A Modified Form of a Vitamin B_{12} Compound Extracted from Whey Fermented by *Lactobacillus helveticus*

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ABSTRACT

The content of vitamin B_{12} in whey was reduced considerably during lactic acid fermentation, which was analogous to the lactic acid fermentation of milk. This apparent B_{12} decrease in whey was caused by B_{12} compounds that were unextractable by conventional extraction with potassium cyanide but that could be extracted by sonication and treatment by proteases such as pepsin and papain. Forms of the extracted B_{12} compounds were examined by bioautographies with *Escherichia coli* 215, coupled with cellulose acetate membrane electrophoresis or HPLC, and were identified as adenosylcobalamin, cyanocobalamin, and hydoroxocobalamin. A considerable amount of unidentified B_{12} was also detected, and this unidentified B_{12} compound seems to be the principal B_{12} compound that was unextractable from the cells. (**Key words**: vitamin B_{12}, lactic acid fermentation of whey, *Lactobacillus helveticus* B-1, bioautography of vitamin B_{12} compounds)

Abbreviation key: **Ado-B_{12}** = adenosylcobalamin, **CN-B_{12}** = cyanocobalamin, **HSO_3-B_{12}** = sulfitocobalamin, **OH-B_{12}** = hydoroxocobalamin, **KCN** = potassium cyanide.

INTRODUCTION

Milk and milk products are a relatively good source of vitamin B_{12}. Lactic acid fermentation of milk has been reported to decrease vitamin B_{12} content, but the cause of this decrease has not been examined in detail (1, 2, 4, 9). We also observed a similar, drastic decrease of vitamin B_{12} content in milk during lactic acid fermentation by *Lactobacillus helveticus* B-1, which is often used in commercial yogurts (7, 10). If vitamin B_{12} is present in fermented milk, it should normally be extractable by a conventional potassium cyanide (**KCN**) extraction method because B_{12} compounds are known to be extracted by this method (6, 8). The unextractability of vitamin B_{12} in fermented milk was partly evaluated in a previous paper (3). In that paper, the vitamin B_{12} content in fermented milk was partially recovered by ultrasonication and pepsin treatment, suggesting that the unextractability might be due to an interaction of vitamin B_{12} with proteins or other components of *L. helveticus* B-1. However, casein in milk interfered with analysis of the vitamin B_{12}, which greatly complicated the assessment of causes for the reduction of vitamin B_{12} during fermentation. As an alternative, whey that had been supplemented with vitamin B_{12} was fermented by using *L. helveticus* B-1 to avoid the inferference of casein.

The present paper reports that vitamin B_{12} concentration apparently decreased in fermented whey as it did in fermented milk, but could be recovered by extraction with KCN after the treatment of fermented whey with sonication and proteases. Analysis of the recovered vitamin B_{12} by bioautography using *Escherichia coli* 215, coupled with cellulose acetate membrane electrophoresis or HPLC, indicated the presence of cyanocobalamin (**CN-B_{12}**), hydroxocobalamin (**OH-B_{12}**), and adenosylcobalamin (**Ado-B_{12}**) and an unidentified form of B_{12}.

MATERIALS AND METHODS

Materials

Cyanocobalamin and pepsin (1:60,000) were purchased from Sigma Chemical Co. (St. Louis, MO), and papain (1:350) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cellulose acetate membrane (70 × 400 mm) was purchased from Sartorius AG (Goettingen, Germany) and was cut to 20 × 400 mm. Acidified whey was prepared from NDM (Snow Brand Milk Products Co., Ltd., Sapporo, Japan). The NDM was reconstituted in distilled water to a concentration of 10% (wt/vol), and pH was adjusted to 4.6 with 85% phosphoric acid for isoelectric precipitation of casein. After refrigeration (ca. 4°C) overnight, the milk was centrifuged at 12,000 × *g* for 20 min. The pH of the supernatant was then adjusted to 6.8 with 0.5*N* NaOH and KOH. The

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supernatant was centrifuged again at 12,000 × *g* for 30 min, and the supernatant was used as whey.

