2-peptide bacteriocins of *Lactobacillus plantarum* NF3 isolated from Nham-pla (traditional Thai indigenous fermented minced fish)

Swetwiwathana, A.[1*], Siripoke, S.[2], Zendo, T.[3], Nakayama, J.[3], and Sonomoto, K.[3]

1. Faculty of Agro-Industry, King Mongkut's Institute of Technology Ladkrabang (KMITL) Bangkok, 10520 Thailand.
2-Department of Biology, Faculty of Science, Srinakarinwirot University, Bangkok 10110 Thailand.
3. Laboratory of Microbial Technology, Department of Bioscience and Biotechnology, Faculty of Agricultural Graduate School, Kyushu University. 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan.
[*]Corresponding author (phone: +66-2-326-4112; fax: +66-2-326-4112; e-mail: adisorns@hotmail.com)

Abstract— **Strain NF3, isolated from Nham-pla (traditional Thai indigenous fermented minced fish), was identified as *Lactobacillus plantarum* based on morphology, sugar fermentation reactions (API 50 CHL), PCR and 16S rDNA sequencing. The strain revealed to produce bacteriocins which active against mostly gram positive bacteria such as lactic acid bacteria, *Bacillus* spp., *Micrococcus luteus* and *Listeria innocua*. The bacteriocins produced by this strain were sensitive to proteolytic enzymes and heat stable under acidic condition. Identification of these purified bacteriocins revealed that the bacteriocins produced by strain NF3 belonged to 2-peptide bactriocins which related to two-peptide lantibiotics known as plantaricin W α (molecular mass of 3,223 Da) and plantaricin W β (molecular mass of 3,099 Da). Thus, the strain NF3 and its bacteriocins have potential for application as starter culture and food preservative, respectively, especially for the safety production of Nham-pla.**

Keywords— **2-peptide bacteriocins, *Lactobacillus plantarum*, Traditional Thai fermented fish, Nham-pla**

I. INTRODUCTION

Lactic acid bacteria (LAB) are known for their production of antimicrobial compounds, including bacteriocins which are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms (De Vuyst and Vandamme, 1994; Nes and Johnsborg, 2004). Their bactericidal mechanism vary and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA, and inhibition of peptidoglycan synthesis (De Vuyst and Vandamme, 1994; James et al., 1991). A number of bacteriocins have been described for *Lactobacillus plantarum* isolated from various fermented products such as meat products, beverages and other food sources (Omar et al., 2006; Powell et al., 2007; Rekhif et al., 1995; Todorov et al., 2009).

Nham-pla, a traditional thai fermented minced fish, is one of local delicious fermented foods consumed mainly in the whole country of Thailand. The preparation of this indigenous fermented product generally depends on a spontaneous or chance inoculation by naturally occurring lactic acid bacteria (LAB) and the use of starter cultures is rare. Although information on bacteriocins in the literature is extensive, it has all been limited to bacteriocins producing strains isolated from foods obtained in the industrialized countries. Little information has emerged regarding fermented foods of Thai origin, especially this traditional Thai indigenous fermented minced fish (Nham-pla). Thus, this study is to confirm the detection and characterization of bacteriocins produced by *Lactobacillus plantarum* NF3 (NP3.8) isolated from a traditional thai fermented fish (Nham-pla) according to the report of Mettametha et al. (2011).

II. MATERIALS AND METHODS

1. Bacterial strains, media and cultivation conditions

NF3 (or NP3.8), the bacteriocin-producer strain, was isolated from Nham-pla (traditional Thai indigenous fermented minced fish). The strain was pre-identified by Gram-stain under microscope, catalase test and API 50 carbohydrate galleries (Biomérieux, Vitek, Inc., Hazelwood, MO, USA) (Schillinger and Lücke, 1989; Garver and Muriana, 1993) as *Lactococcus lactis*, with good correlation at the genus level (99.9 % identity) and acceptable with the results from both of catalase test (negative) and cell morphology (rod shape). The strain revealed to produce bacteriocins which active against mostly gram positive bacteria such as lactic acid bacteria, *Bacillus* spp., *Micrococcus luteus* and *Listeria innocua*. The bacteriocins produced by this strain were also reported sensitive to proteolytic enzymes and heat stable under acidic condition (Mettamethaet al., 2011). *Lb. sakei* subsp. *sakei* JCM 1157 [T] used for the bacteriocin activity tests was propagated in MRS medium at 30[o] C for 24 h. The strain of *Lb. Plantarum* NF3 was stored at -80[o] C in MRS broth containing 15%

(v/v) glycerol. Before use, the strain was cultivated twice for 24 h at 30° C in MRS broth.

