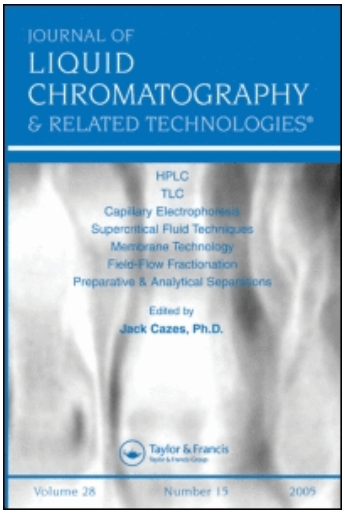

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Analysis of Vitamin B_{12} in Food by Silica Gel 60 TLC and Bioautography with Vitamin B_{12}-Dependent *Escherichia coli* 215

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Analysis of Vitamin B_{12} in Food by Silica Gel 60 TLC and Bioautography with Vitamin B_{12}-Dependent *Escherichia coli* 215

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Abstract: To evaluate whether certain foods contain vitamin B_{12} or inactive corrinoids, a simple technique, bioautography with vitamin B_{12}-dependent *Escherichia coli* mutant after separation of the sample by silica gel 60 thin-layer chromatography, is available. By using the method, vitamin B_{12}-compounds found in some edible cyanobacteria are readily identified. This bioautography has great advantages (simplicity, speed, and inexpensiveness) for the analysis of vitamin B_{12}-compounds in food.

Keywords: Bioautography, Edible cyanobacteria, *Escherichia coli* 215, TLC, Vitamin B_{12}

INTRODUCTION

Vitamin B_{12} (B_{12}) contents of foods have been determined by bioassay with certain B_{12}-requiring microorganisms such as *Lactobacillus delbrueckii* subsp. *lactis* ATCC7830 (formerly *Lactobacillus leichmannii*).[1]

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Radioisotope dilution assay method with radio-labeled B_{12} and hog intrinsic factor (the most specific B_{12}-binding protein) has also been used for the determination of B_{12} contents in foods.[2] Various types of non-RI B_{12} analyzer are being manufactured and clinically used for the routine assay of human serum B_{12} worldwide. We evaluated the applicability of the B_{12} analyzer in food analysis, indicating the excellent correlation coefficient between both methods in most foods tested, although in some specific foods the values determined by the microbiological method were about several-fold greater than the values determined by the analyzer.[3] This difference is due to the fact that *L. delbrueckii* used for the microbiological assay of food B_{12} utilizes corrinoid compounds (such as pseudovitamin B_{12}) inactive for humans as well as B_{12} (Figure 1).

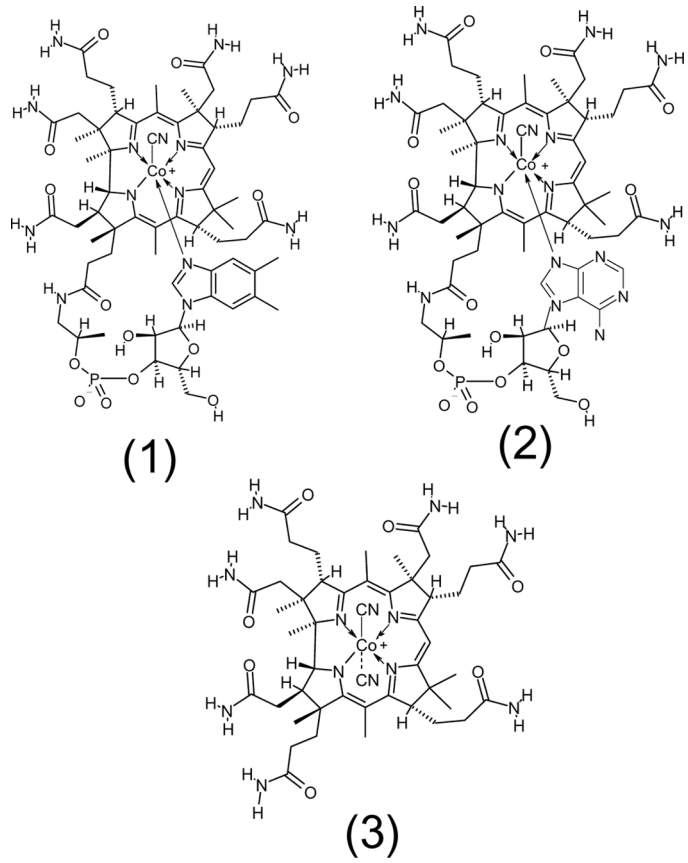

Figure 1. Structures of vitamin B_{12} (1), pseudovitamin B_{12} (2), and dicyanocobinamide (3).

