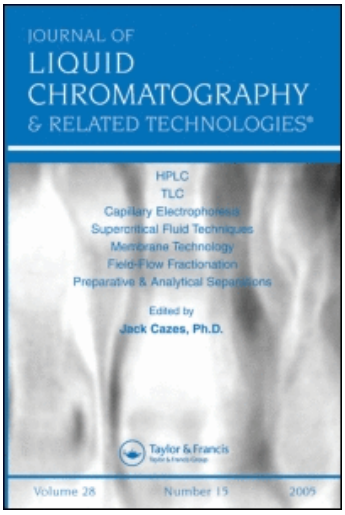


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# Purification and Characterization of Corrinoid Compounds from a Japanese Fish Sauce

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## ABSTRACT

A Japanese fish sauce "Ishiru," which was made from squid by a traditional food manufacturing process, contained the highest amounts ($5.5 \pm 2.3\,\mu g/100\,g$) of $B_{12}$ among various fish sauces tested. Two corrinoid compounds were purified from the fish sauce Ishiru and partially characterized. TLC and HPLC patterns of the main red-colored compound, purified from the fish sauce, were identical to those of authentic vitamin $B_{12}$, but minor compounds could not be identified. Fish sauce may not be suitable for use as a good vitamin $B_{12}$ source, judging from the low daily intake of the sauce and occurrence of the unknown corrinoid-compound.

*Key Words:* TLC; HPLC; Fish sauce; Fermented foods; Vitamin $B_{12}$.



## INTRODUCTION

Various kinds of fish sauces, traditional food supplements in the diet, are widely used in the world as condiments, as flavoring material, and sometimes as a substitute for soy-bean sauce. A fish sauce (Nam-pla) appears to contribute a major source of vitamin $B_{12}$ ($B_{12}$) in Thailand, since it contains considerable amounts of $B_{12}$.[1,2] Although our previous paper[3] has demonstrated that the amounts of $B_{12}$ were several-fold greater in Japanese fish sauces than in some kinds of Nam-pla, thin layer chromatography (TLC) analysis indicated that most $B_{12}$ found in the Japanese fish sauces were derived from unidentified corrinoid compounds. Our unpublished study indicated that a Japanese fish sauce "Ishiru," which was made from squid by a traditional food manufacturing process, contained the highest amounts of $B_{12}$ among various fish sauces tested. It is, however, not clear whether $B_{12}$ found in the Japanese fish sauce Ishiru is actual $B_{12}$ or inactive corrinoids for humans.

Thus, corrinoid compounds found in the fish sauce Ishiru, were characterized by the use of TLC on silica gel as an important purification and analytical tool.

## EXPERIMENTAL

### Materials

$B_{12}$ and a reversed-phase high pressure liquid chromatography (HPLC) column (Wakosil-II 5C18RS, $\phi$ 4.6 $\times$ 150 $mm^2$; particle size, 5 $\mu$m) were

obtained from Wako Pure Chemical Industries (Osaka, Japan). Cosmosil 140C180-OPN was obtained from Nacakai Tesque (Kyoto, Japan). A $B_{12}$ assay medium for *Lactobacillus delbrueckii* subsp. *lactis* (formerly *L. leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). Amberlite XAD-4 was obtained from Japan Organo Co. (Tokyo, Japan). Cyanocobamides (5-hydroxybenzimidazolylcyanocobamide, benzimidazolylcyanocobamide, and 7-adenylcyanocobamide) 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 Japanese fish sauce Ishiru used in the experiments was provided from a local market in Kanazawa-city, Ishikawa-prefecture, Japan.

A Shimadzu (Kyoto, Japan) UV-visible spectrophotometer (UV-1600) was used for measuring turbidity of *L. delbrueckii* test culture in the microbiological $B_{12}$ assay method. A fully automated chemiluminescence $B_{12}$ analyzer ACS 180 (Chiron Diagnostics, East Walpole, MA) was used for $B_{12}$ assay.



## Methods

### Assay of Vitamin $B_{12}$

$B_{12}$ was assayed by the microbiological method with *L. delbrueckii* ATCC 7830 and a $B_{12}$ assay medium (Nissui, Tokyo, Japan), and by the fully automated chemiluminescence $B_{12}$ analyzer ACS 180 (IF-chemiluminescence) as described previously.[4]

### Purification of Corrinoid Compounds from the Fish Sauce Ishiru

One liter of the fish sauce Ishiru was added to 1 L of 0.1 mol/L acetate buffer, pH 4.8, containing 10 mmol/L KCN. Total $B_{12}$ was extracted from the solution by boiling for 30 min, in the dark, at 98°C. The extraction procedures were done in a Dalton (Tokyo, Japan) draught chamber with fume hood. The boiled solution was cooled to room temperature and used for purification of corrinoid compounds. Amberlite XAD-4 resin (500 g), washed with 5 L of methanol and equilibrated with distilled water, was added to the boiled solution and stirred for 3 hr at room temperature in the dark. The resin suspension was passed through a glass funnel (Buchner type) with a glass filter (type 25G1, Iwaki, Tokyo, Japan) and the resin was washed with 5 L of distilled water. The washed resin was added to 1 L of 80% (v/v) methanol solution, and stirred for 3 hr at room temperature in the dark. The resin suspension was passed through the glass funnel.

