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Purification and cloning of lectin that induce cell apoptosis from *Allium chinense*

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ABSTRACT

A 8.7 kDa lectin with high agglutin activity was isolated by affinity chromatography and cloned from *Allium chinense* in this study. For the MTT assay, approximately 60 μg/ml *A. chinense* lectin (ACL) inhibited 50% of the human hepatoma Hep-3B cells grown after 48 h. In addition, no antiproliferative effect was observed on normal human umbilical vein endothelial cells (HUVEC) even at 100 μg/ml concentration. After treatments with ACL on Hep-3B cells, morphologic changes in the nucleus and cytoskeleton were observed under laser scanning confocal microscopy with 4′,6-diamidino-2-phenylindole and tubulin Alexa Fluor 488 staining; whereas, the mitochondrial membrane potential was observed through Mito Tracker Red CMXRos staining. The results showed that ACL led to cell morphology and structure change (e.g., round cell shrinkage). Moreover, ACL resulted in significant change in the shape of the nucleus, damaged the cytoskeleton when tubulin was degraded, and reduced the mitochondrial transmembrane potential. By contrast, no changes were observed on HUVEC cells under the same treatment conditions. DNA fragmentation analysis was used to detect DNA damage. Western blot showed that ACL upregulated caspase-3 and Bax expression during apoptosis and cloned the structural gene of ACL with an open reading frame of 456 bp encoding 151 amino acid residues. The results showed that ACL is a potential anticancer drug.

Introduction

Allium chinense is an edible vegetable and a traditional medicinal plant food against cardio-cerebral vascular diseases. Plant lectins are carbohydrate-binding proteins with non-immune origin with at least one non-catalytic domain that binds reversibly to specific monosaccharides or oligosaccharides (Lam and Ng 2011). Lectins are isolated from a diversity of organisms, such as humans, animals, plants, and microorganisms, which exhibit numerous biological activities (Rudiger and Gabius 2001). Lectins are anti-proliferative (Pohleven et al. 2009), anti-viral (Ding et al. 2010), insecticidal (Banerjee et al. 2011), and so on. Lectins have been classified into 12 different families based on their domain structures and phylogenetic analyses: ABA (Agaricus bisporus agglutinin), amaranthin, chitinase-related agglutinin (CRA), cyanovirin, Euonymus europaeus agglutinin (EEA), Galanthus nivalis agglutinin (GNA), hevein, jacalins, legume lectin, lysin motif (LysM), and nictaba and ricin B families (Jiang et al. 2010). Some lectins with anticancer properties *in vitro* and *in vivo* have been found to bind to cancer cell membranes or receptors, causing cytotoxicity, apoptosis, and tumor growth inhibition even in human case studies (Li et al. 2010). To promote lectin application, its action mechanism must be elucidated. In this work, a lectin isolated from *A. chinense* was evaluated with respect to its antiproliferative properties against human hepatoma Hep-3B cell, a widely used *in vitro* liver function model. As a potential antitumor drug, ACL may be safe and non-poisonous, or has no side-effects, because *A. chinense* is a secure food. We first cloned the ACL gene and expressed in *E. coli* BL21 (DE3). Afterward, the biological activity of recombinant protein was examined. The result would provide theoretical basis and technical guidance for ACL anti-tumor study.

Materials and methods

Plant materials

A. chinense samples were purchased from the Xiangyin *A. chinense* export base in Hunan Province. The bulbs of *A. chinense* were carefully collected and used for protein purification either immediately

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or stored at −20 °C. The bulbs used for RNA isolation were frozen in liquid nitrogen and stored at −70 °C.

Extraction and isolation for A. chinense lectin

The water-soluble proteins of *A. chinense* were extracted using phosphate buffered saline (PBS, pH 7.0). ACL was purified as described (Upadhyay et al. 2010). Total proteins of *A. chinense* were subjected to carbohydrate affinity chromatography using mannose–agarose column (Sigma–Aldrich). The column was equilibrated with buffer I (10 mM imidazole, 150 mM NaCl, and 20 mM $CaCl_2$; pH 7.0). Afterward, the *A. chinense* extract was loaded on the mannose–agarose column and washed with Buffer I to remove the impurities that cannot bind to the column. Elution was conducted using buffer II (10 mM imidazole, 2 M NaCl, and 20 mM $CaCl_2$; pH 7.0). The eluted fractions were desalinated and concentrated with Vivaspin ultrafiltration spin columns. The preparation was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were stained with Coomassie brilliant blue R-250.

Protein digestion and LC–MS analysis

The amino acid sequences of the peptides cleaved by trypsin of the purified ACL were determined by electrospray ionization mass spectrometry (ThermoFisher, San Jose, CA). The target band in SDS-PAGE was collected, destained with Coomassie brilliant blue in 60% acetonitrile (ACN), reduced at 37 °C by 1 M dithiothreitol (DTT) for 0.5 h, and then alkylated by 1 M iodoacetamide in the dark at room temperature for 40 min. Afterward, the gel was washed three times with 50 mM NH_4HCO_3 and incubated with trypsin at a trypsin/protein ratio of 1:100 (wt/wt) at 37 °C overnight. Trypsin activity was quenched using formic acid. The digests were further transferred to a new tube, ultrafiltered by 10 kDa Microcon filtration device (Sartorius, Germany) to remove large molecules, and lyophilized to dryness. Finally, the sample was analyzed by LC–MS using an LTQ XL mass spectrometer (ThermoFisher, San Jose, CA).

