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Full Length Research Paper

Isolation and characterization of bacteriocin with anti-listeria and anti-MRSA activity produced by food and soil isolated bacteria

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The search for a novel peptide-based antibiotic as replacement for currently used antibiotics is promising as it seems to develop less resistant compared to that of other type of antibiotic. Eighteen different food and soil samples were used for screening of a novel bioactive peptide producing bacteria. Among 1,265 isolated bacteria, one hundred and sixty seven isolated colonies showed antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and *Listeria monocytogenes* by co-culture method. The activity of partially purified bacteriocin or PPB prepared from three isolated strains, CR-1-2L, SP-1-36LM and SO-4-1LM against *L. monocytogenes* were 10^5 AU/ml, 2000 AU/ml and 1000 AU/ml, respectively. PPB from three isolated bacteria showed narrow inhibition as they are active only against gram positive bacteria. PPB produced by CR-1-2L, SP-1-36LM and SO-4-1LM was heat stable up to 100°C for 60 min and active within the pH range of 3-9. The activity of PPB prepared from three isolated strains CR-1-2L, SP-1-36LM and SO-4-1LM disappeared when treated with proteinase K, chymotrypsin and trypsin demonstrating their proteinaceous nature. These three isolated strains can be regarded as bacteriocin producing bacteria or BAC. They were all identified as *Bacillus* sp. by 16S rRNA gene sequence. The effect of PPB prepared from strain SO-4-1LM was bactericidal to *L. monocytogenes* and the highest activity was found at six hours after incubation. Tris-Tricine SDS-PAGE analysis revealed that the bacteriocin prepared from *Bacillus* sp. SO-4-1LM had an apparent molecular weight of 2.5 kDa. The bioactive peptide from these three isolated bacteria has a potential for use as an alternative antibacterial agent for the treatment of infection with MRSA and/or use in the food industry in the future.

Key words: bacteriocin, anti-MRSA, anti-listeria, *Bacillus* sp.

INTRODUCTION

Many substances used in pharmaceutical and food industries have been isolated from microorganisms. Recently, antibacterial compound known as bacteriocin have received increasing interest. Bacteriocins are ribosomally synthesized antimicrobial protein or peptide produced by bacteria that usually inhibit closely related species (Klaenhammer, 1988). The most fully studied bacteriocin is nisin A, which is produced by lactic acid

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bacterium (LAB), *Lactococcus lactis*, and has been accepted by World Health Organization as a food biopreservative (Delves-Broughton et al., 1996). Current potential use of bacteriocins are in food industry as natural and safe food preservatives while less research has been conducted on the therapeutic applications as antimicrobial agent (Gray et al., 2006).

Bacteriocin production is common among many Gram-positive bacteria (Pattnaik et al., 2005) especially soil bacteria in the genus *Bacillus* such as *B. cereus*, *B. firmus*, *B. lentus*, *B. licheniformis*, *B. pumilus*, *B. sphaericus*, *B. subtilis* and *B. thuringiensis* (Oscariz et al., 1999; Korenblum et al., 2005; Aunpad and Na-Bangchang 2007; Aunpad et al., 2007; Perez et al., 1992; Galvez et al., 1994; Korenblum et al., 2005; Sharma et al., 2006; Kamoun et al., 2005; Yilmaz et al., 2006). Like LAB, the genus *Bacillus* includes a variety of industrially species which has been granted Generally Recognized as Safe (GRAS) status by the Food and Drug Administration, USA, (Cherif et al., 2003).

The emergence and dissemination of drug resistant pathogenic bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) has been an increasing serious problem in the public health worldwide (Schmitz et al., 1998). New antibacterial agents for controlling these bacteria are urgently needed.

The major concern in using the natural food preservatives instead of chemical preservatives in food products especially in ready-to-eat and preserved seafood product has dramatically increased in recent years. The outbreaks and development of food transmission pathogenic bacteria such as *Listeria monocytogenes*, the causative agent of listeriosis, have been shown to cause severe or even fatal illness worldwide. Due to its severity and high mortality rates have led to widespread public concern (Gahan and Collins, 1991).

