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Antibacterial Activity and Purification of Bacteriocin Produced by *Brevibacillus laterosporus* SA14

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Abstract

A bacteriocin producing strain, *Brevibacillus laterosporus* SA14 was isolated from an air sample at Walailak University, Thailand. The antibacterial activity of *Brev. laterosporus* SA14 was assayed by the cross streak and agar well diffusion method. Results from the cross streak method showed that it can inhibit both Gram positive and Gram negative bacteria. While results from the agar well diffusion method showed that *Micrococcus luteus* TISTR884 exhibited higher sensitivity to bacteriocin produced by *Brev. laterosporus* SA14 than other indicator strains. The highest amount of bacteriocin produced by *Brev. laterosporus* SA14 was detected during day 8 to day 10 of incubation. The crude protein exhibited bacteriocin activity of 800 AU/ml and did not affect cell membranes of *Staphylococcus aureus* TISTR516 and MRSA142. The bacteriocin activity increased from 25 AU/ml in supernatant to 800 AU/ml in crude protein after ammonium sulfate precipitation, corresponding to 88 % recovery and 32-fold purification. The fraction number was 39 to 41 from the Sp-sepharose fast flow column chromatography expressed highest bacteriocin activity with 44.8 % recovery and 128-fold purification. The partially purified bacteriocin was revealed as a single band on SDS-PAGE gel with a molecular weight of 6.9 kDa. The active fractions were finally pooled and purified by Reverse Phase High Performance Liquid Chromatography (RP-HPLC). The result showed a single peak (retention time of 3.466 min) with antibacterial activity.

Keywords: Antibacterial activity, bacteriocin, *Brev. laterosporus* SA14, purification

Introduction

Bacteriocins are cationic and hydrophobic peptides or proteins, produced by bacteria and ribosomally-synthesized generally inhibiting the growth of strains closely related to the producer [1,2]. The inhibitory effect of bacteriocin against other bacterial strains has been investigated for many years, resulting in a number of studies [3-5]. The bacteriocin effects are not always lethal. They may be just bacteriostatic effects, without causing cell death. Some bacteriocins produced by *Bacillus thuringiensis* have a bacteriostatic effect against *Salmonella* sp. [6]. Finally, some producer strains are sensitive to their own bacteriocin, like hyicina 3682 [7]. Nisin from *Lactobacillus lactis* was granted a Generally Recognized as Safe (GRAS) status by the USA Food and Drug Administration. It has been used as a food preservative in forty countries since 1983 [8]. Sisomicin, produced by *Streptomyces* spp. isolated from a soil sample in China, was proved to be active against *Bacillus cereus* and *Escherichia coli* [9,10].

Oligomycin A-like structure produced by *Streptomyces avermitilis* has also been proved to be active against liver cancer and leukeamia [9].

Brev. laterosporus, formerly known as *Bacillus latersporus* was extensively observed because of its ability to produce many peptides that exhibit antibacterial activity [8,11,12]. *Brev. laterosporus* was able to produce various antibiotics such as Espergualin and Bacithrocins A, B, and C, which later proved to be beneficial to the pharmaceutical industry [13-15]. *Brev. laterosporus* SA14 can produce proteins approximately 6.9 kDa in size which is more potent against MRSA than vancomycin and oxacillin [11]. Further, a recent study showed that the crude protein from *Brev. laterosporus* SA14 affected *S. aureus* TISTR517 and MRSA142 by changing their morphology [16].

The objective of this study was to purify bacteriocin produced by *Brev. laterosporus* SA14 and determine its antibacterial activity spectrum.

Materials and methods

Bacterial strains and media

Brev. laterosporus SA14, a bacteriocin producing strain, was isolated from an air sample at Walailak University, Thailand. *Staphylococcus aureus* TISTR517, *Enterococcus faecalis* JCM8726, *Micrococcus luteus* TISTR884, *Salmonella typhimurium* TISTR292, *Escherichia coli* TISTR887, and *Bacillus subtilis* ATCC 6633 were used as indicator strains. Both of *Brev. laterosporus* SA14 and indicator strains were inoculated on LB agar (Luria-Bertani agar) and incubated at 37 °C for 18 h.

Antibacterial activity

The antibacterial activity was assayed by the cross streak method and agar well diffusion method.

