IDENTIFICATION OF PEDIOCIN PA-1 PRODUCING *PEDIOCOCCUS PENTOSACEUS* TISTR 536 ISOLATED FROM NHAM (THAI FERMENTED MEAT)

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Background

Nham, traditional Thai fermented pork, is normally consumed without cooking and considered as a ready-to-eat food after 3-4 days of spontaneous fermentation. The reports on occurrence of the most common contaminant strain of *Salmonella anatum* in the product [1, 2, 3] are therefore a serious public health concern. Since the advantage of using lactic starter cultures was reported to have a positive effect on the microbiological quality and safety of various fermented products [4, 5], thus, the use of lactic starter cultures to control *S. anatum* in Nham was studied [1, 6]. The selection of the most potent bacteriocin-producing lactic acid bacteria (LAB) from the spontaneous fermentation of this thai fermented meat product had been studied [7, 8]. The strain of TISTR 536, which was first isolated from Nham [9] and kept as LAB culture collection at Thailand Institute of Scientific and Technological Research (TISTR), was later studied for bacteriocins production and reported as a bacteriocin-producing strain [8]. The strain exerted the best antimicrobial spectra of their produces on various indicators, which included the opportunistic food pathogens of *Listeria monocytogenes* and *Enterococcus faecalis* [8]. This strain exhibited no effect on *Staphylococcus carnosus,* which may enhance adverse effects on colour and aroma of Nham.

Objectives

According to the report on antagonistic substances produced from LAB strain TISTR 536 [8], thus, this report was conducted to identify the strain of TISTR 536 by using 16S rDNA sequence analyses and commercial kit carbohydrate fermentation patterns of API 50 CHL. In addition, identification of bacteriocins produced from this strain was also reported in the study.

Materials and methods

Bacterial strains and culture media : Bacteriocin-producing strain of TISTR 536 obtained from Bangkok MIRCENS in lyophilized form was used in this study. Before experimental use, the culture was cultivated twice in de Man Rogasa Sharp (MRS) broth [10] and incubated at 30^{o} C for 20-24 hours.
Escherichia coli JM 109 was used as host cell for PCR cloning and sequencing analysis. The strain was propagated in LB medium containing 50 µg/ml of ampicillin at 37^{o} C [11].

Identification of TISTR 536 : Carbohydrate fermentation patterns for the isolated strain of TISTR 536 were determined by using API CHL 50 test (bioMorieux Vitek, Inc., Hazelwood, Mo.) and 16S rRNA sequence analyses were performed by using the following methods :
Partial phenotypic characterization of the strain was performed by firstly preparing overnight cultured of TISTR 536 in MRS broth. 2 ml of the overnight cultured 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 manufacturer. 16S rDNA gene was applied from genomic DNA using primer 1101A (5'- AACGAGCGMRACCC–3') and 1407B 5'-GACGGGCGGTGTGTRC –3') based on primers described by Kawamura, *et al*. Reaction [50 µl contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 0.1% Triton X-100, TOYOBO Co. Ltd.), 200 µmol of each deoxynucleotide triphosphate (TOYOBO Co. Ltd.), 20 pmol of primer and 1 U of Taq polymerase (TOYOBO Co. Ltd.)] was set up in a tube containing 200 ng of template DNA and genomic DNA amplification was then performed using Astec program temp control system PC-800. The program was

3 min at 94° C for 1 cycle followed by 30 cycles of 94° C at 30 sec, 55° C at 30 sec and 72° C at 1 min. The additional step for extending incomplete products was performed at 72° C for 5 min. PCR product was TA cloned and transformed into E. *coli* JM109. Selected white colonies were picked and transferred into 20 µl of PCR solution for confirming the insert using M13 forward and reverse primers (5'-GAGTGGGAACTAGAATAAGCGCGTA–3' and 5'–TTTCACACAGGAAGCTAT GAC –3' respectively). Plasmid DNA of selected transformants was isolated using MagExtracter-Plasmid (TOYOBO). The sequence was determined by using Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Pharmacia Biotech) with an automatic sequencer (ALF express, Pharmacia Biotech). The results from the sequencing analysis were analyzed using catalogued sequences in GenBank with the BLAST tool at National Center for Biotechnology Information (NCBI).

