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Antibacterial effect and cytotoxicity of β-1,3-1,4-glucanase from endophytic *Bacillus subtilis* SWB8

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Abstract: [Objective] We studied the antibiotic activity and selective cytotoxicity of β-1,3-1,4-glucanase from endophytic *Bacillus subtilis* SWB8. **[Methods]** Based on gel permeation chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and liquid chromatography-tandem mass spectrometry methods, protein fragments of β-1,3-1,4-glucanase from endophytic *Bacillus subtilis* strain SWB8 were purified and identified. Then, β-1,3-1,4-glucanase was used to evaluate the antimicrobial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Salmonella paratyphi* A, *Shigella dysenteriae*, *Candida albicans* and *Cryptococcus neoformans* and cytotoxicity against human pulmonary adenocarcinoma cells (A549) and human bone marrow mesenchymal stem cells (MSCs) by using the disc diffusion, methyl thiazolyl tetrazolium and flow cytometry methods, respectively. **[Results]** Bacterial β-1,3-1,4-glucanase showed broad antimicrobial spectrum against all nine bacterial and fungal strains. Furthermore, β-1,3-1,4-glucanase possessed significant anticancer activity against A549 cells that the IC_{50} and IC_{90} values were 11.5 and 20.1 μg/mL, respectively. The percentage of apoptotic A549 cells treated with different concentrations of β-1,3-1,4-glucanase was significantly increased from 4.43% of the control to 43.1% of 19.2 μg/mL glucanase in a dose dependent manner. In contrast, these changes could not be observed in human bone marrow mesenchymal stem cells. **[Conclusion]** β-1,3-1,4-glucanase could be a potential source of desirable antimicrobial agent, or anticancer compounds with higher efficiency and lower toxicity.

Keywords: *Bacillus subtilis*, β-1,3-1,4-glucanase, antimicrobial activity, cytotoxicity

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β-1,3-1,4-glucanase (lichenase, E.C.3.2.1.73) belongs to the glycosyl hydrolases family 16 (GH16) with strict cleavage specificity for β-1,4-linkages adjacent to 3-O-substituted glucopyranose

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residues of barley β-glucans and lichenan, yielding trisaccharide and cellotetraose[1]. It is widely presented in the grain, barley endosperm, and microorganism including *Bacillus*, *Fibrobacter*, *Synechocystis*, *Lysobacter*, *Ruminococcus*, *Achlya*, *Aspergillus*, *Paecilomyces*, *Rhizopus*, *Talaromyces*, *Trichoderma*, *Laetiporus* and *Penicillium*[2-5], et al. The recombinant β-1,3-1,4-glucanase gene has been successfully expressed in yeast, *Escherichia coli* and *Bacillus subtilis*. Furthermore, some species of *Bacillus* such as *B. subtilis*, *B. amyloliquefaciens*, *B. macerans*, *B. circulans*, *B. polymyxa*, *B. licheniformis*, and *B. brevis*[1,6], were the main sources of β-1,3-1,4-glucanase products. Based on the high thermal stability and the hydrolytic activity of plant cell wall β-glucan, β-1,3-1,4-glucanase were spaciously used in malt production, fermentation or lagering in the brew industry.

Plant β-glucanase has been documented to contribute to several physiological processes such as pathogen resistance, cell wall synthesis and plant growth[7-8]. Previous studies indicated that chitinase and β-1, 3-glucanase showed certainly antifungal potential by hydrolyzing or inhibiting the synthesis of fungal cell wall β-1, 3-glucans[9-10], causing cytoplasmic ion leakage[11] or arresting cell cycle[12]. Besides, β-1,3-glucanase or β-1,3-1,4-glucanase was also extracellular antifreeze active protein of plant at the cold temperature[13], and enhanced the salt stress tolerance.

However, there are little reports about antibiotic activity of β-1,3-1,4-glucanase against pathogenic microorganisms, although both of β-1,3-1,4-glucanase and β-1,3-glucanase share from a common ancestry and approximately 50% amino acid sequence, in all likelihood, that β-1,3-1,4-glucanase diverged from β-1, 3-glucanase during the appearance of the graminaceous monocotyledons[10].

Here we reported the antibiotic activities of β-1, 3-1,4-glucanase from endophytic *Bacillus subtilis* strain SWB8 of *Dioscorea zingiberensis* C. H. Wright, which is medicinal twining monocotyledonous angiosperms of the family *Dioscoreaceae*. The protein fragments of bacterial β-1,3-1,4-glucanase showed broad-spectrum inhibition effect against all nine tested microorganism strains and selective cytotoxicity against A549 cells, which could be a potential source of desirable antimicrobial agent or anticancer compounds with higher efficiency and lower toxicity.

