Characterization of Nisin Produced by Lactococcus lactis RP359 Isolated from Kem-Buk-Nud, a Traditional Thai Fermented Food

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Citation

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Abstract

Five hundred colonies of lactic acid bacteria (LAB) isolated from Kem-Buk-Nud, a traditional Thai fermented food, were screened for the presence of nisin gene by using the polymerase chain reaction (PCR) with primers specific to nisin A structural gene. A LAB isolate, RP359, identified as Lactococus lactis subsp. lactis was found to give an expected 231-bp PCR product and to have antimicrobial activity. The inhibition zones produced by RP359 nisin were larger than those produced by nisin A. Sequencing of the PCR product showed that the gene encoding RP359 nisin was a nisin A gene with a silent mutation. The relationship between the mutation and the production of the larger inhibition zones was discussed. To my knowledge, this is the first report presenting the variant of nisin A gene with silent mutation.

INTRODUCTION

Nisin is a bacteriocin produced by some strains of Lactococcus lactis subsp. lactis. It is a 34 amino acid long ribosomally synthesized and post-translationally modified peptide containing five lanthionine rings (Gross and Morell, 1971). According to the lanthonine rings and antimicrobial properties, nisin belongs to a group of bacteriocin called lantibiotics (Schnell et al., 1988). Three nisin variants nisin A, Z and Q show similar antimicrobial properties and differ only in a few amino acids (Twomey et al., 2002; Zendo et al., 2003). Nisin can inhibit growth of a wide range of gram-positive bacteria including Bacillus, Clostridium, Enterococcus, Listeria and Staphylococcus (Tagg et al., 1976). In normal circumstance, gram-negative bacteria are resistant to nisin mainly due to their impermeable outer membranes. However, nisin can be effective against gram-negative bacteria if used in combination with agents destabilizing the outer membrane (Stevens et al., 1992).

Kem-Buk-Nud is a traditional Thai fermented food mainly produced in the northeastern part of Thailand, especially in Ubon Ratchathani province. Its main ingredients are fresh water fish, salt and pineapple. It is consumed raw with vegetables. Since fermentation of Kem-Buk-Nud is still mediated by indigenous bacteria, it is difficult to obtain consistency of product quality and safety. In order to alleviate the difficulties the use of nisin producing lactic acid bacteria as a starter culture in fermentation of Kem-Buk-Nud is now being considered.

The use of nisin producing lactic acid bacteria as starter cultures in fermentation of foods from which they were isolated has been considered to be a practical way in development of starter cultures because no further strain improvement is required. This study is a very first step towards the development of nisin producing lactic acid bacteria to be used as a starter culture in production of Kem-Buk-Nud. This study focused on screening lactic acid bacteria isolated from Kem-Buk-Nud for the production of nisin using PCR technique and on examining some characteristics of the nisin. Nisin producing lactic acid bacteria obtained from this study will be ultimately developed as starter cultures in fermentation of Kem-Buk-Nud.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacteria used in this study are listed in Table 1. Lactococcus lactis subsp. lactis RP359 and Lactococcus lactis subsp. lactis ATCC 11454 are nisin producing strains isolated from Kem-Buk-Nud and obtained from American Type Culture Collection, respectively. All LAB used in this study were grown at 30 ° C in MRS broth and the other bacterial strains, except Clostridium strains, were grown at 37 ° C in BHI broth.

Clostridium strains were propagated anaerobically in Cooked Meat Medium at 37 ° C.

Isolation and screening of nisin producing LAB from Kem-Buk-Nud samples. Kem-Buk-Nud obtained from different shops in Ubon Ratchathani province, Thailand were used as sources of LAB. Liquid parts of the samples were 10-fold serially diluted in 0.85% (w/v) sterile physiological saline. One hundred microliters of appropriate dilutions were spread on MRS agar plates and incubated at 30 ° C for 48 h. Five hundred colonies of LAB were randomly selected from the samples. To assess which LAB isolated from Kem-Buk-Nud carried a nisin gene, DNA coding for nisin was identified by the polymerase chain reaction (PCR) using L. lactis ATCC 11454 as a positive control. Genomic DNA was extracted from all LAB isolated from Kem-Buk-Nud using Genomic DNA extraction kit (Real Biotech Corporation, Taiwan) and used as template DNA for PCR analysis. PCR reaction mixtures of 50 μl prepared in 0.5 ml tubes contained 25 μl of 2x PCR Master Mix (Promega, USA), 5 ng of template DNA and 1 mmol l^{-1} of each primer. PCR amplifications were carried out with the conditions consisting of 1 cycle of 94 ° C for 4 min, followed by 30 cycles of 94 ° C for 45 sec, 55 ° C for 1 min and 72 ° C for 3 min, and 1 cycle of 72 ° C for 10 min. Primers used in this study were designed from nisin A structural gene. The nucleotide sequences of the primers were 5'-ATTATAAGGAGGCACTC-3' and 5'-CATGTCTGAACTAACAA-3' which were complementary to regions 20 bp upstream and 23 bp downstream of the coding sequence, respectively. Amplified PCR products were analyzed by agarose gel electrophoresis on 2.0% (w/v) agarose gel. PCR products were purified using HiYield Gel/PCR DNA fragments Extraction kit (Real Biotech Corporation, Taiwan) and the nucleotide sequences were determined using a Dye Terminator Sequencing kit (Perkin Elmer, USA) and ABI PRISM 377 DNA sequencer (Perkin Elmer, USA) as described by the manufacturer.