2. Identification of the suspected bacteriocin-producing strain NF3 by 16S rDNA sequence

Partial phenotypic characterization of strain NF3 was performed by firstly preparing overnight cultures in MRS broth. 2 ml of overnight culture was harvested by centrifugation. The cells were then suspended in 80 µl of TE buffer (50mM Tris, 50mM EDTA, pH 8). Lysis was initiated by the addition of 5 mg/ml lysozyme. After incubation at 30 °C for 30 min, the mixture was further provided with MagExtracter-Genome (TOYOBO) as specified by the manufacturer. 16S rDNA gene region of strain SKA, corresponding to positions 8 to 1510, was analyzed by PCR (Zendo et al., 2005) using primer 8UA (5'- AGAGTTTGATCCTGGCTCAG –3') and 1510B (5'- GTGAAGCTTACG GCTACCTTGTTACGACTT –3') based on primers described by Martinez-Murcia et al. (1995). 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/).

3. Determination of the concentration of antimicrobial produced from NF3 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°C. The cultures were then centrifuged at 2,700 x g for 10 min. The supernatant from each of 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 on overnight cultures of indicators (Table 1) as recommended by Swetwiwathana et al. (2009) using spot-on-lawn method according to Ennahar et al. (1999) and Mayr-Harting et al. (1972) .

4. Bacteriocins purification

The cell-free supernatant of 1 liters culture incubated at 30 °C of NF3 was purified by a four step procedures as described by Ennahar et al. (1999). The final sample containing the purified bacteriocins was dried by Speed-Vac rotary evaporator (Savant Instruments) and stored at –20°C for molecular mass determination.

5. Mass spectrometric

The molecular masses of purified bacteriocins were determined using a Accu TOF spectrometer, model JMS-T100LC (Agilent Technologies, Germany).

III. RESULTS AND DISCUSSION

1. Identification of the suspected bacteriocin-producing strain NF3 by 16S rDNA sequence

The confirmation results from about 1,500 bp phenotypic characterization of NF3 strain concurred also to early results of strain identification from API 50 CHL commercial kit as *Lb. plantarum*. It was revealed that NF3 showed 99% identity of their DNA sequences to *Lb. plantarum* (Fig. 1). Thus, the antagonistic produces of this strain were used for further studied.

2. Determination of the concentration of antimicrobial produced from NF3 compared to known bacteriocins

The concentration of antimicrobials produced by NF3 was determined in arbitary unit per milliliter (AU/ml) with 13 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 among NF3 strain, pediocin PA-1 and both of nisin A and Z producers was also studied. The results implied that the activity spectra of NF3 was identical to those of nisin A and Z producers by inhibited mostly gram-positive indicators. For cross-reaction between bacteriocin-producers, the activity from antimicrobials produced by NF3 exhibited an effect on pediocin PA-1 producer of TISTR 536 (1,600 Au/ml) and both of nisin A (200 AU/ml) and nisin Z (200 AU/ml) producers, but the bacteriocins produced from NF3 showed no effect on itself. Besides, pediocin PA-1 was shown no effect on the cells of NF3, but both nisin A and nisin Z exhibited the inhibitory effect on NF3 for 800 AU/ml. According to these results we feel realize that bacteriocins produced by NF3 must be different from nisin.

3. Bacteriocins purificatio and mass spectrometric of bacteriocins from NF3

The cell-free supernatant from 1 liters culture incubated at 30° C of *Lb. plantarum* NF3 was purified by a four step procedures as described by Ennahar *et al.* (1999). It was revealed that the bacteriocins produced by this strain might be a family of two-peptide bacteriocins, due to the purified fractions of bacteriocins after reverse phase HPLC exhibited 2 sections of activity (Fig. 2). The first highest activity fraction was shown around 15-16 min of Reverse Phase HPLC profiles and the second highest activity was exhibited around 20-21 min. The molecular masses of these

two purified bacteriocins fractions were estimated to 3,101.7 d (Fig. 3) and 3,226 d (Fig. 4) respectively by using a Accu-TOF spectrometer Model JMS-T100LC (Agilent Technologies, Germany) for molecular weight determination. When compared the molecular weight results of bacteriocins from these 2 fractions, it was revealed that the molecular weight from these 2 purified fractions was concurred to plantaricin W from *Lb. plantarum* which has been reported as a new family of two-peptide lantibiotics (plantaricin W α is 3,223 d and plantaricin W β is 3,099 d) by Holo *et al.* (2001). Thus, this bacteriocin-producing strain is under further study for possibility of using as starter culture for Nham-pla production. Besides, bacteriocins produced from this strain are in confirmative study for their amino acid sequences compared to the known plantaricin.