Preparation of Fermented Whey

The whey was fermented with *L. helveticus* B-1 (obtained from the Japan Dairy Technical Association, Tokyo, Japan) using a method that was similar to that described previously for fermented milk (3, 7, 10). To each 10 ml of whey in test tubes was added 500 ng of vitamin B_{12}, and extraction and identification of vitamin B_{12} compounds were carried out on a 10-fold scale in an Erlenmeyer flask to obtain enough vitamin B_{12} compounds for the identification experiment. The whey that had been supplemented with vitamin B_{12} was pasteurized at 82 to 83°C for 30 min, and, after cooling, the whey was inoculated with 0.1 ml of *L. helveticus* B-1 cultured in pasteurized skim milk (10% reconstituted NDM). The culture was incubated for the indicated times at 37°C. A control sample was prepared by addition of 0.1 ml of lactic acid instead of fermentation by *L. helveticus* B-1.

Assay of Vitamin B_{12} Content

Vitamin B_{12} was extracted by the conventional KCN method, and the vitamin B_{12} content was determined with *Escherichia coli* 215 (5, 6, 8). For extraction of vitamin B_{12}, 9.0 ml of 0.1 *M* acetate buffer (pH 4.5) was added to each 1.0 ml of culture. After the addition of KCN [1 μg/1 ng (estimated) of vitamin B_{12}], vitamin B_{12} compounds were extracted by boiling for 20 min. The volume was adjusted to 100 ml, and the extract was centrifuged at 12,000 × *g* for 20 min to remove cell materials. The contents of vitamin B_{12} in the supernatant fluid were assayed with *E. coli* 215. To increase its extractability, fermented whey was sonicated and treated with proteases (3). For the pepsin treatment, the pH of fermented whey was adjusted to 2.0 with lactic acid. For sonication, the sample was treated three times with a sonic oscillator (output: 40W, Tomy UR-200, Tokyo, Japan) for 3 min, and samples were cooled on ice for 1 min after each irradiation; the pH of samples was adjusted to 2.0. A 0.5 ml of pepsin solution (40 mg/ml) or inactivated pepsin solution, which had been heated at 120°C for 10 min, was added to the samples. For the control, 0.5 ml of distilled water was added instead of the enzyme solution. For the papain treatment, pH was adjusted to ca. 6.0 with 1*N* NaOH. The pH of the sonicated sample was also adjusted to ca. 6.0 after sonication. The procedures with papain (40 mg/ml) were performed in a manner similar to those with pepsin. Treatments with pepsin and papain were carried out for 2 h at 37°C. The pH values of the treated samples were adjusted to 4.5 to 5.0, and vitamin B_{12} was extracted by the KCN method. The vitamin B_{12} content was determined with *E. coli* 215 (6).

Extraction of Vitamin B_{12} from *L. helveticus* B-1

To examine the forms of vitamin B_{12} in *L. helveticus* B-1, 100 ml of fermented whey were cultured for 20 h and then were centrifuged at 12,000 × *g* for 20 min in the dark; subsequent procedures were performed under darkened conditions. The collected cells were suspended in 20 ml of 0.1 *M* potassium phosphate buffer (pH 7.5) and sonicated for a total of 10 min at intervals of 2 min of sonication followed by a pause of 1 min. Papain (400 mg) that had been dissolved in 2 ml of 0.1*M* potassium phosphate buffer (pH 7.5) was added, and the mixture was incubated for 2 h at 37°C. Then, 80 ml of ethanol were added, and vitamin B_{12} compounds were extracted by heat treatment at 90°C for 20 min (5, 6, 7). The ethanol extract of vitamin B_{12} was centrifuged at 12,000 × *g* for 20 min. The supernatant was evaporated to dryness, and 10 ml of distilled water were added to it. The resultant vitamin B_{12} solution was applied to an Amberlite XAD-2 (Rohm and Haas Co., Philadelphia) column (2.0 × 7.5 cm), which had been prewashed with 50 ml of 70% ethanol and then 100 ml of distilled water. The column was washed with 100 ml of distilled water, and vitamin B_{12} compounds were eluted with 50 ml of 50% ethanol. The eluate was evaporated to dryness, and 0.15 ml of distilled water was added to the eluate for bioautography (6).

