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Characterization of bioactive crude from culture broth of *Brevibacillus laterosporus* SA14 against clinical isolates of methicillin-resistant *Staphylococcus aureus* and *in vitro* cytotoxicity to human colon cancer HT-29 cells

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Abstract *Brevibacillus laterosporus* SA14 excreted the antimicrobial peptides into culture broth on the 1st day of cultivation. The antimicrobial peptides in 1-day culture broth were precipitated by using ammonium sulfate of 50% saturation, named as bioactive crude. Its characteristic measurements indicated that the bioactive crude had a relatively gram-positive bacteria with inhibitory spectrum especially *Staphylococcus aureus* TISTR 517 and tested MRSAs by agar well diffusion. The MICs of bioactive crude (ranged from 0.003 to 0.049 mg/ml) against all tested MRSAs was documented. The anti-MRSA activity of bioactive crude was not affected by a wide pH, chemical agents and temperature range. Bioactive crude at the concentration used against MRSA did not cause toxicity to human colon cancer HT-29 cells. Its activity of bioactive compound against MRSAs would provide further evaluation of known chemical structure and may be helpful for its clinical implication.

Keywords: *Brevibacillus laterosporus*, cytotoxicity, HT-29 cell, MIC, MRSA

Introduction

The area of antibacterial peptides is under intense investigation for finding new antimicrobial agents. Polypeptide antibiotics which constitute the *Bacillus* bacteria have been gaining importance as a result of studies. The *Bacillus* species that produced antibiotics were *B. subtilis*, *B. polymyxa*, *B. brevis*, *B. licheniformis*, *B. circulans*, *B. cereus*, *B. thuringiensis* including *Brevibacillus laterosporus* (Tagg *et al*., 1976; Katz and Demain, 1977; Jack *et al*., 1995; Shida *et al*., 1996; Sanders *et al*., 2003). *B. laterosporus,* previously classified as *Bacillus laterosporus*, is an aerobic spore-forming bacterium

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characterized by its ability to produce canoe-shaped lamellar parasporal inclusion adjacent to spore. The species has long been known to include strains toxic to certain invertebrate organisms (Favret and Yousten, 1985; Rivers *et al*., 1991; Orlova *et al*., 1998; Huang *et al*., 2005). In addition, some strains of *B. laterosporus* produced the medically important substances spergualin (Nemoto *et al*., 1987; Umezawa and Takeuchi, 1987), and bacithrocins A, B and C (Kamiyama *et al*., 1994) including a peptide antibiotic with cyanolytic activity (Krachkovskii *et al*., 2002). We have been reported a strain of *B. laterosporus* SA14, an environmental isolate that produced some antibacterial agents and inhibit the growth of a number of pathogenic bacteria. Bioactive crude from. *B. laterosporus* SA14 could show anti-methicillin resistant *Staphylococcus aureus* (MRSA) activity and gave more potent than oxacillin (Chawawisit and Lertcanawanichakul, 2008). In drug discovery, antimicrobial agents are the first identification which determine whether they have clinically relevant antimicrobial activity, then tested for toxicity, and finally tested for their ability to be delivered to the site of infection. Therefore, the objective of this paper was aimed to (i) characterize the corresponding anti-MRSA agent and (ii) test for cytotoxicity of anti-MRSA agent against human cell line.

Materials and methods

Bacterial strains, media, and culture conditions

The clinical isolates of 50 MRSA were collected from patients with skin and soft tissue infections in the Maharaj Nakhon-Si-Thammarat Hospital, Nakhon-Si-Thammarat, Thailand. These isolates were MRSA, identified by using conventional laboratory methods (CLSI, 2006). *S. aureus* TISTR 517 was purchased from Thailand Institute of Scientific and Technological Research, Thailand. *B. laterosporus* SA14, anti-MRSA agent producing, was isolated from air sample at Walailak University, Thailand.

Luria-Bertani (LB: Scharlau) agar media was used in the study as antibiotic-producing medium. Mueller-Hinton (M-H: Merck) was used for plating of the clinical strains of MRSA and also to carry out the anti-MRSA activity assay. The anti-MRSA agent producing SA14 was grown in LB medium. Initially, the seed inoculum was prepared in shake tube condition by transferring only one colony from LB plate in 5 ml of LB medium for 24 h at 37 °C and 150 rpm. The 2% seed culture corresponding to standard McFarland No.0.5 was transferred to a 250-ml flask with 40 ml of LB medium. The experiment was carried out in duplicate. The culture flask was incubated at 37 °C for 5 days at 150 rpm. After every 24 h, 1 ml of the culture broth was drawn from each flask and centrifuged at 10,000 rpm to pellet the cells and the

concentration of protein in culture broth was assayed by Bradford as described elsewhere (Bradford, 1976), using Test Kit (Bio-Rad). The culture broth was collected for every 24 h and was used for the anti-MRSA activity assay.

