Characterization of Vitamin B$_{12}$ Compounds in Fermented Poultry Manure Fertilizers

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Abstract: (1) Background: Currently, no data are available on the vitamin B$_{12}$ content of an organic fertilizer product, viz. fermented poultry manure, or whether the organic fertilizer product contains vitamin B$_{12}$ or inactive corrinoids (or both). (2) Methods: This study conducted a microbiological assay to determine the vitamin B$_{12}$ content of various commercially available fermented poultry manure fertilizer products. (3) Results: The results varied from 1.4 µg to approximately 20 µg per 100 g of dry weight. In the bioautography analysis, selected products had two positive spots with identical $R_f$ values of vitamin B$_{12}$ and pseudovitamin B$_{12}$. High-performance liquid chromatography and liquid chromatography/electrospray ionization–mass spectrometry analyses of the selected products indicated that these fertilizers primarily contained vitamin B$_{12}$. They also contained minor inactive cobamides such as pseudovitamin B$_{12}$, 2-methyladenyl cobamide, and 2-methylmercaptoadenyl cobamide. (4) Conclusions: These results suggested that edible plants would enrich vitamin B$_{12}$ using fermented poultry manure organic fertilizer products.

Keywords: cobalamin; organic fertilizers; fermented poultry manure products; inactive cobamides; vitamin B$_{12}$

1. Introduction

Vitamin B$_{12}$ (B$_{12}$) or cobalamin is synthesized only by certain bacteria and archaea [1]. Animal-derived foods such as fish, shellfish, milk, and meat, are the major dietary B$_{12}$ sources, whereas plant-derived foods, such as fruits, vegetables, and grains, appear to contain none or trace levels of B$_{12}$ [2]. The recommended dietary allowance (RDA) for adults is 2.4 µg of B$_{12}$ per day [3,4], and this value is the lowest among the RDA of all vitamins. Thus, vegetarians and particularly vegans have a low intake of B$_{12}$. Strict vegetarians are at high risk of developing B$_{12}$ deficiency [5,6]. B$_{12}$ deficiency causes hematological, psychiatric, and neurological disorders [7]. Megaloblastic anemia is an early hematological sign. Neuropsychiatric disorders, subacute combined degeneration, and peripheral neuropathy are the most common neurologic manifestations [8]. Hence, production of vegetables containing sufficient quantities of B$_{12}$ is required to prevent vegetarians from developing B$_{12}$ deficiency.

Mozafar [9] indicated that the B$_{12}$ content in vegetables was significantly increased by the addition of cow manure fertilizer. A preliminary study showed that the addition of fermented poultry manure products as organic fertilizers increased the amount of B$_{12}$ (0.43–0.91 µg/100 g wet weight) in rice bran compared with that in the control (0.03–0.23 µg/100 g wet weight) without the fertilizer treatment. Furthermore, the B$_{12}$ contents in rice bran increased with increasing B$_{12}$ content in soils, suggesting that B$_{12}$ would be enriched in rice bran by the addition of fermented poultry manure products.
However, some intestinal bacteria reportedly can synthesize various cobamides with various base moieties in the α-axial ligand of the molecule [10]. Various cobamides that were inactive in humans exist in animal feces [11] and intestinal digesta [12]. Although there are various types of commercially available fermented poultry manure products, there are few reports on the $B_{12}$ level of these products and on whether these products contain inactive cobamides or $B_{12}$.

Therefore, we conducted this study to determine the $B_{12}$ content of commercially available poultry manure organic fertilizer products and characterize the $B_{12}$ compounds found in selected fertilizers.

2. Materials and Methods

2.1. Materials

$B_{12}$ was obtained from Sigma (St. Louis, MO, USA). Pseudovitamin $B_{12}$ (Pseudo$B_{12}$), benzimidazolyl cyanocobamide (BIA), and 5-hydroxybenzimidazolyl cyanocobamide (Factor III) were obtained as described previously [13]. The $B_{12}$ microbial assay medium for *Lactobacillus delbrueckii* ATCC7830 was purchased from Nissui (Tokyo, Japan). We used an ultraviolet (UV)-visible spectrophotometer (Shimadzu, Kyoto, Japan) to determine the turbidity of microbial test cultures. Various types of commercially available fermented poultry manure organic fertilizers and rice brans were purchased from local markets in Japan. Samples A, B, C, D, G, H, and I were obtained from fermented poultry manure fertilizer makers located in Okayama, Fukuoka, Saitama, Hokkaido, Hyogo, Hiroshima, and Kagawa prefectures, and samples E and F were from different makers in Aichi prefecture, Japan.

