RESEARCH ARTICLE

Lipopeptides Extract from *Bacillus Amyloliquefaciens* Induce Human Oral Squamous Cancer Cell Death

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Abstract

A lipopeptide extract of *Bacillus amyloliquefaciens* BACY1 (BLE) was found to induce cell death in human oral squamous cell carcinoma (OSCC) cell lines, SCC4 and SCC25, in this study. The results of MTT assay showed that BLE inhibited OSCC cell proliferation in a dose-dependent manner. BLE was also effective in increasing the sub-G1 phases. Furthermore, when membrane damage in SCC4 cells treated with BLE was monitored by LDH assay, release of LDH was significantly increased. The protein and mRNA levels of pro-apoptotic Bax, and caspase-3 were up-regulated by BLE. Taken together, these results suggest that BLE induces apoptosis and then inhibits the cell proliferation of human OSCC cells.

Keywords: *Bacillus amyloliquefaciens* BACY1 - lipopeptide extract - oral squamous cell carcinoma - apoptosis

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Introduction

Bacillus spp., established by Chon in 1872, is made up of Gram-positive aerobic or facultative endospore-forming rod-shaped bacteria. The optimal temperature for growth is ranged from 30°C to 40°C, with no growth below 15°C or above 50°C. They are widespread in the environment being found in dust, soil, water, air, and vegetable matter, and are an important source of fine biochemicals, antibiotics, and insecticides. Several species of *Bacillus* have been reported to be effective against plant pathogens (Yoshida et al., 2001). They can antagonize pathogens by competing for nutrients and alcove, by stimulating the defensive capacities of the host plant and by producing fungal toxic compounds that render the host more resistant to further pathogen ingress (van Loon et al, 1998; Durrant and Dong, 2004). Antibiotics from *Bacillus* spp. were identified to biosurfactants (Stein, 2005). These compounds, such as lipopeptide, glycolipid, phospholipid, fatty acids, polymeric and particulate biosurfactants, are microbially derived surface-active and amphipathic molecules produced by various microorganisms such as bacteria, filamentous fungi and yeast during growth (Zasloff, 2002; Stein, 2005). Previous studies have indicated that lipopeptides play a major role in disease suppression, because of their high surface activities and antibiotic potential (Romero et al., 2007).

To the best of our knowledge, there are few studies on the cytotoxic activities of marine derived lipopeptides, such as cytotoxicity of *Bacillus vallismortis* metabolites on colon cancer carcinoma cells, the metabolites of *Bacillus* SW31 (similar to *Bacillus firmus*) induced apoptosis of head and neck cancer cells, and the metabolites of *Bacillus pumilus* MB 40 inhibits proliferation of K562 leukemic cell (Jeong et al., 2008; Lim et al., 2010; Moushumi Priya and Jayachandran, 2012). Gradually works are going on regarding *Bacillus* profitable effects on human beings.

The prevalence of oral cancers is high in Asian countries, especially in South and Southeast Asia (Krishna Rao et al., 2013). Oral cancer occurs more often in males compared to females, because of lifestyle differences and hereditary predominantly. Smoking was positively associated with oral cancer risk and a similar direct association was also seen among betel quid chewers. The World Health Organization predicts a rise in worldwide oral cancer incidence in the next decades, and oral squamous cell carcinoma (OSCC) accounts for most malignancies found in the oral cavity (Dantas et al., 2003). Characteristically, OSCC cells, at the later stage of malignancies, often develop drug resistance to against chemotherapeutic agent-mediated apoptosis during the course of tumor progression, leading to poor clinical outcomes (Luqmani, 2005).

Previous studies on *Bacillus* spp. have shown that various strains produced peptide antibiotics with various chemical structures. The chemical and physical diversity of peptide antibiotics makes them ideal candidates not only for therapeutic applications but also in other areas. However, there is no report on the effect of lipopeptide purified from *Bacillus amyloliquefaciens* BACY1 on human OSCC cells. In the present study, an insight of *B. amyloliquefaciens* application was conducted by analyzing anti-proliferative, and apoptotic effects on human SCC4 and SCC25 cells, and to further understand the possible

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regulatory mechanisms at molecular levels.

