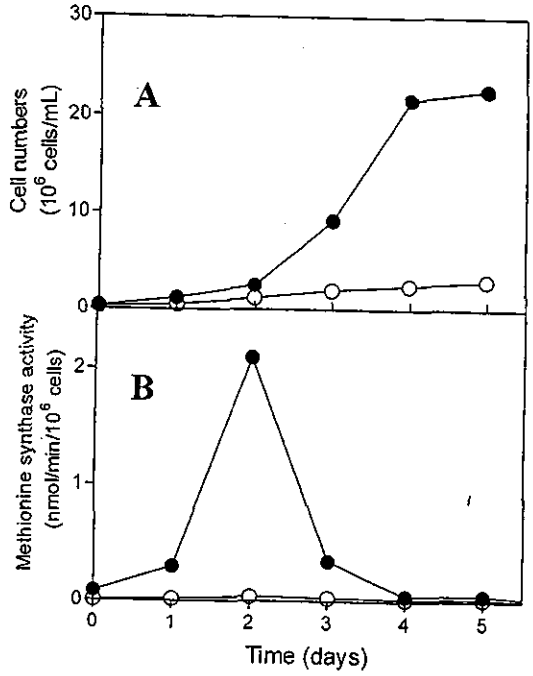

Fig. 1 Changes in the activity of vitamin B_{12}-dependent methionine synthase during cell growth of *E. gracilis* Z.
(A) cell growth, (B) methionine synthase activity.
(●) B_{12}-sufficient cells, (○) B_{12}-defficient cells.

法を用いることができないので*Euglena*細胞を海砂で摩砕することで細胞を破砕し，分画遠心法によりミトコンドリアと葉緑体を分離した。その結果，本酵素活性の約99%が細胞質画分（5.0nmol/min/mg protein）に回収されたが，各オルガネラのマーカー酵素のクロスコンタミネーションによる補正を行っていないので正確な本酵素の細胞内分布とは言えず，今後さらにB_{12}給与細胞の細胞分画方法を含めて再検討する必要がある。また，パーコール精製した無傷のミトコンドリア（19.2nmol/min/mg protein）や葉緑体（1.2nmol/min/mg protein）に本酵素活性が検出されたことから，これらオルガネラにおいても本酵素が存在し，メチオニン代謝に機能していることが推測された。

B_{12}依存性メチオニン合成酵素はヒトを含めた哺乳動物の細胞で細胞質にのみ局在することが報告[6]されているが，今回の実験結果からミトコンドリアにも本酵素が存在する可能性が示唆され，哺乳動物での本酵素の細胞内局在性を再検討する必要があると思われる。

また，*E. gracilis* Zの各オルガネラに存在する本酵素の生理機能や代謝調節機構について生化学的および分子生物学的手法を用いて今後解明する予定である。

謝辞

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Purification and characterization of methylmalonyl-CoA mutase from a photosynthetic coccolithophorid alga, *Pleurochrysis carterae*

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Abstract

Low activity (about 4 mU/mg protein) of 5′ -deoxyadenosylcobalamin-dependent methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) was found in a cell homogenate of a photosynthetic coccolithophorid alga, *Pleurochrysis carterae*. Most of the enzyme occurred as the apo-enzyme, which was labile during purification. The holo-enzyme, which was converted from the apo-enzyme by incubation with 10 μM 5′ -deoxyadenosylcobalamin at 4 °C in the dark, was purified to homogeneity and partially characterized. An apparent molecular mass for the enzyme of 150 ± 5 kDa was calculated by Superdex 200 pg gel filtration. SDS-polyacrylamide gel electrophoresis of the purified enzyme gave a single protein band with an apparent molecular mass of 80 ± 5 kDa, indicating that the *P. carterae* enzyme occurs as a homodimer. Some properties of methylmalonyl-CoA mutase from *P. carterae* were studied.

