Content and Characteristics of Vitamin B₁₂ in Some Seaweeds

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Summary The vitamin B₁₂ (B₁₂) content in seven species of seaweed that are consumed frequently in Hokkaido, Japan, was microbiologically measured using Escherichia coli 215. Asakusanori (Porphyra tenera), maruba-amanori (Porphyra suborbiculata) and akaba-gin-nansou (Rhodoglossum pulcherum) showed higher B₁₂ content than the other species, although the content varied greatly among samples in the same species. A bioautography on a thin-layer plate holding a mixture of silica gel and cellulose, differentiation of B₁₂ and its analogues using a binding specificity of intrinsic factor and haptocorrin, and comparison of the B₁₂ concentration determined by the radioisotope dilution assay method using the intrinsic factor as the B₁₂-binding protein with that by the bioassay method, predominantly showed B₁₂ in maruba-amanori and B₁₂ analogues in wakame (Undaria pinnatifida) and akaba-gin-nansou. The B₁₂ uptake of akaba-gin-nansou from artificial seawater was similar to that of asakusanori that contained only B₁₂.

Key Words vitamin B₁₂, seaweed, B₁₂ analogue, bioassay, radioassay, B₁₂ uptake

The occurrence of vitamin B₁₂ (B₁₂) was believed confined to the animal kingdom until the early 1950s when the B₁₂ content was found to be higher in some seaweeds than beef or fish meat (1). Accordingly, seaweeds were expected to be promising resources of B₁₂ (2) because of their vast production in the world. Most seaweeds contain not only B₁₂ analogues that are active in mammals, but also other

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B₁₂ analogues (2). The biological and nutritional significance of B₁₂ and its analogues in seaweeds have not been reported. However, only Dagnelie et al. reported a deleterious effect on mean corpuscular volume in vegetarian children with a tendency of B₁₂ deficiency when fed some seaweeds including nori (Porphyra family) (3).

The findings of Dagnelie et al. may be due to B₁₂ analogues in the seaweeds. The purpose of this study was to get some clue as to the significance of B₁₂ analogues in some seaweeds for human nutrition. For this reason, B₁₂ content was assayed microbiologically using Escherichia coli 215. It responds to a wider variety of B₁₂ and B₁₂ analogues than any other test organism (4). We measured the B₁₂ contents in some commercially available seaweeds that were consumed frequently in Japan. The structure of B₁₂ and its analogues in maruba-amanori (Porphyra suborbiculata), akaba-gin-nansou (Rhodoglossum pulcherum) and wakame (Undaria pinnatifida) were characterized.

Asakusanori containing only B₁₂ has been known to take up B₁₂ from artificial seawater (5). The relationship between B₁₂ content and B₁₂ uptake by a seaweed was worth studying. The B₁₂ uptake by akaba-gin-nansou, composing exclusively of B₁₂ analogues, was measured and compared with that of asakusanori.

MATERIALS AND METHODS

Seaweeds. Raw asakusanori (Porphyra tenera), raw maruba-amanori (Porphyra suborbiculata), raw funori (Gloiopeltis tenax), raw akaba-gin-nansou (Rhodoglossum pulcherum, known as hotokenomimi), dried kombu (Laminaria angustata), wakame (Undaria pinnatifida) rinsed in warm water at 50°C for 30s and then salted, and dried hijiki (Hijikia fusiforme) were purchased from department stores. The asakusanori was from Ishinomaki, Miyagi, the maruba-amanori was from Otaru, Hokkaido, the funori was from Habomai Island, Hokkaido, the akaba-gin-nansou was from Wakkanai, Hokkaido, the kombu was from Shizunai-cho, Hidaka, Hokkaido, the wakame was from Miyako, Iwate, and the hijiki was from Ise, Mie.

 Extraction and analysis of B₁₂. For the analysis of B₁₂, extract was prepared from 5 g of seaweed and then desalted with a small column of Silanised Silica gel 60 (Merek, Germany) as described by van Kapel et al. (6). All procedures were carried out in the dark to avoid the destruction of B₁₂ by light. B₁₂ content was assayed microbiologically using E. coli 215 (7) and radiologically using a γ-Dual B₁₂ kit (Baxter-Travenol, USA). To test the efficiency of extraction and the microbiological assay method, a known amount of crystalline B₁₂ was added to the seaweed sample. The recovery of B₁₂ usually ranged 100±15%, and in some extreme cases was 60%.

