Quantitative Measurement of Tetrahydromenaquinone-9 in Cheese Fermented by Propionibacteria

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ABSTRACT

Propionibacteria produce tetrahydromenaquinone-9 [MK-9 (4H)] as a major menaquinone (vitamin K₂). This study aimed to determine the MK-9 (4H) concentration in commercial propionibacteria-fermented cheese. The MK-9 (4H) concentration was quantified using an HPLC instrument with a fluorescence detector after postcolumn reduction. Among the various cheese samples, the MK-9 (4H) concentration was highest in Norwegian Jarlsberg cheese, followed by Swiss Emmental cheese. In contrast, the MK-9 (4H) concentrations in Appenzeller or Gruyère cheeses were extremely low or undetected. Likewise, the concentrations in Comte and Raclette cheeses were lower than those in Jarlsberg and Emmental cheeses. In the present study, the MK-9 (4H) concentration in cheese showed a correlation with the viable propionibacterial cell count and propionate concentration. This implies that the increase in propionibacteria contributed to the generation of MK-9 (4H) in cheese. We presumed, based on these results, that Swiss Emmental and Norwegian Jarlsberg cheeses contain a meaningful amount of vitamin K because of their high MK-9 (4H) concentrations (200 to 650 ng/g). **Key words:** vitamin K, menaquinone, propionibacteria, cheese

INTRODUCTION

Vitamin K exists naturally in 2 forms, namely, vitamin K₁ (phylloquinone) in green plants and vitamin K₂ (menaquinone) in animals and bacteria. Vitamin K is an essential cofactor for the formation of γ -carboxyglutamic acid residues in proteins (Shearer, 1990). The γ -carboxyglutamic acid-containing proteins bind calcium ions and influence blood coagulation and tissue calcification. Vitamin K deficiency has been implicated in several clinical ailments such as intracranial hemorrhage in newborn infants (Purves, 2005) and possible

bone fracture resulting from osteoporosis (Iwamoto et al., 2004). To decrease the risk of these diseases, therefore, vitamin K food composition data are required. Further, to appropriately counsel patients taking the antithrombotic medicine warfarin, a vitamin K antagonist, accurate food composition data are of importance.

Early food composition data regarding vitamin K used chick bioassays that were more qualitative than quantitative. Recently, the development of reliable chromatographic procedures has resulted in greater sensitivity and accuracy of vitamin K measurements in foods (Koivu-Tikkanen et al., 2000; Schurgers and Vermeer, 2000). These quantitative analyses have revealed that vitamin K₂ occurs in foods in various molecular forms, such as menaquinone (MK)-4 and MK-7. Therefore, more reliable data are needed on the menaquinone contents in foods.

Numerous types of cheeses are made worldwide, and they are generally considered menaquinone-containing dairy products. Menaquinones are of microbial origin and are classified based on the number of isoprene units in their side chains. The components of menaquinones differ among various types of cheeses. For example, Koivu-Tikkanen et al. (2000) reported that the total amount of menaquinones was higher in Edam-type cheeses than in Emmental-type cheeses because of the higher MK-8 and MK-9 concentrations in the former. According to their study, Emmental cheeses contained substantial amounts of only MK-4 and phylloquinone, regardless of the aging time.

Here, we focused attention on the starter cultures of various cheeses. Swiss-type cheese is generally prepared with lactic acid bacteria and propionibacteria, whereas Edam-type cheese is usually prepared with only lactic acid bacteria. Morishita et al. (1999) indicated, based on the determination of menaquinones in lactic acid bacteria, that *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*, which are the starter cultures of Edam-type cheese, produce mainly MK-8 and MK-9. This information corresponds with the fact that Edam-type cheese contains large amounts of MK-8 and MK-9. On the other hand, propionibacteria have been reported to produce tetrahydromenaquinone-9 [MK-9

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Table 1. Cheese samples in this study

Sample ID	Cheese sample	Origin
A-1	Appenzeller	Swiss
A-2	Appenzeller	Swiss
C-1	Comte	France
C-2	Comte	France
E-1	Emmental	Swiss
E-2	Emmental	Swiss
G-1	Gruyère	Swiss
G-2	Gruyère	Swiss
J-1	Jarlsberg	Norway
R-1	Raclette	Swiss

(4H)] as a major menaquinone (Schwartz, 1973; Furuichi et al., 2006). Propionibacteria are particular to the Swiss-type cheese Emmental and to Gruyère, French Comte, and Norwegian Jarlsberg cheeses (Bouton et al., 1998; Kure and Skaar, 2000; Thierry et al., 2005). Therefore, measurement of the MK-9 (4H) concentration in these cheeses is of interest.