Materials and Methods

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (USA) and methanol was obtained from J.T. Baker Co. (USA). propidium iodide and proteinase inhibitor cocktail was purchased from MILLIPORE (USA). Chemiluminescence Reagent Plus (Western Lightning™) was purchased from PerkinElmer Life Sciences (USA). Primary antibodies: anti-Bax, anti-Bcl-2 and anti-caspase-3 (Cell Signaling Technology, USA) and anti-actin (MILLIPORE, USA) were purchased from the indicated companies. Secondary antibodies: goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were purchased from Sigma-Aldrich (USA).

Isolation of cyclic lipopeptides

The BACY1 isolate of *Bacillus amyloliquefaciens* used in this study was isolated from soil in Chiayi and identified using standard morphological and biochemical tests. The complex medium for cultivation of *B. amyloliquefaciens* BACY1 consisted of Luria-Bertani (LB) medium (Difco Laboratories, USA). Crude cyclic lipopeptides were prepared from culture supernatants of *B. amyloliquefaciens* BACY1 cultured at 28°C with 250 rpm agitation for 48 h. The culture broth was centrifuged (12,000 rpm for 30 min at 4°C) to remove the cells, and followed by acid precipitation 30 min at 4°C. Centrifugation once more, solution was neutralized with 0.1 N NaOH and resuspension with methanol. The lipopeptides extract obtained was named BLE.

Cell lines and cell culture

Human oral squamous cell carcinoma (SCC4 and SCC25) cell lines were cultured in Dulbecco's Modified Eagle's Medium/F12 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (Biological Industries, USA) , 1% 100 U/mL of penicillin and 100 μg/mL of streptomycin and incubated at 37°C under a humidified 5% CO_2 and 95% air at one atmosphere. The medium was renewed every 2 days.

Cell viability assay

Viable cells were detected using the MTT, which forms blue formazan crystals that are reduced by mitochondrial dehydrogenase present in living cells. Cells were inoculated in 96-well plates at a density of 5×10^3 cells per well in 200 μL complete medium. Attached cells were incubated for 24 h prior to the addition of BLE at concentrations of 0, 10, 30, 50, 100, 200, 300, 400 μg/mL), and were then incubated for 24 h, at which time point cells were incubated with 1 mg/mL MTT (50 μL/well) for further 3.5h at 37°C. Reduced MTT was dissolved in DMSO (100 μL/well), and the absorbance was measured at 570 nm excitatory emission wavelength by μQuant (Bio-Tek, USA). The effect of BLE on cell cytotoxicity was appraised as percent cell viability compared to vehicle-treated control cells, which were arbitrarily assigned 100% viability.

Lactate dehydrogenase (LDH) assay

LDH assay is a common assay to estimate a substance's toxicity towards cellular membranes, which irrespective influences on the cells proliferation. It was carried out by the Cytoscan™-LDH Cytotoxicity assay kit (G-Biosciences, USA) according to the manufacturer's protocol. Cells were grown in 96-well microplates at a density of 5×10^3 cells per well in 100 μL of medium. After incubation for 24 h, the cells was treated in quadruplicate with fresh medium containing BLE at 300 μg/mL for 24, 48 and 72h. Control wells contained the cells in the absence of the test compound. After the exposure completed, the 96-well plate was spun at 1800 rpm for 5 min. Thereafter, a 50-μL of supernatant transferred into a new 96-well plate, then add 50 μL reaction mixture from the commercial kit and incubated for 20 min at 37°C in the dark The LDH content was assayed spectrophotometrically that is determined the amount of reduced nicotinamide adenine dinucleotide (NAD) at 490 nm in the presence of lactate and LDH by using a microplate reader (μQuant, BioTek, USA). LDH levels were defined as the light absorption in test wells compared to that in positive (complete cell destruction) and negative (spontaneous cell destruction) control wells.

$$\%\ \text{Cytotoxicity} = \frac{(\text{Experimental } (A_{490}) - \text{Spontaneous } (A_{490}))}{\text{Maximum LDH release } (A_{490})} \times 100$$

Assessment of cell density

SCC4 cells at a density of 2×10^5 cells/well were plated onto 24-well plates and incubated at 37°C for 24 h. Different concentrations of BLE extract (0, 100, 200, 300, 400 μg/mL) were added and the cells were incubated for 24 h. For morphological assessment, the cells were examined under a phase-contrast microscope and were photographed.

