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PURIFICATION OF BIOACTIVE COMPOUNDS PRODUCED BY *BREVIBACILLUS LATEROSPORUS* SA14 AND ITS ANTI-MRSA ACTIVITY

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ABSTRACT

An antimicrobial peptides (AMPs)- producing strain, *Brevibacillus laterosporus* SA14, has been isolated from air. The corresponding AMPs were purified from 4-day culture supernatant to homogeneity by 50% saturated ammonium sulfate precipitation, sequential SP-Sepharose Fast Flow and C18 reverse-phase high performance liquid chromatography. The final purification step, three active fractions were harvested at retention time about 22.90, 23.20 and 26.30 min, designated as SA14-A, SA14-B and SA14-C, respectively. The three AMPs showed antibacterial activity against fifty clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) which had the specific activity of about 300 AU/ml. In this study, the bioactive compounds that produced by *B. laterosporus* SA14 contained AMPs which are medically important substances may be used for alternative treatment of MRSA infection if it has been additionally studied to cytotoxicity against normal human cell.

KEYWORDS: Antimicrobial peptide, Bioactive compound, *Brevibacillus laterosporus* SA14, and methicillin-resistant *Staphylococcus aureus*.

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INTRODUCTION

Currently, the problem of MRSA resisted to the approved antibiotics that increased rapidly of several areas has become a worldwide problem with serious consequences on the treatment of MRSA infection [1-2]. The increased use/misuse of antibiotics in a treatment of MRSA infectious disease is mainly causing to the MRSA develops mechanism of antibiotic resistance. Vancomycin is a main stay for the treatment of infections caused by MRSA [3]. However, there are many researches reported vancomycin failures [4-5]. Such, new antibiotics will have to be developed in order to treat MRSA infections. New and more efficient antibiotics will have to be sought continually because of the capacity of microorganisms to survive their action. Many different strategies for finding new antimicrobial agents are actually proposed and the area of bioactive compounds is under intense investigation. Among the most promising bioactive compounds are antimicrobial peptides (AMP) or bacteriocins that produced by probiotic bacteria [6]. They are bacterial ribosomally synthesized antagonistic extracellular peptides or proteins produced by bacteria which are a natural product or secondary metabolite substance that are produced to kill or to inhibit the growth of the related bacteria. Bacteriocin from gram positive organisms, such as *Bacillus* species, which is an interesting genus to investigate since it produces a diverse array of antimicrobial peptides representing several different basic chemical structures [7]. The production of bacteriocins or bacteriocin-like substances has been already described for some bacilli such as *B. subtilis*, *B. cereus*, *B. stearothermophilus B. polymyxa*, *B. licheniformis*, *B. circulans*, *B. thuringiensis* and other *Bacillus* species including *Brevibacillus laterosporus*, previously classified as *Bacillus laterosporus* (*B. laterosporus*) [8]. *B. laterosporus* is a Gram-positive bacilli and anaerobic spore-forming bacterium characterized by the production of a typical canoe-shaped parasporal body (CSPB) which remains firmly attached to one side of the spore after lysis of the sporangium. It produced different virulence factors such as parasporal crystalline, extracellular protease [9] and lipopeptide antibiotics [10]. Also, the secretion of short-sequence peptides (bacteriocin) with a broad antibiotic spectra, such as laterosporulin [11], loloatin A, including a peptide antibiotic with cyanolytic activity [12] and medically important substance such as spergualin [13-14], bacithrocins A, B and C [15]. Since the mode of action of bacteriocin is remarkably different from conventional antibiotics, they may be considered as a novel antibiotic for the infectious MRSA treatment such as mersacidin, a lantibiotic produced by *Bacillus* sp. strain HIL Y-8554728, inhibited the growth of MRSA strains in mice [16] and bioactive crude from *B. laterosporus* SA14 which inhibited the clinical isolates MRSA more potent than oxacillin [17]. So, the purpose of this research is the purification of bioactive compounds produced by *B. laterosporus* SA14 for investigation of AMP and its anti-MRSA activity for a preliminary scientific data to develop the novel antibiotic in the future.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bioactive compounds-producing strain, *B. laterosporus* SA14, was isolated from air sample in Walailak University campus, Thailand, which identified according to biochemical test by means of API 50CHB fermentation test kit (Bio-merieux, France) as described by Choopan et al. (2008) [18]. It was cultured in Luria bertani (LB: Scharlau) broth with shaking at 150 round per minute (rpm) at 37˚ C for 4 days. The fifty clinical isolates of MRSA were collected from patients at Maharaj Nakhon Si Thammarat Hospital, Thailand, used as indicator strains. They all were cultured in LB broth with shaking at 150 rpm at 37˚ C for 24 h.

