An antitumour lectin from the edible mushroom *Agrocybe aegerita*

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An antitumour lectin (named AAL) consisting of two identical subunits of 15.8 kDa was isolated from the fruiting bodies of the edible mushroom *Agrocybe aegerita* using a procedure which involved precipitating the extract by addition of (NH₄)₂SO₄, ion exchange chromatography on DEAE-Sepharose Fast Flow, gel filtration chromatography on Sephacryl S-200 HR and finally purification on a GF-250 HPLC column. Amino acid analysis of the N-terminus and an internal fragment indicated that the sequences of the two fragments were QGVNIYNI and Q(K)-PDGPWLVEK(Q)R respectively. AAL showed strong inhibition of the growth of human tumour cell lines HeLa, SW480, SGC-7901, MGC80-3, BGC-823, HL-60 and mouse sarcoma S-180. AAL also inhibited the viability of S-180 tumour cells *in vivo*.

Analysis by Hoechst 33258 staining, MitoSensor Kit and flow cytometry showed that AAL induced apoptosis in HeLa cells. TUNEL (terminal transferase deoxytidyl uridine end labelling) analysis of slides of tumour tissues excised from BALB/c mice also demonstrated the apoptosis-induction activity of the lectin. Furthermore, AAL was shown to possess DNase activity in assays using plasmid pCDNA3 and salmon sperm DNA. Based on the results obtained in these assays, we conclude that AAL exerts its antitumour effects via apoptosis-inducing and DNase activities.

Key words: *Agrocybe aegerita*, antitumour, apoptosis, DNase, mushroom lectin.

INTRODUCTION

Mushrooms are well known for their nutritional and medicinal values. A variety of compounds with important pharmacological properties have been isolated from mushrooms, which include polysaccharides, polysaccharopeptides, polysaccharide-proteins with immuno-enhancing and anticancer activities [1], lectins with immunomodulatory, anti-proliferative, antitumour and hypotensive activities [2–4].

During the last decade, there has been a growing interest in fungal lectins, largely due to the discovery that some of these lectins exhibit antitumour activities, e.g. *Volvariella volvacea* lectin shows antitumour activity against sarcoma S-180 cells [5], *Grifola frondosa* lectin is cytotoxic to HeLa cells [6], *Agaricus bisporus* lectin possesses antiproliferation activities against human colon cancer cell line HT29 and breast cancer cell line MCF-7 [7], and *Tricholoma mongolicum* lectin inhibits mouse mastocytoma P815 cells *in vitro* and sarcoma S-180 cells *in vivo* [8]. However, the detailed mechanism of their antitumour activity remains unknown. It is known that the anti-proliferative effect of ABL (*Agaricus bisporus* lectin) is likely to be a consequence of the lectin trafficking to the nuclear periphery, where it blocks NLS (nuclear-localization-sequence)-dependent protein uptake into the nucleus [9].

Several antitumour drugs are now known to induce apoptosis in cancerous cells and thus cell apoptosis is considered to be a primary mechanism against tumour cells [10–12]. A few kinds of plant lectins have been identified which induce apoptosis activity in tumour cells, for example, mistletoe- (*Viscum album* L.) [13,14] and garlic-lectin- [12] induced tumour cell apoptosis. However, there was only one kind of mushroom lectin with tumour cell apoptosis-inducing activity, isolated from the edible mushroom *Kurokawa* (*Boletopsis leucomelas*) [15]. In this paper, we describe an antitumour lectin purified from *Agrocybe aegerita*

(AAL), which exerts its effects via apoptosis induction and DNase activity.

MATERIALS AND METHODS

Mushrooms, tumour cells and agents

The edible mushroom *A. aegerita* was collected from Sanming Institute of Fungi (Sanming, Fujian, P. R. China); human cervical carcinoma (HeLa), humane colonic adenocarcinoma (SW480), human stomach cancer (SGC-7901), human gastric cancer (MGC80-3 and BGC-823), human promyelocytic leukaemia (HL-60) and mouse sarcoma-180 (S-180) cell lines were obtained from CCTCC (China Center for Type Culture Collection, Wuhan, Hubei, People's Republic of China); MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] and Hoechst 33258 were from Sigma, the In Situ Cell Death Detection Kit, POD, was from Roche Diagnostics, ApoAlert™ Mitochondrial Membrane Sensor Kit was from Clontech Laboratories, and foetal calf serum and RPMI 1640 were from Gibco BRL.

Cell cultures

SW480, HeLa, SGC-7901, MGC80-3, BGC-823, HL-60 and S-180 cells were maintained in RPMI 1640 supplemented with 10 % (v/v) foetal calf serum, 100 mg/ml streptomycin and 100 units/ml penicillin. All cell cultures were incubated at 37 °C in a humidified atmosphere of 5 % CO₂.