Colony-forming ability assay

Immediately after drug treatment, the cells were washed with phosphate-buffered saline and trypsinized to determine the cell numbers. The cells were plated at a density of 500-1000 cells on a 60 mm-diameter Petri dish in triplicate for each treatment and cultured for 12-14 days. The cell colonies were stained with 1% crystal violet solution in 30% ethanol. Colonies containing more than 20 cells were scored. Cytotoxicity was determined to be the number of colonies in the treated cells divided by the number of colonies in the untreated control.

Cell cycle distribution and apoptosis determination by flow cytometric assay

SCC4 cells from exponentially growing cultures were seeded (4×10^5 cells) within 6 cm culture plates and incubated with different concentrations of BLE for 0, 12, or 24 and 48h. In each time point, the harvested cells were washed with PBS and centrifuged at 1,500 rpm for 10 min. After fixed with 70% ice methanol at 4°C overnight, the cells were resuspended in PBS including 0.5% RNase A, and 10 mg/mL propidium iodide for 30 min in the dark at room temperature. PI is a highly water soluble, fluorescent compound that cannot pass through intact membranes. It

binds to DNA by intercalating between the bases in double stranded nucleic acids of exposed nuclei while excluded from viable cells. Flow cytometric analysis was performed using a flow cytometer (BDFACS CantoII, USA). The histogram of the cell cycle distribution was analyzed by WinMDI 2.9 software.

Reverse transcription-polymerase chain reaction (RT-PCR)

SCC4 cells at 4×10^5 cell/ml density were treated with 300 μg/mL of BLE for 24, 48, and 72 h and total RNA was extracted using the GeneMark RNA Purification Mini Kit (GeneMark, Taiwan). After extraction, the pellet was resuspended in 20 μL of DEPC (diethyl pyrocarbonate) treated water. cDNA synthesis was carried out using High performance MMLV (Moloney Murine Leukemia Virus) reverse transcriptase cDNA synthesis system (EPICENTRE, USA) at 37°C for 30 min and then 85°C for 1 h according to the standard protocol of the supplier. Single stranded cDNA was amplified by PCR with the following primers.

The sequences of respective primer are: gadph (F-ATCTTCCAGGAGC GAGATCC; R-ACCACTGACACGTTGGCAGT), bcl-2 (F-GATTGATGGGATCGTTGCCT; R-A ATGCCCCAGGATGTACAGA), bax (F-CCAGAGGCGGGGGATGATTG; R-ATCTG AAGATGGGGAGAGGGC), caspase-3 (F-TGAGGCGGTTGTAGAAGAGTT; R-AC AAAATTGTCACATAGAAACACAC). The PCR conditions applied by Mastercycler (Eppendorf, Hamburg, Germany) were: gadph, 35 cycles of denaturation at 94°C for 30 sec, annealing at 56.4°C for 60 sec, and extension at 72°C for 150 sec; Bcl-2, 35 cycles of denaturation at 94°C for 30 sec, annealing at 45.5°C for 60 sec, and extension at 72°C for 150 sec; Bax, 35 cycles of denaturation at 94°C for 120 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec; caspase-3, 35 cycles of denaturation at 94°C for 30 sec, annealing at 54.5°C for 60 sec, and extension at 72°C for 150 sec.

The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (gapdh) was amplified as an internal control. The samples were analyzed in triplicate, and the results of each sample were normalized to gapdh. PCR products were run on 1.2% agarose gels, stained with ethidium bromide and documented. The density of the bands was quantitated by scanning the bands on a gel documentation and analysis system.