Keywords: Cobalamin; Coccolithophorid; 5′ -Deoxyadenosylcobalamin; Food supplement; Marine alga; Methylmalonyl-CoA mutase; *Pleurochrysis carterae*; Vitamin B_{12}

1. Introduction

The photosynthetic coccolithophorid alga, *Pleurochrysis carterae* (formerly *Hymenomonas carterae*) is a unicellular marine calcareous phototroph and produces $CaCO_3$ structures (calcified scales around the cells) (Takenaka et al., 1996). This marine alga absolutely requires vitamin B_{12} (cobalamin, Cbl) for growth (Provasoli and Pintner, 1953). Considerable amounts of Cbl (30–40 pg Cbl/10^5 cells) were taken up and accumulated by the algal cells grown in Cbl (10 μg/l)-supplemented medium. Most Cbl accumulated by the cells was converted to methylcobalamin (MeCbl; 46.1%) and 5′ -deoxyadenosylcobalamin (AdoCbl; 26.0%), which function as coenzyme of methionine synthase (EC 2.1.1.13) and methylmalonyl-CoA mutase (MCM; EC 5.4.99.2), respectively (Miyamoto et al., 2002b).

The AdoCbl-dependent MCM that catalyzes isomerization of (*R*)-methylmalonyl-CoA to succinyl-CoA has been purified and characterized from mammalian tissues (Fenton et al., 1982), an intestinal nematode (Han et al., 1984), and aerobic (Miyamoto et al., 2002a, 2003) and anaerobic bacteria (Francalanci et al., 1986). To elucidate the mechanisms for the enzyme reaction, the anaerobic bacterium *Propionibacterium shermanii* MCM has been studied extensively (Mancia and Evans, 1998; Mancia et al., 1999). However, little information is available on the enzymological properties of MCM in marine algae.

Here, we describe the purification and characterization of MCM from the photosynthetic coccolithophorid alga *P. carterae* from the viewpoint of comparative biochemistry.

2. Materials and methods

2.1. Culture and organism

P. carterae was provided by Prof. M. Okazaki (Tokyo Gakugei University, Japan). Algal cells were cultured for 10 days at 26 °C in a modified Eppley medium (containing 20 μg cyanocobalamin/l; Miyamoto et al., 2002b) prepared with synthetic seawater. The culture (1.5 l) was bubbled with air and illuminated at 40 μmol photon/m^2/s.

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2.2. Enzyme assay

Methylmalonyl-CoA mutase was assayed by a modification of the HPLC method described by Gaire et al. (1999). Briefly, the assay mixture (0.15 ml) for total MCM activity contained 100 mM potassium phosphate buffer, pH 7.5, 30 μM AdoCbl (Sigma, St. Louis, MO, USA), 0.15 mM (*R,S*)-methylmalonyl-CoA (Sigma), and enzyme. AdoCbl was replaced by the same volume of distilled water for measuring holo-MCM activity. Apo-MCM activity was calculated by subtracting the holo-MCM activity from total MCM activity in the sample. The components except for (*R,S*)-methylmalonyl-CoA were mixed in microcentrifuge tubes in the dark and the temperature was equilibrated by incubation in a heating bucket (e-Heating Bucket, Taitec, Saitama, Japan) maintained at 30 °C. The reaction mixture was preincubated for 5 min, started by the addition of (*R,S*)-methylmalonyl-CoA, and left for 5 min. The enzyme reaction was stopped by the addition of 50 μl 10% (w/v) trichloroacetic acid. The reaction mixture was filtered through a 0.45-μm membrane filter (Millex Syringe Driven Filter Unit, LH-type, Millipore, USA). Aliquots (20 μl) of the filtrate were analyzed by HPLC using a Shimadzu HPLC apparatus (two LC-10ADvp pumps, DGV-12A degasser, SCL-10Avp system controller, SPD-10Avvp UV–VIS detector, CTO-10Avp column oven, 100 μl sample loop, C-R6A chromatopac integrator). The sample (20 μl) was put on a reversed-phase HPLC column (Cosmosil 5C18-AR-II, ∅ 3.0 × 150 mm) equilibrated with 50% (v/v) solvent A (100 mM acetic acid in 100 mM potassium phosphate buffer, pH 7.0) and 50% (v/v) solvent B [18% (v/v) methanol in solvent A]. (*R,S*)-Methylmalonyl-CoA and succinyl-CoA were eluted with a linear gradient of methanol [50–100% (v/v) solvent B] for 7.0 min at 40 °C and assayed by measurement of absorbance at 254 nm. The flow rate was 1.0 ml/min. MCM activity was calculated from the amount of succinyl-CoA formed. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of succinyl-CoA at the rate of 1 μmol/min.