Purification of bioactive compounds

(i) Partial Purification of Bioactive Compounds

The *B. laterosporus* SA14, stored in 30% (v/v) glycerol at -20˚C, was streaked onto the

surface of an LB medium plate, and incubated at 37˚C for 24 h. A single colony was inoculated in 5 ml of LB medium, and incubated in shaking incubator at 150 rpm, 37˚C for 24 h. The overnight culture was adjusted with sterile phosphate buffer, approximately to a 0.5 McFarland standard turbidity [19]. Then, the cell suspension was transferred to one liter of LB medium at an inoculum of 1% (v/v) for subculture and incubated in shaking incubator at 150 rpm at 37˚C. After 4 days of incubation period, cells were separated from the culture supernatant by centrifugation at 12,000 rpm, 4˚C for 30 min. The bioactive compounds in culture supernatant were precipitated by ammonium sulphate with 50% saturation at 4˚C for 24 h. The protein precipitates were harvested by centrifugation at 12,000 rpm at 4°C for 20 min, and dissolved in 30 ml of 20 mM sodium phosphate buffer (pH 6.0) and dialyzed against the same buffer at 4°C for 24 h in Spectra/Por dialysis membrane with a molecular weight cut off 3.5 kDa (Bio-Rad, USA) to remove the ammonium sulfate. After this step, the partial purified bioactive compounds solution or bioactive crude was further tested anti-MRSA activity.

(ii) Purification of Bioactive Compounds

The partial purified bioactive compounds or AMP solution was applied to cation exchange chromatography on a SP-Sepharose Fast Flow column (Amersham Pharmacia Biotech), eluted with 0-0.5M linear gradient of sodium chloride over a 20-min period for 24 h. Each active fractions was concentrated by centrifugation passed through the concentrator tube in a molecular weight cut off 3-kDa (Gibco BRL). For final purification, each concentrated active fractions were applied to a C18 reverse-phase HPLC (RP-HPLC) column (Sinochrom ODS-BP, Japan), and performed with 5% acetonitrile (ACN)/ 2mM NH_4FA/ 0.1% H_2O linear gradient containing 0.1% (v/v) trifluoroacetic acid over a 60-min period [6, 20]. The partial purified bioactive compounds were tested anti-MRSA activity.

Anti-MRSA Activity

Anti-MRSA activity was investigated by agar well diffusion technique as described by Sharma et al. (2011) [20]. Briefly, each isolate of indicator strain was approximately adjusted to a 0.5 McFarland standard turbidity [19] by 0.85% NaCl and swabbed on sterilized Mueller Hinton agar (MHA: Himedia) plates using sterilized cotton swab and drilled well by cork-borer which have diameter as 6 mm. Eighty microliter of partial purified bioactive compounds or AMPs were added into the well.. The plates were incubated at 37°C. After 24 h of incubation, the plates were examined the zone of clearance around the individual wells. The antimicrobial activity was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in activity units per ml (AU ml^{-1}) [22]. AU ml^{-1}= highest dilution (or dilution factor) that showed clear well-defined zone of inhibition $\times$ 1000 µl/volume (µl) used in the well. The experiment was done three time independent in duplicate.

