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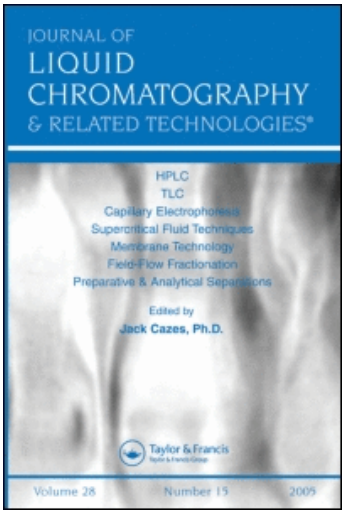

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TLC Analysis of Corrinoid Compounds in the Halophilic Lactic Acid Bacterium *Tetragenococcus halophilus*

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Abstract: Vitamin B_{12} content ($3.1 \pm 0.8\,\mu g/100\,g$ wet weight) of the lactic acid bacterium *Tetragenococcus halophilus* IAC-ks6, isolated from a fermented skipjack viscera, was determined using the microbiological vitamin B_{12} assay method with *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830. On the basis of silica gel 60 TLC and C_{18} reversed-phase HPLC, true vitamin B_{12} predominated in the bacterial cells and functioned as each cofactor of the vitamin B_{12}-dependent enzymes, methionine synthase and ribonucleotide reductase.

Keywords: HPLC, Salted and fermented skipjack viscera, *Tetragenococcus halophilus*, TLC, Vitamin B_{12}

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INTRODUCTION

A gram-positive lactic acid bacterium, *Tetragenococcus halophilus*, is used to ferment soy sauce, a well-known condiment in southeast Asia, China, and Japan.[1,2] *T. halophilus* is a moderately halophilic bacterium and is also associated with other foods (such as cured anchovies) processed under reduced water activity; this organism can grow under both aerobic and anaerobic conditions.[3]

Fish meats and viscera are generally known to contain considerable amounts of vitamin B_{12}.[4] If the bacterium requires and accumulates B_{12} for growth, the vitamin would be an important factor for production of the fermented foods involved in the lactic acid bacterium. There is, however, no information available on whether the bacterium contains true B_{12} or not, and on the physiological role of B_{12}. Here, we describe the analysis of corrinoid compounds found in the bacterial cells by the use of TLC and HPLC.

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EXPERIMENTAL

Materials

B_{12} and a reversed-phase high performance liquid chromatography (HPLC) column (Wakosil-II 5C18RS, $\varphi 4.6 \times 150$ mm; particle size, 5 μm) were obtained from Wako Pure Chemical Industries (Osaka, Japan). 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 (TLC) aluminium sheets were obtained from Merck (Darmstadt, Germany). All other reagents used were of the highest purity commercially available.

A visible spectrophotometer (Ultrospec 10 pro, Amersham Biosciences Corp., Piscataway, NJ, USA) was used for measuring the turbidity of *L. delbreuckii* test cultures in the microbiological B_{12} assay method.

Methods

Organism and Culture

T. halophilus IAC-ks6, which had been isolated from a salted and fermented skipjack viscera "katsuo-shiokara" (Tagomaru Co., Shizuoka, Japan) and identified, was used. The bacterium was cultured in Difco™ M-17 Broth (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 5% (w/v) NaCl and 1% (w/v) glucose without aeration at 27°C for 3 days. The bacterial cells were harvested at the stationary phase and washed twice with 5% (w/v) NaCl solution.

Extraction and Assay of Vitamin B_{12}

After two grams (wet weight) of the bacterial cells were suspended in 40 mL of distilled water and homogenized with a ultrasonic disruptor UD-200 (TOMY, Tokyo, Japan), total B_{12} was extracted from the above suspension with boiling at acidic pH 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 an alkali-resistant factor) as well as B_{12}, the amounts of true B_{12} were calculated by subtracting the values of the alkali-resistant factor from the values of total B_{12}.

Analytical TLC

Each fraction (10 mL) of the bacterial B_{12} extract and alkali-resistant factor described above was evaporated, under reduced pressure, to dryness and dissolved in 1.0 mL of distilled water. The concentrated B_{12} extract, alkali-resistant factor, and 10 mmol/L authentic B_{12} solution (3 μL) were spotted onto silica gel 60 TLC sheet and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) as solvent in the dark at room temperature (25°C). The TLC sheet was dried and cut into small pieces (width 10 × height 5 mm) with scissors. Corrinoid compounds were extracted from each TLC piece with 80% (v/v) methanol overnight. Each extract was filtered, evaporated to dryness under reduced pressure, and dissolved in 1.0 mL of distilled water. In the case of the B_{12} extract and alkali-resistant factor, B_{12} activity was determined by the microbiological B_{12} assay method. Authentic B_{12} was determined by measuring absorbance at 278 nm.

Analytical HPLC

The B_{12}-active fraction with R_f 0.62 on the above-mentioned silica gel TLC and 10 ng/L authentic B_{12} solution (each 100 μL) were analyzed with the C_{18} reversed-phase HPLC column (Wakosil-II 5C18RS, φ4.6 × 150 mm; particle size, 5 μm) and the Shimadzu HPLC apparatus (two LC-10ADvp pumps, DGV-12A degasser, SCL-10Avp system controller, SPD-10Avvp ultraviolet-visible detector, CTO-10Avp column oven, 100 μL sample loop, C-R6A chromatopac integrator). The sample was placed on the reversed-phase HPLC column equilibrated with a 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 35°C. The flow rate was 1.0 mL/min. The fractions eluted from the HPLC column were collected, evaporated to dryness under reduced pressure, and dissolved in 1.0 mL of distilled water. The B_{12} was assayed in these fractions by the microbiological B_{12} assay method.