2.3. Purification of P. carterae MCM

Purification was performed at 0–4 °C with a BioLogic HR chromatography system (Bio-Rad Laboratories, Hercules, CA, USA) in a dark room. The stored *P. carterae* cells (about 15 g wet mass) were suspended in 30 ml, 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose. The cells were disrupted by sonic oscillation (10 kHz, 10 s × 6) and centrifuged at 10,000 × *g* for 10 min. AdoCbl was added to the supernatant fraction at a final concentration of 10 μM and left overnight in the dark at 4 °C to form the holo-enzyme. The treated solution was put on a column (2.4 × 20 cm) of TSKgel QAE-Toyopearl HW55C equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose, and eluted with 300 ml of a linear gradient (0–0.5 M) of KCl in the same buffer. The active fractions were collected and concentrated in a Centriprep-30 centrifugal concentrator (Millipore, Bedford, MA, USA) to a final volume of 1.0 ml. The concentrated solution (1.0 ml) was put on a HiLoad 16/60 Superdex 200 pg column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose and 200 mM KCl, and eluted with the same buffer. The active fractions were combined and dialyzed overnight against 1 l of 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose. The dialyzed solution was concentrated in a Centricon-30 microconcentrator (Millipore) to a final volume of 1.0 ml. The concentrated solution (1.0 ml) was put on a UNO Q-1 column (Bio-Rad) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose, and eluted with 40 ml of a linear gradient (0–0.5 M) of KCl in the same buffer. The active fractions were combined and desalted in Centricon-30 microconcentrators. The solution was put on a Mono Q column HR 5/5 (Amersham Pharmacia Biotech) and eluted with 40 ml of a linear gradient (0–0.5 M) of KCl in the same buffer. The peak fraction of the enzyme activity was concentrated to a final volume of 0.1 ml as above and stored at −80 °C.

2.4. Polyacrylamide gel electrophoresis in the presence or absence of SDS

Polyacrylamide gel electrophoresis (PAGE) was carried out on 5–20% precast Ready Gels J (Bio-Rad, Tokyo, Japan). Purified enzyme (1 μg protein) was electrophoresed in the presence or absence of SDS at constant current (12 mA/gel) with bromophenol blue as a migration marker. After electrophoresis on the slab gel, proteins in the gel were stained with Coomassie brilliant blue R-250 and destained in acetic acid solution according to the manufacturer's instructions. Standard proteins (phosphorylase *b* from rabbit muscle, 97.4 kDa; albumin from bovine serum, 66.2 kDa; ovalubmin, 45.0 kDa; carbonic anhydrase from bovine erythrocyte, 31.0 kDa; trypsin-inhibitor from soybeans, 21.5 kDa; lysozyme from egg whites, 14.4 kDa) in a Bio-Rad kit were used for the calibration of the molecular mass of the subunit of *P. carterae* MCM.

2.5. Gel filtration experiments

The molecular mass of *P. carterae* MCM was determined with a HiLoad 16/60 Superdex 200 pg gel filtration column (Amersham Pharmacia Biotech) using the BioLogic HR chromatography system (Bio-Rad). The column was equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose and 200 mM KCl, and eluted with the same buffer. The molecular mass of the MCM was calibrated with blue dextran (average 2000 kDa), horse spleen apoferritin (480 kDa), yeast alcohol dehydrogenase

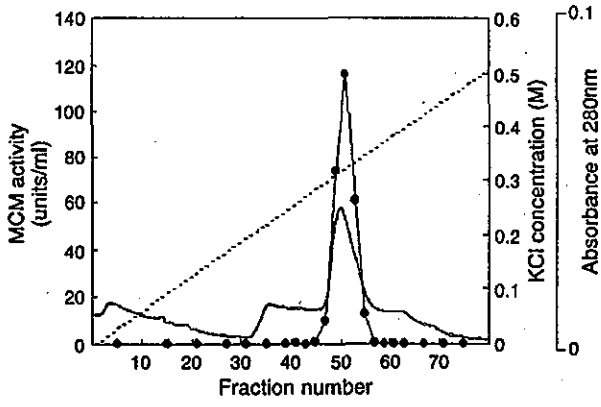

Fig. 1. Elution patterns of *Pleurochrysis carterae* methylmalonyl-CoA mutase activity during Mono-Q column chromatography.(–) absorbance at 280 nm; (●) MCM activity; and (---) KCl concentration. The data are typical elution patterns of the enzyme activity during Mono-Q HR 5/5 column chromatography from four independent experiments.

