3.2 μg/100 g

dry weight). Using a similar B_{12} bioassay method, Finke [41] reported much lower B_{12} content in commercially available crickets (19.3 μg/100 g dry weight), as well as similarly low B_{12} levels in various larvae including mealworms (0.13 μg/100 g dry weight), superworms (0.99 μg/100 g dry weight), and waxworms (< 0.12 μg/100 g dry weight).

Table III-1. B_{12} content in commercially available edible insect products and raw insects

Products	B_{12} content (μg/100 g dry weight)
Diving beetle	89.5 ± 0.6
Cricket	65.8 ± 1.5
Giant water bug	3.2 ± 0.8
Bee larva*	3.0 ± 0.2
Grasshopper**	2.4 ± 0.6
Weaver ant	1.1 ± 0.2

*Raw and **frozen samples were lyophilized before use; all other samples were dry-roasted products. All values represent the mean ± SEM from three independent experiments (n = 3).

B_{12} content of various edible cricket products

I determined the B_{12} content of various widely circulated edible cricket products. The B_{12} content of various house cricket (*A. domesticus*) and two-spotted cricket (*G. bimaculatus*) products was similarly high (approximately 50–75 μg/100 g dry weight) (**Table III-2**). These values were much higher than those of the cricket products described by Finke [41]. They were also higher than the B_{12} content of a frozen cricket (*G. bimaculatus*), which was determined to be 2.88 μg/100 g dry weight in a previous study [43].

Table III-2. B_{12} content in commercially available edible cricket products

Products	B_{12} content (μg/100 g dry weight)	Sample numbers
Cricket	65.8 ± 1.5	Sample 1*
Cricket (organic)	71.9 ± 4.0	Sample 2
Cricket powder	55.0 ± 1.7	Sample 3
Two-spotted cricket	74.5 ± 0.8	Sample 4
Two-spotted cricket powder	50.3 ± 1.8	Sample 5
Mean	63.5 ± 4.2	

Cricket: house cricket *Acheta domesticus*; two-spotted cricket: *Gryllus bimaculatus.* *This sample is the same as listed in **Table III-1**. All values represent the mean ± SEM from three independent experiments (n = 3).

LC/ESI–MS/MS analysis of B_{12} compounds in commercially available edible cricket products

From LC/ESI–MS/MS analysis of commercially available edible cricket products, I found that pseudoB_{12} and B_{12} were eluted as peaks with retention times of 4.6 and 5.4 min, respectively (**Fig. III-1A and B**). The mass spectrum of pseudoB_{12} indicated that a doubly charged ion with an *m/z* of 672.7786 $[M + 2H]^{2+}$ was prominent (**Fig. III-1C**). From its formula ($C_{59}H_{83}CoN_{17}O_{14}P$), its exact mass was calculated as 1343.5374 g/mol; isotope distribution data indicated that pseudoB_{12} was the major doubly charged ion detected under the LC/ESI–MS/MS conditions. As B_{12} has an exact mass of 1354.5674 g/mol ($C_{63}H_{88}CoN_{14}O_{14}P$), a doubly charged ion with an *m/z* of 678.2910 $[M + 2H]^{2+}$ was prominent (**Fig. III-1D**). The MS/MS spectra of pseudoB_{12} and B_{12} indicated that their dominant ions at *m/z* 348.0704 and *m/z* 359.0998, respectively, were ascribed to the nucleotide moieties, and their dominant ions at *m/z* 997.4822 were ascribed to the corrin ring (**Fig. III-1E and F**).

The corrinoids isolated from cricket product sample 1 were eluted as three total ion peaks with retention times of 4.6, 5.5, and 6.2 min (**Fig. III-2A and B**), respectively. The MS

spectrum of the total ion peak with a 4.6 min retention time showed the doubly charged ion given at *m/z* 672.7786, and the MS/MS spectrum of the compound was identical to that of pseudoB_{12} (**Fig. III-2C and F**). The MS spectrum of the total ion peak with a 5.5 min retention time indicated that a major doubly charged ion was given at *m/z* 678.2910, and the MS/MS spectrum of the compound with a singly charged ion at *m/z* 359.0998 was identical to that of B_{12} (**Fig. III-2D and G**). Finally, the MS spectrum of the ion peak with a 6.21 min retention time (**Fig. III-2B**) showed that a major doubly charged ion was formed at *m/z* 695.7720 (**Fig. III-2E**). The MS/MS spectrum of this ion peak showed that the dominant ion at *m/z* 394.0599 was ascribed to the 2-methylmercaptoadenyl nucleotide moiety (**Fig. III-2H**). These spectral data coincide with the calculated mass of factor S (2-methylmercaptoadenyl cobamide: $C_{60}H_{85}CoN_{17}O_{14}PS$, 1389.5252 g/mol). Thus, these results indicate that cricket product sample 1 contained B_{12} and two inactive B_{12} compounds, namely pseudoB_{12} and factor S (**Fig. III-3**).

Relative content of pseudoB_{12}, factor S, and B_{12} in edible cricket products

The relative content (%) of the corrinoid compounds in the edible cricket products is shown in **Table III-3**. According to LC/ESI–MS/MS analysis, the tested edible cricket products contained approximately 74% pseudoB_{12}, 21% factor S, and 5% B_{12}. From this analysis, the B_{12} content of these edible cricket products was calculated as approximately 3.2 μg/100 g dry weight, which is a similar value to that determined in frozen crickets (*G. bimaculatus*) using UPLC [43].

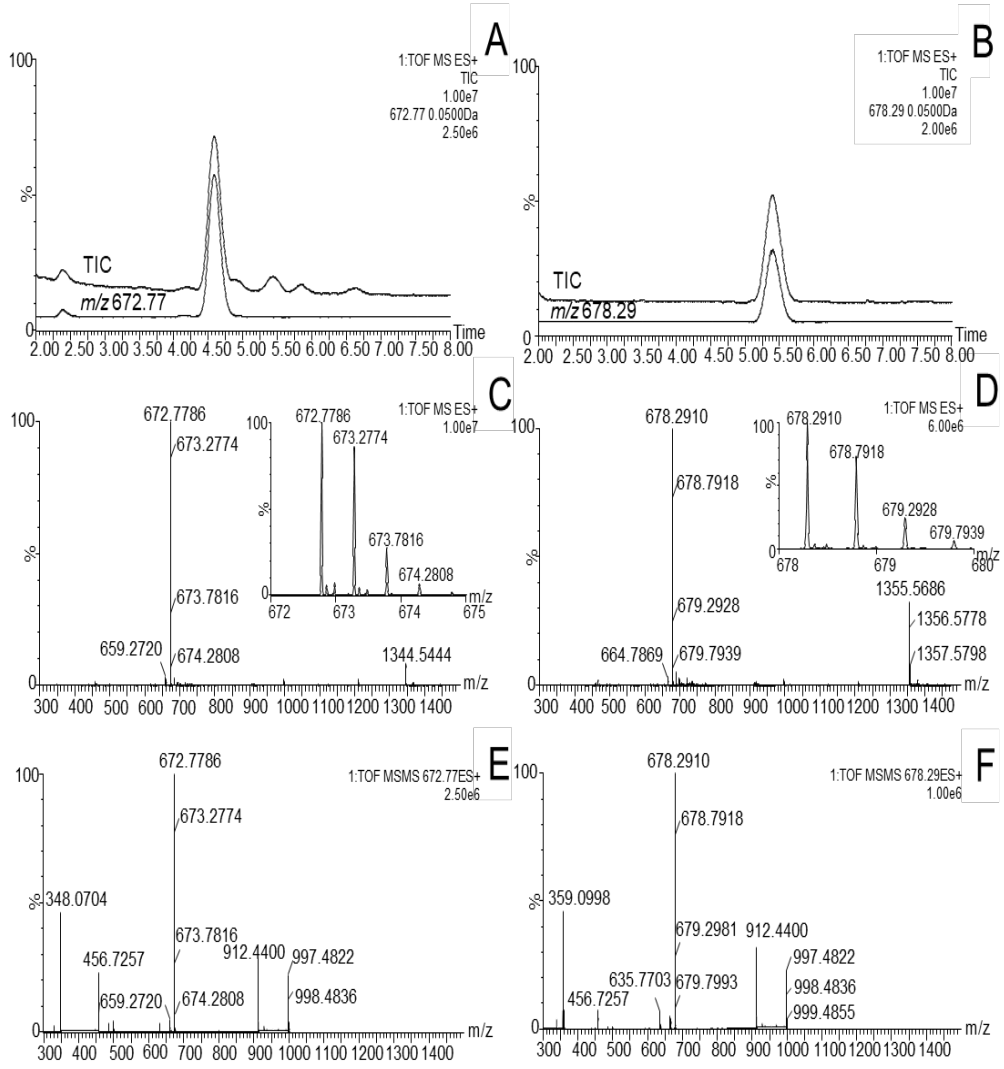

Fig. III-1. LC/ESI–MS/MS chromatograms of pseudoB_{12} and B_{12}. (A) and (B) Total ion chromatograms (TICs) and mass chromatograms of pseudoB_{12} (*m/z* 672.77) and B_{12} (*m/z* 678.29), respectively. **(**C) and (D) MS spectra of pseudoB_{12} (the magnified spectrum from *m/z* 672 to *m/z* 675 is shown as an insert in C) and B_{12} (the magnified spectrum from *m/z* 678 to *m/z* 680 is shown as an insert in D), respectively. (E) and (F) MS/MS spectra of the peak of pseudoB_{12} at *m/z* 348.0704 and B_{12} at *m/z* 359.0998, respectively.

