RESEARCH ARTICLE

Expression and Characterization of Protein Latcripin-3, an Antioxidant and Antitumor Molecule from *Lentinula edodes* C91-3

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Abstract

In this study, an anti-oxidant and anti-tumor protein *Latcripin-3* of *Lentinula edodes* C91-3 was expressed in *Escherichia coli*. for the first time. According to the cDNA library, the full-length gene of *Latcripin-3* was cloned by the methods of 3'-full rapid amplification of cDNA Ends (RACE) and 5'-full RACE. The structural domain gene of *Latcripin-3* was inserted into the pET32 a(+). The functional protein of Latcripin-3 was expressed in *Rosetta-gami* (DE3) E. coli, evaluated by Western blotting and mass spectrometry. DPPH testing showed that the protein *Latcripin-3* can scavenge free radicals remarkably well. The activity of functional protein *Latcripin-3* on A549 cells was studied with flow cytometry and the MTT method. The MTT assay results showed that there was a decreases in cell viability in a dose-dependent and time-dependent manner in protein *Latcripin-3* treated groups. Flow cytometry demonstrated that *Latcripin-3* can induce apoptosis and block S phase dramatically in human A549 lung cancer cells as compared to the control group. At the same time, the cell ultrastructure observed by transmission electron microscopy supported the results of flow cytometry. This research offers new insights and advantages for identifying anti-oxidant and anti-tumor proteins.

Keywords: Antioxidant - antitumor - *Latcripin-3* - *Lentinula edodes* - prokaryotic expression

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Introduction

Valued as both edible and medicinal resources, mushrooms have captured the attention of many investigators on account of the exploitable bioactive constituents that they contain. Mushroom polysaccharides may exhibit hypolipidemic, hypoglycemic, immunostimulant and antitumor activities. *Lentinula edodes* is an edible mushroom isolated from *Basidiomycetes Umbelliferae* fungi, and many strains of *Lentinula edodes* have been researched by the fermentation technology of bioengineering according to the international study of pharmaceutical research regarding *Lentinula edodes*. The extracts contain a variety of proteins in addition to polysaccharides and amino acids. Some direct anti-tumor and antioxidant effects have been found in fermentation broths (Takehara et al., 1981; Huang et al., 2001; Lin et al., 2008). Our group has been committed to the research of the strain of *Lentinula edodes* C91-3. Because the strain was studied for the first time in March 1991, it was named as "C91-3", C referring to China (Liu et al., 2012; Zhang et al., 2013). The extracts contain a variety of proteins in addition to polysaccharides and amino acids (Wasser 2002; Chen et al., 2004; Moradali et al., 2007). The *in vivo* and *in vitro* experiments confirmed that some of the protein components have significant effects on inducing cell apoptosis (Huang et al., 2001; Li et al., 2009).

In this paper we report for the first time details of *Latcripin-3* full length gene cloning by means of the 3'-Full RACE and 5'-Full RACE methods, structural domain expression and purification. The biological activity of functional protein *Latcripin-3* on A549 cells was studied with flow cytometry, the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyl-tetrazolium Bromide (MTT) method, and the DPPH radical scavenging method .

Materials and Methods

Materials

Lentinula edodes strain C91-3 was kept in our laboratory. The Escherichia coli strain JM109, used as the host for cloning the gene Latcripin-3, was purchased from Takara. The Escherichia coli strain Rosetta-gami (DE3) and pET32a (+) vectors used for *Latcripin-3* expression were purchased from Novagen. RNAiso plus kit, the In-FusionTM Advantage PCR Cloning Kit,

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3'-Full RACE Core Set Ver.2.0 kit, 5'-Full RACE Kit, BigDye Terminater V3.1 Cycle Sequencing Kit, Plasmid purification kit, PrimeSTAR® HS DNA Polymerase kit, MiniBEST Agarose Gel DNA Extraction Ver. 3.0 Kit, BamHI, XholI, Protein molecular weight markers and primers were purchased from Takara (Dalian, China). Ampicillin, Chloramphenicol, Tetracycline, Kanamycin sulfate, IPTG and PMSF (protease inhibitor) were purchased from TianGen (Beijing, China). Ultrafiltration centrifuge tubes were purchased from Millipore. DPPH was purchased from Sigma-Aldrich (Shanghai, China). A Ni-NTA-His binding affinity column, mouse anti-his Tag monoclonal antibody and goat anti-mouse IgG peroxidase conjugate were purchased from Novagen. Bicinchoninic Acid (BCA) Protein Assay Kit for Protein Determination and Annexin V-FITC/PI kit were purchased from KeyGen Biotech (Nanjing, China). The integrated potato culture medium was prepared by the staff of our own laboratory. All other reagents were of analytical grade available from commercial suppliers.