Cross streak

The antibacterial activity was tested by the cross streak method as described by Williston *et al*. [17]. *Brev. laterosporus* SA14 was streaked about 7.5 cm in length, 0.6 mm in width across the surface of an LB agar plate and incubated at 37 °C for 48 h. After incubation, all of the indicator strains (*E. coli* TISTR 887, *M. luteus* TISTR 884, *Ent. faecalis* JCM 8726, *S. aureus* TISTR 517, *Sal. typhimurium* TISTR 292 and *B. subtilis* ATCC 6633) were streaked in a perpendicular direction with *Brev. laterosporus* SA14 (0.6 cm in length, 0.6 mm in width) and incubated at 37 °C for 24 h. The LB agar plate containing only indicator strains was used as a control as shown in **Figure 1**. Antibacterial activity was observed as the growth of colonies of indicator strains on the LB plate with/without *Brev. laterosporus* SA14.

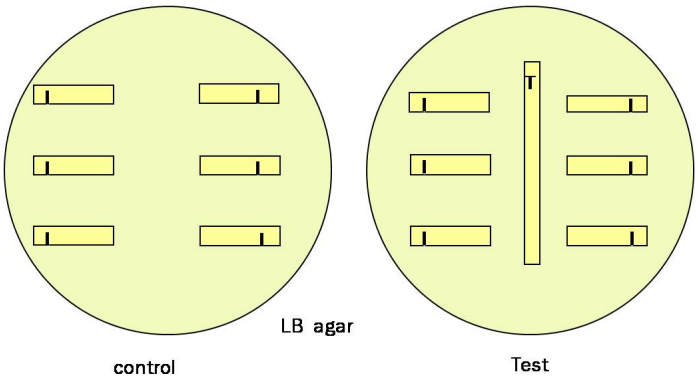

Figure 1 Cross streak method (I, Indicator strain; T, Test strain).

Agar well diffusion method

The antibacterial activity was tested against indicator strains by agar well diffusion as described by [18] with some modifications. Briefly, all indicator strains (*E. coli* TISTR 887, *M. luteus* TISTR 884, *Ent. faecalis* JCM 8726, *S. aureus* TISTR 517, *Sal. typhimurium* TISTR 292 and *B. subtilis* ATCC 6633) were separately streaked on LB agar and incubated at 37 °C for 18 h. A single colony of each indicator strain was inoculated into 5 ml of LB broth and incubated at 37 °C for 18 h. The suspension of each indicator strain was adjusted according to McFarland No. 0.5, and separately swabbed onto the surface of LB agar plates. After that, the swabbed agar was added with a sterile tip from the hole with a diameter of 6 mm, 80 µl (cell free supernatant (cfs) or crude protein) to the well and allowed to diffuse into agar during a 5 h pre-incubation period at room temperature, under aerobically incubation at 37 °C for 24 h. The plate without cfs or crude protein was used as a control. The antibacterial activity was evaluated by measurement of zone of inhibition diameter around the well.

Bacteriocin production

Brev. laterosporus SA14 was grown in 300 ml of LB broth at 37 °C, 150 rpm for 10 days. At the interval time, the cultured medium was harvested daily. Cells were harvested by centrifugation at 4 °C, 10000 rpm for 30 min. CFS of each day was used to test antibacterial activity against *S. aureus* TISTR517 and MRSA142 by agar well diffusion, as previously described.

Ammonium sulfate precipitation

This method as described by Kamoun *et al*. [19] with some modifications. Briefly, *Brev. laterosporus* SA14 was grown in 2000 ml of LB broth at 37 °C, 150 rpm for 8 days. Cells were harvested by centrifugation at 4 °C, 10000 rpm for 30 min. Ammonium sulfate was added to the cfs at 4 °C while it was stirred to reach a saturation of 50 %. The precipitate was collected by centrifugation at 4 °C, 12000 rpm for 30 min. Then, the precipitate was dissolved in 0.15 M phosphate buffer, dialyzed with the same buffer in a dialysis tube (MW cut-off, 3,500) at 4 °C for 24 h. The sample was called crude protein (cp), stored at −20 °C until used for study.