1 MATERIALS AND METHODS

1.1 Microbial strains and cell lines

Bacillus subtilis strain SWB8 isolated from the rhizome of *D zingiberensis* was deposited at China Center for Type Culture Collection (CCTCC M 2010271). The tested microorganisms are *Staphylococcus aureus* (ATCC25923), *Enterococcus faecalis* (ATCC29212), *B. subtilis* (ATCC6633), *E. coli* (ATCC25922) and clinical isolates including *Salmonella typhi*, *Salmonella paratyphi* A, *Shigella dysenteriae*, *Candida albicans* and *Cryptococcus neoformans* identified by the department of medical microbiology, Hu Bei University of Medicine, China. Both of Human pulmonary adenocarcinoma cells (A549) and human bone marrow mesenchymal stem cells (MSCs) were obtained from the Department of Basic Medicine, Hu Bei University of Medicine, China.

1.2 Acquisition of protein extracts

B. subtilis strain SWB8 was inoculated to 250 mL Erlenmeyer flask containing 100 mL of liquid medium (1% peptone, 0.3% beef extract, 0.5% NaCl, pH 7.2) which was incubated at 32 ℃ with the rotation speed of 148 – 182 rpm for 48 h. After centrifugation (5000 rpm, 5 min, 4 ℃), the supernatant of fermenting liquor was treated with chloroform twice (50 mL, 30 min each time). The ivory white material isolated from the middle of two-phase was reserved. Both ethyl acetate and n-butyl alcohol was also used as mentioned before. After dried at room temperature, the ivory white material (IWM) was dissolved in double-distilled water (ddH_2O). By using a saturated ammonium sulfate (SAS) concentration method, a certainly volumes of SAS and IWM solutions resulted in

reaction mixtures of 40% SAS at room temperature. At 4 ℃, the reaction mixture was set aside for 3 h and centrifuged (3000 rpm, 10 min). The precipitated protein was dissolved in double-distilled water and dialyzed (3500MW-CO, Slide-A-Lyzer ® Dialysis Cassette, Thermo) in 0.1 mol/L phosphate-buffered saline (PBS, pH 7.2) until SAS was completely removed. After dried at room temperature, the protein extracts was dissolved in 0.1 mol/L PBS, then sterilized (0.22 μm Millipore filter) and stored at −20 ℃. Liquid medium were also extracted with the same method mentioned before and subject to the following analysis.

1.3 Gel permeation chromatography (GPC)

The condition of GPC included with Waters GPC HPLC SYSTE; TSK-GEL G3000SW × L chromatographic column (300 × 7.8 mm); column temperature 35 ℃; mobile phase: double-distilled water; flow rate: 0.3 mL/min; detection wavelength: 228 nm. According to the conditions, the protein extracts was assayed and separated. The separated protein was dried with cryochem and dissolved in 0.1 mol/L PBS and sterilized (0.22 μm Millipore filter) for antimicrobial or anticancer test, respectively.

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

In SDS-PAGE the protein extracts (20 μg) was layered on top of 12% polyacrylamide separating gel and 5% polyacrylamide stacking gel, meanwhile the electrophoresis was kept at a constant voltage (100 V) at room temperature until the bromophenol blue marker dye reached the bottom of the gel. The Dalton Mark mixture (15 − 200 kDa) was used as standards. The gel was fixed 12 h in the fixing solution (100 mL methanol, 25 mL acetic acid, 125 mL water), then stained in the stain solution (0.25% Coomassie brilliant blue G-250, 50% methanol, 10% acetic acid) overnight on a rocker table. The gel destaining was done by keeping the gels for about 7 h in fresh fixing solution.

1.5 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS method was used for the identification of protein: protein band was excised from gels and destained with 25 mmol/L NH_4HCO_3 and 50% acetonitrile (ACN) for 30 min at 37 ℃. The protein pieces were embedded with ACN and reduced in 25 mmol/L NH_4HCO_3 and subjected to vacuum drying. The dried gel pieces were immersed in 25 mmol/L NH_4HCO_3 with 10 mM dithiothreitol (DTT) at 57 ℃ for 1 h, the solution was drawn out and then the equal volumes 25 mmol/L NH_4HCO_3 with 55 mM iodoacetamide was added in the dark for 45 min. After washing with 25 mmol/L NH_4HCO_3, 50% ACN and 100% ACN, the gel pieces were added with 8 μL trypsin (0.02 μg/μL) at 37 ℃ overnight. The peptides were extracted with 50 μL 67% ACN (including 5% formic acid) two times, lyophilized and stored at −20 ℃.

The peptides were desalted with C-18 pipet tips (300 μm ID × 5 mm, 5 μm). The desalted peptides were analyzed by LC-MS/MS. By using Data Analysis software version 3.2, the peptide mapping fingerprint and tandem mass spectrometry 2 map were integrated and the MS data were compared with the sequences of proteins in the National Center for Biotechnology Information (NCBI) database to identify proteins.