Furthermore, both deoxyribosides and deoxynucleotides (known as an alkali-resistant factor) can substitute B_{12} in the lactic bacterium.[4] To evaluate whether certain foods contain B_{12} or inactive corrinoids, some B_{12}-compounds have been purified to homogeneity and identified: [5–7] the evaluation procedures take a lot of time and cost. These problems would be easily resolved by the use of a simple technique, bioautography with B_{12}-dependent *E. coli* mutant after separation of the sample by silica gel 60 thin-layer chromatography (TLC).[8] The bioautography has great advantages (simplicity, speed, and inexpensiveness) for the analysis of B_{12}-compounds in food.

EXPERIMENTAL

Materials

B_{12} and dicyanocobinamide were obtained from Sigma (St Louis, USA). Silica gel 60 TLC aluminium sheets were obtained from Merck (Darmstadt, Germany). All other reagents used were of the highest purity commercially available. The edible cyanobacteria tested were provided from the MicroAlgae Corporation, Japan.

Methods

Extraction and Assay of Vitamin B_{12}

After 2 g of each sample of edible algae were suspended in 40 mL of distilled water and homogenized with an ultrasonic disruptor UD-200 (Tomy, Tokyo, Japan). Total B_{12} was extracted with boiling at acidic pH range and assayed by the microbiological method with *L. delbrueckii* ATCC 7830 according to the method described in the Japanese Standard Tables of Food Composition.[4] Since *L. delbrueckii* ATCC 7830 can utilize both deoxyribosides and deoxyribonucleotides (known as the 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

Bioautograpy of B_{12}-compounds was done according to the modified method of the reference cited.[8] The B_{12} extract (10 mL) prepared above

was partially purified with Sep-pak Plus C_{18} cartridge (Waters Corp. Milford, USA) which had been washed with 5 mL of 75% (v/v) ethanol and then equilibrated with 5 mL of distilled water. The C_{18} cartridge was washed with 5 mL of distilled water and then B_{12}-compounds were eluted with 2 mL of 75% (v/v) ethanol. The eluate was evaporated with a centrifugal concentrator (Integrated Speed Vac® System ISS110, Savant Instruments Inc., NY, USA). The residual fraction was dissolved with 1 mL of distilled water. Two micro-liters of the concentrated B_{12} extract and authentic B_{12} (cyanocobalamin, 10 μg/L) were spotted on the silica gel 60 TLC sheet (10 × 10 cm) 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 completely dried, it was overlaid on 1.5% (w/v) agar containing a basal medium (KH_2PO_4, 3 g/L; K_2HPO_4, 7 g/L; sodium citrate, 0.5 g/L; $MgSO_4$, 0. 1 g/L; $(NH_4)_2SO_4$, 1 g/L; NaCl, 5 g/L; glucose, 3 g/L at pH 7.2) and small volume (~100 μL) of *E. coli* 215 culture [grown for 20 h in a pre-culture medium (peptone, 40 g/L; glucose, 2 g/L; NaCl, 5 g/L at pH 7.2)] in a disposable Petri dish (144 × 104 × 16 mm) and then incubated at 30°C for about 20 h.

After being sprayed with a methanol solution of 4% (w/v) 2,3,5-triphenyltetrazolium salt on the gel plate and then left for 1 h at 30°C, 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.

RESULTS AND DISCUSSION

Specificity of Vitamin B_{12} Structure

Figure 2 shows structural specificities of B_{12}-compounds in the growth of B_{12}-dependent *E. coli* 215. A concentrated solution of authentic dicyanocobiamide (Figure 1), which lacks the cobalt-coordinated nucleotide from B_{12}, was separated three red-colored spots with silica gel 60 TLC, suggesting that a portion of dicyanocobinamide might be converted into diaquocobinamide and aquocyanocobinamide in aqueous solution.