Method of determining the ORF region of Lentinula edodes C91-3 Latcripin-3 full-length gene

Total RNA from *Lentinula edodes* C91-3 mycelia, which had been cultivated for 18 days in the integrated potato culture medium, was isolated using the RNAiso plus kit (Takara), chloroform and isopropanol. The RNA was washed with 75% ethanol, dissolved in nuclease-free water, and checked for integrity by 1.0% agarose gel electrophoresisy. The primers for the 3'-RACE and 5'-RACE experiments were designed with primer 5.0 software according to the information of transcriptome, and all primers required for the research are listed in Table 1. The sequences of 3'-Full RACE and 5'-Full RACE were analyzed and stitched, and then primers F and R were designed accordingly. The cDNA was synthesized by Takara 3'-Full Race Core Ver. 2.0. The cDNA was amplified by PCR with primers F and R. The sequence of the amplified gene was analyzed and confirmed by the ABI PRISMTM 3730XL DNA Sequencer and Applied Biosystem.

cDNA synthesis, cloning, and plasmid constructs of Latcripin-3

The cDNA structure segments synthesis of the peroxidase domain was performed using 3'-Full RACE Core Set Ver. 2.0 kit (Takara) and PrimeSTAR® HS DNA Polymerase kit (Takara), strictly according to the manufacturer's instructions. The primers 1F and 1R (shown in Table 1) were designed by us and synthesized by Takara. The restriction sites were introduced to facilitate cloning into the pET32a (+) vector, and the randomly added extra GC and CC nucleotides in the forward and reverse primers, respectively, provided support for the restriction enzymes. A 6×His tag was added at the 3' end for later verification. The amplified cDNA (738 bp) separated on 1% agarose gel was eluted by using the MiniBEST Agarose Gel DNA Extraction Ver. 3.0 Kit (Takara), and cloned into the BamH I/Xhol I sites of the pET32a(+) vector by using the In-Fusion® HD Cloning Kit (Clontech). In-Fusion production was transformed into JM109 E. Coli competent cells, and grown on ampicillin-containing a 100 ug/ml LB-agar plate by streaking. The positive white colonies, confirmed by DNA sequencing from Takara (Dalian), were inoculated into 100 ug/ml ampicillin-containing medium at 37°C. The plasmid was isolated from the overnight mini-culture, and transformed into Rosetta-gami (DE3) E. coli competent cells (Novagen) for expression. The positive recombinant clones were identified by means of 1.0% agarose gel electrophoresis of the double restriction enzyme digestion of the plasmid.

Table 1. Primers Used in the Experiments

Primer Name	Primer Sequence (5'→3')	Length (bp)
3'-RACE Outer Primer F	TATTTGCTGTGATTTTATTCCG	22
3'-RACE Outer Primer R	CACCGGTGTGGAAGGCGTATT	21
3'-RACE Inner Primer F	GCCCATGAAGTGCTTCGTTT	20
3'-RACE Inner Primer R	TAGATGCCAGGGACCGCTTCT	21
5'-RACE Outer Primer F	TCTTCGCCAGGGACCGTAGAT	21
5'-RACE Outer Primer R	GGTGGACAATGCAAGGGCTACGA	23
5'-RACE Inner Primer F	CTGAGCGTCGGTAGCATTTGC	21
5'-RACE Inner Primer R	TGCAAGGGCTACGAAAGGGA	20
F Primer	ATGAGTGGCGGATTTGAACG	20
R Primer	ATAACAGCCATCTTTGCCAT	20
	BamH I	
Primer 1 F	GGCTGATATCGGATCCCTTGCCCAAGATCTTACCG	35
Primer 1 R	GGTGGTGGTGCTCGAGCTGGTTGATCATTGATTGC	35
	Xhol I	

Culture cultivation and expression of the recombinant protein

An overnight culture of E. coli bearing pET32a-*Latcripin-3* was diluted with a 1:100 ratio in 100 ml TB medium supplemented with 34 ug/ml chloramphenicol, 12.5 ug/ml tetracycline, 15 ug/ml kanamycin sulfate, 100 ug/ml Ampicillin and 1% glucose, and cultured at 37°C. The culture was induced with IPTG (final concentration =0.8 mM) when the OD600 reached 0.6, and incubated for another 2 h. After induction, 5 ml was withdrawn from the culture at 1 h intervals, centrifuged to collect the cells, lysed using lysozyme, and evaluated using Sodium Dodecylsulfonate Polyacrylate Gel Electrophoresis (SDS-PAGE). The expression of the fusion protein was confirmed by Western blotting using an anti-his antibody, and then it was compared to negative control (non-recombinant clones).

