Purification of Antimicrobial peptides produced by *Brevibacillus laterosporus* SA14 and its anti methicillin-resistant *Staphylococcus aureus* (MRSA) activity

Kittisak Chawawisit and Monthon Lertcanawanichakul*

School of Allied Health Sciences and Public Health, Walailak University, Nakhon Si Thammarat 80161, Thailand

ABSTRACT

A bacterial strain *Brevibacillus laterosporus* (*Brev. laterosporus*) SA14, which was isolated from air, could produce antimicrobial peptides that showed a strong anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity. The antimicrobial peptides were prepared from 4-day cultured supernatant of *Brev. laterosporus* SA14 by 50% saturated ammonium sulfate precipitation, cation exchange chromatography on a SP-Sepharose Fast Flow column and high performance liquid chromatography on a C18 reverse-phase column. After the final purification step, three active fractions were obtained at 22.90-, 23.20- and 26.30-minute retention time which were eluted by 0-100% acetonitrile/H_2O linear gradient containing 0.1% (v/v) trifluoroacetic acid and then designated as antimicrobial peptide SA14-A, SA14-B and SA14-C, respectively. The SA14-A, SA14-B and SA14-C showed anti-MRSA activity, which had specific activity at 200, 250 and 300 AU/ml, respectively. This present study is the first report of novel antimicrobial peptides produced by *Brev. laterosporus* SA14 which are medically important substances and potential for use as an alternative antibacterial agent in treatment of MRSA infection.

INTRODUCTION

Currently, the problem of MRSA resisted to the approved antibiotics that increased rapidly of several areas with serious consequences on the treatment of MRSA infection (Fig1a). Including vancomycin antibiotic is unavailable for treatment of infectious MRSA (Oancea & Stoia, 2010). Thereby, new efficient antimicrobial agents will have to be continually researched and developed for use as an alternative antimicrobial agent. Many different strategies for finding new antimicrobial agents are actually proposed and the area of antimicrobial peptides is under intense investigation. Antimicrobial peptides are bacterial ribosomally synthesized antagonistic extracellular peptides or secondary metabolite substance produced for kill or inhibits the growth of the related bacteria. *Brevibacillus laterosporus* (*Brev. laterosporus*) is a Gram-positive bacilli and an aerobic spore-forming bacterium (Fig1b.) characterized by the production of a typical canoe-shaped parasporal body (CSPB). It can produced different medically important substance such as laterosporulin, loloatin A, and bacithrocins A, B and C (Singh et al. 2012). Since the mode of action of antimicrobial peptides is remarkably different from conventional antibiotics (Fig1c.), they may be considered as a novel antibiotic for the infectious MRSA treatment. So, the purpose of this research is the purification of antimicrobial peptides produced by *Brev. laterosporus* SA14 and investigated anti-MRSA activity for as preliminary scientific data and development to antibiotic in the future.

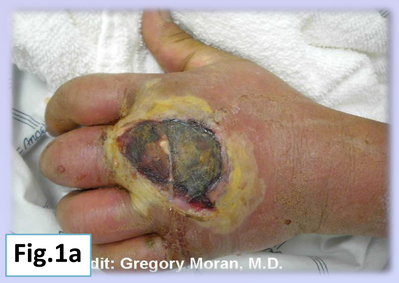

Fig.1a dit: Gregory Moran, M.D.

