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Broth from Canned Clams Is Suitable for Use as an Excellent Source of Free Vitamin B_{12}

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ABSTRACT: Vitamin B_{12} was assayed and characterized in the broth of canned clams (boiled plain). The broth contained considerable amounts of vitamin B_{12} (2.7−14.1 μg/100 g, 1.3−6.7 μg/can). HPLC and LC/ESI-MS/MS chromatograms demonstrated that the clam broth contained true vitamin B_{12}. Gel filtration experiments indicated that most (72%) of the vitamin B_{12} found in the broth was recovered in free vitamin B_{12} fractions. These results indicate that the clam broth would be suitable for use an excellent source of free vitamin B_{12} for elderly persons with food-bound vitamin B_{12} malabsorption.

KEYWORDS: canned clam, boiled plain, broth, free vitamin B_{12}, vitamin B_{12} deficiency

INTRODUCTION

A considerable proportion of the people who have low serum vitamin B_{12} (B_{12}) levels but who do not have pernicious anemia exhibit malabsorption of protein-bound B_{12} (food-bound B_{12} malabsorption).[1] Food-bound B_{12} malabsorption is found in persons with certain gastric dysfunctions, especially atrophic gastritis with low stomach acid secretion, which prevails in the elderly (Figure 1).[2,3] Because the bioavailability of crystalline (free) B_{12} is not altered in people with atrophic gastritis,[4] the Institute of Medicine has recommended that most of the recommended dietary allowance (RDA) (2.4 μg/day) should be obtained by consuming foods fortified with B_{12} or a B_{12}-containing supplement.[5]

The usual dietary sources of B_{12} are animal food products (i.e., meat, milk, egg, fish, and shellfish).[6] Japanese people obtain most (~84%) of their daily B_{12} intake from both fish and shellfish.[7] Shellfish, which siphon large quantities of B_{12}-synthesizing bacteria in the sea, are known to be excellent sources of B_{12}; especially bivalves contain substantial amounts of B_{12} (28−62 μg/100 g).[8] The B_{12}-synthesizing bacteria can also synthesize various corrinoids (including corrinoid compounds inactive for humans) with different bases in the lower ligand. In some shellfish, B_{12} contents determined by the microbiological method are shown to be several fold greater than the values determined by a chemiluminescence method.[9] Although corrinoid compounds have been isolated and identified from popular shellfish,[9] our preliminary experiments suggest that certain edible shellfish contain substantial amounts of pseudovitamin B_{12} inactive for humans. Fresh clams and their processed food products (e.g., canned clams) are popular food items for peoples in various countries. Canned clams are readily accessible to all peoples among clam products. If the broth from canned clams contains substantial amounts of true and free B_{12}, it would be a natural and excellent source of free B_{12} for elderly persons with food-bound B_{12} malabsorption. Here, we characterize B_{12} compounds from the broth of commercially available canned clams (boiled plain).

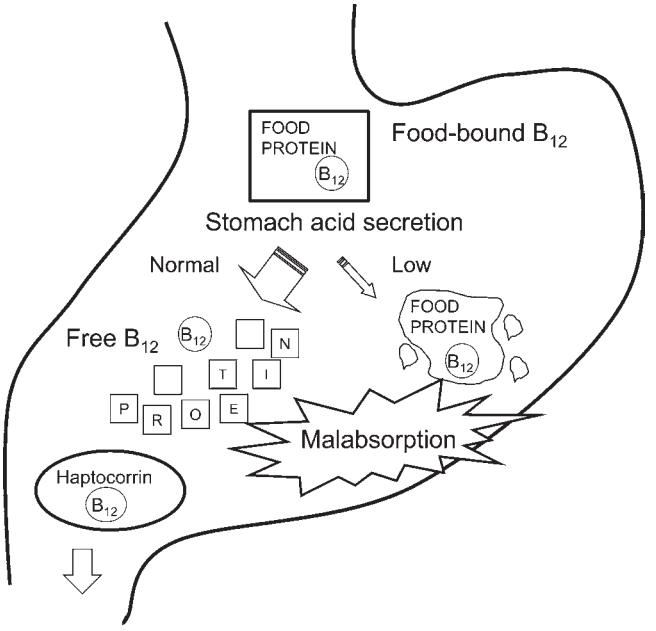

Figure 1. Outline of food-bound vitamin B_{12} malabsorption.

