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Research Article

Identification of Nisin Z producing *Lactococcus lactis* N12 associated with traditional Thai fermented rice noodle (Kanom Jien)

Adisorn Swetwiwathana[1*], Takeshi Zendo[2], Jiro Nakayama[2] and Kenji Sonomoto[2]

[1]Faculty of Agro-Industry, King Mongkut's Institute of Technology Ladkrabang (KMITL) Chalong-krung Road, Bangkok 10520, Thailand

[2]Laboratory of Microbial Technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka 812-8581, Japan

*Author to whom correspondence should be addressed, email: adisorns@hotmail.com

Abstract: Fifty isolates of lactic acid bacteria (LAB) from traditional Thai fermented rice noodle (Kanom Jien) were tested for bacteriocin production. The results revealed that only the bacteriocin produced from strain N12 was shown as being active against mostly gram-positive bacterial indicators. The bacteriocin produced also exhibited a strong inhibitory effect on the foodborne pathogen, *Staphylococcus aureus,* which is mostly reported as the dominant foodborne pathogen in this Thai fermented rice noodle product. This bacteriocin-producing LAB strain was preliminarily identified as *Lactococcus lactis* subsp. *lactis* by using API 50 CHL test kit. The identification by partial 16S rDNA confirmed that N12 were 99% identical to *Lc. lactis* subsp. *lactis*. When comparing the inhibitory spectrum of the antagonist produced by this strain with those of nisin A and Z producers (*Lc. lactis* subsp. *lactis* NCDO 497 and IO-1 JCM 7638 respectively), it was implied that *Lc. lactis* subsp. *lactis* N12 produced an antagonistic substance related to nisin. Further analysis was carried out on antagonists produced from N12 by direct application of sterile cultured supernatant to liquid chromatography/mass spectrometry (LC/MS) and PCR analysis of the structural genes of this bacteriocin-producing strain. The results from both LC/MS and PCR analysis confirmed that N12 could produce nisin Z. This potent nisin Z producer strain was thus selected for further study aimed on the potential use as a starter culture in safer traditional Thai fermented rice noodle (Kanom Jien) production.
Keywords: food, pathogen, bacteriocin, lactic acid bacteria, Thailand

Introduction

Kanom Jien, a traditional Thai fermented rice noodle, is an easy to prepare dish consumed usually as a substitute for rice with various types of curry or curry sauce. The dish is believed to have originated in China. Non-sterilized rice grains are soaked and fermented by lactic acid bacteria such as *Lactobacillus plantarum* [1], at the beginning of spontaneous fermentation before milling and processing into rice noodles.

LAB have for centuries been responsible for the fermentative processing and preservation of many food products including dairy, meat, vegetables and bakery products [2]. They have also been a subject of interest with respect to the production of growth inhibition compounds, including bacteriocins [3]. Bacteriocins are antimicrobial protenaceous compounds that are generally inhibitory towards related bacterial strains [4, 5]. The use of bacteriocins has attracted increased attention as potential bio-preservatives and as a possible substitute for chemical preservation [6], since nisin was accepted by the U.S. Food and Drug Administration in 1987 as a generally recognized safe food additive in dairy products. Today nisin is a permitted preservative in at least 48 countries, in which it is used in a variety of products, including cheese, canned food and cured meat [7]. Although information on nisin in the literature is extensive, little information has emerged regarding potential use in traditional fermented products. Hence this study is to report on the detection and characterization of nisin Z producing *Lactococcus lactis* N12 isolated from a traditional Thai fermented rice noodle (Kanom Jien).

Materials and Methods

Isolation of lactic acid bacteria (LAB) from Kanom Jien and activity assay

LAB were isolated from 10 samples of traditional Thai fermented rice noodle sold in retail markets in Bangkok. Samples (25 g) of food were homogenized with 225 ml of 0.85% (w/v) sterile normal saline, 10-fold serially diluted, plated on MRS [8], + 0.5 % Calcium carbonate ($CaCO_3$) agar plates and followed culture condition as described by Swetwiwathana [9]. Colonies were either selected randomly or all colonies were sampled if the plate contained less than 10, according to Leisner *et al*. [10]. The purity of the isolates was checked by repeated streaking on fresh MRS + $CaCO_3$ agar plates, followed by microscopic determination. All selected strains of LAB were maintained in MRS broth with 20% glycerol at -20^o C.