Anti-MRSA activity assay

In vitro anti-MRSA activity was measured for 5 days by agar well diffusion assay with plates overseeded with representative strains of MRSA as described by Cintas *et al*. (The culture broth was collected everyday, for 5 days, then agar wells were filled with 80 µl of culture broth and corresponding plates were incubated at 37 °C overnight. The inhibition zone around the well was documented.

Bioactive crude preparation of strain SA14

B. laterosporus SA14 was grown in LB broth at 37 °C until it reached late log phase. Cells were removed by centrifugation (4°C, 10,000 rpm, 10 min) and peptides in the culture broth were precipitated at 4 °C overnight with ammonium sulfate of 50% saturation. The resulting precipitate was harvested by centrifugation (4 °C, 10,000 rpm, 30 min), resuspended in phosphate buffer (pH 7.2) and dialysed, using SnakeSkin Dialysis Tubing (Bio-Active) cutoff of 3.5 K, against the same buffer at 4 °C for 24 h.

Effect of enzymes, temperature, pH, and surfactants

Bioactive crude preparation (1.56 mg protein/ml) was incubated at 37 °C with proteinase K, pronase, trypsin, chymotrypsin, lipase, lysozyme or amylase (Sigma, USA) to a final concentration of 1 µg/ml for 3 h at room temperature or was incubated at -20 °C for a day. To determine the effect of temperature at different pH values, the bioactive crude preparation (1.56 mg protein/ml) was adjusted to pH 2.0, 7.0 or 10.0 using 1 M HCl or 1 M NaOH. These were then incubated either at 37 °C for 5 h or at 121 °C for 15 min and readjusted to pH 7.0 for the residual anti-MRSA activity assay. The effect of SDS, Tween 80 and Triton X-100 [at 1 v/v%] and EDTA (10 mM) was determined by incubating the bioactive crude with any of these agents at 37 °C for 5 h. The sample then was diluted 10-fold and assayed for residual anti-MRSA activity. The results were documented by multiplying of the dilution factor 10. Untreated bioactive crude was used as control.

The residual anti-MRSA activity was determined by a dilution as described elsewhere (Cintas *et al*., 1995). Briefly, agar wells were filled with 80

μl serial twofold dilutions of treated bioactive crude and corresponding plates were incubated at 37 °C for 24 h. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution yielding a definite zone of inhibition on the MRSA lawn.

Minimum inhibitory concentration (MIC) determination

The MIC was determined by using a microplate assay (NCCLS, 1991). The tested MRSAs were grown to mid-log phase in M-H medium in a condition of 37 °C for 24 h. The overnight cultures were separately adjusted to a 0.5 McFarland standard (Parrot *et al*., 1989). The inoculum was diluted to obtain approximately 1.5×10^6 cells/ml. The number of viable cells in the inoculum was not determined for the standard suspension and it probably varied depending on the strains tested. However, it was the same for bioactive crude tested simultaneously.

Twofold dilutions of the bioactive crude preparation (0.003-1.56 mg protein/ml) were freshly prepared with microtiter plates (Nunc). The wells finally containing 100 μl of M-H medium with or without (control) bioactive crude. One hundred micolitres of standardized each MRSA suspension was added to each well to a final volume of 200 μl then the plates were incubated at 37 °C for 24 h. After incubation period, the OD was measured with a microplate reader (model Reader 250, biomerieux) using double wavelength at 620 nm and 690 nm. The OD obtained from the latter wavelength was used for background subtraction. The blank was the M-H medium alone by incubation of the same condition. The MIC was calculated from the highest dilution showing complete inhibition of the tested MRSAs (OD equals OD of the balnk). The MIC determinations were repeated independently 3 times, always with *Staphylococcus aureus* TISTR 517 as a control. The results are presented as the mean±SD.

Cytotoxicity test using MTT assay

The effect of bioactive crude on the inhibition of HT-29 cells (kindly supplied by Dr.Walee Chamulitrat, Heidelberg, Germany) was determined. The cells were grown in DMEM supplementing with 10% FCS and 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37^oC. For MTT assay, HT-29 cells were trypsinized and plated with a density of $5x10^4$ cells/9.4 cm^2 of 6-well plate. Cells were allowed to grow for 36 h to obtain 70% confluent cells. Bioactive crude (1.56 mg protein/ml) from strain SA14 was prepared at various concentrations and was added into the culture. After incubation for 24 h,

complete culture medium containing 0.024-1.56 mg/ml concentration of bioactive crude were removed. MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] at final concentration of 0.5 mg/ml in complete medium was added and incubated for 4 h. The medium was removed and MTT-formazan crystals were solubilized by adding DMSO. The absorbance was recorded at the wavelength of 560 nm. The effect of bioactive crude on growth inhibition was assessed as percent cytotoxicity. Data were expressed as mean±SD from three-independent experiment in duplicate. Bioactive crude concentration inhibiting 50% (IC_{50}) growth compared to control (untreated cells) was calculated.