2.2. Extraction and Assay of Vitamin $B_{12}$

We added rice brans or fermented poultry manure fertilizer products (10 g each) were added to 100 mL of 5 mmol/L acetate buffer (pH 4.5) containing 0.01% (w/v) potassium cyanide (KCN). Total $B_{12}$ was extracted by boiling this mixture for 30 min in a fume hood (Dalton Co., Tokyo, Japan). The $B_{12}$ content was evaluated by the microbiological assay using *L. leichmannii* ATCC 7830. The obtained $B_{12}$ extracts were diluted with distilled water and used as samples. The turbidity (% T) of *Lactobacillus* test culture was determined at 600 nm using a Shimadzu spectrophotometer. This bacterium can use an alkali-resistant factor (deoxyribosides or deoxyribonucleotides) and $B_{12}$ [14]. We calculated the amount of $B_{12}$ by subtracting the values of the alkali-resistant factor from the total $B_{12}$ values.

2.3. Thin-Layer Chromatography (TLC) Bioautography Assay Using $B_{12}$-Dependent *Escherichia coli* 215

The extracts of selected fertilizer A, B, and C products listed in Table 1 were placed on C18 Cartridges (Sep-Pak® Vac 20 cc (5 g), Waters Corp., Milford, MA, USA), which was washed with 30 mL of distilled water. The $B_{12}$ compounds were eluted with 30 mL of 75% (v/v) ethanol. We evaporated the eluate to dryness under reduced pressure and dissolved the residual fraction in a small amount of distilled water. These extracts (1 µL) were placed onto silica gel 60 TLC plates and treated with 2-propanol/NH$_4$OH (28%)/water (7:1.2 v/v/v) as a solvent in the dark at room temperature (25 °C). $B_{12}$ compounds on the TLC plate was visualized with 2,3,5-triphenyltetrazolium salt using the method of Tanioka et al. [15].
Table 1. Vitamin B$_{12}$ contents in nine types of commercially available fermented poultry manure organic fertilizer products. B$_{12}$ was extracted from various fermented poultry manure products by the KCN-boiling method and determined by the microbiological method as described in the text.

| Fertilizer Products | Manufacture’s Location in Japan | Vitamin B$_{12}$ Contents (µg/100 g Dry Weight) |
|---------------------|--------------------------------|-----------------------------------------------|
| A                   | Okayama prefecture             | 20.6 ± 0.8                                   |
| B                   | Fukuoka prefecture             | 12.6 ± 0.5                                   |
| C                   | Saitama prefecture             | 8.5 ± 0.2                                    |
| D                   | Hokkaido prefecture            | 7.4 ± 0.2                                    |
| E                   | Aichi prefecture               | 4.8 ± 0.2                                    |
| F                   | Aichi prefecture               | 12.5 ± 0.5                                   |
| G                   | Hyogo prefecture               | 10.1 ± 0.3                                   |
| H                   | Hiroshima prefecture           | 6.8 ± 0.1                                    |
| I                   | Kagawa prefecture              | 1.4 ± 0.0                                    |
| Mean ± SD           |                                | 9.4 ± 5.5                                    |

2.4. High-Performance Liquid Chromatography (HPLC)

The selected fertilizer extracts were treated with Sep-Pak $^®$ Vac 20 cc (5 g) C18 Cartridges (Waters Corp.) under the same above-described conditions. The eluate (approximately 30 mL) was evaporated to dryness under reduced pressure, dissolved in 5 mL of distilled water, and centrifuged for 10 min at 10,000 g. We loaded the supernatant onto an EASI-EXTRACT $^®$ B$_{12}$ Immunoaffinity Column (P80) (R-Biopharm AG, Darmstadt, Germany) and then purified the B$_{12}$ compounds according to the manufacturer’s recommended protocol. The purified samples were dissolved in distilled water and filtered using a Nanosep MF centrifuge device (0.4 µm, Pall Corp., Tokyo, Japan) to remove small particles. The purified compounds were dissolved in 80 µL of Milli-Q water, filtered using a membrane filter, and then analyzed using an HPLC apparatus (SPD-10AV UV-Vis detector, SCL-10A VP system controller, DGU-20A degasser, LC-10Ai liquid chromatography, and CTO-20A column oven). An aliquot (35 µL) of the samples was placed on a reversed-phase HPLC column (Wakosil-II 5C18RS, φ 4.6 × 150 mm; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) equilibrated with 20% (v/v) methanol containing 1% (v/v) acetic acid. The corrinoids were isocratically eluted with the same solution at a flow rate of 1.0 mL/min at 40 °C and monitored by determining the absorbance at 361 nm. We analyzed authentic B$_{12}$, BIA, Factor III, and PseudoB$_{12}$ were analyzed under the same conditions. The retention times of the authentic Factor III, BIA, PseudoB$_{12}$, and B$_{12}$ were 7.7, 8.0, 8.5, and 10.3 min, respectively.