Identification of nisin producing lactic acid bacteria. The nisin producing LAB was identified by using the API 50CHL system as specified by the manufacturer (Bio-Merieux, France).

Detection of antimicrobial activity. Antimicrobial activity of nisins produced by L. lactis RP359 and L. lactis ATCC 11454 against a wide range of indicator organisms was examined by agar diffusion assay. Plates were prepared by adding a 0.1% inoculum of an exponentially growing culture of the indicator strain to 30 ml of medium containing 1.5% agar. After solidification in plates, wells (0.6 mm in diameter) were made with a sterile cork borer and filled with 40 μl of tested solution. After incubation at appropriate conditions, the diameters of inhibition zones were measured.

Determination of minimal inhibitory concentrations (MICs). To determine the MICs of nisins produced by L. lactis RP359 and L. lactis ATCC 11454, exponentially growing cultures of indicator strains were diluted 100-fold in appropriate media containing no (control) or different concentrations of purified nisin and further incubated until control cultures were grown to saturation. The MIC was taken as the lowest concentration of nisin that resulted in the absence of detectable growth. Each MIC was determined from two experiments.

Effect of enzymes and heat on bacteriocin activity. To evaluate the effect of enzymes on bacteriocin activity, each bacteriocin preparation was treated with the following enzymes at final concentration of 1 mg ml^{-1} : -chymotrypsin, trypsin, proteinase K, lipase and -amylase. Following incubation at 37 ° C for 2 h, enzyme activity was terminated by heating at 100 ° C for 5 min. The residual activity was assayed against L. sake ATCC 15521, a nisin A sensitive strain. Untreated samples were used as control. To determine the effect of heat on bacteriocin activity, each bacteriocin preparation was heated at 100 ° C for 10, 20 and 30 min. The residual activity was assayed against L. sake ATCC 15521. Untreated samples were included as control.

Purification and gel electroporesis of nisins. Purification of the nisins produced by L. lactis RP359 and L. lactis ATCC 11454 were performed according to the method described by Yang et al. (Yang et al., 1992). Protein content of each product was determined using method described by Lowry et al. (1951). The purified nisins were analyzed by SDS-PAGE using Tris-Tricine system. The stacking gel and separating gel used in this study were 10% and 16.5%, respectively.

Nucleotide sequence accession number: The nucleotide sequence described in this study has been deposited in the EMBL/GenBank/DDBJ databases under the accession number AM410671.

RESULTS

By using PCR with primers specific to nisin A structural gene, one LAB isolate, designated RP359 was shown to give a PCR product corresponding to a 231 bp nisin gene amplified from genomic DNA of L. lactis ATCC 11454, a

nisin A producing strain. The culture supernatant of the nisin gene carrying LAB isolate was found to be able to inhibit L. sake ATCC 15521, a nisin A sensitive strain. The bacterial isolate strain RP359 was identified as L. lactis subsp. lactis.

To analyze the amplified PCR product of LAB strain RP359, it was subjected to nucleotide sequencing. Results showed that the sequence was 231 bp long with an open reading frame (ORF) (Figure 1). The comparison between the sequence of nisin A gene and that of the ORF revealed a single nucleotide substitution from G to A at the position corresponding to the third base of the fifteenth codon of the ORF. To confirm the nucleotide substitution, PCR amplification and nucleotide sequencing of the PCR product were repeated for LAB strain RP359 and the same results were obtained. This point mutation was considered to be a silent mutation because it did not cause difference between amino acid sequence deduced from the ORF sequence and that of nisin A (Fig. 1).

To examine whether the silent mutation in nisin gene of L. lactis RP359 resulted in alteration of some characteristics of the nisin, experiments were conducted to compare production level, sensitivity to enzymes and heat and antimicrobial spectrum of the nisin produced by L. lactis RP359 (RP359 nisin) and nisin A.

Purified nisins of L. lactis RP359 and L. lactis ATCC 11454 were subjected to protein content determination and gel electrophoresis. Protein contents in both tested samples were found to be very similar at around 50 mg. Before analyzing the purified nisins by Tris-Tricine SDS-PAGE, their antimicrobial activities against L. sake ATCC 15521 were confirmed by agar diffusion assay. On a Coomassie stained SDS-PAGE gel, it was showed that each of the purified samples contained only a single protein band with a molecular weight of about 3.5 indicating the purity of the tested samples (Fig. 2).

