Food Additives (Hypochlorous Acid Water, Sodium Metabisulfite, and Sodium Sulfite) Strongly Affect the Chemical and Biological Properties of Vitamin B₁₂ in Aqueous Solution

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ABSTRACT: Food additives, such as hypochlorous acid water, sodium metabisulfite, and sodium sulfate, strongly affect the chemical and biological properties of vitamin B₁₂ (cyanocobalamin) in aqueous solution. When cyanocobalamin (10 μmol/L) was treated with these compounds, hypochlorous acid water (an effective chlorine concentration of 30 ppm) rapidly reacted with cyanocobalamin. The maximum absorptions at 361 and 550 nm completely disappeared by 1 h, and vitamin B₁₂ activity was lost. There were no significant changes observed in the absorption spectra of cyanocobalamin for 0.01% (w/v) sodium metabisulfite; however, a small amount of the reaction product was formed within 48 h, which was subsequently identified as sulftocobalamin through high-performance liquid chromatography. Similar results were shown for sodium sulfate. The effects of these food additives on the vitamin B₁₂ content of red shrimp and beef meats were determined, revealing no significant difference in vitamin B₁₂ 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 vitamin B₁₂ in food, as most of this vitamin present in food is its protein-bound form rather than the free form.

INTRODUCTION

Vitamin B₁₂ or cobalamin (B₁₂) is a well-established member of corrinoids.¹ B₁₂ is synthesized by certain archaea and bacteria but not by animals or plants.² B₁₂ synthesized by bacteria and archaea is accumulated in animal tissues through the natural food chain.² Thus, animal-derived foods are good sources of B₁₂ for humans.³,⁴ Consequently, strict vegetarians are at a greater risk of developing B₁₂ deficiency.⁵

Following analysis of B₁₂ compounds in various foods using liquid chromatography-tandem mass spectrometry, certain foods contained a substantial amount of pseudovitamin B₁₂,⁶-⁸ which has adenine in place of 5,6-dimethylbenzimidazole as base in the lower nucleotide of the molecule.¹ Moreover, an unnatural and inactive B₁₂ compound, B₁₂ [c-lactone], was found in some edible mushrooms.⁵,¹⁰ B₁₂ [c-lactone] was readily formed from B₁₂ by treatment with the organochlorine antibacterial agent chloramine-T.¹⁰ In addition, preliminary experiments indicate that B₁₂ is completely inactivated upon treatment with hypochlorous acid water. Hypochlorous acid is usually used to sanitize food products such as vegetables, fruits, and meat.¹¹-¹³ This acid is widely used in the food industry as the disinfection agents of food processing equipment.¹²,¹³ However, hypochlorous acid readily reacts with proteins to form an aggregate and oxidizes specific amino acids.¹⁴,¹⁵ High hypochlorous acid treatment reportedly induces a significant decrease of vitamin C content in vegetables.¹⁶ There is limited information on the disadvantages associated with the use of hypochlorous acid. If hypochlorous acid has the ability to readily degrade B₁₂, ingestion of the formed products might induce B₁₂ deficiency in humans. To study whether such B₁₂ degradation products formed by hypochlorous acid are harmful to humans, some of these compounds, which were unstable, were purified and characterized in this study.

Furthermore, other preliminary experiments indicate that food additives such as sodium metabisulfite,¹⁵ and sodium sulfate,¹⁷,¹⁸ had the ability to change the ultraviolet-visible (UV-visible) absorption maxima of B₁₂ in aqueous solution. These results and observations suggest the possibility that B₁₂ is readily inactivated by treatment with these food additives; hence, its nutritional values may be significantly reduced.

In this study, we evaluated the effects of hypochlorous acid water, sodium metabisulfite, and sodium sulfate on the chemical and biological properties of B₁₂ under aqueous conditions. Furthermore, we examined whether significant loss of B₁₂ occurs in food treated with these food additives.
MATERIALS AND METHODS

Materials. Cyanocobalamin (CN-B12) and hydroxocobalamin were purchased from Sigma-Aldrich (St. Louis, MO). Sulfuricobalamin was synthesized from hydroxocobalamin by treatment with sodium sulfite according to the method described by Suarez-Moreira et al.10 Food additives, such as hypochlorous acid water, sodium metabisulfite, and sodium sulfite were purchased from markets in Japan. Argentine red shrimp (Pleoticus muelleri [Bate]) was purchased from a local market in Tottori City, Japan.

UV-Visible Spectra of CN-B12 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-B12 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 timepoints, the UV–visible absorption spectra of these solutions were measured using a UV–visible spectrophotometer (UV-2550; SHIMAD-ZU Corp., Kyoto, Japan).