RESULTS

1. Purification of Bioactive Compounds

The AMPs were sequentially obtained from culture supernatant of 4-days culture of *B. laterosporus* SA14 by ammonium sulfate precipitation, sequential cation exchange chromatography on a SP-Sepharose Fast Flow column, and C18 RP-HPLC (Table 1). Three active peptides were eluted at retention time 22.90, 23.20 and 26.30 min and designated as SA14-A, SA14-B and SA14-C, respectively (Fig 1).

2. Anti-MRSA Activity of Bioactive Compounds

The anti-MRSA activity of 4-days culture supernatant, partial - and purified bioactive compounds (AMP) of *B. laterosporus* SA14 was investigated by agar well diffusion technique. All of samples have been showed anti-MRSA activity against all the clinical isolates of MRSA tested. The 4-days culture supernatant of B. laterosporus SA14, partial purified and bioactive compounds gave the specific activity at 120, 200 and 300 AU/ml, respectively (Table 1).

Table 1

Summary of the purification profile for AMP from B. laterosporus SA14

Purification stages	Volume (ml)	Specific activity (AU/ml)	Total activity (AU)	Recovery (%)
Culture supernatant	1000	120	1.2×10^5	100
Ammonium sulphate SP-Sepharose /	20	200	0.04×10^5	3.3
C18 RP-HPLC	10	300	0.03×10^5	2.5

Total activity was calculated by (specific activity × volume) % Recovery was calculated by [(Total activity of ammonium sulphate or SP-Sepharose × 100) / Total activity of culture supernatant.]