Release of UV-absorbing material

To determine the leakage of ultraviolet light-absorbing material, the method was used Motta and Brandelli [20]. The cell suspension of *S. aureus* TISTR 517 and MRSA142 were adjusted to McFarland No. 0.5 (1 - 2 $\times 10^8$ CFU/ml) then mixed with crude protein (800 AU/ml) at a 1:1 ratio (v/v) whereas the suspension that did not contain the crude protein was used as a control. The crude protein mixture was incubated at 37 °C for 24 h prior to centrifugation at 4 °C, 6000 rpm for 30 min. The UV absorbance of the cfs was measured at 260 and 280 nm with a spectrophotometer (EON, Bio-Tex, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The crude protein was examined on SDS-PAGE by the method described in Laemmli [21]. The prestained SDS-PAGE standards broad range (Bio-rad) were used as protein markers. After electrophoresis, the gel was divided into 2 parts. One part was stained with Coomassie brilliant blue R-250 (10 % acetic acid in water, containing 60 mg/L of Coomassie Blue R-250) and the other part was washed three times with sterile water and used to test antibacterial activity in the gel [4].

Determination of the antibacterial activity in gel

To determine antibacterial activity in the gel a method described by Bhunia *et al.* [22] was used. After electrophoresis, the gel was washed 3 times with sterile distilled water and placed on the surface of an LB agar plate and then overlaid with soft agar containing 500 µl of indicator strain solution (MRSA142) which was prepared and adjusted to McFarland No. 0.5. After that, it was incubated at 37 °C for 18 h and the inhibition zone on the gel observed.

Sp-sepharose fast flow column chromatography

To purify the crude protein the method described by Wu *et al*. [23] was used. The crude protein was applied to the activated and pre-equilibrated (0.15 M phosphate buffer pH 7.2) Sp-sepharose fast flow column. The column was washed by 0.15M phosphate buffer pH 7.2. The elution was carried out using 0 - 0.5M NaCl and 0.15M phosphate buffer pH 7.2, at a flow rate 1 ml/min. Sixty fractions (each of 5 ml) were collected, checked for antibacterial activity by the agar well diffusion method. The protein concentration was measured at OD 280 nm with a spectrophotometer (EON, Bio-Tex, USA) and analyzed molecular weight (MW) by SDS-PAGE.

Reverse phase high performance liquid chromatography (RP-HPLC)

The purity of the crude protein was determined with some modifications by the method described by Wu *et al*. [23]. The active fractions from Sp-sepharose fast flow column chromatography were pooled, concentrated and then applied to LochroCART 250-4 Lichrospher RP-18 column (Merck) washed with buffer A (water; 0.1 % trifluoroacetic acid (Fisher)). Bound protein was eluted with buffer B (acetonitrile 99.8 % (Maclon); 0.1 % trifluoroacetic acid) at a flow rate of 0.9 ml/min and the following gradient: 0 to 2 min, 50 % buffer A; 2 to 15 min, 80 % buffer A. The fractions were collected at one min intervals (each of 0.9 ml), and checked for antibacterial activity. Protein concentration was measured at OD 280 nm with a spectrophotometer (EON, Bio-Tex, USA).

Determination of the Arbitrary Units (AU)

The Arbitrary Units (AU) were determined by agar well diffusion method using *S. aureus* TISTR 517 and MRSA142 as indicator strains. The protein sample (crude protein; from cfs, from ammonium sulfate precipitate and partial purified protein; from Sp-sepharose fast flow column chromatography and from RP-HPLC) were diluted along with 2-fold dilution. Both of indicator strains, *S. aureus* TISTR 517 and MRSA142, were grown in 5 ml of LB both at 37 °C, 150 rpm for 24 h. The cell of each indicator strain was adjusted to McFarland standard No. 0.5, swabbed on the surface of LB agar and left at room temperature for 10 min for the agar surface to dry. The wells were made with sterile tip (1 ml). Eighty µl of each two-fold dilution of protein sample was added into the well of each plate, incubated at 37 °C for 24 h. The zone of inhibition was observed. The AU was defined as the reciprocal highest dilution showing a clear zone of growth inhibition. The bacteriocin-like protein activity was determined as AU/ml (AU/ml was defined as the reciprocal of the highest bacteriocin-like protein dilution factor that inhibited the growth of the indicator strain). The activity was calculated by using the formula below [24].