Purification of the protein and analysis by mass spectrometry

All the purification steps were performed at 4°C. The cells were collected by centrifugation at 10,000 rpm for 10 min, then resuspended in buffer A (20 mM Tris, 500 mM NaCl, and 2 0mM imidazole, pH7.9) to a concentration of 0.1 g/ml, with 1 mg/ml lysozyme and protease inhibitor (PMSF) added according to the manufacturer's instructions. Resuspended cells were sonicated and the cell lysate was collected by centrifugation at 16,000 g for 20 min. The supernatant was removed after centrifugation, and the precipitate was dissolved in extraction buffer B (20 mM Tris, 500 mM NaCl, 8 M Urea and 20 mM imidazole, pH 7.9). After centrifugation, the supernatant was loaded on a Ni-NTA resin affinity column, and the target protein was eluted using 20 mM Tris-HCl supplemented with 8 M urea, 500 mM NaCl, and a linear gradient of imidazole

from 50 to 500 mM with different concentrations of imidazole, pH 7.9. The eluted protein was analyzed on 12% SDS-PAGE. After SDS-PAGE, the Coomassie stained protein band corresponding to the correct mass for the target fusion protein was extracted from the gel and the protein digested using restriction grade trypsin. A hybrid linear ion trap-Orbitrap (LTQ Orbitrap XL, Thermo) mass spectrometer equipped with an Eksigent NanoLC-Ultra 2D Plus HPLC system was used for the analysis of the tryptic fragments.

Refolding and concentration of the fusion protein

An appropriate volume of eluate containing the fusion protein was diluted with the extraction buffer B to a final protein concentration of 0.05 mg/mL. The dilute solution was dialyzed against a renaturation buffer [PBS supplemented with 0.5mM GSH (glutathi-one) and 0.05 mM GSSG (glutathione dimmer)] containing different concentrations of urea. Refolding occurred during dialysis at 10°C with stirring at 120 rpm; the buffer was changed every 8 h, and the urea concentration was decreased in a stepwise manner. Finally, the diluted solution was dialyzed against PBS at pH 7.4. The diluted solution was concentrated using a UFC 901096 (15 ml, 10 kda) ultrafilter. The final protein concentration was measured by a BCA Protein Assay kit.

Antioxidant activity assay

The antioxidant activity of *Latcripin-3* was examined by a 1,1-diphenyl-2-picrylhydrazyl(DPPH) radical scavenging assay. Microplate DPPH assay was performed as described by Brand-Williams et al. (1995) and modified by Brem et al. (2004). Briefly, in a 96-well plate, successive sample dilutions (100 μL/well, 15, 30, 60, 120, 240 and 360 μg/mL) and 100 μL/well ethanol as a blank were tested in triplicate, received DPPH solution (50 μM in absolute ethanol, 100 μL/well), and allowed to stand for 30 min in the dark at room temperature to enable any reaction to take place. L-Ascorbic acid was used as the positive control. The absorbance was measured at 517 nm with a microplate reader. A decrease in the absorbance of the DPPH solution indicated an increase in the DPPH radical scavenging activity. This activity is given as a percentage of DPPH radical scavenging, which is calculated with the following equation:

DPPH radical scavenging (%)=[(Ab-As)/Ab]×100

Where Ab is the average absorbance of the blank, and As is the average absorbance of the sample (Amiri, 2011).
Antitumor activity assay

Cell culture

A549 human lung adenocarcinoma epithelial cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were seeded in a 25 mL culture flask at a density of 2×10^6, then maintained in DMEM/F12 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 units/mL of penicillin and 100 mg/mL streptomycin at 37°C in a humidified atmosphere under 5% CO_2. The medium was changed every 2 d during the experimental period. The cells were treated with the *Latcripin-3* in different concentrations.