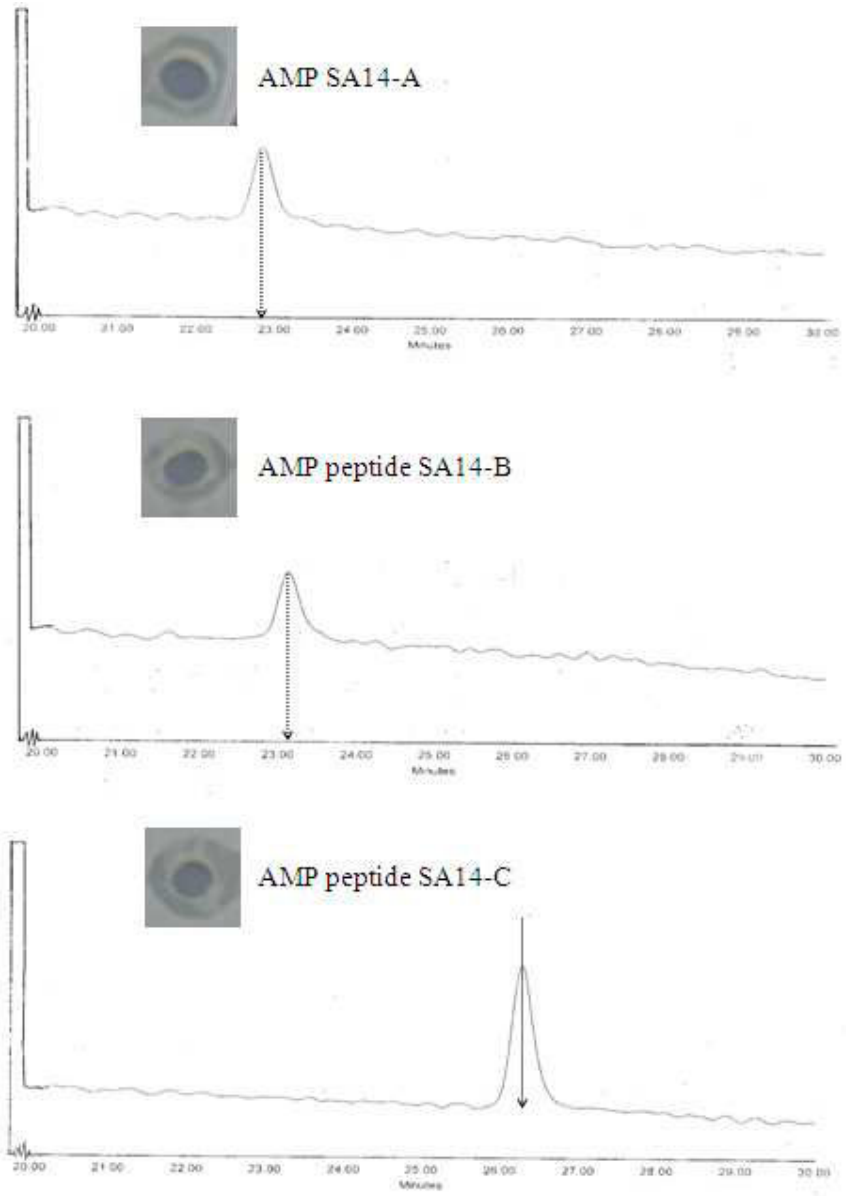

Figure 1

C18 RP-HPLC chromatogram of purified bioactive compounds. Elution was performed by 5% ACN/ 2mM NH_4FA/ 0.1% H_2O linear gradient over a 60-min period. Three active fractions were eluted at retention time 22.90 min (AMP SA14-A), 23.20 min (AMP SA14-B) and 26.30 min (AMP SA14-C), respectively.

DISCUSSION

In this study, the extracellular bioactive compounds or AMP was accumulated in the culture broth of 2-days culture of *B. laterosporus* SA14, which has been showed the anti-MRSA activity. The 4-days culture supernatant showed the highest anti-MRSA activity which the specific activity was 120 AU/ml. The bioactive compounds, which produced in those cultured periods, were considered as secondary metabolites or natural peptides that secreted for the purpose of killing other bacteria. This provides them with a competitive advantage in their environment, eliminating competitors to gain resources [23]. Gram-positive endospore forming bacilli have long been associated with the production of antimicrobial peptides, which is secondary metabolite, such as bacitracin, gramicidin, polymyxin and lichenin have been recovered from *B. subtilis*, *B. brevis*, *B. polymyxa*, and *B. licheniformis*, respectively [24]. Thuricin and cerein that produced by *B. thuringiensis* [25] and *B. cereus* [26], respectively. Laterosporulin [11] and loloatin A [12] that produced by *B. laterosporus*. In this experiment, the AMP was partial purified by ammonium sulfate precipitation followed by cation exchange chromatography. Each separated fraction was tasted anti-MRSA activity. Only three active fractions, which was eluted by 0.1 M sodium chloride, had the specific activity is 300 AU/ml and higher than specific activity of 4-days old culture supernatant and partial purified bioactive compounds of strain SA14 about 2.5 and 1.5-fold, respectively. Surprisingly, all the three active fractions have been shown a single peak on chromatogram which confirmed by RP-HPLC. Therefore we designated the bioactive compounds as AMP SA14-A, SA14-B and SA14-C for single peak on chromatogram which showed at retention time about 22.90, 23.20 and 26.30 min, respectively. The AMP SA14-A should be possessed polarity (hydrophilic amino acid) higher than the antimicrobial peptide SA14-B and SA14-C, respectively. In contrast, the antimicrobial peptide SA14-C should be possessed positive charge (cationic amino acid) higher than the SA14-B and SA14-A, respectively because it was before eluted by 0.1M sodium chloride in cation exchange chromatography step, which according with two novel antimicrobial peptides, Subpeptin JM4-A and Subpeptin JM4-B, that produced by *B. subtilis* JM4 6 and a novel antimicrobial peptide BL-A60 that produced by *B. laterosporus* strain A60 [31]. The several study reported mechanism of action of active peptide may be interacted with the target cell membrane of MRSA through the formation of ion channels and transmembrane pores causing extensive membrane rupture and eventually leading to the lysis of the cells 27-28. In other words, the total net positive charge from cationic amino acid of active peptide were attracted to the negative charge that existed on the outer envelope of the bacterial target cells (i.e. anionic phospholipids, phosphate groups on lipopolysaccharide and teichoic acids) and another of the active peptide was inserted into the bilayer forming transmembrane pores and crossed through the pores to interact/disturb with the intracellular targets [29], such as (i) bind to DNA (ii) inhibit DNA, RNA and protein synthesis and (iii) inhibit enzymatic activity [30].