All selected LAB strains were precultured in MRS broth (Oxoid) overnight at 30^{o}C. Each overnight culture was spotted onto the surface of a special bacteriocin screening medium (BSM) [11], and grown for 20-24 h at 30^{o}C under anaerobic conditions in order to minimize the formation of hydrogen peroxide and acetic acid [12]. The agar spot assay (direct method) was performed for antagonistic potential screening as described by Fleming *et al*. [13] and Uhlman *et al*. [14]. A top layer of 5-ml Lactobacilli agar AOAC (LAA, Difco) inoculated with 2% of an overnight culture of the indicator strain (Table 1) was poured onto the bottom layer of the BSM base and incubated at the culture condition for each indicator strain [9]. Antimicrobial producers were examined after 24 h for zone of inhibition. The most potent strains, which were active against more than 5 indicators and exhibited an inhibitory effect on food pathogens such as *E. coli, L. monocytogenes, Staph. aureus* and *S. anatum*, were selected for further study.

Determination of the concentration of antimicrobials produced compared to known bacteriocins

The study was conducted by inoculating 1% of overnight culture of the selected potent LAB strain and known bacteriocin-producers such as pediocin PA-1 from *P. pentosaceus* TISTR 536, nisin A from *Lc. lactis* NCDO 497 and nisin Z from *Lc. lactis* IO-1 JCM 7638 in MRS broth, and culturing for 20-22 h at 30^{o}C. The cultures were then centrifuged at 2,700 x g for 10 min. The supernatant from each of the cultures was adjusted to pH 6.5 with 5.0 N NaOH and then filter-sterilized with 0.20 µm pore-size polysulfone membrane (Cica, Tokyo). The cell-free supernatant was determined for antagonistic activity by using spot-on-lawn method according to Ennahar *et al*. [15] and Mayr-Harting *et al*. [16].

Identification of the suspected bacteriocin-producing strain and DNA sequence

The suspected bacteriocin-producing isolates were identified based on carbohydrate fermentation patterns by using API 50 CHL test kit (BioMorieux Vitek, Inc., Hazelwood, MO, USA). Cell morphology of each isolate was studied with gram-stains under microscope. Catalase test for each strain as recommended by Schillinger and Luecke [12] was also performed in the study. Partial phenotypic characterization of each suspected strain was performed by firstly preparing overnight cultures in MRS broth. 2 ml of the overnight culture was harvested by centrifugation. The cells were then resuspended in 80 µl of TE buffer (50 mM Tris, 50 mM EDTA, pH 8). Lysis was initiated by the addition of 5 mg/ml lysozyme. After incubation at 37^{o} C for 30 min, the mixture was further provided with MagExtracter-Genome (TOYOBO) as specified by the manufacturer. 16S rDNA gene region of suspected strains, corresponding to positions 8 to 1510, was analyzed by PCR [17], using primer 8UA (5'- AGAGTTTGAT CCTGGCTCAG –3') and 1510B (5'-GTGAAGCTTACG GCTACCTTGTTACGACTT –3') based on primers described by Martinez-Murcia *et al*. [18]. PCR product was then purified by using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Purified PCR product was used for DNA sequencing (Macrogen, Seoul, Korea). The obtained DNA sequences were analyzed using GENETYX-WIN software (GENETYX, Tokyo, Japan). Database searches were performed using BLAST of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/ BLAST/).

Liquid Chromatography/Mass Spectrometry (LC/MS) analysis

The molecular mass of bacteriocin in cultured supernatants produced by the isolated bacteriocin-producing LAB from Kanom Jien was determined using LC/MS as described by Zendo *et al*. [19]. The total ion chromatograms were taken in a mass range from *m/z* 500 to 3000. To detect and identify a bacteriocin from supernatants, a mass chromatogram was taken in a mass range from *m/z* 1000 to 3000. The data acquisition was performed using a JOEL MassCenter program (JEOL).

PCR analysis and DNA sequencing of bacteriocin from bacteriocin-producing LAB

The purified genomic DNA of the potent strain provided by the MagExtracter-Genome (TOYOBO, Osaka, Japan) as mentioned previously, was prepared. Nisin A primers (Forward primer: 5'– CCGGAATTCATAAGGAGGCACTCAAAATG -3' and Reverse primer: 5' – CGGGGTACCTACTATCCTTTGATTTGGTT – 3') were designed and synthesized (Hokkaido System Science Co. Ltd., Hokkaido, Japan) while PCR amplification was performed according to Swetwiwathana [9]. PCR product was then purified by using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Purified PCR product was used for DNA sequencing (Macrogen, Seoul, Korea). The obtained DNA sequences were analyzed using GENETYX-WIN software (GENETYX, Tokyo, Japan). Database searches were performed using BLAST of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/).