MTT assay

Cell viability was assessed by MTT colorimetric 3-(4, 5-dimethylthia-zol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay, which is based on the reduction of an MTT into formazan dye by active mitochondria (Mosmann, 1983). Briefly, the cells were placed in 96-well culture dishes at a density of 5×10^4 cells/well in DMEM/F12 culture medium containing 10% FBS, then incubated at 37°C under 5% CO_2. After 24 h, the cells were washed and placed in a culture medium with different concentrations of *Latcripin-3* (7.5 μg/mL, 15 μg/mL, 30 μg/mL and 60 μg/mL), and 10% FBS DMEM/F12 was used as a negative control, after 24 h Next, 20 mL of MTT solution (5 mg/mL in PBS) was added to each well of a microtiter plate, and the samples were then incubated for 4 h at 37°C. After washing, the formazan dye precipitates, which are proportional to the number of live cells, were dissolved in 150 mL of DMSO. The absorbance at 492 nm was then read using a microtiter plate reader (Thermo Electron, Vantaa, Finland). The rate of cell growth inhibition was calculated using the following formula:

Inhibition ratio (%)=[(A0-At)/A0]×100%

Where A0 is the average absorbance of the control group, and At is the average absorbance of the test group. The effects of each concentration were analyzed in triplicate.

Flow cytometry assay and cell morphology

The logarithmic phase of the A549 cells were plated in two 25 mL culture flasks at 3×10^5 cells/flask, then cultured in 4 mL 10% FBS DMEM/F12. After 24 h of incubation, the medium was replaced with the medium supplemented with 30 μg/mL and 60 μg/mL protein Latcripin-3, and 10% FBS DMEM/F12 was used as a negative control. After another 48 h of incubation, the cells were trypsinizated with 0.25% EDTA (Ethylenediamine Tetraacetic Acid)-trypsogen and washed with PBS three times. All the cells were divided into three equal parts. Annexin V-FITC/PI dye was added into one part according to the instructions of the kit. First, 200 μL Binding Buffer was added, then 10 μL Annexin V-FITC and 5 μL PI were added. After 15 min of dark incubation, the apoptosis function of *Latcripin-3* was analyzed with FAC-Scan Flow Cytometry (Becton Dickinson), and analyzed by Cell Quest software (Cell Quest Software, Version 3.3, 2007, Cell Quest, Inc.: Tokyo, Japan). The second part was used to quantitatively detect the cell-cycle distribution. Cells were fixed in 70% ethanol overnight at 4°C and stained with 50 μg/mL propidium iodide (PI) by incubation at 4°C for 30 min (Ye et al., 2005). The stained cells were also analyzed with FAC-Scan Flow Cytometry (Becton Dickinson) and analyzed by Cell Quest software. Finally, the last part of the cells were fixed with 2% glutaric dialdehyde and prepared for transmission electron microscopy. After having been fixed at 4°C for 24 h, the cells were fixed with 1% osmic acid for 3 h. After washing, the cells were dehydrated by being placed in increasingly higher concentrations of alcohol at 4°C. Then, 100% acetone and embedding solution (2:1) were added at 25°C for 4 h. 100% acetone and embedding solution (1:2) were added at 25°C for 12 h. Embedding solution was added at 37°C

for 12 h, then incubated at 45°C for 12 h, and at 60°C for 24 h. When the embedding medium became hard, the sections were cut on an ultramicrotome. Then the sections were stained with 3% uranyl acetate-lead citrate. The cells' ultrastructure was observed with an electron microscope.

Annexin V-FITC and PI double staining were used to analyze apoptosis, and PI staining was used the cell-cycle distribution by flow cytometry assay. Changes of cell ultrastructure were observed by transmission electron microscopy.

Statistical analysis

SPSS10.0 software (SPSS10.0 Software, Version 10.0, 2007, SPSS Statistical Program for Social Sciences, Inc.: Chicago, IL, USA) was used for statistical analysis. Independent-sample T-testing was used to compare the differences between the two groups. $p<0.05$ was considered to be statistically significant between the two groups

Results

ORF region of Lentinula edodes C91-3 Latcripin-3 full-length gene

In order to investigate the protein in *Lentinula edodes* C91-3, the total RNA of *Lentinula edodes* C91-3 was extracted and a cDNA library was prepared. The short reads were obtained by using the Solexa High-flux Sequencing Technique. These short reads were assembled de novo with the sequence assembly software SOAP2. The output was the Unigene sequences, which could not be extended at both ends. At the same time, the given information was parsed and the gene function was annotated (Takehara et al., 1981; Wouter et al., 2008). The Unigene sequences were blasted with the blastx method. All of the information on the *Lentinula edodes* C91-3 transcriptome was obtained. *Latcripin-3* was found to have a peroxidase domain using the online tools Sanger Pfam (Pfam-ID: PF00141). According to the results of the gene functional annotation, *Latcripin-3* as a novel member of the peroxidase Family was screened out of the Unigene sequences. The name *Latcripin-3* was derived from three words: *Lentinula edodes*, transcriptome and protein. The stem "La tcri pin" represents the novel molecule (Liu et al., 2012).