Recently, Furuichi et al. (2006) found that the *Propionibacterium freudenreichii* strain isolated from Swiss-type cheese produced a large amount of MK-9 (4H) in milk whey medium. They suggested that the menaquinone-producing ability of *P. freudenreichii* was equivalent to that of *Bacillus subtilis*, which is used for making natto, a Japanese fermented soybean food. However, to the best of our knowledge, no information is available on the MK-9 (4H) concentrations in various cheeses. This study was aimed at performing quantitative measurements of MK-9 (4H) concentrations in several Swiss-type cheeses. In addition, to examine whether a biomass of propionibacteria was associated with the MK-9 (4H) concentration, we measured the concentrations of short-chain fatty acids and the viable cell counts of lactic acid bacteria and propionibacteria in Swiss-type cheeses.

MATERIALS AND METHODS

Cheese Samples

Cheese samples were purchased in 2006 from several retail stores in Tokyo, Japan. Only propionibacteria-fermented cheese samples were selected. Table 1 shows the types of cheeses used in the study. The samples were vacuum-packed in plastic bags and stored at 4°C in the dark until analyzed.

Bacteria

Propionibacterium freudenreichii ET-3 was used in this study. The strain was isolated from a Swiss-type cheese and stocked in the Food Science Institute of Meiji Dairies, Odawara, Japan.

Chemicals and Reagents

Vitamin K₁ (phyloquinone), MK-4 standards, and all reagents used for vitamin K determination were of HPLC grade and were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Enumeration of Lactic Acid Bacteria and Propionibacteria

The cheese samples were diluted with saline (0.85% NaCl), and a series of 10-fold dilutions was prepared. The series were plated onto the following 3 selective agar plates: M17 (Difco, Becton Dickinson, Sparks, MD) with 0.5% (wt/wt) lactose and 1.5% (wt/wt) agar for streptococci, DeMan, Rogosa, Sharpe (MRS, Difco) for lactobacilli, and yeast-extract lactate (YEL; Britz and Riedel, 1994) for propionibacteria. The M17 and MRS agar plates were incubated aerobically at 37°C for 48 h. The YEL agar plates were incubated anaerobically at 30°C for 72 h. After incubation, the colonies were counted according to the morphology of each colony, and representatives were checked using the catalase reaction, gram reaction, and cellular morphology. Measurement was performed twice independently, and the results were averaged.

Assays of Short-Chain Fatty Acids

Short-chain fatty acids were determined with an HPLC system as described by Niwa et al. (2002). Ten microliters of each sample was injected into an HPLC system equipped with an electric conductivity detector (Shimadzu Co., Kyoto, Japan) after removing the proteins by treatment with Carrez-1 solution (53.5 g of ZnSO₄·7H₂O/100 mL of distilled water) and Carrez-2 solution (17.2 g of K₄[Fe(CN)₆]·3H₂O/100 mL of distilled water). The proteins in 4 mL of 10 diluted cheese samples were precipitated by adding 0.5 mL of Carrez-1 solution and 0.5 mL of Carrez-2 solution. Measurement was performed twice independently, and the results were averaged.

Preparation of the MK-9 (4H) Standard

Tetrahydromenaquinone-9 was extracted from *P. freudenreichii* ET-3 according to a procedure previously described by Furuichi et al. (2006). Crude extracts were placed on a silica gel column [Silica SG120 column, 20 × 250 mm (Shiseido, Tokyo, Japan); mobile phase, hexane and diethyl ether (100:0.8 vol/vol); temperature, 35°C; and flow rate, 9.9 mL/min]. The fraction containing MK-9 (4H) was separated by spectrophotometric monitoring at 254 nm. The putative MK-9 (4H) peak was manually collected into a test tube. The collected

fraction was evaporated and then redissolved in a small amount of acetone. This crude solution of MK-9 (4H) was subsequently purified on an octadecylsilica column [Capcell pack C₁₈ UG80 column, 20 × 250 mm (Shiseido); mobile phase, acetonitrile and isopropyl alcohol (1:1 vol/vol); temperature, 35°C; and flow rate, 9.9 mL/min]. Purified MK-9 (4H) was ascertained as a single spot on a thin-layer chromatography plate (silica gel 60 F254 plate; Merck, Darmstadt, Germany). In addition, the purity of the MK-9 (4H) sample was determined by HPLC with the octadecylsilica column [Capcell pack C₁₈ SG 120 column, 4.6 × 250 mm; mobile phase, methyl alcohol and ethyl alcohol (1:1 vol/vol); temperature, 40°C; and flow rate, 1.0 mL/min] and monitoring at 254 nm. The UV and mass spectra were measured with a Shimadzu UV-1600 spectrophotometer and a mass spectrometer (JMS-AM II; Japan Electron Optics, Tokyo, Japan), respectively.

