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Characteristic and the mode of action of Bacteriocin produced by *Brevibacillus laterosporus* SA14 which isolated from the air

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Brevibacillus laterosporus SA14 which isolated from the air sample at Walailak University in Thailand which is used as tester strain for antibacteria activity and was found highly produced bacteriocin at 8th days of culturing. Bacteriocin which inhibited gram positive bacteria caused hospital-acquired and community-acquired infections, such as, *Staphylococcus aureus* TISTR 517 and MRSA1424 (methicillin-resistant *Staphylococcus aureus*) by agar well diffusion method. It has shown a higher activity against indicator strains when compared with oxacillin and cell free supernatant which is not ammonium precipitation. In addition, the specific activity increased to 800 AU/ml, corresponding 88% of recoveries, 32 fold of purification. The mode of action of bacteriocin which has shown bacteriostatic activity with Optical density (OD) at 600 nm and Colony-Forming Units (CFU), were continuously decreased. When the study under electron microscope has found cells morphology alteration, such as, enlarged, blistered and hollowed. The molecular weight of this bacteriocin belows 6.9 kDa determined by SDS-PAGE. Moreover, the antibacterial activity of this bacteriocin is not affected by SDS, Triton X-100, pH value 8-9 and the range of different temperature while antibacterial activity slightly lost when it was treated with pH value 2-7, 10-11, proteinase K, pronase E, EDTA and Tween80.

Keywords: Bacteriocin, *Brev. laterosporus*, *S. aureus*, MRSA, mode of action.

Introduction

Bacteriocins or bacteriocin-like substances (BLS) are small ribosomal cationic peptides synthesized by gram positive and negative bacteria. Their spectrum activity is narrow, which is generally against closely related strains (Tagg *et al*., 1976). *Escherichia coli* producing colicin is the first bacteriocin discovered by Gratia and co-worker in 1925. For gram positive bacteria, bacteriocin and bacteriocin producing strain of the lactic acid bacteria have been investigated in food industry because they are the preservative food

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against infection bacteria because they enhance food safety and also it is generally regarded as safe (GRAS) (Ray and Daeschel, 1994). Most of bacteriocins were produced by a small lactic acid bacteria, cationic and amphipathic peptides (Klaenhammer, 1993). Nowadays, bacteriocins were divided by 5 classes. Class I - III are the main class ((Nissen-Meyer *et al*., 1997). Class I is a post translationally modified lantibiotics peptides, and it is stable to heat and low molecular mass (Sahl *et al*., 1995). Class II unmodified peptides is stable to heat as same as class I (Stoddard *et al*., 1992). Class III, is a large heat labile peptide. Class IV being a peptide has lipid or chabohydrate in the structure. Class V being a peptide has cicular structure because of its form from linkage between N-terminal and C-terminal. Now, *Bacillus* species can produce total 167 antibiotic compounds (Katz and Demain, 1977) and 68 for antibiotic peptides, such as, bacillomycin, iturin, mycosubtilin, as well as the other compounds, for example, amylase and protease (Harwood, 1989; Boer and Diderichsen, 1991). *Bacillus* species produced many antimicrobial peptides by using biopreservatives in food and beverage industry (Zheng and Slavic, 1999). In some case, antimicrobial peptide is used as natural compound for biocontrol of phytopathogents (Bais *et al*., 2004). Moreover, Shida *et al*., 1996 changed its class'*Bacillus laterosporus'*into '*Brevibacillus laterosporus'* which produced many peptides against bacteria. In the recent year, *Brev. Laterosporus* is able to produce many antibiotics, such as, Espergualin and Bacithrocins (A, B and C) which important using for making medicine (Nemoto *et al*., 1987; Umezawa *et al*., 1987; Kamiyamaetal, 1994). *Brev. laterosporus* G4 produced protein with molecular weight approximately 28.7 kDa and has shown to kills free-living nematodes (*Panagrellus redivius*) and also, plant-parasite nematodes (*Bursaphelenchus xylophilus*) (Tain *et al*., 2006). Zubasheva *et al*., 2010 has found that *Brev. laterosporus* produced protein which exhibited larvacidal activity against larva of mosquitoes *Anophelis stephensi* and *Aedes aegypti*. Whereas, *Brev. laterosporus* MTCC 2298 has produced lignin peroxidase which degradingly debased of sulfonated azo dyes (Gomare *et al*., 2008). From the study of Prasanna *et al*., 2012, they have reported that the novel strain *Brev. laterosporus* Lak1210 has produced chitinase which has shown an activity against the phytopathogenic fungus *Fusarium equiseti*. It also exhibited toxicity to larvae of diamondback moths (*Plutelia xylostella*). For general mechanism of bacteriocin, it is against the target cell and it is also able to make cells die.