Determination of the concentration of antimicrobial produced from TISTR 536 : The study was conducted by inoculating 1 % an overnight culture of the selected potent LAB strains and culturing for 24 hours at 30° C. The culture of TISTR 536 were then centrifuged at 2,700 x g for 10 minutes. The supernatant from the culture was adjusted to pH 7.0 with 5.0 N NaOH and then filter-sterilized with 0.20 µm pore-size polysulfone (Cica, Tokyo). The cell-free supernatant was determined for antagonistic activity by using spot on lown method [12, 13] against the indicator strains (Table 1). The titer expressed in activity units/ml (AU/ml) was defined as the highest dilution factor of bacteriocin preparation that still caused a clearly visible zone of inhibition in the indicator lawn.

Bacteriocins purification : The cell-free supernatant of 2 liters culture incubated at 30° C of TISTR 536 was purified by a four step procedures as described by Ennahar *et al.* (1999) [12]. The final sample containing the purified bacteriocins was dried by Speed-Vac rotary evaporator (Savant Instruments) and stored at –20°C.

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

PCR analysis and DNA sequencing of bacteriocin from TISTR 536 : The total DNA of TISTR 536 was isolated by using the method described by Anderson and McKay [14]. Pediocin PA-1 primer designed and synthesized (Hokkaido System Science Co. Ltd., Hokkaido, Japan) for PCR amplification are Pedi-1F (5' –GAGTGGGAACTAGAATAAGCGCGT A –3') and Pedi-1R (5' –TTACTCTTATTCATAAAATCACCCC –3'). DNA analyses and sequence alignments were carried out using the BLAST program [15].

Results and Discussion

Identification of TISTR 536 : Microscopic results of TISTR 536 revealed that this strain was gram-positve, coccus-shape bacteria and existed in pairs and tetrads. Based on the microscopic results and the rapid API carbohydrate fermentation patterns (Table 2) implied that the microorganism was 92.4 % identical to *Pediococcus pentosaceus*. By the complete DNA sequence analysis, the results showed the 100% of identity related to *P. pentosaceus*. Thus, this was confirmed that LAB strain TISTR 536 is classified as *P. pentosaceus*.

Determination of the concentration of antimicrobial produced from TISTR 536 : The confirmation of antagonistic produced from TISTR 536 was reexamined with 20 indicator strains using MRS cell-free cultured supernatant spot on lawn (Table 3). The results revealed that the LAB strain TISTR 536 could produce bactericidal substances to inhibit various bacterial indicators and exhibited most effect on *Kocuria varians*, *Lactobacillus plantarum* (ATCC 14917T), *L. sakei* subsp. *sakei*, *Listeria innocua* and *Lis. monocytogenes.*

Table 1 : Bacterial strains used as indicators in this study

Indicator	Source [a]	Culture condition [b]
Gram positive		
Pediococcus pentosaceus	JCM 5885 and 5890 T	MRS 30° C anaerobic
Lactobacillus plantarum	ATCC 8014	MRS 30° C anaerobic
L. plantarum	ATCC 14917 T	MRS 30° C anaerobic
L. sakei subsp. *sakei*	JCM 1157 T	MRS 30° C anaerobic
Lactococcus lactis subsp. *lactis*	ATCC 19435 T	MRS 30° C anaerobic
Lc. lactis subsp. *lactis*	NCDO 497	MRS 30° C anaerobic
Lc. lactis subsp. *lactis*	JCM 7638	MRS 30° C anaerobic
Lc. lactis subsp. *cremoris*	TUA 1344L	MRS 30° C anaerobic
Leuconostoc mesenteroides subsp. *mesenteroides*	JCM 6124 T	MRS 30° C anaerobic
Micrococcus luteus	IFO 12708	TSB-YE 37° C aerobic
Listeria innocua	ATCC 33090 T	TSB-YE 37° C aerobic
Lis. monocytogenes	ATCC 19117	TSB-YE 37° C aerobic
Enterococcus faecalis	JCM 5803 T	TSB-YE 37° C aerobic
Kokuria varians	LTH 1545	TSB-YE 37° C aerobic
Staphylococcus carnosus	LTH 2102	TSB-YE 37° C aerobic
S. aureus subsp. *aureus*	ATCC 12600 T	TSB-YE 37° C aerobic
Bacillus circulans	JCM 2504 T	TSB-YE 30° C aerobic
B. coagulans	JCM 2257 T	TSB-YE 37° C aerobic
B subtilis	JCM 1465 T	TSB-YE 30° C aerobic
Gram negative		
Escherichia coli	JCM 109	TSB 37° C aerobic
Salmonella anatum	WHO-BKK	TSB 37° C aerobic

