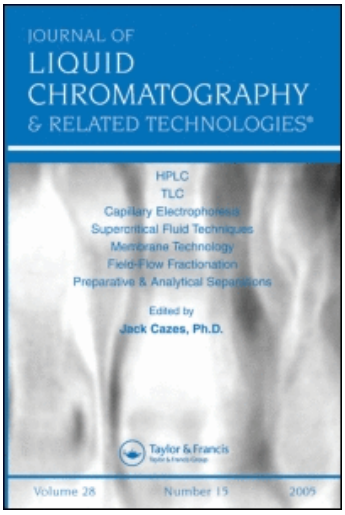

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Purification and Characterization of a Corrinoid Compound from a Japanese Salted and Fermented Salmon Kidney "Mefun"

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Purification and Characterization of a Corrinoid Compound from a Japanese Salted and Fermented Salmon Kidney "Mefun"

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Abstract: Significant amounts of vitamin B_{12} (about $116.3 \sim 556.3\,\mu g/100\,g$) were determined in a Japanese salted and fermented salmon kidney "Mefun." A corrinoid compound was purified to homogeneity from Mefun and partially characterized. TLC and HPLC patterns of the purified corrinoid compound were identical to those of authentic vitamin B_{12}, but not to inactive corrinoids. The vitamin B_{12} found in Mefun was not derived from concomitant vitamin B_{12}-synthesizing bacteria, but had accumulated in the salmon kidney. Gel filtration experiments demonstrated that most of the vitamin B_{12} found in Mefun was recovered in the free vitamin B_{12} fractions.

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These results indicate that Mefun would be an excellent vitamin B_{12} (free form) source for elderly persons with food-bound vitamin B_{12} malabsorption.

Keywords: Salted and fermented salmon kidney, HPLC, TLC, Vitamin B_{12}

INTRODUCTION

Many people with low serum vitamin B_{12} (B_{12}) levels, but who do not have pernicious anemia, become malabsorptive for protein-bound B_{12} (food-bound B_{12} malabsorption).[1] The food-bound B_{12} malabsorption is found in persons with certain gastric dysfunctions, especially atrophic gastritis with low stomach acid secretion, which prevails in elderly people.[2] Because the bioavailability of crystalline B_{12} is not altered in people with atrophic gastritis, the Institute of Medicine has suggested that most of the recommended dietary allowance (RDA) (2.4 μg/day) should be obtained by consuming foods fortified with B_{12} or B_{12}-containing supplement.[3]

The food containing the highest amount (327.6 μg/100 g) of B_{12} among those described in the Japanese Standard Tables of Food Composition[4] is a Japanese salted and fermented salmon kidney "Mefun," which has a delicate flavor. Feeding of only 0.8 g of Mefun can supply the RDA (2.4 μg/day) for adults. If Mefun contains substantial amounts of true and free B_{12}, it would be an excellent B_{12} source for elderly persons with food-bound B_{12} malabsorption.

Another salted and fermented fish product, fish sauce, has been reported to contain considerable amounts of B_{12},[5] which is an inactive or unidentified corrinoid compound.[6] Certain bacteria can synthesize various corrinoid compounds, some of which are not active for humans, but are assayable in the B_{12} determination systems.[7,8] There is no information available on whether B_{12} found in the salted and fermented fish product, Mefun, is true B_{12} or inactive corrinoids for humans. Thus, a corrinoid compound is purified and characterized to clarify the bioavailabilty of B_{12} found in Mefun.

EXPERIMENTAL

Materials

B_{12} and a reversed-phase high pressure liquid chromatography (HPLC) column (Wakosil-II 5C18RS, φ4.6 × 150 mm; particle size, 5 μm) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Cosmosil 140C18-OPN was obtained from Nacakai Tesque (Kyoto, Japan). A B_{12} assay medium for *Lactobacillus delbrueckii* subspecies *lactis* (formerly *L. leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 thin layer chromatography (TLC) aluminium sheets were obtained from Merck (Darmstadt, Germany). Amberlite XAD-4 was obtained from

Japan Organo Co. (Tokyo, Japan). Cyanocobamides (5-hydroxybenzimidazolylcyanocobamide, benzimidazolylcyanocobamide, and 7-adeninylcyanocobaminde) isolated from bacteria were kindly provided by Dr. E. Stupperich, Ulm University, Germany. All other reagents used were of the highest purity commercially available. The tested samples of the Japanese salted and fermented salmon kidney "Mefun" were provided from a local market in Niigata-, Aomori-, and Hokkaido-prefectures, Japan.