The use of bacteriocins as replacement for currently used antibiotics is promising, and the isolated bacterium in the genus *Bacillus* might be a potential candidate strain for using as an alternative source of peptide antibiotics in the future. These bacteriocins might be also used as a food preservative for controlling food transmission pathogenic bacteria in the food industry.

MATERIALS AND METHODS

Sample collection, bacterial isolation and growth conditions

Eighteen different food and soil samples were collected from three different areas from Thailand. The samples were taken aseptically in sterile polypropylene tube and kept at 4°C during t ransportation. For soil sample, each 1.0 g of the sample was resuspended in 9.0 ml Tryptic soy broth (TSB, Difco, USA) and shaken by vortexing for 2 min. For food sample, each 1.0 g of the sample was homogenized in 9.0 ml of de Man Rogosa broth (MRS, Difco, USA). The suspension was incubated at 37°C overnig ht and serially diluted from 10^{-1} to 10^{-3} in 0.1% peptone. One hundred μl of the liquid sample was directly spread on TSA (Difco, USA) and MRS agar (Difco, USA). Then plates were incubated at 37°C for 18 to 24 h.

Screening of bacteriocin producing strains

The single colonies obtained from each sample were screened for their antibacterial activity against two indicator strains, methicillin resistant *S. aureus* (MRSA, DMST 5199) and *L. monocytogenes* by co-culture method as described by Rosado and Seldin (1993).

Bacterial strain identification

Three isolated strain CR-1-2L, SP-1-36LM and SO-4-1LM were identified according to its 16S rRNA gene sequence (Brosius et al., 1978). The nucleotide sequence was compared with genbank nucleotide database using Blastn search (http://www.ncbi.nlm.nih.gov/blastn) in order to identify the strain.

Determination of bacteriocin activity

The antibacterial activity of bacteriocin was detected by an agar-well diffusion method (Tagg and Mac-Given, 1971) and bacteriocin activity (AU/ml) was determined by the serial dilution method (Jansen and Hirschmann, 1994). The assay for each sample was done in triplicate.

Preparation of partially purified bacteriocin (PPB)

Three isolated strain CR-1-2L, SP-1-36LM and SO-4-1LM were selected for further study. A 200 ml TSB medium was inoculated with 1% (10^6 CFU/ml) of an overnight culture of each isolate. The cultures were incubated at 37°C for 16-18 h with shak ing. Following cultivation, cell-free culture supernatant was obtained through centrifugation at 8,000 × g for 20 min (Sorvall Biofu ge, Mandel Scientific, Canada) followed by sterile filtration. Ammonium sulfate (103.2 g) was added to the supernatant while stirring to reach 80% saturation and left overnight at 4°C. The sample was centrifuged at 8000 × g for 50 min. Then the supernatant was discarded and the precipitate was dissolved in 5 ml of sterile distilled water and dialyzed against 1.5 L of sterile distilled water for 16 to 18 h. The active supernatant was designated as partially purified bacteriocin or PPB.

Spectrum of inhibitory activity

Each PPB was used to assess the antibacterial activity against a total of 17 selected Gram-positive and Gram-negative test bacteria by the agar-well diffusion method (Tagg and Mac-Given, 1971). Equal volume of sterile distilled water was used as control solution. The appearance of the inhibition zone was determined after 18 h of incubation.

Enzyme sensitivity, heat and pH stability

The PPB was treated at 37°C for 1 h with 1 mg/ml final concen-tration of the following enzymes: trypsin, α–chymotrypsin and proteinase K (Sigma-Aldrich, USA). After incubation, the reaction

mixtures were boiled for 10 min to inactivate the enzymes and the residual antibacterial activity was measured by agar-well diffusion (Tagg and Mac-Given, 1971). Thermal stability of bacteriocin was investigated by determination of the residual antibacterial activity after incubation of PPB at different temperature ranging from 40 to 100°C for 30 and 60 min, and at 121°C for 15 min. To investigate the effect of pH, antibacterial activity was measured following the pH adjustment of the bacteriocin with 0.1 N NaOH or 0.1 N HCl and incubation at 4ºC for 1 h.