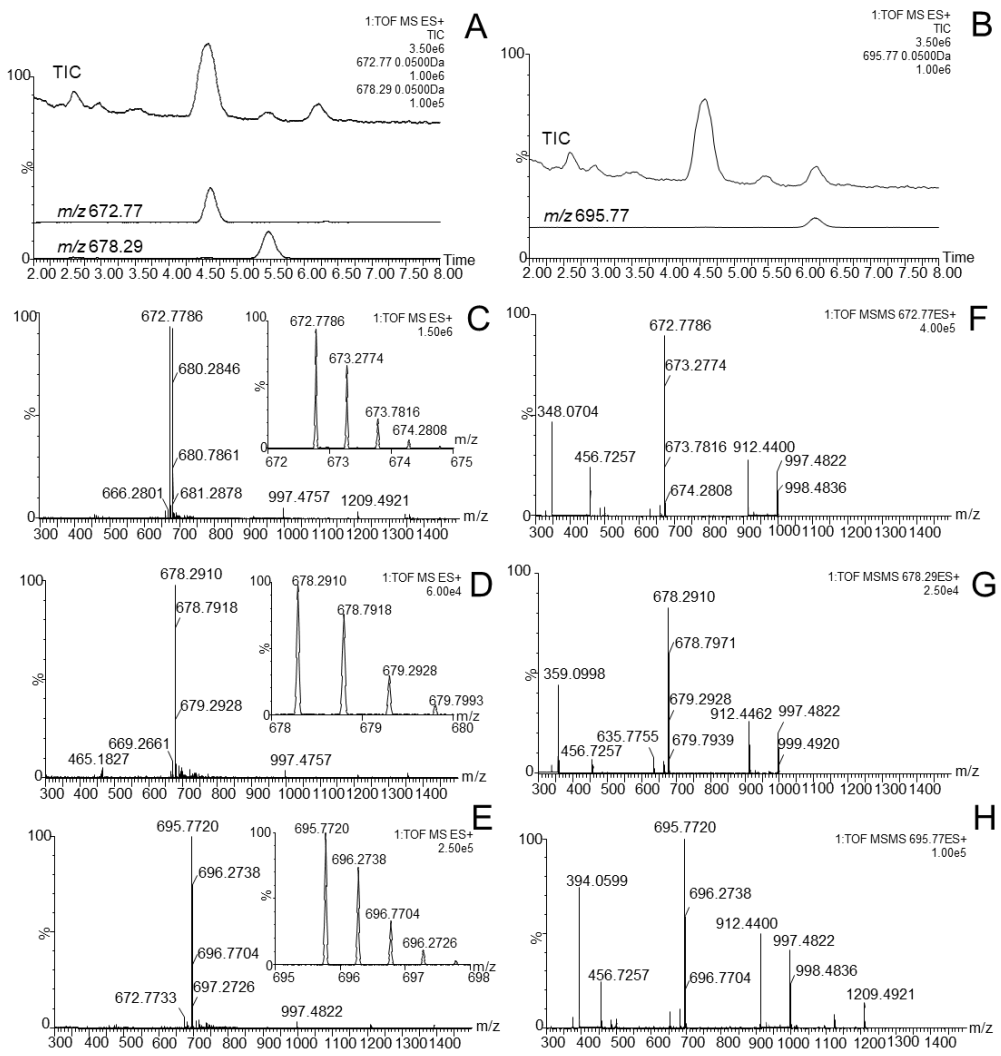

Fig. III-2. LC/ESI–MS/MS chromatograms of the corrinoid compounds purified from a cricket product. (A) and (B) Total ion chromatogram (TIC) and mass chromatogram of the corrinoid compounds (*m/z* 672.77, 678.29, and 695.77). (C)–(E) MS spectra of the corrinoid compounds with retention times of 4.57 min (the magnified spectrum from *m/z* 672 to *m/z* 675 is shown as an insert in C), 5.47 min (the magnified spectrum from *m/z* 678 to *m/z* 680 is shown as an insert in D), and 6.21 min (the magnified spectrum from *m/z* 695 to *m/z* 698 is shown as an insert in E), respectively. (F)–(H) MS/MS spectra of the peaks of the corrinoid compounds with retention times of 4.57 min (*m/z* 348.0704), 5.47 min (*m/z* 359.0998), and 6.21 min (*m/z* 394.0599), respectively.

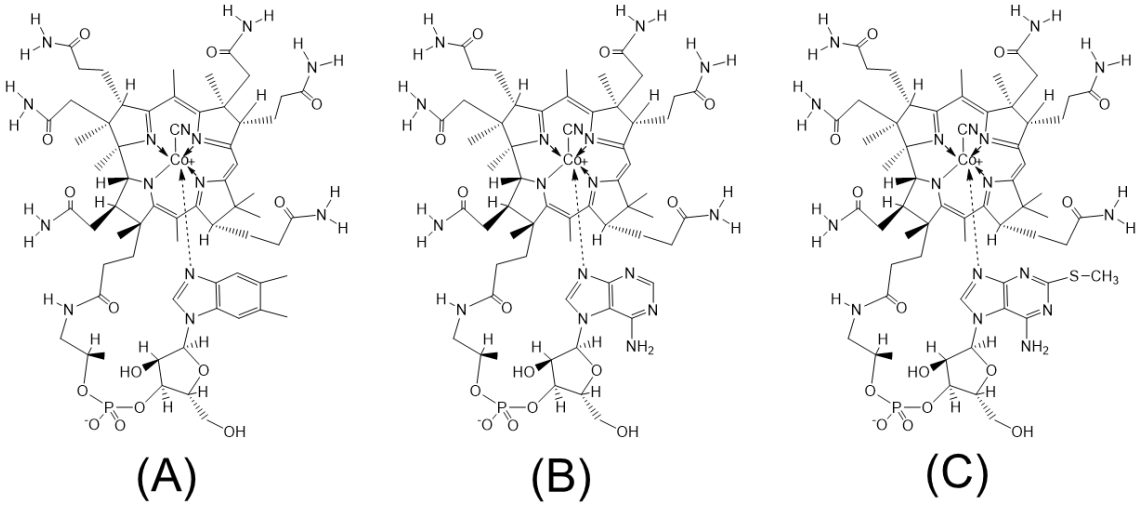

Fig. III-3. Structures of the corrinoid compounds found in commercially available edible cricket products. (A) Authentic B_{12}, (B) pseudoB_{12}, and (C) factor S.

Table III-3. Relative content (%) of pseudoB_{12}, factor S, and B_{12} in commercially available edible cricket products

Cricket samples	Relative content (%)		
	PseudoB_{12}	Factor S	B_{12}
Sample 1	74.6 ± 1.8	19.2 ± 1.4	6.2 ± 0.5
Sample 2	74.8 ± 1.0	20.3 ± 1.3	4.8 ± 0.5
Sample 3	71.3 ± 1.9	24.9 ± 2.0	3.8 ± 0.6
Sample 4	73.5 ± 1.6	21.8 ± 1.1	4.7 ± 0.5
Sample 5	76.4 ± 2.3	17.7 ± 1.7	5.9 ± 0.8
Mean	74.1 ± 0.8	20.8 ± 1.1	5.1 ± 0.4

Relative contents were calculated using LC/ESI–MS/MS. All values represent the mean ± SEM of triplicate experiments.

B_{12} content and compounds in adult crickets (A. domesticus) fed with cricket food A and B

When newly-emerged crickets were fed with cricket food A (approximately 4.1 μg of B_{12} per 100 g fresh weight) or B (approximately 15.5 μg of B_{12} per 100 g fresh weight) for 23

days, there was no significant difference in their body sizes (food A-fed crickets: 14.3 ± 0.1 mm; food B-fed crickets: 14.2 ± 0.4 mm). Significant differences in B_{12} content between food A and B were observed; however, B_{12} content in food A or B-fed crickets was not significantly changed. (**Table III-4**). The corrinoid compounds purified from food A-fed crickets using LC/ESI–MS/MS (**Fig. III-4**) were identical to those found in edible cricket product sample 1 (**Fig. III-2**). While food A-fed crickets contained approximately 76% pseudoB_{12}, 19% factor S, and 5% B_{12}, food B-fed crickets contained approximately 56% pseudoB_{12}, 34% factor S, and 10% B_{12}. As shown in **Table III-4**, cricket food B contained B_{12} but not pseudoB_{12} or factor S. Similarly, cricket food A contained only a small amount (approximately 3%) of pseudoB_{12}, while the remaining corrinoid was B_{12}. These results suggest that pseudoB_{12} and factor S might be synthesized in the cricket's bodies, perhaps due to intestinal bacteria.

Table III-4. Total B_{12} content and relative content (%) of pseudoB_{12}, factor S, and B_{12} in adult crickets (*Acheta domesticus*) fed with cricket food A and B (see Materials and methods for ingredients)

	Total B_{12} content (μg/100 g fresh weight)	Corrinoid compounds (percentage of total corrinoids)		
		PseudoB_{12}	Factor S	B_{12}
Food A	4.1 ± 0.6	2.9 ± 0.2	n.d.	97.1 ± 0.2
Food B	15.5 ± 0.6[a]	n.d.	n.d.	100
Food A-fed cricket	28.6 ± 3.1	76.0 ± 2.6	19.3 ± 1.5	4.7 ± 1.0
Food B-fed cricket	21.3 ± 2.9	56.1 ± 3.7	33.7 ± 3.1	10.3 ± 0.9

Relative contents were calculated using LC/ESI–MS/MS. All values represent the mean ± SEM of triplicate experiments. n.d. represents not detected. [a] indicates significant difference between the values of cricket foods and adult crickets grown in the laboratory.

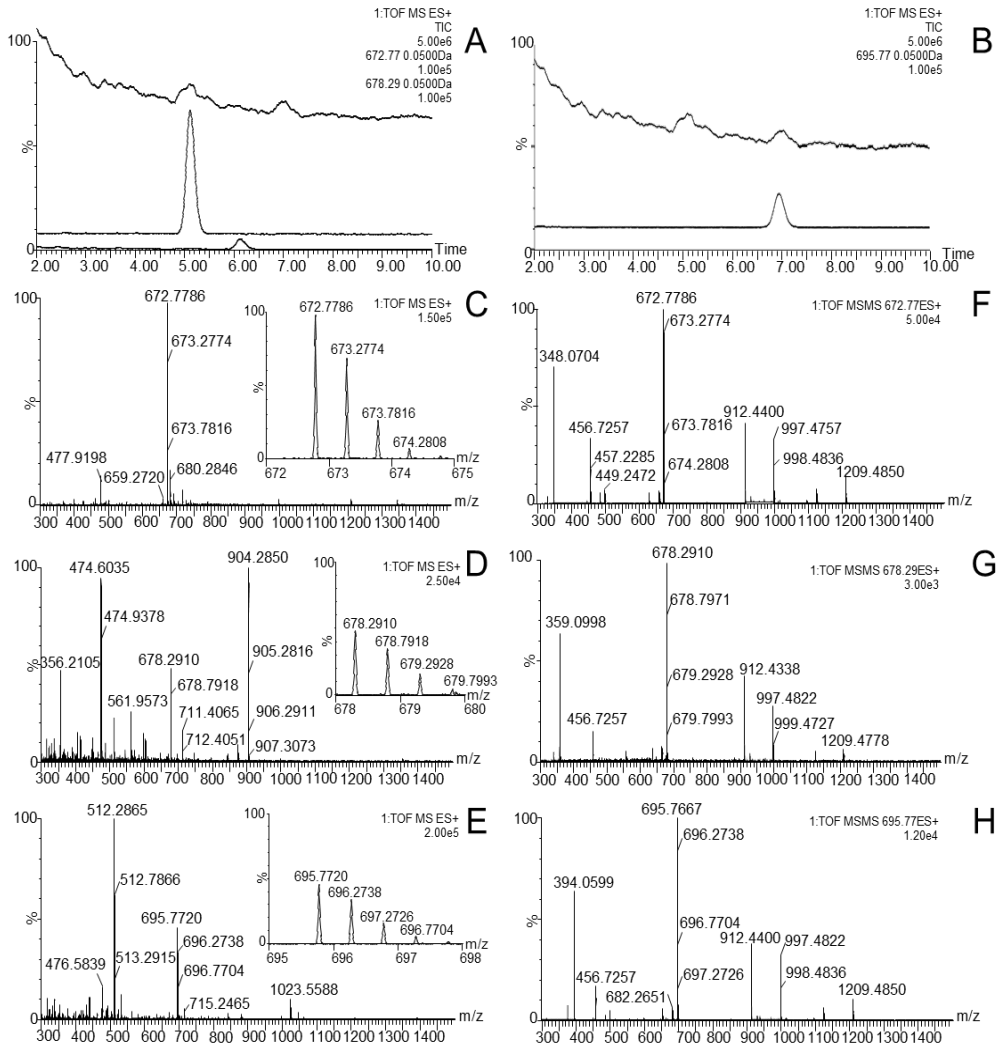

Fig. III-4. LC/ESI–MS/MS chromatograms of the corrinoid compounds purified from adult crickets fed with cricket food A. (A) and (B) Total ion chromatogram (TIC) and mass chromatogram of the corrinoid compounds (*m/z* 672.77, 678.29, and 695.77). (C)–(E) MS spectra of the corrinoid compounds with retention times of 5.07 min (the magnified spectrum from *m/z* 672 to *m/z* 675 is shown as an insert in C), 6.10 min (the magnified spectrum from *m/z* 678 to *m/z* 680 is shown as an insert in D), and 6.95 min (the magnified spectrum from *m/z* 695 to *m/z* 698 is shown as an insert in E), respectively. (F)–(H) MS/MS spectra of the peaks of the corrinoid compounds with retention times of 5.07 min (*m/z* 348.0704), 6.10 min (*m/z* 359.0998), and 6.95 min (*m/z* 394.0599), respectively.

