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Characterization of Vitamin B_{12} Compounds from Korean Purple Laver (*Porphyra* sp.) Products

EMI MIYAMOTO,[†] YUKINORI YABUTA,[‡] CHUNG SHIL KWAK,[§] TOSHIKI ENOMOTO,[||] AND FUMIO WATANABE*,[‡]

Department of Health and Nutrition, Nagasaki International University, Sasebo 859-3298, Japan, School of Agricultural, Biological and Environmental Sciences, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan, Institute on Aging, Seoul National University, Seoul 110-510, Korea, and Department of Food Science, Ishikawa Prefectural University, Ishikawa 921-8836, Japan

Vitamin B_{12} contents of various Korean purple laver products were determined with the microbiological vitamin B_{12} assay method. Although a substantial amount (133.8 μg/100 g) of vitamin B_{12} was found in dried purple laver, seasoned and toasted laver products contained lesser vitamin B_{12} contents (about 51.7 μg/100 g). The decreased vitamin B_{12} contents in the seasoned and toasted laver products, however, were not due to loss or destruction of vitamin B_{12} during the toasting process. Silica gel 60 thin layer chromatography−bioautogram analysis indicated that all Korean laver products tested contain true vitamin B_{12}, but not inactive corrinoid compounds. In vitro gastrointestinal digestion experiments indicated that digestion rate of vitamin B_{12} from the dried Korean purple laver was estimated to be 50% under pH 2.0 conditions (as a model of normal gastric function). These results suggest that Korean purple laver products would be excellent vitamin B_{12} sources for humans, especially vegetarians.

KEYWORDS: In vitro digestion; Iwa-nori; Korean purple laver; *Phorphyra* sp.; vitamin B_{12}

INTRODUCTION

Vitamin B_{12} (B_{12}) is synthesized only in certain bacteria (*1*). The B_{12} synthesized by bacteria is concentrated mainly in the bodies of higher predatory organisms in the natural food chain system. Animal foods (i.e., meat, milk, egg, fish, and shellfish), but not plant foods, are considered to be the major dietary sources of B_{12} (*2*). Some plant foods, such as edible algae, however, contain large amounts of B_{12} (*3*). Croft et al. (*4*) have demonstrated that algae acquire B_{12} through a symbiotic relationship with B_{12}-synthesizing bacteria in the natural environment, since the concentration of B_{12} in natural seawater appears to be very low.

Various types of edible algae are available as food items. Certain purple lavers (*Porphyra* sp.) appear to be the most widely consumed among the eible algae worldwide. Especially, Japanese people enjoy many kinds of purple laver products (dried, seasoned and toasted, and so on), which were made from Susabi-nori (*Porphyra yezoensis*), Asakusa-nori *(Porphyra tenera*), and Iwa-nori (*Porphyra* sp.). Several sheets of dried purple lavers (nori) are often served with steamed rice and can be consumed from certain sushi, vinegared rice rolled in nori.

Early studies (*5, 6*) have reported that algal B_{12} appears to be inactive for mammals. Although the purple laver (*P. yezoensis*) contains a substantial amount of true B_{12} (*7*), most of the edible algae (Arame, Hijiki, Kombu, Wakame and so on) contain none or only traces of B_{12} (*3*). Takenaka et al. (*8*) have demonstrated that the dried purple laver (*P. yezoensis*) is bioavailable to mammals. However, the B_{12} found in another purple laver (*P. tenera*) has been reported to be changed into unidentified B_{12} analogues by the drying process (*9*). Thus, it is still unclear whether B_{12} compounds found in the dried purple lavers are true B_{12} and bioavailable to mammals.

Recently many Korean purple laver products (dried, and seasoned and toasted) have been also consumed worldwide. The Korean purple lavers appear to be very similar to Japanese Iwa-nori (*Porphyra* sp.). There is, however, little information available on characterization and bioavailablity of Korean purple lavers as well as Japanease Iwa-nori.

We characterized B_{12} compounds of various Korean purple laver products and determined effects of in vitro gastroinestinal digestion on release of B_{12} from the dried Korean laver to estimate the bioavailablity of B_{12} from purple laver in humans.