When a diluted solution of authentic dicyanocobinamide was analyzed by the bioautography, three spots of *E. coli* 215 growth were found in the identical R_f values. The result indicates that structural specificity of B_{12} in *E. coli* 215 growth is very low so that a variety of B_{12}-compounds would be detected with the bioautography.

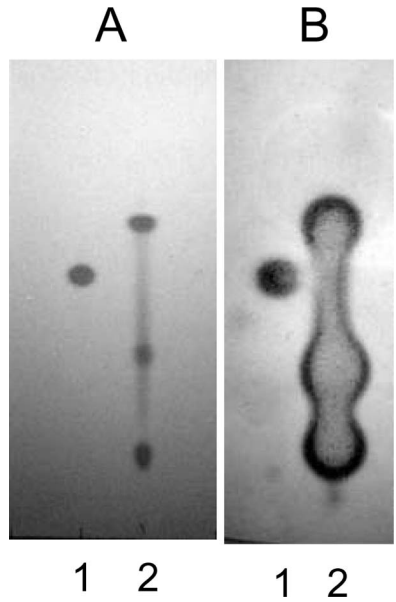

Figure 2. Silica gel 60 TLC patterns and bioautograms of authentic vitamin B_{12} and dicyanocobinamide. A, silica gel 60 TLC patterns of B_{12} (1) and dicyanocobinamide (2). Two microliters of 10 mmol/L authentic B_{12} and dicyanocobinamide were spotted on the silica gel 60 TLC sheet (10×10 cm) and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) in the dark at room temperature (25°C). B, *E. coli* 215-bioautograms of B_{12} (1) and dicyanocobinamde (2). Two microliters of diluted solutions of B_{12} and dicyanocobinamide were treated with TLC under the same conditions and then visualized by the bioautography.

Effects of *L*-Methionine and Deoxynucleotide

Effects of *L*-methionine and 2-deoxyadenosine monophosphate on the *E. coli* 215 bioautography were studied (Figure 3). Although *E. coli* 215 was sensitive to *L*-methionine as well as B_{12}, a spot of the *E. coli* growth was not found in the *L*-methionine solution treated with Sep-pak C_{18} Plus cartridge. The result indicates that the partially purification step requires both to remove *L*-methionine from the B_{12} extract and to concentrate B_{12} in the extract. *E. coli* 215 was insensitive to 2-deoxyadenosine monophosphate as an alkali-resistant factor.

Sensitivity to Vitamin B_{12}

Figure 4 shows effects of B_{12} concentrations on *E. coli* 215 growth. When each area of the spots of *E. coli* 215 growth was calculated, it was

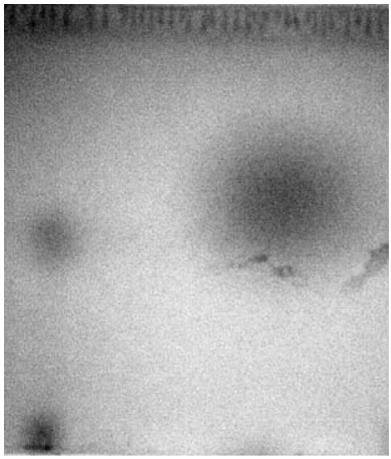

Figure 3. Effects of *L*-methionine and deoxynucleotide on the *E. coli* 215-bioautography. Authentic B_{12} (10 pg) (1) and 2-deoxyadenosine monophosphate (3 μg) (2) were analyzed with the TLC-bioautography under the same conditions. Ten mmol/L *L*-methionine solution were treated with (3) or without (4) Sep-pak C_{18} Plus cartridge and then analyzed.

increased in proportion to increase in B_{12} concentrations. The detection limit of B_{12} was ~10 pg of B_{12} on this bioautography.

Analysis of Vitamin B_{12}-Compounds in Some Edible Cyanobacteria

Our previous studies[9,10] have demonstrated that some edible cyanobacteria, *Nostoc commune* "Ishikurage" and *Aphanothece sacrum* "Suizenji-nori" contained substantial amounts of pseudo-B_{12}, which has been already purified and identified from these dried bacterial cells. To evaluate applicability for food B_{12} analysis, the B_{12} extracts prepared from these cyanobacteria were analyzed with B_{12}-dependent *E. coli* 215 bioautography (Figure 5A and B). The B_{12}-active compounds found in the *N. commune* cells were separated completely as two (main and minor) spots, whose R_f values were identical to those of authentic pseudo-B_{12} and B_{12}, respectively. The similar result was given in the *A. sacrum* cells. These results indicate that it was easy to identify B_{12}-compounds found in both edible cyanobacteria using this method.