CONCLUSION

The bioactive compounds which produced by *B. laterosporus* SA14 was sequentially purified by ammonium sulfate precipitation, cation exchange chromatography and C18 RP-HPLC. Three antimicrobial peptides could inhibit all the clinical isolates of MRSA tested and were confirmed by single peak that showed on HPLC chromatogram. The three AMPs are medical important substance which can be developed into antibiotic for infectious MRSA treatment. Our future efforts will focus on characterization and cytotoxicity against normal human cell line of three AMPs and its chemical structure.

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Conflict of Interest declared none.

REFFERENCES

1. Alanis AJ, Resistance to Antibiotics: Are We in the post-antibiotic era? J Arch Medical Res, 36: 697-705, (2005).
2. Oancea S and Stoia M, Antibiotic resistance of bacterial pathogens: the magnitude of the problem from two perspectives-Romanian and worldwide. J Romanian Biotechnol Lett, 15: 5519-5529. (2010).
3. Bush K, Antimicrobial agents. J Curr Opin Chem Biol, 1: 169-175. (1997).
4. Hiramatsua K, Hanakia H, Inob T, Yabutab K, Oguric T and Tenover FC, Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother, 40: 135-146. (1997).
5. Chang S, Sievert DM, Hageman JC, Boulton ML, Tenover FC, Downes FP, Shah S, Rudrik JT, Pupp GR, Brown WJ, Cardo D and Fridkin SK, Infection with vancomycin-resistant *Staphylococcus aureus* containing the *van*A resistance Gene. J Med, 348: 1342-1347. (2003).
6. Wu S, Jia S, Sun D, Chen M, Chen X, Zhong J and Huan L, Purification and characterization of two novel antimicrobial peptides Subpeptin JM4-A and Subpeptin JM4-B produced by *Bacillus subtilis* JM4. J Curr Microbiol, 51: 292-296. (2005).
7. Bizani D and Brandelli A, Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. Strain 8 A. J Appl Microbiol, 93: 512-519. (2002).
8. Shida O, Takagi H, Kadowaki K and Komagata K, Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. J System Bacteriol, 46(4): 939-946. (1996).
9. Huang X, Tian B, Niu Q, Yang J, Zhang L and Zhang K, An extracellular protease from *Brevibacillus laterosporus* G4 without parasporal crystals can serve as a pathogenic factor in infection of nematodes. J Res Microbiol, 156: 719-727. (2005).
10. Desjardine K, Pereira A, Wright H, Matainaho T, Kelly M and Andersen RJ, Tauramamide, a lipopeptide antibiotic produced in culture by *Brevibacillus laterosporus* isolated from a marine habitat: structure elucidation and synthesis. J Nat Pro, 70: 1850-1853. (2007).
11. Singh PK, Chittpurna A, Sharma V, Patil PB and Korpole S, Identification, purification and characterization of Laterosporulin, a novel bacteriocin produced by *Brevibacillus* sp. strain GI-9. PLoSONE , 7: 1-8. (2012).
12. Krachkovskii SA, Sobol AG, Ovchinnikova TV, Tagaev AA, Yakimenko ZA, Azizbekyan RR, Kuznetsova NI, Shamshina TN and Arseniev AS, Isolation, biological properties, and partial structure of antibiotic loloatin A. Russ J Bioorg Chem, 28(4): 269-273. (2002).
13. Nemoto K, Abe F and Nakamura T, Effects of spergualin and 15-deoxyspergualin on the development of graftversus-host disease in mice. Transplant Proc, 19: 3520-3521. (1987).
14. Umezawa K and Takeuchi T, Spergualin: a new antitumor antibiotic. J Biomedical Pharmacother, 41: 227-232. (1987).
15. Kamiyama T, Umino T, Nakamura Y, Itezono Y, Sawairi S, Satoh T and Yokose K, Bacithrocins A, B and C, novel thrombin inhibitors. J Antibiot, 47: 959-968. (1994).
16. Kruszewska D, Sahl HG, Bierbaum G, Pag U, Hynes SO and Ljungh A, Mersacidin eradicates methicillin-resistant *Staphylococcus aureus* (MRSA) in a mouse rhinitis model. J Antimicrob Chemother, 54: 648-653. (2004).

