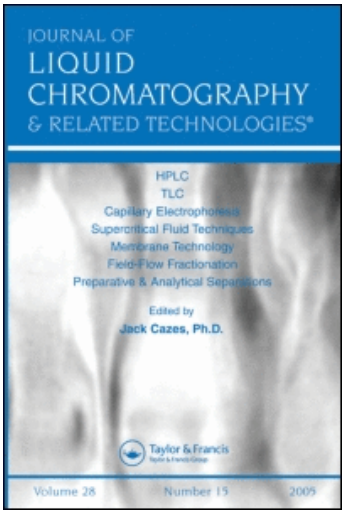

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TLC-BIOAUTOGRAPHY ANALYSIS OF VITAMIN B_{12} COMPOUND FROM THE SHORT-NECKED CLAM (*RUDITAPES PHILIPPINARUM*) EXTRACT USED AS A FLAVORING

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□ *To determine whether certain shellfish extracts used as flavorings became excellent food sources of free vitamin B_{12}, their vitamin B_{12} contents were assayed and characterized. Although vitamin B_{12} contents of scallop and freshwater clam extracts were none and very low (0.1 μg/100 g), respectively, short-necked clam extract contained substantial amounts of vitamin B_{12} (131.8 μg/100 g). A vitamin B_{12} compound was purified and characterized with silica gel 60 TLC and a reversed-phase HPLC. The purified red-colored compound was identical to true vitamin B_{12}, but not to inactive corrinoid compounds. Sephadex™ G-50 gel filtration experiments indicated that most of vitamin B_{12} found in the short-necked clam extract was recovered in the free vitamin B_{12} fractions. These results indicate that the short-necked clam extract would be a natural source of free vitamin B_{12} for elderly persons with food-bound vitamin B_{12} malabsorption.*

Keywords flavoring, *Ruditapes philippinarum*, shellfish, short-necked clam, vitamin B_{12}

INTRODUCTION

A considerable proportion of the people who have low serum vitamin B_{12} levels show malabsorption of protein-bound B_{12}.[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 peoples.[2,3] Because the bioavailability of crystalline (free) B_{12} is not altered in peoples with atrophic gastritis,[4] the Institute of Medicine has recommended that most of the recommended dietary allowance

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(RDA; 2.4 μg/day) should be obtained by consuming some foods fortified with free B_{12} or free B_{12}-containing supplement.[5]

The B_{12} compounds are only synthesized by certain bacteria and then concentrated mainly in the bodies of higher predatory animals in natural food chain system.[6] The usual dietary sources of B_{12} are animal food products (i.e., meat, milk, egg, fish, and shellfish).[7] As shellfishes can siphon large quantities of microorganisms in the sea and freshwater, they are known to be excellent sources of B_{12}. Indeed, our previous study has demonstrated that edible shellfishes contain substantial amounts of true B_{12}.[8] In Asian countries, especially Japan, various types of shellfish extracts are manufactured to use as seasonings or flavorings. If the shellfish extracts contain substantial amount of free B_{12}, they would became natural and excellent sources of free B_{12} for elderly persons with food-bound B_{12} malabsorption. Here, we describe purification and characterization of B_{12} compound from the short-necked clam extract.

EXPERIMENTAL

Materials

B_{12} was purchased from Sigma (St. Louis, Missouri, USA). 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). A reversed-phase HPLC column (Wakosil-II 5C18RS, $\phi 4.6 \times 150$ mm; particle size 5 μm) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Cosmosil 140C18-OPN was obtained from Nacalai Tesque (Kyoto, Japan). All other reagents used were of the highest purity commercially available. The shellfish extracts used in this study were given from Maruhahi Muramatsu Corp., Shizuoka, Japan. A Shimadzu (Kyoto, Japan) ultraviolet-visible spectrophotometer UV-2550 was used for this study.

Methods

Extraction and assay of vitamin B_{12}

Two grams of each shellfish extract were used for a sample. Total B_{12} was extracted with boiling at pH 4.8 in the presence of KCN and assayed by the microbiological method with *L. delbrueckii* ATCC 7830 according to the method adopted in the Japanese Standard Tables of Food Composition.[9] Since *L. delbrueckii* ATCC 7830 can utilize both deoxyribosides and deoxyribonucleotides (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}.

Bioautography of vitamin B_{12} compound with vitamin B_{12}-dependent **Escherichia coli** *215*

Bioautograpy of B_{12} compound was done according to the method of the reference cited.[10] After the above sample of the short-necked clam extract was concentrated and partially purified with Sep-pack C18 cartridge (Waters Corp., Milford, USA), 2 μL of the purified B_{12} extract and authentic B_{12} (cyanocobalamin, 10 μg/L) were spotted on the silica gel 60 TLC sheet and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) in the dark at 25°C. After the TLC sheet was dried, agar containing basal medium and pre-cultured *E. coli* 215 was overlaid and then incubated at 30°C for 20 h. After being sprayed with a methanol solution of 2,3,5-triphenyltetrazolium salt on the gel plate, B_{12} compounds were visualized as red in color indicating *E. coli* growth.