The 80% (v/v) methanol eluant (about 1 L) containing corrinoid compounds was pooled, evaporated to dryness under reduced pressure, and dissolved in 30 mL of distilled water.

After a column ($24 \times 120\,mm^2$) of Cosmosil 140C18-OPN (Nacalai Tesque, Kyoto, Japan) was washed with 75% (v/v) ethanol solution and equilibrated with distilled water, the solution was put on the column and eluted with a linear gradient (0–90% v/v) of ethanol. The $B_{12}$-active fractions were assayed by the IF-chemiluminescence method, pooled, evaporated to dryness under reduced pressure, and dissolved with a small amount of distilled water. The concentrated solution was purified by silica gel 60 TLC, which was developed with 2-propanol/$NH_4OH$ (28%)/water (7 : 1 : 2 v/v/v) as a solvent, in the dark, at room temperature. The dried TLC sheets were fractionated by cutting them into small pieces. Corrinoid compounds were extracted from the pieces with 80% (v/v) methanol, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The $B_{12}$-active fractions were assayed by the IF-chemiluminescence method. The concentrated solution was further 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 on a reversed-phase HPLC column (Wakosil-II 5C18RS, $\phi$ $4.6 \times 150\,mm^2$; 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 mL/min. The corrinoid compounds were isocratically eluted with the same solution, monitored by measuring absorbance at 278 nm, and collected at 1 mL with a Bio-Rad Laboratories fraction collector (Model 2110). The $B_{12}$-active fractions were assayed by both microbiological and IF-chemiluminescence methods. $B_{12}$-active fractions were separated as two peaks. Each peak was pooled, evaporated to dryness under reduced pressure, and dissolved in 0.1 mL of distilled water. Each concentrated solution was put on a silica gel 60 TLC sheet and developed with 2-propanol/$NH_4OH$ (28%)/water (7 : 1 : 2 v/v/v) as the mobile phase, in the dark, at 25°C. Each pink-colored spot on the dried TLC sheet was collected, extracted with 80% (v/v) methanol, evaporated to dryness under reduced pressure, and dissolved in 20 μL of distilled water, and used as a purified corrinoid compound.

## Analytical TLC and HPLC

The concentrated solutions (2 μL) of each corrinoid compound purified from the fish sauce, and cyanocobamides (benzimidazolyl-, 5-hydroxybenzimidazolyl-, and 7-adenyl-cyanocobamides) were spotted on the silica gel 60 TLC sheet and developed with 2-propanol/$NH_4OH$ (28%)/water (7 : 1 : 2 v/v/v) as the mobile phase, in the dark, at 25°C. The TLC sheet was dried and $R_f$ values of the pink-colored spots of the corrinoids were determined.

In the case of HPLC, the concentrated solutions (2 μL) of each purified corrinoid compound and these cyanocobamides, were analyzed with the reversed-phase HPLC column (Wakosil-II 5C18RS, $\phi$ 4.6 × 150 mm$^2$; 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 absorbance at 278 nm. The retention times of these corrinoids were determined at the flow rate of 1 mL/min.

## RESULTS AND DISCUSSION

The Japanese fish sauce Ishiru, which was made from squid by a traditional food manufacturing process, contained the highest amount of $B_{12}$ ($5.5 \pm 2.3$ μg/100 g) among various fish sauces tested using the IF-chemiluminescence method.

To determine whether the corrinoid compounds found in the fish sauce "Ishiru" are true $B_{12}$ or inactive corrinoid compounds for humans, corrinoid compounds were purified and characterized. Figure 1 shows elution profiles of corrinoid compounds from the fish sauce Ishiru on a reversed-phase HPLC during purification. Corrinoid compounds were eluted as two peaks (main and minor) when assayed by both microbiological and IF-chemiluminescence methods. Each final purified preparation gave a single pink-colored spot by TLC on silica gel 60 (Fig. 2).

The purified corrinoid compounds, authentic $B_{12}$, and cyanocobamides (7-adenyl-, 5-hydroxybenzimidazolyl-, and benzimidazolyl-cyanocobamides), which occur in bacteria, were analyzed by silica gel 60 TLC and reversed-phase HPLC (Table 1). The $R_f$ value (0.61) of the main corrinoid compound I was identical to the value of authentic $B_{12}$, of which the retention time (9.4 min) was also identical to that of the main corrinoid compound in reversed-phase HPLC. $R_f$ value and retention time of the minor corrinoid compound II were not identical to those of any authentic corrinoids tested.