Firstly, mechanism bacteriocin can be the permeabilization and it make pore at cell membrane of target cell (Heerklotz *et al*., 2004). Some cases of bacteriocin need a receptor on surface of target cell (Hechard and Sahl, 2002). Secondly, DNA synthesis was inhibited by bacteriocin and it is able to make cell die (Blondelle *et al*., 1999). Thirdly, bacteriocin inhibited correct cell wall

synthesis which found in gram positive bacteria and leading to death. The objective of study is to investigate the characterization of bacteriocin from *Brev. laterosporus* SA14 and to study the mode of action in order to inhibit the growth of other bacteria ,such as, *Staphylococcus aureus* TISTR 517 and MRSA1424 (methicillin-resistant *Staphylococcus aureus*).

Materials and methods

Bacterial strains and media

Brev. laterosporus SA14, a bacteriocin producing strain was isolated from the air sample at Walailak University, Thailand, and cultured in Luria-Bertani (LB: Scharlau) medium at 37°C. *S. aureus* TISTR 517 as well as MRSA142 (methicillin-resistant *Staphylococcus aureus*) were grown in Luria-Bertani (LB: Scharlau) medium at 37°C.

Bacteriocin production

Brev. Laterosporus SA14 was cultured in LB broth for 10 days. At the interval time, the medium culture was harvested for everyday. Cells were removed by centrifugation at 10000 rpm, 4°C for 30 min. Cell free supernatant of each day was used for testing antibacterial activity against *S. aureus* TISTR 517 and MRSA142 with agar well diffusion method.

Antibacterial activity

Brev. laterosporus SA14 was cultured in LB broth at 37 °C, 150 rpm. Bacteriocin activity was determined with the agar well diffusion method using 10^8 cells of the indicator strains and 80 µl of the bacteriocin. The assays were performed by using *S. aureus* TISTR 517 and MRSA142 as the indicator strains.

Ammonium precipitation

Brev. Laterosporus SA14 was cultured in LB broth for 8 days. Cells were removed by centrifugation at 10000 rpm, 4°C for 30 min. Ammonium sulfate was added to the cell free supernatant while it was stirring to reach a saturation of 50%. The precipitate was collected by centrifugation at 12000 rpm, 4°C for 30 min, which dissolved in phosphate buffer. After that, it dialysed against the same buffer overnight in dialysis tube (MW cut-off, 3,500). The solution obtained that it contained the antimicrobial activity, designated as the

ammonium sulphate precipitate (ASP), crude protein. The sample was stored at −20 °C until further use.

Determination of the Arbitrary Units (AU)

Crude protein and cell free supernatant were diluted to two-fold dilution. Both indicator strains, *S. aureus* TISTR 517 and MRSA142 were cultured in LB both at 37 °C for 24 h. The culture medium of each indicator strain was adjusted to McFarland standard No. 0.5 and it was used for swabbing on surface of LB agar. It took 10 minutes for the agar surface to well dry. It was filled with 80 µl of two-fold dilution of crude protein or cell free supernatant.

Polyacrylamide gel electrophoresis

The crude protein was examined on SDS-PAGE (5% and 15% acrylamide for the stacking and separating gels, respectively) as described by Laemmli, 1970. Prestained SDS-PAGE standards broad range (Bio-rad) was used as protein markers. After electrophoresis, the gel was divided into two parts. One part was stained with Coomassie brilliant blue R-250 and the other part was washed three times with sterile water and was used to test activity by placed the gel on LB agar and overlaid with soft agar containing indicator strain.