$$AU/ml = (X \times 1{,}000)/V \quad (1)$$

X is the highest dilution exhibiting inhibition
V is the volume of filled sample in to each well

Results and discussion

Antibacterial activity

The result from the cross streak method showed the inhibitory effect of *Brev. laterosporus* SA14 against six indicator strains as there was no colony of any indicator strain found near the colony of *Brev. laterosporus* SA14 in **Figure 2**. The result from the agar well diffusion method also showed the inhibitory effect of *Brev. laterosporus* SA14. According to the inhibition zone of each indicator strain recorded in **Table 1**, *M. luteus* TISTR884 was the most susceptible to bacteriocin produced by *Brev. laterosporus* SA14. This result indicating that the bacteriocin showed more effect against to gram positive bacteria than gram negative bacteria. It can be concluded that the bacteriocin produced by *Brev. laterosporus* SA14 showed broad inhibitory spectrum against all indicator strains. Similarly, bacteriocin produced by *Brev. brevis* exhibited broad inhibitory spectrum against all the pathogenic indicator [25]. Most bacteriocins of Gram positive bacteria affect a wider range of bacteria (including various species of both Gram positive and Gram negative bacteria) than colicins (bacteriocin of Gram negative bacteria)

[26]. However, the mode of action of bacteriocin from gram positive bacteria was not clear. The mode of action of gram positive bacteria by the peptides can interact and interfere with the cell membrane which leads to destruction of the proton motive force, leakage of the inner essential molecules and cell death. BLS P34 produced by Bacillus sp. P34 shown the bactericidal and damage cell membrane of *Listeria monocytogenes* provoked the UV-absorbing materials [27]. Further, they may use bacteriocin produced by *Brev. laterosporus* SA14 as food preservatives in the food industry or for use as antibiotics in hospital. Similarly, Sisomicin produced by *Streptomyces* spp. isolated from soil samples in China, was proved to be active against *B. cereus* and *E. coli* [10]. Oligomycin A-like structures produced by *Streptomyces avermitilis* was also proved to be active against liver cancer and leukeamia [9].

Bacteriocin production

The cell free supernatant from the 5 - 10 day old of *Brev. laterosporus* SA14 exhibited antibacterial activity against *S. aureus* TISTR517 and MRSA142 when determined by agar well diffusion. The cell free supernatant from the 8 - 10 day old of *Brev. laterosporus* SA14 exhibited the highest antibacterial activity against *S. aureus* TISTR517 and MRSA142 observed from the zone of inhibition as shown in **Table 2**.

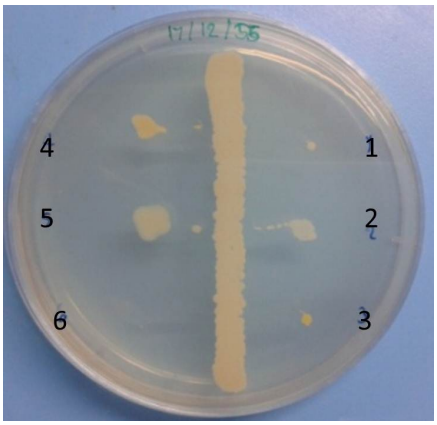

Figure 2 The antibacterial activity of *Brev. laterosporus* SA14 against indicator strains by the cross streak method. The indicator strains used in this test were *S. aureus* TISTR517, *Ent. faecalis* JCM8726, *M. luteus* TISTR884, *Sal. typhimurium* TISTR292, *E. coli* TISTR887, and *B. subtilis* ATCC 6633, which were defined as 1, 2, 3, 4, 5 and 6, respectively.

Table 1 The antibacterial activity of cell-free supernatant obtained from *Brev. laterosporus* SA14.

S. no.	Indicator strain	Zone of inhibition (mean of 3 trials) (mm)
1	*S. aureus* TISTR517	17
2	*Ent. faecalis* JCM8726	18
3	*M. luteus* TISTR884	19
4	*Sal. typhimurium* TISTR292	12
5	*E. coli* TISTR887	14
6	*B. subtilis* ATCC 6633	17

Table 2 Zone of inhibition of cell free supernatant from *Brev. laterosporus* SA14 against indicator strains.