Results and Discussion

Isolation of bacteriocin-producing LAB from Kanom Jien and strain identification

An agar spot test was used to screen 50 LAB strains isolated from Kanom Jien for antagonistic activity (data not shown). It was revealed that only a strain of N12 exhibited an inhibitory effect on various indicators, which mostly were gram-positive, including food pathogens such as *L. monocytogenes* and *Staph. aureus*. The concentration of antimicrobials produced by this strain was determined in arbitrary unit per millilitre (AU/ml) with 21 indicators and compared to those of pediocin PA-1 producer of *P. pentosaceus* TISTR 536, nisin A producer of *Lc. lactis* NCDO 497 and nisin Z producer of *Lc. lactis* IO-1 JCM 7638 (Table 1). Cross-reaction of the produced bacteriocin among N12 strain, pediocin PA-1 and both of nisin A and Z producers was also studied. The results implied that the activity spectra of N12 was identical to those of nisin A and Z producers. The activity from antimicrobials produced by N12 exhibited an effect only on pediocin PA-1 producer of TISTR 536, but there was no effect among nisin producer strains.

From the preliminary results of catalase test, cell morphology and carbohydrate fermentation (API 50 CHL test kit) pattern of N12 (Table 2) indicated that N12 was gram positive, coccoid shape and catalase negative. The strain showed 93.2% of identity to *Lc. lactis* subsp. *lactis* which concurred to the prior morphology result. The confirmation results from about 1,500 bp phenotypic characterization of this LAB strain concurred also to early results of strain identification from API 50 CHL commercial kit. It was revealed that N12 showed 99% identity of their DNA sequences to *L. lactis* subsp. *lactis* (Fig. 1). Thus, the produced antagonists and the nisin producing genes of this strain were further studied.

gb [EU483103.1]
***Lactococcus lactis* subsp. *lactis* strain R-30 16S ribosomal RNA gene. Partial sequence**
Length = 1461

Score = 1511 bits (818), Expect = 0.0
Identities = 828/832 (99 %), Gaps = 3/832 (0 %)
Strand = Plus/Plus

Figure 1. 16S rDNA sequences result of bacteriocin-producing strain N12 by database searches from NCBI.

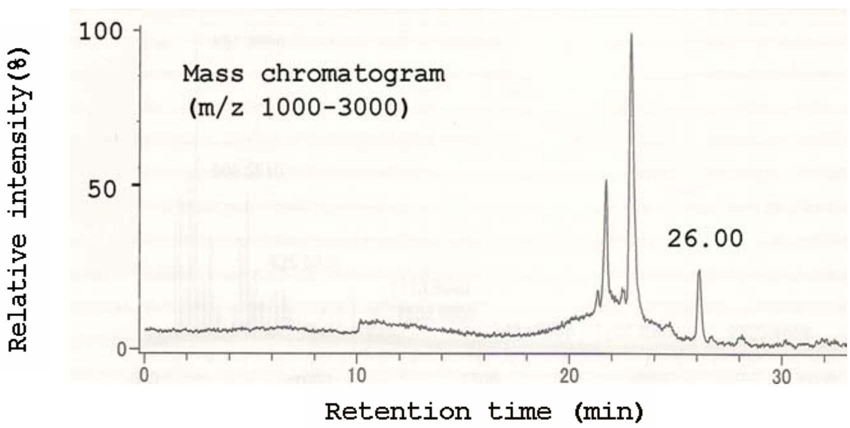
LC/MS analysis of bacteriocin produces and DNA sequencing of bacteriocin genes from N12