(150 kDa), bovine serum albumin (66.0 kDa), and horse heart cytochrome *c* (12.4 kDa). Blue dextran and the proteins were monitored by measuring the absorbance at 280 nm.

2.6. Properties of MCM

2.6.1. Optimum temperature and pH

The optimum temperature of the enzyme was determined by incubating at temperatures ranging from 5 to 50 °C for 5 min at pH 7.5. In the case of the thermal stability of the enzyme, the enzyme activity was assayed at 30 °C for 5 min at pH 7.5 after the purified enzyme was preincubated at temperatures ranging from 5 to 50 °C for 10 min at pH 7.0.

The optimum pH of the enzyme was determined by incubation at various pH values ranging from 5.0 to 9.5 in 10 mM Tris–acetate buffer for 5 min at 30 °C.

2.6.2. Effect of SH-inhibitors

Enzyme activity was assayed at 30 °C for 5 min at pH 7.5 using the purified enzyme treated with 3 mM of SH-inhibitors [$HgCl_2$, *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, 5,5′ -dithiobis-(2-nitrobenzoic acid), and iodoacetoamide] at 30 °C for 10 min at pH 7.0.

2.6.3. Effect of monovalent and divalent cations

Enzyme activity was assayed at 30 °C for 5 min at pH 7.5 in the presence of each monovalent and divalent cation (NaCl, KCl, NH_4Cl, $CoCl_2$, $MnCl_2$, $FeCl_2$ and $MgCl_2$ all at 3 mM).

2.6.4. Effect of substrate concentrations

In the case of determination of K_m values for methylmalonyl-CoA and succinyl-CoA (reverse reaction), the enzyme activity was assayed at various concentrations of (*R,S*)-methylmalonyl-CoA or succinyl-CoA under the standard assay conditions.

2.7. Protein assay

Protein was assayed by the use of Bio-Rad protein assay kit, with ovalbumin as a standard according to the manufacturer's instructions.

3. Results and discussion

3.1. MCM activity in a cell homogenate of P. carterae

MCM activity was considerably lower in a cell homogenate of *P. carterae* (about 4 mU/mg protein) grown for 10 days (stationary phase) than in those of the other organisms (about 25–50 mU/mg protein) previously reported (Kellermeyer et al., 1964; Han et al., 1984; Miyamoto et al., 2002a). Low specific activity (2.6 mU/mg protein) of this enzyme is also found in a cell homogenate of *Euglena gracilis* Z (a fresh water-Cbl-requiring alga) grown photoautotrophically; it is significantly increased in those cells adapted to propionate-supplemented medium (Watanabe et al., 1996).

Most (about 95%) of the *P. carterae* enzyme occurred as the apo-enzyme. Our preliminary experiments indicated that the enzyme activity, when assayed in the presence of AdoCbl, was completely lost through several column chromatographies. As a consequence of the lability of the apo-enzyme, the enzyme protein could not be purified to homogeneity. The holo-enzyme, which was converted from the apo- enzyme by incubation with 10 μM AdoCbl at 4 °C overnight in the dark, was considerably more stable relative to the apo-enzyme. Thus, the holo-enzyme was purified to homogeneity.

3.2. Purification of P. carterae MCM

Fig. 1 shows the elution profile of *P. carterae* MCM during Mono-Q HR 5/5 column chromatography (the final purification step). The enzyme activity gave a single peak at 0.35 M KCl and most of the protein was recovered in this fraction. The purification procedures for MCM from a homogenate of *P. carterae* are summarized in Table 1.