Assessment of mode of action

To determine the mode of action of PPB from strain SO-4-1LM, bacteriocin (final concentration 50 AU/ml) was added to mid-logarithmic growth phase cells of *L. monocytogenes* in 200 ml TSBYE. Equal volume of sterile distilled water was added to the control flask. The culture was incubated at 37°C and samples were taken at different time points and OD was measured at 600 nm. Number of viable cells (CFU/ml) was measured by standard plate counting method on TSAYE plates.

Molecular weight determination

The molecular weight of bacteriocin from isolated strain SO-4-1LM were determined by the Tris-Tricine SDS–PAGE with 5% stacking gel and 16% separating gel (Schagger and von Jagow, 1987). The first half of the gel (protein gel) was stained with PageBlue Protein staining solution (Fermentas, USA) whereas another half of the gel (activity gel) was washed in sterile distilled water for 30 min and overlaid with TSBYE (0.8% agar) seeded with 1% (v/v) *L. monocytogenes* and incubated at 37°C for 16 to 18 h. The formation of clear halo or inhibition zone was observed and compared with protein gel.

RESULTS

Among 1,265 bacteria with different colony morphology isolated from fourteen food and four soil samples, one hundred and sixty seven isolated colonies showed antibacterial activity against methicillin resistant *S. aureus* (MRSA) and/or *L. monocytogenes* by co-culture method. There were three Gram-positive isolated bacteria strain CR-1-2L, SP-1-36LM and SO-4-1LM showing antimicrobial activity against *L. monocytogenes* and/or MRSA with zone of inhibition larger than 5 mm. All were identified as *Bacillus* sp. with 99% identity according to their partial 16S rRNA gene sequence and selected for further study.

The antimicrobial activity spectrum of partially purified bacteriocin (PPB) from three isolated *Bacillus* sp. against 17 test microorganisms was examined by agar diffusion method. All of them showed antibacterial activity against only Gram-positive test bacteria however they also showed weak inhibitory effect to some of Gram-negative test microorganisms (Table 1). *Bacillus* sp. CR-1-2L displayed the broadest spectrum of activity as it inhibited nearly all of Gram-positive microorganisms (Table 1).

The PPB prepared from three isolated strains was tested for sensitivity to various proteolytic enzymes (trypsin, α–chymotrypsin and proteinase K), a key criterion for bacteriocin characterization. The complete inactivation was observed after treatment with all proteolytic enzymes (Table 2). Temperature stability experiment revealed that PPB from three isolated *Bacillus* sp. was completely stable at high temperature up to 100°C for 30 min (Table 2). With regard to pH sensitivity, antibacterial activity of PPB was highest at pH 7-8 and the activity was maintained at high level within the pH range of 3.0 to 9.0 (Table 2). They lost only 20% of activity when being exposure to pH below 4.0.

To determine whether the bacteriocin of isolated *Bacillus* sp. SO-4-1LM was bactericidal or bacteriostatic, a 50 AU/ml final concentration of PPB was added to a mid-logarithmic culture of *L. monocytogenes*. A strong decrease in the viability of *L. monocytogenes* was observed over a period of 6 h (Figure 1). No regrowth was observed after 18 h in the presence of bacteriocin and the O.D. reading of indicator strain was nearly constant during this period (data not shown).

The molecular weight of bacteriocin from isolated *Bacillus* sp. SO-4-1LM was determined by Tris-Tricine SDS-PAGE analysis of PPB. As shown in Figure 2b, a single protein band with clear halo revealed a bacteriocin activity. The band had an apparent molecular mass of 2.5 kDa.