High-Performance Liquid Chromatography (HPLC) Analysis of B12 Compounds Formed by Treatment with These Food Additives. CN-B12 was treated with hypochlorous acid water (an effective chlorine concentration of 10 ppm) for 1 h as described above. Subsequently, the treated CN-B12 solution was immediately loaded onto a Sep-Pak Vac (5 g) C18 cartridge (Waters Corp., Milford, MA) equilibrated with 20 mL of distilled water after washing with 20 mL of 75% (v/v) ethanol solution. The C18 cartridge was washed with 20 mL of distilled water, and the B12 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 UV-visible absorption. The UV-visible absorption spectra of these solutions were measured using a UV-visible spectrophotometer (UV-2550; SHIMAD-ZU Corp., Kyoto, Japan).

Effect of These Food Additives on the B12 Content of Red Shrimp Meat. The edible portion (approximately 150 g) of Argentine red shrimps was collected and homogenized using a 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 cm³) from each sample were formed and subsequently allowed to stand at 4 °C for 48 h in the dark. B12 was extracted from aliquots (2.0 g) of each stored sample and determined using Lactobacillus delbrueckii American Type Culture Collection (ATCC® 8014) bioassay as previously described.21

Effect of Various Concentrations of Hypochlorous Acid Water on the B12 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 cm³) from each beef meat were formed and subsequently allowed to stand at 4 °C for 48 h in the dark. B12 was extracted and assayed as described above.

Statistical Analysis. One-way analysis of variance and post-hoc analysis were performed using Dunnnett’s multiple comparison tests to evaluate the effect of food additives on the B12 content of red shrimp meat and to determine the effect of various concentrations of hypochlorous acid water on the B12 content of ground beef meat. Analyses were performed using GraphPad Prism 3 for Windows Version 2.01 (GraphPad Software Inc., La Jolla, CA). Data are presented as the mean ± standard error of the mean (SEM). p < 0.05 denoted statistically significant differences.
**RESULTS**

UV–Visible Spectra of CN-B₁₂ Treated with or without Selected Food Additives in an Aqueous Solution. On the basis of preliminary experiments, hypochlorous acid water, sodium metabisulphite, and sodium sulfite were selected as food additives. Changes in the UV–visible spectra of CN-B₁₂ 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-B₁₂ 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-B₁₂, 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 (Figure 1A). These specific absorption maxima of CN-B₁₂ completely disappeared by 1 h.

Addition of 0.01% (w/v) sodium metabisulphite 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-B₁₂ were hardly changed for 48 h (Figure 1B). Sodium sulfite also showed similar spectroscopic behavior (Figure 1C).

B₁₂ Compounds Formed during Treatment with Hypochlorous Acid Water. The B₁₂ 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-B₁₂. When CN-B₁₂ 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-B₁₂ and B₁₂ (ε-lactone), respectively. Instead, the B₁₂ 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) (Figure 2). The compounds P-2, P-3, and P-6 could be purified and subjected to ¹H NMR spectroscopy.

**Figure 1.** UV–visible absorption spectra of CN-B₁₂ after treatment with these food additives. CN-B₁₂ was dissolved in hypochlorous acid water (at an effective chlorine concentration of 30 ppm) (A), 0.01% (w/v) sodium metabisulphite (B), and 0.01% (w/v) solution sodium sulfite (C) solutions at a final concentration of 10 µmol/L. These solutions were left for 0 h (--), 1 h (− − −), 24 h (− − −), and 48 h (− − − −) at room temperature (25 °C). A solution of CN-B₁₂ treated without these food additives was used as control (− −). At the indicated time points, the UV–visible absorption spectra of these solutions were measured using a UV–visible spectrophotometer. These are typical spectrophotometric data obtained from three independent experiments.

**Figure 2.** HPLC patterns of the B₁₂ compounds treated with hypochlorous acid water. CN-B₁₂ was dissolved in hypochlorous acid water (at an effective chlorine concentration of 10 ppm) at a final concentration of 10 µmol/L and left for 1 h at room temperature (25 °C) in the dark. The CN-B₁₂ solution was loaded onto a Sep-Pak Vac (5 g) C18 cartridge to remove the hypochlorous acid. The fraction of the B₁₂ compounds was dissolved in a small amount of water and subsequently used as a sample for HPLC analysis. An aliquot (30 µL) of the sample was placed on a reversed-phase HPLC column. The B₁₂ 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 HPLC patterns of the B₁₂ compounds are typical data obtained from three independent experiments.