Western blot analysis

Cells were plated at a density of 1×10^6 cells in 100-mm culture dishes. After incubation of cells for the indicated time periods with or without the treatment of BLE, the cells were harvested, washed with cold PBS and lysed in whole cell extract buffer (20 mM HEPES pH 7.6, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na_3VO_4, 50 mM NaF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and supplemented with 1% proteinase inhibitor cocktail). Protein concentration was determined by the Bradford assay and BSA was used as a protein standard. Proteins (20 μg) were separated on 4-12% acrylamide gel by SDS-PAGE and transferred onto activated nitrocellulose membranes (Pall, USA). The membranes were washed with TBST (Tris-buffered saline/0.1% Tween 20) followed by blocking with TBST containing 5% (w/v) non-fat dried milk. The membranes were incubated overnight with primary antibodies specific for cleaved caspase-3 (1:1000), Bcl-2 (1:1000), Bax (1:1000) or Actin (1:5000) at 4°C. The membranes were then exposed to secondary antibodies coupled to horseradish peroxidase for 1h at room temperature. The membranes were washed three times with TBST at room temperature. Immunoreactivities were detected by ECL reagents. Equal protein loading was assessed by the expression level of actin protein.

Statistical analyses

For each protocol, three independent experiments were performed. Results were presented as mean±standard error of the mean (SEM). Statistical calculations were performed using the SigmaPlot 9.0 software (Systat Software, CA). Differences in measured variables between experimental and control groups were assessed using an unpaired t test. $p<0.05$ was considered statistically significant.

Results

Bacillus amyloliquefaciens BACY1 lipopeptides extract has inhibitory effects on human oral squamous cell carcinoma cell lines

To evaluate whether lipopeptides extract from *Bacillus amyloliquefaciens* BACY1 has the potential to inhibit the growth of OSCC cell lines, they were treated with different concentrations of BLE extract for 24 h to monitor the progression of growth by MTT assay. As shown in Figure 1, BLE had a dramatic suppression proliferation on both of SCC4 and SCC25 cells in a dose-dependent manner, whereas untreated cells were maintained in a state of exponential proliferation. IC_{50} of BLE against SCC4 and SCC25 cells was found to be 180 μg/mL and 300 μg/

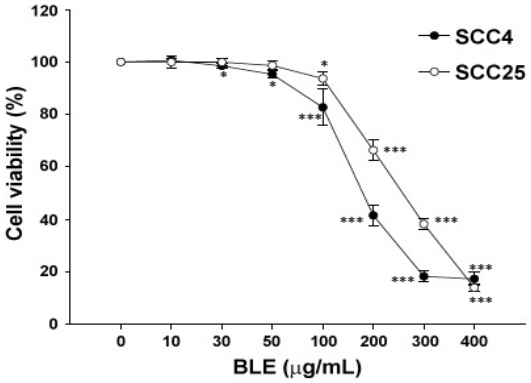

Figure 1. Dose Dependent Response of Viability in Human Oral Squamous Carcinoma Cell Lines Exposed to BLE. SCC4 and SCC25 were treated with 0, 10, 30, 50, 100, 200, 300, or 400 μg/mL of BLE for 24 h. Cytotoxicity was determined by assessment with the MTT assay. The data are described as means ± SE of three independent experiments. The results are expressed as the percent change from the controlled condition. *$p<0.05$; ***$p<0.001$

mL at 24 h, respectively. These results indicated that the extract killed the SCC4 cells predominantly, but it had lower cytotoxicity on the SCC25 cells.

BLE induces alteration of the membrane integrity in SCC 4 cells

Lactate dehydrogenase (LDH) is an enzyme widely present in cytosol that converts lactate to pyruvate. The enzyme may be leaked through pores forming in the cell membrane, including membrane rupture or complete membrane destruction caused by the tested substances. Thus, LDH assay was to evaluate the SCC4 cell membrane integrity after treatment. LDH leaks into media and its extracellular levels elevate depending upon the BLE cytotoxicity and the higher LDH values in the medium indicate higher cytotoxicity levels. In Figure 2, BLE treatment significantly decreased the LDH activity in a time-dependent manner.