1.6 Antimicrobial test

By using the disc diffusion method[14], those tested microorganisms were used to determine the antimicrobial activity of the separated protein by GPC at biosafety laboratory (BSL-2). The paper discs that each contained 12 μg of protein were placed on the surface of solid medium (bacterial medium: 1% peptone, 0.3% beef extract, 2.0% agar, 0.5% NaCl, pH 7.2. fungal medium: 1% peptone, 4% glucose, 1.5% agar, distilled water, pH 7.2) coated with the tested microorganism (10^7 − 10^8 CFU per plate) which already incubated for 48 h. PBS (0.1 mol/L, 20 μL), *Gentamicin sulphate* (10 μg) and fluconazole (15 μg) were used as negative or positive control to evaluate inhibition of gram positive, gram negative bacteria and fungus, respectively. The bioassay plates were incubated at 37 ℃ (bacteria) or 32 ℃ (fungus) for 24 h, to measure the diameter of

the inhibition zone (mm) to evaluate antimicrobial effect. Each experiment was repeated for 5 times, and the data were presented in the form of mean ± standard deviations (SD).

1.7 Evaluation of cytotoxic activity

Both of A549 and MSCs cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, U. S. A) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Gibco, U. S. A) and *penicillin* G (100 U/mL) or *streptomycin* (100 μg/mL) maintained in a humidified incubator of 5% CO_2 at 37 ℃, respectively. The separated protein was diluted into DMEM at a final ratio of 14.4, 19.2, 24.0, 28.8, 33.6, 38.4, 43.2 or 48.0 μg/mL.

The cell viability was evaluated using MTT method [15]. A volume of 100 μL of cell suspension containing 5×10^4 cells was seeded in each well of 96-well microtiter plates and incubated for 48 h (A549) or 72 h (MSCs). Then the cells were treated with 100 μL protein of different concentrations for another 24 h. After that, 25 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added in each well and incubated for 4 h. The cell inhibition ratio was evaluated by absorbance value at 570 nm. All experiments were repeated for 4 times, and the data were presented in the form of mean ± SD.

A549 and MSCs cells were cultured in 6-well microtiter plates at a density of 5×10^4 cells per well with the volume of 2 mL, respectively. After 48 h (A549) or 72 h (MSCs), the cells were treated with protein at a final concentration of 4.8, 12.0 or 19.2 μg/mL for 24 h. According to the User's Manual of Annexin V-FITC Kit (Sigma, USA), the treated A549 and MSCs cells were subjected to fluorescence labeling with fluorescein isothiocyanate (FITC) and propidium iodide (PI), respectively. The apoptotic ratio of the treated A549 and MSCs cells were evaluated using flow cytometry (FCM) [16]. Based on the same method, A549 cells were treated with protein of 24.0 μg/mL for 10, 20, 50, 90 and 130 min, respectively, then subjected to fluorescence labeling to record the morphologic features. All experiments were repeated for three times with similar results.

1.8 Statistic analysis

The data are presented as mean ± SD. The significant test was assessed by analysis of variance (ANOVA). A *P*-value of less than 0.05 was considered to indicate significance.

2 RESULTS

2.1 Isolation and identification of β-1, 3-1, 4-glucanase

Protein extracts from *Bacillus subtilis* SWB8 showed five peaks with the retention time of 31.46, 32.36, 41.26, 43.48 and 52.6 min in GPC analysis (Fig. 1-A). By using one-dimensional SDS-PAGE, the protein extracts showed five different gel bands of approximately 70, 56, 40, 30, 25 kDa (Fig. 1-B). Taken the GFC and SDS data together, the retention time of 70, 56, 40, 30, 25 kDa proteins would be 31.46, 32.36, 41.26, 43.48 and 52.6 min in GPC, respectively. Because liquid medium also showed the same 70 and 56 kDa protein bands (Fig. 1-B), only 41.26, 43.48 and 52.6 min β-1, 3-1, 4-glucanase fragments separated by GPC were reserved for further analysis. Those gel bands were then cut into slices and subjected to LC-MS/MS analysis. Based on the score level, the partial amino acid sequences of 40, 30 and 25 kDa proteins (ANNVSVTSSGEMR—SAQTYGYG-LYEVR, ANNVSVTSSGEMR—SAQTYGYGLYEVR and LALTSPSYNKFDCGENR—SAQTYGYGLYEVR) showed the same composition with β-1, 3-1, 4-glucanase of *B. cereus* (GB: AAK16547) and shared high identity to the β-1,3-1,4-glucanase derived from other *B. subtilis* isolates (Fig. 2). The 3 proteins also possessed the same amino acid sequences (SAQTYGYGLYEVR). All the three separated proteins were identified as β-1, 3-1, 4-glucanase, suggesting that the β-1, 3-1, 4-glucanase precursor would be cleaved into three fragments of 40, 30 or 25 kDa with the retention time of 41.26, 43.48 or 52.6 min in GPC, respectively.