After the 3'-Full, 5'-Full RACE reaction, the RT-PCR reaction with primer F and R, 5 μL of RT-PCR product was tested by 1.0% agarose gel for electrophoresis. Consistent with the sequencing results, a clear band was shown at about 1100 bp (Figure 1a) which is in line with the *Lentinula edodes* C91-3 *Latcripin-3* full-length gene Open Reading Frame (ORF) region (GenBank Accession Number: KF682440).

Protein structure analysis of protein Latcripin-3

The secondary structure of the protein *Latcripin-3* was analyzed with the Swiss-Model database (Arnold et al., 2006) and the Pfam database (Pfam Database. Available online: http://www.pfam.sanger.ac.uk/ accessed on 26 November 2012).

The results showed that two Pfam-A matched the search sequence (Figure 1b). Therefore, the tertiary structure of *Latcripin-3* was predicted by structure modeling on phery2 website online tools. The tertiary structure of *Latcripin-3* showed that the proteins with multiple consecutive alpha helix structure eventually formed a three-dimensional structure of a pocket by RasMol software (Figure 1c).

Construction of recombinant plasmid

PCR amplification and double enzyme digestion were conducted to examine the recombinant plasmid. The double restriction enzyme digestion of pET32a(+) vector production and recombinant plasmid was separated on 1% agarose gel. The size of the target fragment from restriction enzyme digestion of recombinant plasmid was about 800 bp, the same as the structural gene fragment we designed. The recombinant gene was reconstructed successfully.

Expression and affinity purification of protein Latcripin-3

As shown in Figure 2a, *Latcripin-3* was expressed as insoluble inclusion bodies with a molecular weight of approximately 44.5 kDa on SDS-PAGE. The inclusion bodies were dissolved in a denaturation buffer, loaded onto a nickel ion affinity column, then washed with an elution buffer containing different concentrations of imidazole. Figure 2b shows the purification of the Latcripin-3. After dialysis with the refolding buffer containing different concentrations of urea, about 30 ml intermediate was obtained as the 45-kDa final product of refolding. We propose that autocatalytic activation occurred during dialysis at 10°C. After centrifugal concentration using the ultrafiltration centrifuge, the protein thickened. Measured with the Bicinchoninic Acid (BCA) method, the protein concentration was 720 μg/mL.

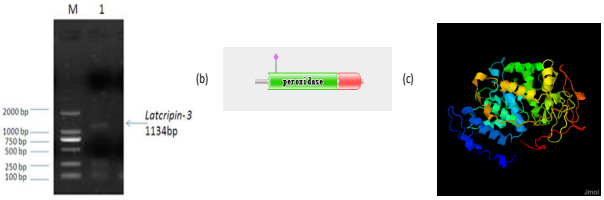

Figure 1. A) Agarose gel Electrophoresis of Latcripin-3 Full-Length of Gene. M: DNA Marker DL2000, 1: Full-length of Latcripin-3 Gene PCR 2:Nnegative control; B) The Structural Domain Analysis of Protein Latcripin-3 with Pfam Database (significant Pfam-A Matches); C) The tertiary structure of protein Latcripin-3

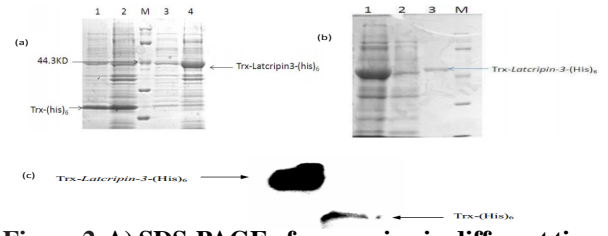

Figure 2. A) SDS-PAGE of expression in different time spans. M: Premixed Protein Marker (Low). lane 1, supernatant of control group; lane 2, precipitation of control group; lane 3, supernatant of sample induced for 3 h; lane 4, precipitation of sample induced for 3 h. B) SDS-PAGE of affinity purification of protein Latcripin-3. M: Premixed Protein Marker (Low). Lane 1, inclusion bodies were dissolved in denaturation buffer; lane 2, flow through solution; lane 3, purified target protein. C) Western blot of purified protein Latcripin-3