Dose-dependent effects of BLE on the SCC4 cell density

In Figure 3, the exposure to elevated concentration of BLE for 24 h caused detectable morphological change in cell viability by phase-contrast microscope. The control cells had a well spread squamous shape. In contrast, after 300 μg/mL BLE treatment, the SCC4 cells significantly began to decrease by 60% and the viable cells exhibited star-like morphology, the contacted among cells loosened and dose-dependent decrease in cell density (Figure 3).

BLE inhibits clonogenic survival in SCC 4 cells

Next, the colony forming ability assay was performed to examine the long-time SCC4 cell survival and to evaluate cancer cell killing efficiency. In Figure 4, the values on colony forming ability were: 34%, 14.8%, 0.1%, after 250 μg/mL, 300 μg/mL, 350 μg/mL BLE treatments, respectively.

Induction of sub-G1 phase by BLE on SCC 4 cells

To define whether the growth inhibition mechanism was related to cell cycle arrest or an apoptotic mechanism, BLE were used for further mechanistic study on SCC4 cells. The hypoploid cells have less cellular DNA content than cell that are typically found in the G0/G1, S or G2/M stages of the cell cycle. Flow cytometric analysis showed that exposure of SCC4 cells to doses of BLE for 12 h promoted approximately the same percentage of hypoploid cells observed in the control and treatment of SCC 4 cells with 300 μg/mL of BLE led to profound changes of the cell cycle profiles after incubation up to 24 h, a significant accumulation of cells in the sub-G1 phase (Figure 5).

BLE up-regulates pro-apoptotic bax and caspase-3 expression and down-regulates anti-apoptotic bcl-2 expression in SCC 4 cells

To gain insight into relevant mechanisms of BLE induced anti-proliferation and apoptosis in human oral squamous cell carcinoma cell. The expression of Bax, caspase-3, and Bcl-2 as a regulator involved in apoptosis were examined by RT-PCR and Western blot analysis. In Figure 6, BLE treatment elevated Bax and caspase-3 at both transcriptional and translational levels in a time-

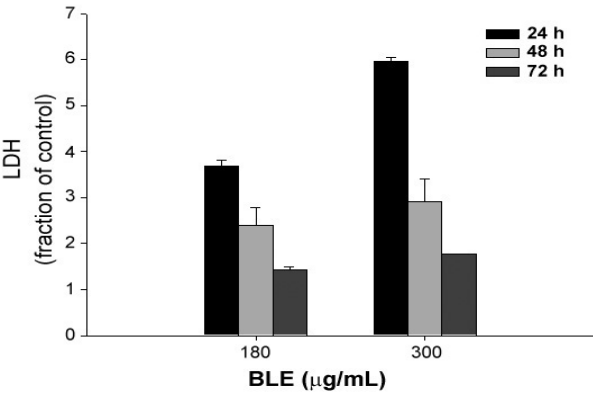

Figure 2. BLE Decreased the LDH Activity in SCC 4 Cells. Cells were treated with BLE (180, 300 μg/mL) for 24, 48, 72 h. LDH membrane integrity was measured as describe in material and method section

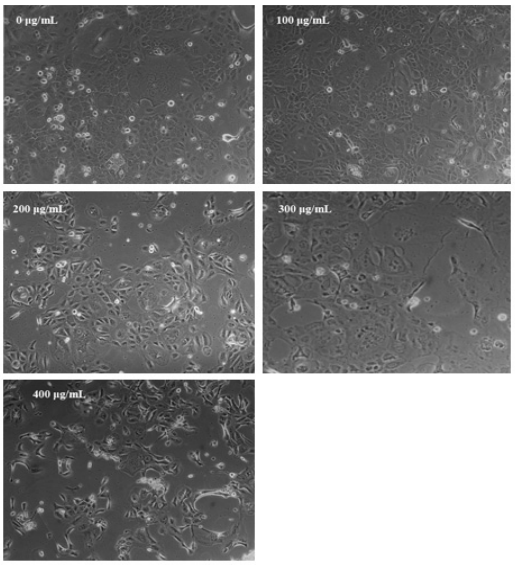

Figure 3. Assessment of Cell Density Exposed to doses of BLE for 24h. (200×magnification)

