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A Modification of A Microbiological Assay of Vitamin B_{12} In Fermented Vegetables

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ABSTRACT

A modification of a microbiological assay of vitamin B_{12} was made and used to determine the vitamin levels during kimchi fermentation. A cyanide-buffer solution of pH 6.0 replaced the metabisufite-buffer specified in the A.O.A.C. method. The vitamin B_{12} activity was decreased by blending kimchi samples for 5 minutes and retained the activity by steaming for 10 minutes before blending. The alkali hydrolysis of kimchi at pH 12.0 for 30 minutes at 121°C was sufficient to destroy the vitamin B_{12} and permit the detection of analogs with the same assay organism. Vitamin B_{12} reached a maximum of 47 ng/100 g during the fermentation of kimchi 15 4°C. Inoculation of the kimchi with *Propionibacterium shermanii* (ATCC 13673) increased the vitamin production to a maximum of 102 ng/100 g at 1 week of fermentation. Soy flour (0.5%) or beef extract (0.05%), which were regarded as protein sources, added to the inoculated kimchi further increased the vitamin B_{12} activity to 197 and 203 ng/100 g.

INTRODUCTION

Vitamin B_{12} (cyanocobalamin), the antipernicious anemia factor, is known to be a dietary requirement for the growth of humans, a variety of animals, and microorganisms. Since the vitamin was first isolated in crystalline form from liver in 1948 by Rickes et al.[1] and by Smith[2], it has been found in most animal tissues and muscles, milk, and eggs. It appears to be absent from most of the higher plants, although some plant roots show small amounts which may derive from contamination with microorganisms in the soil[3]. The vitamin is not synthesized by animals, but it is produced by bacterial or fungal fermentation within the ruminants' own digestive tract and then absorbed. Humans are unable to utilize the intestinally synthesized vitamin[4].

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The extraordinarily complex structure of the vitamin was determined in 1955 by Hodgkin et al.[5]. It was found to have a molecular weight of 1355, being the largest vitamin yet discovered. The vitamin has been quantitated by measurement of growth of chicks and rats [6][7]. The vitamin also promoted the growth of microbiological test organisms such as *Lactobacillus lactis* Dorner[8], *Lactobacillus leichmannii* [9] and *Escherichia coli* [10], and algae such as *Euglena gracilis* [11] and *Ochramonas malhamensis*[12].

Estimation of vitamin B_{12} in foods and food supplements can also be done by colorimetric methods[13] [14], isotope and isotope dilution methods[15], and chemical methods[16] [17]. Thus several types of assays have been developed for assessing vitamin B_{12} activity, each method has specific advantages and well as some limitations.

Difficulties have been observed with each and the extraordinarily small amount of this vitamin found in

foods is one of the problems in the assay. The extraction step in the assay procedure may also be a source of problem. Most of the noncyanocobalamins (coenzyme forms of cyanocobalamin) present in natural substances are less stable than cyanocobalamin and are readily destroyed by steaming or heating during the microbiological assay.

Lactobacillus leichmannii and *Ochramonas malhamensis* have been widely used as the most appropriate organisms for the assay of vitamin B_{12} in various complex products. Lichtenstein *et al*[18]. reported that the *L. leichmannii* assay is better suited to routine practice and the assay has been more widely accepted because of its rapidity, uniformity of response, and precision of results.

Farquharson and Adams[19] have found five forms of vitamin B_{12} in foods: adenosylcobalamin, hydroxocobalamin, methylcobalamin, cyanocobalamin, and sulphitocobalamin. Adenosylcobalamin and hydroxocobalamin were predominant. A number of other cobalt-containing compounds have been shown to be present in natural materials and to differ from vitamin B_{12} in lacking the 5,6-dimethylbenzimidazole group. These compounds are characteristically inactive for animals but active biologically in microbiological assays.

In this study, an attempt was made to modify the vitamin B_{12} extracting procedure in the microbiological assay method with *Lactobacillus leichmannii* as the test organism

EXPERIMENTAL PROCEDURES

PREPARATION OF KIMCHI SAMPLES: A typical winter kimchi was made and studied in this experiment, as it is the major fermented vegetables in Korea and contains small amounts of vitamin B_{12} from microbial production. The ingredients and their proportions are shown in Table 1. Soy flour (0.5% hammer milled) or beef extract (0.05%, BBL, Division of BioOuest) was added as the protein supplements to increase the vitamin production in some experimental samples. The salted and stuffed cabbages were packed tightly into a 2-gal glass jar and pressed with a weight (a bottle filled with water) to exclude air. The jar was covered and kept in a refrigerator at 4°C for a 10-wk fermentation period. Vitamin B_{12} was assayed at 0 and 4 days; 1,2,3,4,5, and 10 wk of fermentation. During the fermentation of kimchi, pH of the juice was measured on each sample. Total acidity also was determined by titration with 0.0990 N NaOH solution and expressed in percent as lactic acid.