Days	Zone of inhibition (mean of 3 trials) (mm)	
	***S. aureus* TISTR517**	**Methicillin resistant *S. aureus* 142 (MRSA142)**
1	0±0.00	0±0.00
2	0±0.00	0±0.00
3	0±0.00	0±0.00
4	0±0.00	0±0.00
5	10±1.82	10±1.82
6	15±2.73	15±2.73
7	18±3.27	19±3.45
8	20±3.64	21±3.82
9	20±3.64	21±3.82
10	20±3.64	21±3.82

Value are means (n = 5) ± SE, Data were analyzed using the pair simple T-test ($P < 0.05$); *Diameter of well = 6 mm; *All experiments were done in triplicate.

Table 3 Release of intracellular UV-absorbing material *S. aureus* TISTR517 and MRSA142.

	Untreated cells		**Treated cells**	
	***S. aureus* TISTR517**	**MRSA142**	***S. aureus* TISTR517**	**MRSA142**
Nucleic acids (A_{260} nm)	0.066±0.012	0.059±0.011	0.122±0.012	0.109±0.011
Protein (A_{280} nm)	0.058±0.009	0.047±0.007	0.103±0.009	0.087±0.007

Value are means (n = 5) ± SE, Data were analyzed using the pair simple T-test ($P < 0.05$).

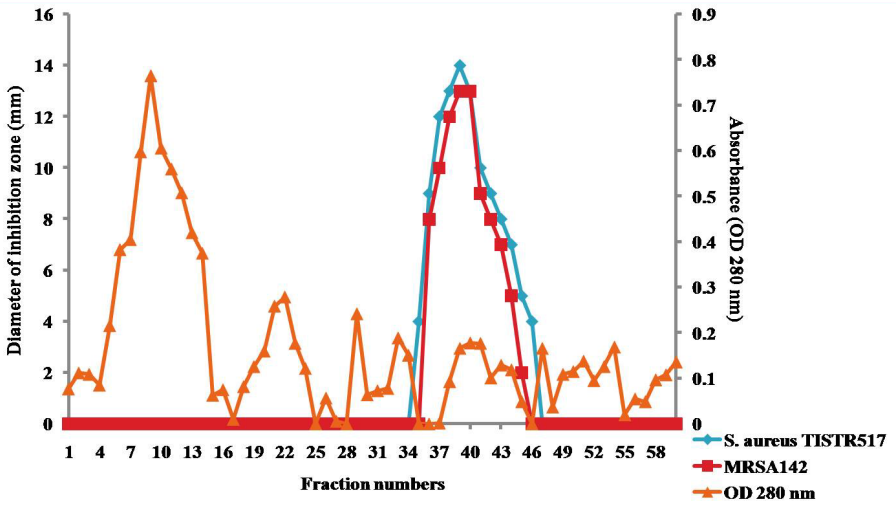

Figure 3 Elution profile of bacteriocin from *Brev. laterosporus* SA14 by the sp-sepharose fast flow column chromatography.

Ammonium sulfate precipitation

Brev. laterosporus can produce substances for medical purposes such as Espergualin [14,15] and Bacithrocins A, B and C [13]. Bacteriocin produced by *Brev. laterosporus* SA14 exhibited antibacterial activity against *S. aureus* TISTR517 and MRSA142. After precipitating with 50 % ammonium sulfate the partially purified bacteriocin showed specific activity increased from 25 AU/ml in cfs to 800 AU/ml in crude protein (after precipitating with ammonium sulfate), corresponding to 88 % recovery and 32 purification fold and then the partially purified compound was further purified by Sp-sepharose fast flow column chromatography and RP-HPLC.

