Analysis method for determination of nisin A and nisin Z in cow milk by using liquid chromatography-tandem mass spectrometry

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ABSTRACT

Nisin, a polypeptide with antimicrobial properties, is known as a natural preservative. It is used in various foods, including dairy products. This study validated a novel procedure using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the determination of nisin A and nisin Z in cow milk. An extraction solution of 0.1 M acetate buffer containing 1 M NaCl (pH 2.0) and MeOH (1:1) was used to extract nisin A and nisin Z from milk samples. After the addition of extraction buffers, the samples were homogenized and centrifuged. The supernatant was filtered and injected for LC-MS/MS analysis. The linearity of the analytical method had a high correlation coefficient ($r \geq 0.9987$). The limits of quantitation of nisin A and nisin Z were approximately 12.9 and 10.9 $\mu\text{g/kg}$, respectively. The accuracy of the analytical method in milk ranged from 90.6 to 103.4% for nisin A and from 83.8 to 104.4% for nisin Z. The coefficient of variation values of intra- and interday in milk determined to be less than 5% in both nisin A and nisin Z. Because the proposed method has comparatively high recovery and low coefficient of variation, it seems appropriate for the determination of nisin A and nisin Z in milk samples. As the quantification of nisin A and nisin Z in milk samples by using LC-MS/MS has only been rarely reported until now, this study provides a meaningful technological advance for the dairy industry.

Key words: nisin A, nisin Z, liquid chromatography-tandem mass spectrometry, milk

INTRODUCTION

Nisin is produced by certain strains of *Lactococcus lactis* ssp. *lactis*. It is among the best-known and most-studied bacteriocins and antimicrobial polypeptides and is composed of 34 AA, with a molecular mass of 3.5 kDa (Hurst, 1981; Kaletta and Entian, 1989; Delves-

Broughton et al., 1996). Because nisin has antimicrobial activity against gram-positive spoilage and pathogenic bacteria such as *Listeria monocytogenes*, *Clostridium botulinum*, and *Staphylococcus aureus* (Falahee et al., 1990; Thomas et al., 2000), it is used as a preservative by food manufacturers to inhibit the growth of gram-positive pathogens related to food-borne illness (Sobrinho-López and Martín-Belloso, 2008).

In 1969, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives defined the identity and purity of commercial nisin, Nisaplin (WHO, 1969), which contains 2.5% nisin A. Nisin Z, which differs from nisin A only at position 27, where Asn replaces His (Reunanen, 2007), was identified as a natural nisin variant by Mulders et al. (1991). Since the reporting of nisin A and nisin Z, other nisin variants including Q, F, and U have been identified (Piper et al., 2011).

Nisin can be produced in a sterilized medium of nonfat milk solids or non-milk-based fermentation compounds, including yeast extract and carbohydrate solids (WHO, 2013). It is also naturally produced in cheese during fermentation (EU, 2008). Since the World Health Organization approved nisin as a preservative, it has been used in various products (Hurst, 1981; Delves-Broughton, 1990), including cheese, salad dressing, pudding, vegetables, and even beer (Delves-Broughton et al., 1996), in more than 40 countries. The standards for nisin use and the products in which it is used vary by country. In particular, nisin can be used in milk and dairy products in China (National Health Family Planning Commission of China, 2011), and the US Food and Drug Administration permits its use in dairy products in accordance with good manufacturing practice standards.

Researchers have reported a variety of analytical methods for nisin detection, including agar diffusion (Tramer and Fowler, 1964; Pongtharangkul and Demirci, 2004), ELISA (Falahee et al., 1990; Falahee and Adams, 1992; Daoudi et al., 2001), bioassay (Walstrom and Saris, 1992; Reunanen and Saris, 2003; Immonen and Karp, 2007), HPLC (Chan et al., 1989; Mulders et al., 1991; de Vos et al., 1993), liquid chromatography-