Discussion

Despite the bioassay detecting high B_{12} content (approximately 50–75 μg/100 g dry weight) in edible cricket products, LC/ESI–MS/MS analysis indicated that pseudoB_{12} and factor S were actually the predominant corrinoid compounds, with B_{12} making up only 5% of total corrinoids. PseudoB_{12} is the dominant corrinoid compound formed by *Clostridium cochlearium* [20], *Lactobacillus reuteri* [21], *Propionibacterium acidipropionici* [22], and cyanobacteria [18]. Furthermore, pseudoB_{12} and factor S have been found in human feces [19]. In humans, some ingested B_{12} is converted into various corrinoids with different bases, including pseudoB_{12} and factor S, by intestinal bacteria. Like humans, crickets are omnivores that eat both plants and animals [44]. Thus, crickets are likely to ingest B_{12} from animal-derived foods. Although the intestinal absorption mechanism of B_{12} is unknown in insects, B_{12} synthesized by intestinal bacteria might be absorbed into the cricket's bodies. Bawa et al. [42] reported that B_{12} content significantly increased in crickets fed with a dry pumpkin pulp-supplemented basal cricket diet relative to a base diet. Although pumpkin pulp does not appear to contain B_{12}, certain components of pumpkin pulp might stimulate the intestinal bacteria to induce the synthesis of corrinoid compounds. In the present study, total B_{12} content was greater in crickets fed with food A, which had a lower B_{12} content (approximately 4 μg/100 g) than that of food B (16 μg/100 g). In addition, neither food contained factor S nor did cricket food B contain any pseudoB_{12} (a small amount was found in cricket food A). These results suggest that the inactive corrinoids found in these cricket products might be synthesized by cricket intestinal bacteria such as *Citrobactor, Klebisiella,* and *Bacteroides* [45,46].

In mammals, pseudoB_{12} apparently does not act as a B_{12} antagonist to inhibit the gastrointestinal absorption of B_{12} because the IF involved in the gastrointestinal absorption of B_{12} in mammals preferentially binds B_{12} in the presence of pseudoB_{12} under physiological conditions [23]. Currently, there is no data on the effects of factor S on B_{12} binding in humans.

However, I speculate that factor S does not affect human gastrointestinal absorption of B_{12} because the lower ligand of the factor S molecule has a similar basic structure (adenine) to that of pseudoB_{12}. Given that the B_{12} content of edible cricket products is approximately 3 μg /100 g dry weight, consumption of approximately 80 g of cricket products would provide the RDA for adults (2.4 μg/day).

Summary

In this study, I determined the B_{12} content of commercially available edible insect products using a bioassay based on *L. delbrueckii* ATCC 7830. Although the vitamin content of giant water bug, bee larva, grasshopper, and weaver ant products was low, I found that diving beetle and cricket products contained relatively high amounts of B_{12} (approximately 89.5 and 65.8 μg/100 g dry weight, respectively). In the cricket products most widely circulated as foods, specific corrinoid compounds were extracted and identified using LC/ESI–MS/MS. Despite the bioassay detecting high B_{12} content (approximately 50–75 μg/100 g dry weight) in these cricket products, LC/ESI–MS/MS analysis indicated that pseudoB_{12} and factor S were actually the predominant corrinoid compounds (approximately 74% and 21%, respectively), with B_{12} making up only 5% of total corrinoids.

Chapter IV

Characterization of vitamin B_{12} compounds formed by the treatment with food additives

Introduction

An unnatural and inactive B_{12} compound, B_{12}[*c*-lactone], was reportedly found in some edible mushrooms [29,30]. B_{12}[*c*-lactone] was readily formed from B_{12} by the treatment with the organochlorine antibacterial agent chloramine-T [30]. In addition, preliminary experiments indicate that B_{12} is completely inactivated on treatment with hypochlorous acid water. Hypochlorous acid is usually used to sanitize food products such as vegetables, fruits and meat [47–49]. This acid is widely used in food industry as the disinfection agents of food processing equipment [48,49]. However, hypochlorous acid readily reacts proteins to form an aggregation and oxidizes specific amino acids [50,51]. High hypochlorous acid treatment reportedly induces a significant decrease of vitamin C content in vegetables [52]. There is limited information on disadvantages associated with the use of hypochlorous acid. If hypochlorous acid has the ability to readily degrade B_{12}, ingestion of the formed products might induce B_{12} deficiency in humans.

Furthermore, preliminary experiments also indicate that food additives such as sodium metabisulfite [51] and sodium sulfite [53,54] had the ability to change the ultraviolet–visible (UV–visible) absorption maxima of B_{12} in aqueous solution, suggesting the possibility that B_{12} is readily inactivated by the treatment with these food additives.

In this chapter, I evaluated the effects of hypochlorous acid water, sodium metabisulfite, and sodium sulfite on the chemical and biological properties of B_{12} under aqueous conditions. Furthermore, I examined whether significant loss of B_{12} occurs in food treated with these food additives.

Materials and Methods

Materials

CN-B_{12} and OH-B_{12} were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sulfitocobalamin (SO_3^--B_{12}) was synthesized from OH-B_{12} by treatment with sodium sulfite according to the method described by Suarez-Moreira et al [55]. Food additives, such as hypochlorous acid water, sodium metabisulfite, and sodium sulfite were purchased from markets in Japan. Argentine red shrimp (*Pleoticus muelleri* [Bate]) and grounded beef meat were purchased from a local market in Tottori City, Japan.

UV-visible spectra of CN-B_{12} treated with or without food additives in aqueous solution

Hypochlorous acid water was adjusted to an effective chlorine concentration of 30 ppm using an effective chlorine concentration measurement kit (AQ-102P; SIBATA Scientific Technology, Ltd., Saitama, Japan) The other food additives (i.e., sodium metabisulfite and sodium sulfite) were dissolved in distilled water at a concentration of 0.01% (w/v). CN-B_{12} was dissolved in these food additive solutions at a final concentration of 10 μmol/L. These solutions were left for 0, 1, 24, and 48 h at room temperature (25˚C). At the indicated time points, the UV–visible absorption spectra of these solutions were measured using a UV–visible spectrophotometer (UV-2550; Shimadzu Corp.).

HPLC analysis of B_{12} compounds formed by treatment with these food additives

The CN-B_{12} was treated with hypochlorous acid water (an effective chlorine concentration of 10 ppm) for 1 h as described above. Subsequently, the treated CN-B_{12} solution was immediately loaded onto a Sep-Pak® Vac (5 g) C18 cartridge (Waters Corp.) equilibrated with 20 mL of Milli-Q water after washing with 20 mL of 75% (v/v) ethanol solution. The C18 cartridge was washed with 20 mL of Milli-Q water, and the B_{12} compounds were eluted with 75% (v/v) ethanol solution. The eluate was allowed to

evaporate to dryness under reduced pressure, dissolved in a small amount of water, and subsequently used as a sample for HPLC analysis. The Shimadzu HPLC apparatus (SPD-10AV UV–Visible detector, SCL-10A VP system controller, DGU-20A3 degasser, LC-10Ai Pumps, CTO-6A column oven) and CDS ver. 5 chromato-data processing system (LAsoft, Ltd, Chiba, Japan) were used. An aliquot (30 μL) of the sample was placed on a reversed-phase HPLC column (Wakosil®-II 5C18RS, φ 4.6 × 150 mm; particle size 5 μm; FUJIFILM Wako Pure Chemical Corp.), which was equilibrated at 40˚C with 20% (v/v) methanol containing 1% (v/v) acetic acid at a flow rate of 1.0 mL/min. The B_{12} compounds were eluted for 20 min with a linear gradient of 20–90% (v/v) methanol solution containing 1% (v/v) acetic acid, followed by elution with 90% (v/v) methanol solution containing 1% (v/v) acetic acid for 10 min, and were monitored by measuring the absorbance at 361 nm. The B_{12} compounds were eluted as major six peaks with retention times of 8.6 (P-1), 10.9 (P-2), 12.3 (P-3), 14.7 (P-4), 15.9 (P-5), and 17.1 min (P-6). These peak fractions were collected and evaporated to dryness under reduced pressure and dissolved in a small amount of water. Each peak fraction was further purified using a reversed-phase HPLC under the same conditions. The B_{12} compounds P-2, P-3, and P-6 were homogeneously purified; however, we failed to purify the remaining B_{12} compounds owing to their instability.

The CN-B_{12} was treated with sodium metabisulfite or sodium sulfite for 48 h as described above and immediately placed on a reversed-phase HPLC column (Wakosil®-II 5C18RS, φ 4.6 × 150 mm; particle size 5 μm; FUJIFILM Wako Pure Chemical Corp.), which was equilibrated at 40˚C with 20% (v/v) methanol containing 1% (v/v) acetic acid at a flow rate of 1.0 mL/min. The B_{12} compounds were isocratically eluted under the same conditions and monitored by measuring the absorbance at 361 nm.

B_{12}-dependent Escherichia coli 215 bioautography of the B_{12} compounds formed by hypochlorous acid water

Bioautography of the B_{12} compounds was performed as previously described [56]. An

aliquot (2 μL) of the partially purified sample (50 μg/L) using the Sep-Pak® Vac (5 g) C18 cartridge as described above. The authentic CN-B_{12} (50 μg/L) were spotted on filter papers (circle; 8 mm), which were placed on 1.5% (w/v) agar containing a basal medium of *E. coli* 215 and incubated at 37˚C for approximately 20 h. The agar 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.

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy of the B_{12} compounds formed by hypochlorous acid water

Purified B_{12} compounds with respective retention times of 10.9 (P-2), 12.3 (P-3), and 17.1 min (P-6) were analyzed using ^{1}H NMR spectroscopy to characterize their chemical structures formed after treatment with hypochlorous acid water. NMR spectra were measured with a Bruker DRX500 (Bruker Daltonics, Billerica, MA, USA). The spectra of these compounds (P-2, P-3, and P-6) were obtained in deuterium oxide at room temperature (25˚C). Chemical shifts are given on a δ (ppm) scale with 3-(trimethylsilyl) propionic acid-d_4 sodium salt (TSP) used as an internal standard.

Effect of these food additives on the B_{12} content of red shrimp meat

The edible portion (approximately 150 g) of Argentine red shrimps was collected and homogenized using mortar and pestle. Subsequently, 1.0 mL of hypochlorous acid water (an effective chlorine concentration of 30 ppm), 0.1% (w/v) sodium metabisulfite solution, or 0.1% (w/v) sodium sulfite solution, and distilled water (control) were added to each 10 g of the shrimp meat homogenate, followed by thorough mixing. Three sets of putties (3 × 3 × 1 cm) from each sample were formed and subsequently allowed to stand at 4˚C for 48 h in the dark. B_{12} was extracted from aliquots (2.0 g) of each stored sample and determined using the method described in **Chapter II**.