MATERIALS AND METHODS

Materials. B_{12} was purchased from Sigma (St. Louis, MO). A B_{12} assay medium for *Lactobacillus delbrueckii* subspecies *lactis* (formerly *Lactobacillus leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 thin layer chromatography (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). A Shimadzu (Kyoto, Japan) UV−visible spectrophotometer (UV-2550) was used for measuring

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Table 1. Vitamin B_{12} Contents of Broth and Clam from Various Canned Clams (Boiled Plain)[a]

	A (n = 5)	B (n = 5)	C (n = 5)	D (n = 5)	E (n = 5)	F (n = 5)	G (n = 4)
weight (g)							
solid substance, clam	66.5 ± 0.8	68.2 ± 3.0	51.2 ± 1.7	40.4 ± 1.5	41.0 ± 1.1	416.9 ± 3.5	
broth	63.2 ± 0.8	53.9 ± 3.6	56.1 ± 2.0	47.6 ± 1.5	46.6 ± 0.9	429.3 ± 27.4	1400.8 ± 4.3
vitamin B_{12} contents (μg/ 100 g)							
solid substance, clam	17.4 ± 1.3	39.4 ± 1.9	27.5 ± 1.4	34.4 ± 3.2	33.3 ± 6.5	34.1 ± 5.3	
broth	6.3 ± 0.5	8.8 ± 0.5	10.3 ± 1.0	14.1 ± 3.1	2.7 ± 1.3	3.1 ± 0.7	ND
vitamin B_{12} contents (μg/can)							
solid substance, clam	11.6 ± 0.8	27.0 ± 2.2	14.2 ± 1.1	13.9 ± 1.1	13.8 ± 1.2	142.4 ± 10.1	
broth	4.0 ± 0.3	4.8 ± 0.6	5.9 ± 0.8	6.7 ± 1.4	1.3 ± 0.3	13.1 ± 1.2	ND
total	15.5 ± 0.9	31.8 ± 2.5	20.1 ± 1.6	20.6 ± 2.0	15.2 ± 1.0	155.5 ± 11.3	ND

[a] Canned clams A−E were made in Japan. Canned clam F and clam juice G were made in China and the United States, respectively. ND, not detectable. Values represent the mean ± SEM (n = 5).

the turbidity of *L. delbreuckii* test cultures in the microbiological B_{12} assay method.

All other reagents used were of the highest purity commercially available. The tested canned clams (boiled plain) and clam juice were provided from local markets in Japan.

Methods. *Extraction and Assay of Vitamin B_{12}.* Two grams each of broth and clams of various canned clams (boiled plain) were used for the sample. Total B_{12} was extracted with boiling at acidic pH range in the presence of KCN and assayed by a microbiological method with *L. delbrueckii* ATCC 7830 according to the method described in the *Standard Tables of Food Composition in Japan*.[8] Becausee *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}. B_{12} was assayed at least five times independently, and the data represent as the mean ± SEM.

To evaluate the effect of the autoclave treatment on B_{12} contents of the clam broth, 10 raw short-necked clams (without shells) (20.9 ± 1.1 g) were added to the same grams of water and then treated by heating with an autoclave (MC-23, ALP Co., Ltd., Tokyo, Japan) at 121 °C for 10 min. Untreated clams were used as control. The treated clams were immediately cooled to 25 °C. The broth and clams were separated and weighed. B_{12} was extracted and assayed in the broth with or without the autoclave treatment as described above.