Extraction of Vitamin K from Cheese

Vitamin K in the cheese was extracted according to the method of Kojima et al. (2004). Briefly, 2.5 g of cheese was homogenized with 45 mL of distilled water, followed by addition of 100 mL of phosphate buffer (pH 7.7) and 1.25 g of lipase. The sample solution was incubated at 37°C for 3 h while stirring with a magnetic stirrer. The hydrolysate was transferred to a 500-mL amber separatory funnel containing 2.5 mL of 10 N NaOH and 100 mL of 99.5% ethanol. Vitamin K was extracted with three 100-mL portions of *n*-hexane. The resultant hexane layer was collected and subsequently evaporated. The dried residue was dissolved in 10 mL of *n*-hexane and applied on a Sep-Pak silica cartridge (Nihon Waters Co. Ltd., Tokyo, Japan). The quinones were eluted with 15 mL of 8% (vol/vol) diethyl ether in *n*-hexane. The eluate was evaporated and redissolved by adding a small amount of acetone. A small aliquot of this solution was filtered and used for vitamin K analysis with HPLC. The measurement was performed twice independently.

Analytical HPLC of MK-9 (4H)

Vitamin K was determined with an HPLC system (Kojima et al., 2004). The HPLC instrument was equipped with a postcolumn electrochemical reducer (Environmental Science Associates, Chelmsford, MA) and a fluorescence detector [FP-1520S (Jasco Co., Tokyo, Japan); Capcell pack C₁₈ SG 120 column, 4.6 × 250 mm (Shiseido, Kyoto, Japan); mobile phase, methyl alcohol and ethyl alcohol (6:4 vol/vol), with NaClO₄ (7 g/L); temperature, 45°C; flow rate, 1.0 mL/min; and injection volume, 10 µL]. Detection was carried out at

an excitation wavelength of 240 nm and an emission wavelength of 430 nm. Vitamin K concentration was calculated by the external standard method. The repeatability of MK-9 (4H) measurement was determined in Norwegian Jarlsberg cheese. In the present study, the recovery of MK-9 (4H) was not determined; however, Kojima et al. (2004) reported that the average recovery of MK-9 from milk samples was approximately 85% or more.

Statistical Methods

A correlation analysis was performed to examine whether a biomass of propionibacteria was associated with MK-9 (4H) concentration. Spearman's rank correlation was used for the correlation analysis.

RESULTS

MK-9 (4H) Standard

We previously found that *P. freudenreichii* ET-3 produced a large amount of MK-9 (4H) (Furuichi et al., 2006); therefore, the MK-9 (4H) standard was purified from *P. freudenreichii*. The purified menaquinone sample showed a single spot on the thin-layer chromatography plate, and its UV spectrum showed 4 sharp absorption peaks at 243.0, 248.5, 260.5, and 269.5 nm. The mass spectrum of the purified sample indicated the molecular weight at *m/z* 788 and fragmented peaks at *m/z* 719, 651, 584, 516, 450, 380, and 227 (Figure 1). These spectra were almost the same as those for MK-9 (4H) previously reported by Schwartz (1973). We checked the purity of this sample by a conventional method with HPLC. The chromatogram of this sample indicated a single large peak whose area was more than 95% that of all the peaks on the chromatogram. Therefore, we used this sample as the standard for HPLC analysis.

Bacterial Counts and Short-Chain Fatty Acid Concentrations in Cheeses

Because MK-9 (4H) is produced by propionibacteria and not lactic acid bacteria, we assessed whether the cheeses used in this study were fermented by propionibacteria. Table 2 shows the bacterial counts in the tested cheeses. The viable propionibacterial cell counts were higher in the Swiss Emmental E-1 and Norwegian Jarlsberg J-1 samples than in the other cheese samples. Moreover, the E-1 and J-1 cheese samples contained a large amount of propionate (Table 3). In contrast, both propionibacteria cell counts and propionate concentrations in Swiss Appenzeller and Gruyère cheese samples were low. With regard to propionate concentration, the

Figure 1. Electron impact mass spectra and chemical structure of tetrahydromenaquinone-9.

values in the French Comte and Swiss Raclette cheese samples appeared to be moderate when compared with the values of all the other cheese samples. In the present study, the propionibacteria cell count and propionate concentration in the cheese samples exhibited a positive correlation ($r^2 = 0.60$, $P = 0.07$).