Effect of various concentrations of hypochlorous acid water on the B_{12} content of beef meat

Ground beef meat was treated with 1.0 mL of hypochlorous acid water (effective chlorine concentrations of 30, 60, and 80 ppm), followed by thorough mixing. Three sets of putties (3 × 3 × 1 cm) from each beef meat were formed and subsequently allowed to stand at 4˚C for 48 h in the dark. B_{12} was extracted and assayed as described above.

Statistical analysis

One-way analysis of variance and a post-hoc analysis were performed using Dunnett's multiple comparison tests to evaluate the effect of food additives on the B_{12} content of red shrimp meat and to determine the effect of various concentrations of hypochlorous acid water on the B_{12} content of ground beef meat. Analyses were performed using GraphPad Prism 3 for Windows version 2.01 (GraphPad software Inc.). Data are presented as the mean ± standard error of the mean (SEM). A $p < 0.05$ denoted statistically significant differences.

Results

UV–visible spectra of CN-B_{12} treated with or without food additives in an aqueous solution

On the basis of preliminary experiments, hypochlorous acid water, sodium metabisulfite, and sodium sulfite were selected as food additives. Changes in the UV–visible spectra of CN-B_{12} after treatment with these food additives were monitored for 48 h to determine the degree of reaction in aqueous solution. The UV–visible absorption spectrum of a reaction mixture containing CN-B_{12} and hypochlorous acid water (an effective chlorine concentration of 30 ppm) showed that the absorption peak at 278 nm, which is the specific wavelength of CN-B_{12}, disappeared from 0 h. Other specific absorption peaks at 361 and 550 nm were also shifted to 365 and to 586 nm, respectively, and thereafter were significantly decreased (**Fig. IV-1A**). These specific absorption maxima of CN-B_{12} completely disappeared by 1 h.

Addition of 0.01% (w/v) sodium metabisulfite indicated that the specific absorption peaks

at 278 and 361 nm were slightly increased and thereafter decreased during the time course; however, these specific absorption maxima of CN-B_{12} were hardly changed for 48 h (**Fig. IV-1B**). Sodium sulfite also showed similar spectroscopic behavior (**Fig. IV-1C**).

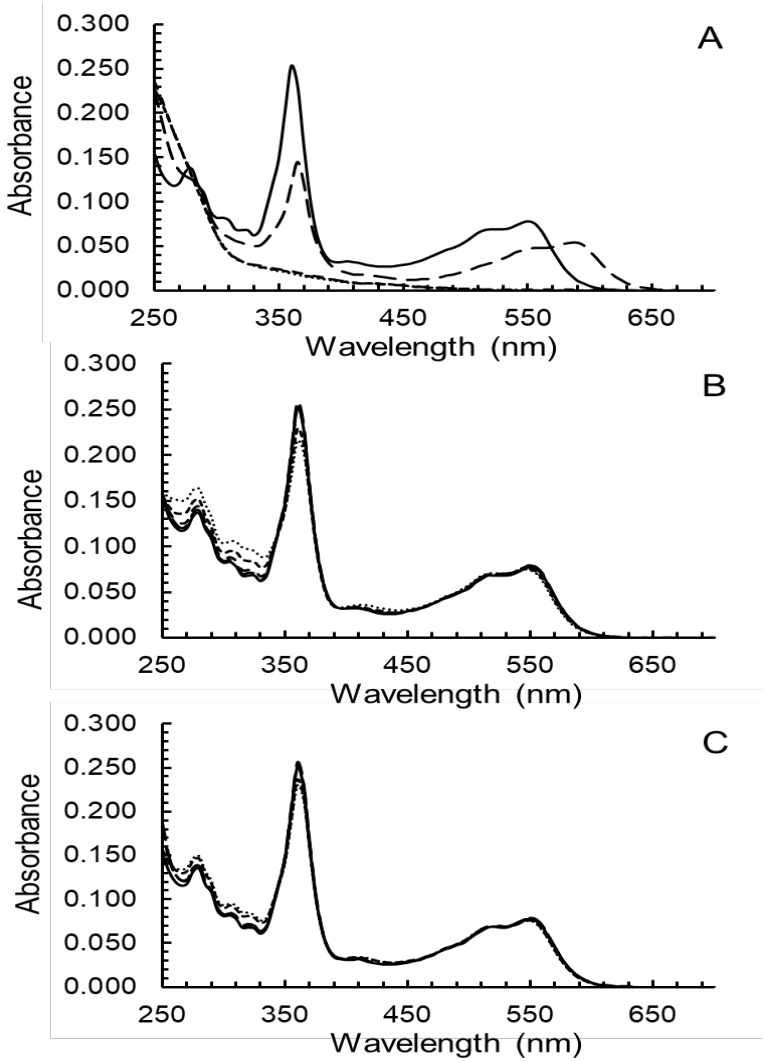

Fig. IV-1. UV–visible absorption spectra of CN-B_{12} after treatment with these food additives. (A) Hypochlorous acid water (at an effective chlorine concentration of 30 ppm), (B) 0.01% (w/v) sodium metabisulfite, and (C) 0.01% (w/v) solution sodium sulfite. These solutions were left for 0 h (— — —), 1 h (－ － －), 24 h (----------), and 48 h (……) at room temperature (25˚C). A solution of CN-B_{12} treated without these food additives was used as control (———).

HPLC analysis of B_{12} compounds formed by the treatment with hypochlorous acid water.

B_{12} compounds formed after treatment with these compounds were analyzed using HPLC to determine the effects of these food additives on the chemical structure and biological activity of CN-B_{12}. When CN-B_{12} treated with hypochlorous acid water for 60 min was analyzed using a reversed-phase HPLC, there were no peaks observed with retention times of 7.2 and 7.7 min, which are derived from authentic CN-B_{12} and B_{12}[*c*-lactone], respectively, were found. Instead, the B_{12} compounds were eluted as major six peaks with respective retention times of 8.6 (P-1), 10.9 (P-2), 12.3 (P-3), 14.7 (P-4), 15.9 (P-5), and 17.1 min (P-6) (**Fig. IV-2**). The compounds P-2, P-3, and P-6 could be purified and subjected to ^{1}H NMR spectroscopy.

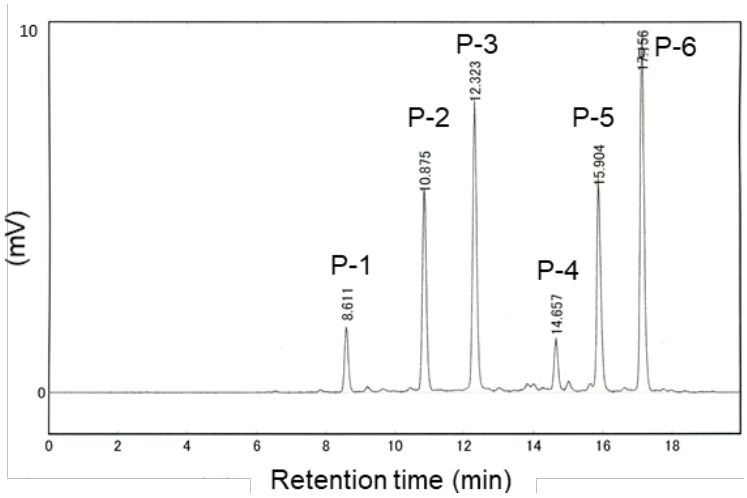

Fig. IV-2. HPLC chromatograms of the B_{12} compounds treated with hypochlorous acid water. The HPLC patterns of the B_{12} compounds are typical data obtained from three independent experiments.

^{1}H NMR spectroscopy of the B_{12} compounds formed by hypochlorous acid water.

I compared the ^{1}H NMR spectra of the purified compounds with that of authentic CN-B_{12} (**Fig. IV-3**). In the spectrum of the compound P-2 (**Fig. IV-4A**), the signal corresponding to the olefinic proton at C10 disappeared, whereas the characteristic signals coupled with each

other at δ_H 3.86 (1H, d, J = 19.0 Hz) and δ_H 3.15 (1H, d, J = 19.0 Hz) appeared. Thus, the carbon at this position may have been saturated by the treatment. The signal corresponding to R1 (δ_H 6.39) indicated the presence of a ribose group. Furthermore, signals corresponding to methyl groups B10, B11, and Pr3 were also detected, indicating the presence of dimethylbenzimidazole and β-aminoisopropyl groups. All of the remaining methyl groups (C53, C35, C25, C36, C54, C47, C46, and C20) on the corrin ring appeared at chemical shifts similar to authentic B_{12}.

The spectra of compounds P-2 and P-3 (**Fig. IV-4A and B**) were very similar to each other; however, the values of the chemical shift of the signals were different. The signals corresponding to B2, B4, B7, and R1 indicated the presence of dimethylbenzimidazole and a ribose group. All methyl groups corresponding to the those of B_{12} were detected in the spectrum, indicating that the corrin ring skeleton and β-aminoisopropyl groups were retained in compound B. Therefore, the compounds P-2 and P-3 may be structural isomers.

The signal corresponding to B4 at δ_H 7.32 remained on the dimethylbenzimidazole ring in the spectrum of the compound P-6 (**Fig. IV-4C**), whereas the signal corresponding to B7 disappeared. Thus, the B7 position on the dimethylbenzimidazole group was likely substituted by the treatment. The chemical shifts of the signals of methyl groups at C35 and C53 (δ_H 2.62 and 2.61 ppm) showed high-magnetic field shifts. Additionally, the signal corresponding to the methyl group at C20 disappeared. Thus, the conjugation system of the corrin ring was modified by the treatment.

HPLC analysis of B_{12} compounds formed by the treatment with sodium metabisulfite and sodium sulfite.

Following the treatment of CN-B_{12} with sodium metabisulfite for 48 h and analysis using reversed-phase HPLC, the B_{12} compounds were isocratically eluted as major and minor peaks with retention times of 8.7 and 27.5 min, respectively (**Fig. IV-5A**).

The major peak with a retention time of 8.7 min was identical to that of authentic CN-B_{12}

(data not shown), suggesting that most CN-B_{12} was not changed by treatment with sodium metabisulfite. Previous studies reported that OH-B_{12} was readily converted to SO_3^--B_{12} by the addition of sodium sulfite [55,57]. When SO_3^--B_{12} was prepared and analyzed using HPLC, the minor peak with a retention time of 27.5 min was identical to that of SO_3^--B_{12} (**Fig. IV-5B**). Similar results were obtained for sodium sulfite (data not shown).

