A newly discovered bacteriocin from *Weissella cibaria* KMITL-QU 21 associated in traditional thai fermented meat-rice sausage (Sai-krog Isan)

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Abstract

A total of 60 strains of lactic acid bacteria (LAB) were daily randomly isolated from the 2 batches of 3-day fermentation traditional thai fermented meat-rice sausage (Sai-krog Isan) produced by one fermented meat industry in Bangkok and screened for bacteriocin-producing strains. Only the LAB strain KMITL-QU 21, which implied to produce bacteriocin and displayed the strongest effect on mostly gram-positive bacteria, was later identified at the DNA level as *Weissella cibaria*. This bacteriocin activity was totally eliminated by exposure to high temperature (100^{o} C) for 5 min. Molecular mass of the purification to homogeneity of the bacteriocin produced by this strain showed that it was about 5,975 Da. N-terminal amino acid sequence analysis of this bacteriocin was performed and implied that the amino acid sequence of this bacteriocin was belonged to N-terminal blocking group. The further clarification of amino acid sequence and characterization as well as the applying of this bacteriocin as a biopreservative for traditional thai fermented meat are under studying. This is the first report of *Weissella cibaria* strain in this traditional thai fermented meat rice sausage and as well the high molecular weight of bacteriocin produced by this strain.

Introduction

Sai-krog Isan, a traditional thai fermented meat-rice sausage, is normally made of minced pork, cooked rice, cooked salt, garlic and food additives, mixed well and stuffed tightly in edible casing. The product is left to ferment at the room temperature for 2-3 days. The most important microorganisms during the spontaneous fermentation of this product belong to the LAB genera Lactobacillus, Pediococcus and Micrococcus. According to numerous reports on using LAB and bacteriocin-producing LAB as starter cultures to harm various pathogens in fermented foods (Hammes and Knauf, 1994; Ennahar et al., 1996) and traditional thai fermented meat such as Nham (Swetwiwathana, 2005), thus, an attempt on finding the most potent bacteriocin-producing LAB strains from meat-rice sausage and use of these potent strains as starter cultures in order to improve the quality and safety during the fermentation of this product was studied and reported in this paper.

Materials and methods

Isolation of bacteriocin-producing LAB and determination of the concentration of antimicrobial produced : A total of 60 strains of LAB were daily randomly isolated from the 2 batches of 3-day fermentation Sai-krog Isan produced by a meat industry in Bangkok and screened for their bacteriocins production on bacteriocin-screening medium (BSM, Tichaczek *et al*., 1992) with 14 indicators (Table 1) as described by Swetwiwathana (2005). The most potent LAB strain was cultured and determined its antagonistic activity with 14 indicators as desbribed by Ennahar *et al*. (1996) and Swetwiwathana (2005). An antagonistic spectrum was compared with known bacteriocin producers of *P. pentosaceus* TISTR 536 (pediocin PA-1- Swetwiwathana, 2005), *Lc. lactis* IO-1 (nisin Z – JCM 7638) and *Lc. lactis* QU 14 (lacticin Z – Iwatani et al., 2007).

***Determination of heat treatment on antimicrobial activity* :** The cell-free cultured supernatant of each selected strain was divided into 2 parts and then adjusted to pH 3.0 for the first part and to 6.5 for the latter. Thermostability of the antimicrobial activity at each pH was conducted by heating the culture supernatant at 100^{o}C for 5 and 10 min and they were then cooled. Culture supernatant with pH 3.0 was adjusted to 6.5 before filter-sterilization. The remaining activities of all the sample aliquots were determined by the spot-on-lawn assay as describes above and compared to those of the control aliquots of pH 6.5 from each strain without heat treatment.

***Identification of bacteriocin-producing strains by using 16S rDNA* :** DNA extraction and PCR analysis were performed as described by Zendo et al. (2005). Primers using for 16S rDNA sequence were primer 8UA (5'- AGAGTTTGATCCTGGCTCAG –3') and 1510B (5'- GTGAAGCTTACG GCTACCTTGTTACGACTT –3') based on primers described by Martinez-Murcia et al. (1995). 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/).

