Peptide extracts from Lentinus squarrosulus Mont. induces toxicity and oxidative stress in human cancer cells

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Introduction
Cancer has become one of major health problems since the last decade. It causes a high rate of death worldwide [1]. The mortality in cancer patients mostly results from the spreading or metastasis of cancer pathology from original tumour tissue to secondary vital organs [2]. Metastasis cancer cells possess a critical capability to survive under the detachment condition in blood and lymphatic circulation named anoikis resistance [3]. Despite many chemotherapeutic drugs have been approved for cancer treatment, serious side effects, especially toxicity to normal cells, limit the benefits of the drugs [4]. Furthermore, chemotherapy with anti-metastasis activity has not been established. The attempt in searching for anti-cancer drugs with selective toxicity to cancer cells and high safety profile in human has been proceeded for long time. Among these, therapeutic compounds from natural resources gain a lot of attention [5].

Reactive oxygen species (ROS) play an important role in both augmentation and inhibition on cancer development allowing for the design of chemotherapeutic drugs [6, 7]. Here in, we aimed to investigate the toxic activity of peptide extracts from Thai edible mushroom, Lentinus squarrosulus Mont. in both human cancer and normal cells. The therapeutic potential on anti-cancer, anti-metastasis and generation of oxidative stress was also evaluated in cancer cells. The results from this study would lead to the design of biological compound that selectively and effectively inhibits cancer disease.

Methods
Cell culture
Human lung cancer H460, H292, A549 and colon cancer HCT116 cells (ATCC, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, Meanwhile, human hair follicle dermal papilla cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM). All culture medium were supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 100 units/ml of penicillin/streptomycin from Gibco (Gaithersburg, MA, USA). The cells were maintained at condition of 37°C and 5% CO2 until reach 70-80% confluency before used for further experiments.

Preparation of peptide extracts
Fresh fruiting bodies of Lentinus squarrosulus Mont. were homogenized with deionized sterile water at the ratio of mushroom 1 g/3 ml water. Ammonium sulfate was added to the supernatant to reach 40-80% saturation. The peptide pellets were collected by centrifugation and dialyzed with phosphate buffered saline (PBS) without NaCl and KCl pH 7.4. Peptide extracts were further purified by ion-exchange chromatography on a diethylaminoethyl (DEAE)-cellulose (Sigma Chemical, St. Louis, MO, USA) column (5×30 cm) which was pre-equilibrated with phosphate buffered saline (PBS) without NaCl and KCl pH 7.4. Peptide extracts waseluted usingPBS pH 7.4 containing 0 - 0.5 M NaCl at a flow rate of 0.2 ml/min. The peptide composition of each fraction
was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% (w/v) gel stained with 0.1% Coomassie brilliant blue R-250 solution. Bicinchoninic acid (BCA) protein assay kit (Thermo scientific, Waltham, MA USA) was used for analysis of total peptide content.

**Cytotoxicity assay**

Cells were seeded into 96 well-plate at a density of 8,000 cells/well. Peptide extracts at 0-100 μg/ml were added into culture medium and further incubated for 24 h. In order to evaluation on cell viability, the cells were incubated with 0.4 mg/ml of MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St. Louis, MO, USA) in dark place at 37°C for 4 h. Then, the formazan crystal was dissolved with dimethylsulfoxide (DMSO) and examined for the intensity by microplate reader (Anthros, Durham, NC, USA) at wavelength of 570 nm.

**Anoikis assay**

Cells were detached and made into single-cell suspension at a density of 150,000 cells/ml in RPMI-serum free medium. Then, they were culture with or without non-toxic concentrations of peptide extracts for 0, 3, 6, 9 and 12 h in ultra-low attachment plate. Cell viability at indicated time points was determined by MTT assay.

**Cellular ROS detection**

ROS were determined by 2,7-dichlororourescein diacetate (DCFH2-DA) fluorescent probe (Sigma, St. Louis, MO, USA). Cells were pre-incubated with 10 μM DCFH2-DA for 15 min at 4°C prior treatment with peptide extracts for 30 min-3 h. The alteration on cellular ROS level was evaluated through the measurement of DCFH2-DA fluorescence intensity using microplate reader at the excitation and emission wavelengths of 488 and 538 nm, respectively.

**Statistical analysis**

Mean data will be averaged from at least three independent experiments. Statistical analysis will be performed using one-way ANOVA followed by Bonferroni’s post hoc test. A p-value ≤ 0.05 will be considered as statistical significance.

**Results**

**Peptide extracts from L. squarrosulus Mont, induces toxicity in human cancer cells**

The composition of peptide extracts obtained from ion-exchange chromatography presented in SDS-PAGE analysis (Fig. 1a). The extracts containing various peptides with molecular weight varied from <10 to 75 kDa was showed in 0, 0.1, 0.3 and 0.4 M NaCl fraction. Notably, the most purified peptide extracts were demonstrated in the fraction of 0.2 M NaCl. The toxicity of peptide extracts was firstly investigated in human lung cancer H460 cells. Figure 1b showed that the remaining of 50% cell viability was found in H460 cells treated with peptide extracts from 0 and 0.3 M NaCl at 25 μg/ml for 24 h.