Lactic acid bacteria were detected in all the cheese samples. However, the viable cell counts of lactococci were very low in almost all samples except Appenzeller A-2 and Raclette. Therefore, it is probable that the lac-

tococci died and the cell counts largely decreased during cheese ripening and storage.

MK-9 (4H) Concentration in Cheeses

In the present study, the detection limit of MK-9 (4H) was 500 pg, and the coefficient of variation for the peak area of MK-9 (4H) in Jarlsberg cheese among triplicate analyses was 1.1%. Table 4 shows the concentrations of phyloquinone, MK-4, and MK-9 (4H) in the cheese samples. Among the cheese samples tested, Jarlsberg

Table 2. Bacterial counts in cheese

Sample ID	Viable cell counts (cfu/g)			
	Total count	Lactococci	Lactobacilli	Propionibacteria
A-1	1.8×10^6	3.0×10^5	1.3×10^6	1.5×10^5
A-2	5.3×10^7	5.2×10^7	1.4×10^5	1.3×10^6
C-1	4.1×10^6	$<10^3$	3.8×10^6	3.0×10^5
C-2	2.0×10^6	$<10^3$	9.0×10^5	1.1×10^6
E-1	9.4×10^7	1.5×10^4	6.9×10^7	2.5×10^7
E-2	2.6×10^6	$<10^3$	2.6×10^6	$<10^5$
G-1	7.8×10^7	4.0×10^3	7.8×10^7	4.0×10^5
G-2	3.0×10^6	$<10^3$	3.0×10^6	$<10^5$
J-1	7.5×10^7	4.5×10^4	3.2×10^5	7.5×10^7
R-1	7.8×10^8	5.7×10^8	2.1×10^8	2.0×10^5

Table 3. Short-chain fatty acids in cheese

Sample ID	Short-chain fatty acid (mmol/g)			
	Succinate	Lactate	Acetate	Propionate
A-1	3.7	42.2	24.6	4.0
A-2	0.8	48.0	11.0	6.5
C-1	5.1	139.2	29.6	23.7
C-2	19.1	62.8	43.3	45.2
E-1	7.1	27.2	59.5	89.7
E-2	2.4	61.2	49.3	31.8
G-1	1.4	113.5	12.3	11.2
G-2	0.8	113.5	10.0	1.0
J-1	7.1	0.5	53.4	106.3
R-1	5.8	24.5	29.0	24.7

Table 4. Concentrations of phyloquinone (VK₁), menaquinone-4 (MK-4), and tetrahydromenaquinone-9 [(MK-9 (4H))] in cheese

Sample ID	Vitamin K concentration (ng/g)			Total
	VK ₁	MK-4	MK-9 (4H)	
A-1	58	43	ND ¹	101
A-2	47	52	20	119
C-1	51	84	52	187
C-2	33	55	60	148
E-1	52	81	314	447
E-2	51	86	222	359
G-1	58	81	ND	139
G-2	33	96	ND	129
J-1	60	84	652	796
R-1	26	50	47	123

¹ND = not detected.

cheese contained the largest amount of MK-9 (4H) (652 ng/g). Moreover, the analytical HPLC chromatogram showed the existence of presumptive MK-8 and MK-9 peaks (Figure 2). We assumed, based on these results, that Jarlsberg cheese contained a large amount of vitamin K. In contrast, Emmental cheeses E-1 and E-2 contained a large amount of MK-9 (4H) and only small amounts of phyloquinone and MK-4 (Table 4), and the presumptive MK-8 and MK-9 peaks for these cheeses were not detected. In a previous investigation, MK-8 and MK-9 were not detected in Emmental-type cheeses (Koivu-Tikkanen et al., 2000). Therefore, it is probable

Figure 2. Analytical HPLC chromatogram of Norwegian Jarlsberg cheese. Peaks: 1, menaquinone (MK)-4; 2, phyloquinone; 3, presumptive MK-8; 4, presumptive MK-9; 5, tetrahydromenaquinone-9 [MK-9 (4H)].

that the major menaquinone in Emmental cheese is MK-9 (4H). In the present study, MK-9 (4H) concentrations were lower in the Comte and Raclette cheeses than in the Jarlsberg and Emmental cheeses. In the Appenzeller A-1 and Gruyère cheeses, MK-9 (4H) was not detected.

In the present study, the MK-9 (4H) concentration in the cheese demonstrated a positive correlation with the propionibacteria cell count and propionate concentration ($r = 0.53$, $P = 0.11$; $r = 0.94$, $P = 0.005$, respectively).