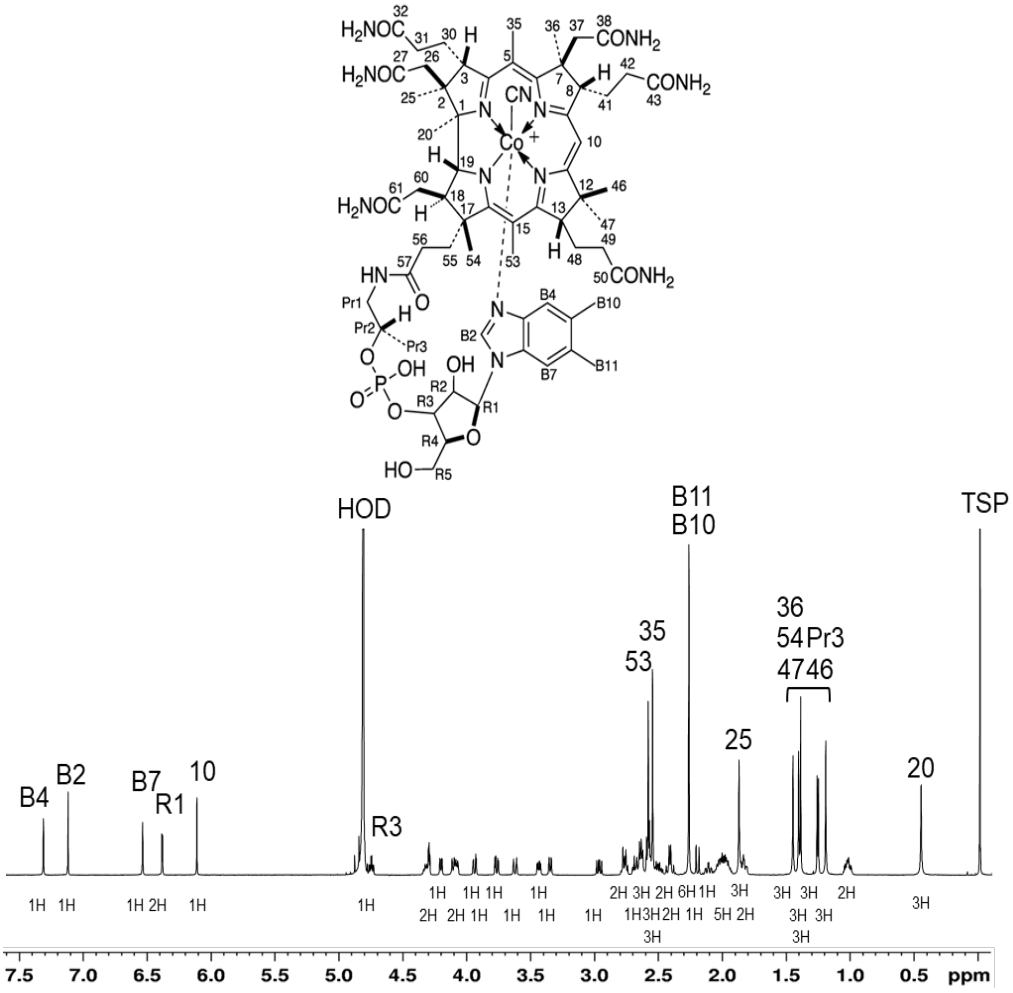

Fig. IV-3. ^{1}H NMR spectrum of authentic CN-B_{12}.

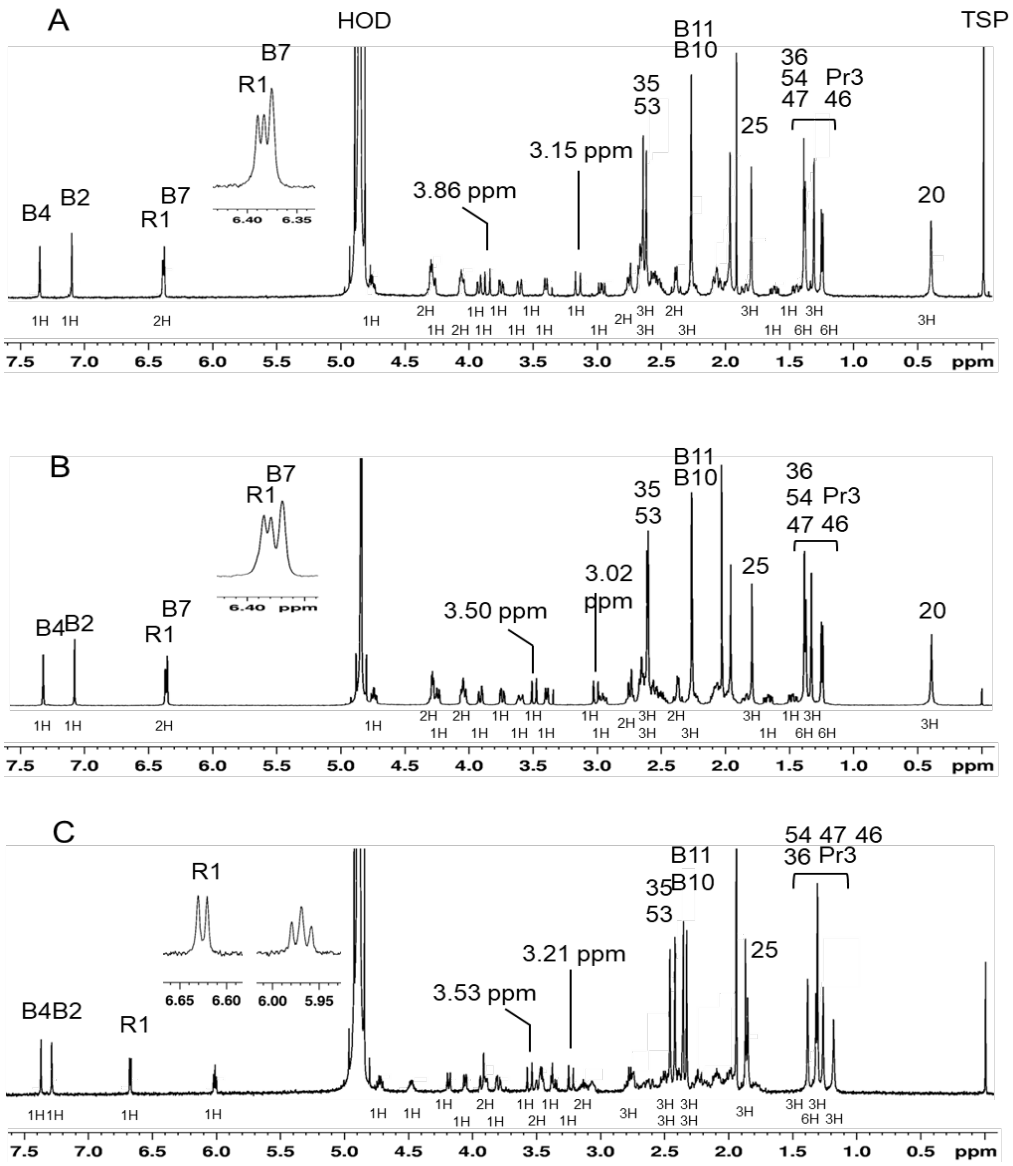

Fig. IV-4. ^{1}H NMR spectra of B_{12} compounds formed during treatment with hypochlorous acid water. (A) Compound P-2 (with a retention time of 10.9 min), (B) compound P-3 (with a retention time of 12.3 min), and (C) compound P-6 (with a retention time of 17.1 min).

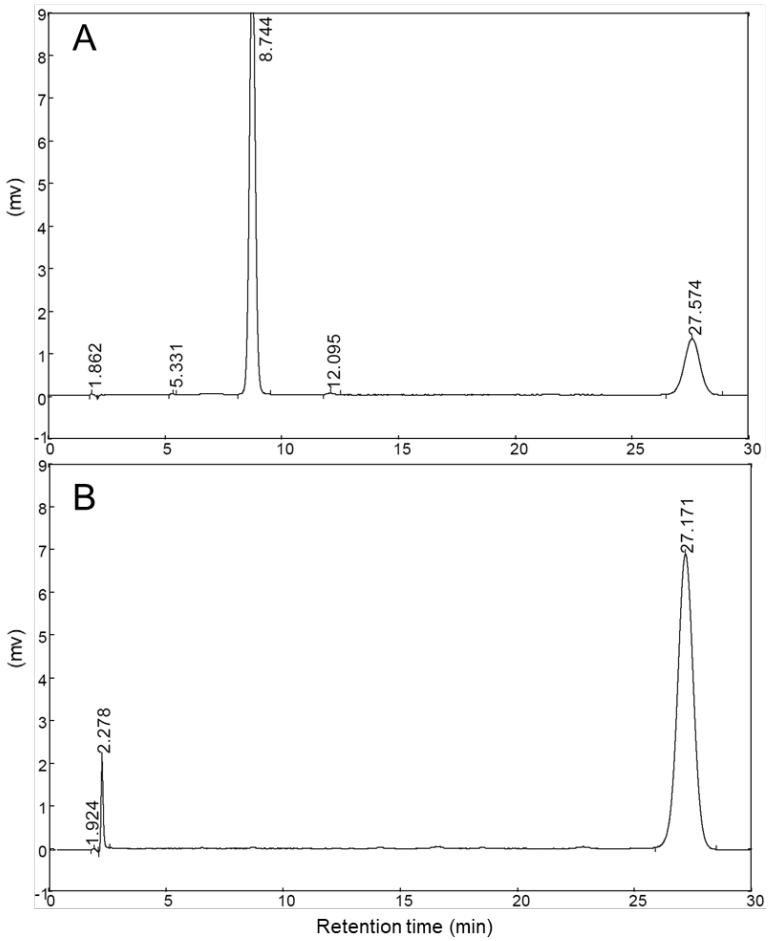

Fig. IV-5. HPLC patterns of the B_{12} compounds treated with 0.01 (%) sodium metabisulfite. (A) Treated CN-B_{12} solution (50 μL) and (B) authentic SO_3^--B_{12} (20 μL of 130 mg/L). Data are typical HPLC patterns of the B_{12} compounds treated with sodium metabisulfite or authentic SO_3^--B_{12} obtained from three independent experiments.

Biological activity of the B_{12} compounds formed by the treatment with the food additives.

SO_3^--B_{12} is well established as a naturally occurring biologically active B_{12} [58]. The minor peak (with a retention time of 27.5 min) formed by the addition of sodium metabisulfite or sodium sulfite (**Fig. IV-5A**) was active in B_{12}-dependent *E. coli* 215 (data not shown). However, based on the absence of bacterial growth, B_{12}-dependent *E. coli* 215 bioautography of hypochlorous acid-treated B_{12} compounds indicated that hypochlorous acid water was readily inactivated CN-B_{12} in aqueous solution (**Fig. IV-6**).

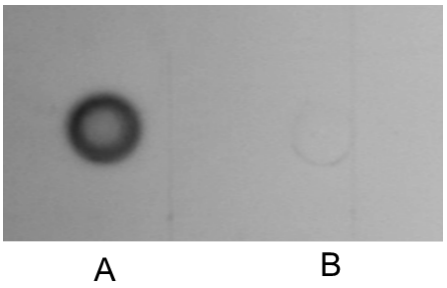

Fig. IV-6. Effects of the B_{12} compounds treated with hypochlorous acid water on B_{12}-dependent *E. coli* 215. (A) Authentic B_{12} (100 pg) and (B) hypochlorous acid-treated B_{12} compounds.

Effect of the food additives on the B_{12} content of red shrimp meat.

In Japan, sodium metabisulfite and sodium sulfite are typically used to prevent black discoloration in shrimps. Hypochlorous acid water (an effective chlorine concentration of 30 ppm), 0.1% (w/v) sodium metabisulfite solution, 0.1% (w/v) sodium sulfite solution, and distilled water (control) were added to red shrimp meats and then stored for 48 h at 4˚C in the dark to determine whether these food additives can reduce the B_{12} content in food. B_{12} was extracted from the red shrimp meat treated with or without these food additives, and its amount was determined using the *L. delbrueckii* ATCC 7830 bioassay. There was no significant decrease detected in the B_{12} content of the red shrimp meat treated with these food additives (**Fig. IV-7**).