![SDS-PAGE analysis](image1)

![Bar graph](image2)

**Figure 1** a) SDS-PAGE analysis of peptide composition in the fractions of 0-0.5 M NaCl separated by ion-exchange chromatography. b) Toxic effect of peptide extracts at 25 μg/ml in human lung cancer H460 cells. Values are means of the independent triplicate experiments ± SD. * p ≤ 0.05 versus non-treated control.
Selective toxicity of peptide extracts from *L. squarrosulus* Mont.

The selective toxicity to cancer cells was further evaluated in peptide extracts of 0 and 0.3 M NaCl fractions. Half maximal inhibitory concentration (IC50) of the peptide extracts in both human cancer and normal cells was demonstrated in figure 2. Low IC50 of the peptide extracts from 0 and 0.3 M NaCl fraction was showed in various human cancer cells including H460 (0 M NaCl: 22.69 ± 1.31 μg/ml and 0.3 M NaCl: 22.38 ± 3.33 μg/ml), H292 (0 M NaCl: 13.34 ± 0.16 μg/ml and 0.3 M NaCl: 26.00 ± 0.30 μg/ml), A549 (0 M NaCl: 12.60 ± 0.58 μg/ml and 0.3 M NaCl: 16.41 ± 0.19 μg/ml), HCT116 (0 M NaCl: <5 μg/ml and 0.3 M NaCl: 16.41 ± 0.19 μg/ml). Selective toxicity effect of the peptide extracts from 0 and 0.3 M NaCl fraction was illustrated by higher IC50 which were approximately >100 μg/ml in human dermal papilla (DP) cells.

Peptide extracts from *L. squarrosulus* Mont. generates oxidative stress in cancer cells

In order to investigate the possible toxic mechanisms, the oxidative stress was evaluated in cancer cells treated with peptide extracts from *L. squarrosulus* Mont. After culture with peptide extract of 0 and 0.3 M NaCl fraction at 25 μg/ml, there was an obvious increase of cellular ROS level in H460 lung cancer cells (Fig. 3). The significant augmentation of ROS level was early observed after incubation with peptide extracts for 30 min. Meanwhile, the decrease of relative ROS level was showed in the cells treated with antioxidant, N-acetylcystein (NAC) and glutathione (GSH) for 1-3 h as negative control.

Anoikis sensitizing effect of peptide extracts from *L. squarrosulus* Mont.

According to high metastasis character of lung cancer cells [4], the ability to induced cell death under detachment condition of peptide extracts was investigated in H460 cells. Anoikis sensitizing effect of peptide extracts of 0 M NaCl fraction was presented in figure 4. The significant decrease of %cell viability was observed in the cells treated with non-toxic concentration (0.05-0.5 μg/ml) of 0 M NaCl peptide extracts for 6-12 h.
Discussion

In searching for a novel compound from natural resources, peptide extracts from mushroom have been highlighted as a new biologic substance for many therapeutic potentials including anti-cancer. Pore formation on cytoplasmic and mitochondrial membrane has been proposed as a major mechanism of anti-cancer peptides extracted from mushroom (8). However, the dramatic increase of cellular ROS in cancer cells incubated with peptide extracts from L. squarrosulus Mont. revealed another possible mechanism of anti-cancer peptides. The relation between oxidative stress and toxic effect of peptides from L. squarrosulus Mont. should be further investigated. Among various advantages of chemotherapeutic drugs, the deficiency of cancer selectivity limits the therapeutic use of the drugs (4). Safety profile of peptide extracts from L. squarrosulus Mont. was presented with very high IC50 in human dermal papilla cells compare with IC50 in various cancer cells. The induction of cell death in both attachment and detachment condition strengthens the benefits of peptide extracts from L. squarrosulus Mont. for cancer treatment. Most of cancer patients are diagnosed at the late stage of disease which is the spreading of cancer to vital organs (1, 4). Sensitizing anoikis has been demonstrated as an effective

![Graph](image)

Figure 4 Peptide extracts of 0 M NaCl fraction at 0-0.5 µg/ml significantly induced cell death under detachment condition in human lung cancer H460 cells. Values are means of the independent triplicate experiments ± SD.

* * * * * * * * * * * p ≤ 0.05 versus non-treated control.

Conclusion

The results from this study disclose the possibility to develop peptide extracts from L. squarrosulus Mont. as an anti-cancer drug. Human safety profile, anti-cancer and anti-metastasis potential are considerable characters of peptide extracts from L. squarrosulus Mont.. Nevertheless, the purification and amino acid sequencing of the active peptide has to be further evaluated,

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