Bioautography of Vitamin B_{12} Compound with Vitamin B_{12}-Dependent Escherichia coli 215. Bioautograpy of the B_{12} compound was done according to the method of the reference cited.[10] After the selected B_{12} extracts were concentrated and partially purified with a Sep-Pak C18 cartridge (Waters Corp., Milford, MA), 2 μL of the purified B_{12} extracts and authentic B_{12} [cyanocobalamin (CN-B_{12}), 10 μg/L] was 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 precultured *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. The broth of selected canned clams (boiled plain) was centrifuged at 10000*g* for 10 min at 25 °C to remove insoluble materials. To an aliquot (10 mL) of the supernatant were added 1 mL of 0.57 mol/L acetic buffer, pH 4.5, and 50 μL of 1% (w/v) KCN, and the mixture was left for 2 h at 25 °C to convert various B_{12} compounds with the different upper ligands to CN-B_{12}. The treated clam broth was loaded onto an immunoaffinity column (EASI-EXTRACT Vitamin B_{12} Immunoaffinity Column (P80) R-Biopharm AG, Darmstadt, Germany), and then B_{12} was purified according to the manufacturer's recommended protocol. The purified B_{12} solution was analyzed by HPLC using a JASCO HPLC apparatus (PU-2080 Plus Pump, UV-2070 Plus Spectrophotometer, DG-2080-53 Degasser, CO-2065 column oven) and CDS ver. 5 chromato-data processing system (LAsoft, Ltd., Chiba, Japan). The sample (100 μL) was put on a reversed-phase HPLC column (Wakosil-II 5C18RS, ∅ 4.6 × 150 mm; particle size, 5 μm) equilibrated with 20% (v/v) methanol containing 1%

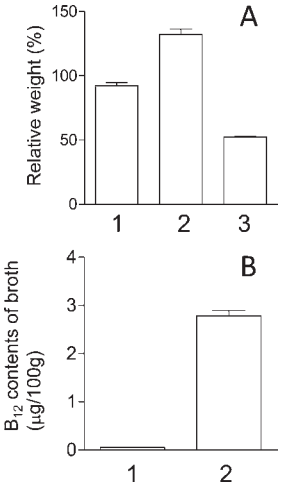

Figure 2. Effects of autoclave treatment on weights and B_{12} contents of the clam broth: (A) relative weights of total (clam body and broth) (1), broth (2), and clam body (3) are expressed as percent of the controls; (B) B_{12} contents of broth were assayed in the controls (1) and the treated clams (2). Ten raw short-necked clams (without shells) (20.9 ± 1.1 g) were added to the same grams of water and then treated by heating with an autoclave at 121 °C for 10 min. Untreated clams were used as controls.

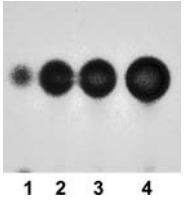

Figure 3. *E. coli* 215 bioautogram after silica gel 60 TLC of the B_{12} extracts of the broth of selected canned clam (boiled plain) and authentic B_{12}. Lanes: 1, authentic B_{12}; 2, broth of the canned clam A; 3, broth of the canned clam B; 4, broth of the canned clam C. The data are typical bioautograms from five independent experiments.