Mode of Action

The mode of action was according to the method suggested by Kayalvizhi and Gunasekaran (2010). The crude protein (final concentration at 800AU/mL) was added to mid-log phase growing cells of *S. aureus* TISTR517 and MRSA142 in LB broth. Growing cells of *S. aureus* TISTR517 and MRSA142 in LB broth without crude protein was used as control. The optical density of cultures broth were recorded at 600 nm and the number of viable cells were done by plating on LB agar.

Scanning electron microscopy

Samples were taken from exponentially growing cultures of *S. aureus* TISTR517 and MRSA142 treated and untreated with crude protein (800 AU/ml). Cells were harvested by centrifugation and washed twich and 0.15M phosphate buffer pH 7.2. The cells were fixed with 2.5 % Glutaraldehyde ($C_5H_8O_2$) for 1-2 h and then washed twich with phosphate buffer and washed twich with distilled water. Dehydration was done in a graded acetone series (5—100%). The samples were dried by Critical Point Drying method and the

samples were laid on Stub by fixed with carbon tape, carbon paint. The samples were coated with gold by using sputter Coater and observed with scanning electron microscope.

Stability of the antimicrobial activity

Stability of antimicrobial activity was slightly modified from the method seggested by Bendjeddou *et al*. (2012). To study heat stability, 500 µl of crude protein was treated at temperature range from 30°C, 50°C, 70°C for 30 min, 100°C for 5, 10, 20, 30, 40, 50 and 60 min, and 121°C for 5, 10 and 15 min. For pH stability test, the sample was adjusted to the different of pH values with 1M NaOH or 1M HCl (pH 2.0~11.0) and incubated for 1 h at 37°C, neutralized to pH 7.0. It was tested for antimicrobial activity. Similarly, to analyse the effect of detergent, the crude protein was incubated with Tween80, EDTA, Triton X-100 and SDS for 1 h at 37°C. For the effect study of enzyme on antibacterial activity, 100 µl of crude protein was incubated with proteinase K and pronase E (final concentration 1 mg/ml) at 37°C for 1 h. And then, the reaction were stopped by boiling for 3 min. After that, it was tested for antimicrobial activity. The untreated crude protein was used as like the control.

Results

Antibacterial activity

The sterile cell free supernatant of *Brev. Laterosporus* SA14 exhibited highest antibacterial activity against *S. aureus* TISTR517 and MRSA142 from the 8^{th} day to the 10^{th} day. When total protein in supernatant were calculated, it was found that was not different from the 8^{th} day to the 10^{th} day. However, no zone of inhibition was observed around the LB broth. So, the observation of inhibition did not due to the culture media (Data not shown). The culture medium from the 8^{th} day of *Brev. Laterosporus* SA14 after ammonium precipitation at 50% have saturated and dialysed. Crude protein has shown more activity against indicator strains when compared with supernatant (Data not shown). Moreover, when compared antibacterial activity between crude protein and oxacillin which is against *S. aureus* TISTR517 and MRSA142, found that *S. aureus* TISTR517 and MRSA142 are susceptible to crude protein but resistant to oxacillin (Data not shown). **Table 1.** Effect of heats, chemicals, enzymes and pH on the antimicrobial activity of crude protein from *Brev. laterosporus* SA14

Treatment	Activity	
	S. aureus TISTR517	MRSA142
Heat		
30°C, 50°C, 70°C /30 min	+++	+++
100°C/5, 10, 20, 30, 40, 50, 60 min	+++	+++
121°C/5, 10, 15 min	+++	+++
Chemical		
EDTA, Tween80	++	++
SDS, Triton X-100	+++	+++
Enzyme		
Proteinase K	++	++
Pronase E	++	++
pH		
2-5	++	++
6-7	++	+++
8-9	+++	++
10-11	++	++

Activity was determined by agar well diffusion method against *S. aureus*TISTR 517 and MRSA142. +++ = zone of inhibition ≥ 20 mm; ++ = zone of inhibition 10-19 mm; + = zone of inhibition< 10 mm; - = none zone of inhibition was observed. All experiment were done in triplicate

Determination of the Arbitrary Units (AU)

For the determination of AU in cell free supernatant or crude protein was calculated from highest dilution that exhibited inhibition zone to against indicator strains. From this experiment which has shown the specific activity, it increased from 25 AU/ml in supernatant to 800 AU/ml in crude protein, corresponding to 88% recovery and 32 purification fold (Table 2).