DISCUSSION

In the present study, we found large amounts of MK-9 (4H) in a few cheeses. Among the cheeses tested, the MK-9 (4H) concentration was higher in Swiss Emmental and Norwegian Jarlsberg cheeses than in the other cheeses (Table 4). Interestingly, MK-9 (4H) appears to be a major menaquinone in Jarlsberg and Emmental cheeses. Several studies on vitamin K determination in food have been carried out, and MK-8 and MK-9 have been considered the major vitamin K types in cheese (Koivu-Tikkanen et al., 2000; Schurgers and Vermeer, 2000). This is because the common starter *Lc. lactis* produces these menaquinones. Koivu-Tikkanen et al. (2000) reported that in Edam-type cheese, the concentration of vitamin K, which was almost the sum of MK-8 (~100 ng/g) and MK-9 (~300 ng/g), was 494 ng/g. According to their results, the vitamin K concentrations in Emmental samples ranged from 80 to 90 ng/g. In our study, the vitamin K concentrations, that is, the total concentrations of phyloquinone, MK-4, and MK-9 (4H), in the Emmental E-1 and E-2 cheese samples were 447 and 359 ng/g, respectively (Table 4). More surprisingly, the Jarlsberg cheese sample contained 60 ng/g of phyloquinone, 84 ng/g of MK-4, and 652 ng/g of MK-9 (4H) (Table 4); moreover, presumptive MK-8 and MK-9 peaks were observed on the chromatogram (Figure 2). It is probable, based on these results, that the vitamin K concentrations in these natural cheeses are currently underestimated.

In the present study, vitamin K concentrations in Emmental and Jarlsberg cheeses were moderate when compared with those in green vegetables (>1,000 ng/g of phyloquinone). However, according to a previous report (Schurgers and Vermeer, 2000), phyloquinone absorption from vegetables is very poor (5 to 15%), whereas menaquinone (MK-4, MK-7, MK-8, and MK-9) absorption from dairy products and the Japanese food natto is considerably better (probably almost complete). Consequently, we can presume that Emmental and Jarlsberg cheeses contain meaningful amounts of vitamin K because of their high MK-9 (4H) concentra-

tions. To our knowledge, the MK-9 (4H) concentration in cheese has not been reported previously; therefore, this paper is the first to describe the quantitative determination of MK-9 (4H) in cheese.

In this study, the MK-9 (4H) concentration in cheese showed a correlation with the viable propionibacterial cell count ($r = 0.53$, $P = 0.11$) and the propionate concentration ($r = 0.94$, $P = 0.005$). In fact, the MK-9 (4H) concentration in the Jarlsberg cheese sample, which showed the highest propionibacteria cell count and propionate concentration, was the highest among all the cheese samples. In contrast, both propionibacteria and MK-9 (4H) were not detected in Gruyère cheese G-2. Therefore, from these results it is evident that the increase in propionibacteria in cheese contributed to the generation of MK-9 (4H).

During cheese ripening and storage, the number of propionibacteria decreases with cell death and subsequent autolysis (Bouton et al., 1998; Treimo et al., 2006). Furuichi et al. (2006) suggested that *P. freudenreichii* ET-3 cells accumulate MK-9 (4H) during the growth period and release it after their lysis. Although cheese generally has a long expiration date, information about the stability of vitamin K in dairy products is far from complete. Accordingly, further studies are required to examine whether MK-9 (4H) is stable in cheese stored for a long time.

Previously, it was believed that vitamin K affected only blood coagulation (Shearer, 1995). A further spur to vitamin K research has been the discovery of a diverse group of vitamin K-dependent proteins that are unrelated to blood coagulation but implicated in calcium homeostasis (Kaneki et al., 2006). According to several articles (Iwamoto et al., 2004; Kaneki et al., 2006), vitamin K deficiency may be associated with low bone mineral density and increased risk of bone fracture. Therefore, vitamin K has emerged as a potential protector against osteoporosis. We presumed, based on the present study, that various types of natural cheeses may contain meaningful amounts of vitamin K because of the presence of menaquinones derived from the bacterial species grown in cheese. Moreover, dairy products, including cheese, generally contain abundant calcium. Therefore, we believe that the consumption of natural cheese would contribute to human bone health.

In conclusion, the results in this study suggest that the quantity of vitamin K in Swiss Emmental and Norwegian Jarlsberg cheeses is greater than that estimated conventionally, and that the main constituent of vita-

min K in cheese is MK-9 (4H). We believe that several natural cheeses may become candidate dairy products for the prevention of osteoporosis because of their abundant menaquinone and calcium contents.

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