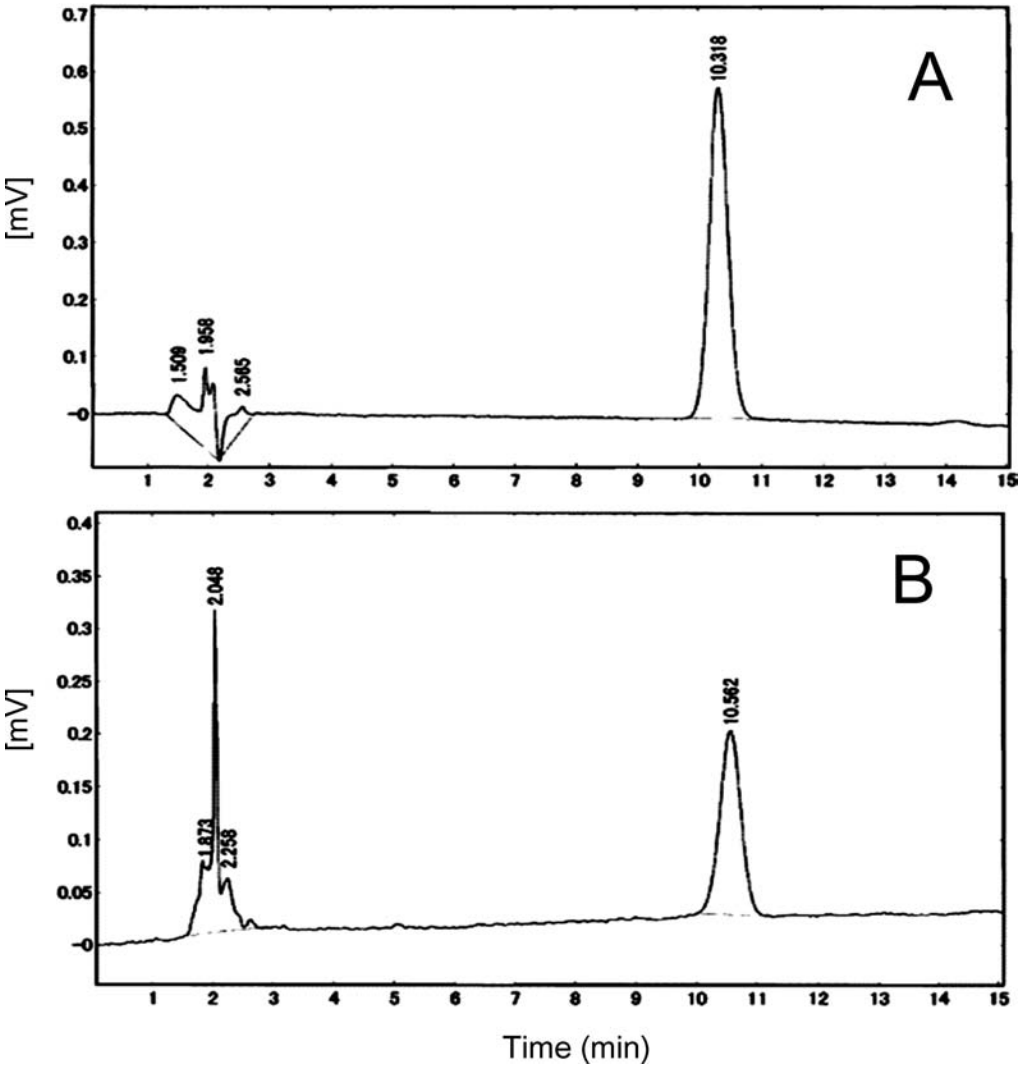

Figure 4. HPLC patterns of the B_{12} compound purified from the broth of the selected canned clam (boiled plain) by an immunoaffinity column: (A) authentic B_{12}; (B) purified compound from canned clam broth. The B_{12} compound was analyzed as described in the text. The data are typical HPLC patterns of the clam broth B_{12} compound from four independent experiments.

(v/v) acetic acid at 40 °C. The flow rate was 1.0 mL/min. The B_{12} compound and authentic B_{12} were isocratically eluted with the same solution and monitored by measuring absorbance at 361 nm. The retention time of authentic B_{12} was 10.3 min.

LC/ESI-MS/MS. The purified B_{12} compound and authentic B_{12} were dissolved in 0.1% (v/v) acetic acid and filtered with a Nanosep MF centrifuge device (0.4 μm, Pall Corp., Tokyo, Japan) to remove small particles. We analyzed an aliquot (2 μL) of the filtrate using a LCMS-IT-TOF coupled with an Ultra-Fast LC system (Shimadzu, Kyoto, Japan). The purified compound was injected in an InertSustain column (3 μm, 2.0 × 100 mm, GL Science, Tokyo, Japan) and equilibrated with 85% solvent A [0.1% (v/v) acetic acid)] and 15% solvent B (100% methanol) at 40 °C. B_{12} compounds were eluted with a linear gradient of methanol (15% solvent B for 0−5 min, 15−90% solvent B for 5−11 min, and 90−15% solvent B for 11−15 min). The flow rate was 0.2 mL/min. ESI conditions were determined by injection of authentic B_{12} to the MS detector to achieve optimum parameters to detect the parent and daughter ions of B_{12} compounds. The ESI-MS was operated in positive ion mode. Argon was used as the collision gas. The identification of B_{12} (m/z 678.2939) representing $[M + 2H]^{2+}$ was confirmed by comparison of the observed molecular ions and the retention times.

Sephadex G-50 Gel Filtration Experiment. The free B_{12} was separated from the clam broth using a column (1.4 × 10 cm, econo-pack column, Bio-Rad) of Sephadex G-50 fine (GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, U.K.) and then assayed. The selected clam broth was centrifuged at 10000*g* for 10 min at 25 °C to remove insoluble materials. An aliquot (1.0 mL) of the supernatant was applied on the column, which had been equilibrated with 100 mmol/L potassium phosphate buffer, pH 7.0. 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 0.5 mL. To each fraction of the eluate were added 100 μL of 0.57 mol/L acetate buffer, pH 4.5, and 40 μL of 0.5% (w/v) KCN, and the mixture was mixed vigorously and left overnight at 4 °C in the dark. B_{12} was assayed by the microbiological method. The macromolecular and free B_{12} fractions were estimated with blue dextran and authentic B_{12}, respectively, by measuring the absorbance at 280 nm.