Purification of vitamin B_{12} compound from the short-necked clam extract

About 500 g of the short-necked clam extract was added to 4 L of 0.57 mmol/L sodium acetate buffer, pH 4.8, containing 0.05% (w/v) KCN. B_{12} compounds were extracted from the solution by boiling for 30 min at 98°C in the dark. The extraction procedures were done in a Dalton (DF-11AK; Tokyo, Japan) draught chamber with a fume hood. After the boiled solution was cooled to room temperature (25°C), it was put on a column (50 × 400 mm) of Amberlite XAD-4 resin which had been washed with 5 L of methanol and then equilibrated with distilled water. After the column was washed with 3 L of distilled water, B_{12} compounds were eluted with 2.0 L of 30% (v/v) methanol solution. The 30% (v/v) methanol eluate was pooled, evaporated to dryness under reduced pressure, and dissolved in 50 mL of distilled water. The solution was placed on a column (24 × 150 mm) of Cosmosil 140C18-OPN (Nacalai Tesque) which had been washed with 75% (v/v) methanol solution and equilibrated with distilled water. The B_{12} compounds were eluted with a stepwise gradient (0, 10, 20, and 30% v/v) of methanol. These four fractions were separately evaporated to dryness under reduced pressure, and dissolved with a small amount of distilled water. The 10% methanol fraction was purified with silica gel 60 TLC, which was developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) as the solvent in dark at room temperature (25°C). A spot with red-tint on the dried TLC sheet was collected, extracted with 80% (v/v) methanol solution, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was further purified by TLC under the same conditions. The

concentrated solution was further purified by HPLC (JASCO PU-2080 Plus Intelligent HPLC Pump, UV-2075 Plus Intelligent UV/Vis-detector, and DG-2080-53 Degassor; and Shimadzu CTO-6A column oven and C-R6A Chromatopac). The sample (100 μL) was put on a reversed-phase HPLC column (Wakosil-II 5C18RS) 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, dissolved in a small amount of distilled water, and used as a purified B_{12} compound.

Analytical TLC and HPLC

Concentrated solutions (2 μL each) of the purified compound and authentic B_{12} were spotted on silica gel 60 TLC sheets and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) and 1-butanol/2-propanol/water (10:7:10 v/v) as solvents in the dark at room temperature (25°C).

In the case of HPLC, the diluted solutions (20 μL each) of the purified compound and authentic B_{12} were analyzed with the reversed-phase HPLC column (Wakosil-II 5C18RS) using the HPLC apparatus. The B_{12} compounds 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 compounds were determined at a flow rate of 1.0 mL/min.

Ultraviolet-visible spectrum

The purified B_{12} compound was dissolved in 2.0 mL of distilled water. The spectrum of the purified compound was measured with a Shimadzu spectrophotometer (UV-2550) at room temperature (25°C). Quartz cuvettes (2.0 mL, $d = 1$ cm) were used.

Sephadex™ G-50 gel filtration experiment

The free B_{12} found in the clam extract was separated with Sephadex™ G-50 fine (GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, England) gel filtration column (15 × 140 mm) and then microbiologically assayed. The short-necked clam extract was diluted seven times with 1.0 mmol/L potassium phosphate buffer, pH 7.0, containing 0.2 mol/L KCl. Aliquot (1.0 mL) of the diluted sample was applied on the column which had been equilibrated with 1.0 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 had been 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 described above and assayed by the microbiological assay method.

RESULTS AND DISCUSSION

As shown in Table 1, the Japanese Standard Tables of Food Composition[9] has described that raw shot-necked and freshwater clams contain substantial amount of B_{12}. The B_{12} contents of certain shellfish extracts used as flavorings in food manufactures were determined by the microbiological method. Although B_{12} contents of scallop and freshwater clam extracts were none and very low, respectively, short-necked clam extract contained substantially amounts of B_{12} (131.8 μg/100 g). These results suggest that significant loss of B_{12} is shown during preparation of their extracts from the raw shellfishes.

The B_{12} sample of the short-necked clam extract was analyzed with the *E. coli* 215 bioautography after being separated by silica gel 60 TLC (Fig. 1). The B_{12}-activity found in the clam extract was given as a single spot, whose R_f value (0.58) was identical to that of authentic B_{12}.