Effect of various concentrations of hypochlorous acid water on the B_{12} content of ground beef meat.

Hypochlorous acid is usually used to sanitize vegetables, fruits and meat [47–49]. As animal-derived foods are good sources of B_{12} for humans [59,60], effect of various concentrations of hypochlorous acid on the B_{12} content of beef meat was evaluated. Effective chlorine concentrations of 30, 60, and 80 ppm of hypochlorous acid water and distilled water (control) were added to ground beef meats and then stored for 48 h at 4˚C in the dark. B_{12}

was extracted from the ground beef meat treated with or without hypochlorous acid water, was extracted from the ground beef meat treated with or without hypochlorous acid water, and its amount was determined using the bioassay. The B_{12} content of the ground beef meat gradually decreased with increased effective chlorine concentrations of hypochlorous acid but there was no significant difference among these values (**Fig. IV-8**).

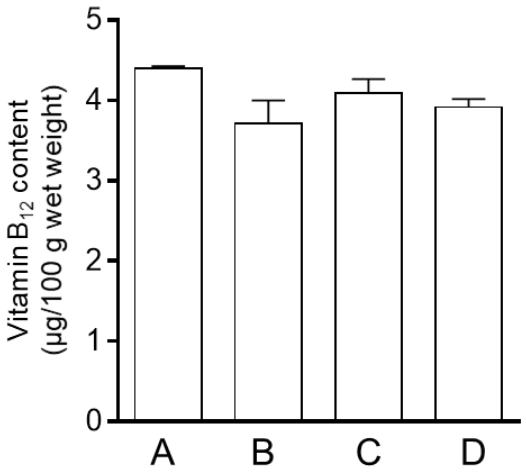

Fig. IV-7. Effects of treatment with hypochlorous acid water, sodium metabisulfite, and sodium sulfite on the B_{12} content of red shrimp meat. (A) Control, (B) hypochlorous acid water (at an effective chlorine concentration of 30 ppm), (C) 0.1% (w/v) sodium metabisulfite solution, and (D) 0.1% (w/v) sodium sulfite solution. B_{12} content was assayed in triplicate. Data are represented as means ± SEM (n = 3).

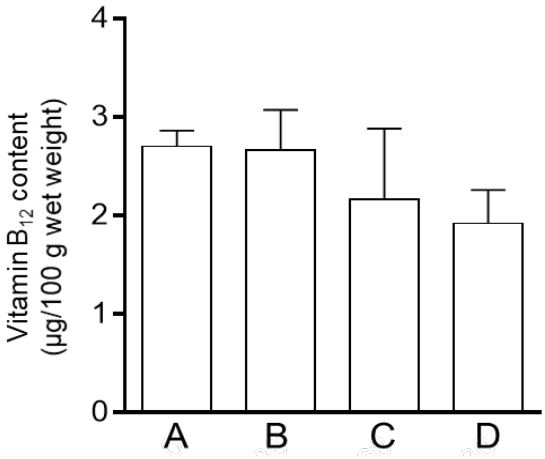

Fig. IV-8. Effects of various concentrations of hypochlorous acid water on the B_{12} content of ground beef meat. (A) Control, (B) hypochlorous acid water at effective chlorine concentrations of 30 ppm, (C) 60 ppm, and (D) 80 ppm. B_{12} content was assayed in triplicates. Data are represented as means ± SEM (n = 3).

Discussion

Food additives that may inactivate B_{12} were screened for their ability to change the UV−visible absorption spectra of CN-B_{12}. According to the results of my preliminary experiments, hypochlorous acid water, sodium metabisulfite, and sodium sulfite were selected. Hypochlorous acid water is a highly safe antimicrobial agent and widely used as a food additive [47−49]. However, treatment with hypochlorous acid water (an effective chlorine concentration of 30 ppm) resulted in immediate and significant decreases at the absorption peaks at 278, 365, and 586 nm. Notably, these absorption maxima of CN-B_{12} completely disappeared by 1 h (**Fig. IV-1A**). This finding suggested the destruction of the corrin ring and the liberation of the central cobalt ion of CN-B_{12} following treatment with hypochlorous acid water. High concentration of hypochlorous acid reportedly induced the oxidative cleavage of the corrin ring and porphyrin ring, destroying these compounds [61,62].

Although these observations suggest that treatment of food with hypochlorous acid water induces significant loss of B_{12}, this agent did not affect the level of B_{12} in this study **(Fig. IV-7 and -8**). These results imply that hypochlorous acid water could not react with food B_{12}, which is present in its protein-bound form in food [15,63]. Hypochlorous acid is generated by myeloperoxidase and plays an important role in the innate immune system of mammals [64]. Nevertheless, it was suggested that highly generated hypochlorous acid leads to various diseases due to the action of a potent oxidant protein and induces chlorination and protein aggregation [62,65]. This observation suggests that hypochlorous acid water as a food additive adversely affects food proteins in foods, but not B_{12}.

Through HPLC analysis, five major peaks as degradation products were detected. Among them, I analyzed three compounds eluted from the HPLC column at 10.9 (P-2), 12.3 (P-3), and 17.1 min (P-6), respectively, using ^{1}H NMR spectroscopy. On the basis of the ^{1}H NMR spectra, the compounds P-2 and P-3 may be structural isomers, which lacked the olefinic proton at C10. On the other hand, the compound P-6 lacked the B7 proton on the dimethylbenzimidazole ring instead of C10. Thus, the C10 position in the corrin ring appears to be the first site of reaction with hypochlorous acid water. Compound P-6 appears to be largely affected by treatment with hypochlorous acid water versus compared to compounds P-2 and P-3. These degradation products have not been detected in the oxidation process of B_{12}. Determination of the chemical structures of these compounds would lead to a better understanding of the degradation mechanism of B_{12} by hypochlorous acid water.

When CN-B_{12} was treated with sodium metabisulfite or sodium sulfite for 48 h, small amount of SO_3^--B_{12} was formed. SO_3^--B_{12} was formed from glutathionylcobalamin, a product formed by the decyanation reaction of CN-B_{12} in the presence of reduced glutathione in mammalian cells [66,67]. SO_3^--B_{12}, which is one of the naturally occurring B_{12} in foods, is biologically active in humans. However, it has been reported that, in humans, the intestinal absorption of SO_3^--B_{12} was lower than that of CN-B_{12} [58].

The results of this study indicate that these food additives have the ability to significantly

change the properties of B_{12} in aqueous solution; however, they are unable to reduce the B_{12} content of food.

Summary

Food additives such as hypochlorous acid water, sodium metabisulfite, and sodium sulfite, strongly affect the chemical and biological properties of B_{12} in aqueous solution. When CN-B_{12} (10 μmol/L) was treated with these compounds, hypochlorous acid water (an effective chlorine concentration of 30 ppm) rapidly reacted with CN-B_{12}. The maximum absorptions at 361 and 550 nm were completely disappeared by 1 h, and B_{12} activity was lost. There were no significant changes observed in the absorption spectra of CN-B_{12} for 0.01% (w/v) sodium metabisulfite; however, a small amount of reaction product was formed within 48 h, which was subsequently identified as SO_3^--B_{12} through HPLC. Similar results were shown for sodium sulfite. The effects of these food additives on the B_{12} content of red shrimp and beef meats were determined, revealing no significant difference in B_{12} content of shrimp and beef meats with or without the treatment even in hypochlorous acid water. The results suggest that these food additives could not react with food B_{12} in food, as most of this vitamin present in food is its protein-bound form rather than the free form.

Chapter V
Conclusions

Plants and animals cannot synthesize B_{12}; however, bacteria and archaea can synthesize it [9]. More than twenty years have passed since the discovery of pseudoB_{12} in the edible cyanobacterium *Spirulina* sp. [18], and for the last 10 years, advanced analytical techniques such as LC–MS/MS have been used to identify inactive corrinoids in food [24,26,27,29,30,68]. In this thesis, I characterized various inactive corrinoids found in edible shrimp and cricket products.

As shown in the preceding section, humans have a complex intestinal absorption system for dietary B_{12}, with the gastric B_{12}-transport protein (IF), playing a key role because of its binding specificity for biologically active B_{12} [13,69]. Thus, humans have the ability to select biologically active B_{12} from the various corrinoids found in food using the IF-mediated intestinal absorption system [70]. Given that inactive corrinoid compounds show no or very little affinity to human IF, their bioavailability is believed to be minimal in humans (**Fig. V-1**).

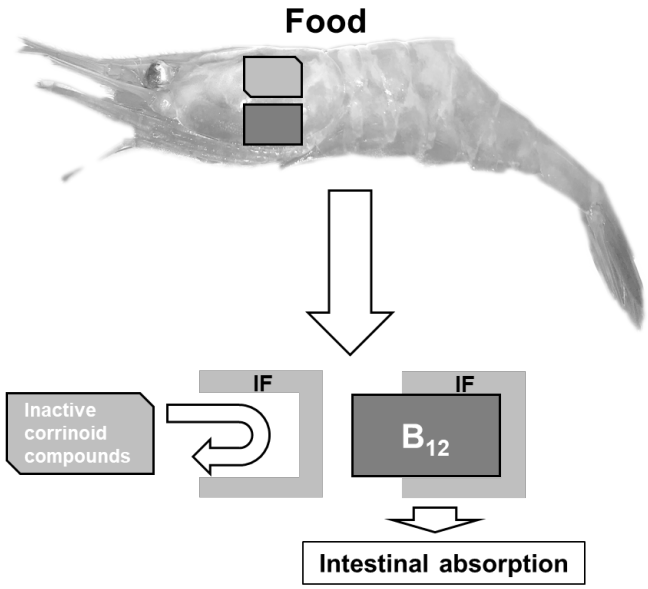

Fig. V-1. Intrinsic factor can select inactive corrinoids in humans.

Naturally occurring inactive cobamides

PseudoB$_{12}$ is the dominant corrinoid compound synthesized by certain bacteria [20–22] (**Fig. V-2**) and is found in human feces [19]. Shellfish is a major dietary source of B$_{12}$ in humans [25]. However, certain types of edible shellfish (e.g., herbivorous sea snails such as abalone and turban shell) contain substantial amounts of pseudoB$_{12}$, which is abundant in their viscera [26,27]. As shown in **Chapter III**, edible cricket products contain substantial amounts of pseudoB$_{12}$ and factor S, which has been reported to be found in human feces [19] and apple snail (escargot) products [24].

Factor IIIm is present in high quantities in escargot products [24]. Certain types, but not all, of *Chlorella* products used as human supplements, contain substantial amounts of factor IIIm [68]. Nevertheless, the occurrence of factor IIIm has not been reported in other foods. These findings indicate that these naturally occurring inactive cobamides are absent in major dietary B$_{12}$ sources, such meat, milk, and fish **(Table V-1**).

PseudoB$_{12}$ seldom binds to IF because of its very low affinity [23]. Additionally, although there is no information on the binding affinities of factor S and factor IIIm, studies evaluating the binding abilities of other corrinoid compounds with similar structures suggest that factor S is inactive [23] whereas factor IIIm is active [23].