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mass spectrometry (Zendo et al., 2008; ISO, 2009), and liquid chromatography-tandem mass spectrometry (LC-MS/MS; ISO, 2009; Schneider et al., 2011; Fuselli et al., 2012). Agar diffusion techniques are the most widely used; however, they have many limitations, such as low sensitivity due to interfering substances in food extracts and long microbial culture times (Tramer and Fowler, 1964; Pongtharangkul and Demirci, 2004) as well as the formation of false inhibitory zones related to the low pH of samples (Wolf and Gibbons, 1996). The ELISA methods are more sensitive than agar diffusion techniques and traditional bioassays (Falahee et al., 1990; Falahee and Adams, 1992), but require extended time for completion and can be confounded by cross-reactions with subtilisin (Falahee and Adams, 1992; Walstrom and Saris, 1992). Bioassays using bioluminescence (Walstrom and Saris, 1992) and green fluorescent protein (Reunanen and Saris, 2003) have been used to overcome these obstacles. These methods are relatively sensitive and provide rapid analysis; however, they are limited by the duration for which these enzymes can be used and the low recoveries related to bioavailability (Walstrom and Saris, 1992; Immonen and Karp, 2007).

Liquid chromatography-mass spectrometry, which enables accurate molecular mass determination of target molecules in crude samples, is an effective method for the detection of bacteriocins (Mehlis and Kertscher, 1997; Zendo et al., 2008). Recently, the use of LC-MS/MS to quantitate nisin in cheese has been reported (ISO, 2009; Schneider et al., 2011; Fuselli et al., 2012); however, most of these procedures are limited in that they detect only nisin A (ISO, 2009; Fuselli et al., 2012). To improve this technique, Schneider et al. (2011) developed an analytical method for nisin A and nisin Z detection using LC-MS/MS. However, this method has a low recovery rate that should be increased before its application.

Because nisin is allowed in dairy products such as milk in some countries, an analytical procedure for determining nisin A and nisin Z content in milk is required to control the addition of nisin to foodstuffs and prevent its fraudulent use. The current study developed a new pretreatment for LC-MS/MS analysis to determine nisin A and nisin Z content and validated its use for determination of these bacteriocins in cow milk.

MATERIALS AND METHODS

Standards and Reagents

Nisin A and nisin Z were purchased from Sigma-Aldrich (St. Louis, MO; assay 2.5%) and Zhejiang Silver-Elephant Bio-Engineering Co. Ltd. (Tian Tai, Zhejiang, China; assay 99%), respectively. The metha-

nol (MeOH) used as the extraction solvent was HPLC grade, and the acetonitrile used as the mobile phase was purchased from Merck (Whitehouse Station, NJ). Formic acid used for the mobile phase was purchased from Wako (Tokyo, Japan), and the isopropanol used for washing needles was purchased from Merck. The cow milk samples were purchased at a local market in Chungbuk, Korea.

Sample and Standard Preparation

Milk samples (2.0 g) were weighed, placed in a tube, and mixed with 30 mL of extraction solvent consisting of 0.1 M acetate buffer containing 1 M NaCl (pH 2.0) and MeOH (1:1). The samples were homogenized for 1 min at maximum level (CH/PT10–35GT, Kinematica, Luzern, Switzerland). The homogenized samples were sonicated (AJC-4020, OMAX, Buchun, Korea) for 10 min at room temperature and centrifuged at $2,500 \times g$ for 10 min at 4°C (Supra-22K, Hanil Corp., Korea). After centrifugation, the supernatant was transferred to a 50-mL volumetric flask and the precipitate was re-extracted with 10 mL of extraction solvent. After centrifugation under the same conditions, the supernatant was separated and 10 mL of extraction buffer was added to the precipitate for re-extraction. A third extraction was performed by following the same procedure as that used for the second extraction. The supernatant was collected in a 50-mL volumetric flask, and the volume of the solution was made up to 50 mL by using extraction buffer. The aqueous extract supernatant was filtered with a 0.20- μ m membrane filter, and the filtered sample solution was used for LC-MS/MS analysis (Figure 1).

For the standard stock solutions, 40 mg of nisin A standard (2.5% assay) was dissolved in 20% acetonitrile containing 0.5% formic acid in a 10-mL volumetric flask to make a concentration of 100 mg/kg. Similarly, 10 mg of nisin Z was dissolved in the same solvent to be a concentration of 1,000 mg/kg. The standard solutions were diluted adequately and mixed immediately preceding LC-MS/MS analysis to contain identical concentrations of nisin A and nisin Z.

