Characterization of a Vitamin B\textsubscript{12} Compound in the Edible Purple Laver, *Porphyra yezoensis*

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The edible purple laver, *Porphyra yezoensis*, contained 51.49±1.51 μg of vitamin B\textsubscript{12} compounds per 100 g dry weight of the laver (mean±SEM, n=4). A vitamin B\textsubscript{12} compound was purified from the lyophilized purple laver and partially characterized. The silica gel 60 TLC and reversed-phase HPLC patterns of the purified pink-colored compound were identical to those of authentic vitamin B\textsubscript{12}, but not to those of vitamin B\textsubscript{12} analogues inactive for humans.

Key words: vitamin B\textsubscript{12}; methylcobalamin; purple laver; *Porphyra yezoensis*; intrinsic factor

Various types of edible seaweed are available in the world. Edible seaweed is known to be rich in vitamins, minerals and dietary fiber.\textsuperscript{1} Dried laver sheet (nori), which appears to be most widely eaten among the edible seaweed, contains substantial amounts of B\textsubscript{12}, which is an essential nutrient for all animals and some microorganisms, and is known to be synthesized in certain bacteria, but not in animals or plants.\textsuperscript{2} Several studies have indicated that most of the types of B\textsubscript{12} in seaweed are B\textsubscript{12} analogues so that they may not be bioavailable to mammals.\textsuperscript{2,4,5} Rau et al.\textsuperscript{7}, however, have described that some types of seaweed can supply adequate amounts of bioavailable B\textsubscript{12} to strict vegetarians. Our previous study\textsuperscript{6} has also demonstrated that some dried green and purple lavers contain large amounts of B\textsubscript{12} by both a *Lactobacillus* bioassay and chemiluminescence B\textsubscript{12} analysis. Thus, it is still unclear whether B\textsubscript{12} found in edible seaweed is true B\textsubscript{12} or an inactive B\textsubscript{12} analogue, because there is no information available on the chemical properties of the algal B\textsubscript{12}. In this paper, a B\textsubscript{12} compound was purified and partially characterized from the edible purple laver, *Porphyra yezoensis*, to evaluate the bioavailability of the intrinsic B\textsubscript{12}.

Fresh purple laver (*Porphyra yezoensis*) was obtained from the fishermen's association of Ishinoura in Akashi city (Hyogo prefecture, Japan), immediately dried with a freeze-dryer (FD-550R, Eyela, Tokyo, Japan) and then stored at -80°C until needed for use.

Total B\textsubscript{12} was assayed by an ACS 180 fully automated B\textsubscript{12} chemiluminescence analyzer (Chiron Diagnostics, East Walpole, MA, U.S.A.) as described previously.\textsuperscript{9} The B\textsubscript{12} extracts subsequently described were directly applied to the B\textsubscript{12} chemiluminescence analyzer. The amount of B\textsubscript{12} was determined in triplicate.

The lyophilized purple laver was powdered in a food mill (MX-X51, National, Osaka, Japan). About 500 g of the lyophilized purple laver powder was added to 8 liters of a 63 mM acetate buffer at pH 4.8. Total B\textsubscript{12} was extracted from the suspension by boiling with KCN at an acidic pH value;\textsuperscript{10} KCN was added to the suspension at a final concentration of 10 mM. The suspension was boiled for 30 min at 98°C in the dark, the extraction procedures being conducted in a draught chamber. The boiled suspension was passed through a double layer of gauze, and the resulting filtrate was used for the B\textsubscript{12} assay. Amberlite XAD-4 resin (2 kg; Japan Organ Co., Tokyo, Japan), which had been washed with 10 liters of methanol and then equilibrated with distilled water, was added to the filtrate, and the mixture stirred for 3 h 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 liters of dis-