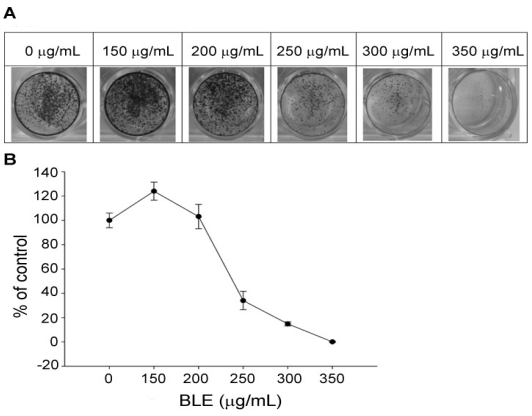

Figure 4. The Effect of Growth Inhibition on SCC4 Cells was Examined by Colony Forming Ability Assay. BLE were added to cells for 24 h, and cytotoxicity was determined by colony-forming ability assay. (A) The photograph of representative dishes shows colony formation in SCC4 cells. (B) The growth of BLE treated groups is expressed as percentage of the control. Values are means ± SD of triplicates of each experiment. *p<0.05, ***p<0.001

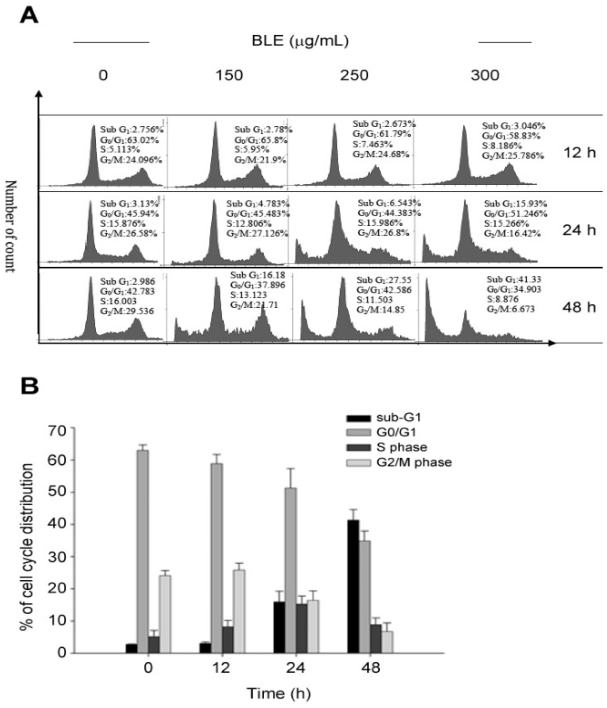

Figure 5. Flow Cytometric Detection of the Cell- cycle Profiles in BLE-treated or Non-treated SCC4 Cells after 12, 24, and 48h. (A) Histogram presentation analysis by WinMDI software shows sub-G1, G0/G1, S and G2/M stages of the cell cycle. **(B)** Time-dependent effect of BLE (300 μg/mL) on the induction of sub-G1 phase in SCC 4 cells. The control cells were treated with medium. The figure is the representative of three independent experiments. **p*<0.05, ****p*<0.001

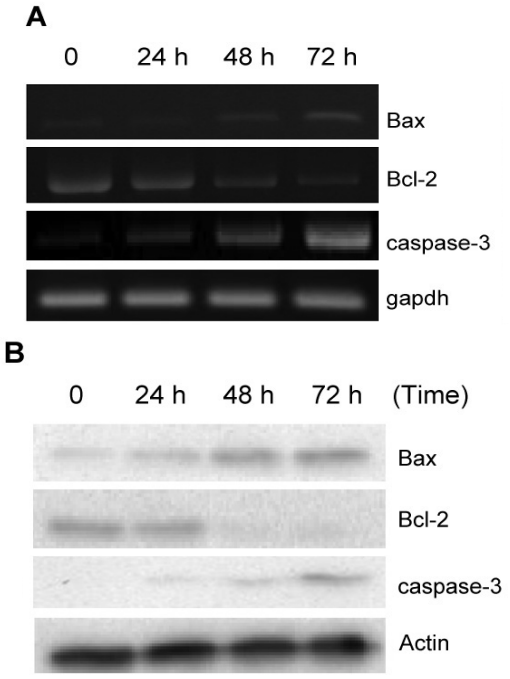

Figure 6. BLE Treatment Modulated the Bax, Caspase 3, Bcl-2 mRNA and Protein Expression in SCC4 Cells. The total RNA and protein of SCC 4 cells was extracted after 24, 48 and 72h of incubation with or without 300 μg/mL of BLE. The expression of the genes of interest was measured by reverse transcriptase PCR **(A)** and the associated-protein levels were carried out by Western blotting **(B)**