RESULTS AND DISCUSSION

Table 1 shows the B_{12} contents determined by the microbiological method in commercially available canned clams (boiled plain). B_{12} contents of the solid substance, the clams themselves,

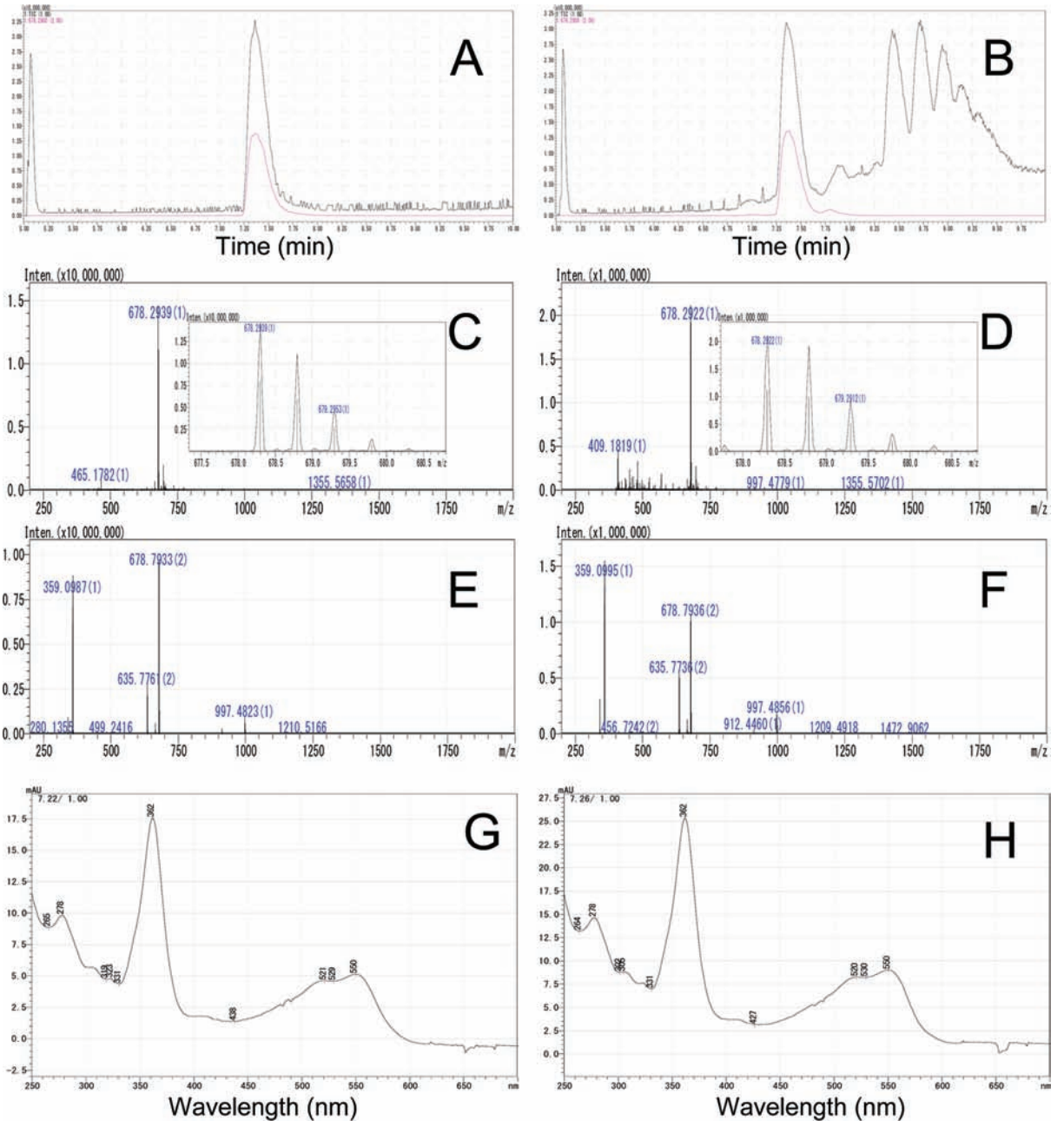

Figure 5. LC/ESI-MS/MS analysis of the B_{12} compound purified from the broth of the selected canned clam (boiled plain). B_{12} was analyzed with LCMS-IT-TOF (Shimadzu) as described in the text. The total ion chromatograms of authentic B_{12} and the purified compound from the canned clam broth are shown in panels A and B, respectively (black, TIC; red, m/z 678.293). The mass spectra of the ion peak with retention time of 7.2 min are shown in panel C for authentic B_{12} (m/z 678.2939) and in panel D for the purified compound (m/z 678.2922); the enlarged spectra of the B_{12} divalent ion are inserted. The MS/MS spectra of the authentic B_{12} (m/z 678.2939) and the purified compound (m/z 678.2922) are shown in panels E and F, respectively. The photodiode-array spectra of the ion peak with a retention time of 7.2 min from the authentic B_{12} and the purified compound are shown in panels G and H, respectively.

in the canned clam were considerably lower (17.4−39.4 μg/100 g) relative to the values (63.8 μg/100 g) described in the *Standard Tables of Food Composition in Japan*.[8] The broths of the canned clams, however, contained considerable amounts of B_{12} (2.7−14.1 μg/100 g and 1.3−6.7 μg/can; sample F (large can), 3.1 μg/100 g and 13.1 μg/can). B_{12} was undetectable in certain canned clam juice used as a flavoring (G).