MATERIALS AND METHODS

Materials. B_{12}, pepsin (P7012, from porcine gastric mucosa), and pancreatine (P8096, from porcine pancreas) were purchased from Sigma (St. Louis, MO). 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

* To whom correspondence should be addressed. Tel and fax: (+81)857-31-5412. E-mail: watanabe@muses.tottori-u.ac.jp.
[†] Nagasaki International University.
[‡] Tottori University.
[§] Seoul National University.
[||] Ishikawa Prefectural University.

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(TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). An ultraviolet/visible spectrophotometer (460, JASCO Corporation, Tokyo, Japan) was used for measuring the turbidity of *L. delbrueckii* test cultures in the microbiological B_{12} assay.

All other reagents used were of the highest purity commercially available. The tested samples of Korean purple lavers (dried, and seasoned and toasted) were provided from local markets in Seoul-city, Korea. Japanese purple lavers (Iwa-nori, dried) were purchased from local markets in Kanazawa-city and Tottori-city, Japan.

Extraction and Assay of Vitamin B_{12}. Two grams of various purple lavers were used for the samples. Total B_{12} was extracted by boiling with 0.005% (w/v) KCN at pH 4.5 to convert various B_{12} compounds with different α-ligands (e.g., coenzyme forms of B_{12}) to cyanocobalamin ($CN\text{-}B_{12}$) and assayed by the microbiological method with *L. delbrueckii* ATCC 7830 according to the method described in the Japanese Standard Tables of Food Composition (*10*). 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} Compounds with Vitamin B_{12}-Dependent *Escherichia coli* 215. Bioautography of B_{12} compounds was done according to the method of the reference cited (*11*). After the B_{12} extracts prepared above were partially purified with a Sep-Pak Plus C18 cartridge (Waters Corporation, Milford, MA), 0.5 μL of the purified B_{12} extracts and 2 μL of 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 room temperature (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. After the treated agar plate was photographed with a digital camera (Coolpix 4300, Nikon, Japan), an area of the spot of *E. coli* 215 growth was calculated by the use of ImageJ software.

Toasting Treatment. A sheet of the dried Korean purple laver was toasted for a few minutes over a fire with a gas range until the laver's color was changed from purple to green. The toasted laver sheet was left for 3 h at 25 °C. B_{12} was extracted from the dried laver sheets with or without the toasting treatment and analyzed with TLC−bioautography as described above.

Analysis of Coenzyme Forms of Vitamin B_{12}. B_{12} compounds were extracted from the dried Korean purple laver by the method of boiling with 80% (v/v) ethanol solution (*7*). One hundred milliliters of 80% (v/v) ethanol solution was added to the purple laver (2 g weight), heated at 98 °C for 30 min under reflux conditions, and cooled to room temperature. The solution was centrifuged at 10000*g* for 10 min. The supernatant was allowed to evaporate to dryness under reduced pressure and dissolved in 20 mL of distilled water. The solution was centrifuged at 10000*g* for 10 min to remove insoluble materials. A portion of the supernatant fraction was put on a Sep-Pak Plus C18 cartridge (Waters Corporation) which had been washed with 5 mL of 75% (v/v) ethanol solution and then equilibrated with distilled water, washed with 10 mL of distilled water, and eluted with 2 mL of 50% (v/v) ethanol solution. The eluate was used as a sample for TLC−bioautogram analysis. All procedures were done in the dark.

In Vitro Gastrointestinal Digestion. The in vitro digestion method (**Figure 1**) used in this experiment was based on the method cited (*12*). Thirty grams of the dried Korean purple laver was broken into small pieces with a mortar and pestle. A portion (5.0 g) of the broken laver was added to 90 mL of distilled water. The pH was adjusted to 2.0 or 4.0 with 1 mol/L HCl, and was done to 7.0 with 1 mol/L NaOH. Freshly prepared pepsin solution (1 g of pepsin in 10 mL of 0.1 mol/L HCl) was added to provide 0.01 g (21900 units) of pepsin/5 g of the laver. After 15 min, the pH values were checked and if necessary readjusted to the respective pH. The sample was made up to 100 g with distilled water and incubated in a shaking water bath (120 strokes/min) at 37 °C for 2 h.

Prior to the intestinal digestion step, the pH of the gastric digests (pH 2.0 and 4.0) was raised to pH 6.5 by the addition of 1 mol/L $NaHCO_3$. Freshly prepared pancreatin solution (0.2 g of pancreatin in 50 mL of 0.1 mol/L $NaHCO_3$) was added to provide 2.5 mg of pancreatin/5 g of the laver. The sample was incubated in a shaking water bath (120 strokes/min) at 37 °C for 2 h. The pH was then adjusted to 7.2 by the addition of 0.5 mol/L NaOH.