We compared the ¹H NMR spectra of the purified compounds with that of authentic CN-B₁₂ (Figure 3). In the spectrum of compound P-2 (Figure 4A), the signal corresponding to the olefinic proton at C10 disappeared, whereas the characteristic signals coupled with each other at δH 3.86 (1H, d, J = 19.0 Hz) and δH 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 (δH 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-aminosipryl 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 vitamin B₁₂.

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The spectra of compounds P-2 and P-3 (Figures 4A,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 B12 were detected in the spectrum, indicating that the corrin ring skeleton and β-aminoisopropyl groups were retained in compound P-3. Therefore, compounds P-2 and P-3 may be structural isomers.

The signal corresponding to B4 at δH 7.32 remained on the dimethylbenzimidazole ring in the spectrum of compound P-6 (Figure 4C), whereas the signal corresponding to B7 disappeared. Thus, the B7 position on the dimethylbenzimidazole group was likely substituted by treatment. The chemical shifts of the signals of methyl groups at C35 and C53 (δH 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 treatment.

**B12 Compounds Formed during Treatment with Sodium Metabisulfite and Sodium Sulfite.** Following the treatment of CN-B12 with sodium metabisulfite for 48 h and analysis using reversed-phase HPLC, the B12 compounds were isocratically eluted as major and minor peaks with retention times of 8.7 and 27.5 min, respectively (Figure 5A). The major peak with a retention time of 8.7 min was identical to that of authentic CN-B12 (data not shown), suggesting that most CN-B12 was not changed by treatment with sodium metabisulfite. Previous studies reported that hydroxocobalamin was readily converted to sulfotocobalamin by the addition of sodium metabisulfite. When sulfotocobalamin was prepared and analyzed using HPLC, the minor peak with a retention time of 27.5 min was identical to that of sulfotocobalamin (Figure 5B). Similar results were obtained for sodium sulfite (data not shown).

**Biological Activity of the B12 Compounds Formed during Treatment with Selected Food Additives.** Sulfotocobalamin is well established as a naturally occurring biologically active B12. The minor peak (with a retention time of 27.5 min) formed by the addition of sodium metabisulfite or sodium sulfite (Figure 5A) was active in B12-dependent E. coli 215 (data not shown). However, based on the absence of bacterial growth, B12-dependent E. coli 215 bioautography of hypochlorous acid-treated B12 compounds indicated that hypochlorous acid water readily inactivated CN-B12 in aqueous solution (Figure 6).

**Effect of the Selected Food Additives on the B12 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 B12 content in food. B12 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. To evaluate the effect of food additives on the B12 content of red shrimp meat, one-way analysis of variance and post-hoc analysis were performed using Dunnett’s multiple comparison tests. When p < 0.05 denoted statistically significant differences, there was no significant decrease detected in the B12 content of the red shrimp meat treated with these food additives (Figure 7).

**Effect of Various Concentrations of Hypochlorous Acid Water on the B12 Content of Ground Beef Meat.** Hypochlorous acid is usually used to sanitize vegetables, fruits, and meats. As animal-derived foods are good sources of B12 for humans, the effect of various concentrations of hypochlorous acid on the B12 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 to determine whether these food additives can reduce the B12 content in food. B12 was extracted from the ground beef meat treated with or without these food additives, and its amount was determined using the *L. delbrueckii* ATCC 7830 bioassay.
°C in the dark. B12 was extracted from the ground beef meat treated with or without hypochlorous acid water, and its amount was determined using the bioassay. The B12 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 (Figure 8).

**DISCUSSION**

An unnatural and inactive B12 compound, B12 [β-lactone], has been formed from B12 and the organochlorine antibacterial agent chloramine T.¹⁰ Thus, food additives that may inactivate B12 were screened for their ability to change the UV–visible absorption spectra of CN-B12. According to the results of these preliminary experiments, hypochlorous acid water, sodium metabisulfite, and sodium sulfitre were selected. Hypochlorous acid water is a highly safe antimicrobial agent and widely used as a food additive.¹¹–¹³ However, treatment with hypochlorous acid water (an effective chlorine concentration of 30 ppm) resulted in immediate and significant decreases of the absorption peaks at 278, 365, and 586 nm. Notably, these absorption maxima of CN-B12 completely disappeared by 1 h (Figure 1A). This finding suggested the destruction of the corrin ring and the liberation of the central cobalt ion of CN-B12 following treatment with hypochlorous acid water. A high concentration of hypochlorous acid reportedly induced the oxidative cleavage of the corrin ring and the porphyrin ring, destroying these compounds.²⁴,²⁵ Although these observations suggest that the treatment of food with hypochlorous acid water induces significant loss of B12, this agent did not affect the level of B12 in this study (Figures 7 and 8). These results imply that hypochlorous acid water could not react with food B12, which is present in its protein-bound form in food.²⁶,²⁷

Hypochlorous acid is generated by myeloperoxidase and plays an important role in the innate immune system of mammals.²⁸ 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.²⁵,²⁹ This observation suggests that hypochlorous acid water as a food additive adversely affects food proteins in foods, but not B12.