IV. CONCLUSIONS

Consequently, the strain identification of about 1,500 base pairs of 16S rDNA sequences of the bacteriocin producedr strain NF3 is *Lb. plantarum.* and can produce bacteriocin which identified as plantaricin W-like bacteriocins. Hence, this potent plantaricin W-like producer strain was selected for our further study aimed on the potential use as starter culture in safety traditional Thai fermented minced fish (Nham-pla) production. Besides, the purified bacteriocin fractions are now under amino acid sequences determination.

ACKNOWLEDGEMENT

This work was supported in part by the Japan Society for the Promotion of Science (JSPS) and National Research Council of Thailand (NRCT). We also acknowledge Mr. Noppadol Mettametha for obtaining the NF3 strain and preliminary results data of NF3 identification from API 50 CHL.

REFERENCES

De Vuyst, L., & Vandamme, E. 1994. Bacteriocins of Lactic Acid Bacteria. Blackie London, UK.

Ennahar, S., Zendo, T., Sonomoto, K., & Ishizaki, A. 1999. Investigation of Bacteriocin Production and Purification from *Nukadoko* Isolates Displaying Antimicrobial Activity. Japanese J. Lactic Acid Bacteria. 10 : 29 - 37.

Garver, K.I., &Muriana, M. 1993. Detection and Characterization of Bacteriocin Producing Lactic Acid Bacteria from Retail Food Products. Int. J. Food Microbiol. 19 : 241-258.

Holo, H., Jeknic, Z., Daeschel, M., Stevanovic, S., & Nes, I.F. 2001. Plantaricin W from *Lactobacillus plantarum* Belongs to a New Family of Two-peptide Lantibiotics. Microbiol. 147 : 543 – 651.

James, R., Lazdunski, C., & Pattis, F. 1991. Bacteriocins, Microcins and Lantibiotics. New York : Springer-Verlag.

Mayr-Harting, A., Hedges, A. J., & Berkeley, R. C. W. 1972. Methods for Studying Bacteriocins. Method in Microbiol. 7A : 315 – 422.

Mettametha, N., Siripoke, S., and Swetwiwathana, A. 2011. Isolation and Identification of Bacteriocin-producing Lactic Acid Bacteria from Minced Fermented Fish (Nham-pla). Srinakharinwirot Science Journal. 27(1) : 107-127.

Nes, I.F., & Johnsborg, O. 2004. Exploration of Antimicrobial Potential in LAB by Genomics. Current Opinion in Biotechnology. 15 : 100-104.

Omar, N.B., Abriouel, H., Lucas, R., Martínez-Cañamero, M., Guyot, J-P., & Gálvez, A. 2006. Isolation of Bacteriocinogenic *Lactobacillus plantarum* Strains from Ben Saalga, a Traditional Fermented Gruel from Burkina Faso. Int. J. of Food Microbiol. 112(2006) : 44 – 50.

Powell, J.E., Witthuhn, R.C., Todorov, S.D., & Dicks, L.M.T. 2007. Characterization of Bacteriocin ST8KF Produced by a Kefir Isolate *Lactobacillus plantarum* ST8KF. Int. Dairy J. 17 : 190 – 198.

Rekhif, N., Atrih, A., Michel, M., & Lefebvre, G. 1995. Activity of Plantaricin SA6, a Bacteriocin Produced by *Lactobacillus plantarum* SA6 Isolated from Fermented Sausage. J. of Appl. Bacteriol. 78 : 349 – 358.

Schillinger, U., & Lücke, F.-K. 1989. Antibacterial Activity of *Lactobacillus sake* Isolated from Meat. Appl. Environ. Microbiol. 55 : 1901 - 1906.

Swetwiwathana, A., Zendo, T., Nakayama, J., & Sonomoto, K. 2009. Identification of Nisin Z Producing *Lactococcus lactis* N12 Associated with Traditional Thai Fermented Rice Noodle (Kanom Jien). Asian J. Of Food and Agro-Industry. 2(2) : 116-125.

Todorov, S.D., Ho, P., Vaz-Velho, M., & Dicks, L.M.T. 2009. Characterization of Bacteriocins Produced by Two Strains of *Lactobacillus plantarum* Isolated from *Beloura* and *Chouriço*, Traditional Pork Products from Portugal. Meat Sci. 2009. doi : 10.1016/j.meatsci. 2009.08.053

Zendo, T., Eungruttanagorn, N., Fujioka, S., Tashihiro, Y., Nomura, K., Sera, Y., Kobayashi, G., Nakayama, J., Ishizaki, A., & Sonomoto, K. 2005. Identification and Production of a Bacteriocin from *Enterococcus mundtii* QU 2 Isolated from Soybean. J. Appl. Microbiol. 99 : 1181-119

Figure 1 : 16S rDNA sequences result of bacteriocin-producing strain NF3 by database searches from NCBI

gb[DQ239698.1] ***Lactobacillus plantarum*** **strain L5 16S ribosomal RNA gene. Partial sequence**
Length = 1532 Score = 967 bits (488), Expect = 0.0
Identities = 494/496 (99 %), Gaps = 0/496 (0 %) Strand = Plus/Minus

Table 1 : Antimicrobial spectrum of bacteriocin (AU/ml) from NF3 compared to pediocin PA-1 (TISTR 536) nisin A (NCDO 497) and nisin Z (IO-1 JCM 7638) producers.