A visible spectrophotometer (Ultrospec 10 pro, Amersham Biosciences Corp., Piscataway, NJ, USA) was used for measuring the turbidity of *L. delbreuckii* test cultures in the microbiological B_{12} assay method. A Shimadzu (Kyoto, Japan) UV-visible spectrophotometer (UV-1600) was used for spectral analysis of the purified corrinoid compound.

Methods

Extraction and Assay of Vitamin B_{12} in Mefun

After about 50 g of each Mefun sample was homogenized with a mixer (MX-X51-H, National, Osaka, Japan), a portion (2 g) of each homogenate was used for the sample. Total B_{12} was extracted with boiling at acidic pH and assayed by the microbiological method with *Lactobacillus delbrueckii* subspecies *lactis* ATCC 7830, according to the method described in the Japanese Standard Tables of Food Composition.[4] Since *L. delbrueckii* ATCC 7830 can utilize both deoxyribosides and deoxynucleotides (known as an alkali-resistant factor) as well as B_{12}, the amount of true B_{12} was calculated by subtracting the values of the alkali-resistant factor from the values of total B_{12}.

Purification of Corrinoid Compound from Mefun

About 500 g of Mefun (made in Hokkaido-Prefecture) was homogenized with the mixer (National) and added to 4 L of 10 mmol/L acetate buffer, pH 4.8, containing 10 mmol/L KCN. Corrinoid compound was extracted from the solution by boiling for 30 min at 98°C in the dark. The extraction procedures were done in a Dalton (Tokyo, Japan) draught chamber with a fume hood. The boiled solution was cooled to room temperature (25°C) and centrifuged at 10,000 × *g* for 10 min. The supernatant fraction was put onto a column (5.0 × 45.0 cm) of Amberlite XAD-4 resin which had been washed with 5 L of methanol and equilibrated with distilled water. After the column was washed with 2 L of distilled water, the corrinoid compound was eluted with 1.5 L of 80% (v/v) ethanol. The 80% (v/v) ethanol eluant was pooled, evaporated to dryness under reduced pressure, and dissolved in 30 mL of distilled water. The solution was put onto a column (24 × 150 mm) of Cosmosil 140C18-OPN (Nacalai Tesque, Kyoto, Japan) which had been washed with 75% (v/v) ethanol solution and equilibrated with distilled

water. The corrinoid compound was eluted with a stepwise gradient (0, 10, 20, 30, and 80% v/v) of ethanol. These five fractions were separately evaporated to dryness under reduced pressure, and dissolved with a small amount of distilled water. Each concentrated solution was purified by silica gel 60 TLC, which was developed with 2-propanol/NH_4OH (28%)/water (7 : 1 : 2 v/v) as the solvent, in the dark, at room temperature (25°C). A spot with red-tint on the dried TLC sheet was collected, extracted with 80% (v/v) methanol, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was purified by HPLC using a Shimadzu HPLC apparatus (LC-6A Pump, SPD-6A Spectrophotometer, CTO-6A column oven, C-R6A Chromatopac). The sample (100 μL) was put onto a reversed-phase HPLC column (Wakosil-II 5C18RS, $\varphi 4.6 \times 150$ mm; particle size, 5 μm) equilibrated with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 35°C. The flow rate was 1.0 mL/min. The compound with the red-tint was isocratically eluted with the same solution, monitored by measuring absorbance at 278 nm, and collected at 1.0 mL with a Bio-Rad Laboratories fraction collector (model 2110). The fractions with the red-tint were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was further purified by HPLC under the same conditions. The peak fraction of the eluant with the red-tint was evaporated to dryness under reduced pressure, and dissolved in 100 μL of distilled water, and used as a purified corrinoid compound.