Another edible cyanobacterium *Nostoc flagelliforme* (black hair-like form) grows naturally on Chinese inland semi-desert ground and

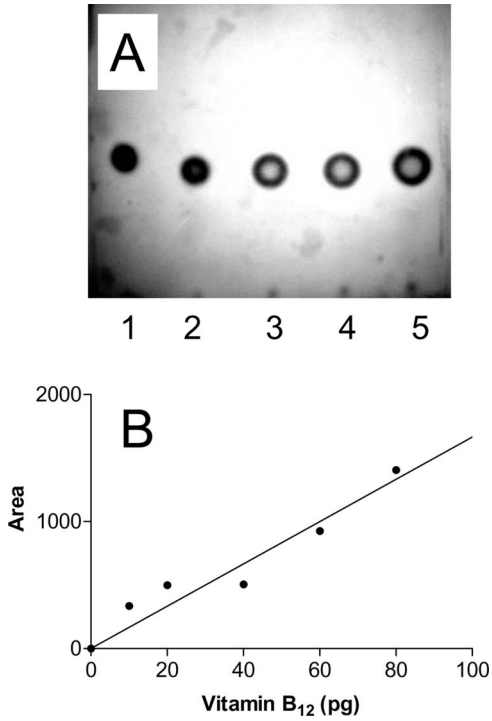

Figure 4. Effects of vitamin B_{12} concentrations on the *E. coli* 215-bioautography. Various concentrations [20 pg (1), 40 pg (2), 60 pg (3), 80 pg (4), and 100 pg (5)] of authentic B_{12} were analyzed with the TLC-bioautography under the same conditions as described in the text. A, a typical bioautogram; B, relationship between areas of the spots of *E. coli* growth and various B_{12} concentrations added.

is available on a cooking ingredient of Chinese food. The dried *N. flagelliforme* cells contained 113 μg of B_{12} per 100 g of the cells by the microbiological B_{12} assay. Although we tried to purify B_{12}-compounds to clarity whether this edible cyanobacterium contains true B_{12} or not, the experiment ended in failure. Therefore, the bacterial B_{12} extract was analyzed with the *E. coli* 215 bioautography (Figure 5C). The B_{12}-active compounds found in the *N. flagelliforme* cells were separated completely as two spots, whose R_f values were identical to those of authentic pseudo-B_{12} and B_{12}, respectively. The area of the B_{12} spot was calculated to be about 45.3% of the sum of the area of the two spots, suggesting that the bacterial cells contained about 51.2 μg of true B_{12} per 100 g of the dried cells.

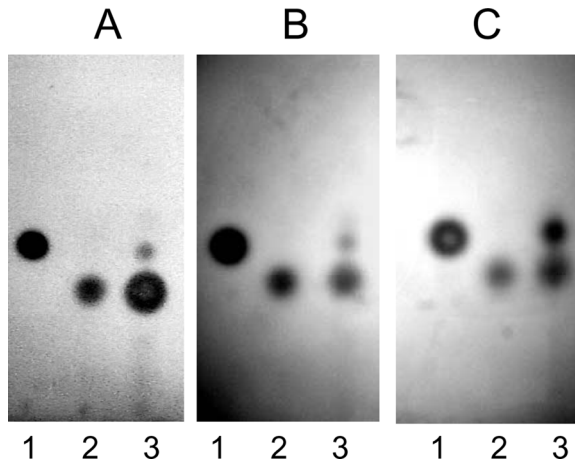

Figure 5. Bioautography of authentic vitamin B_{12}, pseudovitamin B_{12}, and extracts of some edible cyanobacteria. Authentic B_{12} (1), pseudo-B_{12} (1), and each of the B_{12} extracts (3) of *Aphanothece sacrum* (A), *Nostoc commune* (B), and *Nostoc flagelliforme* (C) were analyzed with the TLC-bioautography under the same conditions as described in the text.

These results presented here indicate that the improved bioautography has great advantages (simplicity, speed, and inexpensiveness) for the analysis of B_{12}-compounds in food.

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