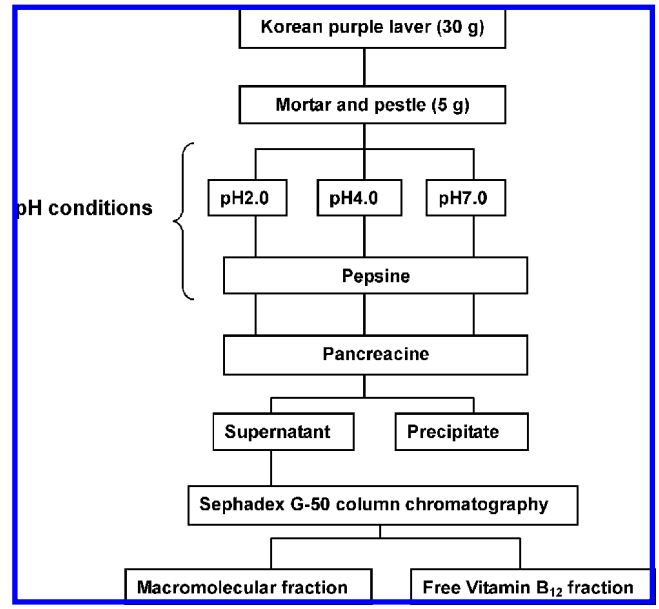

Figure 1. Scheme of the in vitro gastrointestinal digestion used in the experiment.

The intestinal digests were centrifuged at 10000*g* for 30 min at 4 °C to separate soluble and precipitate fractions. Aliquot (1.0 mL) of the soluble fraction was applied on a Sephadex G-50 fine (GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, England) gel filtration column (1.5 × 14 cm) 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 were estimated with blue dextran and authentic B_{12} by measuring absorbance at 600 and 551 nm, respectively. B_{12} was extracted from these fractions under the same conditions described above and assayed by the microbiological B_{12} assay method.

RESULTS AND DISCUSSION

B_{12} Contents of Various Korean Purple Laver Products. B_{12} contents of various Korean purple lavers were determined with the microbiological B_{12} assay method. Although substantial amounts (133.8 μg/100 g) of B_{12} were found in the dried laver (A), seasoned and toasted laver products (B, C, and D) contained 51.7 ± 15.2 μg of B_{12} per 100 g weight. Kwak et al. (*13*) have reported that Korean laver contained 66.76 μg of B_{12} per 100 g weight. The dried Japanese purple lavers (Iwa-nori) (E and F) contained larger amounts (86.5 and 120.7 μg/100 g, respectively) of B_{12} than the values (39.9 μg/100 g) described in the Standard Tables of Food Composition in Japan (*10*).

These results suggest that B_{12} contents of the seasoned and toasted laver products are about half of the values of the dried laver products.

Identification of B_{12} Compounds in Various Korean Purple Laver Products. We tried to purify B_{12} compounds to clarify whether Korean purple lavers contain true B_{12} or inactive corrinoid compound (pseudo-B_{12}) which has been found in edible blue-green algae (cyanobacteria) (*14*). The experiment, however, ended in failure, since a substantial amount of algal purple pigment (phycoerythrin) may interfere with purification of B_{12} compounds. Therefore, each B_{12} extract of the purple

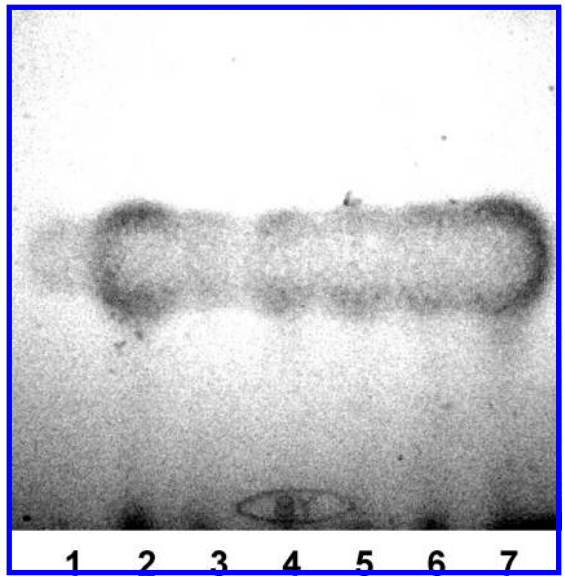

Figure 2. *E. coli* 215 bioautogram after silica gel 60 TLC of the B_{12} extracts of selected purple laver products and authentic B_{12}: 1, authentic B_{12}; 2, Korean dried laver A; 3, Korean seasoned and toasted laver B; 4, Korean seasoned and toasted laver C; 5, Korean seasoned and toasted laver D; 6, Japanese dried laver E; 7, Japanese dried laver F. The data are a typical bioautogram from five independent experiments.