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\textsuperscript{3} Abbreviations: AdoB\textsubscript{12}, adenosyl-vitamin B\textsubscript{12} or adenosylcobalamin; B\textsubscript{12} vitamin B\textsubscript{12} or cobalamin; CN-B\textsubscript{12}, cyano-vitamin B\textsubscript{12} or cyanocobalamin; MeB\textsubscript{12}, methyl-vitamin B\textsubscript{12} or methylcobalamin; OH-B\textsubscript{12}, hydroxo-vitamin B\textsubscript{12} or hydroxocobalamin.
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tilled water. The washed resin was added to 5 liters of an 80% (v/v) methanol solution and stirred for 3 h at room temperature in the dark. The resin suspension was then passed again through the glass funnel. The combined eluates containing the B₁₂ compound were pooled, evaporated to dryness under reduced pressure, and dissolved in 20 ml of distilled water. The solution was loaded into a column (24 × 70 mm) of Cosmosil 140C18-OPN (Nacalai Tesque, Kyoto, Japan), which had been washed with a 75% (v/v) ethanol solution and then equilibrated with distilled water, and eluted with a linear gradient (0-25% v/v) of ethanol. The B₁₂-active fractions were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was further purified by HPLC with Shimadzu (Kyoto, Japan) apparatus (two LC-10ADvp pumps, a DVG-12A degasser, SCL-10Avp system controller, SPD-10Avvp ultraviolet-visible detector, CTO-10Avp column oven, 100 μl sample loop, and C-R6A chromatopac integrator). The sample (50 μl) was loaded into a reversed-phase HPLC column (Wakosil-II 5C18RS, φ4.6 × 150 mm; 5 μm particle size; Wako Pure Chemical Industries, Osaka, Japan) which had been equilibrated with a 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40°C. The flow rate was 1 ml/min. The B₁₂ compound was isocratically eluted with the same solution, monitoring by measuring the absorbance at 361 nm, and collected in 1 ml fractions. The B₁₂-active fractions were pooled, evaporated to dryness under reduced pressure, and dissolved in a small amount of distilled water. The concentrated solution was put on to a silica gel 60 TLC sheet (Merck, Darmstadt, Germany) and developed with 2-propanol/NH₄OH (28%)/water (7:1:2 v/v) as a solvent in the dark at room temperature. The pink-colored spot on the TLC sheet was dried, collected, extracted with an 80% (v/v) methanol solution, evaporated to dryness under reduced pressure, and dissolved in 50 μl of distilled water. The concentrated solution was further purified by HPLC under the same conditions. The final pink-colored fractions were collected, evaporated to dryness under reduced pressure, dissolved in 20 μl of distilled water, and used as the purified B₁₂ compound.

The B₁₂ concentration in the edible purple laver, Porphyra yezoensis, was determined by the B₁₂ chemiluminescence analyzer with hog intrinsic factor (IF), the most specific B₁₂-binding protein. The lyophilized purple laver contained 51.49 ± 1.51 μg of B₁₂ compounds per 100 g dry weight of the laver (mean ± SEM, n = 4). Van den Berg et al.² have indicated that some dried edible purple lavers (Porphyra sp.) contained 12.0–68.8 μg of B₁₂ compounds per 100 g dry weight of each laver; these values were determined by a radioisotope dilution assay with IF. A similar B₁₂ concentration (83.6 μg of B₁₂ compounds per 100 g dry weight of the laver) in an edible purple

![Fig. 1. Ultraviolet-visible Spectrum of the Purified B₁₂ Compound from the Purple Laver.](image)

A portion of the purified preparation was dissolved in 0.1 ml of distilled water. The spectrum was measured with a Shimadzu spectrophotometer (UV-1600) at room temperature, supermicro quartz cuvettes (0.1 ml, d = 1 cm) being used.

Table 1. Rf Values and Retention Times of the B₁₂ Compound Purified from the Purple Laver, CN-B₁₂, and Cyanocobamides by TLC and HPLC

<table>
<thead>
<tr>
<th>Compound</th>
<th>TLC (Rf value)</th>
<th>HPLC (retention time, min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solvent I</td>
<td>Solvent II</td>
</tr>
<tr>
<td>B₁₂ compound from purple laver</td>
<td>0.18</td>
<td>0.59</td>
</tr>
<tr>
<td>CN-B₁₂</td>
<td>0.18</td>
<td>0.59</td>
</tr>
<tr>
<td>Benzimidazolylcyanocobamide</td>
<td>0.15</td>
<td>0.55</td>
</tr>
<tr>
<td>5-Hydroxybenzimidazolylcyanocobamide</td>
<td>0.16</td>
<td>0.47</td>
</tr>
<tr>
<td>Pseudovitamin B₁₂</td>
<td>0.14</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Concentrated solutions (2 μl) of the B₁₂ compound purified from the purple laver, and of cyanocobamides (benzimidazolyl and 5-hydroxybenzimidazolyl cyanocobamides, and pseudovitamin B₁₂) were spotted on silica gel 60 TLC sheets and developed with 1-butanol/2-propanol/water (10:7:10 v/v) and 2-propanol/NH₄OH (28%)/water (7:1:2 v/v) as solvents I and II, respectively, in the dark at room temperature.

In the case of HPLC, concentrated solutions (2 μl) of the purified B₁₂ compound and of the cyanocobamides were analyzed in a reversed-phase HPLC column (Wakosil-II 5C18RS). The corrinoids were isocratically eluted with a 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 40°C, and monitored by measuring the absorbance at 361 nm. The solvent flow rate was 1 ml/min.
laver (*Porphyra* sp.) has also been shown by a *Lactobacillus delbrueckii* ATCC 7830 microbiological B12 assay. These results indicate that edible purple lavers, including *Porphyra yezoensis*, contain substantial amounts of B12 compounds that are active in binding IF involved in the mammalian intestinal absorption of dietary B12.