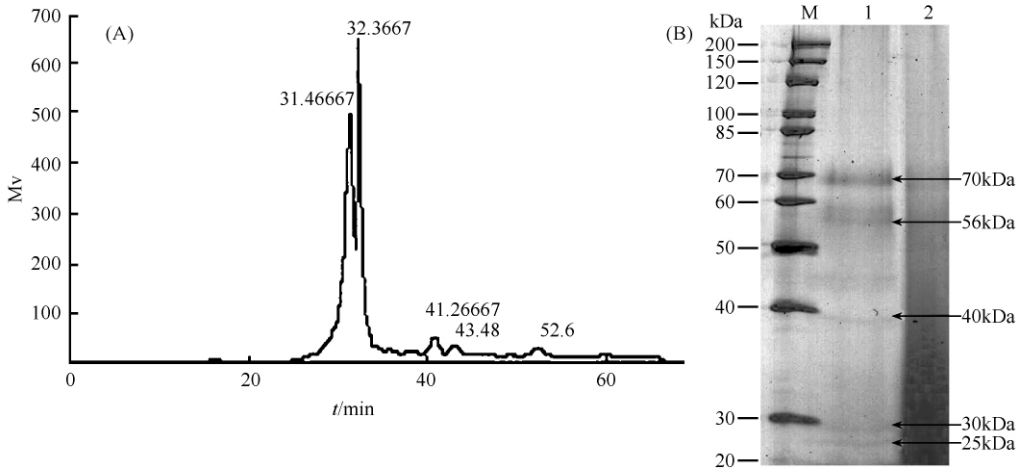

Fig. 1 Analysis of protein extracts on a GPC and an SDS-PAGE (insert). A: The protein extracts was applied onto a TSK-GEL G3000SW × L chromatographic column (300 × 7.8 mm) as described in Materials and Methods. B: The protein extracts (20 μg) was electrophoresed on a 12% polyacrylamide gel. Lane M: standard proteins. Lane 1: protein extracts from *Bacillus subtilis* SWB8. Lane 2: protein extracts from medium.

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                    10         20         30         40         50
B.cereus    ---------- MKRVLLILVT GLFLSLCAIT SAASAQTGGS FFEPFNSYNS
SWB8-a      ---------- ---------- ---------- ---------- ----------
SWB8-b      ---------- ---------- ---------- ---------- ----------
SWB8-c      ---------- ---------- ---------- ---------- ----------
B.subtilis  -------MPY LKRVLLLLVT GLFMSLFAVT STASAQTGGS FFDPFNGYNS
B.lichenif  ---------- ---------- ---------- -----QTGGS FYEPFNNYNT
B.circulan  --MTKWMSIR VKMTALLAAA ALLISVS--A TQPIQAQGAA FTEPLNGPNP
B.amyloliq  ---------- MKRVLLILVT GLFMSLCGIT SSVSAQTGGS FFEPFNSYNS
B.brevis    MVKSKYLVFI SVFSLLFGVF VVGFSHQGVK AEEERPMGTA FYESFDAFDD

                    60         70         80         90        100
B.cereus    GFWQKANGYS NGDMFNCTWR ANN-VSVTSS GEMRLA---- LTSPSYNKFD
SWB8-a      ---------- ---------- ANN-VSVTSS GEMR------ ----------
SWB8-b      ---------- ---------- ANN-VSVTSS GEMR------ ----------
SWB8-c      ---------- ---------- ---------- ----LA---- LTSPSYNKFD
B.subtilis  GFWQKADGYS NGNMFNCTWR ANN-VSVTSL GEMRLA---- LTSPSYNKFD
B.lichenif  GLWQKADGYS NGNMFNCTWR ANN-VSMTSL GEMRLS---- LTSPSYNKFD
B.circulan  GMFYTSDGWG NGVDFGVGWK ASNQEFSNGI MALRLDNVGC PSNCSGKPYA
B.amyloliq  GLWQKADGYS NGDMFNCTWR ANN-VSMTSL GEMRLA---- LTSPSYNKFD
B.brevis    ERWSKAGVWT NGQMFNATWY PEQ-VTADGL MRLTIA---- KKTTSARNYK