Separation of B₁₂ analogues and characterization. Four forms of B₁₂ (cyanocobalamin, hydroxocobalamin, methylcobalamin and 5′-deoxyadenosylcobalamin), a B₁₂ analogue (cobinamide) and the seaweed extract were separated by thin-layer
chromatography using solvent solution of 2-butanol–28% ammonia water–water (75:2:25), and then the chromatogram was analyzed by bioautography according to the method of Gimsing and Nexø (8). The dye 2,3,5-triphenyltetrazolium salt, however, was not included from the beginning of incubation because the dye retarded the growth of E. coli 215. The solution of the dye was sprayed over the agar plate after 20 h of culturing.

**Materials.** In this report, B12 refers to forms that are active in mammals. It includes cyanocobalamin, hydroxocobalamin, methylcobalamin and 5′-deoxyadenosylcobalamin. Cyanocobalamin was used as the standard B12 for bioassays in this study because of its stability. Corrinoid compounds that can not be utilized by mammals, but can be utilized by E. coli 215 are referred to as B12 analogues. Monocarboxylic acid derivatives of B12 and cobamides are the main components of analogues. In this experiment, cobinamide was used as a B12 analogue. The B12 and cobinamide were from Sigma, USA, and Calbiochem, USA, respectively.

**Characterization of B12.** B12 and its analogues were determined by utilizing B12 binding proteins of hog intrinsic factor (IF) (purchased from Sigma) and haptocorrin (HC, formerly designated as R-binder), prepared from hog gastric mucosa by the method of Allen and Mehlman (9). The former binds only B12, but the latter binds both B12 and its analogues. One unit of IF or HC binds 1,000 pg of B12 or its analogues. A 0.1 ml solution containing 0.6 units of IF or HC was added to the seaweed extract or to the authentic solution containing 300 pg of B12 or its analogues in a volume of 0.1–0.3 ml. The mixture was incubated at room temperature for 15 min. Following incubation, 0.025 ml of glycerine and 0.02 ml of a 2.5% solution of Blue Dextran 2,000 (Pharmacia, Sweden) were added. The mixture was then applied on a small column (7 × 55 mm) of Sephadex G-75 (Pharmacia). The protein-bound B12 and its analogues were eluted in a void volume. Fractions of the analogues free from the protein were collected and assayed microbiologically. The amounts of analogues remaining after treatment with IF and HC were termed I and H, respectively. The amount of free B12 and its analogues in the untreated extract was termed N. N minus I gives the B12 amount, and N minus H gives the sum of B12 and its analogues. I minus H gives the amount of B12 analogues.

**Uptake experiment of B12.** Uptake of B12 by the seaweeds was performed as follows (5): Approximately 0.5 g of each seaweed (fresh) was placed in 20 ml of artificial seawater prepared for marine fish breeding (Top-Marine, product of Nankai Gyoen Co. Ltd., Japan). According to the manufacturer, it contains no B12. 57Co-labeled cyanocobalamin (57Co-B12) (purchased from Ciba–Corning Diagnostics, USA) was added at a concentration of 1.1 or 50.6 pg/ml and incubated in a bright room at room temperature of approximately 25°C for 4 h with gentle shaking. The seaweeds were then removed, washed briefly with fresh artificial seawater and 57Co radioactivity was counted by a Wallac 1282 Compugamma counter.
RESULTS

Contents of B12

Table 1 shows the B12 content determined microbiologically in each of the seven species of seaweed. The variation was large among individual samples in the same species. However, the highest mean B12 content was found in asakusanori and akaba-gin-nansou, followed by maruba-amanori. Kombu and hijiki had lower contents of B12 than other species.

Characterization of B12

The bioautographies of four forms of B12, cobinamide and seaweed extracts on a thin layer of silica gel-cellulose are shown in Fig. 1. This shows a typical result of two experiments. Four authentic forms of B12 and cobinamide were clearly separated from each other (five lanes on the left side). A dominant spot of maruba-amanori migrated to the same position as methylcobalamin (methy-B12), and the faint one as that of cyanocobalamin (cyano-B12). A single spot corresponding to cyano-B12 existed in wakame. For akaba-gin-nansou, a dominant spot corresponded to cyano-B12 and a faint one to methy-B12. Since cyano-B12 is not a natural form and no cyanogen compounds were used in the extraction procedure, the cyano-B12-like spot may be an analogue with a similar relative mobility $(R_f)$ to cyano-B12 (i.e., not cyano-B12). To attest this, extracts of the seaweeds were treated with B12-binding proteins and the remaining amounts of the analogues were measured as described under Materials and Methods. Table 2 is made up from an average of the two experimental results. As shown in the top part of the table, both IF and HC treatments completely removed cyano-B12. The HC removed cobinamide. Therefore, the amount of unbound B12 and its analogues that remained after