To determine whether the B_{12}-activity detected in the short-necked clam extract by the microbiological assay method is derived from true B_{12} or not, a B_{12} compound was purified and characterized from the short-necked clam extract. The red-colored compound was easily separated during silica gel 60 TLC (Fig. 2). The final purified preparation gave a single red-colored spot on the silica gel 60 TLC and a single peak by the

TABLE 1 Vitamin B_{12} Contents of Some Shellfish Extracts Used for Flavorings

	Vitamin B_{12} content (μg/100 g)	Reference
Raw materials		
Short-necked clams (*Ruditapes philippinarum*)	52.4	9
	37.0	8
Freshwater clams (*Corbicula japonica*)	62.4	9
	38.4	8
Scallops (*Patinopecten yessoensis*)	11.4	9
	13.4	8
Concentrated extracts		
Short-neck clams	131.8*	This study
Freshwater clams	0.1*	This study
Scallops	ND	This study

ND, not detected.

*The values presented mean values from five independent experiments.

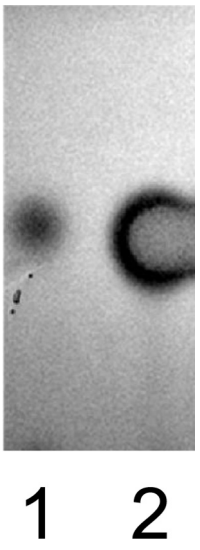

FIGURE 1 *E. coli* 215-bioautogram after silica gel 60 TLC of the B_{12} sample of the short-necked clam extract and authentic B_{12}. 1, Authentic B_{12} and 2, the short-necked clam extract. The data are the typical bioautogram from four independent experiments.

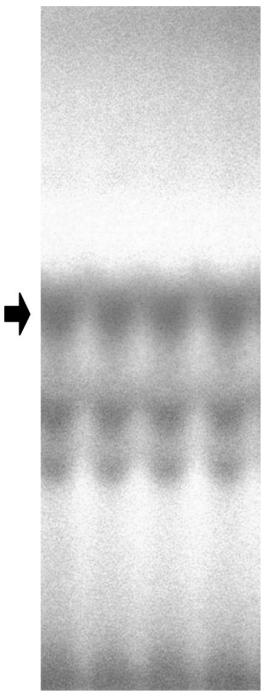

FIGURE 2 Migration patterns of B_{12} (red-colored) compound during 2nd-Silica gel 60 TLC in the purification steps. Data present a typical migration pattern of the compound on the TLC. Arrow shows red-colored spots.

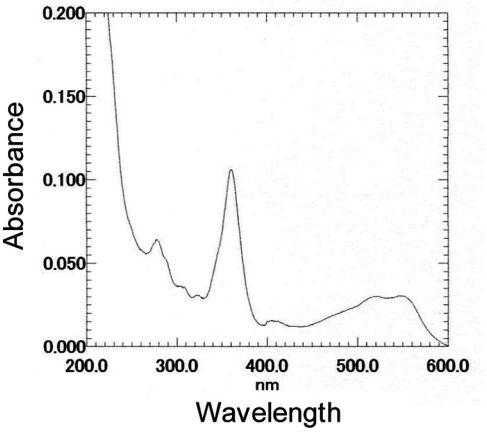

FIGURE 3 Ultraviolet-visible spectrum of the red-colored compound purified from the short-necked clam extract.

reversed-phase HPLC, indicating that the B_{12} compound was purified to homogeneity. The ultraviolet-visible spectrum of the purified compound showed a typical absorption spectrum of cobalt-containing corrinoid (Fig. 3); λ_{max} nm (absorbance) of the compound was at 547.5 (0.031), 405.0 (0.016), 360.0 (0.106), and 278.0 (0.064). The R_f values (0.58 and 0.12) of the purified compound were identical to the values of authentic B_{12}, of which the retention time (8.7 min) was also identical to that of the purified compound.

The Sephadex^{TM}-G50 gel filtration of the clam extract demonstrated that most (>98.5%) of the B_{12} found in the clam extract was recovered in the free B_{12} fractions (Table 2). These results suggest that the short-necked clam extract is available on a natural source of free B_{12} (as a functional seasoning or flavoring) for humans.

As about 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),[1] Institute of Medicine (1998) has recommended that most of the RDA (2.4 μg/day) should be obtained by consuming some foods fortified with free B_{12} or free

TABLE 2 Occurrence of Free Vitamin B_{12} in the Short-necked Clam Extract

Percentage of vitamin B_{12}*	
Macromolecular fraction	Free vitamin B_{12} fraction
1.5 ± 0.6	98.5 ± 0.6

*Percentage against vitamin B_{12} content of the shellfish extract without the treatment. The values presented mean ± SD from five independent experiments.

B_{12}-containing supplement.[5] Feeding of only 1.8 g of the clam extract can supply the RDA for adults.

The results presented here indicate that certain foods (soup, noodles, and so on) manufactured with the short-necked clam extract would be an excellent and natural source of free B_{12} for elderly persons with food-bound B_{12} malabsorption.

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