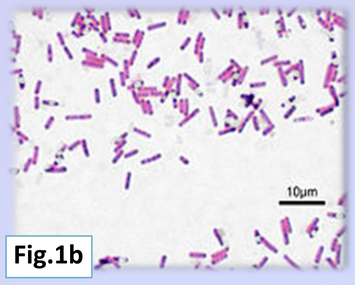

Fig.1b

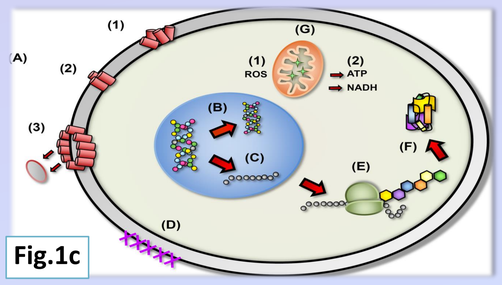

Fig.1c

MATERIALS AND METHODS

1. MEDIA, BACTERIAL STRAINS AND CULTURE CONDITIONS

- ***Brev. laterosporus* SA14** was isolated from air sample in WU, Thailand, cultured in Luria bertani (LB: Scharlau) broth with shaking at 150 rpm, 37˚C, 96 h and confirmed biochemically by means of API 50 CHB fermentation test kit (Bio-merieux, France)
- **MRSA** were collected from patients at Maharaj Nakhon Si Thammarat Hospital, Thailand, cultured in LB broth with shaking at 150 rpm, 37˚C, 24 h.

2. PURIFICATION OF ANTIMICROBIAL PEPTIDES

2.1 PARTIAL PURIFIED ANTIMICROBIAL PEPTIDES

- A single colony of *Brev. laterosporus* SA14 was inoculated in 5 ml LB medium, and incubated in shaking incubator at 150 rpm, 37 ˚C, 24 h.
- The overnight culture was adjusted with sterile phosphate buffer, approximately to a 0.5 McFarland standard and transferred to 1% (v/v) for subculture in 1 liter of LB medium to incubated in shaking incubator at 150 rpm at 37˚C, 96 h.
- Supernatant was precipitated by ammonium sulphate at 50% saturation. The protein precipitates were harvested by centrifugation, and dissolved in sodium phosphate buffer (pH 6.0) and dialyzed against the same buffer in Spectra/Por dialysis membrane with a molecular weight cut off 3.5 kDa to remove the ammonium sulfate.
- After this step, the partial purified antimicrobial peptides solution was tested anti-MRSA activity by agar well diffusion technique

2.2 COMPLETE PURIFIED ANTIMICROBIAL PEPTIDES

- The antimicrobial peptides solution from 2.1 was passed through cation exchange chromatography on a SP-Sepharose Fast Flow column, which was eluted by 0-0.5 M linear gradient of sodium chloride.
- Each fraction was tested anti-MRSA activity by agar well diffusion technique.
- Active fractions were applied to high performance liquid chromatography (HPLC) on a C18 reverse-phase column, and performed by 0 - 100% acetonitrile/H_2O linear gradient containing 0.1% (v/v) trifluoroacetic acid over a 60-min period.
- The complete purified antimicrobial peptides was confirmed by single peak on chromatogram .

3. ANTI-MRSA ACTIVITY ASSAY

- Each isolate of clinical MRSA was adjusted to a 0.5 McFarland standard and swabbed on sterilized Mueller Hinton agar plates.
- The swabbed plates was drilled and added 80 µl of partial and complete purified antimicrobial peptides and incubated at 37 ˚C, 24 h.
- The inhibition zone was measured and expressed as arbitrary units per ml (AU/ml).

RESULTS

The antimicrobial peptides was purified by ammonium sulfate precipitation, cation exchange chromatography on a SP-Sepharose Fast Flow column and high performance liquid chromatography on a C18 reverse-phase column. The final step, 3 active peptide peaks were exhibited on chromatogram at retention time about 22.90-, 23.20- and 26.30- min (Fig. 2) and showed anti-MRSA activity, which had specific activity at 200, 250 and 300 AU/ml, respectively (Table 1).