Western blotting and mass spectrometry

To validate the purified protein, the Western blot method and mass spectrometry were used, an anti-his-tag positive band which appeared in the position of about 45 kda (Figure 2c) was the Trx-Latcripin-3-(His)6 protein. The resulting protein product was subjected to identification by a hybrid linear ion trap-Orbitrap (LTQ Orbitrap XL, Thermo) mass spectrometer. In accord with the primary mass spectrogram and the results of the analysis comparison, a manganese peroxidase fragment gained the highest score, and 65.83 s is the appearance time of the prominent peak. As shown in the secondary mass spectrogram of 65.83 s, there is a steeple-crowned peak indicated in the active protein fragment.

Antioxidant activity assay

The DPPH radical is a stable organic free radical with an absorption band at 517, and thus it is a useful reagent for investigating the free radical scavenging activities of different compounds. The results of the DPPH free radicals scavenging activity is shown in Figure 3. The *Latcripin-3* was capable of neutralizing the DPPH free radicals via hydrogen donating activity by 10. 27, 15.89, 18.45, 21.07, 30.16 and 35.18% at respective concentrations of 15, 30, 60, 120, 240 and 360 mg/ml; DPPH scavenging was increased in a concentration dependent manner compared to ascorbic acid, which was used as the positive antioxidant control in this investigation.

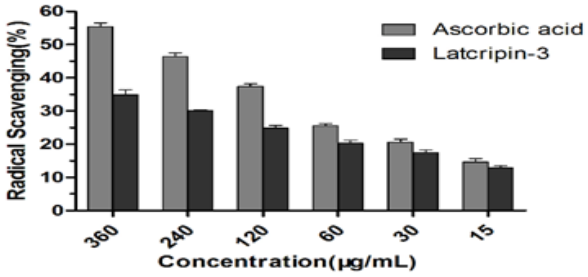

Figure 3. DPPH Radical Scavenging Activity of Latcripin-3

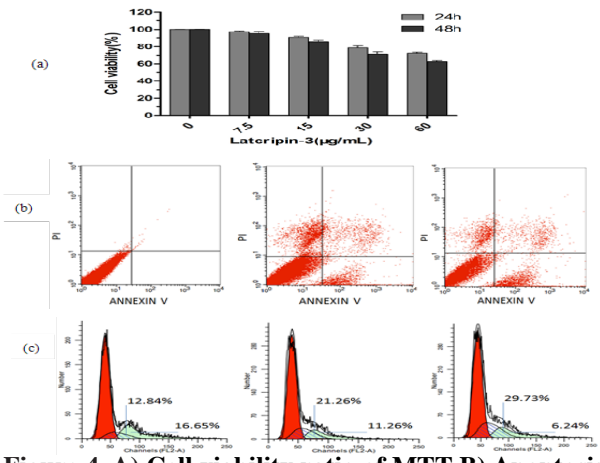

Figure 4. A) Cell viability ratio of MTT B) Apoptosis rate of Latcripin-3 on A549 cell, (1) negative control group, (2) 30 μg/mL Latcripin-3 group, (3) 60 μg/mL Latcripin-3 group. C) Cell cycle analyses of Latcripin-3 treated cells A549. (1) negative control group, (2) 30 μg/mL Latcripin-3 group, (3) 60 μg/mL Latcripin-3 group

Antitumor activity assay

MTT assay of protein Latcripin-3: To evaluate the effects of *Latcripin-3* on the growth and survival of A549 cells, the cells were exposed to various concentrations of *Latcripin-3* for 24 h and 48 h, and their viability was then evaluated by an MTT assay. As shown in Figure 4a, treatment with *Latcripin-3* led to decreases in cell viability in a dose-dependent and time-dependent manner. Furthermore, the decrease induced by *Latcripin-3* (60 μg/mL) at 48 h was significantly greater than the decrease induced by *Latcripin-3* (60 μg/mL) at 24 h (46.67% *vs* 30.21%). Therefore, further experiments were conducted using *Latcripin-3* (30 μg/mL and 60 μg/mL) for 48 h.

Apoptosis function detection by flow cytometry

After 48 h of incubation, the A549 cells were stained with the Annexin/PI. The cell apoptosis rate was measured by flow cytometry in triplicate with the Cell Quest software. In Figure 4b, the upper right quadrant shows the advanced stage apoptotic cells, the lower right quadrant shows early stage apoptotic cells, the upper left quadrant shows dead cells, and the lower left quadrant shows normal cells. The results illustrated that 60 μg/mL *Latcripin-3* induced A549 cell apoptosis increased. Treated by Latcripin-3, the tolerance to physical and chemical effects of the A549 cell was decreased, the cell debris was increased and the number of dead cells was increased significantly.