INOCULATION WITH VITAMIN B_{12}-PRODUCING ORGANISM: To determine the possibility of increasing the production of vitamin B_{12} during the kimchi fermentation, *Propionibacterium shermanii* was chosen for this study as an inoculum at the beginning of the fermentation. A culture of *P. shermanii* (ATCC No. 13673) in dehydrated form was rehydrated with 10 ml of agar stabs for stock cultures grown at the same temperature. For inoculation of the organism into a kimchi sample, the stab culture was again transferred into the broth, grown at 30°C for 48 hr, and a dilution to give 10^6 cells (according to optical density measurements standardized with cell counts) was inoculated per g of the sample at the time the kimchi was made.

DETERMINATION OF VITAMIN B_{12}: Vitamin B_{12} produced in kimchi fermentation was determined by a microbiological assay method with *Lactobacillus leichmannii* as the test organism. The basic assay procedure followed the Official Methods of Analysis of A.O.A.C.[20]. and the buffer extracting solution was substituted to modify the procedure as described below.

TEST ORGANISM: *Lactobacillus leichmannii* (ATCC No. 7830) was obtained from the American Type Culture Collection (ATCC) and transferred into culture medium (Difco Lactobacilli broth for AOAC microbiological assays). The organism was grown at 37 ± 0.5°C for 24 hr and stored in a refrigerator at 7°C after having grown for 18 - 20 hr. Before using the new culture in the assay, successive daily transfer of the stab culbure were made in a 2-week period at the incubation temperature of 37 ± 0.5°C for 18 - 30 hr.

STANDARD CONCENTRATION – RESPONSE CURVE: To read %T of each assay tube, a Spectronic 20 was used and the photometer was calibrated as mg dried cell weight per tube. Using the inoculum of *L. leichmannii* grown in sterile suspension medium with

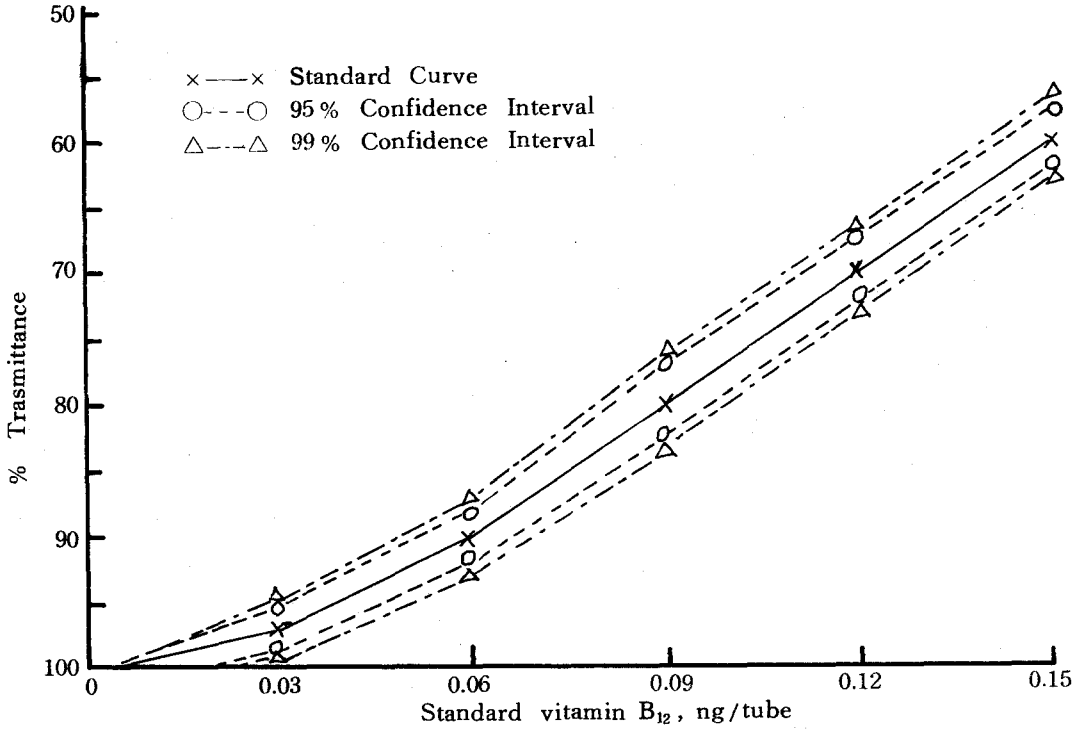

Fig. 1. Standard curve based on values of 20 replications.