We evaluated how much B_{12} is extracted from a clam's body to broth during pressurized sterilization. Figure 2A shows the changes in weights of the raw short-necked clam body and broth during autoclave treatment (at 121 °C for 10 min). Although the treated clam body weights were decreased to 52.5% of the control (untreated clam body weights), the treated broth weights were increased significantly relative to the control broth weights, suggesting that various compounds (nutrients, amino acids, organic acids, and so on) were extracted from the clam's body into the broth during autoclave treatment. A considerable amount of B_{12} was found in the treated broth, but not in the control (Figure 2B). These results indicated that the clam's B_{12} was readily extracted into the broth during pressuring sterilization.

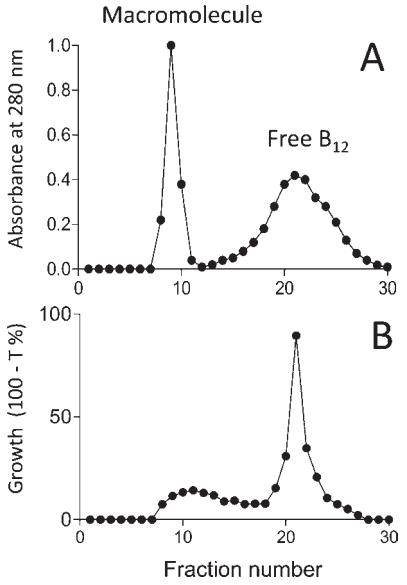

Figure 6. Elution patterns of B_{12}-active compounds during Sephadex-G50 gel filtration of the selected clam broth: (A) authentic blue dextran and B_{12} mixture; (B) selected clam broth. B_{12} was assayed by the microbiological method. The macromolecular and free B_{12} fractions were estimated with blue dextran and authentic B_{12}, respectively, by measuring absorbance at 280 nm.

Our preliminary experiments suggest that certain edible shellfish contain substantial amounts of pseudovitamin B_{12} inactive for humans. To evaluate whether the broths of the canned clams contain true B_{12} or pseudovitamin B_{12}, the clam broths selected from Table 1 (A−C) were analyzed with the *E. coli* 215 bioautography after being separated by silica gel 60 TLC (Figure 3). The B_{12}-active compound found in each broth of canned clams was given as a single spot, the R_f value (0.56) of which was identical to that of authentic B_{12}. The selected clam broth was purified by an immunoaffinity column and then analyzed by reversed-phase HPLC; the retention time (10.5 min) of the purified compound was similar to that of authentic B_{12} (Figure 4).