Through HPLC analysis, we detected five major peaks as degradation products from B12 by HPLC analysis. Among them, we 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 ¹H NMR spectroscopy. On the basis of the ¹H NMR spectra, compounds P-2 and P-3 may be structural isomers, which lacked the olefinic proton at C10. On the other hand, compound P-6 lacked the B7 proton on the dimethybenzoimidazole ring instead of C10. Thus, the C10

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**Figure 4.** ¹H NMR spectra of B12 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).
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 B12. Determination of the chemical structures of these compounds would lead to a better understanding of the degradation mechanism of B12 by hypochlorous acid water.

When CN-B12 was treated with sodium metabisulfite or sodium sulfite for 48 h, a small amount of sulftocobalamin was formed. Sulftocobalamin was formed from glutathionylcobalamin, a product formed by the decyanation reaction of CN-B12 in the presence of reduced glutathione in mammalian cells. When CN-B12 was treated with sodium metabisulfite or sodium sulfite for 48 h, a small amount of sulftocobalamin was formed. Sulftocobalamin was formed from glutathionylcobalamin, a product formed by the decyanation reaction of CN-B12 in the presence of reduced glutathione in mammalian cells. Sulftocobalamin, which is one of the naturally occurring cobalamins in foods, is biologically active in humans. However, it has been reported that, in humans, the intestinal absorption of sulftocobalamin was lower than that of CN-B12.

The results of this study indicate that these food additives have the ability to significantly change the properties of B12 in aqueous solution; however, they are unable to reduce the B12 content of food.

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Figure 5. HPLC patterns of the B12 compounds treated with 0.01% (w/v) sodium metabisulfite. CN-B12 was dissolved in 0.01% (w/v) sodium metabisulfite solution at a final concentration of 10 μmol/L and left for 48 h at room temperature (25 °C) in the dark. (A) Treated CN-B12 solution (50 μL) or (B) authentic sulftocobalamin (20 μL of 130 mg/L) was loaded onto a reversed-phase HPLC column. The B12 compounds were isocratically eluted and monitored by measuring the absorbance at 361 nm. Data are typical HPLC patterns of the B12 compounds treated with sodium metabisulfite or authentic sulftocobalamin obtained from three independent experiments.

Figure 6. Effects of the B12 compounds treated with hypochlorous acid water on B12-dependent E. coli 215. (A) Authentic B12 (100 pg) and (B) hypochlorous acid-treated B12 compounds after purification using a Sep-Pak Vac (5 g) C18 cartridge.

Figure 7. Effects of treatment with hypochlorous acid water, sodium metabisulfite, and sodium sulfite on the B12 content of red shrimp meat. The edible portion of Argentine red shrimps was collected and homogenized using a mortar and pestle. The shrimp meat homogenate (10 g) was treated with 1.0 mL of distilled water as a control (1), hypochlorous acid water (at an effective chlorine concentration of 30 ppm) (2), 0.1% (w/v) sodium metabisulfite solution (3), and 0.1% (w/v) sodium sulfite solution (4) and subsequently mixed. Three sets of putties (3 × 3 × 1 cm³) from each sample were formed and then allowed to stand at 4 °C for 48 h in the dark. The B12 was extracted from each stored sample, and its amount was determined using L. delbrueckii ATCC 7830 bioassay. B12 content was assayed in triplicate. Data are represented as means ± SEM (n = 3).

Figure 8. Effects of various concentrations of hypochlorous acid water on the B12 content of ground beef meat. Ground beef meat (10 g) was treated with 1.0 mL of distilled water as a control (1) and treated with 1.0 mL of hypochlorous acid water at effective chlorine concentrations of 30 ppm (2), 60 ppm (3), and 80 ppm (4) and subsequently mixed. Three sets of putties (3 × 3 × 1 cm³) from each sample were formed and then allowed to stand at 4 °C for 48 h in the dark. B12 was extracted from each stored sample, and its amount was determined using L. delbrueckii ATCC 7830 bioassay. B12 content was assayed in triplicate. Data are represented as means ± SEM (n = 3).
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Author Contributions
N.O., A.Y., M.I., and Y.B. performed most experiments. T.F. and A.I. analyzed the cobalamin products formed by the experiments. T.F., N.O., T.B., Y.Y., A.I., and F.W. designed the experiments, interpreted the results, and wrote and approved the manuscript. All authors commented on the manuscript and approved the final version.

Notes
The authors declare no competing financial interest.

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