Results

Anti-MRSA activity of bioactive crude

The anti-MRSA production by SA14 was found after 24 h of incubation and remained up to the 5th day of the preliminary experiment. After every 24 h, the culture broth from the SA14 culture flask was tested for its anti-MRSA activity. An initial zone of inhibition measuring was observed on the 1st day and remained constant for anti-MRSA activity for at least 5 days with a zone of inhibition measuring in range of 16-18 mm for 80 µl of culture broth. The bioactive crude precipitated from 1 day-old culture broth showed adverse effect against all the tested MRSAs (MIC range from 0.003 to 0.049 mg protein/ml) with an average of 0.022±0.01 mg protein/ml (Table 1).

Sensitivity to temperature, pH, enzymes, and surfactants

The anti-MRSA activity of the bioactive crude revealed a reduction of 50% in the activity for all the heat treated samples (121°C). In context with the different pH values (2.0, 7.0 or 10.0), the anti-MRSA activity remained unaffected at both pH (2.0 or 7.0), exceptionally, the activity was decreased 50% at pH 10.0. The anti-MRSA agent was sensitive to digestion with different enzymes especially after incubation at -20 °C for a day. The anti MRSA was retained its full activity following exposure to SDS, Tween 80, Triton X-100, and EDTA (Table 2).

Biocompatibility of bioactive crude with human colon cancer HT-29 cells

Biocompatibility was performed for therapeutic potential of the bioactive crude against representative strains of tested MRSAs. It was tested whether the concentration used caused toxicity to human cells. Bioactive crude treatment

(0.024-1.56 mg protein/ml for 24 h) of HT-29 colon cancer cell line was performed for cytotoxicity. The results showed that bioactive crude at 0.024-0.195 mg protein/ml did not inhibit growth of HT-29 cells. Higher concentration (0.39-1.56 mg protein/ml) of bioactive crude resulted in a dose-dependent inhibition of cell growth (Fig. 1). Mean IC_{50} of bioactive crude was 0.36±0.006 mg protein/ml. This concentration was approximately 16.4-fold higher than that concentration used against MRSAs. It was suggested that the concentration of 0.022±0.01 mg protein/ml did not cause cytotoxicity to human cells.

Table 1. Activities of bioactive crude of strain SA14 against tested MRSAs

Organism(s)	MIC (mg/ml) of bioactive crude
Staphylococcus aureus TISTR 517	0.006
MRSA	
304	0.025
1282	0.025
6634	0.025
1636	0.025
1065	0.025
1436	0.025
423	0.025
1383	0.025
8176	0.012
2559	0.049
1388	0.049
1925	0.025
189	0.012
1424	0.003
7181	0.012
7645	0.025
438	0.025
1479	0.025
392	0.025
1606	0.025
2468	0.003
1965	0.025
1890	0.025
1195	0.012
1438	0.025
1181	0.025
2499	0.049
1096	0.025
1400	0.025
1541	0.025
696	0.025
2508	0.012

382	0.025
239	0.025
7535	0.025
2503	0.025
1801	0.025
931	0.025
7669	0.025
1995	0.012
644	0.012
1891	0.012
7613	0.025
7234	0.006
8161	0.006
8164	0.025
7672	0.025
106	0.006
1610	0.025
142	0.025
Mean±SD	0.022±0.01

TISTR, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand
MRSA, Methicillin resistant *Staphylococcus aureus*

Table 2. Effect of different enzymes, temperature, pH and surfactants on inhibitory activities of bioactive crude from strain SA14

Treatment	Inhibitory activities of bioactive crude (AU/ml)
Enzymes	
Control	50
Amylase	25
Lipase	25
Lysozyme	25
Chymotrypsin	25
Pronase	25
Proteinase K	25
Trypsin	50
pH, and Temperature (°C)	
Control	50
2.0, and 37	50
2.0, and 121	25
7.0, and 37	50
7.0, and 121	25
10.0, and 37	25
10.0, and 121	25
Surfactants	
Control	32
SDS	32
Tween 80	32