To evaluate whether the B12 compounds found in *Porphyra yezoensis* were biologically active, the IF-active B12 compound was purified and partially characterized. The final purified preparation gave a single pink-colored spot by silica gel 60 TLC and a single peak by reversed-phase HPLC, indicating that the B12 compound had been purified to homogeneity.

The ultraviolet-visible spectrum of the B12 compound purified from the purple laver showed a typical absorption of cobalt-containing corrinoid (Fig. 1); 270 nm (absorbance) was at 550.0 (0.483), 520.0 (0.431), 361.5 (1.612) and 278.5 (0.964). The purified B12 compound, authentic CN-B12, and cyanocobamides (pseudovitamin B12 and 5-hydroxybenzimidazolyl, and benzimidazolyl cyanocobamides; all kindly provided by Dr. E. Stupperich, Ulm University, Germany) which occur in bacteria were compared by silica gel 60 TLC and reversed-phase HPLC (Table 1). The *R* values of 0.18 and 0.59 in solvents I and II, respectively, by TLC) for the pink-colored compound that had been purified from the purple laver were identical to the values for authentic CN-B12, whose retention time (0.4 min by HPLC) was also identical to that of the purified B12 compound. These results indicate that the pink-colored compound purified from the purple laver was CN-B12, and not an inactive B12 analogue. No further detailed information on the B12 compound is available because only a small amount of the purified sample was obtained (for the NMR study).

The lyophilized purple laver contained four known types of biologically active B12 compounds (approximate values: OH-B12, 1.6%; CN-B12, 4.5%; AdoB12, 28.6%; and MeB12, 65%), the coenzyme forms (AdoB12 and MeB12) of B12 been predominant (Fig. 2). Most (~80%) of the B12 compounds found in some dried purple laver (*Porphyra* sp.) sheets that were commercially available in a local market have been recovered in the OH-B12 fraction. These results suggest that the B12 coenzymes, which are light-labile, were converted to OH-B12 during the drying process (probably by exposure to sunlight) of the purple laver, and that lyophilization would be an effective drying method without loss of the B12 coenzymes in the edible purple laver. Although the fact that the B12 compounds found in *Porphyra yezoensis* could bind to hog IF substrates that the compounds would likely be absorbed in the mammalian intestines, the bioavailability of the laver B12 compounds remains to be determined in detail by using B12-deficient rats.

Fig. 2. Reversed-phase HPLC Pattern of B12 Compounds from the Purple Laver.

The B12 compounds were extracted from the lyophilized purple laver powder (10 g) by boiling with 80% (v/v) ethanol solution. Four hundred ml of an 80% (v/v) ethanol was added to the laver powder, and the solution was heated at 98°C for 30 min under reflux conditions and then cooled to room temperature. The solution was passed through 5B-type filter paper, the resulting filtrate being evaporated to dryness under reduced pressure and the residue being dissolved in 50 ml of distilled water. The solution was loaded into a C18 cartridge (Sep-Pak Vac 20 cm; Waters Corporation, Milford, Massachusetts, U.S.A.) which had been washed with 50 ml of a 75% (v/v) ethanol solution and then equilibrated with distilled water, washed with 20 ml of distilled water, and eluted with 50 ml of a 25% (v/v) ethanol solution. The eluate was evaporated to dryness under reduced pressure and dissolved in 3 ml of distilled water. The resulting solution was centrifuged at 10000 × g for 5 min, and the supernatant was passed through a membrane filter (0.5 µm, LCR13-LH; Millipore, Bedford, MA, U.S.A.). The filtrate was used as a sample for HPLC. The sample preparation for HPLC was done in the dark. The sample (50 µl) was loaded into a reversed-phase HPLC column (Wakosil-II 5C18RS) that had been equilibrated with solvent A [a 5% (v/v) methanol solution containing 0.1% (v/v) acetic acid] at 40°C. The solvent flow rate was 1 ml/min. The B12 compounds were eluted with a linear gradient of methanol [from 0 to 90% of a 50% (v/v) methanol solution containing 0.1% (v/v) acetic acid] for 30 min. The retention times of authentic OH-B12, CN-B12, AdoB12 and MeB12 were 10.4, 20.0, 23.5, and 28.7 min, respectively. Fractions (1 ml) were collected from the HPLC column, evaporated to dryness under reduced pressure, and dissolved in 1 ml of distilled water. These fractions were used for the B12 assay. Arrows indicate peak fractions of the authentic B12 compounds eluted from the HPLC column. The data are the typical elution pattern of B12 compounds from three independent experiments.

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References