                   110        120        130        140        150
B.cereus    CGENRSAQTY GYGLYEVRMK PAKNTGIVSS FFTYTGPTE- ----------
SWB8-a      -----SAQTY GYGLYEVR-- ---------- ---------- ----------
SWB8-b      -----SAQTY GYGLYEVR-- ---------- ---------- ----------
SWB8-c      CGENRSAQTY GYGLYEVR-- ---------- ---------- ----------
B.subtilis  CGENRSVQTY GYGLYEVRMK PAKNTGIVSS FFTYTGPTDG --TPWDEIDI
B.lichenif  CGENRSVQTY GYGLYEVNMK PAKNVGIVSS FFTYTGPTDG --TPWDEIDI
B.circulan  SAEYATTQKY GYGRVEARIK AAKGTGLVTS LFTYSG--AA PGTSNDEIDI
B.amyloliq  CGENRSVQTY GYGLYEVRMK PAKNTGIVSS FFTYTGPTEG --TPWDEIDI
B.brevis    AGELRTNDFY HYGLFEVSMK PAKVEGTVSS FFTYTGEWDW DGDPWDEIDI
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Fig. 2 Multiple alignment of the partial amino acid sequence of 40, 30 or 25 kDa protein fragments with β-1,3-1,4-glucanases from other sources. The multiple amino acid sequence alignments were built by using BioEdit Sequence Alignment Editor with the following sequences: *B. cereus* (GB: AAK16547), *B. subtilis* (GB: AAM08358), *B. Licheniformis* (PDB: 1GBG), *B. circulans* (GB: AAG53947), *B. amyloliquefaciens* (SP: P07980), *B. brevis* (SP: P37073), and the amino acid sequence of 40, 30 and 25 kDa protein fragments (SWB8-a, SWB8-b, SWB8-c) from SWB8 protein extracts. Pane indicate the partial amino acid sequence alignment sites of 40, 30 and 25 kDa protein fragments with β-1,3-1,4-glucanases from other sources.

2.2 Antibacterial Activity

The separated protein with the retention time of 41.26, 43.48 and 52.6 min in GPC were dissolved in 0.1 mol/L PBS and sterilized (0.22 μm Millipore filter) for antimicrobial test. The inhibition zone was presented on the plates which were coated with microorganisms tested and reached the maximum value after incubation for 8 h (bacteria) or 24 h (fungus), respectively. The results (Fig. 3) showed that all the microorganisms tested were inhibited by β-1,3-1,4-glucanase ($P<0.05$). *E. faecalis* (21.8 ± 0.3 mm) ($P<0.05$), *E. coli* (19.0 ± 1.0 mm) ($P<0.05$), *C. albicans* (22.5 ± 0.6 mm) and *C. neoformans* (24 ± 1 mm) ($P<0.05$) were more sensitive to β-1,3-1,4-glucanase. All the 3 proteins showed the similar antimicrobial activity.

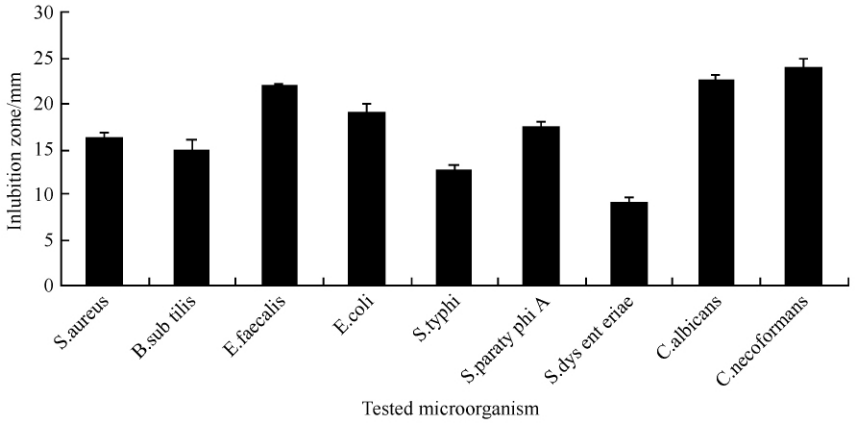

Fig. 3 The antimicrobial activity of β-1,3-1,4-glucanase. The data represent diameter of the inhibition zones which were not recorded until the inhibition zone reached the maximum value. *Penicillin* G (60 000 U), *Gentamicin sulphate* (10 μg) and fluconazole (15 μg) were used as positive control. Positive standard: full transparent, un-colony and the diameter of inhibition zone larger than 6 mm. The data were presented as mean ± SD of five independent experiments ($n=5$) (P-value relative to negative control group: $P<0.05$). Error bars represent SD.

2.3 Anticancer activity

The separated protein with the retention time of 41.26, 43.48 and 52.6 min in GPC were dissolved in 0.1 mol/L PBS and sterilized (0.22 μm Millipore filter) for anticancer test.

2.3.1 MTT assay: MTT assay showed that the cytotoxicity of β-1,3-1,4-glucanase selectively acted on A549 cells (Fig. 4). The inhibition ratio of A549 cells showed positive correlation with the increasing concentrations of the protein. The IC_{50} and IC_{90} values of the protein were 11.5 and 20.1 μg/mL ($P<0.05$), respectively. The inhibition ratio of MSCs cells was only 20.4% even treated with the highest concentration of 24.0 μg/mL.