standard cyanocobalamin (100 ng/300 ml), a standard concentration-response curve was drawn relating %T at 660 nm to mg drid cell weight (Figure 1),. This curve was prepared for each assay and the growth of the test organism in the assay tube was read in percent transmittance (%T) to determine the vitamin B_{12} activity from the standard curve. It was found in the preliminary tests that the most definitive standard curve was obtained at 660 nm.

MEDIA AND BUFFER SOLUTION: Basal medium stock solution was prepared from vitamin B_{12} assay medium U.S.P.(Difco, Detroit, Michigan) and used as the basal medium for the assay. Suspension media were prepared for stock cultures. Lactobacilli broth A.O.A.C. (Difco, Detroit, Michgan) was used to make liquid culture medium and agar culture medium with the addition of 1.5% agar. Basal medium diluted with an equal volume of water was prepared to wash the culture. All of the prepared media were cooled rapidly in a cold water bath to prevent color formation after sterilization and stored in the dark at 7°C.

Metabisulfite-buffer solution was recommended as the vitamin B_{12}-extracting solution in the A.O.A.C. method. However, preliminary studies showed tht kimchi samples extracted with metabisulfite-buffer solution. Cyanide-buffer solution was made and used to extract vitamin B_{12} from the fermented vegetables, resulting in higher values of the vitamin activity as compared to those obtained from samples extracted with metabisulfite buffer or water. The cyanide-buffer solution was formulated with 3.25 g Na_2HPO_4 (dibasic sodium phosphate), 1.5 g citric acid•H_2O and 10 mg KCN per 250 ml.

ASSAY PROCEDURES: Assay tubes and other necessary glassware were meticulously washed with sodium lauryl sulfate, and carefully rinsed with distilled water. All glassware and tubes were then sterilized by dry heat for 2 hr at 250°C. The assay procedure fol-

Table 1. Proportions of kimchi ingredients

Ingredients	Distribution % by weight
Korean cabbage (Chinese cabbage)	81
Radish roots (white)	9
Salt (pickling salt)	3.5
Green onion	2.7
Salted shrimp	1.8
Red pepper powder (dried)	0.8
Garlic (fresh)	0.7
Ginger (fresh)	0.5

lowed the principles of the AOAC method except for the extracting procedure modified as described above.

CORRECTION FOR VITAMIN B_{12}-ANALOGS: Pseudovitamin B_{12} ←and deoxyribosides were determined, in the preliminary studies, to be present in large quantities in kimchi. Hydrolysis of food extracts at pH 12.0 for 30 min at 121°C was used to destroy vitamin B_{12} ←[21]. The microbiological activity remaining after this hydrolysis was considered to be growth factors for the assay organism that are not cobalamins. Therefore, the difference in the results before and after the alkali treatment is the activity of vitamin B_{12} assayed in the samples. The non-vitamin B_{12} activity was determined and used as a correction factor in this study with kimchi.

RESULTS AND DISCUSSION

Metabisulfite which was recommended as the vitamin B_{12}-extracting buffer in the A.O.A.C. method inhibited the growth of the test organism. The fermented vegetables extracted with water had higher values of vitamin B_{12} activity than the samples extracted with metabisulfite-buffer solution (Table 2). The vitamin in kimchi may be in the free form rather than bound. Increased destruction of vitamin B_{12} by addition of ascorbic acid was observed in kimchi samples extracted with water or metabisulfite-buffer solution(Table 3). The addition of iron in levels of 12 and 17 ppm with the same extracting solutions showed a little protective effect for vitamin B_{12} (Table 3 and 4). It has been noted that cyanocobalamin is decomposed in the presence of ascorbic acid unless a suitable stabilizer, such as a trace amount of iron is added [22)23)]. Bartilucci and Foss[24)] and Skeggs[22)] recommended the addition of equal volumes of propylene glycol and glycerine to protect vitamin B_{12} from the destruction during its assay. However, as shown in Table 3, the mixed solution did not exhibit any protective effect in kimchi samples.