Cell cycle analyses by flow cytometry

To investigate the effects of *Latcripin-3* on cell-cycle status, the A549 cells were treated with different concentrations of *Latcripin-3* for 48 h, then analyzed for cell-cycle alteration by flow cytometry. We observed that protein *Latcripin-3* caused a dose-dependent accumulation of cells in S phase. The percentage of S cells increased from 12.84 in the control to 21.26% in populations treated with 30 μg/mL *Latcripin-3* and to 29.73% in populations treated with 60 μg/mL. Consequently, fewer cells progressed to G2/M phase, from 16.65% in the untreated group to 11.26%, and 6.24% in the treated group (Figure 4c). This finding indicated that cell cycle progression was dramatically blocked in S phase when the cells were treated with Latcripin-3.

Discussion

This is the first attempt to express the protein of *Lentinula edodes* C91-3 with the prokaryotic expression system. The results of this research show that *Latcripin-3* expressed by this system has high biological activity, which proves that this prokaryotic expression system is suitable for the expression of fungus proteins.

This is also the first time to produce an antioxidant and antitumor protein from *Lentinula edodes* C91-3. According to the transcriptome sequence, a peroxidase gene attracted our attention, due to the prediction of the structure function domain. There are two structure domains, i.e. peroxidase domain and DUF 3415 domain (Figure 1b), and we found that the protein's tertiary structure is very distinctive. With a multiple consecutive

alpha helix structure, the protein can properly combine with the receptor.

Reactive Oxygen Species (ROS) are produced during normal cellular function (Kathiria et al., 2012), and ROS can induce DNA sequence changes in the form of mutations, deletions, gene amplification and rearrangements (Mate´s et al., 2008). In addition to the obvious link between tobacco consumption and cancer of the lung, there is increasing evidence that carcinogens in smoke many also have an impact in many other organ sites (Moor, 2002). Smoking can produce mass ROS, and do harm to human health. These alterations may result in the initiation of signaling leading to cell death or the activation of several proto-oncogenes and/or the inactivation of some tumor suppressor genes (Raj et al., 2011). As a type of antioxidant enzyme, peroxidase can eliminate the free radicals of ROS, resist damage caused by oxidative stress, and sometimes possess an antitumor function (Benabadji et al., 2004; Cakar et al., 2012).

Reactive oxygen species and free radicals are involved in a variety of pathological events including cancer. The anti-oxidant defense enzymes have been suggestive of playing an important role in maintaining physiological levels of oxygen and hydrogen peroxide and eliminating peroxides generated from inadvertent exposure to xenobiotics and drugs. Any natural compound with anti-oxidant properties may help in maintaining health when continuously taken as components of dietary foods, spices or drugs. The increase in the levels of antioxidant profiles i.e. GPx, GR, SOD and Catalase by Moringa oleifera, Lam drumstick extract may be attributed to have biological significance in eliminating reactive free radicals that may affect the normal functioning of cells (Bharali et al., 2003; Moor, 2013). Therefore we cloned and expressed this gene, and the research results proved that the protein has strong antioxidant and antitumor functions.

We speculated that such a molecular structure posses a peroxidase biological activity in some aspect. It can also be seen from Figure 4 that the biological function of *Latcripin-3* is obverse. The A549 cells were markedly induced to apoptosis, and at the same time the cell cycle of the A549 cells changed significantly. The improved understanding of these functional domains will potentially provide further understanding of the apoptosis mechanism of Latcripin-3. Further research is required to elucidate the relationship of these two functional domains (peroxidase and DUF 3415).

In conclusion, in this study a novel antioxidant and antitumor molecule, namely protein *Latcripin-3* from *Lentinula edodes* C91-3, was successfully induced and expressed by means of a prokaryotic expression system. The protein can scavenge free radicals, induce them to apoptosis, and change cell cycle in human lung cancer cells (A549). Although further research is required to elucidate the apoptosis mechanism induced by Latcripin-3, this is the first time to determine and study this new protein (GenBank Accession Number: KF682440). This study reveals new insights and advantages in finding antitumor proteins, and addresses the possibilities of solving screening or purification problems, further building the foundation for future antitumor studies of *Lentinula edodes* C91-3.