dependent manner. In addition, the anti-apoptotic Bcl-2 mRNA and protein were decreased by 300 μg/mL BLE for 48h.

Discussion

Since a great promising chemotherapeutic source for new natural products that have not been well-known in terrestrial micro-organisms, studies of the anti-tumor activities of lipoproteins have been gained intense interest over the last decade. Previous research provided the foundation for the use of microbial extracellular lipoproteins as novel reagents for the treatment of cancer cells (Jeong et al., 2008; Cao et al., 2009; Lim et al., 2010). Although lipoproteins have been tested for potential anti-tumor effects, the mechanism is not clear. *Bacillus* spp. are widely distributed in nature, and due to the passive distribution of endospores, their occurrence is not restricted to their natural habitat. In the present work, *Bacillus amyloliquefaciens* BACY1 proved to produce a secondary metabolite that has been suggested to have potent anticancer activity on OSCC cells.

LDH is commonly used to evaluate the degree of cell membrane damage. If target cells are incubated with a cytotoxic agent, cytoplasmic LDH is released into the culture from dead cells as a result of plasma-membrane damage (Heerklotz and Seelig, 2007). Based on the results demonstrated that there were significantly increased in LDH activity on SCC4 cells at IC_{50} and IC_{80} for BLE. In addition, our results showed that BLE induced the cell shrinkage phenomena suggested that hydrophobic residues of the lipopeptide moieties penetrate rather deeply into the membrane interface, leading to membrane permeabilization but not surface activity (Kim et al., 2007). Consistent with previous reports (Silva et al., 2007), such detergent-like activity was strikingly observed to stabilize leaks due to the compound's ability to construct multiple-structured polymers (Kim et al., 2007). BLE may be acting as a molecule disturbing a biochemical phenomenon that occurs at a specific site (e.g. lipid raft) of the membrane (Kerr et al., 1994). The remarkable modulation of cell survival and death signaling by BLE seems to occur by its intense membrane effects. That is BLE may display detergent-like activity on cell membranes leading to membrane permeabilization.

This study demonstrated that BLE indeed induced the apoptosis of SCC4 cells. The cell cycle distribution demonstrates that BLE sensitized the cells with a concomitant profound increase in the sub-G1 fraction. In this study, the caspase-3, and Bax level were increased in a time-dependent manner in BLE treated SCC 4 cells, in agreement with data in RNA level. It is well known that the ratio between pro- and anti- apoptotic proteins determines in part the susceptibility of cells to a death signal then leads to apoptosis (Vander Heiden et al., 2001). Accordingly, the result in Figure 6 showed that Bcl-2 was significantly decreased and Bax was increased in BLE treated cancer cells. The increased Bax/Bcl-2 ratio indicates that mitochondria-mediated apoptosis is involved in BLE-induced cell death in OSSC cells.

One interesting point of this study was that lots of

reports have shown that several of the biological properties of lipopeptide, including its antiviral and antibacterial activities, due mainly to its interactions with membranes. It can cause the membrane system to disintegrate and finally, result in target cells to burst (Sheppard et al., 1991). Previous studies with protoplasts and eukaryotic cells also proved that surfactin binds readily to cell membranes with a high degree of selectivity, depending on the membrane lipid composition (Rodrigues et al., 2006; Jeong et al., 2008; Lim et al., 2010).

Taken together, the central finding of the present study is that the lipopeptide extract of *Bacillus amyloliquefaciens* BACY1 induces apoptotic death of SCC4 cells. Furthermore, detailed investigation showed that BLE could induce cell death involved up-regulating of caspase-3 and Bax expression.

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