Chromatography

Dry fruiting bodies were crushed into a powder, and 15 g of this powder was extracted twice with 150 ml of distilled water at 4 °C for 10 h. The extract was combined and centrifuged at

Abbreviations used: AAL, *Agrocybe aegerita* lectin; MMT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; TUNEL, terminal transferase deoxytidyl uridine end labelling.

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10 000 g for 20 min. Solid ammonium sulphate was added to the supernatant to 40 % saturation and centrifuged again. Solid ammonium sulphate was then added into the supernatant to 80 % saturation, and the 40–80 % precipitate was collected by centrifugation, dissolved in a small volume of distilled water and dialysed extensively against 10 mM phosphate buffer (pH 6.0). The dialysate was loaded on to a DEAE-Sephacryl Fast Flow column (1.6 cm × 10 cm), and the column was eluted with a linearly increasing concentration of NaCl from 0–0.3 mol/l in the starting buffer. The main eluate was further purified by gel filtration on a Sephacryl S-200 HR column (1.6 cm × 80 cm) followed by elution with 0.025 M phosphate buffer (pH 7.1). The resulting component was finally purified on a GF-250 HPLC column, which was eluted with 150 mmol/l NaCl in 20 mmol/l Tris/HCl (pH 8.0).

Molecular mass estimation and isoelectric focusing

The molecular mass of the isolate was determined by both SDS/PAGE and gel filtration on a Superdex 75 HR column. The isoelectric point was determined on a 5 % (w/v) polyacrylamide gel containing 2 % (w/v) ampholine, selected to establish a pH gradient from 3.5 to 9.5.

Amino acid composition analysis and amino acid sequence analysis

Amino acid analysis was performed using a Hitachi model 835 analyser. Samples (50 µg) were hydrolysed in 6 M HCl in sealed evacuated tubes for 24 h at 110 °C. The N-terminal amino acid residues were analysed using an Applied Biosystems 470A automatic protein sequencer. In order to obtain more information about the amino acid sequence, the isolate was digested by trypsin and separated by HPLC; one large fragment (1324 Da) was sequenced by mass spectrography.

MTT assays

Cell suspensions were prepared at a concentration of 3×10^4 cells/ml, and incubated in a 96-multiwell plate for 24 h. After discarding the culture medium and washing the cells with PBS, 0.1 ml of medium [supplemented with 2 % (v/v) foetal calf serum], containing the sample at various concentrations, was then incubated for another 48 h. For positive control experiments, cells were treated with 0.1 ml of medium (supplemented with 2 % foetal calf serum) containing 100 µg/ml of adriamycin, and the negative control group was treated with the same medium without adriamycin. The number of surviving cells was determined by the MTT method [16]. The inhibition ratio was calculated according to the following equation:

$$\text{Inhibition ratio (\%)} = (A_{\text{control group}} - A_{\text{AAL treated group}}) / A_{\text{control group}} \times 100 \%$$

In vivo studies

S-180 cells maintained in the peritoneal cavity of male BALB/c mice were used for the antitumour activity assay. The cell suspension was diluted to 1.18×10^8 cells/ml, and 0.1 ml of the suspension was subcutaneously inoculated into the oter area of the BALB/c mice (7 weeks old, 20 g). The tumour-bearing mice were treated, 3 days after inoculation, by *in situ* tumour injection

of 0.06 ml of the mushroom isolate (1.67 mg/ml in physiological saline solution) at the dosage of 0.1 mg/mouse every other day. The control group was treated with 0.9 % NaCl. The mice were killed 20 days after the inoculation of tumour cells, and their solid tumours were excised and weighed. The tumour inhibition ratio was calculated as follows [17–19]:

$$\text{Inhibition ratio (\%)} = [(C - T) / C] \times 100 \%$$

where *C* is the average tumour weight of the control group and *T* is the average tumour weight of the AAL-treated group [20]. Tumour tissues were submitted for standard paraffin embedding and sectioned into 5 µm slices. Some parts of the slides were stained with haematoxylin/eosin, and the other parts were used in TUNEL (terminal transferase deoxytidyl uridine end labelling) assays.

Hoechst 33258 staining

HeLa cells were exposed to 100 µg/ml samples for 48 h, washed with PBS and fixed in fresh Carnoy's fixative solution (methanol/acetic acid, 3:1 by vol.). After staining with Hoechst 33258 (0.5 µg/ml in PBS) for 30 min, representative photomicrographs were taken with a Leica DM/LM microscope under UV illumination (380 nm).

ApoAlert™ Mitochondrial Membrane Sensor Kit

HeLa cells were incubated in a 6-well plate for 24 h, in which the mushroom isolate at a final concentration of 100 µg/ml was added. The ApoAlert™ Mitochondrial Membrane Sensor Kit was used to detect cell apoptosis 48 h later. The cells were examined under a microscope using a band-pass filter. The adherent HeLa cells were removed from the plate and approx. 1×10^6 cells were aliquoted into flow cytometry tubes. MitoSensor monomers were detectable in the 525 nm channel on Coulter EpicsXL™ Flow Cytometer [21].