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References

Amiri H (2011). The *in vitro* antioxidative properties of the essential oils and methanol extracts of *Satureja macrosiphonia* Bornm. *Nat Prod Res*, **25**, 232-43.

Arnold K, Bordoli L, Kopp J, et al (2006). The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. *Bioinformatics*, **22**, 195-201.

Benabadji SH, Wen R, Zheng JB, et al (2004). Anticarcinogenic and antioxidant activity of diindolylmethane derivatives. *Acta Pharmacol Sin*, **25**, 666-71.

Bharali R, Tabassum J, Azad MR (2003). Chemomodulatory effect of *Moringa oleifera*, lam, on hepatic carcinogen metabolising enzymes, antioxidant parameters and skin papillomagenesis in mice. *Asian Pac J Cancer Prev*, **4**, 131-9.

Brand-Williams W, Cuvelier ME, Berset C (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci Technol*, **28**, 25-30.

Brem B, Seger C, Pacher T, et al (2004). Antioxidant dehydrotocopherols as a new chemical character of Stemona species. *Phytochemistry*, **65**, 2719-29.

Cakar J, Paric A, Maksimovic M, et al (2012). Antioxidative and antitumor properties of *in vitro* -cultivated broccoli (Brassica oleracea var. italica) *Pharm Biol*, **50**, 175-81.

Chen YY, Chang HM (2004). Antiproliferative and differentiating effects of polysaccharide fraction from fu-ling (Poria cocos) on human leukemic U937 and HL-60 cells. *Food Chem Toxicol*, **42**, 759-69.

de Jong WK, Blaauwgeers JL, Schaapveld M, et al (2008). Thymic epithelial tumours: A population-based study of the incidence, diagnostic procedures and therapy. *Eur J Cancer*, **44**, 123-30.

Huang Y, Wang J, Li G, et al (2001). Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants Taxus mairei, Cephalataxus fortunei and Torreya grandis. *FEMS Immunol Med Microbiol*, **31**, 163-7.

Kathiria AS, Butcher LD, Feagins LA, et al (2012). Prohibitin 1 modulates mitochondrial stress-related autophagy in human colonic epithelial cells. *PLoS One*, **7**, 3123.

Li R, Yu C, Li Y, et al (2009). SOAP2: An improved ultrafast tool for short read alignment. *Bioinformatics*, **25**, 1966-67.

Lin YW, Chiang BH (2008). Anti-tumor activity of the fermentation broth of Cordyceps militaris cultured in the medium of Radix astragali. *Process Biochem*, **43**, 244-50.

Liu B, Zhong M, Lun Y, et al (2012). A Novel Apoptosis Correlated Molecule: Expression and Characterization of Protein Latcripin-1 from *Lentinula edodes* C (91-3). *Int J Mol Sci*, **13**, 6246-65.

Mate´s JM, Segura JA, Alonso FJ, et al (2008). Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis, and carcinogenesis. *Arch Toxicol*, **82**, 273-99.

Moor MA, Tsuda H (2002). Smoking-related research in the Asian-Pacific Countries. *Asian Pac J Cancer Prev*, **3**, 291-304.

Moor MA (2013). Overview of cancer registration research in the Asian Pacific from 2008-2013. *Asian Pac J Cancer Prev*, **14**, 4461-84.

Moradali MF, Mostafavi H, Ghods S, et al (2007). Immunomodulating and anticancer agents in the realm of

macromycetes fungi (macrofungi). *Int Immunopharmacol*, **7**, 701-24.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, **65**, 55-63.

Raj L, Ide T, Gurkar AU, et al (2011). Selective killing of cancer cells by a small molecule targeting the stress response to ROS. *Nature*, **475**, 231-9.

Takehara M, Mori K, Kuida K, et al (1981). Antitumor effect of virus-like particles from *Lentinus edodes* (Shiitake) on Ehrlich ascites carcinoma in mice. *Arch Virol*, **68**, 297-301.

Wasser SP (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl Microbiol Biotechnol*, **60**, 258-74.

Ye M, Liu JK, Lu ZX, et al (2005). Grifolin, a potential antitumor natural product from the mushroom *Albatrellus confluens*, inhibits tumor cell growth by inducing apoptosis *in vitro*. *FEBS Lett*, **579**, 3437-43.

Zhong M, Liu B, Wang X, et al (2013). De novo characterization of *Lentinula edodes* C91-3 transcriptome by deep Solexa sequencing. *Biochem Biophys Res Commun*, **431**, 111-15.