Indicator	TISTR536	NF	NCDO	IO-1
P. pentosaceus JCM 5885	200	1,600	3,200	1,600
Lb. plantarum ATCC 14917 ^{T}	6,400	1,600	1,600	1,600
Lb. sakei subsp. *sakei* JCM 1157 ^{T}	6,400	3,200	25,600	25,600
Lc. lactis subsp. *lactis* ATCC 19435 ^{T}	0	1,600	1,600	1,600
Enterococcus faecium TUA 1344L	6,400	1,600	1,600	1,600
Leuconostoc mesenteroides JCM 6124 ^{T}	800	800	3,200	1,600
Micrococcus luteus IFO 12708	0	100	800	800
Listeria innocua ATCC 33090 ^{T}	12,800	200	1,600	800
Enterococcus faecalis JCM 5803 ^{T}	800	800	800	400
Bacillus circulans JCM 2504 ^{T}	0	800	6,400	6,400
B. coagulans JCM 2257 ^{T}	0	800	6,400	6,400
B subtilis JCM 1465 ^{T}	0	400	3,200	1,600
Escherichia coli JCM 109	0	0	0	0
TISTR536	0	1,600	3,200	3,200
NF	0	0	800	800
NCDO	0	200	0	0
IO-1	0	200	0	0

ATCC, American Type Culture Collection, Rockville, Md; JCM, Japanese Culture of Microorganisms, Japan; JM, commercial strain from Toyobo, Osaka, Japan; IFO, Institute for Fermentation, Osaka, Japan; TUA, Tokyu University of Agriculture, Japan.

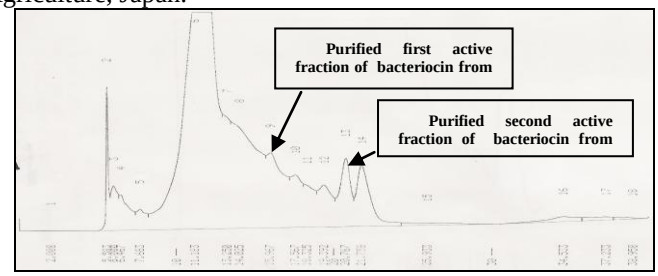

Figure 2 : Reverse Phase HPLC profiles of bacteriocins produced by NF3 (Gradient condition : 0-6 min 28% acetonitrile [ACN]; 6-36 min 28-55% ACN; 36-40 min 55-100 % ACN)

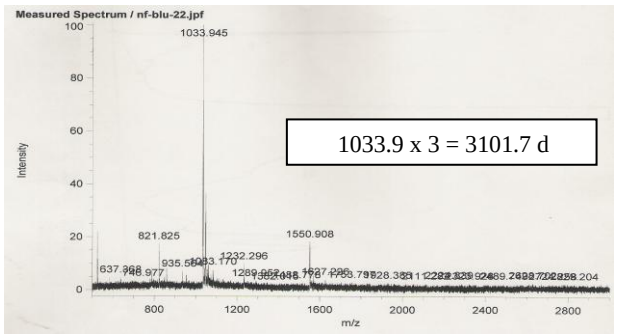

Figure 3 : Molecular mass of purified bacteriocin (first) fraction from NF by Accu-TOF spectrometer Model JMS-T100LC

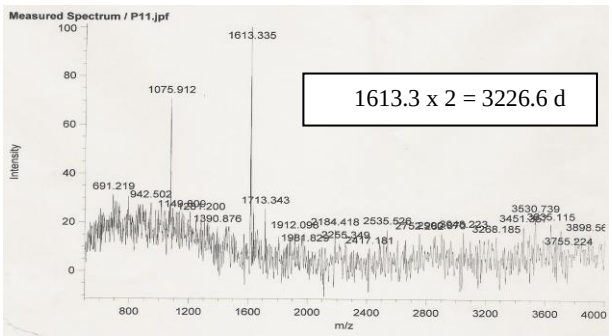

Figure 4 : Molecular mass of purified bacteriocin (second) fraction from NF by Accu-TOF spectrometer Model JMS-T100LC