LC-MS/MS Instruments and Conditions

The identification and quantification of analytes were carried out using an HPLC system (1200 series, Agilent, Santa Clara, CA) and triple quadrupole mass spectrometer (Agilent 6410), equipped with an electrospray ionization source, binary pump, degasser, and autosampler. Chromatographic analysis was performed on a CW-C₁₈ column (50 $\times$ 2 mm, 3 μ m; Imtakt, Portland, OR), and the column oven temperature was held at

Figure 1. Flowchart of the pretreatment for nisin extraction in milk for the analysis of liquid chromatography-tandem mass spectrometry (LC-MS/MS).

40°C. The mobile phase consisted of 0.1% formic acid (A) and 100% acetonitrile (B), and a gradient elution was applied as follows: A:B (80:20) for 0.5 min; reaching A:B (10:90) over 6 min; A:B (10:90) for 2 min; reaching A:B (80:20) over 8.1 min; return to the initial conditions and re-equilibrate the column for 4 min. The injection volume was set at 10 μ L, and the flow rate of the mobile phase was 0.3 mL/min. Instrumental source parameters, such as nebulization, drying gas temperature, drying gas flow, and spray voltage, were optimized by injecting nisin standard solution (1 μ g/mL) in the mobile phase at a flow rate of 10 μ L/min as follows: drying gas flow, 6 L/min; drying gas temperature, 300°C; spray voltage, 4,000 V; and nebulization, 40 psi.

For quantitative purposes, LC-MS/MS analysis was performed with positive mode electrospray ionization and the multiple reaction-monitoring parameters for determination of nisin A and nisin Z content in milk are shown in Table 1. The parent ions were initially determined by a full scan, and the optimal voltages of fragmentor were determined when the parent ions had the highest intensity. The fragment ions were also selected through scanning and the optimal settings of collision energy were achieved when fragment ions had the highest intensity. During MS/MS analysis, we used nitrogen as the nebulization and collision gas, and the

run time was 12 min. Data were analyzed with Agilent 6410 Quantitative analysis version (B. 02. 01) analyst data-processing software.

Validation Protocol

The quantification method was validated for linearity, accuracy, precision, and sensitivity. To estimate the limit of detection (LOD), limit of quantitation (LOQ), and linearity of our developed method, we distributed the standard stock solutions of nisin A and nisin Z homogeneously in the milk samples, which then had final concentrations of 12.5, 25, 50, 100, or 200 μ g/kg. Each solution was injected into the LC-MS/MS system and analyzed in triplicate. The linearity of the method was determined by analyzing the standard plots associated with a 5-point standard calibration curve. The LOD was calculated as 3.3 $\times$ the SD of the intercept or slope of the calibration curve, and LOQ was calculated as 10 $\times$ the SD of the intercept or slope of the calibration curve.

Accuracy and precision were estimated by calculating the recovery rate (%) and CV (%), respectively. Recovery rate was determined as follows. Each standard stock solution of nisin A and nisin Z (1.0, 2.5, or 5.0 mg/kg) was spiked into milk samples that were

Table 1. Multiple reaction monitoring parameters of nisin A and nisin Z for liquid chromatography-tandem mass spectrometry analysis

Analyte	Average mass (Da)	Parent ion (m/z)	Fragment ion (m/z)	Fragmentor (V)	Collision energy (eV)
Nisin A	3,354.1	671.6	810.9	105	10
	3,354.1	671.6	790.3	105	10
Nisin Z	3,330.4	667.0	804.9	125	10
	3,330.4	667.0	738.4	115	10

confirmed to be negative for these bacteriocins, and the final concentration of nisin in the samples corresponded to 20, 50, or 100 $\mu\text{g/kg}$. The spiked samples were kept at room temperature for 10 min and then pretreated and analyzed in triplicate by using the procedure described above. To determine precision, we measured both intra- and interday CV values. These measurements were carried out by spiking milk samples with the same concentrations of standard stock nisin A and nisin Z solutions used in the recovery test. The final solutions obtained from samples spiked with high concentrations of the standard solutions were adequately diluted with a mobile phase before LC-MS/MS analysis so that the concentration fell within working range of the calibration curve.