Cloning of A. chinense lectin

RNA was isolated from *A. chinense* bulbs using acid guanidine thiocyanate method, and cDNAs were prepared using RevertAid™ First Strand cDNA synthesis kit (Fermentas, CA). Two specific primers were synthesized based on the amino acid sequence determined by LC–MS and on the conserved sequences of the MBL family: lectin-p1 (5-CATATGATGGCCAGGAACCTACTG-3) and lectin-p2 (5-CTCGAGGTACCAGTAGACCAAATATC-3). The conserved sequences of acl were amplified through PCR using *A. chinense* bulb cDNA as template. PCR products of the expected size were cloned into pMD18-T vector (Takara Biotechnology, JP) and subsequently sequenced. Afterward, high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) (Liu and Chen 2007) was used to obtain the unknown DNA sequences flanked by known sequences of acl. Another two specific primers were synthesized based on the sequence obtained from the conserved acl sequences and hiTAIL-PCR result: lectin-p3 (5-TACGGATCCATGGCCAGGAACCTAC-3) and lectin-p4 (5-CCGCTCGAGTCAAGGAGCAGCAGCAGCCTC-3). In addition, the open reading frame (ORF) of acl was amplified through PCR using *A. chinense* bulb cDNA as template.

Sequence analysis

Similar studies were performed using BLASTn and BLASTp algorithms at the NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), as well as FASTA3 and PSI-BLAST programs at the EBI server (http://www.ebi.ac.uk/Tools/similarity.html). The cDNA-deduced amino acid sequence of ACL was analyzed using online proteomics tools at the ExPASy server (http://www.expasy.org/tools/).

Antiproliferative activity assay

Human hepatoma (Hep-3B, stored in our laboratory) and human umbilical vein endothelial (HUVEC; kindly provided by a professor from University of South China, HengYang, China) cell lines were used. The cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 100 U/ml penicillin–streptomycin in a cell constant temperature incubator under humidified atmosphere of 5% CO_2 at 37 °C. After pancreatic enzyme digestion, the cells were resuspended in the appropriate growth media to a final concentration of 4×10^4 cells/ml seeded onto a 96-well culture plate (Costar, Schiphol-Rijk, NLD). Serial dilutions of the ACL solution (final concentrations of 10, 20, 40, 80, and 100 μg/ml, respectively), 5-fluorouracil (5-Fu, BBI, USA, 80 μg/ml) and Concanavalin A (Con A, Sigma, USA, 60 μg/ml) as positive control in 100 μl phosphate buffer solution (PBS, pH 7.4) were added. The untreated cells were used as blank control. After 24 h of incubation, the cells were washed, and their viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Solarbio, China) according to the instructions provided by the manufacturer. The attenuance of the samples at 492 nm was measured, and the percentages of cell proliferation were determined as (A_{test} cells/$A_{control}$ cells) × 100; where A_{test} cells and $A_{control}$ cells are the absorbances of the formazan measured for cells treated with different ACL concentrations and for non-treated control cells, respectively. All values reported are the means for triplicate experiments.

Apoptosis evaluation by DNA fragmentation detection assay

DNA fragmentation was used to determine apoptosis induction by observing biochemical changes (Kalinina et al. 2002). Hep-3B and HUVEC cell lines were treated with ACL (100 μg/ml), 5-Fu (80 μg/ml), Con A(60 μg/ml) respectively. In addition, the untreated cells were used as blank control, followed by 24 h incubation. The cells were washed three times with PBS, and then harvested after pancreatic enzyme digestion. Each sample containing 2×10^6 cells was resuspended in 500 μl lysis buffer [20 mM EDTA, 50 mM Tris–HCl, pH 8.0, 0.8% (w/v), sodium dodecyl sulfate (SDS), 20 mg/ml proteinase K, 0.5% deoxysodium cholate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton-X-100]. The samples were then incubated at 55 °C for 2 h and digested with RNase A (0.2 mg/ml) for 1 h at 37 °C. Total DNA was extracted, and then washed twice with 70% ethanol (v/v). The DNA precipitate was air-dried and resuspended in 30 μl sterile water. The total DNA was detected on 1.0% agarose gel.

Subcellular structure observation by fluorescence microscopy

Hep-3B and HUVEC cell lines were seeded on 25 mm coverslips in a six-well plate (3×10^5 cells/well). The cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 100 U/ml penicillin–streptomycin in a cell constant temperature incubator under humidified atmosphere of 5% CO_2 at 37 °C. ACL (100 μg/ml), 5-Fu (80 μg/ml) and Con A (60 μg/ml) were added after 24 h. After the treatment, the cells were washed with cold PBS, fixed with 4% paraformaldehyde for 10 min, and permeabilized in 0.1% Triton X-100 for about 10 min. Subsequently, cells were washed again with PBS and incubated with 0.1 μg/ml tubulin-Alex Fluor 488 (Invitrogen, USA) for 30 min, Mito Tracker Red CMXRos (Invitrogen, USA) for 20 min, and 4′,6-diamidino-2-phenylindole (DAPI, 0.1 μg/ml) for 20 min in a dark enclosed space, successively. Cells were visualized by laser confocal scanning microscopy (LCSM, Carl Zeiss, GER).

Location of ACL in Hep-3B cells

Hep-3B cell lines were seeded on 25 mm coverslips in a six-well plate (3×10^5 cells/well). After ACL (100 μg/ml) was treated for 24 h,