A cyanide buffer solution at pH 6.0[25)] replaced the metabisulfite-buffer solution to extract vitamin B_{12} in the samples (Table 4). added vitamin B_{12} (1-2 ng/ml) was fully recovered from samples extracted with the

Table 2. Effects of buffer solutions and heat treatments on vitamin B_{12} extraction of kimchi

Solution	Heat treatment	Replication 1 Total ng/100g	Replication 2 Total ng/100g	Replication 3 Total ng/100g	Replication 3 B_{12} analog ng/100g	Replication 3 B_{12} ng/100g
H_2O	15 min steaming	245	368	366	344	22
H_2O	10 min steaming	235	350	322	323	19
S_2O_5 – solution	15 min steaming	280	310	341	203	18
S_2O_5 – solution	10 min autoclaving[a]	235	296	254	449	5
CN– solution	15 min steaming	239	361	NT[b]	NT[b]	NT[b]
CN– solution	10 min autoclaving	243	383	378	334	44

[a] Autoclaving was at 121°C.
[b] Not tested.

Table 3. Effect of added ascorbic acid and iron on vitamin B_{12} assay in kimchi

Extraction solution	pH of kimchi	Added ascorbic acid (mg/g)	Protector added	Vitamin B_{12} level found (%)[a]
Water	4.40	0.13	—	87
Water	4.60	0.25	—	89
Water	4.60	0.50	—	83
Water	4.60	—	Fe^{++}[b], 10 ppm	100
Water	4.40	0.13	Fe^{++}[b], 12 ppm	101
Water	4.40	0.13	Fe^{++}[b], 17 ppm	104
Water	4.35	0.25	Fe^{++}[b], 17 ppm	103
Water	4.40	—	Pg-gly[c], 0.1 ml/ml	76
Water	4.35	—	Pg-gly[c], 0.2 ml/ml	69
Metabisulfite-buffer (10^{-1} dil.)	4.35	0.13	—	77
Metabisulfite-buffer (10^{-1} dil.)	4.35	0.25	—	71
Metabisulfite-buffer (10^{-1} dil.)	4.35	0.50	—	66

[a]Vitamin B_{12} content of control sample with no additional ascorbic acid or protective agents was used as 100% base.
[b]$FeSO_4$ was used.
[c]Mixture of equat volumes of propylene glycol and glycerin.

Table 4. Recovery of vitamin B_{12} added to kimchi samples

Extraction solution	pH of kimchi	Vitamin B_{12} added ng/g	Ascorbic acid added mg/g	Recovery of added vitamin B_{12} %
Water	5.55	2	0.13	68
Water	5.55	2	0.25	36
Water	5.55	2	0.25 + Fe^{++}[a], 17 ppm	56
Cyanide-buffer	5.55	2	—	103
Cyanide-buffer	4.49	1	—	110

[a]$FeSO_4$ was used.

buffer solution. Cyanide-buffer solution have been recommended to convert the less stable noncyanocobalamins to the more stable cyanocobalamin[25)26)] Wuest and Perlman[27)] noted that cyanide solution is also active in releasing vitamin B_{12} retained in the cell materials of the organism. It was also found, in this experiment, that the buffer solution resulted in higher values of vitamin B_{12} assay from kimchi samples and showed no inhibitory effect on the growth of the test organism.

Blending kimchi samples for 5 min with an Osterizer

Table 5. Effects of blending of blending and steaming before blending of kimchi on vitamin B_{12} content

Sample	Treatment	Total ng/100g	Analogs[a] ng/100g	B_{12} ng/100g
Kimchi juice	None(not blended)	383	338	45
Kimchi juice	Blended	354	324	30
Kimchi juice	Steamed and blended	375	334	41
Kimchi juice + solids	Blended	356	335	21
Kimchi juice + solids	Steamed and blended	389	351	38

[a]Values obtained after hydrolysis of the kimchi extracts at pH 12.0 for 30 min at 121°C.