RESULTS AND DISCUSSION

We examined the solubility of nisin in several solvents before selecting an appropriate extraction solvent. Nisin A and nisin Z dissolved incompletely in 100% MeOH and acetate buffer containing 1 M NaCl. However, they dissolved well in a mixture of acetate buffer containing 1 M NaCl and MeOH (1:1). Overall, nisin A (assay 2.5%), which contained salt compounds such as NaCl, dissolved better than nisin Z, which had high purity (assay 99%). Most of analysis methods related to nisin, such as agar diffusion or ELISA, have focused on the detection or estimation of approximate amount of the bacteriocin present in culture media. Only a few studies to quantify nisin A or nisin Z in food using LC-MS/MS have been reported (ISO, 2009; Schneider et al., 2011; Fuselli et al., 2012), and they have only targeted cheese samples. As milk contains lipid besides milk proteins, which are the major components of cheese, it seems to be necessary to confirm that the method is suitable for milk samples even if certain analysis method is available to determine nisin in cheese samples. However, analytical methods for determining nisin A and nisin Z content in milk have been rarely reported until now. Fuselli et al. (2012) used a solution of acetate buffer containing 1 M NaCl (pH 4.5) and MeOH (2:1) to extract nisin from cheese samples and a Zorbax 300SB-C₈ column (150 $\times$ 4.6 mm, 5 μm ; Agilent) for LC-MS/MS analysis. We adjusted the optimal pH and composition

of the extraction solvent used by Fuselli et al. (2012) to obtain excellent recovery. We found that acetate buffer containing 1 M NaCl (pH 2.0) and MeOH (1:1) yielded higher recovery than acetate buffer containing 1 M NaCl (pH 4.5) and MeOH (2:1) when the tests were performed under the same analytical conditions.

Some countries prefer nisin Z to nisin A as a natural preservative according to a major nisin manufacturer in China (Zhejiang Silver-Elephant Bio-Engineering). Therefore, the simultaneous quantitation of nisin A and nisin Z in food is a crucial task. From this point of view, the analytical methods reported by Fuselli et al. (2012) and ISO (2009) are limited because they determine only nisin A in cheese samples. Therefore, the aim of the present study was to establish optimal conditions for LC-MS/MS analysis to determine nisin A and nisin Z content in milk samples simultaneously. Recently, Schneider et al. (2011) reported an analytical method for the determination of nisin A and nisin Z content in cheese samples using LC-MS/MS. They used 0.5% formic acid including 2 $\mu\text{g/mL}$ of BSA and 20% acetonitrile in distilled water as the extraction solvent and a PLRP-S column (150 $\times$ 2.1 mm, 3 μm ; Agilent) for LC-MS/MS analysis. The parent ions for nisin A and nisin Z selected for their study were 671.7 and 667.2 m/z , respectively, whereas the fragment ions for nisin A were 811.2 and 790.5 m/z and those for nisin Z were 805.4 and 739.4 m/z . Although Schneider et al. (2011) established the first advanced procedure for the simultaneous analysis of nisin A and nisin Z in cheese samples, their method has disadvantages, such as low recovery rate and complex pretreatment.

To validate the proposed method, we examined linearity, sensitivity, recovery, and CV. The linearity of the method was evaluated at concentrations ranging from 12.5 to 200 $\mu\text{g/kg}$ (5 levels). The external standard calibration curve of nisin A and nisin Z had linearity, with correlation coefficients of ≥ 0.9986 and ≥ 0.9984 , respectively (Figure 2). The LOD values for nisin A and nisin Z in cow milk were 4.3 and 3.6 $\mu\text{g/kg}$, respectively, whereas the LOQ values were 12.9 and 10.9 $\mu\text{g/kg}$, respectively.

The sensitivity of the developed procedure was evaluated using the chromatograms and spectra obtained after the LC-MS/MS analysis of spiked milk samples.

Figure 2. Calibration curve (linearity) of nisin A and nisin Z in milk; the x-axis indicates the concentration of nisin, and the y-axis indicates the area of the chromatogram.