RP359 nisin and nisin A exhibited the same response to enzymes and heat treatments. They were sensitive to -chymotrypsin and proteinase K but resistant the other enzymes including trypsin, lipase and -amylase. No change in their antimicrobial activities was detected from the samples treated with heat at 100 ° C for 10, 20 and 30 min.

To investigate antimicrobial spectrum of RP359 nisin and nisin A, they were tested for antimicrobial activity using agar diffusion assay against a wide range of indicator organisms comprising LAB and food-borne pathogens. It was found that all strains that were sensitive to RP359 nisin were also sensitive to nisin A, and vice versa. The sizes of the inhibition zones varied considerably between the different indicator strains. However, among all of the sensitive strains, the inhibition zones produced by RP359 nisin were larger than those produced by nisin A (Table 1). When L. lactis RP359 and L. lactis ATCC 11454 were used as indicator strains in this assay, they were shown not to be inhibited by both nisins, indicating self-protection or immunity of the producers and cross-immunity between them.

The MICs of RP359 nisin and nisin A were determined in liquid cultures of nisin-sensitive indicator strains. It was found that the MICs of both nisins were identical in all cases (Table 1). These results demonstrate that there is no difference in specific activities of RP359 nisin and nisin A against all tested indicator strains.

Figure 1

Figure 1: Nucleotide sequence and deduced amino acid sequence of the PCR product amplified from genomic DNA of RP359 using primers specific to nisin A structural gene. The open reading frame is in underline. The nucleotide in the ORF sequence that differs with that in nisin A gene sequence is in bold.

Figure 2

Table 1: Inhibition zones and MICs obtained for nisin produced by subsp. RP359 and subsp. ATCC 11454, using various strains of indicator organism

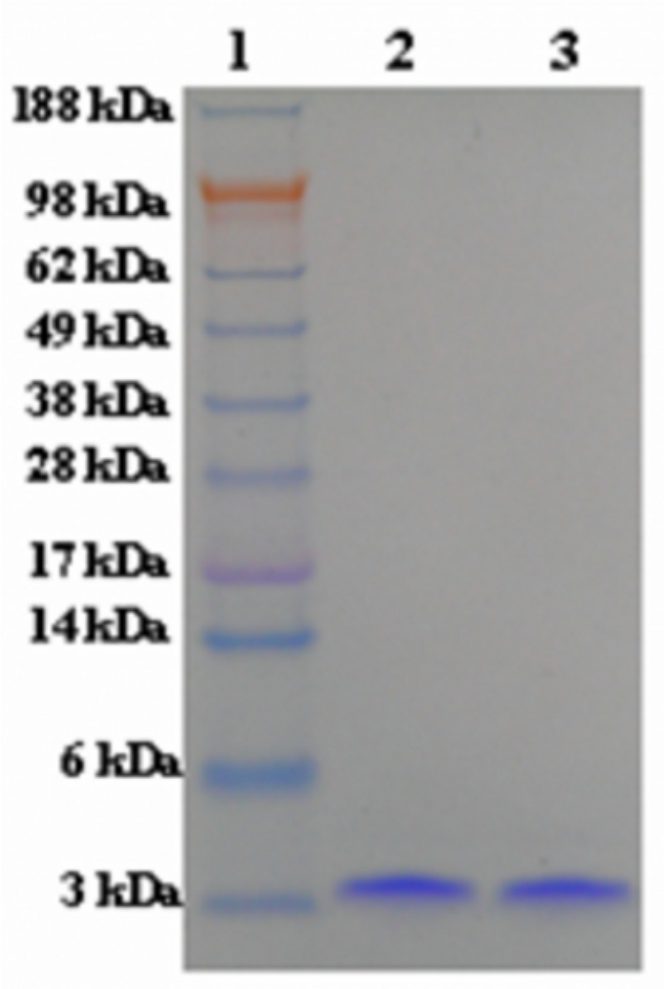
Indicator organism[a]	Diameter of inhibition zone (mm)[b]		MIC (μg ml^{-1})	
	RP359	ATCC 11454	RP359	ATCC 11454
Enterococcus faecalis TISTR 927	15.1	8.6	0.4	0.4
Enterococcus hirae TISTR 928	13.3	7.6	0.8	0.8
Lactobacillus acidophilus ATCC 4356	18.7	10.9	0.06	0.06
Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 11842	16.6	9.7	0.09	0.09
Lactobacillus casei ATCC 334	18.1	10.6	0.07	0.07
Lactobacillus curvatus ATCC 25601	14.8	8.6	0.5	0.5
Lactobacillus sake ATCC 15521	20.7	12.1	0.02	0.02
Lactococcus lactis subsp. *cremoris* ATCC 19257	14.6	8.2	0.5	0.5
Lactococcus lactis subsp. *lactis* ATCC 11007	13.9	8.1	0.6	0.6
Lactococcus lactis subsp. *lactis* ATCC 11454	0	0	-	-
Lactococcus lactis subsp. *lactis* RP359	0	0	-	-
Leuconostoc cremoris ATCC 19254	15.0	8.6	0.4	0.4
Leuconostoc mesenteroides TISTR 473	14.2	8.2	0.6	0.6
Pediococcus pentosaceus TISTR 374	17.2	10.0	0.08	0.08
Bacillus cereus ATCC 1178	15.7	9.3	0.2	0.2
Clostridium perfringens DMST 11147	15.3	9.0	0.3	0.3
Escherichia coli ATCC 25922	0	0	-	-
Escherichia coli O157:H7 ATCC 35150	0	0	-	-
Listeria monocytogenes DSM 20600	14.7	8.5	0.5	0.5
Listeria innocua DSM 20649	13.9	8.0	0.6	0.6
Salmonella enterica serovar Paratyphi A ATCC 9150	0	0	-	-
Salmonella enterica serovar Typhi ATCC 6539	0	0	-	-
Staphylococcus aureus ATCC 14458	16.8	9.8	0.09	0.09
Staphylococcus aureus TISTR 118	15.2	8.9	0.4	0.4