Table 2. Antibacterial activity recoveries of crude protein of *Brev. latersporus* SA14

Stage	Supernatant	Ammonium sulfate precipitate (crude protein)
Volume (ml)	2000	55
Specific activity (AU/ml)	25	800
Total activity (AU)	50000	44000
Purification (fold)	1	32
Recovery (%)	100	88

Mode of action

The Mode of action of crude protein produced by *Brev. Laterosporus* SA14 against *S. aureus* TISTR517 and MRSA142 were investigated. The addition of crude protein at 800 AU/mL concentrations to mid-log phase of culture medium has been found that cells viability of *S. aureus* TISTR517 and MRSA142 were continuously decreased with optical density at 600 nm. When counting the number of cell viability of indicator strains by plating on LB agar, it caused the reduction in Colony Forming Unit (CFU) of growing cultures with the passage time (Fig 1).

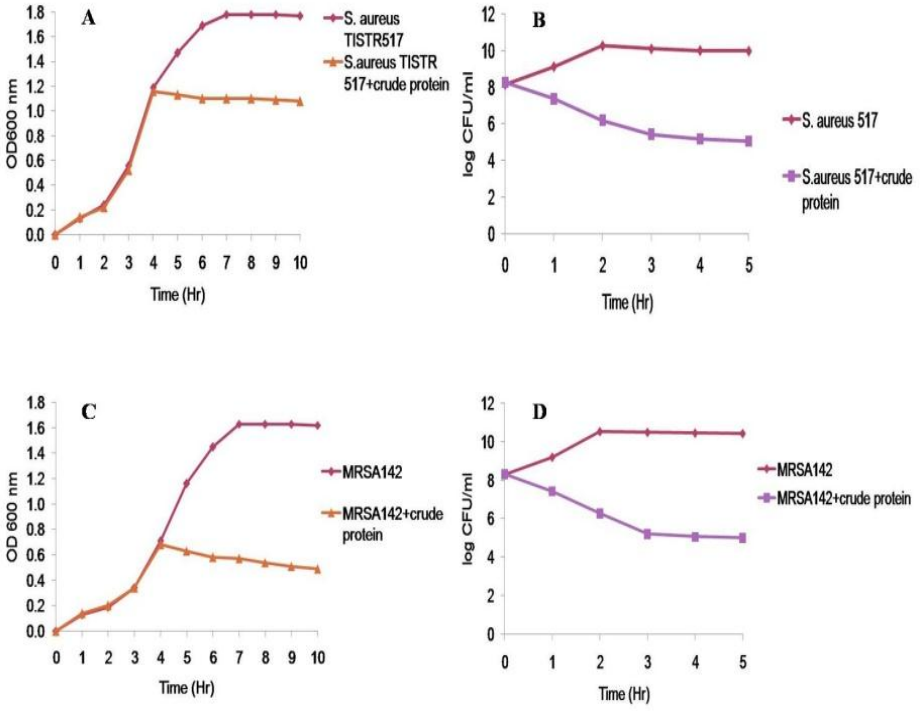

Fig. 1. Antibacterial activity of crude protein from *Brev. laterosporus* SA14. Crude protein (final concentration, 800 AU/ml (▲)) was added to the growing cells of the indicator strains (A, B) *S. aureus* TISTR517 and (C, D) MRSA142 after 4 h of growth. For control (♦), culture of indicator strains were grown without the addition of crude protein.

Polyacrylamide gel electrophoresis

The supernatant was precipitated with ammonium sulfate at saturaton of 50%. The precipitant or crude protein showing the antimicrobial activity was resolved in SDS-PAGE. Molecular weight was estimated below 6.9 kDa. For

the gel assay of inhibition activity of this crude protein against MRSA142, it was confirmed for its antibacterial activity (Fig 2).