Zendo et al. [19], advised that ions from known bacteriocins such as nisin A, nisin Z, nisin Q, lacticin 481 or mundticin KS could be detected in a range higher than *m/z* 1000 by using LC/MS. Therefore, extraction of ions higher than *m/z* 1000 is applicable for initial attempt to detect unknown bacteriocins, without any information on their molecular mass. Thus, culture supernatant of bacteriocin-producing *Lc. lactis* subsp. *lactis* N12 was analysed for nisin Z mass spectrum and retention time by using LC/MS after pretreatment to eliminate impurities in the culture supernatants and a total ion chromatogram was obtained. The potent bacteriocin-derived ions were then scanned by extracting ion with *m/z* 1000-3000 (Fig. 2a). A peak at around 26 min could be identified as a potent bacteriocin-derived peak. The mass spectrum at this retention time showed that this molecule was detected as *m/z* 1111.58 and 1665.93, which corresponded to $[M+3H]^{+3}$ and $[M+2H]^{+2}$, respectively (Fig. 2b). Therefore, the molecular mass of the bacteriocin was determined to be 3332, which was in complete agreement with those of nisin Z. The results of identification of the bacteriocin produced from N12 through LC/MS concurred with the identification of nisin Z produced by *Lc. lactis* QU1 by using LC/MS [19]. In order to prove that the bacteriocin produced by *Lc. lactis* subsp. *lactis* N12 was nisin Z, PCR analysis using the known sequences of the nisin A structural genes was performed. The expected 300 bp fragment containing the structural genes of nisin was amplified and then sequenced (Fig. 3). The results indicated that the 300 bp of nisin structural gene sequences from *Lc. lactis* subsp. *lactis* N12 were 96% identical to nisin Z.

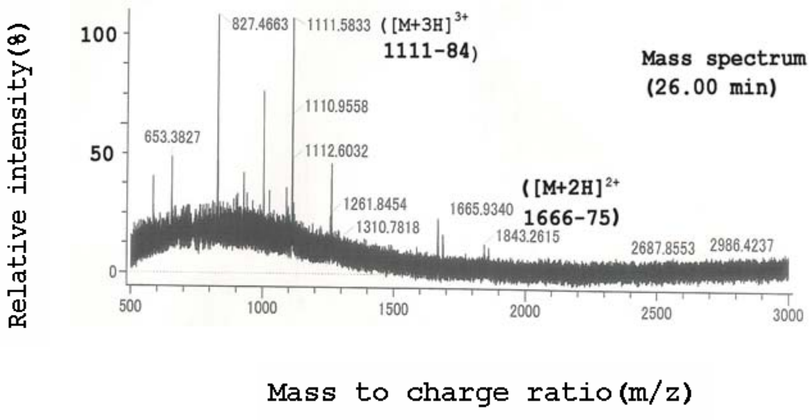
Table 1. Inhibitory spectrum of antimicrobials (AU/ml) by N12 from Khanom-Jien compared to nisin A (NCDO 497) and nisin Z (IO-1 JCM7638) producers.

Indicator strain[a]	N12	TISTR536	NCDO 497	IO-1 JCM7638
B. circulans JCM 2504 [T]	6,400	0	800	3,200
B. coagulans JCM 2257[T]	6,400	0	3,200	6,400
B. subtilis JCM 1465 [T]	800	0	200	800
E. faecalis JCM 5803 [T]	800	800	100	400
E. coli JM 109	0	0	0	0
K. varians LTH 1545	400	6,400	400	400
Lb. plantarum ATCC 8014	800	0	200	400
Lb. plantarum ATCC 14917 [T]	800	6,400	100	400
Lb. sakei subsp. *sakei* JCM 1157 [T]	6,400	6,400	6,400	12,800
Lc. lactis subsp. *lactis* ATCC 19435 [T]	200	0	100	200
Lc. lactis subsp. *cremoris* TUA 1344L	800	1,600	100	400
Leu. mesenteroides subsp. *mesenteroides* JCM 6124 [T]	1,600	1,600	800	1,600
Lis. innocua ATCC 33090 [T]	1,600	6,400	100	800
Lis. monocytogenes ATCC 19117	1,600	6,400	1,600	1,600
M. luteus IFO 12708	1,600	0	200	800
P. pentosaceus JCM 5885	800	400	200	400
P. pentosaceus JCM 5890 [T]	200	200	0	100
Salmonella anatum WHO-BKK	0	0	0	0
Staphylococcus aureus subsp. *aureus* ATCC 12600 [T]	400	0	100	200
Staph. carnosus LTH 2102	400	0	100	200
Nisin A producer NCDO 497	0	0	0	100
Nisin Z producer strain IO-1 JCM 7638	0	0	0	0
TISTR 536	400	0	200	400
N12	0	0	0	0

[a] ATCC, American Type Culture Collection, Rockville, Md; JCM, Japanese Culture of Microorganisms, Wako, Japan; JM, commercial strain from Toyobo, Osaka, Japan; LTH, Lebensmitteltechnologie Hohenheim University, Stuttgart, Germany; TUA, Tokyu University of Agriculture, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; WHO-BKK, World Health Organization, Salmonella-Shigella Center, Bangkok, Thailand.