Release of UV-absorbing material

The effect of crude protein on the cell membrane of *S. aureus* TISTR517 and MRSA142 was investigated by detecting the leakage of ultraviolet light-absorbing material using a spectrophotometer (EON, Bio-Tex, USA). The UV absorbance of cfs of both treated and untreated samples were measured at 260 and 280 nm with a spectrophotometer. The results showed that crude protein at 800 AU/ml did not affect the treated cells when compared the absorbance value at 260 and 280 nm as shown in **Table 3**. The finding published in [16] reported an alteration in morphology of *S. aureus* TISTR517 and MRSA142 after being treated with bacteriocin obtained from *Brev. laterosporus* SA14 with hollowed, blistered and enlarged cells. Furthermore, cell death was induced by concentration and time used [28]. However, the mode of action of bacteriocin from gram positive bacteria was not clear. The mode of action of gram positive bacteria by the peptides can interact and interfere with the cell membrane which leads to destruction of the proton motive force, leakage of the inner essential molecules and cell death. BLS P34 produced by *Bacillus* sp. P34 showed bactericidal and damage cell membrane of *L. monocytogenes* provoked the UV-absorbing materials [27]. So, it can be concluded that the mode of action of bacteriocin produced by *Brev. laterosporus* SA14 is bacteriostatic and bacteriocin does not interact and interfere with the cell membrane because no leakage of the inner essential molecules was found.

Sp-sepharose fast flow column chromatography

It was found that crude protein eluted with 0.4M NaCl and phosphate buffer. The fraction number 39 - 41 exhibited antibacterial activity against *S. aureus* TISTR517 and MRSA142 as shown in **Figure 3**. Then, active fractions were pooled and concentrated by evaporation. The pooled fraction gave higher specific activity of bacteriocin increased to 3,200 AU/ml, corresponding to 44.8 % recovery and 128 purification fold as shown in **Table 4**. Similarly, 2 novel antimicrobial peptides, Subpeptin JM4-A and Subpeptin JM4-B, produced by *B. subtilis* JM4 and a novel antimicrobial peptide BL-A60 produced by *Brev. laterosporus* strain A60 [29] were observed.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The partially purified protein from Sp-sepharose fast flow column chromatography showed a MW of approximately 6.9 kDa as analysed by SDS-PAGE. The antimicrobial activity in the gel showed a clear zone of inhibition on gel against MRSA142 as shown in **Figure 4**. It was significantly different from bacteriocin produced by *Brev. brevis* strain GM100 [30]. Bacteriocin produced by *Brev. laterosporus* SA14 display a bacteriostatic mode but *Brev. brevis* strain GM100 displayed a bactericidal mode.

Reverse phase high performance liquid chromatography (RP-HPLC)

The pooled fractions from the Sp-sepharose fast flow column were applied to LochroCART 250-4 Lichrospher RP-18 column (Merck) and washed with buffer A (water; 0.1 % Trifluoroacetic acid (Fisher)). The bound protein was eluted with buffer B (acetonitrile 99.8 % (Maclon); 0.1 % trifluoroacetic acid) at a flow rate 0.9 ml/min. The protein sample was eluted at the time of 3.466 min. Then, the fraction at this time was collected and checked for antibacterial activity. The protein exhibited adverse effects against MRSA142 as shown in **Figure 5**.

Determination of the Arbitrary Units (AU)

The number of AU was calculated per milliliter of sample. After precipitation with 50 % ammonium sulfate the AU showed a specific activity of a partially purified form as proteinaceus, it increased from 25 AU/ml in cfs to 800 AU/ml, corresponding to 88 % recovery and 32 purification fold and showed strong activity against *S. aureus* TISTR517 and MRSA142 depending upon the increasing of the concentration (dose-depending fashion) [16]. At stage of Sp-sepharose fast flow column chromatography found that the specific activity increased to 3200 AU/ml, corresponding to 44.8 % recovery and 128 purification fold. The final stage of purification, RP-HPLC, showed the specific activity decreased to 800 AU/ml, corresponding to 6.48 % recovery and 32 purification fold as shown in **Table 4**. In this study there was a low recovery rate of bacteriocin. Other researchers have also obtained low recovery rates of bacteriocin, for example the lactacin B (2.4 % recovery) [31,32] and acidocin 8912, (13.6 % recovery) [33]. On the other hand, high recovery rates (41 % recovery, 369-fold purification) were obtained for lactacin F [34]. It may be caused by different conditions or the device such as the column that used for purification.