17. Chawawisit K and Lertcanawanichakul M, Minimum inhibitory concentration (MIC) of crude preparations of *Brevibacillus laterosporus* SA14 bioactive material compared to vancomycin and oxacillin, against clinical isolates of methicillin-resistant *Staphylococcus aureus*. J Microbiol Biotechnol, 24: 2199-2204. (2008).
18. Choopan A, Nakbud K, Dawveerakul K, Chawawisit K and Lertcanawanichakul M, Anti-methicillin resistant *Staphylococcus aureus* activity of *Brevibacillus laterosporus* strain SA14. Walailak J Sci Technol, 5(1): 47-56. (2008).
19. CLSI, Performance Standards for Antimicrobial Susceptibility Testing. Clinical and Laboratory Standards Institute: M100-S16. (2006).
20. Ahern M, Verschueren S and Sinderen DV, Isolation and characterization of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. FEMS Microbiol lett, 220: 127-131. (2003).
21. Sharma N, Kapoor R, Gautam N and Kumari R, Purification and characterization of bacteriocin produced by *Bacillus subtilis* R75 isolated from fermented chunks of mung bean (*Phaseolus radiatus*). J Food Technol Biotechnol, 49(2): 169-176. (2011).
22. Graciela M, Vignolo M, de Kairuz N, Aida AP, de Ruiz H, Oilver G, Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705. J Appl Bacteriol, 78: 5-10. (1995).
23. Lohans CT and Vederas JC, Development of class IIa bacteriocins as therapeutic agents. Int J Microbiol, 2012: 1-13. (2012).
24. Oppegard C, Rogne P, Emanuelsen L, Kristiansen PE, Fimland G and Meyer JN, The two-peptide class II bacteriocins: structure, production and mode of action. J Molec Microbiol Biotechnol, 13: 210-219. (2007).
25. Cherif A, Quazri H, Daffonchio D, Cherif H, Siama KB, Hassen A, Japua S and Boudabous A, Thurin 7: A novel bacteriocin produced by *Bacillus thuringenesis* BMG 1.7, a new strain isolated from soil. Lett Appl Microbiol, 32: 2432-2247. (2001).
26. Oscariz JC, Lasa I and Pisabarro AG, Detection and characterization of cerein 7, a new bacteriocin produced by *Bacillus cereus* with a broad spectrum of activity. Fed Eur Microbiol Soc, Microbiol Lett, 178, 337-341. (1999).
27. Jenssen H, Hamill P and Hancock REW, Peptide antimicrobial agents. J Clin Microbiol rev, 19: 491-511. (2006).
28. Giuliani A, Pirri G and Nicoletto SF, Antimicrobial peptides: an overview of a promising class of therapeutics. Cent Eur J Biol, 2(1): 1-33. (2007).
29. Zhou T, Zeng H, Qiu D, Yang X, Wang B, Chen M, Guo L and Wang S, Global transcriptional responses of *Bacillus subtilis* to xenocoumacin 1. J Appl Microbiol, 111: 652-662. (2011).
30. Brogden KA, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria. J Nat Rev Microbiol, 3: 238-250. (2005).
31. Zhao J, Guo L, Zeng H, Yang X, Yuan J, Shi H, Xiong Y, Chen M, Han L, Qiu D, Purification and characterization of a novel antimicrobial peptide from *Brevibacillus laterosporus* strain A60. Peptides, 33: 206-211. (2012).