Bioautography Coupled with Cellulose Acetate Membrane Electrophoresis or HPLC

Bioautography, coupled with cellulose acetate membrane electrophoresis or HPLC and microbiological assay, was used to investigate the forms of B_{12} compounds. For electrophoresis with cellulose acetate membrane, the sample obtained from the B_{12} supplemented fermented whey (2 μl of ca. 10 μg/ml) and authentic vitamin B_{12} compounds (each 2 μl of ca. 2 mg/ml solution) were applied on the center (origin) of the membrane. After electrophoresis, the position of authentic B_{12} was detected by the reddish color, and, for the detection of vitamin B_{12} in the sample, the membrane was cut at 0.5-cm intervals from origin toward both sides. Vitamin B_{12} activity of each 0.5-cm membrane strip was assayed microbiologically; strips were placed in test tubes with the assay

TABLE 1. Effect of sonication and protease treatments in vitamin B_{12} content of fermented whey.

Whey[1]	Treatment	Vitamin B_{12} content			
		Pepsin[2]		Papain[2]	
		(ng/ml)	(%)[3]	(ng/ml)	(%)[3]
Unfermented (lactic acid)	None	51.7	100	52.1	100
Fermented	None	15.6	30	15.8	30
Fermented	Protease	23.1	45	23.1	44
Fermented	Heated protease	15.5	30	16.1	31
Fermented	Sonication and protease	32.6	63	35.7	69
Fermented	Sonication and heated protease	26.2	51	24.7	47

[1]Incubation was at 37°C for 24 h.

[2]Pepsin or papain was the protease used.

[3]Percentages of recovery of vitamin B_{12}.

medium (6). The HPLC was performed with a system (JASCO, Tokyo, Japan) consisting of a degasser (model DG-980-50), a ternary gradient unit (model LG-980-02), a pump (model 880-PU), a detector (model 970-UV/VIS), and an integrator (model 807-IT). A CrestPak C18S column (5 μm), 4.6 × 150 mm (JASCO) was used, and two different isocratic solvent systems were used for elution of vitamin B_{12} compounds. Injection volume was 4 μl for the sample (ca. 10 μg/ml, estimated microbiologically) and 1 μl for each authentic vitamin B_{12} sample (ca 0.5 mg/ml). The first eluting solvent consisted of 20% methanol in 0.05 *M* acetate buffer (pH 4.0), and the next was 35% methanol in the same buffer. The flow rate was 1 ml/min. The effluents from the detector were collected in 1.0-ml fractions and the vitamin B_{12} activity of each fraction was assayed microbiologically.

RESULTS AND DISCUSSION

Change of Vitamin B_{12} Content During Whey Fermentation

Previous research (3) suggested that the concentration of vitamin B_{12} in whey (1.5 ng/ml) was not sufficient to permit identification of the vitamin B_{12} compounds after fermentation with *L. helveticus*. Consequently, whey was fortified by adding the equivalent of 50 ng/ml of vitamin B_{12}. As shown in Figure 1, the vitamin B_{12} content, as determined by the traditional assay, of the supplemented whey decreased considerably from ca. 55 to 15 ng/ml (27% of original vitamin B_{12} content) during fermentation with *L. helveticus* B-1. This decrease was similar to that of fermented milk (3).

The decrease of vitamin B_{12} content in fermented whey or fermented milk seems to be caused only by specific strains of lactic acid bacteria such as *L. helveticus* B-1. No reduction of vitamin B_{12} content was observed during fermentation with *Streptococcus thermophilus* (10) or *Lactobacillus casei* (data are not shown), indicating that vitamin B_{12} could be extracted from those cultures by the conventional KCN extraction method.