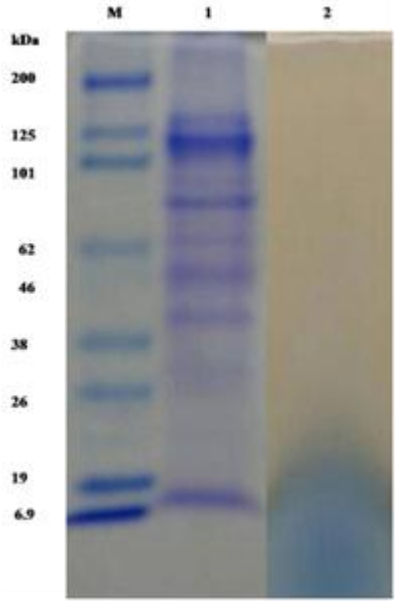

Fig. 2. Tricine SDS-PAGE analysis of antimicrobial protein from *Brev. Laterosporus* SA14. Lane M, Molecular weight marker; Lane1, crude protein from *Brev. Laterosporus* SA14 by ammonium precipitation; Lane2, Direct overlay of SDS-PAGE gel showing clear zone against MRSA142

Scanning electron microscopy

The impact of the crude protein on morphology of exponential phase *S. aureus* TISTR517 and MRSA142 cells were investigated with scanning electron microscopy (magnification of x30000). Untreated cells has shown intact cells of *S. aureus* TISTR517 and MRSA142, which are typical cocci (Fig 3A, 3D). When it was held in contact with crude protein (800 AU/ml) for 3 and 5 h, it has shown the alteration in morphology, with hollowed, turgided, blistered and enlarged (Fig 3B, 3C, 3E, 3F).

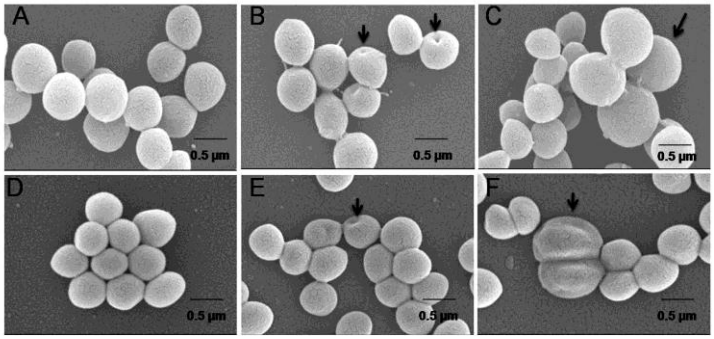

Fig. 3. Scanning electron micrographs of untreated *S. aureus* TISTR517 and MRSA142 (A and D) and treated cells with 800 AU/ml of crude protein from *Brev. laterosporus*SA14 after 3h (B and E) 5h (C and F). Magnification of x 30000. (Upper panel, *S. aureus* TISTR517; Lower panel, MRSA142)

Stability of the antimicrobial activity

The part of stability test was found that crude protein still retained activity when treated with at temperatures ranging from 30°C, 50°C, 70 °C for 30 min, 100°C for 5, 10, 20, 30, 40, 50 and 60 min, and 121°C for 5, 10 and 15 min. The activity of crude protein is stable after incubated at pH 8-9. It was treated with Triton X-100 and SDS while the activity has slightly lost at pH 2-7, 10-11. Then, it was treated with EDTA, Tween80. Similarly, the results was treated with enzymes (Table 1).

Discussions

In this study, it used *Brev. laterosporus* SA14 as bacteriocin producing strain. From the result, it has shown high bacteriocin production at the 8th day of culturing in LB broth at 37°C, 150 rpm. After ammonium precipitation, crude protein, which has shown high activity when compared with cell free supernatant, it might be the crude protein being partial purify and increasing of the concentrate. When the test of antibacterial activity between crude protein and oxacillin were found, the indicator strains resistant to oxacillin but sensitive to crude protein. Similarly result from bacteriocin was produced by *Lactobacillus paracasei* subsp. *Paracasei* BMK2005. It has shown inhibition zone against multridrug-resistant pathogens (Bendjeddou *et al*., 2012). The mode of action of bacteriocin was found the bacteriostatic because the OD at 600 nm of culture medium and the CFU continuously decreased after treating with bacteriocin and changing in morphology with hollowed, turgided and enlarged. Similarly, the action of bacteriocin from *Lacbacillus acidophilus* TS1 by inducing the death cell of indicator strains resulted of marked reduction in