DISCUSSION

The number of methicillin resistant *S. aureus* or MRSA infections has been increasing and become a serious problem in public heath worldwide. Novel antibacterial agents are urgently needed to combat this drug resistant problem. Recently, a variety of bacteriocins have attracted attention for their potential use as food preservatives while less research has been conducted on the therapeutic applications as antimicrobial agent. The use of bacteriocin as an alternative agent to overcome the problem is promising (Papagianni, 2003). Hence in this study, new bacteriocin-producing bacteria (BAC) were isolated and their biochemical properties were characterized.

Although the colony morphology of three Gram positive isolated bacteria strain CR-1-2L, SP-1-36LM and SO-4-1LM showing high anti-MRSA and/or anti-listeria activity was different, all were identified as *Bacillus* sp. with 99% identity according to their partial 16S rRNA gene sequence. It is well documented that bacteriocin or bacteriocin-like production is common among different *Bacillus* species (Oscariz et al., 1999; Korenblum et al., 2005; Aunpad and Na-Bangchang 2007; Aunpad et al., 2007; Perez et al., 1992; Galvez et al., 1994; Korenblum et al., 2005; Sharma et al., 2006; Kamoun et al., 2005; Yilmaz et al., 2006). Most of them can inhibit only Gram positive bacteria and less effective against Gram negative

Table 1. Inhibitory spectrum of PPB from three isolated *Bacillus* sp.

Strain	Source*	Growth medium	Incubation temperature (ºC)	Inhibitiory activity†		
				SO-4-1LM	CR-1-2L	SP-1-36LM
Gram-positive						
Bacillus cereus	MT	TSA	37	w	+	w
Bacillus pumilus	WAPB4	TSA	37	++	+	w
Bacillus subtilis	ATCC6633	TSA	37	-	-	-
Bacillus sphearicus	SOPB1	TSA	37	-	++	++
Enterococcus sp.	MT	BHI	37	-	-	-
Vancomycin resistant						
Enterococcus (VRE)	DMST4737	BHI	37	-	-	-
Staphylococcus aureus	MT	TSA	37	-	w	-
Methicillin resistant						
S. aureus (MRSA)	DMST5199	TSA	37	+	w	+
Listeria monocytogenes	MT	MRS	37	++	++	++
Gram-negative						
Escherichia coli	O157	TSA	37	w	-	-
Ampicillin resistant						
E.coli (ARE)	DMST19374	TSA	37	-	-	-
Salmonella typhi	MT	TSA	37	w	w	-
Salmonella typhimurium	MT	TSA	37	-	-	-
Shigella dysenteriae	MT	TSA	37	-	-	-
Shigella flexneri	MT	TSA	37	-	-	-
Shigella boydii	MT	TSA	37	-	-	-
Shigella sonnei	MT	TSA	37	w	-	-

*ATCC, American Type Culture Collection; MT, Department of Medical Technology, Thammasat University, Thailand; DMST, Department of Medical Sciences, Ministry of Public Health Thailand.

†(-) no inhibition, (w) weak inhibition (less than 1 mm of inhibition zone), (+) mild inhibition (1-5 mm of inhibition zone), (++) strong inhibition (more than 5 mm of inhibition zone).

strains. The partially purified bacteriocin (PPB) prepared from three gram positive isolated *Bacillus* sp. strain CR-1-2L, SP-1-36LM and SO-4-1LM was active only against Gram positive bacteria under investigation with high anti-bacterial activity against MRSA and *L. monocytogenes*. Two of them, SO-4-1LM and SP-1-36LM, can inhibit MRSA but no antagonistic activity observed against drug sensitive *S. aureus*. These strains might be used as an alternative source for specifically curing MRSA in the future.