Table 1
Purification of methylmalonyl-CoA mutase from *P. carterae*

Step	Total protein (mg)	Total activity (units/min)	Specific activity (units/mg protein)	Yield (%)
Crude homogenate	871.9	3.54	0.004	100.0
TSKgel QAE-Toyopearl 550	84.6	2.33	0.03	65.9
HiLoad 16/60 Superdex 200 pg	2.72	0.51	0.19	14.5
UNO Q-1	0.30	0.17	0.58	4.9
Mono-Q	0.003	0.03	11.94	0.87

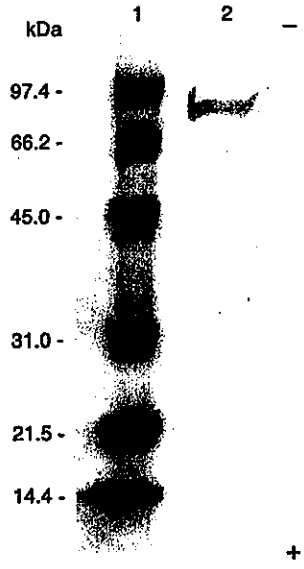

Fig. 2. PAGE of methylmalonyl-CoA mutase from the final purification step. (1) Molecular mass standard proteins; (2) the purified enzyme in the presence of SDS. Typical migration patterns of the purified enzyme after polyacrylamide slab gel electrophoresis in the presence of SDS from three independent experiments.

MCM was purified about 2985-fold over the homogenate with a yield of 0.87%. PAGE of the purified enzyme in the absence of SDS using a precast gel showed a single protein band (data not shown).

3.3. Molecular mass

An apparent molecular mass of the enzyme was calculated to be 150 ± 5 kDa by Superdex 200 pg gel filtration. SDS-PAGE of the purified enzyme gave a single protein band with an apparent molecular mass of 80 ± 5 kDa, indicating that the *P. carterae* enzyme is composed of two identical subunits (Fig. 2).

P. carterae MCM has a similar subunit structure to the mammalian (Fenton et al., 1982) and worm (Han et al., 1984) enzymes, while the *P. shermanii* (Francalanci et al., 1986) and *Methylobacterium extorquens* NR-1 (Miyamoto et al., 2002a) enzymes consist of two non-identical subunits with molecular masses of 79–85 and 67–70 kDa, respectively.

3.4. Some properties of MCM

The optimum temperature for MCM activity was 30 °C. The enzyme, when incubated at various temperatures for 10 min at pH 7.5, was stable up to 10 °C; activity was completely lost at 50 °C. The optimum pH of the enzyme was 7.5.

The apparent K_m values of the enzyme were 0.59 mM for (*R,S*)-methylmalonyl-CoA and 5.5 mM for succinyl-CoA

The purified enzyme activity was inhibited completely by the addition of 3 mM $HgCl_2$, but not by the addition of commonly used SH-inhibitors, iodoacetamide, *N*-ethylmaleimide, *p*-chloromercuribenzoic acid and 5,5′ -dithiobis-(2-nitrobenzoic acid).

MCM activities of *P. shermanii* (Kellermeyer et al., 1964), *M. extorquens* NR-1 (Miyamoto et al., 2002a) and intestinal worm (Han et al., 1984) are not affected by the SH-inhibitors, but those of *Sinorhizobium meliloti* (Miyamoto et al., 2003) and mammals (Fenton et al., 1982) are considerably inhibited.

Although the *S. meliloti* MCM (Miyamoto et al., 2003) is activated by the addition of some monovalent cations (NH_4^+, K^+ and so on), the addition of monovalent (Na^+, K^+ and NH_4^+) and divalent cations (Co^{2+}, Mn^{2+}, Fe^{2+} and Mg^{2+}) at 3 mM did not affect the enzyme activity of *P. carterae*. The identical result has been reported in MCM of other organisms (Fenton et al., 1982; Kellermeyer et al., 1964; Miyamoto et al., 2002a).

Due to the low specific activity of the enzyme in the cell homogenate and lability of the enzyme during purification, we could not obtain enough enzyme to study further detailed enzyme properties. However, these results presented here indicate for the first time that the marine alga *P. carterae* contains an AdoCbl-dependent homodimeric MCM.