[a] ATCC, American Type Culture Collection, Rockville, Md; JCM, Japanese Culture of Microorganisms, Japan; JM, commercial strain from Toyobo, Osaka, Japan; LTH, Lebensmitteltechnologie Hohenheim University, Stuttgart, Germany; TUA, Tokyu University of Agriculture, Japan; IFO, Institute for Fermentation, Osaka, Japan; WHO-BKK, World Health Organization, Salmonella-Shigella Center, Bangkok, Thailand.
[b] MRS medium (Oxoid); TSBYE, Trypticase soy broth (Difco) + 0.6 % Yeast extract (Difco)

Table 2 : API CHI 50 carbohydrate fermentation pattern of TISTR 536

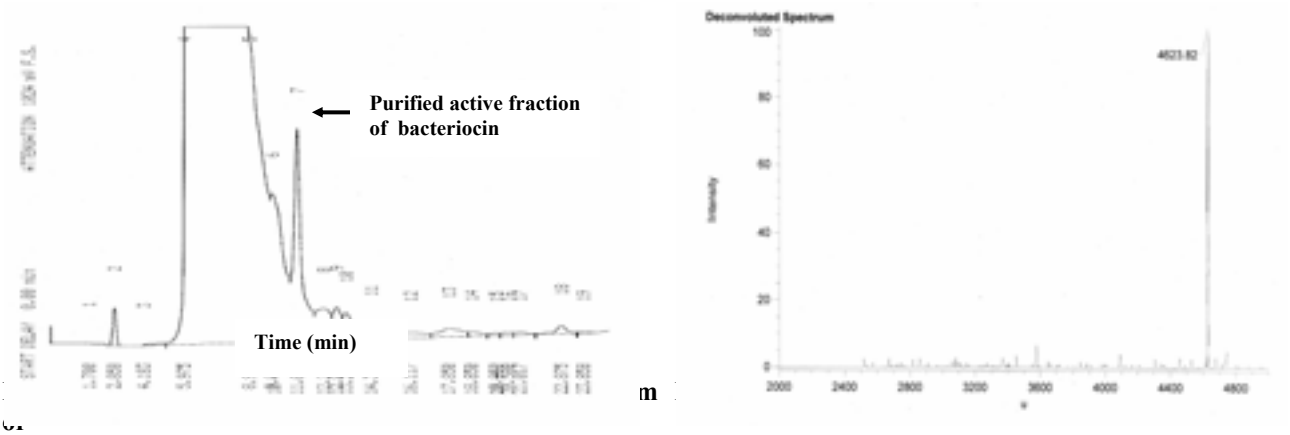
Carbohydrate	Result	Carbohydrate	Results	Carbohydrate	Results
Glycerol	-	Mannitol	-	Melezitose	-
Erythritol	-	Sorbitol	-	D-Raffinose	-
D-Arabinose	-	α Methyl-D-mannoside	-	Amidon	-
L-Arabinose	-	α Methyl-D-glucoside	-	Glycogene	-
Ribose	+	N Acetyl glucosamine	+	Xylitol	-
D-Xylose	-	Amygdaline	+	β Gentiobiose	+
L-Xylose	-	Arbutine	+	D-Turanose	-
Adonitol	-	Esculine	+	D-Lyxose	-
β Methyl-xyloside	-	Salocine	+	D-Tagatose	+
Galactose	+	Cellobiose	+	D-Fucose	-
D-Glucose	+	Maltose	+	L-Fucose	-
D-Fructose	+	Lactose	-	D-Arabitol	-
D-Mannose	+	Melibiose	-	L-Arabitol	-
L-Sorbose	-	Saccharose	-	Gluconate	-
Rhamnose	-	Trehalose	+	2 ceto-gluconate	-
Dulcitol	-	Inulin	-	5 ceto-gluconate	-
Inositol	-				

+ = positive result of Carbohydrate fermentation after incubating at 35° C for 48 hours ,
- = no fermentation occurred after incubating at 35° C for 48 hours

Table 3 : Inhibitory spectrum results of antagonistic substances produced by TISTR 536 using MRS cell-free cultured supernatant spot on lawn (AU/ml)