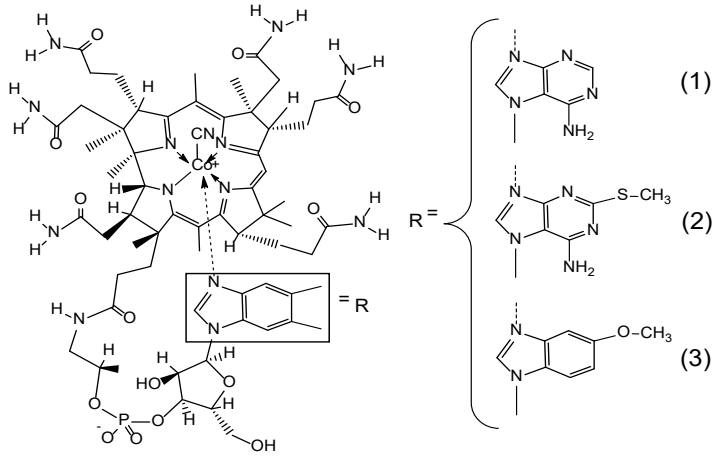

Fig. V-2. Naturally occurring cobamides as inactive corrinoids found in food. (1) Adenyl cobamide (pseudoB$_{12}$ or factor IV), (2) 2-methylmercaptoadenyl cobamide (factor S), and (3) 5-methoxybenzimidazolylcobamide (factor IIIm).

Table V-1. Naturally occurring inactive corrinoids in foods

Naturally occurring inactive cobamides	Food	Reference
PseudoB_{12}	Abalone innards	26
	Turban shell muscles and innards	27
	Cricket	This study
Factor S	Apple snail (escargot)	24
	Cricket	This study
Factor IIIm	Apple snail (escargot)	24
	Chlorella	68

Inactive corrinoids with a chemically modified corrin ring

Chloramine-T is an active chlorine compound with strong oxidative activity and is widely used as a biocide [71] and also produces a lactone form of B_{12}, B_{12}[*c*-lactone] [72]. Certain mushrooms contain considerable amounts of B_{12}[*c*-lactone] (**Table V-2 and -3**) [29,30]. The harmful effects of B_{12}[*c*-lactone] remain to be determined. As shown in the **Chapter II**, B_{12}-*d*-monocarboxylic acid and B_{12}-dicarboxylic acids have been found in shrimp head innards and their products. Oral and intravenous administrations of B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids have failed to improve B_{12} status in B_{12}-deficient rats [38], suggesting that both B_{12}-*d*-monocarboxylic acid and B_{12}-dicarboxylic acids are not absorbed in humans. These B_{12} compounds have not been reported in other foods.

Table V-2. Inactive corrinoids with a chemically modified corrin ring

Chemically modified cobalamins	Food	Reference
B_{12}[c-lactone]	dried mushroom	29,30
B_{12}-*d*-monocarboxylic acid B_{12}-dicarboxylic acid	shrimp head innards	This study

As shown in **Chapter IV**, food additives, such as hypochlorous acid water, sodium metabisulfite, and sodium sulfite, strongly affect the chemical and biological properties of B_{12} in aqueous solutions. However, the effects of these food additives on B_{12} content in red shrimp and beef meat have been determined, revealing no significant difference in B_{12} content in shrimp and beef meat regardless of treatment with hypochlorous acid water. The results suggest that these food additives may not react with B_{12} in food as most of it is present in a protein-bound form instead of the free form.

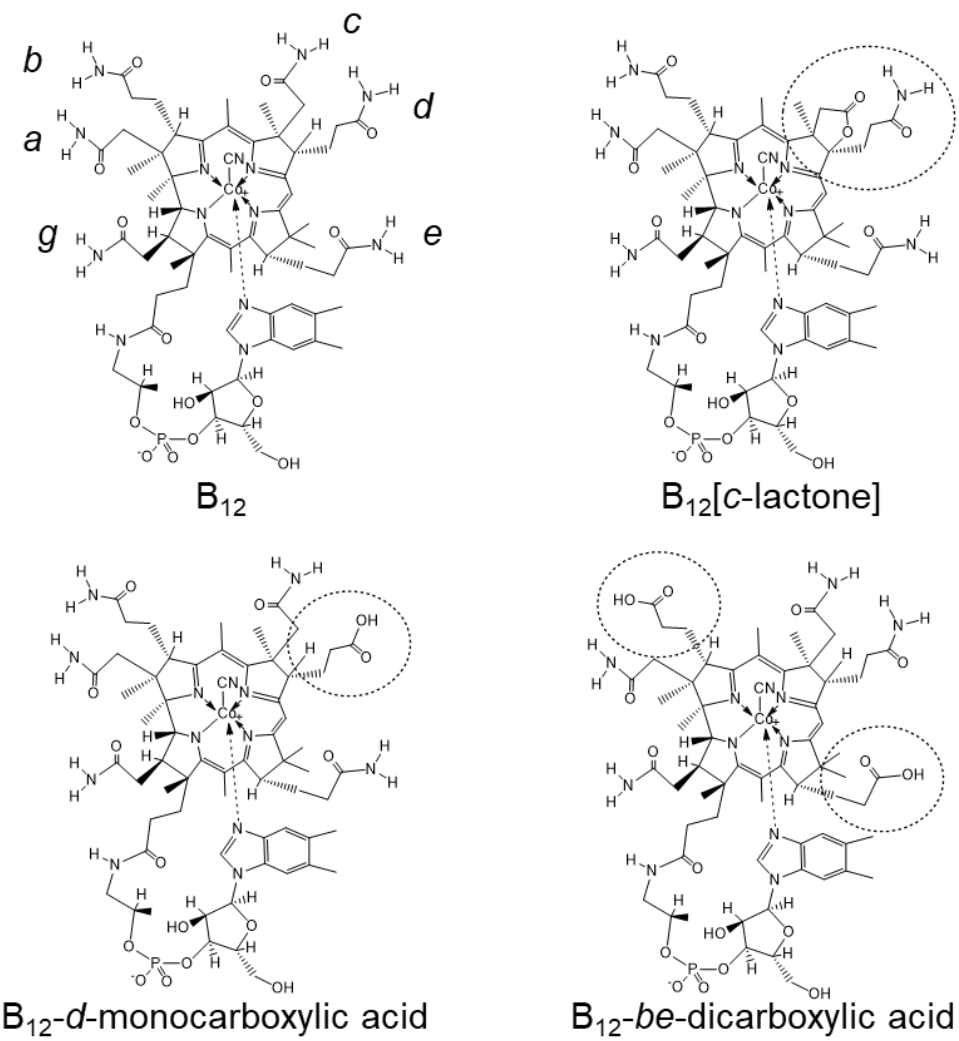

Fig. V-3. Chemically modified cobalamins in food. B_{12}-*be*-dicarboxylic acid represents one of the three B_{12}-dicarboxylic acids and has not been completely identified in shrimp head innards at this time.

Effects of inactive corrinoids on cellular B_{12} metabolism in mammalian cells

Recently, it has been reported that pseudoB_{12} may function as a coenzyme of MS in mammalian cultured cells [73]. Moreover, human MS apoenzyme reportedly has little specificity for alterations in various moieties of the B_{12} molecule [74]. Although B_{12}[*c*-lactone] and intact B_{12} show identical affinity for MS, naturally occurring cobamides with different base moieties (e.g., pseudoB_{12}) and B_{12} compounds with a modified corrin ring (e.g., B_{12}-*d*-monocarboxylic acid) can bind to human MS with a greater affinity than intact B_{12}, thereby fully activating it [74]. These results suggest that these corrinoid compounds function as MS coenzymes if absorbed in the intestine and transferred into the target cells. Unlike intact B_{12}, pseudoB_{12} cannot induce the expression of MS mRNA [73]. Therefore, it is probably not associated with the internal ribosome entry site [73].

Small changes in the lower nucleotides of B_{12} have significantly decreased the compound's binding affinity to human MCM, preventing adenosyl forms of pseudoB_{12} from serving as coenzymes or inhibitors [73]. Although this has not been investigated in related compounds, it is possible that the outcomes for factor S and factor IIIm would be comparable because the former has an adenine base and the adenosyl form of the latter has a much lower affinity for MCM than AdoB_{12}.

During B_{12} deficiency, a significant quantity of a stable MCM apoenzyme is accumulated in mammalian cells. In this case, pseudoB_{12} would neither function as a coenzyme of MCM nor induce the expression of MS mRNA in the cells. Yet, the activity of MS in vivo might decrease significantly, even though pseudoB_{12} can fully function as a coenzyme. PseudoB_{12} can bind TC II at a lower affinity than B_{12} [75]. Bito et al. [73] suggested that a substantially higher concentration of pseudoB_{12} inhibits the TC II-mediated uptake of B_{12} in mammalian cells. These results suggest that if a substantial amount of pseudoB_{12} is absorbed in the intestine, it may accelerate the development of B_{12} deficiency during B_{12}-deprived conditions. Bacteria or their products cross the intestinal barrier *via* bacterial translocation [76,77]. Moreover, alcohol and stress increase the membrane permeability of the intestinal

tract [78,79]. Thus, inactive corrinoids (pseduoB_{12}, factor S, and factor IIIm) synthesized by intestinal bacteria or ingested from edible cyanobacteria, snails, and crickets, as well as B_{12}-mono and -dicarboxylic acids that exist in shrimp head innards, can penetrate the intestinal wall and enter the bloodstream. Such unusual absorption of these inactive corrinoids might result in B_{12} deficiency, probably leading to the symptoms (multiple sclerosis and so on) known as B_{12}-analog diseases [80] (**Fig. V-4**).

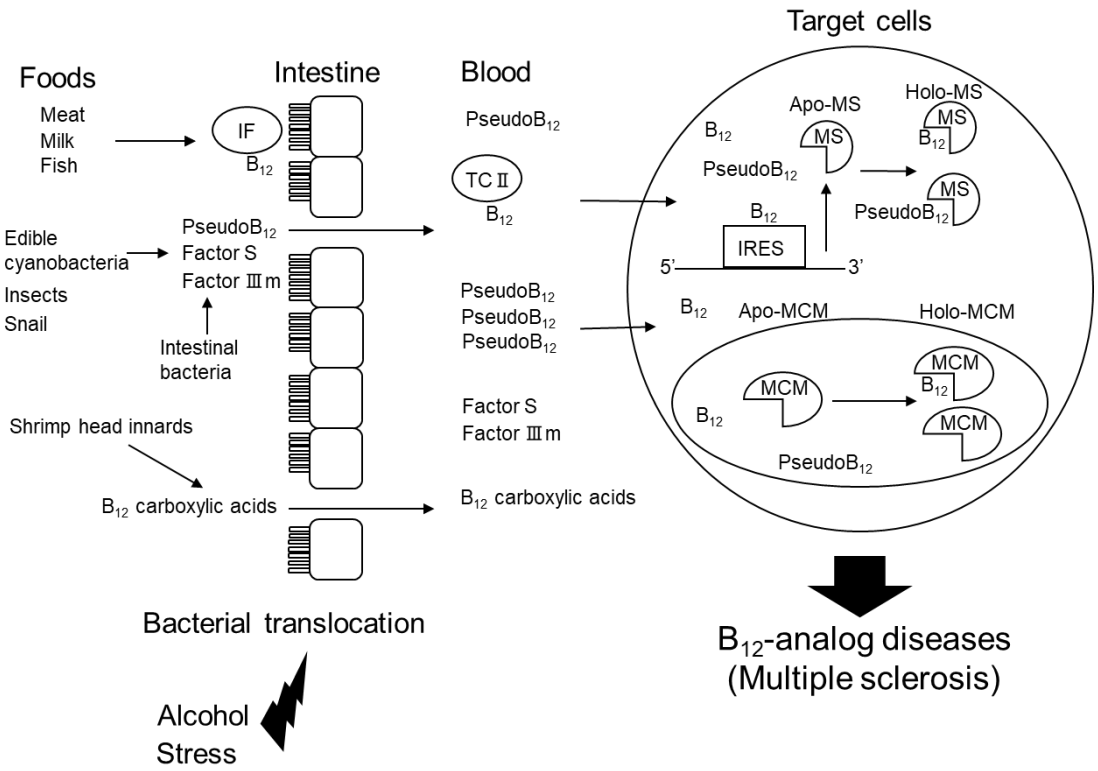

Fig. V-4. Effect of inactive corrinoids on cellular B_{12} metabolism of mammalian cells. MS, methionine synthase; MCM, methylmalonyl-CoA mutase; IRES, internal ribosome entry site.