2.5. Liquid Chromatography–Mass Spectrometry (LC–MS)

We analyzed an aliquot (2 µL) of the compounds purified using the above-described immunoaffinity column by a LCMS-IT-TOF system (Shimadzu) using the method of Okamoto et al. [14]. The identification of B$_{12}$ or cyanocobalamin (C$_{63}$H$_{88}$CoN$_{14}$O$_{14}$P) (m/z 678.2914), BIA (C$_{61}$H$_{84}$CoN$_{14}$O$_{14}$P) (m/z 664.2748), Factor III (C$_{61}$H$_{84}$CoN$_{14}$O$_{15}$P) (m/z 672.2715), adenyl cyanocobamide or PseudoB$_{12}$ (C$_{59}$H$_{83}$CoN$_{17}$O$_{14}$P) (m/z 672.7749), 2-methyladenyl cyanocobamide or Factor A (C$_{60}$H$_{85}$CoN$_{17}$O$_{14}$P) (m/z 679.7834), 2-methylmercaptoadenyl cyanocobamide or Factor S (C$_{60}$H$_{85}$CoN$_{17}$O$_{14}$P) (m/z 695.7657), and p-cresolyl dicyanocobamide (C$_{62}$H$_{86}$CoN$_{13}$O$_{15}$P) (m/z 659.2783) and representing [M + 2H]$^{2+}$ was confirmed by comparison of the observed molecular ions.

3. Results and Discussion

The amount of B$_{12}$ in nine types of commercially available fermented poultry manure fertilizer products was determined using a microbiological method (Table 1). The B$_{12}$ content of the fermented poultry manure fertilizer products varied from 1.4 µg to approximately 20 µg per 100 g of dry weight. The B$_{12}$ contents of organic wastes (cow dung and sheep feces) used as organic fertilizers were 14–100 and 186 µg/100 g, respectively [9].
The B₁₂ contents of the fermented poultry manure fertilizers tested in the present study were lower than those of these organic wastes. During the fermentation process of the poultry manure, uric acid present in the manure is degraded into urea and ammonia, and heat is generated at approximately 60 °C. B₁₂ is unstable under alkaline and heat conditions [16]. Low B₁₂ contents of the poultry manure fertilizers might be due to the loss of B₁₂ during the fermentation process.

Human feces reportedly contain various inactive cobamides such as Factor A (60.6%), p-cresol cobamide (16.3%), PseudoB₁₂ (12.5%), Factor S (15.5%), Factor III (1.8%), and phenol cobamide (0.1%) [11]. To clarify whether the fermented poultry manure fertilizer products contained B₁₂ or inactive cobamindes, the corrinoids observed in the selected fertilizer products (A, B, and C) listed in Table 1 were analyzed using an *E. coli* 215 bioautogram after separation by silica gel 60 TLC. The corrinoids observed in fertilizer A and B products produced obvious spots with \( R_f \) values identical to those of B₁₂ and PseudoB₁₂. However, the fertilizer C product showed only the spot with an \( R_f \) value identical to that of B₁₂ (Figure 1).

Figure 1. Bioautogram analysis of B₁₂ compounds detected in selected fermented poultry manure fertilizer products. A, B, and C represent the products listed in Table 1. Typical bioautogram data are obtained from three independent experiments.

Corrinoids were purified from the extracts of the selected fertilizer A, B, and C products using a B₁₂ immunoaffinity column and then identified by C18 reversed-phase HPLC (Figure 2). The retention times of Factor III, BIA, PseudoB₁₂, and B₁₂ were 7.7, 8.0, 8.5, and 10.3 min, respectively. The retention times of the three main peaks found in the fertilizer A product were similar to those of Factor III (peak d), PseudoB₁₂ (peak b), and B₁₂ (peak a). Two peaks with retention times of 8.9 (peak e) and 11.9 (peak f) min were detected as unidentified compounds. We obtained similar results in the fertilizer B product. However, the fertilizer C product showed a single major peak with a retention time of 10.2 min, which was the identical retention time of B₁₂.
LC-MS analysis precisely identified corrinoid compounds in the selected fertilizer products. As described previously [17,18], the MS results of authentic B$_{12}$, Factor III, and PseudoB$_{12}$ indicated major doubly charged ions of $m/z$ 678.2937, $m/z$ 672.2715, and $m/z$ 672.7749, respectively. We eluted selected ion monitoring chromatograms of various cobamides with different base moieties (Figure 3) revealed that PseudoB$_{12}$ and B$_{12}$ at retention times of 7.3 and 7.4 min, respectively, whereas Factor III was undetectable. Furthermore, Factor A ($m/z$ 679.7834) occurred at the identical retention time of PseudoB$_{12}$. We eluted Factor S ($m/z$ 695.7657) at a retention time of 7.5 min. However, there is no information regarding the retention times of Factors A and S as these authentic compounds were not available. These results indicated that the corrinoid compounds presented in the fertilizer A product were completely identified as B$_{12}$ and PseudoB$_{12}$ and tentatively identified as Factor A and S. We obtained similar results in the fertilizer B product. However, the fertilizer C product contained only B$_{12}$, judging from the selected ion monitoring chromatogram data.
TLC-bioautography, HPLC, and LC-MS analyses indicated that B12 was the predominant corrinoid in all the selected fertilizer products, and minor inactive cobamides such as PseudoB12, Factor A, and Factor S appeared in the fertilizer A and B products.