Further detailed information on the fish sauce corrinoid compounds was not available because large amounts of the purified samples were not obtained for NMR study.

Although some (5-hydroxybenzimidazolyl- and benzimidazolyl-cyanocobamides) naturally occurring corrinoid compounds are fully active for the binding of IF[5] and growth of *L. delbrueckii* ATCC7830,[6] 7-adenylcyanocobamide reveals moderate affinity to IF[5] and is inactive for pernicious anemia.[6] Although corrinoid compounds inactive for the binding of IF are probably not absorbed in mammalian intestine by the IF-mediated system, the minor corrinoid compound II was capable of binding to IF. We have no

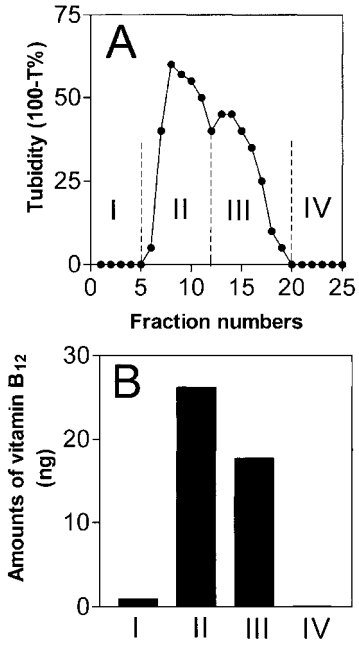


***Figure 1.*** Elution profiles of corrinoid compounds from a Japanese traditional fish sauce "Ishiru," during a reversed-phase HPLC in the purification steps. Corrinoid compounds were determined by the microbiological method. (A) Fractions 1–5 (I), 6–12 (II), 13–20 (III), and 21–25 (IV) were combined and assayed for corrinoid compounds by the IF-chemiluminescence method. (B) Data present a typical elution pattern of corrinoid compounds by HPLC from three experiments.

information available on whether the minor corrinoid compound II is active or inactive for humans.

Areekul et al.[1] have reported that a human would obtain 0.1–0.4 μg of $B_{12}$ per day from fish sauce in Thailand. Fish sauce may not be suitable for use as a good source of $B_{12}$, judging from the low daily intake [4.2–16.7% of

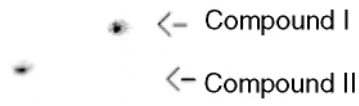


***Figure 2.*** Silica gel 60 TLC pattern of the purified corrinoid compounds. Data present a typical migration pattern of the purified corrinoid compounds by TLC from three experiments.

***Table 1.*** $R_f$ values and retention times of the purified corrinoid compounds, authentic $B_{12}$, and cyanocobamides on TLC and HPLC.

| | TLC ($R_f$) | HPLC (min) |
|---|---|---|
| Main compound I | 0.61 | 9.4 |
| Minor compound II | 0.55 | 14.5 |
| $B_{12}$ | 0.61 | 9.4 |
| Benzimidazolylcyanocobamide | 0.57 | 7.3 |
| 5-Hydroxybenzimidazolylcyanocobamide | 0.49 | 7.0 |
| 7-Adenylcyanocobamide | 0.48 | 7.7 |

the recommended dietary allowance for adults (2.4 μg/day)] and the possibility that the unidentified corrinoid compounds generally occur in various fish sauces.[3]



## REFERENCES

1. Areekul, S.; Boonyananta, C.; Matrakul, D.; Chantachum, Y. Determination of vitamin $B_{12}$ in fish sauce in Thailand. J. Med. Assoc. Thai. **1972**, *55*, 243–248.
2. Areekul, S.; Thearawibul, R.; Matrakul, D. Vitamin $B_{12}$ contents in fermented fish, fish sauce, and soya-bean sauce. Southeast Asian J. Trop. Med. Public Health **1974**, *5*, 461.
3. Takenaka, S.; Enomoto, T.; Tsuyama, S.; Watanabe, F. TLC analysis of corrinoid compounds in fish sauce. J. Liq. Chromatogr. Rel. Technol. **2002**, *26*, 2685–2689.
4. Watanabe, F.; Takenaka, S.; Abe, K.; Tamura, Y.; Nakano, Y. Comparison of a microbiological assay and a fully automated chemiluminescent system for the determination of vitamin $B_{12}$ in food. J. Agric. Food Chem. **1998**, *46*, 1433–1436.
5. Stupperich, E.; Nexo, E. Effect of the cobalt-N coordination on the cobamide recognition by the human vitamin $B_{12}$ binding proteins intrinsic factor, transcobalamin and haptocorrin. Eur. J. Biochem. **1991**, *199*, 299–303.
6. Schneider, Z.; Stroinski, A. Biosynthesis of vitamin $B_{12}$. In *Comprehensive $B_{12}$*; Schneider, Z., Stroinski, A., Eds.; Walter de Gruyter: Berlin, 1987; 93–110.

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