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Summary

Vitamin B_{12} (B_{12}) is synthesized by specific archaea and bacteria and is concentrated mainly in the bodies of higher predatory organisms through the natural food chain. Thus, animal-derived foods (i.e., meat, milk, egg, fish, and shellfish) contained considerable amounts of B_{12}. In particular, fish and shell fish are reported to be important nutritional sources of B_{12} for humans. However, some species of edible snails apparently contain pseudoB_{12} inactive in humans. Shrimp are popular foods worldwide, but there is no information available on whether they contain pseudoB_{12}. Thus, I characterized B_{12} compounds in edible shrimp muscles and head innards (**Chapter II**). B_{12} contents in both the muscles and head innards of various shrimp species were determined using a microbiological assay based on *L. delbrueckii* subsp. *lactis* ATCC7830. A considerable amount of B_{12} (approximately 2.4–4.3 μg/100 g wet weight) was detected in shrimp muscles. The shrimp head innards contained significantly higher levels of B_{12} (approximately 12.5–33.2 μg/100 g wet weight). Commercially available shrimp innards products contained approximately 30 μg B_{12}/100 g wet weight. The muscle extract contained only one corrinoid compound, which was identified as B_{12} using LC/ESI-MS/MS, whereas the shrimp head innards contained three corrinoid compounds, which included large amounts of B_{12} and two smaller amounts of B_{12}-*d*-monocarboxylic acid and tentatively identified B_{12}-dicarboxylic acids.

Insects have a high nutritive value because they are rich in macronutrients, thus they can contribute to world food security and may replace animal-derived protein (e.g., meat and fish). However, there is little information on B_{12} content in edible insects. As shown in **Chapter III**, I determined the B_{12} content of commercially available edible insect products by the microbiological assay. Although B_{12} content of giant water bug, bee larva, grasshopper, and weaver ant products was low, diving beetle and cricket products contained high amounts of B_{12} (approximately 89.5 and 65.8 μg/100 g dry weight, respectively). In the cricket

products most widely circulated as foods, corrinoid compounds were identified using LC/ESI–MS/MS, indicating that PseudoB_{12} and factor S were the predominant corrinoid compounds (approximately 74% and 21%, respectively), with B_{12} making up only 5% of total corrinoids. Therefore, edible cricket products might not be a suitable source of B_{12}. These results indicated that inactive corrinoid compounds found in foods are divided into two categories: 1) naturally occurring cobamides such as pseudoB_{12} and factor S and 2) B_{12} compounds with chemically modified side chains of the corrin ring such as B_{12} carboxylic acids.

An unnatural and inactive B_{12} compound, B_{12}[*c*-lactone] formed by treatment with an organochlorine antibacterial agent, was found in some edible mushrooms. Moreover, preliminary experiments indicated that B_{12} is completely inactivated by treatment with hypochlorous acid water used to sanitize food products. The effects of some food additives on the chemical and biological properties of B_{12} under aqueous conditions were evaluated (**Chapter IV**). Food additives such as hypochlorous acid water, sodium metabisulfite, and sodium sulfite, strongly affect the chemical and biological properties of B_{12} in aqueous solution. When CN-B_{12} (10 μmol/L) was treated with these compounds, hypochlorous acid water (an effective chlorine concentration of 30 ppm) rapidly reacted with CN-B_{12}. The maximum absorptions at 361 and 550 nm were completely disappeared by 1 h, and B_{12} activity was lost. There were no significant changes observed in the absorption spectra of CN-B_{12} for 0.01% (w/v) sodium metabisulfite; however, a small amount of reaction product was formed within 48 h, which was subsequently identified as SO_3^--B_{12} through HPLC. Similar results were shown for sodium sulfite. The effects of these food additives on the B_{12} content of red shrimp and beef meats were determined, revealing no significant difference in B_{12} content of shrimp and beef meats with or without the treatment even in hypochlorous acid water. The results suggest that these food additives could not react with food B_{12} in food, as most of this vitamin present in food is its protein-bound form rather than the free form.

摘要

本研究では、食品に含まれるビタミン B_{12}(B_{12})化合物に焦点をあて、その特性を詳細に分析した。B_{12} は一部の細菌でのみ生合成され、食物連鎖により高等動物の体内に蓄積されるため、動物性食品(畜肉、牛乳、鶏卵、魚介類)が B_{12} の良い供給源となっている。これまでに、あらゆる食品において B_{12} 化合物種が特定されているが、一部の食品にはヒトにおいて不活性なシュード B_{12} が含まれていることが明らかになっている。本研究では、日本人にとって特に主要な B_{12} 供給源の一つである食用エビの筋肉部と頭部内臓部に含まれる B_{12} 化合物の特性を分析した。また、近年世界の食用安全保障に寄与する可能性を持つと注目されている食用昆虫に着目した。食用昆虫はタンパク質や脂質などの栄養素を豊富に含むことが知られているが、ビタミンに関する情報は限られており、含まれる B_{12} 化合物種についてはほとんど知られていない。したがって、食用昆虫の B_{12} 含量並びに食用として世界的に流通している食用コオロギの B_{12} 化合物を分析した。さらに、食品の加工段階で用いられる塩素系消毒剤との反応により生成する不活性型 B_{12} 化合物が、一部の乾燥キノコから検出された研究背景から、食品添加物処理によって生成する B_{12} 化合物の特性について詳細に分析した。

各食品中の B_{12} 含量は、日本食品標準成分表に準じて *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830 を用いた微生物学的定量法により測定した。また、LC/ESI–MS/MS を用いて各食品に含まれる B_{12} 化合物を分析した。

アマエビを始めとした 4 種の食用エビの B_{12} 含量は湿重量 100 g あたり約 2.4–4.3 μg であり、日本食品標準成分表に記載されている B_{12} 含量とほぼ同程度であった。一方、頭部内臓部には湿重量 100 g あたり約 12.5–33.2 μg の B_{12} が含まれており、内臓部には高濃度の B_{12} が蓄積していた。また、内臓部の加工品においても同程度の B_{12} 含量を示した。さらに、LC/ESI–MS/MS を用いて食用エビに含まれる B_{12} 化合物を詳細に分析した結果、筋肉部に含まれる B_{12} 化合物は B_{12} のみであったが、頭部内臓部では B_{12} と 2 種類の B_{12} 化合物(化合物 A と B)の存在が確認され、それらは B_{12} のコリン環の質量が 1 及び 2 大きい化合物であったことから、コリン環の側鎖が修飾されていることが明らかになった。化合物 A と B の分子量から B_{12} モノカルボン酸と B_{12} ジカルボン酸が推察され、HPLC 及び LC/ESI–MS/MS により分析した結果か

ら、化合物 A は B_{12}-*d*-モノカルボン酸と同定した。一方、化合物 B は同定することができなかったが、B_{12} ジカルボン酸であると推察した。また、食用エビの頭部内臓部に含まれる B_{12} 化合物の約 18–65%が B_{12} モノカルボン酸とジカルボン酸であり、それらの含量はエビの種類に依存すると考えられる。さらに、B_{12}-*d*-モノカルボン酸は *L. delbrueckii* ATCC 7830 において活性を示さなかったため、頭部内臓部の B_{12} 含量には影響を与えないと考えられる。

また、食用コオロギに含まれる B_{12} 化合物について検討した。市販されている 6 種類の食用昆虫製品の B_{12} 含量を測定した結果、タガメ、ハチノコ、イナゴ、アリでは、乾燥重量 100 g あたり約 1.1–3.2 µg と低い値を示したのに対して、ゲンゴロウとコオロギは約 89.5–65.8 µg と多量の B_{12} を含んでいた。さらに、食品としての流通が盛んであるコオロギ製品の B_{12} 化合物を分析した結果、コオロギ製品に含まれる B_{12} 化合物はシュード B_{12} が約 74%、factor S が約 21%、そして B_{12} は約 5%程であり、コオロギ製品の主要なコリノイド化合物は、ヒトにおいて生理的に不活性なシュード B_{12} と factor S であることが明らかになった。これらの結果から、コオロギ製品の B_{12} 含量は乾燥重量 100 g あたり約 3.2 µg であり、一日の推奨量（2.4 µg/日）を満たすには約 80 g を消費する必要があることから、コオロギ製品は B_{12} の供給源として適していないことが明らかになった。

最後に、食品添加物の処理によって生成される B_{12} 化合物の化学的および生物学的特性について検討した。次亜塩素酸水(有効塩素濃度 30 ppm)処理によって、B_{12} に特有な 361 nm と 551 nm の最大吸収が反応時間 1 h で完全に消失した。また、次亜塩素酸水と B_{12} との反応によって生成する化合物を ^{1}H NMR を用いて分析した結果、コリン環の側鎖が修飾された構造を有しており、これらは生理活性を示さない化合物であった。ピロ亜硫酸ナトリウムと亜硫酸ナトリウム処理においては著しい吸収スペクトルの変化は確認されなかったが、48 h 以内に少量の反応生成物が生成され、HPLC 分析により生物活性を有するスルフィト B_{12} と同定した。さらに、これらの食品添加物が食品中の B_{12} 含量に及ぼす影響を検討するため、赤エビと牛ミンチ肉を各食品添加物で処理した。その結果、いずれの食品添加物処理においても各食品中の B_{12} 含量の減少は認められなかった。これは食品中の B_{12} のほとんどがタンパク質結合型として存在しているため、食品中の B_{12} との反応が緩和され B_{12} 含量が保持されたものと推察される。

学位論文の基礎となる学会誌公表論文のリスト

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2. Naho Okamoto, Tomohiro Bito, Nanami Hiura, Ayaka Yamamoto, Mayu Iida, Yasuhiro Baba, Tomoyuki Fujita, Atsushi Ishihara, Yukinori Yabuta, and Fumio Watanabe (2020) Food Additives (Hypochlorous Acid Water, Sodium Metabisulfite, and Sodium Sulfite) Strongly Affect the Chemical and Biological Properties of Vitamin B_{12} in Aqueous Solution. *ACS Omega*, 5 (11): 6207−6214. (**Chapter IV**)