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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, ϕ 4.6 × 150 mm^2; particle size, 5 μm) 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 × 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, ϕ 4.6 × 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, ϕ 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 ± 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

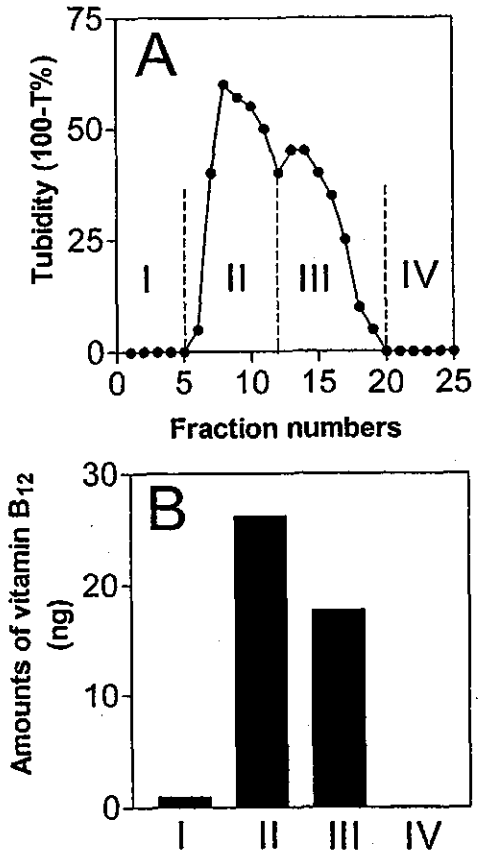

Figure 1. Elution profiles of corrinoid compounds from a Japanese traditional fish sauce "Ishiru," during a reversed-phase HPLC in the purification steps. Corrinoid compounds were determined by the microbiological method. (A) Fractions 1–5 (I), 6–12 (II), 13–20 (III), and 21–25 (IV) were combined and 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

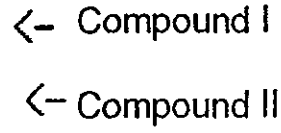

Figure 2. Silica gel 60 TLC pattern of the purified corrinoid compounds. Data present a typical migration pattern of the purified corrinoid compounds by TLC from three experiments.

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]

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日本人の母乳中ビタミンB_6含量

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The Vitamin B_6 Content in Milk of Japanese Women

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The recommended dietary allowance (RDA) of vitamin B_6 (B_6) for Japanese was formulated for the first time in the 6th revised National Reference Intake in Japan. The RDA of infant is 0.1mg/day in the 6th revision. Milk intake of infant was calculated to be 850 ml/day in the 5th revised RDA, however it was set at 750 ml/day based on investigation in the 6th revised RDA. The B_6 intake of infant deeply depended on the content of B_6 in breast milk. Thus we determined the B_6 contents in breast milk of Japanese women.

Milk samples were collected from 25 healthy nursing women whose infants were 2 ～ 5 ages in month. Total B_6 content in milk was quantified by HPLC. The average content of B_6 was 0.25 mg/*l* breast milk. This value was not different from those of other studies and the datum in 6th revised RDA.

Key Words: vitamin B_6 , human milk , Japanese women , infant , RDA

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緒 言

日本人の栄養所要量は，昭和45年に初回の策定が施行されて以来，日本人の体格，生活習慣などに合わせて5年ごとに改定されている．平成12年度(2000年)から実施されている第6次改定[1]では，食事摂取基準が設けられ，さらにビタミン6項目，ミネラル7項目が新規に追加された．ビタミンB_6(B_6)は第6次改定により初めて策定されたビタミンのひとつである．成人の所要量は，疫学調査を基に求められているが乳児の所要量は摂取する母乳に依存する．母乳は3大栄養素の

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略語：PCA, Perchloric acid; PL, Pyridoxal; PLP, Pyridoxal 5'-phosphate.