[a] ATCC, American Type Culture Collection; DMST, Department of Medical Science Thailand; DSM, Deutsche Sammlung von Mikroorganismen; TISTR, Thailand Institute of Scientific and Technological Research.

[b] Values were obtained with identical concentrations (1μg ml^{-1}) of bacteriocins in the agar diffusion assay. They were the average of two experiments.

Figure 3

Figure 2: Coomassie stained SDS-PAGE gel of identical amounts (1μg) of nisin A (lane 2) and RP359 nisin (lane 3). Lane 1, SeeBlue Plus2 protein standard.



DISCUSSION

In this study, we intended to screen LAB isolated from Kem-Buk-Nud for the production of nisin, not other bacteriocins because of beneficial properties of nisin and its suitability for our future uses. Nisin is the only bacteriocin accepted as safe to be used as a food preservative. It has been well tested and confirmed as non-toxic when consumed orally and has proved to be a safe food preservative (Delves-Broughton, 1990). The susceptibility of nisin to enzymatic degradation is an advantage for its use in food, as nisin is quickly digested and would not affect the intestinal flora or be absorbed into the blood stream.

The detection frequencies of nisin producing strains from LAB isolated from various foods were substantially different. Among traditional Thai fermented foods, one nisin producing strain was found from 500 LAB isolated from

Kem-Buk-Nud whereas the same number of nisin producing strain was found from 14,020 LAB isolated from nham, a Thai style fermented meat (Noonpakdee et al, 2003). For other fermented foods, one, two and two nisin producing strains were detected from 4,608 LAB isolated from dry fermented sausages (Rodriguez et al., 1995), from 153 LAB isolated from sauerkraut (Harris et al., 1992) and from 123 LAB isolated from vegetables (Uhlman et al., 1992), respectively. Many factors may be responsible for the difference in the detection frequencies including nature of food sources, screening methods and sensitivity of indicator strains used in screening steps.

Nucleotide sequences of the coding regions of nisin A gene and RP359 nisin gene differed in only one nucleotide. This nucleotide substitution did not cause difference in amino acid sequences and some characteristics (spectrum of antimicrobial activity and sensitivity to enzymes and heat) between both nisins. Only difference between nisin A and RP359 nisin that could be detected was the size of the inhibition zones obtained from agar diffusion assays. From the results, it could be concluded that this difference was not resulted from the difference in production level or specific antimicrobial activity between nisin A and RP359 nisin. It is possible that the nucleotide substitution (silent mutation) in RP359 nisin gene may affect the physicochemical properties (eg., solubility, stability, aggregation behavior, and diffusion) of the nisin which in turn result in ability to produce large size of inhibition zones. Silent mutations in several genes have been reported to be responsible for changing in folding, solubility and aggregation behavior of the proteins encoded by the mutated genes (Cortazzo et al., 2002; Komar et al., 1999). However, more studies need to be done to elucidate the relationship silent mutation in RP359 nisin gene and the ability of the nisin to produce large size of the inhibition zones.

L. lactis subsp. lactis RP359 obtained from this study was shown to be interesting as a potential protective culture in fermentation of Kem-Buk-Nud and other Thai fermented foods because of its nisin production ability and its natural occurrance in Kem-Buk-Nud. However, the use of the nisin producing strain in the production of Thai fermented food requires more studies that have been currently in progress in our laboratory.

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