Triton X-100	32
EDTA	32

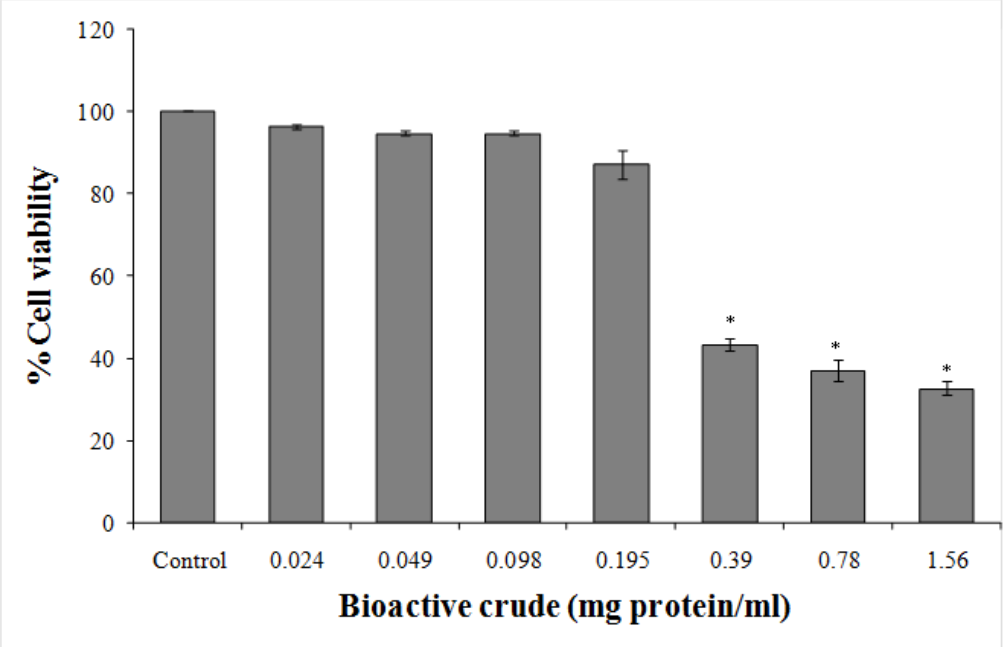

Fig. 1. Effect of bioactive crude treatment on HT-29 cell growth. The cells were treated with SA14 bioactive crude varied from 0.024-1.56 mg protein/ml for 24 h. The growth inhibition of HT-29 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For control, HT-29 cells were cultured in culture medium without bioactive crude. Data shown are mean±SD of three-independent experiments in duplicate (* p<0.001 vs. control).

Discussion and Conclusion

In this study, the extracellular antibacterial agent was shown to accumulate in the growth medium as seen on the 1st day of the culture period. The activity of this antibacterial agent was the most effective against gram-positive bacteria, *S. aureus* and MRSAs. An attempt was made to precipitate this antibacterial agent in the 1 day-old culture broth using ammonium sulfate (50% saturation). Its inhibitory activity showed similar patterns of spectra as 1 day-old culture broth.

The inhibitory activity of bioactive crude of *B. laterosporus* SA14 was decreased upon treatment with proteolytic enzymes such as, chymotrypsin, pronase or proteinase K but was not affected by trypsin. Surprisingly, non-proteolytic enzymes also reduced the inhibitory activity. Some strains of *Bacillus* genus often produced a series of nonribosomal peptide isoforms which contained unusual residues such as amino acids of formylated, acylated, and covalent linked to another function group in nonribosomal peptide gramicidin,

surfactin, iturin and tauramamide (Cosmina *et al*., 1993; Tsuge *et al*., 2001; Kessler *et al*., 2004; Desjardine *et al*., 2007), as well as post translational modified amino acid in ribosomal peptide subtilin and sublactin (Entian and de Vos WM, 1996; Paik *et al*., 1998). Therefore, the lost of inhibitory activity after exposed with enzymes might be a result in the presence of one or more of these elements, also they could interfere with peptide sequencing. The anti-MRSA activity is the most stable at pH 7.0 after incubating at 37°C for 5 h and its inhibitory activity was still observed at pH 2.0 or 10.0 after either incubating at 37°C or at 121°C (Table 1). The lower the pH the higher is the stability of anti-MRSA agent to high temperature, similar to previous findings for subpeptin (Wu *et al*., 2005).

The biocompatibility of bioactive crude with human HT-29 colon cancer cells was assessed because its potential for toxicity is a major obstacle limiting their clinical use. Bioactive crude from SA14 caused minimal toxicity seen in this study. It has been shown that 50% inhibitory dose of pexiganan with A549 lung epithelial cells was approximately 4.08 µM and MICs of the compound against *S. aureus* ATCC 33591 was 6.5-13 µM (Chongsiriwatana *et al*., 2008). This suggested that bioactive crude from SA14 may serve as an alternative to antibiotics in the prevention and treatment of MRSA infection. The results reported the potential use of bioactive crude as anti-MRSA agent in the future.

The strain SA14 appears to have a potential to produce anti-MRSA agent, which is resisting to acidic or basic environment. Stability of the anti-MRSA agent to high temperature is considered to be very important. Cytotoxicity to human cells was minimal and that emphasizes their therapeutic potential. These characteristics make it good candidates for the food industry and human health in future.

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