Bacteriocins purification, mass spectrometric and amino acid sequences: The cell-free supernatant of 1 liters culture incubated at 30^{o} C of M 13-5 was purified by a four step procedures as described by Ennahar et al. (1996). The final sample containing the purified bacteriocins was dried by Speed-Vac rotary evaporator (Savant Instruments) and stored at –20^{o}C for molecular mass determination. The molecular masses of purified bacteriocins were determined using a Accu TOF spectrometer, model JMS-T100LC (Agilent Technologies, Germany).

Results and discussions

KMITL-QU 21, was the only strain of 60 isolated LAB from Sai-krog Isan, that implied to exhibit an inhibition activities against the tested indicators on bacteriocin-screening medium (data not shown). When compared its antagonistic activity from MRS cultured cell-free supernatant of KMITL-QU 21 with 3 known bacteriocins of pediocin PA-1, nisin Z and lacticin Z (Table 1), it was revealed that antagonistic spectrum of KMITL-QU 21 cell-free supernatant exhibited different spectrum from these 3 known bacteriocins. Its antagonistic produced exhibited an inhibitory effect to nisin Z producer strain of *Lc. lactis* IO-1. Moreover, the KMITL-QU 21 strain was sensitive to lacticin Z and nisin Z which produced from *Lc. lactis* QU 14 and IO-1, respectively. Antimicrobial activities of the produced from KMITL-QU 21 was completely affected by heating at 100^{o}C for 5 min at pH 6.5 and 10 min at pH 3.0 (Table 2), while all known bacteriocins were partially affected by the same time and temperature at both studied pH. By the partial DNA sequence analysis (about 1,500 base pairs) for strain identification (data not shown), the results showed the 99% of identity related to *Weisella cibaria.*

Table 1. Antimicrobial spectrum of bacteriocin like substance (AU/ml) from KMITL-QU 21 compared to other known bacteriocins

Indicator	**TISTR536**	**QU 14**	**KMITL-QU 21**	**IO-1**
Pediococcus dextrinicus	NT	25,600	**400**	NT
P. pentosaceus JCM 5885	200	200	**200**	1,600
Lb. plantarum ATCC 14917 ^{T}	6,400	800	**0**	1,600
Lb. sakei subsp. *sakei* JCM 1157 ^{T}	6,400	3,200	**6,400**	25,600
Lc. lactis subsp. *lactis* ATCC 19435 ^{T}	0	12,800	**200**	1,600
Leuconostoc mesenteroides JCM 6124 ^{T}	800	100	**400**	1,600
Micrococcus luteus IFO 12708	0	100	**100**	800
Listeria innocua ATCC 33090 ^{T}	3,200	200	**0**	800
Enterococcus faecalis JCM 5803 ^{T}	800	800	**0**	400
Ent. faecium TUA 1344L	6,400	800	**0**	1,600
Bacillus circulans JCM 2504 ^{T}	0	200	**100**	6,400
B. coagulans JCM 2257 ^{T}	0	12,800	**1,600**	6,400
B subtilis JCM 1465 ^{T}	0	100	**0**	1,600
Escherichia coli JM 109	0	0	**0**	0
TISTR536 (produced pediocin PA-1)	0	0	**0**	0
QU 14 (produced lacticin Z)	0	0	**0**	400
KMITL-QU 21	**0**	**400**	**0**	**400**
IO-1 (produced nisin Z) JCM 7638	0	400	**400**	0

ATCC, American Type Culture Collection, Rockville, Md; JCM, Japanese Culture of Microorganisms, Japan; JM, commercial strain from Toyobo, Osaka, Japan; TUA, Tokyu University of Agriculture, Japan; TISTR 536, *P. pentosaceus*-pediocin PA-1 producer; IO-1, *Lc. lactis*- nisin Z producer.

The antagonistic substances produced by KMITL-QU 21 was later purified and determined for the molecular weight (MW) (Fig. 1), it was found that MW of the purified fraction was about 5,975 d (Fig. 2) which

related to lactizin Z (MW = 5,968.9 d) produced from *Lactococcus lactis* QU14 (Iwatani et al., 2007). This molecular weight of bacteriocin had never been reported to produce by *W. cibaria*, hence, we realized that this bacteriocin from *W. cibaria* KMITL-QU 21 might be a novel bacteriocin. This bacteriocin is under further studying for some characterizations and amino acid sequence determination.