Table 1. Vitamin B12 content in some species of seaweed. B12 contents were assayed microbiologically using *E. coli* 215 (7) on the extract of seaweeds prepared as described by van Kapel et al. (6).

| Species             | Scientific name       | Number of samples* | Vitamin B12 content M±SD (range) (µg/100 g wet weight) |
|---------------------|-----------------------|--------------------|--------------------------------------------------------|
| Asakusanori         | *Porphyra tenera*     | 24                 | 3.19±3.84 (0.209–12.1)                                  |
| Maruba-amanori      | *Porphyra suborbiculata* | 24                 | 0.417±0.376 (0.038–1.39)                                |
| Funori              | *Gloiopeitlis tenax*  | 10                 | 0.169±0.131 (0.057–0.445)                               |
| Akaba-gin-nansou    | *Rhodoglossum pulcherum* | 17                 | 1.50±3.03 (0.013–12.3)                                  |
| Kombu               | *Laminaria angustata* | 11                 | 0.012±0.003 (0.004–0.026)                               |
| Wakame              | *Undaria pinnatifida*  | 21                 | 0.082±0.095 (0.006–0.398)                               |
| Hijiki              | *Hijikia fusiforme*    | 8                  | 0.011±0.014 (0.002–0.043)                               |

*Number of samples denotes number of packages from which the seaweed was taken.

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Fig. 1. Bioautogram of vitamin B12 and its analogues in the seaweeds. A thin-layer plate was prepared from a slurry mixture of three weights of powdered cellulose (Whatman, Microgranular Cellulose, CC41) and one weight of silica gel (Merck, Silica gel G, Type 60). Extracts of the seaweeds, solutions of authentic B12 (cyanocobalamin, hydroxocobalamin, methylcobalamin and 5'-deoxyadenosylcobalamin) and cobinamide were applied at one end of the plate, and developed with 2-butanol-28% ammonia water-water (75:2:25). After drying the cellulose-silica gel plate, agar containing basal medium and E. coli 215 was overlayed and then incubated at 30°C for 20h. After spraying methanol solution of 2,3,5-triphenyltetrazolium salt on the gel plate, the position of B12 was visualized as red color by E. coli growth.

treatment with IF and disappeared after treatment with HC corresponds to that of cobinamide, a B12 analogue. A small amount of B12 and its analogues in maruba-amanori was similar to cyano-B12, and so the major B12 activity of Rf 0.37 was CH3-B12. The pattern of the remaining ratio after treatment in akaba-gin-nansou showed essentially the same one as the B12 analogues exemplified by cobinamide. This suggests that the seaweed predominantly contains analogues. Some B12 activity with E. coli 215 remained (23%) in wakame after treatment with HC. The cause of the remaining activity is not known at present.

B12 concentrations determined by the microbiological assay method were compared with those measured by the radioisotope dilution assay method on the same seaweed extracts. B12 and some B12 analogues such as cobamides and...
Table 2. Determination of vitamin B\textsubscript{12} and its analogues by intrinsic factor and haptocorrin. Seaweed extract, solution of B\textsubscript{12} and cobinamide were treated with hog intrinsic factor (IF) that binds only B\textsubscript{12} or hog haptocorrin (HC) that binds both B\textsubscript{12} and its analogues. The amount of binding protein was twice the amount of all B\textsubscript{12} and analogue contents. The mixture was then filtered through a small column of Sephadex G-75 (Pharmacia, Sweden) to collect the free form fraction and assayed with \textit{E. coli} 215.

| Sample                          | Relative remaining B\textsubscript{12} and its analogues to that of control (%) |
|---------------------------------|----------------------------------------------------------------------------------|
|                                 | Control (without treatment) | Treatment with IF | Treatment with HC |
| Cyanocobalamin                  | 100                         | 0* (0, 0)**       | 0 (0, 0)          |
| Cobinamide                      | 100                         | 103 (100, 106)    | 0 (0, 0)          |
| Wakame (\textit{Undaria pinnatifida}) | 100                       | 88 (99, 79)       | 23 (25, 20)       |
| Akaba-gin-nansou (\textit{Rhodoglossum pulcherum}) | 100                       | 101 (117, 84)    | 0 (0, 0)          |
| Maruba-amanori (\textit{Porphyra suborbiculata}) | 100                       | 0 (0, 0)         | 0.3 (0.6, 0)      |

* Mean values of two experiments.
** Values in the parentheses denote individual measurements.