2.3.2 FCM analysis: FCM analysis showed that the percentage of apoptotic A549 cells treated with β-1,3-1,4-glucanase at different concentrations (0, 4.8, 12.0 and 19.2 μg/mL) was significantly increased from 4.43% (the control) to 43.1% ($P<0.05$) of

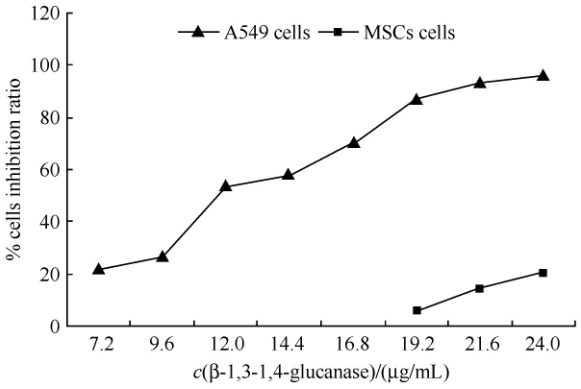

Fig. 4 The cell viability of A549 and MSCs cells induced by β-1,3-1,4-glucanase. The inhibition ratio of treated A549 cells (▲) and MSCs (■) were presented as mean ± SD of four independent experiments ($n=4$) (P-value relative to control group treated without β-1,3-1,4-glucanase: $P<0.05$), respectively. Error bars represent SD.

19.2 μg/mL in a dose-dependent manner. Meanwhile, the late apoptotic or necrotic ratio was also significantly increased from 2.57% to 14.7% ($P<0.05$) (Fig. 5 upper part). In contrast, these changes

could not be observed in MSCs cells (Fig. 5 lower part). Although the apoptosis and necrotic ratios of FCM analysis was far lower than the data of MTT assay at the same concentration of protein, this was mainly due to discard the floating or fragmentation cells of culture plates.

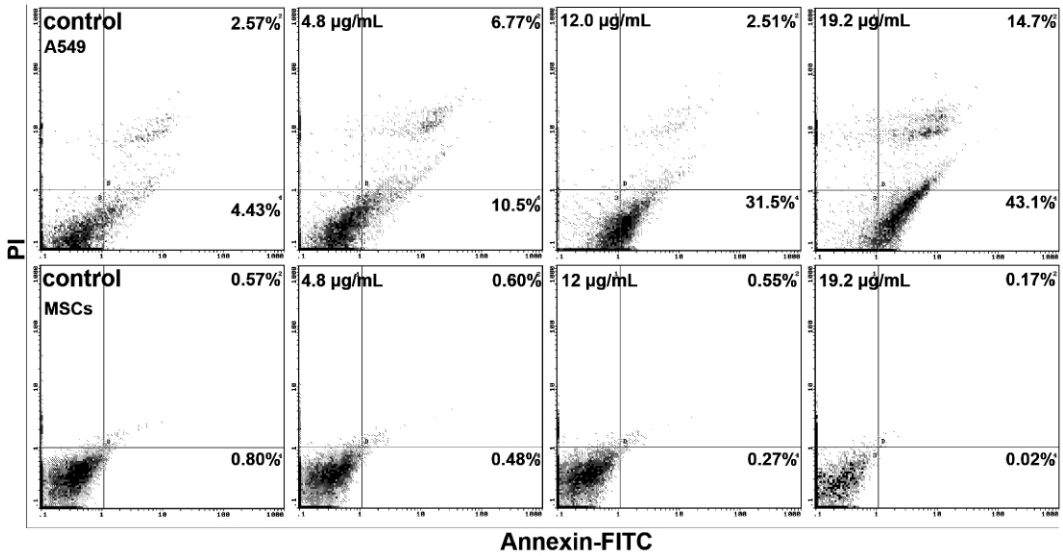

Fig. 5 FCM analysis of A549 cells and MSCs treated with or without β-1,3-1,4-glucanase. The graphic upper and lower part represents A549 cells and MSCs treated with β-1,3-1,4-glucanase of 0 (control), 4.8, 12.0 and 19.2 μg/mL for 24 h, respectively. The upper right and lower right quadrants represent late apoptotic or necrotic cells (Annexin V-FITC + /PI +) and early apoptotic (Annexin V-FITC + /PI-), respectively. (*P*-value relative to control group without β-1,3-1,4-glucanase: $P < 0.05$).

2.3.3 Morphological feature Morphological changes, such as cell shrink, cytoplasmic blebbing and condensation [17] which occurred on A549 cells while treated with β-1,3-1,4-glucanase indicated apoptosis of A549 cells (Fig. 6). Within 10 min, the adherent A549 cells started to shrink, and then the