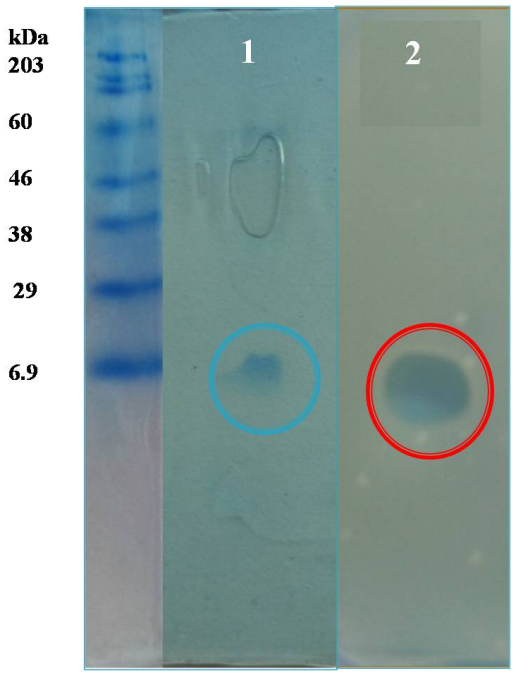

Figure 4 SDS-PAGE of the partially purified bacteriocin (after Sp-sepharose fast flow column) (1) and antibacterial activity against MRSA142 in gel (2).

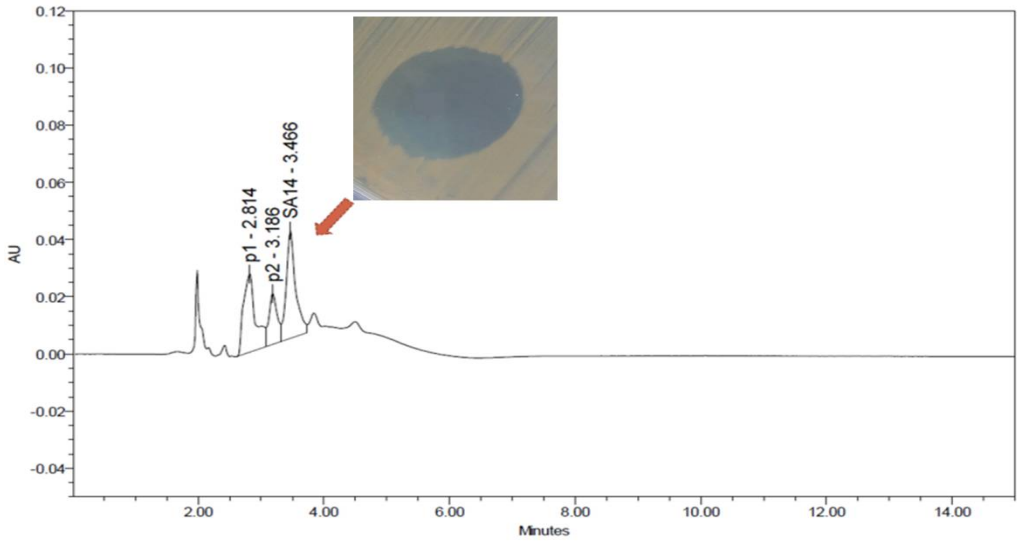

Figure 5 RP-HPLC elution profile of antimicrobial peptide purification.

Table 4 The antibacterial activity recoveries of bacteriocin of *Brev. latersporus* SA14 at different purification stages.

Purification stage	Volume (ml)	Specific activity (AU/ml)	Total activity (AU)	Purification (fold)	Recovery (%)
Supernatant	2000	25	50000	1	100
Ammonium sulfate precipitate	55	800	44000	32	88
Sp-sepharose fast flow column	15	3200	24600	128	44.8
RP-HPLC	8.1	800	3240	32	6.48

Conclusions

Brev. laterosporus SA14 showed high bacteriocin production at 8 days of culturing time. After ammonium sulfate precipitation, the protein sample called the crude protein, shows high activity against *S. aureus* TISTR517 and MRSA142. The stage of Sp-sepharose fast flow column chromatography showed the fraction number 39 - 41 gave high antibacterial activity and an increase in specific activity. At the stage of RP-HPLC a single peak, eluted at a time of 3.466 min, exhibited against MRSA showed the zone of inhibition and specific activity decreased. The molecular weight of bacteriocin is approximately 6.9 kDa when analysed by SDS-PAGE. Further studies may concern the bacteriocin encoding gene from *Brev. laterosporus* SA14 by genetic engineering.

Acknowledgments

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