Table 6. Content of vitamin B_{12} and its analogs in kimchi fermented at 4°C

Fermentation time	Replication	Control kimchi, ng/100g			Inoculated kimchi, ng/100g		
		Total	Analog	B_{12}	Total	Analog	B_{12}
0 day	1	196	189	7	240	211	29
	2	253	241	12	274	249	25
	3	247	230	17	275	230	45
	Ave.	232	220	12	263	230	33
1 week	1	371	332	59	401	288	113
	2	423	390	33	456	366	90
	3	373	323	50	421	318	103
	Ave.	389	342	47	426	324	102
10 week	1	310	296	14	334	301	33
	2	336	324	12	344	305	39
	3	308	282	26	331	281	50
	Ave.	318	301	17	336	295	41

blender destroyed vitamin B_{12}. However, steaming the kimchi for 10 min before blending (endpoint temperature of 85°C) decreased this loss (Table 5). The 10-min steaming apparently destroyed the oxidases. From the results of hydrolysis of kimchi at pH 12.0 for 30 min at 121°C, vitamin B_{12} analogs, which would be pseudovitamin B_{12} and deoxyribosides, were found in large quantities in the samples (Table 6). The alkali-treatment of kimchi samples was sufficient to destroy the vitamin B_{12} and permit the detection of analogs with the same assay. In laboratory tests with added vitamin B_{12}, none of the production of hydrolysis of the vitamin served to stimulate growth of the assay organism.

Figure 2 shows the production of vitamin B_{12} in kimchi fermented at 4°C. Vitamin B_{12} was found at a maximum concentration of 47 ng/100g in control kimchi, which had not been inoculated with *Propioni bacterium shermanii*. Inoculation of kimchi samples

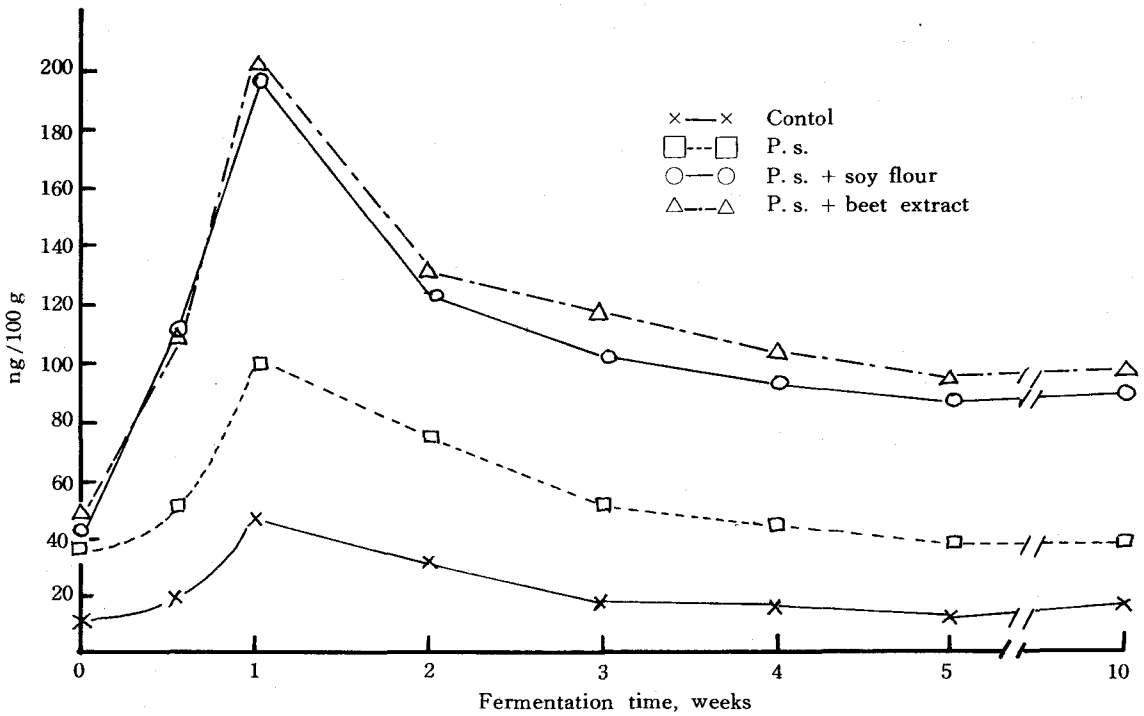

Fig. 2. Content of vitamin B_{12} in control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C.