The sensitivity of PPB from three isolated *Bacillus* sp. to proteinase K, trypsin and α-chymotrypsin suggests the proteinaceous nature of these antimicrobial substances. Therefore, these isolated bacteria can be regarded as bacteriocin producing bacteria or BAC. The bacteriocin from these three isolated *Bacillus* sp. was heat stable as evidenced by its ability to reserve the activity at 100°C. In addition, it was stable within a wide range of pH (3 to 9). The heat stable property was also observed in two bacteriocins, that is, entomocin 9 and AMS T6-5, produced by *B. thuringienesis* HD9 (Cherif et al., 2003) and *B. licheniformis* T6-5 (Korenblum et al., 2005), respectively. The stability of bacteriocin from these three isolated *Bacillus* sp. at high temperature and over a wide range of pH indicates the potential application in agro-industries as they could preserve their activity at extreme conditions.

Although three isolated strain CR-1-2L, SP-1-36LM and SO-4-1LM were identified as *Bacillus* sp., the bacteriocin prepared from each strain showed different properties and spectrum of inhibitory. *Bacillus* sp. strain SO-4-1LM was selected for further study due to its good physiochemical properties and also high anti-MRSA and anti-listeria activity. The decline in the number of living

Table 2. Effect of enzymes, temperature and pH on PPB.

Treatments and conditions	Residual activity (%)		
	SO-4-1LM	CR-1-2L	SP-1-36LM
None (control)	100	100	100
Enzyme treatment			
Trypsin	20	5	5
α–chymotrypsin	5	5	5
Proteinase K	0	10	0
Temperature			
40°C, 30 min	100	100	100
40°C, 60 min	100	100	100
60°C, 30 min	95	93	92
60°C, 60 min	93	93	77
80°C, 30 min	95	93	69
80°C, 60 min	95	86	77
100°C, 30 min	80	71	69
100°C, 60 min	76	0	0
121°C, 15 min	0	0	0
pH			
3.0	92	96	78
4.0	80	96	89
5.0	100	93	93
6.0	88	91	100
7.0	100	100	100
8.0	100	94	100
9.0	88	93	100

cell of *L. monocytogenes* over a period of 6 h after the addition of PPB suggested that the bacteriocin prepared from *Bacillus* sp. strain SO-4-1LM has a bacteriocidal effect without cell lysis. This was supported by the nearly constant O.D. readings recorded after the addition of the bacteriocin (data not shown). The stable O.D. indicated that the cells of *L. monocytogenes* were not lysed. Most of bacteriocin from *Bacillus* sp. showed a bactericidal effect with or without cell lysis (Aunpad and Na-Bangchang, 2007; Hyronimus et al., 1998; Cherif et al., 2003; Gray et al., 2006).

The molecular weight of bacteriocin from *Bacillus* sp. strain SO-4-1LM as determined by Tris-Tricine SDS-PAGE analysis was 2.5 kDa. The low molecular masses have been reported for several *Bacilli* bacteriocins such as 3.160 kDa bacthuricin F4 (Kamoun et al., 2005), 3.162 kDa thuricin 17 (Gray et al., 2006) and 3.4 kDa coagulin (Hyronimus et al., 1998), 2.97 and 3.44 kDa ericin A and S (Stein et al., 2002), 1.02 kDa iturins, 1.46 kDa fengycin and 1.03 kDa surfactin (Kim et al., 2010). It is to our knowledge, there was no data revealing this size of bacteriocin produced by *Bacillus* sp. Therefore, this is the novel bacteriocin produced by *Bacillus* sp.

Conclusion

Many bacteriocin producing bacteria (BAC) with high anti-MRSA and/or anti-listeria activity was isolated from food and soil samples in Thailand. These BAC strains are non-pathogenic and derived from the food and nature. The biochemical properties such as thermal stability and wide range pH stability are remarkable. The bacteriocin produced by these microorganisms might be used as an alternative source for controlling drug resistant *S. aureus* or using in the food industry as food preservatives in the future.