ほかビタミン，ミネラルなどバランスよく含む食品であり[2]，乳児の成育には最も適した栄養源である．

第 6 次改定日本人の栄養所要量での乳児の B_6 所要量は，0.1 mg/日と定められている[1]．これは 2.5 mg/日以下の B_6 摂取量の健康な母親の母乳中の B_6 含量が0.13 mg/*l* であるという West と Kirksey の報告[3]を基に設定されている．また第 5 次改定までは乳児の哺乳量を 850 ml/日として所要量を算定していたが，第 6 次改定においてこれまでの哺乳量の調査に基づいて，乳児の哺乳量は 750 ml/日と 100 ml 削減された．

乳児の所要量は母乳に含まれるビタミン B_6 含量と哺乳量に依存するため，これらを明らかにすることにより乳児の所要量を明確にできると考えられる．また，これまでのところ母乳中の B_6 含量については諸外国のデータがほとんどであり，食習慣や体格の違いを考慮すると日本人を対象にしたデータを用いて十分に検討する必要があると考えられる．そこで今回は，わが国の授乳婦から採取した母乳中の B_6 含量を測定した．

実　験　方　法

1．被験者

被験者は妊娠並びに出産が正常な経過で満期出産し，満月齢で 2 ～ 5ヶ月の乳児を完全母乳哺育している日本人授乳婦を対象にした．対象者の摂取している食事組成については不明であるが，栄養に対する興味も高くバランスのとれた食事を摂取していることを前提とし，本趣意に同意し体調の良いボランティアから採取した母乳 25 検体を使用した．

また今回の研究は，全てヘルシンキ宣言に従って実施され，昭和女子大学における倫理委員会より承認を得て実施したものである（承認番号 *01-06*　平成 14 年 2 月 4 日承認）．

2．母乳採取

母乳は，ほぼ 14 時～ 16 時の授乳後に，乳房をマッサージした後の後乳を採取して，冷凍母乳パック（カネソン本舗社製）に保存し，分析に供するまで－20℃にて保存した．

3．ビタミン B_6 の分析

母乳中の PLP 濃度および B_6 ビタマー濃度は，HPLC 法を用いて測定した[4]．母乳サンプル 0.5 ml は 3*N* 過塩素酸（PCA）0.25 ml で除タンパク質処理を行った．この上清に 1 M リン酸ナトリウム緩衝液（pH 5.5）を 0.2 ml 加え，5N KOH で pH を 3.5 に調整したものを PCA 抽出液とした．PLP の検出は PCA 抽出液を pH 7.5 に調整してからシアン化カリウムで処理を行い，再び pH 3.5 に調整してから HPLC 分析に供した．また他の B_6 ビタマーは，PCA 抽出液を 0.45 μM のメンブランフィルターに通した後，HPLC 分析に供した．HPLC 分析の条件は以下の通りである．

HPLC分析条件
検出波長；
　＜PLP＞励起波長 320 nm，蛍光波長 420 nm
　＜PLP 以外の B_6 ビタマー＞
　　励起波長 305 nm，蛍光波長 390 nm
流速；0.5 ml/min
温度；30℃
移動相；CH_3CN / 0.1M $KHPO_4$ - 0.1M $NaClO_4$（pH 3.5）
　　= 1 : 99 (v/v)
カラム；TOSOH TSKgel ODS-120A (4.6 mm ID 25 cm)

結　　果

表 1 および図 1 は，日本人授乳婦より採取した母乳に含まれる B_6 含量の平均値と 25 検体の総 B_6 量の分布を示したものである．分析の結果，PLP と PL 以外のビタマーは検出されなかった．分析した 25 検体の母乳中 B_6 含量は，全平均で PLP が 1.15±0.09 μM，PL が 0.30± 0.02 μM であり，全 B_6 濃度として 1.45±0.09 μM (0.25 mg/*l* PN 換算量）であった．

考　　察

これまでに報告されている母乳中の B_6 含量を表 2 に示した．母乳中に含まれる B_6 濃度は，母親の B_6 摂取量に依存して変化することが報告[3]されている．Borschel ら[5]は，母親が 2.5 mg/日の B_6 摂取量であると，乳児の B_6 摂取量は 0.1 mg/日であるとしている．また West と Kirksey[3]によると 2.5 mg/日以下の B_6 摂取量の母親の母乳中の B_6 含量は，*0.13 mg/l* であるとし，Thomas ら[6]は，0.21 mg/*l* であると報告している．第 6 次改定日本人の栄養所要量では，2.5 mg/日以下の B_6 摂取をしている母親

表 1．Content of Vitamin B_6 in Human Milk.