Table 3. Vitamin B\textsubscript{12} uptake by asakusanori and akaba-gin-nansou. Approximately 0.5 g of fresh seaweed was placed in artificial seawater containing \textsuperscript{57}Co-B\textsubscript{12}. After 4 h of incubation with gentle shaking, the seaweed was removed, washed briefly with fresh artificial seawater and \textsuperscript{57}Co radioactivity counted.

| Species                              | Concentration of \textsuperscript{57}Co-B\textsubscript{12} (pg/ml) | Incubation temperature (°C) | Vitamin B\textsubscript{12} uptake M±SD (ng/g wet weight) |
|--------------------------------------|---------------------------------------------------------------------|-----------------------------|----------------------------------------------------------|
| Asakusanori (\textit{Porphyra tenera}) | 1.1                                                                 | 25                          | 0.050±0.004                                              |
|                                      | 50.6                                                                | 25                          | 2.34±0.05                                                |
|                                      | 50.6                                                                | 0                           | 2.00±0.30                                                |
| Akaba-gin-nansou (\textit{Rhodoglossum pulcherum}) | 1.1                                                                 | 25                          | 0.029±0.006                                              |
|                                      | 50.6                                                                | 0                           | 0.021±0.003                                              |
|                                      | 50.6                                                                | 25                          | 2.05±0.19                                                |

Each condition represents the total of three experiments.

cobinamide were estimated by a previous method (4). By the latter method, which utilizes pure IF, only B\textsubscript{12} was detected according to the manufacturer's description. Therefore, the concentrations of B\textsubscript{12} determined by microbioassay were equal to that determined by radioassay when only B\textsubscript{12} existed in the extracts. The concen-
trations estimated by the former method become larger when the samples include analogues. The ratio of the concentration by the former method to that by the latter method was $1.09 \pm 0.23$ ($n=3$) for maruba-amanori, $1.71 \pm 1.32$ ($n=3$) for akaba-gin-nansou and $1.44 \pm 1.24$ ($n=7$) for wakame. This indicates that maruba-amanori contains only $\text{B}_{12}$ and akaba-gin-nansou contains a significant amount of $\text{B}_{12}$ in addition to analogues. These results prompted us to compare the characteristics of $\text{B}_{12}$ uptake by asakusanori, which contained only $\text{B}_{12}$ (1) and is similar to maruba-amanori and akaba-gin-nansou.

Table 3 shows the uptake of $\text{B}_{12}$ by two species of seaweed at different temperatures and in two concentrations of $\text{B}_{12}$. The $\text{B}_{12}$ uptake by akaba-gin-nansou was somewhat lower than that by asakusanori under both conditions. However, the difference was trivial. Therefore, it was concluded that akaba-gin-nansou possesses the ability to take up $\text{B}_{12}$.

**DISCUSSION**

The deviations in $\text{B}_{12}$ content between individual samples of the same species of seaweed were large both in this report and the report by Kanazawa (2). This may be due to the season and the place in which the seaweeds were harvested. The $\text{B}_{12}$ content of seawater fluctuates seasonally (10) although the interrelationship between the $\text{B}_{12}$ contents of seawater and seaweeds is not known.

The $\text{B}_{12}$ contents in seaweeds reported by Hashimoto and Maeda (1) were 1.5–3.5 for asakusanori, 0.3 for kombu, 0.7 for wakame and 1.2 for hijiki, in $\mu\text{g} \text{B}_{12}/100 \text{g}$ wet weight of seaweed. According to the review by Kanazawa (2), they were 2.9 for asakusanori, 0.15 for funori, 0.03 for kombu and 0.057 for hijiki. The former authors utilized *Euglena gracilis* and the latter author *E. gracilis* and *Ochromonas malhamensis* as assay organisms for $\text{B}_{12}$. Our data reported in this paper are similar to those of Kanazawa (2), but generally much lower than those of Hashimoto and Maeda (1) except for asakusanori. These differences depend on the test organism used for the assay of $\text{B}_{12}$ and its analogues.