Effect of Treatment Such as Proteases Digestion on Vitamin B_{12} Content

Attempts were made to recover vitamin B_{12} from fermented whey, and the results are shown in Table

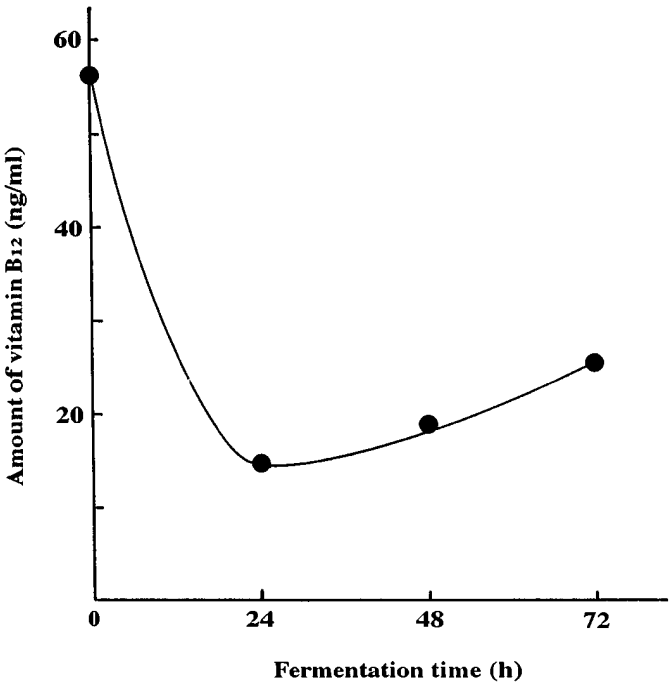

Figure 1. The vitamin B_{12} content during fermentation of whey at 37°C with *Lactobacillus helveticus* B-1.

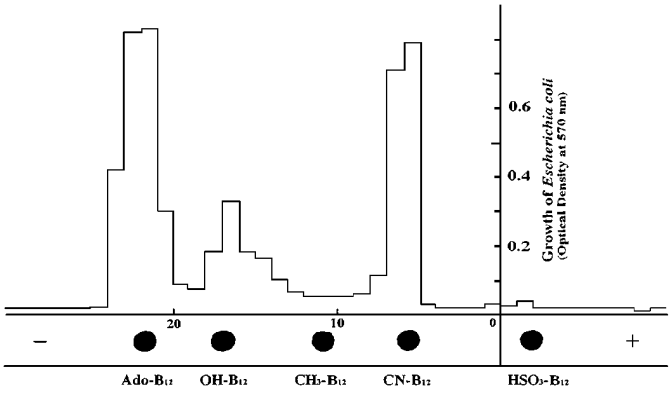

Figure 2. Cellulose acetate membrane electrophoresis bioautography of vitamin B_{12} compounds that were extracted from fermented whey after sonication and papain treatment. Electrophoresis was carried out at 0.5*N* acetic acid for 2 h. Authentic vitamin B_{12} compounds used were adenosylcobalamin (Ado-B_{12}), hydroxocobalamin (OH-B_{12}), methylcobalamin (CH_3-B_{12}), cyanocobalamin (CN-B_{12}), and sulfitocobalamin (HSO_3-B_{12}); compound positions are shown by the closed circles. The positions of vitamin B_{12} activities of the sample were detected by microbiological assay with *Escherichia coli* as described in the Materials and Methods.

1. All of the supplemented vitamin B_{12} (51.7 or 52.1 ng/ml) was recovered from the control whey acidified with lactic acid. The vitamin B_{12} content of fermented whey was lowered by 70% to 15.6 or 15.8 ng/ml. Treatment of fermented whey with pepsin or papain increased the recovery of vitamin B_{12} to about 45% (23.1 ng/ml), but no increase was obtained with inactivated protease. Sonication followed by protease treatment caused a maximum recovery of vitamin B_{12} of 63% (32.6 ng/ml) with pepsin and 69% (35.5 ng/ml) with papain.