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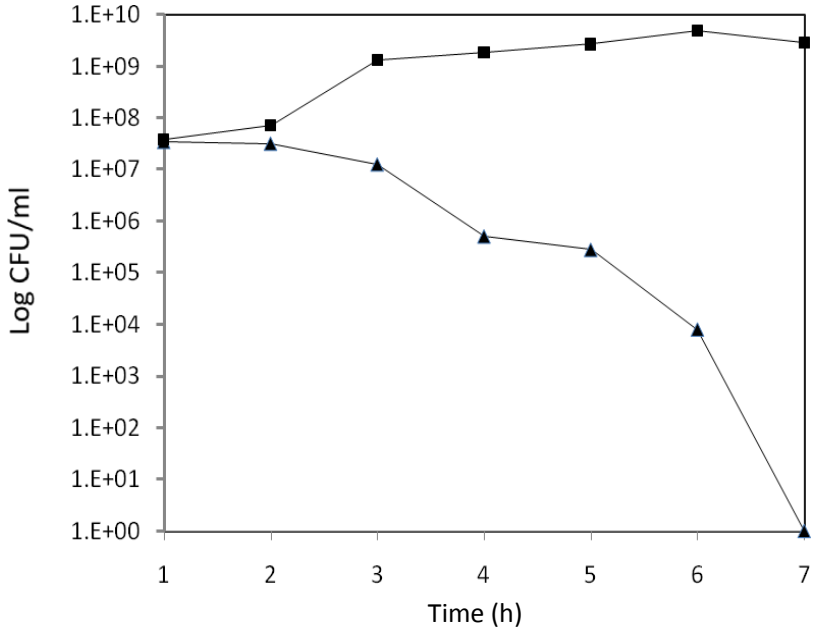

Figure 1. Effect of PPB prepared from *Bacillus* sp. SO-4-1LM on the growth of *L. monocytogenes*. The results are expressed as log CFU/ml counted in the presence of 50 AU/ml (GRSA) of PPB after addition to mid logarithmic growth phase cells. Control was incubated with DW (GRSA).

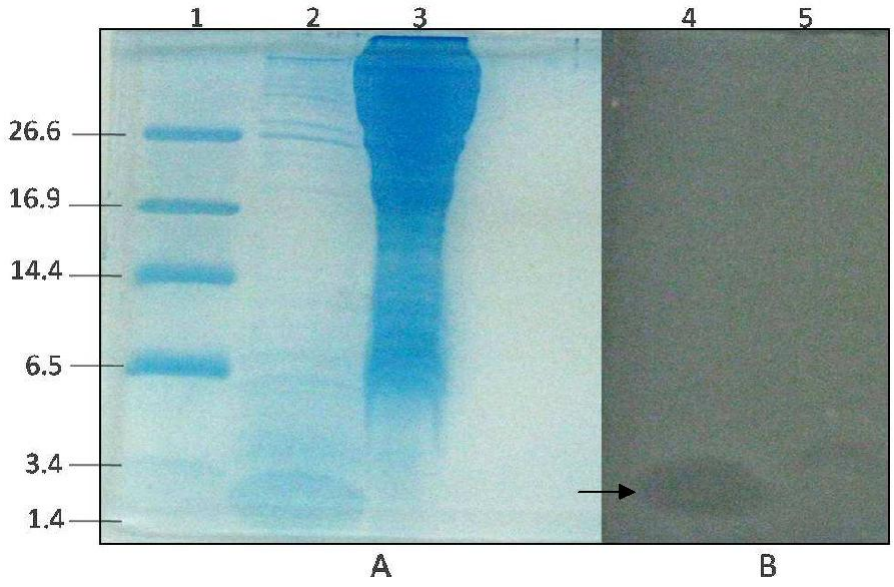

Figure 2. Tris-Tricine SDS PAGE analysis of PPB prepared from *Bacillus* sp. SO-4-1LM. (A) Coomassie brilliant blue stained gel (B) The activity gel shows the clear zone (arrow) after overlaid with TSBYE (0.8% agar) seeded with *L. monocytogenes* and incubated overnight. Lane 1: Peptide molecular weight marker; lane 2 and 4: PPB; lane 3 and 5: cell supernatant after sonication.

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