The review by Kanazawa (2) showed that certain kinds of seaweed contain only $\text{B}_{12}$, but others also contain some analogues such as pseudo-$\text{B}_{12}$, which has adenosyl moiety instead of 5,6-dimethylbenzimidazole of the $\text{B}_{12}$ molecule, factor A, which contains 2-methyladenosynl moiety in place of 5,6-dimethylbenzimidazole and cobinamide, which lacks the nucleotide portion of the $\text{B}_{12}$ molecule. *E. gracilis* and *O. malhamensis* were used for the assay of $\text{B}_{12}$. These organisms respond to $\text{B}_{12}$ and a limited variety of $\text{B}_{12}$ analogues. This study aimed to measure $\text{B}_{12}$ and its analogues in seaweeds. The use of the organism which responds to a wide spectrum of analogues was a prerequisite. We used *E. coli* 215 as reported by Toraya (4).

The $\text{B}_{12}$ content in asakusanori was measured by bioassay with *E. coli* 215 for the first time in this study. Different microorganisms respond to $\text{B}_{12}$ and its analogues differently: *O. malhamensis* responds to only $\text{B}_{12}$, *E. gracilis* to $\text{B}_{12}$ and other cobamides, and *E. coli* 215 to $\text{B}_{12}$, other cobamides and cobinamide (4).
B12 contents in asakusanori as measured with these three kinds of microorganisms were quite consistent in spite of the remarkable difference in response to B12 analogues. This suggests that asakusanori contains only B12.

The observations by Hashimoto and Maeda (1), and our observations in this report, indicate that the nori (Porphyra) family contains B12. This observation conflicts with the deleterious effect of nori on B12 availability reported by Dagnelie et al. (3). The exact reason of this conflict is not clear at present. It was not cited in the article of Dagnelie et al. (3) whether they used raw seaweeds or dried ones. If they used dried nori, B12 may have been converted into its analogues by the drying process of the seaweeds and by light. Recently, Rauma et al. (11) reported that serum B12 concentration in vegans taking nori seaweed was twice that of those not taking the seaweed. However, the serum B12 concentration was not associated with the mean corpuscular volume of erythrocytes.

The characterization of B12 analogues (2) has been done by the specificity of test organisms on the different analogues of B12. Further, B12 and its analogues have been separated by chromatographic techniques (I). The method is based on the differences in the distribution coefficients of B12 and its analogues between the solid phase and the solvent. Some analogues of B12 may be mistaken to be B12 by the chromatographic method. This is exemplified by a B12 analogue in akaba-ginnansou in Fig. 1 in this report. In this study, B12 was discriminated from its analogues using mammalian IF. The results obtained by the binding method reflect the biological availability of B12 and its analogues more efficiently than chromatographic methods.

Akaba-ginnansou predominantly contains B12 analogues. This prompted us to compare the B12 uptake properties of akaba-ginnansou and asakusanori, which contains predominantly B12 according to Hashimoto and Maeda (1). In spite of striking differences in the form of B12 found in these two species, the characteristics of the B12 uptake by both algae were similar. Namely, they took up similar amounts of B12 from artificial seawater, and the uptake was somewhat less at 0°C than at 25°C in both species. There was no relationship between the fact that akaba-ginnansou contains predominantly analogues of B12 and that it possesses the ability to take up B12. Akaba-ginnansou may be able to take up both B12 and its analogues. The samples of seaweed that we used for determination of B12 and its analogues contained only B12 analogues by chance. This possibility will be examined by measuring the content of B12 and its analogues in akaba-ginnansou after taking up B12. Otherwise, akaba-ginnansou may convert B12 into its analogues after it takes up B12.

The ratio of B12 concentration measured by bioassay to that measured by radioassay, which is presumed to reflect the ratio of B12 plus its analogues to B12, were 1.71 in akaba-ginnansou and 1.44 in wakame. The values indicate that the amount of B12 analogues was 0.71 times the amount of B12 in akaba-ginnansou and 0.44 times in wakame, contrasting with the predominancy of analogues in akaba-ginnansou and wakame. The purity of the IF in the radioassay kit is always a
subject of debate.

Our results indicate that rhodophyta phylum (red algae) such as asakusanori, maruba-amanori and akaba-gin-nansou contained B_{12} and its analogues at a much higher content than phaeophyta phylum (brown algae) such as hijiki and kombu. Maruba-amanori contained almost only B_{12}, but akaba-gin-nansou contained exclusively analogues as detected by methods utilizing the specificity of two B_{12}-binding proteins.

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