The purified compound was further analyzed by LC/ESI-MS/MS (Figure 5). Authentic B_{12} was eluted as a peak with a retention time of 7.2 min. The mass spectrum of authentic B_{12} indicated that a divalent ion of m/z 678.2939 $[M + 2H]^{2+}$ was major (Figure 5A,C); its exact mass calculated from its formula ($C_{63}H_{88}CoN_{14}O_{14}P$) was 1354.5674, and isotope distribution data supported that B_{12} formed predominantly the divalent ion under LC/ESI-MS conditions. The purified compound from canned clam was eluted as several ion peaks, indicating impurities still existed; the mass spectrum of the peak with a retention time of 7.2 min showed the B_{12} divalent ion of m/z 678.2922 (Figure 5B, D). MS/MS (Figure 5E, F) and photodiode array (Figure 5G,H) spectra of the purified compound were identical to those of authentic B_{12}.

Sephadex-G50 gel filtration of the selected clam broth demonstrated that most (72%) of the B_{12} found in the broth of canned clam (boiled plain) was recovered in the free B_{12} fractions (Figure 6). These results suggest that the clam broth is available as an excellent source of free B_{12} (1.0−4.8 μg of free B_{12} per can).

As about 30% of people over 50 years of age 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] The Institute of Medicine (1998) 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 a B_{12}-containing supplement.[5]

The results presented here indicate that the broth of canned clams (boiled plain) would be an excellent and natural source of free B_{12} for humans.

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REFERENCES

(1) Baik, H. W.; Russell, R. M. Vitamin B_{12} deficiency in the elderly. *Annu. Rev. Nutr.* **1999**, *19*, 357–377.

(2) van Asselt, D. Z.; van den Broek, W. J.; Lamers, C. B.; Corstens, F. H.; Hoefnagels, W. H. Free and protein-bound cobalamin absorption in healthy middle-aged and older subjects. *J. Am. Gerontol. Soc.* **1996**, *44*, 949–953.

(3) Krasinski, S. D.; Russell, R. M.; Samloff, I. M.; Jacob, R. A.; Dallal, G. E.; McGrandy, R. B.; Hartz, S. C. Fundic atrophic gastritis in an elderly population: effect on hemoglobin and several serum nutritional indicators. *J. Am. Gerontol. Soc.* **1986**, *34*, 800–806.

(4) McEvoy, A. W.; Fenwick, J. D.; Boddy, K.; James, O. F. Vitamin B_{12} absorption from the gut does not decline with age in normal elderly humans. *Age Ageing* **1982**, *11*, 180–183.

(5) Institute of Medicine. Vitamin B12. In *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*; Institute of Medicine, National Academy Press: Washington, DC, 1998; pp 306−356.

(6) Watanabe, F. Vitamin B_{12} sources and bioavailability. *Exp. Biol. Med.* **2007**, *232*, 1266–1274.

(7) Yoshino, K.; Inagawa, M.; Oshima, M.; Yokota, K.; Umesawa, M.; Endo, M.; Yamagishi, K.; Tanigawa, T.; Sato, S.; Shimamoto, T.; Iso, H. Trends in dietary intake of folate, vitamin B_6, and vitamin B_{12} among Japanese adults in two rural communities from 1971 through 2001. *J. Epidemiol.* **2005**, *15*, 29–37.

(8) Resources Council, Science and Technology Agency. In *Standard Tables of Food Composition in Japan − Vitamin K, B6 and B12*; Resources Council, Science, and Technology Agency: Tokyo, Japan, 1995; pp 6−56.

(9) Watanabe, F.; Katsura, H.; Takenaka, S.; Enomoto, E.; Miyatomo, E.; Nakatsuka, T.; Nakano, Y. Characterization of vitamin B_{12} compounds from edible shellfish, clam, oyster, and mussel. *Int. J. Food Sci. Nutr.* **2001**, *52*, 263–268.

(10) Tanioka, Y.; Yabuta, Y.; Miyamoto, E.; Inui, H.; Watanabe, F. Analysis of vitamin B_{12} in food by silica gel 60 TLC and bioautography with vitamin B_{12}-dependent *Escherichia coli* 215. *J. Liq. Chrom. Rel. Technol.* **2008**, *31*, 1977−1985.