(a)



(b)

Figure 2. Detection and identification of a bacteriocin from *Lc. lactis* N12

(a) Mass chromatogram extracted the ions with *m/z* 1000-3000 to specify possible bacteriocin-derived peaks. In the mass chromatogram, the retention time of a possible bacteriocin was determined to be 26.00 min. Peaks around 21-23 min were impurities derived from medium components.

(b) Mass spectrum at the retention time showed that the bacteriocin was detected as $[M+3H]^{3+}$ (*m/z* 1111.58) and $[M+3H]^{2+}$ (*m/z* 1665.93) ions related to nisin Z from *Lc. lactis* QU 1 which showed $[M+3H]^{3+}$ (*m/z* 1111.84) and $[M+3H]^{2+}$ (*m/z* 1666.75) ions [19].

```
>emb|X61144.1|LLNISZ  L.lactis nisZ gene for nisin Z
Length=360

 Score =  363 bits (196),  Expect = 2e-97
 Identities = 214/221 (96%), Gaps = 7/221 (3%)
 Strand=Plus/Minus

Query  8    TGG-TATTTGCTT-CGTG-AT-CTAC-ATTAC-AGTTGCTGTTTTC-TGTTACAACCCAT  60
            ||| ||||||||| |||| || |||| ||||| ||||||||||||| |||||||||||||
Sbjct  297  TGGTTATTTGCTTACGTGAATACTACAATTACAAGTTGCTGTTTTCATGTTACAACCCAT  238

Query  61   CAGAGCTCCTGTTTTACAACCGGGTGTACATAGCGAAATACTTGTAATGCGTGGTGATGC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  237  CAGAGCTCCTGTTTTACAACCGGGTGTACATAGCGAAATACTTGTAATGCGTGGTGATGC  178

Query  121  ACCTGAATCTTTCTTCGAAACAGATACCAAATCCAAGTTAAAATCTTTTGTACTCATTTT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  177  ACCTGAATCTTTCTTCGAAACAGATACCAAATCCAAGTTAAAATCTTTTGTACTCATTTT  118

Query  181  GAGTGCCTCCTTATAATTTATTTTGTAGTTCCTTCGAACGA  221
            |||||||||||||||||||||||||||||||||||||||||
Sbjct  117  GAGTGCCTCCTTATAATTTATTTTGTAGTTCCTTCGAACGA  77
```

Figure 3. Nisin Z structural genes of *Lc. lactis* subsp. *Lactis* N12 by database searches from NCBI.

Table 2. Catalase test, morphology and carbohydrate fermentation pattern of N12.

Test	N 12	Test	N 12
Carbohydrate fermentation pattern		**Carbohydrate fermentation pattern**	
Control	-	Esculin	+
Glycerol	-	Salicin	+
Erythritol	-	Cellobiose	+
D-Arabinose	-	Maltose	+
L-Arabinose	-	Lactose	-
Ribose	+	Melibiose	?
D-Xylose	-	Saccharose	+
L-Xylose	-	Trehalose	+
Adonitol	-	Inulin	-
β Methyl-xyloside	-	Melezitose	-
Galactose	?	D-Raffinose	+
D-Glucose	+	Amidon	?
D-Fructose	+	Glycogene	-
D-Mannose	+	Xylitol	-
L-Sorbose	-	β-Gentiobiose	+
Rhamnose	-	D-Turanose	-
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	-
Mannitol	+	D-Fucose	-
Sorbitol	-	L-Fucose	-
α-Methyl-D-mannoside	-	D-Arabitol	-
α-Methyl-D-glucoside	-	L-Arabitol	-
N-Acetyl glucosamine	+	Gluconate	-
Amygdalin	?	2-Ketogluconate	-
Arbutin	+	5-Ketogluconate	-
Catalase	-		
Cell morphology	coccoid		
Gram-stain	+		

+ = positive; - = negative; ? = doubtful

Conclusion

Consequently, the strain identification of N12 by both carbohydrate fermentation kit of API 50 CHL and about 1,500 base pairs of 16S rDNA sequences, inhibitory spectrum of the bacteriocin produced from N12, the identification of bacteriocin produced from N12 by direct application of sterile culture supernatant through LC/MS and PCR analysis of the nisin Z structural genes of this strain, all lead to the conclusion that LAB strain N12 isolated from traditional Thai fermented rice noodle (Kanom Jien) is *Lc. lactis* subsp. lactis and can produce bacteriocin which have been identified as nisin Z. As a result, this potent nisin Z producer strain has been selected for further study aimed at its potential use as a starter culture in traditional Thai fermented rice noodle (Kanom Jien) production.

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