lavers was analyzed with a B_{12}-dependent *E. coli* 215 bioautogram after being separated by silica gel 60 TLC (**Figure 2**). The B_{12}-activity found in each purple laver was given as a single spot, whose R_f value (0.56) was identical to that of authentic B_{12}. The result indicated that Korean purple lavers contain true B_{12}, but not pseudo-B_{12} inactive for humans. Although Yamada et al. (*9*) have reported that a portion of the B_{12} found in raw purple laver (Asakusa-nori, *P. tenera*) was converted into certain harmful and unidentified B_{12} compounds during the drying process, all purple lavers used in the experiments did not contain any unidentified B_{12} compounds.

Effects of Toasting Treatment on B_{12} Contents of Dried Korean Purple Laver. To clarify whether the decreased B_{12} contents of the seasoned and toasted lavers in comparison with the dried lavers were due to destruction of B_{12} during the toasting process, the dried laver was treated with toasting until the laver's color was changed from purple to green (**Figure 3A**). B_{12} was extracted from the lavers treated with or without toasting and analyzed with TLC−bioautogram. The area of the B_{12} spot in the laver treated with toasting was identical with that in the laver treated without toasting (**Figure 3B**). The result indicated that the toasting treatment did not affect B_{12} contents in the dried purple lavers. The decreased B_{12} contents of the seasoned and toasted lavers appear to be not due to destruction of B_{12} during the toasting process, but due to decrease in contents of the laver itself per 100 g of the laver product by the addition of various seasonings (salt, sesame oil, and so on). There is no information available on how much decrease in B_{12} contents of the laver products is caused by the addition of various seasonings, since any seasoning contents used are not given on these product packages.

Occurrence of Coenzyme Forms of B_{12} in Dried Korean Purple Laver. The coenzyme forms of B_{12} were extracted with 80% (v/v) ethanol and analyzed by the TLC−bioautogram method. The B_{12} compounds found in the dried purple laver were separated as three spots, whose R_f values were identical to those of authentic hydroxocobalamin (OH-B_{12}), 5′-deoxyadenosylcobalamin (AdoB_{12}), and methylcobalamin (CH_3-B_{12}),

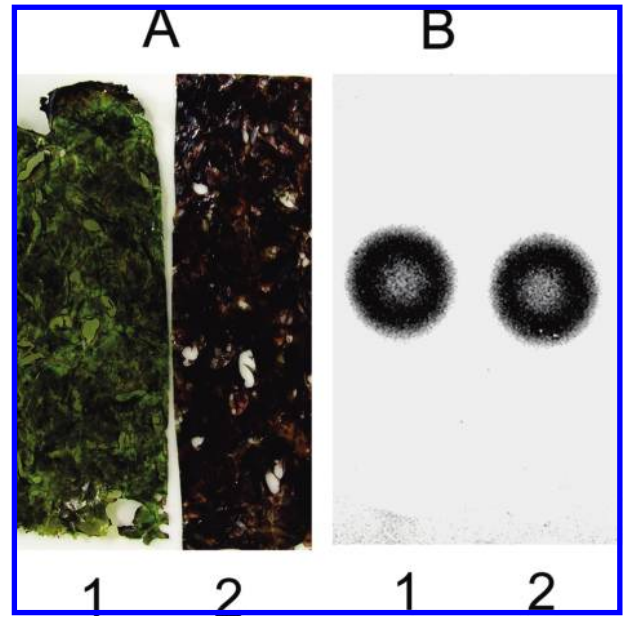

Figure 3. Effects of toasting treatment on B_{12} contents of dried Korean purple laver: **A**, color of the dried laver treated with (1) or without (2) toasting; **B**, B_{12} was extracted from the lavers treated with (1) or without (2) toasting and then analyzed with the *E. coli* 215 bioautogram analysis. The data are a typical bioautogram from three independent experiments.