Gel Filtration Experiment

One gram of Mefun was homogenized in 10 mL of 10 mmol/L potassium phosphate buffer, pH 7.0, containing 0.2 mol/L KCl by the use of a universal homogenizer (Nihon Seiki Seisaku-Sho Co., Tokyo, Japan), and centrifuged at 10,000 × *g* for 10 min. The supernatant was used for a homogenate of Mefun. A portion (1.0 mL) of the homogenate was placed onto a column (1.4 × 25 cm) of Sephadex G-50 which had been equilibrated with 10 mmol/L potassium phosphate buffer, pH 7.0, containing 0.2 mol/L KCl. The column was eluted with the same buffer at a flow rate of 1.0 mL/min. The eluate from the column was fractionated at 1.0 mL. The macromolecular and free B_{12} fractions, which were estimated with blue dextran and authentic B_{12} by measuring absorbance at 600 and 551 nm, respectively, were pooled. B_{12} was extracted from these fractions under the same conditions and assayed by the microbiological method.

Analytical TLC and HPLC

Concentrated solutions (2 μL) of the purified compound and cyanocobamides (benzimidazolyl, 5-hydroxybenzimidazolyl, and 7-adeninyl cyanocobamides) were spotted onto silica gel 60 TLC sheets and developed with 1-butanol/2-propanol/water (10 : 7 : 10 v/v) and 2-propanol/NH_4OH (28%)/water

(7 : 1 : 2 v/v) as solvents I and II, respectively, in the dark, at room temperature (25°C).

In the case of HPLC, the concentrated solutions (2 μL) of the purified compound and these cyanocobamides were analyzed with the reversed-phase HPLC column (Wakosil-II 5C18RS, $\varphi 4.6 \times 150$ mm; particle size, 5 μm) and the Shimadzu HPLC apparatus. The corrinoids were isocratically eluted with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 35°C, and monitored by measuring the absorbance at 278 nm. The retention times of these corrinoids were determined at a flow rate of 1.0 mL/min.

Ultraviolet-Visible Spectrum

The purified preparation was dissolved in 0.1 mL of distilled water. The spectrum was measured with a Shimadzu spectrophotometer (UV-16000) at room temperature (25°C). Super micro quartz cuvettes (0.1 mL, $d = 1$ cm) were used.

Bacterial Counts

The colony forming units (c.f.u.) as the number of viable counts were estimated with the agar surface plating method[9] with GAM agar (Nissui, Tokyo, Japan) containing 0 or 20% (w/v) NaCl. Samples were diluted with saline containing 0.1% (w/v) agar and plated on the agar plates. The agar plates were then incubated at 30°C for 6 days with or without the AnaeroPack system (Mitsubishi Gas, Tokyo, Japan).

RESULTS AND DISCUSSION

Table 1 shows the amounts of B_{12} in a Japanese salted and fermented salmon kidney "Mefun." Substantial amounts (116.3 ~ 556.3 μg/100 g) were found in three samples of Mefun; the values were similar to those described in Standard Tables of Food Composition in Japan.[4] To clarify bioavailability of B_{12} found in Mefun, a corrinoid compound was purified and characterized.

Figure 1 shows silica gel 60 TLC patterns of corrinoid compound during Cosmosil 140 C18 OPN column chromatography in the purification steps. The

Table 1. Amounts of vitamin B_{12} in a Japanese salted and fermented salmon kidney "Mefun". B_{12} was assayed in triplicate in each Mefun sample ($n = 4$).

Mefun	Vitamin B_{12} concentration (μg/100 g)
Sample 1 (made in Niigata Prefecture, Japan)	116.3 ± 13.4
Sample 2 (made in Aomori Prefecture, Japan)	365.7 ± 80.7
Sample 3 (made in Hokkaido Prefecture, Japan)	556.3 ± 91.85

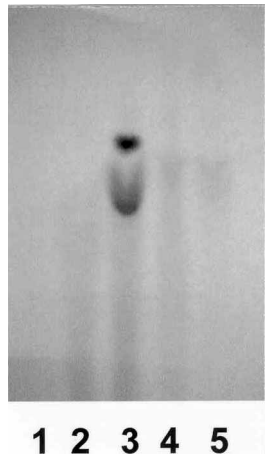

Figure 1. Silica gel 60 TLC patterns of corrinoid compound during Cosmosil 140C18-OPN column chromatography in the purification steps. The corrinoid compound (R_f value 0.6) was eluted with a stepwise gradient [0 (lane 1), 10 (lane 2), 20 (lane 3), 30 (lane 4), and 80% (v/v) (lane 5)] of ethanol. Data present a typical migration pattern of the corrinoid compounds on the chromatography from three experiments.

spot with the red-tint (corrinoid compound) was found only in the 20% (v/v) ethanol fraction. The spot with the red-tint compound was easily purified with silica gel 60 TLC and reversed-phase HPLC.