Indicator strains (Source)	Cell-free cultured spot on lawn (AU/ml)
Bacillus circulans (JCM 2504^T)	0
B. coagulans (JCM 2257^T)	0
B. subtilis (JCM 1465^T)	0
Enterococcus faecalis (JCM 5803 ^{T})	800
Escherichia coli (JM 109)	0
Kocuria varians (LTH 1545)	6,400
Lactobacillus plantarum (ATCC 8014)	0
L. plantarum (ATCC 14917^T)	6,400
L. sakei subsp. *sakei* (JCM 1157 ^{T})	6,400
Lactococcus lactis subsp. *lactis* (ATCC 19435^T)	0
Lc. lactis subsp. *cremoris* (TUA 1344L)	1,600
Leuconostoc mesenteroides (JCM 6124^T)	1,600
Listeria innocua (ATCC 33090 ^{T})	6,400
Lis monocytogenes (ATCC 19117)	6,400
Micrococcus luteus (IFO 12708)	0
Pediococcus pentosaceus (JCM 5885)	400
P. pentosaceus (JCM 5890 ^{T})	200
Salmonella anatum (WHO-BKK)	0
Staphylococcus aureus (ATCC 12600 ^{T})	0
S. carnosus (LTH 2102)	0

Bacteriocin purification and Molecular mass determination : The bacteriocins from TISTR 536 were purified after subjecting to C_2/C_{18} reverse-phase (PepRPC HR 5/5, Amersham Pharmacia Biotech) chromatography. The active fraction of purified bacteriocin (Fig. 1) were then analyzed by ion spray mass spectrometry. The results (Fig. 2) implied that the molecular mass of this bacteriocin was related to pediocin PA-1 (4,623.82 Da) produced from *P. acidilactici* PAC1.0 [16] and *L. plantarum* WHE 92 [17].



TISTR 536 after subjected to C_2/C_{18} reverse-phase HPLC

bacteriocin from TISTR 536

PCR analysis and DNA sequencing of bacteriocin from TISTR 536 : In order to prove that the bacteriocin produced by strain TISTR 536 was pediocin PA-1, PCR analysis using the known sequences of the pediocin structural gene was performed. The expected 300 bp fragment containing the structural gene of pediocin PA-1 of TISTR 536 was amplified and then sequenced. The results (Fig. 3) indicated that the sequences were 100% identical to that of pediocin PA-1.

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1    GCGTTGATAGGCCAGGTTTCAAAAATTGACCAAGATCGTTAACCAGTTTGGTGCGAAAAT    60

61   ATCTAACTAATACTTGATATTTAAATTGAGTGGGAACTAGAATAAGCGCGTATTAAGGAT   120

121  AATTTAAGAAGAAGGAGATTTTTGTGATGAAAAAAATTGAAAAATTAACTGAAAAAGAAA   180
                                   K   K   I   E   K   L   T   E   K   E   M

181  TGGCCAATATCATTGGTGGTAAATACTACGGTAATGGGGTTACTTGTGGCAAACATTCCT   240
       A   N   I   I   G   G   K   Y   Y   G   N   G   V   T   C   G   K   H   S   C

241  GCTCTGTTGACTGGGGTAAGGCTACCACTTGCATAATCAATAATGGAGCTATGGCATGGG   300
       S   V   D   W   G   K   A   T   T   C   I   I   N   N   G   A   M   A   W   A

301  CTACTGGTGGACATCAAGGTAATCATAAATGCTAGCATTATGCTGAGCTGGCATCAATAA   360
       T   G   G   H   Q   G   N   H   K   C   *

361  AGGGGTGATTTTATGAATAAGACTAAGTCGGAACATATTAAACAACAAGCTTTGGACTTA   420

421  TTTACTAGGCTACAGTTTTTACTACAGAAGC                                451
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BACTERIOCIN PEDIOCIN PA-1 PRECURSOR

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Identities = 61/61 (100%), Positives = 61/61 (100%)
Score =  138 bits (347), Expect = 7e-32

Query :   KKIEKLTEKEMANIIGGKYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHK
      :   KKIEKLTEKEMANIIGGKYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHK
Subject :KKIEKLTEKEMANIIGGKYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHK
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Figure 3 : Nucleotide sequence and deduced amino acid sequence of the pediocin PA-1 gene isolated from *P. pentosaceus* TISTR 536. Primers are in bold and underline. Stop codon is shown by asterisk.

Conclusions :

The study concluded that the strain TISTR 536 is *P. pentosaceus* and this strain can produce pediocin PA-1. *P. pentosaceus* TISTR 536 is the first pediocin PA-1 producer strain isolated from Nham, a type of thai traditional fermented meat products.

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