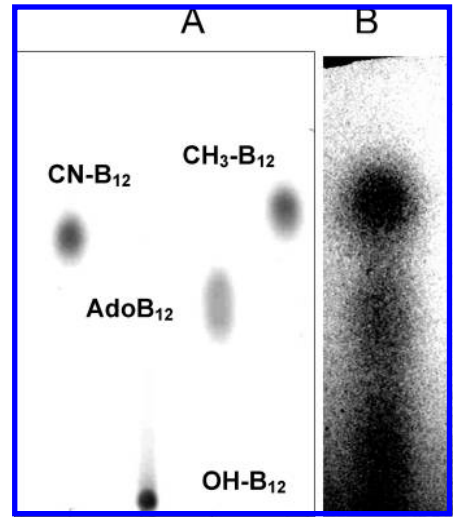

Figure 4. Occurrence of coenzyme forms of B_{12} in dried Korean purple laver: **A**, authentic B_{12} compounds; **B**, B_{12} compounds were extracted from the lavers and then analyzed with the *E. coli* 215 bioautogram analysis. The data are a typical bioautogram from three independent experiments.

respectively (**Figure 4**). The area of each B_{12} spot in the Korean purple laver was calculated to be about 49% OH-B_{12}, 33% CH_3-B_{12}, and 18% AdoB_{12}, indicating that half of the B_{12} found in the Korean purple laver was the coenzyme forms (CH_3-B_{12} and AdoB_{12}). It has been reported that most (∼80%) of the B_{12} found in commercially available dried Japanese purple laver sheets is recovered in OH-B_{12} (*7*).

In Vitro Gastrointestinal Digestion of the Dried Korean Purple Laver. Bioavailablity of the B_{12} found in the Korean purple laver was determined under various pH conditions using an in vitro gastrointestinal digestion method. A chromophore (phycoerythrobilin) (*15*) of the purple pigment protein was significantly released from the laver by the in vitro digestion

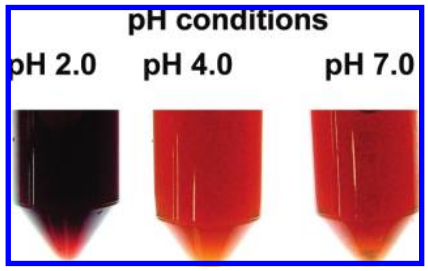

Figure 5. Purple pigment chromophore in soluble fractions after in vitro gastrointestinal digestion under pH 2.0, pH 4.0, and pH 7.0 conditions. The depth of purple color represents the degree of content of the pigment chromophore (phycoerythrobilin) released from the laver.

Table 1. Effects of pH Values on the Relative Contents of Free Vitamin B_{12} during the in Vitro Gastrointestinal Digestion of the Dried Korean Purple Laver[a]

	pH conditions		
fraction	pH 2.0	pH 4.0	pH 7.0
macromolecular	1.5 ± 0.4[b]	4.1 ± 0.3	3.6 ± 0.2
free vitamin B_{12}	50.8 ± 2.1	47.4 ± 2.1	2.5 ± 0.3

[a] The values represent mean ± SEM from three independent experiments. [b] Percentage against B_{12} contents of the dried purple laver treated without the in vitro digestion.

under the pH 2.0 conditions, but there was only a little release of the phycoerythrobilin under the pH 4.0 or pH 7.0 conditions (**Figure 5**). These results indicated that the dried purple laver could be well digested only under the pH 2.0 conditions, but not under the pH 4.0 and 7.0 conditions. Under the pH 2.0 and 4.0 conditions, about half of the B_{12} found in the dried purple laver was recovered in the free B_{12} fractions during the in vitro digestion (**Table 1**). Release of free B_{12} from the purple laver was significantly decreased under the pH 7.0 conditions as a model for severe atrophic gastritis, which prevails in elderly people (*16*). Under all pH conditions, only small amounts of B_{12} were recovered in the macromolecular fractions during Sephadex G-50 gel filtration. These results suggest that digestion rate of B_{12} in the Korean purple laver would be estimated to be 50% in persons with normal gastric function. Moreover, even in the pH 4.0 conditions (as a model for subnormal gastric function), a relatively higher digestive ratio (about 47%) was shown in these experiments.

The results presented here suggest that Korean purple laver products are excellent B_{12} sources for humans, especially vegetarians.

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