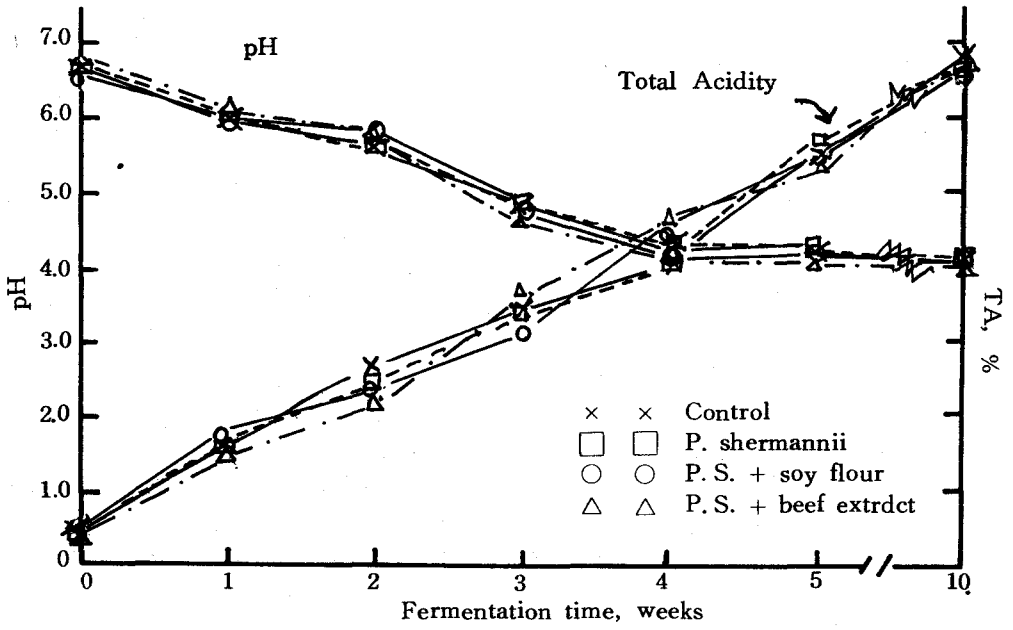

Fig. 3. The pH and total acidity of control and inoculated kimchi, with or without soy flour or beef extract, and fermented at 4°C.

with *Propionibacterium* increased the vitamin production to 102 ng/100 g. The addition of soy flour (0.5%) or beef extract (0.05%) further increased the vitamin content to 197 and 203 ng/100 g, respectively. Maximum production of vitamin B_{12} was achieved, in all samples, at 1 week of fermentation. After 3 weeks, the vitamin decreased gradually to the 5 weeks of fermentation; no further decrease was seen at 10 weeks of fermentation.

Figure 3 shows the pH measurements and total acidity of the samples. The pH of all samples decreased during the fermentation; control and inoculated kimchi with or without soy flour or beef extract showed no significant difference in values. In the total acidity, again no significant difference was observed between control and the treated samples. The pH and total acidity did not appear related to vitamin B_{12} production or destruction. Vitamin B_{12} should be stable at the pH of kimchi, since the pH was above 4.0 even after 10 weeks of fermentation.

SUMMARY

To determine vitamin B_{12} levels during kimchi fermentation, a microbiological assay method with *Lactobacillus leichmannii* (ATCC No. 7830) was modified. The assay procedure followed the principles of the Official Method of Analysis of A.O.A.C., 1975 except for the extracting procedure modified. A cyanide-buffer solution of pH 6.0 extracted vitamin B_{12} satisfactorily in kimchi samples and avoided the inhibitory effect on the growth of the test organism shown by the standard buffer. Added vitamin B_{12} (1 - 2 ng/ml) was fully recovered from samples extracted with the new buffer solution. This buffer replaced the metabisulfite-buffer specified in the Official Method. The loss of vitamin B_{12} activity by blending kimchi samples for 5 min was minimized by steaming for 10 min before blending (end-point temperature of 85°C). Pseudovitamin B_{12} and deoxyribosides which are not cobalamins but resemble them in structure and act as growth stimulating factors for the test organism were determined after hydrolysis at pH 12.0 for 30 min at 121°C. This factor was used to correct the total vitamin B_{12} activity.

To increase the production of vitamin B_{12} during kimchi fermentation, *Propionibacterium shermanii* (ATCC No. 13673) was inoculated on the basis of 1 x 10^6 cells per g of product at the beginning of fermentation at 4°C. Maximum levels of vitamin B_{12} in all samples were produced by 1 week of fermentation. Control kimchi samples which were not inoculated had a maximum vitamin B_{12} content of 47 ng/100 g. Kimchi inoculated with the *Propionibacterium* had an increased vitamin level, 102 ng/100 g. The addition of soy flour (0.5%) or beef extract (0.05%) to the inoculated kimchi increased the vitamin production to 197 and 203 ng/100g, respectively, more than four times that of the control kimchi.

Changes in pH and total acidity were not significantly defferent between the control and inoculated kimchi with or without soy flour or beef extract. The kimchi samples at the optimum fermentation period of 3 weeks had a pH of 4.5 - 4.8 and total acidity of 0.30 - 0.40% as lactic acid.

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