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Enzyme Assay

Total (apo- and holo-enzyme) activity of B_{12}-dependent enzymes, ribonucleotide reductase (EC 1.17.4.2),[5] methylmalonyl-CoA mutase (EC 5.4.99.2),[6] and methionine synthase (EC 2.1.1.13)[7] were assayed in each reaction mixture containing excess amounts of the respective B_{12} coenzymes by the methods cited in the references.

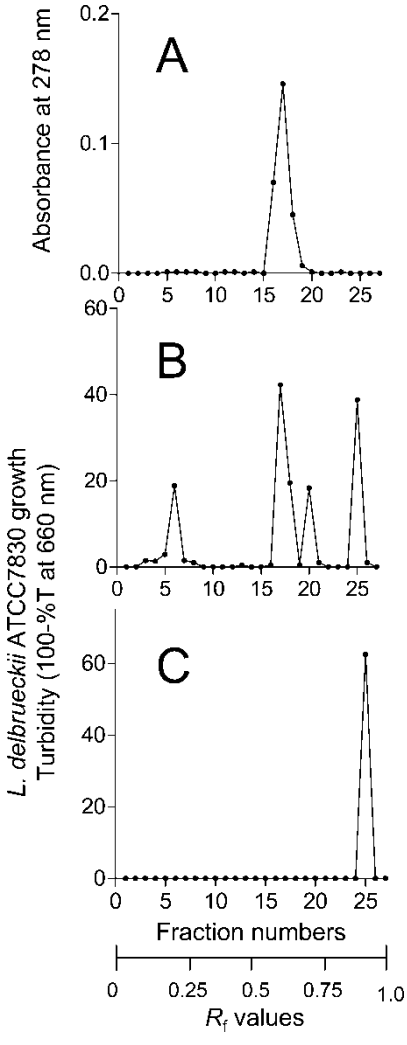

Figure 1. Silica gel 60 TLC patterns of the bacterial B_{12}-active compounds in the fractions of the B_{12} extract and alkali-resistant factor, and authentic B_{12}. (A) authentic B_{12}, (B) B_{12} extract, (C) alkali-resistant factor.

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RESULTS AND DISCUSSION

The amount of B_{12} in the lactic acid bacterium *Tetragenococcus halophilus* IAC-ks6, which had been isolated from a salted and fermented skipjack viscera, was assayed by the microbiological method adopted in the Japanese Standard Tables of Food Composition.[4] The bacterial cells contained 3.1 ± 0.8 (mean $\pm$ SEM) μg of B_{12} per 100 g of the cells (wet weight). We could not purify and characterize any corrinoid compounds from the cells because of it's low B_{12} concentration and low cell yield.

To clarify whether corrinoid compounds found in the lactic acid bacterium were true B_{12} or inactive corrrionid compounds, each fraction of the B_{12} extract and alkali-resistant factor from the bacterial cells was treated with silica gel 60 TLC and then B_{12} activity was determined by the microbiological B_{12} assay method. Fig. 1 shows silica gel 60 TLC patterns of B_{12} activity in the B_{12} extract and the alkali-resistant factor of the lactic acid bacterium, and authentic B_{12}. The B_{12} activity of the B_{12} extract was separated as four peaks with R_f values of 0.25, 0.62, 0.75, and 0.93. The B_{12}-active fractions with R_f of 0.62 and 0.93 were identical to the values for authentic B_{12} and alkali-resistant factor, respectively. The result indicates that the B_{12}-active fraction with R_f of 0.93 is derived from the alkali-resistant factor (probably deoxyribosides or deoxyribonucleotides).

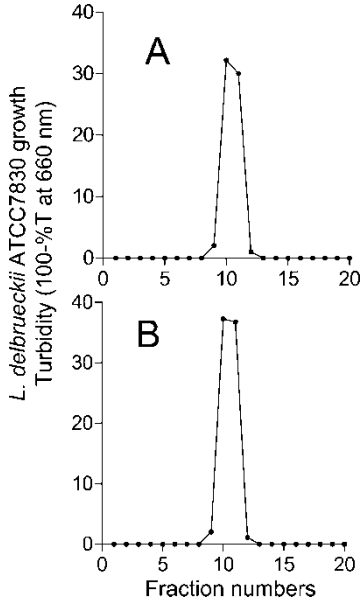

Figure 2. C_{18}-reversed-phase HPLC patterns of the main B_{12}-active fraction (with R_f 0.62) separated by TLC and authentic B_{12}. (A) authentic B_{12}, (B) main B_{12}-active fraction.

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The B_{12}-active fraction with R_f 0.62 on the silica gel TLC was further analyzed with C_{18} reversed-phase HPLC (Fig. 2). Retention time (9.5 min) of authentic B_{12} was identical to that of the fraction with R_f 0.62. These results suggest that the B_{12}-active compound with R_f of 0.62 is true B_{12}. Two minor B_{12}-active fractions with R_f 0.25 and 0.75 on silica gel TLC (Fig. 1) would be derived from unidentified corrinoid compounds.

To determine the physiological function of B_{12} in the lactic acid bacterium, some B_{12}-dependent enzyme activities were assayed. Considerably high activity of methionine synthase (1.5 nmol/min/mg protein) was found in a cell homogenate of the bacterium. Although, a trace ($<$0.01 nmol/min/mg protein) of ribonucleotide reductase activity was detected, methylmalonyl CoA mutase could not be detected.

Our results indicate that the lactic acid bacterium *T. halophilus* IAC-ks6 has the ability to accumulate B_{12}, which functions as each cofactor of methionine synthase and ribonucleotide reductase.

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