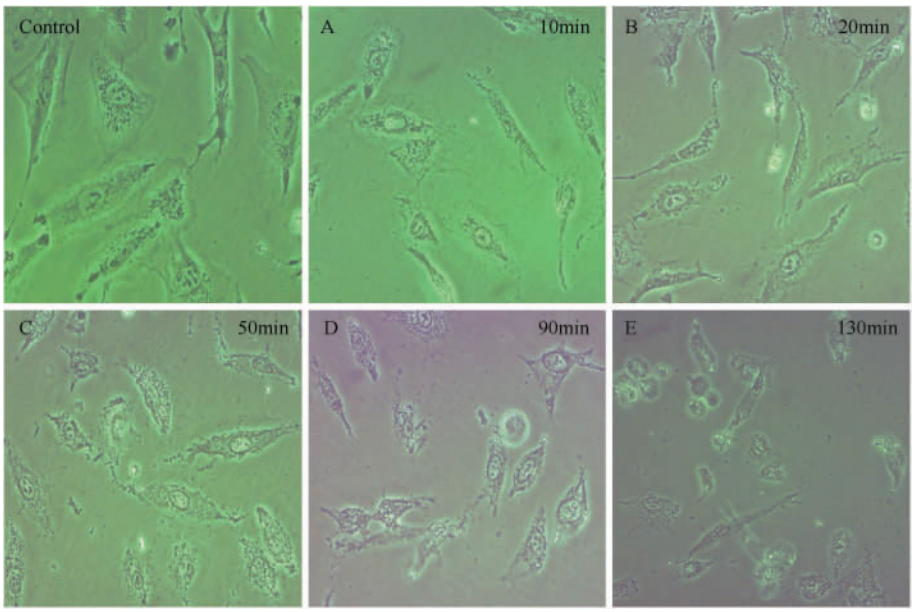

Fig. 6 The morphological features of A549 treated with β-1,3-1,4-glucanase. Control: A549 cells treated without β-1,3-1,4-glucanase. The morphologic features of A549 cells treated with β-1,3-1,4-glucanase of 24.0 μg/mL for 10, 20, 50, 90, 130 min, respectively, were recorded (inverted microscopic).

other morphological changes such as cytoplasmic blebbing (50 min), cytoplasm condensation (90 min) were presented. After 130 min incubated with β-1,3-1,4-glucanase, the A549 cells were floated with the fragmented nucleus. In contrast, these morphological changes could not be observed in MSCs cells.

LSCM analysis indicated (Fig. 7) that A549 cells stained with green fluorescence or red fluorescence were continually increased in a time-dependent manner. At 10 min (Fig. 7-A), the plasmalemma of early apoptotic cells were stained with unsymmetrical green fluorescence. At 20min (Fig. 7-B), apoptotic cells were observed as green shrinkage membrane and red condense nucleic acid. Particularly, the cells that the nucleic acid integrated with PI after 130 min incubation (Fig. 7-E) were distinguished increased than 40 min earlier(Fig. 7-D).

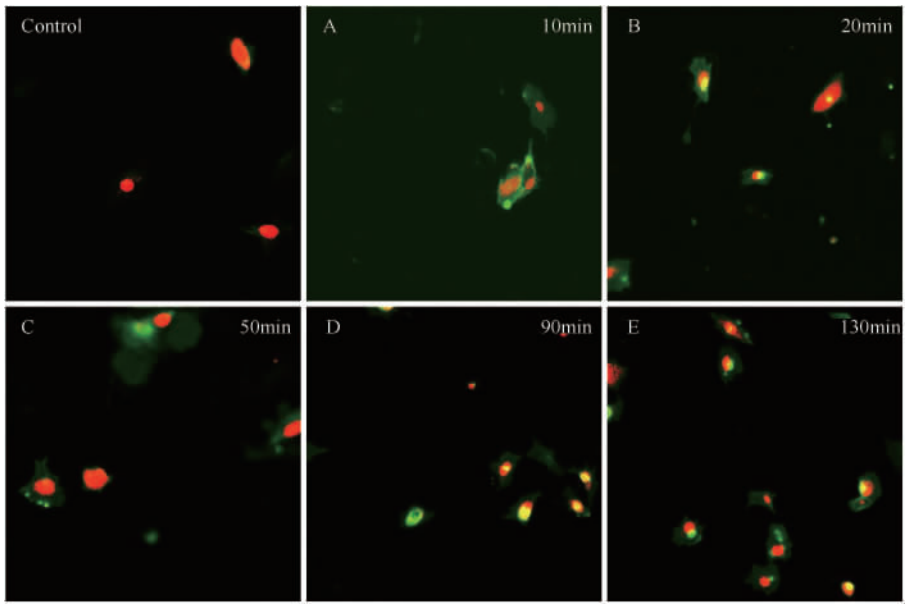

Fig. 7 The morphological features of A549 cells treated with or without β-1,3-1,4-glucanase. Control: A549 cells treated without β-1,3-1,4-glucanase. The morphologic features of A549 cells treated with β-1,3-1,4-glucanase of 24.0 μg/mL for 10, 20, 50, 90, 130 min, respectively, were recorded. Early apoptotic cells: green fluorescence; late apoptotic or necrotic cells: green and red fluorescence (laser scanning confocal microscope, LSCM).

It is clear that β-1,3-1,4-glucanase induce apoptosis of A549 cells within a shot time. Furthermore, all the 3 proteins showed the similar anticancer activity.

