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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 μg/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 (Sobrino-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 methanol (**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 × *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-μm 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 quadruple mass spectrometer (Agilent 6410), equipped with an electrospray ionization source, binary pump, degasser, and autosampler. Chromatographic analysis was performed on a CW-C_{18} column (50 × 2 mm, 3 μm; Imtakt, Portland, OR), and the column oven temperature was held at