Forms of Vitamin B_{12} Compounds Extracted from Fermented Whey

For characterization of vitamin B_{12} compounds, treatment with papain was used rather than pepsin because neutral pH would be more suitable for the separation and identification of the forms of vitamin B_{12}. Samples that were obtained by vitamin B_{12} extraction and partial purification were examined by cellulose acetate membrane electrophoresis bioautography. Figure 2 shows the results of bioautography of segments of the membrane after electrophoresis with 0.5*N* acetic acid. In this bioautography, vitamin B_{12} activity with *E. coli* 215 was detected in the vicinity of positions equivalent to the migrations of Ado-B_{12}, OH-B_{12}, and CN-B_{12}. Trace amounts of sulfitocobalamin (HSO_3-B_{12}) also seemed to be present.

Results of HPLC bioautography are shown in Figure 3. In addition to the vitamin B_{12} compounds detected in cellulose acetate membrane electrophoresis bioautography, a new unidentified vitamin B_{12} compound was eluted at 42 to 43 min just prior to Ado-B_{12}. In cellulose acetate membrane electrophoresis (Figure 2), this unidentified vitamin B_{12} seems to have co-migrated with the known vitamin B_{12} compounds such as OH-B_{12}, but further investigation is necessary to confirm this. Results of bioautography with *L. delbrueckii* ATCC 7830, another test microorganism of microbiological assay of vitamin B_{12}, were very similar to those with *E. coli*.

The unidentified vitamin B_{12} compound appears to be extractable after disruption of the cells by sonication and papain treatment. The identification of this unknown form of vitamin B_{12} and the mode of presence in the cell are under investigation.

CONCLUSIONS

Vitamin B_{12} content is decreased considerably during fermentation of whey with *L. helveticus* B-1. The decrease apparently resulted from incorporation of vitamin B_{12} into the bacterial cells, because most of the vitamin in the cells could be extracted by sonication and papain treatment. Forms of the extracted vitamin B_{12} compounds were Ado-B_{12}, OH-B_{12}, and an unidentified B_{12}. Both OH-B_{12} and the unidentified form were newly detected compounds obtained by degradation of cells and cellular proteins. The unidentified B_{12} compound appeared to be one of the major vitamin B_{12} compounds extracted.

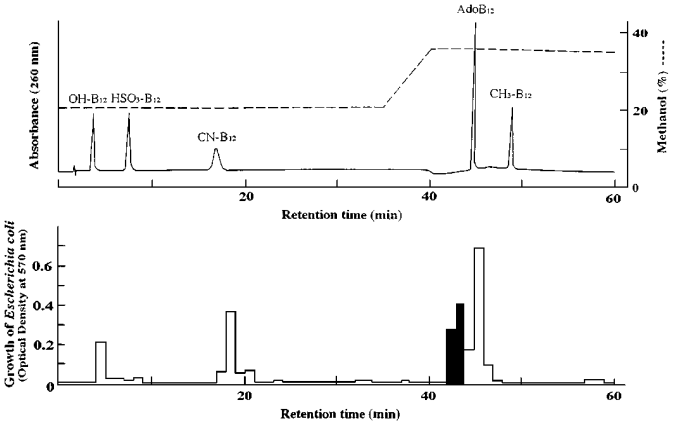

Figure 3. The HPLC bioautography of vitamin B_{12} compounds extracted from fermented whey after sonication and papain treatment. Authentic vitamin B_{12} compounds that are the same as those described in the legend of Figure 2 were detected by measuring UV absorption at 260 nm. The vitamin B_{12} activity of the effluents from the detector in HPLC was assayed microbiologically. The position of the unidentified vitamin B_{12} compound (retention time 42 to 43 min) is marked with black.

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