Table 2. Sensitivity of the antimicrobial compounds produced by KMITL-QU 21to heat treatments

Strain	Control	Residual activity (AU/ml)			
		pH 6.5		pH 3.0	
		100°C, 5 min	100°C, 10 min	100°C, 5 min	100°C, 10 min
KMITL-QU 21	6,400	0	0	100	0
TISTR 536	6,400	3,200	1,600	6,400	3,200
IO-1	25,600	6,400	1,600	12,800	12,800
QU 14	3,200	800	0	1,600	400

L. sakei subsp. *sakei* JCM 1157 ^{T} was used as an indicator strain for the remaining activity

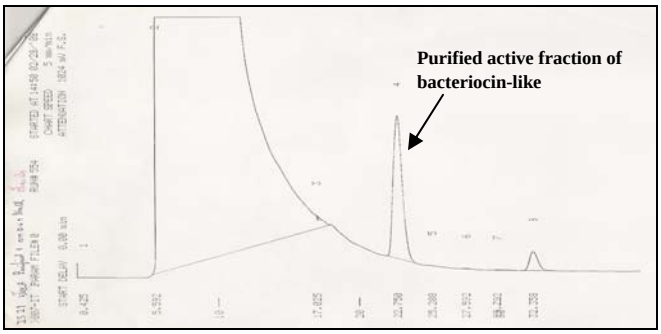

Figure 1. Reverse Phase HPLC profiles of bacteriocin-like produced from *W. cibaria* KMITL-QU 21.

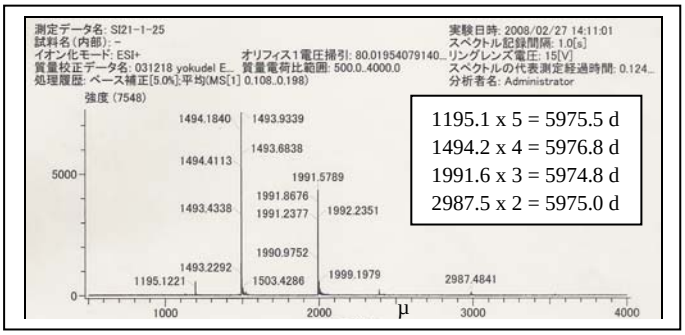

Figure 2. Molecualr mass of purified bacteriocin fraction from *W. cibaria* KMITL-QU 21.

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References

Ennahar, S., Aoude-Werner, D., Sorokine, O., Van Dorsselaer, A., Bringel, F., Hubert, J-C. & Hasselmann, C.1996. Production of pediocin AcH by *Lactobacillus plantarum* WHE 92 isolated from cheese. Appl. Environ. Microbiol. 62(12):4381–4387.

Hammes, W. P. & Knauf, H. J. 1994. Starters in the processing of meat products. *Meat Sci.* 36 : 155-168.

Iwatani, S., Zendo, T., Yoneyama, F., Nakayama, J. & Sonomoto, K. 2007. Characterization and structure analysis of a novel bacteriocin, lacticin Z, produced by *Lactococcus lactis* QU 14. Biosci. Biotechnol. Biochem. 71(8) : 1984-1992.

Martínez-Murcia, A.J., Acinas, S.G. & Rodriguez-Valera, F. 1995. Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol. Ecology. 17(4):247-255.

Swetwiwathana, A. 2005. Microbiological quality enhancement of Thai fermented meat product (Nham) using Nham-associated pediocin-producing lactic acid bacteria (*Pediococcus pentosaceus* TISTR 536). Ph.D. Thesis of Department of Bioscience and Biotechnology, Kyushu Univ., Japan.

Tichaczek, P. S., Nissen-Meyer, J., Nes, I. F., Vogel, R. F. & Hammes., W. P. 1992. Characterization of the Bacteriocins Curvacin A from *Lactobacillus curvatus* LTH 1174 and Sakacin P from *L. sake* LTH 673. System. Appl. Microbiol. 15 : 460 - 468.

Zendo, T., Eungruttanagorn, N., Fujioka, S., Tashiro, 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 : 1181-1190.