The ultraviolet-visible spectrum of the purified compound showed a typical absorption of cobalt-containing corrinoid compound (Fig. 2); λmax nm (absorption) was at 550.5 (0.497), 521.0 (0.441), 361.0 (1.631), and 279.0 (0.880).

The purified compound, authentic B_{12}, and cyanocobamides (7-adeninyl-, 5-hydroxybenzimidazolyl-, and benzimidazolyl-cyanocobamides) which occur in bacteria were analyzed by silica gel 60 TLC and reversed-phase HPLC (Table 2). The R_f values of the purified compound were identical to the values of authentic B_{12}, of which the retention time was also identical to

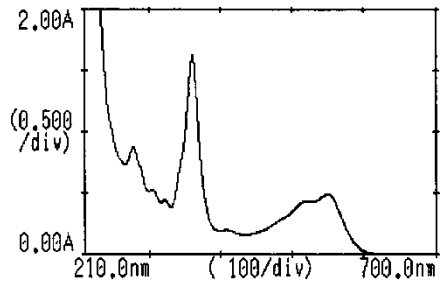

Figure 2. Ultraviolet-visible spectrum of the purified compound.

Table 2. R_f values and retention times of the purified compound, authentic B_{12}, and cyanocobamides on TLC and HPLC

	TLC (R_f)		
	Solvent I	Solvent II	HPLC (min)
Purified compound	0.24	0.61	9.4
Vitamin B_{12}	0.24	0.61	9.4
Benzimidazolylcyanocobamide	0.18	0.57	7.3
5-Hydroxybenzimidazolylcyanocobamide	0.20	0.49	7.0
7-Adeninylcyanocobamide	0.14	0.48	7.7

that of the purified compound in reversed-phase HPLC. These results indicate that Mefun contains substantial amounts of true B_{12}.

Table 3 shows the bacterial counts in Mefun samples using two different salinity media. The bacterial counts varied (from 10^2 to 10^4 c.f.u./g) among samples. The high salinity-tolerant bacteria (halophilic bacteria) were detected in only one sample (sample 3). Although this sample showed the highest content of B_{12}, the number of bacteria was not as high, compared with one of other fermented foods. The halophilic-anaerobic bacteria were not detected in any Mefun samples (containing about 15% (w/w) NaCl). Kuda et al.[10] reported that the halophilic lactococci *Tetragenococcus*, which can grow in anaerobic conditions, is dominant in some salted fish products.

In preliminary experiments, a fresh salmon kidney also contained substantial amounts of B_{12} ($128.5 \pm 4.9\,\mu g/100\,g$). As Mefun is dehydrated

Table 3. Bacterial counts in a Japanese salted and fermented salmon kidney "Mefun" (log colony forming units/g)

	Media			
	NaCl 0% (w/w)		NaCl 15% (w/w)	
Mefun	Aerobe	Anaerobe	Aerobe	Anaerobe
Sample 1 (made in Niigata Prefecture, Japan)	—*	—	—	—
Sample 2 (made in Aomori Prefecture, Japan)	4.09	2.95	—	—
Sample 3 (made in Hokkaido Prefecture, Japan)	3.15	—	2.70	—

* <2.0.

considerably by the addition of salt, the B_{12} contents of Mefun samples would be higher relative to those of the fresh salmon kidney. These results indicate that most of the B_{12} found in Mefun is not derived from concomitant B_{12}-synthesizing bacteria, but has accumulated in the salmon kidney.

The Sephadex G-50 gel filtration of a homogenate of Mefun demonstrated that most (84.2%) of the B_{12} was recovered in the free B_{12} fractions; the remaining B_{12} (15.8%) was associated with macromolecules.

Because approximately 30% of people older than 50 years are estimated to have atrophic gastritis with low stomach acid secretion and have decreased bioavailability of B_{12} from food (food-bound B_{12} malabsorption), the Institute of Medicine has recommended that most of the 2.4 μg of B_{12} per day (RDA) should be obtained by consuming foods fortified with B_{12} or B_{12}-containing supplement.[3] The results presented here indicate that a Japanese salted and fermented salmon kidney "Mefun" would be an excellent free form B_{12} source for elderly persons with food-bound B_{12} malabsorption.

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