As depicted in Figure 2, the peak d, showing the identical retention time (7.7 min) of Factor III, was detected during the HPLC of fertilizer A and B products. However, Factor III (m/z 672.2715) was not detectable by LC-MS (Figure 4), suggesting that the peak with a retention time of 7.7 min does not come from corrinoid compounds. However, the unidentified compounds with retention times of 8.9 (peak e) and 11.9 (peak f) min detected by HPLC (Figure 2) appear to be derived from Factors A and S, respectively, judging from the data of selected ion monitoring chromatograms by LC-MS (Figure 4). The relative contents (%) of B12 (56.9% and 61.5%), PseudoB12 (15.4% and 13.2%), Factor A (7.2% and 8.0%), and Factor S (20.5% and 17.3%) were calculated in fertilizer A and B products, respectively, using HPLC data. These inactive cobamides would be synthesized by poultry intestinal bacteria and/or formed during the fermentation process of the manure by concomitant bacteria [19]. Differences in the corrinoid compounds found in fertilizers A, B, and C might be due to the different types of corrinoid-synthesizing bacteria in the poultry intestine and fermentation process.

Biofertilizers containing purple photosynthetic bacteria reportedly contain B12 (main) and Factor III (minor) [18,20]. Studies have also reported that cyanobacteria that are responsible for biological nitrogen fixation in flood rice fields primarily contained PseudoB12 [21,22]. However, no information is available on regarding other organic fertilizers and wastes such as cow dung and sheep feces. Several plants, such as lettuces, can absorb B12 from soils [9] or hydroponic solutions [23].
Figure 4. LC-MS chromatograms of B\textsubscript{12} compounds from fermented poultry manure fertilizer A, B, and C products. Panels (A) (fertilizer A product), (B) (fertilizer B product), and (C) (fertilizer C product) show total ion chromatograms (—) and selected ion monitoring chromatograms (—) of (1) B\textsubscript{12} (m/z 678.2914), (2) PseudoB\textsubscript{12} (m/z 672.7749), (3) BIA (m/z 664.2748), (4) Factor III (m/z 672.2715), (5) p-cresolyl dicyanocobamide (m/z 659.2783), (6) Factor A (m/z 679.7834), and (7) Factor S (m/z 695.7657). Typical ion chromatogram data are obtained from three independent experiments.

4. Conclusions

A preliminary study indicated that the addition of fermented poultry manure fertilizers increased the amount of B\textsubscript{12} in rice bran. However, the B\textsubscript{12} content of the fermented poultry manure fertilizers varied from 1.4 µg to approximately 20 µg per 100 g of dry weight. LC-MS analyses showed that the fermented poultry manure fertilizers A, B, and C primarily contained B\textsubscript{12}.

Therefore, by utilizing such fermented poultry manure fertilizers, edible plants could be enriched in B\textsubscript{12}. Even if the fertilizer products contain PseudoB\textsubscript{12}, Factor A, and Factor S, these inactive corrinoids would not be absorbed into the human intestine because of their low affinity to the human intrinsic factor, a gastric B\textsubscript{12} transport protein [24]. Furthermore, there is a fertilizer–soil–microbial ecosystem in nature. Further studies are required to clarify the effects of these fermented fertilizers in edible plants by enriching them with B\textsubscript{12}.

Author Contributions: All authors contributed significantly to the development of the, procedure and writing of the manuscript—reviewing and editing. H.K., K.K., S.T. and F.W. conducted the experiment, investigation, formal analysis, visualization, writing—original draft of the manuscript. Editing and approval of the final version of the manuscript was by K.K., T.B. and F.W. All authors have read and agreed to the published version of the manuscript.
**Funding:** The authors received no specific funding for this work.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

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