3 DISCUSSION

MS analysis proved that 40, 30 or 25 kDa proteins possess the same amino acid sequence (ANNVSVTSSGEMR, LALTSPSYNKFDCGENR or SAQTYGYGLYEVR), corresponding to that of β-1,3-1,4-glucanase from *B. cereus* between Ala71 and Arg118, suggesting that the three fragments were obviously derived from the same β-1,3-1,4-glucanase. Thus, we supposed that the 3 protein fragments may splice from the same protein precursor during the post-translational modification process.

The metabolites of *B. subtilis* have been reported with antibacterial, antimycotic, anticancer and antiviral activities [18-22]. This is mainly due to the lysis of surfactin, which is a cycle lipoheptapeptide containing seven amino acids and a β-hydroxy fatty acid [23]. Based on surfactin amphiphilic structure, it may induces the formation of membrane channel and efficiently penetrate into biomembranes, ultimately leading to disintegration of organism membrane structure [21-22]. Our studies showed that polypeptides from β-1,3-1,4-glucanase arrested bacterial growth and gemmation of *C. albicans* and *C. neoformans*. It is

interested that polypeptides from β-1,3-1,4-glucanase showed certainly inhibition effect on *B. subtilis* ATCC6633 (Fig. 3). Because of the strict cleavage specificity for β-1,4-linkages adjacent to β-1,3-linkages of β-glucan, there was lack of evidence that β-1,3-1,4-glucanase or its polypeptides may hydrolyze the β-1,3-glucan linkage of fungal walls or β-1,4-glucosid bond of bacterial wall peptidoglycan. Although the antimicrobial mechanisms of β-1,3-1,4-glucanase remain largely unknown, based on the fact that polypeptides of β-1,3-1,4-glucanase inhibited tested microorganisms growth and the amphiphilic structure, it is presumed that the broad-spectrum antimicrobial activity is caused by formation of cytoplasm membrane channel or programmed death of cells [11-12].

Polypeptides of β-1,3-1,4-glucanase exhibited high effectiveness cytotoxicity on A549 cells, and it is involved in the process of apoptosis. In a short incubation time (Fig. 6,7), it trigger the apoptotic procedure such as PS turning out from ento-membrane, intracellular fluid release, dilated intracellular spaces, cytoplasmic blebbing, cytoplasm condensation and condense nucleic acid, suggesting the formation of cytoplasm membrane channel on cancer cells and occurrence of apoptotic events. Interestedly, it does not display the same inhibition effect in MSCs cells which are multipotential progenitor cells capable of differentiating into multiple lineages of the mesenchyme that sensitive to regular anticancer agent, such as cyclophosphamide, 5-fluorouracil and taxol [24-26]. Our data indicates MSCs cells can tolerate high concentration of β-1,3-1,4-glucanase (16.8 μg/mL) which reveals the selective cytotoxicity against malignant cells. Perhaps, the fragments of β-1,3-1,4-glucanase or β-1,3-1,4-glucanase from *B. subtilis* SWB8 or *B. subtilis* strain SWB8 could be a potential source of desirable anticancer compounds with higher efficiency and lower toxicity. Furthermore, *B. subtilis* strain SWB8, which is a endophytes residing in the tissues of living plants without inflicting negative effects [27], could become a beneficial sources of natural biologic products for exploitation in medicine, agriculture, and industry.

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内生枯草芽孢杆菌 SWB8 菌株 β-1,3-1,4-葡聚糖酶的抗菌作用及细胞毒性

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摘要:【目的】本文研究从药用植物黄姜中分离的内生枯草芽孢杆菌菌株 SWB8 分泌的 β-1,3-1,4-葡聚糖酶的抗菌活性和细胞毒性。**【方法】**利用液体发酵、凝胶渗透色谱(GPC)、十二烷基-聚丙烯酰胺凝胶电泳(SDS-PAGE)和液相层析串联质谱(LC-MS/MS)等方法纯化和鉴定枯草芽孢杆菌株 SWB8 合成的 β-1,3-1,4-葡聚糖酶;利用纸片扩散法,检测葡聚糖酶抑制临床致病性细菌和真菌生长的活性;应用 MTT 法和流式细胞术(FCM)评估此葡聚糖酶对人肺腺癌细胞(A549)和骨髓间质干细胞(MSCs)的细胞毒性。**【结果】**细菌性 β-1,3-1,4-葡聚糖酶显示了广谱的抗菌活性;抗肿瘤活性主要以细胞凋亡的方式选择性的抑制人肺腺癌细胞系 A549 细胞的增殖,而对人骨髓间质干细胞系 MSC 细胞无明显影响。**【结论】**首次报道 β-1,3-1,4-葡聚糖酶的抗菌和抗肿瘤细胞的活性。内生枯草芽孢杆菌 SWB8 菌株有可能成为抗菌和高效低毒的抗肿瘤药物的潜在来源。

关键词: 枯草芽孢杆菌,β-1,3-1,4-葡聚糖酶,抗菌作用,细胞毒性

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