As shown in Figure 3, an apparent peak in the nisin A chromatogram appeared at a retention time of 2.95 min, whereas the peak in the nisin Z chromatogram appeared at a retention time of 3.06 min. The retention times of nisin A- and nisin Z-spiked samples were identical to those of the standard stock solutions. We also found that the spectra obtained from spiked milk samples had product ions and fragmentation ratios of the 2 product ions identical to those of the standard stock solutions. That is, nisin A had product ions of 810.9 and 790.3 m/z , whereas nisin Z had product ions of 804.9 and 738.4 m/z . The chromatograms obtained

in the present study had peak tailing. We adjusted pH and used organic salts to enlarge the retention time of the peaks, induce peak symmetry, and obtain high recovery rates; however, achieving perfect symmetry was difficult, as is the case with most compounds with large molecular weights. The present study used a reverse-phase CW-C₁₈ column (50 $\times$ 2 mm, 3 μ m; Intakt) with large pore size, as the molecular weight of nisin is comparatively large (3.5 kDa). Additionally, nisin has multiple charges and binds easily with various functional groups. Therefore, peak tailing seemed to be occurring naturally. However, we confirmed that

Figure 3. Chromatograms and mass spectra obtained after liquid chromatography-tandem mass spectrometry analysis of milk samples spiked with nisin A and nisin Z. In the right-hand panels, the diamonds (◆) indicate the precursor ions. Color version available online.

no problems were observed in the reproducibility and quantification of the results obtained using the developed analytical method.

The recoveries in cow milk fortified with 20, 50, or 100 $\mu\text{g/kg}$ of standard solutions ranged from 90.6 to 103.4% for nisin A and from 83.8 to 104.4% for nisin Z. Although the parent and fragment ions selected in pres-

ent study are similar to those reported by Schneider et al. (2011), our method was disclosed to yield higher recovery value (80–113%), compared with the recovery value (48–68%) obtained from their study. The CV values of nisin A and nisin Z were <0.6 and <3.1%, respectively (Table 2). As the standard stock solution concentrations increased, the recoveries of nisin A and

Table 2. Recovery and coefficient of variation (CV) of the analytical method for the detection of nisin in milk

Item	Nisin A (concentration; $\mu\text{g/kg}$, $n = 3$)			Nisin Z (concentration; $\mu\text{g/kg}$, $n = 3$)		
	20	50	100	20	50	100
Recovery (%)	103.4	95	90.6	104.4	92.8	83.8
CV	0.6	0.3	0.5	3.1	0.4	0.8

Table 3. Precision of the analytical method in the detection of nisin A and nisin Z in milk

Spiked level ($\mu\text{g/kg}$)	Precision (CV, %; n = 3)			
	Intraday		Interday	
	Nisin A	Nisin Z	Nisin A	Nisin Z
20	0.63	3.12	2.64	2.90
50	0.31	0.39	1.66	0.97
100	0.55	0.05	0.96	0.76

nisin Z decreased. The intraday CV values ranged from 0.31 to 0.63% for nisin A and from 0.05 to 3.12% for nisin Z, whereas the interday values ranged from 0.96 to 2.64% for nisin A and from 0.76 to 2.90% for nisin Z (Table 3). Compared with traditional methods, the proposed analytical method seems to be economical because analysis time is significantly decreased by using a shorter column, rapid pretreatment, and simultaneous determination of nisin A and nisin Z content.

CONCLUSIONS

A suitable method for the determination of nisin A and nisin Z content in cow milk is required because nisin is used as an antimicrobial agent in milk. In the current study, we developed an analytical method with novel pretreatment and LC-MS/MS conditions that yielded a high recovery rate and validated the method for application in milk samples. The proposed method had excellent recovery (80–113%) and low CV values (<5%), and therefore seems to be an appropriate tool for the determination of nisin A and nisin Z content in cow milk and milk products. Analytical methods for the determination of nisin A and nisin Z content in milk have been rarely reported; therefore, our study is likely to provide a meaningful technological advance for this task. Furthermore, this procedure may be an efficient means of preventing the fraudulent use of nisin in the dairy industry.

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