OD of growing cells (Maqsood, 2008). Ennahar *et al*., 2000 reported that death cell was induced by concentration and time was used. However, the mode of action of bacteriocin from gram positive bacteria was not clear. From the proposal of Montville and Bruno, 1994; Brotz and Sahl, 2000, they mentioned that the mode of action of gram positive bacteria by the peptides can interacts and interfere the cell membrane which leads to destruct the proton motive force, leakage of the inner essential molecules and cell death. BLS P34 produced by *Bacillus* sp. P34, it has shown that the bactericidal and the damage of the cell membrane of *Listeria monocytigenes* provoked the uv-absorbing materials (Motta *et al*., 2008). Moreover, from the research of Bendali and co-worker in 2008 found that *L. monocytogenes* cells after treat with bacteriocin as like substance has shown alteration in morphology with elongated shape. In addition, Gonzales *et al*., 1996, they reported that plantaricin C induced of mesosome-like membraneous formation protruding into cytoplasm of sensitive cells. The effect of mersacidin on morphological of *Staphylococcus simulans* 22 showing cell morphology alteration by thickness, blistered, roughened and reduction of biosynthesis cell wall. In some cells, mersacidin increased vast alterations such as halted in their attempt to be divided because septa of dividing cells had ceased but the membrane biosynthesis went on by the stringy membrane extensions at each of the end for the progressing septum (Molitor *et al*., 1996). In the case of Lactococcin 972 (Lcn972) inhibits septum biosynthesis in *Lactococcus lactis* more than made pores in cytoplasmic membrane (Martinez *et al*., 2008). For the stability test, bacteriocin from *Brev. Laterosporus* SA14 retained activity at pH value range and the high activity at pH8-9. This bacteriocin exhibited stability at alkaline condition. Bacthuricin F103 is bacteriocin produced by New *Bacillus thuringiensis*, it has shown the maximum activity at pH7 and activity retained at pH9 (Kamoun *et al*., 2011).

Similarly, the results of Pumilicin 4 produced by Newly Isolated Bacteria *Bacillus pumilus* strain WAPB4 which is not affected at pH value 8-9 and the activity of bacteriocin did not lost at different temperature range (Aunpad and Na-Bangchang, 2007). For test the effect of enzyme proteinase K and pronase E on activity of bacteriocin found that the activity slightly lost. It characterized general bacteriocin. Several authors reported that bacteriocin was degraded by protease enzyme. Antibacterial activity of Bacthuricin F103 lost about 20% and 30% after treat with trypsin and neutrase, respectively (Kamoun *et al*., 2011). Bacteriocin from *Bacillus licheniformis* MKU3 has resistant against to proteinase K and pronase E (Kayalvizhi and Gunasekaran, 2010). After determination, the effect of SDS, Triton X-100, EDTA and Tween80 on antibacterial activity has shown that the activity is not affected by SDS and Triton X-100. The activity decreased when treated with EDTA and Tween80.

Similarly, bacteriocin produced by *L. lactis* ssp. Lactis LL171 has resistant against SDS, Triton X-100, EDTA and Tween80 at 1% final concentration used (Kumari *et al*., 2012). The specific activity increased to 800 AU/ml in crude protein, corresponding to 88% recovery and 32 purification fold then, assay by SDS-PAGE which revealed the molecular weight lower than 6.9 kDa and in gel assay of inhibition of this crude protein against MRSA142. Its activity was confirmed. Suggesting that the position of inhibition zone in gel still stay at the same position on protein band by SDS-PAGE.

Conclusion

In conclusion, *Brev. laterosporus* SA14 was isolated from the air sample at Walailak University showing high produced bacteriocin at the 8^{th} day. This bacteriocin has shown bacteriostatic activity against *S. aureus* TISTR517 and MRSA142 at 800 AU/ml resulting to alteration morphology of *S. aureus* TISTR517 and MRSA142. The molecular weight of bacteriocin below 6.9 kDa and in gel SDS-PAGE demonstrated that is against *S. aureus*TISTR517 and MRSA142. The bacteriocin is stable at temperature range and pH value. The activity of this bacteriocin still retained after treated with enzymes and chemicals.

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