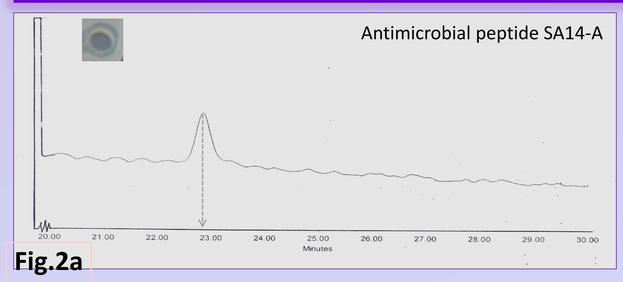

Fig.2a

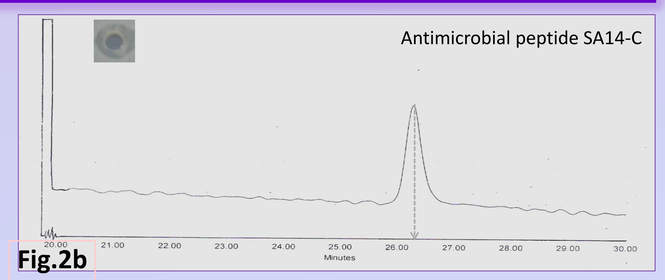

Fig.2b

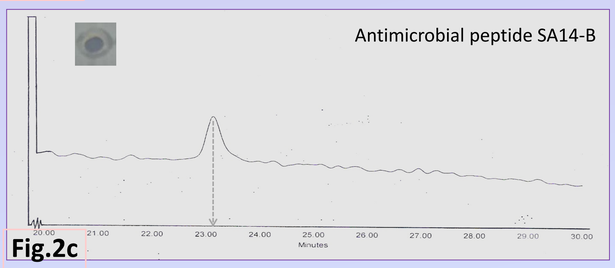

Fig.2c

Table 1

Purification stages	Volume (ml)	Specific activity (AU/ml)	Total activity (AU)	Purification fold	Recovery (%)
Culture supernatant	1000	120	1.2×10^5	1	100
Ammonium sulfate	20	200	0.04×10^5	1.6	3.3
SP Sepharose	10	300	0.03×10^5	2.5	2.5

DISCUSSION

In this study, the extracellular antimicrobial peptide was accumulated in the culture broth of 2-days culture of *Brev*. *laterosporus* SA14, which has been showed the anti-MRSA activity. However, the anti-MRSA activity was higher when the *Brev. laterosporus* SA14 was cultured in a longer time (4-days) which showed the specific activity is 120 AU/ml. Gram-positive endospore forming bacilli have long been associated with the production of antimicrobial peptides, which is secondary metabolite, such as, thuricin, cerein, gramicidin, polymyxin and lichenin have been recovered from *Bacillus* sp. including laterosporulin, bacitracin and loloatin A which recovered from *Brev. laterosporus*. The antimicrobial peptide SA14-A, SA14-B and SA14-C were obtained by a three-steps of purification procedure including (i) ammonium sulfate precipitation (ii) cation exchange chroma-tography and (iii) C18 reverse-phase high performance liquid chromatography. They showed anti-MRSA activity, which had the specific activity is 200, 250 and 300 AU/ml, respectively and higher than the specific activity of 4-days old culture supernatant in a range of 1.6 - 2.5-fold, The SA14-A, SA14-B and SA14-C is a cationic antimicrobial peptides because they can be adsorbed by a cation exchanger. Furthermore they can be dissolved in water and organic solvents such as acetonitrile, which indicated that SA14-A, SA14-B, and SA14-C are amphipathic, containing both hydrophilic and hydrophobic amino acid residues, and may form alpha helices. The secondary structure prediction analysis also showed the same results, in other words the increased alpha helical content correlates with stronger antimicrobial activities. Several researches revealed that antimicrobial peptides interact with the target cell membrane through the formation of ion channels and transmembrane pores, causing extensive membrane rupture and eventually leading to the lysis of the microbial cells (Brogden et al., 2005). The active peptides reported here showed a total net positive charge, indicating it is likely to be initially attracted to the net negative charges that exist on the outer envelope of the MRSA cells such as anionic phospholipids, phosphate groups on lipopolysaccharide LPS and teichoic acids. Another part of active peptides, which is hydrophobic amino acid residues, attach with MRSA cell membrane and insert into the bilayer, forming transmembrane pores, and across through the pores to interact with the intracellular targets. Once in the cytoplasm, translocated active peptides can bind to DNA, and then inhibit DNA, RNA and protein synthesis including inhibit enzymatic activity, finally leading to the lysis of the target cell.

CONCLUSION

The 3 antimicrobial peptides, produced by *Brev. laterosporus* SA14, achieved after three-step purification and confirmed by single peak which showed on chromatogram. They can be inhibited all the clinical isolates of tested MRSA. The 3 antimicrobial peptides is medical important substance which may be developed into antibiotic for infectious MRSA treatment. Our future efforts will focus on characterization and cytotoxicity against normal human cell line of 3 antimicrobial peptides and its chemical structure.

ACKNOWLEDGEMENT

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