(μM)	PLP	PL	Total B_6
	1.15±0.09	0.30±0.02	1.45±0.09

Mean ± S.E. (n=25)

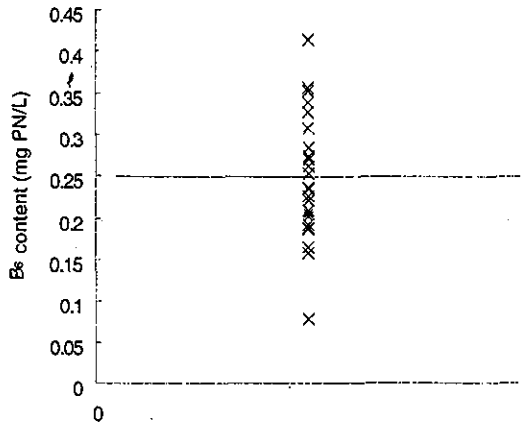

図 1. Distribution of B_6 content in human milk. ※ Line shows mean value (n=25).

表 2. Content of Vitamin B_6 in Human Milk.

Literature	Year	B_6 content in human milk (mg/*l*)	Methods of analysis
West and Kirksey[3]	1976	0.13	microbiological assay
Thomas *et al.*[10]	1979	0.204	microbiological assay
Thomas *et al.*[6]	1980	0.21	microbiological assay
Börschel *et al.*[5]	1986	0.11～0.33	microbiological assay
Andon *et al.*[7]	1989	0.124※	microbiological assay
Morrison and Driskell[8]	1985	0.162※	HPLC
Present study	2004	0.25	HPLC

※文献では nM 表記であったが，PN 換算の数値として示した．

の母乳中の B_6 含量は 0.13 mg/*l* であるという報告に基づき，一日の哺乳量が 750 ml として 0～5ヶ月齢の乳児の所要量は 0.1 mg/日と設定されている．

一般に食品中の B_6 含量を測定する方法は，*Saccharomyces cerevisiae* ATCC9080 を用いた微生物定量法が主である．この方法は全 B_6 化合物を一括して定量することができるため簡便である．しかし，*S. cerevisiae* はリン酸エステル型を含めた結合型誘導体を利用できないため，定量に先立ち試料を加水分解し遊離型に変換するための前処理操作が必要である．最近では HPLC 法による B_6 定量法が用いられており，微生物定量法を用いた Andon ら[7]の報告と，HPLC 法を用いた Morrison と Driskell[8]の測定法による差異は認められない．本研究では Tsuge[4]の方法による HPLC 法を用いて母乳中の B_6 含量を測定した．

West と Kirksey[3]は産後の日数の違いによる母乳中 B_6 含量についても報告している．これによると 2.5 mg/日あるいはそれ以上の B_6 摂取をしている母親において，産後 3ヶ月以内の母乳中 B_6 含量は，0.26 mg/*l*，3～7 ヶ月では 0.29 mg/*l*，7 ヶ月以上では 0.25 mg/*l* と，産後の日数の違いによる変動は見られていない．今回の母乳は産後 2～5ヶ月であり，母乳中の B_6 含量は平均で 0.25 mg/*l* であった．これは，これまでに報告されている同時期の母乳中の含量とほぼ同値であった．

わが国の調製粉乳に含まれる B_6 量[9]は，一般調製粉乳で製品 100 g 当たり 0.3～0.6 mg(60～120 μg /100 kcal，あるいは 14 % 調製乳液 100 ml 当たり 42～84 μg)である．この人工乳を 1 日 750 ml 与えると，0.32～0.63 mg/日の B_6 を摂取できることになる．この値は，第 6 次改定日本人の栄養所要量に示されている 0.1 mg/日を十分に満たしていることになる．Borschel ら[5]の報告によると，人工乳により 0.45～0.58 mg/日の B_6 を摂取している乳児と，B_6 の摂取量が 2.5 mg/日以下の母親の母乳により 0.11～0.33 mg/日の B_6 量を摂取している乳児の成

育は，6ヶ月齢までは身長，体重共に有意な差は見られなかったと報告している．これより第6次改定日本人の栄養所要量で定められている乳児の所要量 0.1 mg/日は成育には適していると考えられる．今回の調査は日本人の乳児の B_6 所要量に対する基礎データとして有用であると考えられる．

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文　献

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