Cell cycle analysis

HeLa cells (2×10^5) cultured with or without the samples (100 µg/ml) at 37 °C for 24 h and 48 h were harvested, washed with PBS, and fixed with 75 % ethanol at 4 °C for 2 h. Cells were then treated with RNase A (0.25 mg/ml) at 37 °C for 1 h. After washing, the cells were stained with 50 mg/ml propidium iodide at room temperature for 10 min. Cell cycle analysis was performed on Coulter EpicsXL™ Flow Cytometer.

TUNEL assay

TUNEL tests of paraffin slides were performed using a commercial kit (In Situ Cell Death Detection Kit, POD), following the manufacturer's instructions. The slides were counterstained with haematoxylin and viewed under a light microscope.

DNase activity assay

DNase activity of the mushroom isolate was measured using a plasmid digestion assay, using pCDNA3 as the substrate. The incubation was performed with 2 µg of plasmid in 19 µl of digestion buffer (6 mM Tris/HCl, 6 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol), to which 1 µl of sample was added (at a final concentration of 50 µg/ml, 5 µg/ml or 0.5 µg/ml). After 30 min

Figure 1 Purification of AAL

Ion-exchange chromatography of the crude protein extract on a DEAE-Sepharose Fast Flow column (1.6 cm × 10 cm). A gradient of 0–0.3 mol/l NaCl in 10 mmol/l phosphate buffer (pH 6.0) was used for elution of the three peaks, D₁, D₂ and D₃.

at 37 °C, the integrity of the pCDNA3 was monitored by gel electrophoresis. Alternatively, the DNase activity was followed semi-quantitatively by incubation of the above substrate (40 µl or 1.6 µg of salmon sperm DNA) together with 10 µl of sample (samples were diluted in 100 mM Tris/HCl, pH 7.1). After an incubation of 30 min at room temperature, the integrity of the salmon sperm DNA was monitored by gel electrophoresis of the DNA in a 0.7% (w/v) agarose gel.

RESULTS

Purification and biochemical properties of the lectin

The crude protein prepared from ammonium sulphate precipitation (40–80% saturation) was applied on a DEAE-Sepharose Fast Flow column (1.6 cm × 10 cm) and three peaks (D₁, D₂ and D₃) were eluted with a linear gradient of 0–0.3 mol/l NaCl in phosphate buffer (Figure 1), in which the D₂ peak corresponded to 0.15 mol/l NaCl. After pooling and concentrating, the fractions containing peak D₂ were applied to a Sephacryl S-200 HR column. The fractions corresponding to the main peak were collected. The homogeneity was estimated by HPLC on a GF-250 column and there was only one peak, indicating that the main peak from the Sephacryl S-200 HR column had been well purified. We named this AAL (*A. aegerita* lectin).

AAL appeared to be homogeneous in both SDS/PAGE and isoelectric focusing. As shown in Figure 2(A), purified AAL migrated as a single peptide with a molecular mass of 15.8 kDa in SDS/PAGE, whereas using gel filtration on a Superdex 75 HR column, AAL was shown to have a molecular mass of 32 kDa in 0.05 mol/l phosphate buffer (results not shown), which indicated that native AAL is most likely a homodimeric protein. The isoelectric point of AAL was 3.8 (Figure 2B).

The amino acid composition of AAL, given in Table 1, showed a high content of neutral non-polar amino acids (glycine, alanine, valine) and acidic amino acids, a low content of methionine, arginine, lysine and histidine residues, and traces of cysteine. This result was consistent with the low isoelectric point of AAL.

Figure 2 Molecular mass and isoelectric point of AAL

(A) SDS/PAGE analysis of AAL for molecular mass. Lane 1: protein molecular mass markers (94 kDa, 67 kDa, 43 kDa, 30 kDa, 14.4 kDa); lane 2: *A. aegerita* lectin (AAL). (B) Isoelectric point determination in 5% PAGE containing ampholine, pH range 3.0–9.5.

Table 1 Amino acid composition of AAL

Glx, sum of Glu and Gln; Asx, Asp plus Asn; ND, not determined; tr., trace.

Amino acid	Content	Residues in one molecule
Asx	8.183	16
Thr	5.637	10
Ser	4.488	8
Glx	6.546	12
Pro	1.674	3
Gly	6.119	11
Ala	6.381	12
Cys	tr.	0
Val	7.496	14
Ile	4.273	8
Leu	5.448	10
Tyr	2.447	5
Phe	3.435	6
Met	0.323	1
Lys	2.201	4
His	0.904	2
Trp	ND	ND
Arg	1.780	3

The N-terminus of native AAL was blocked. By using several methods, it was determined that the N-terminus of AAL was pyroglutamyl. After treatment with pyroglutamate aminopeptidase, AAL was sequenced from the N-terminus up to only 8 amino acids, QGVNIYNI. After digesting by trypsin and separating on an HPLC column, one big fragment (1324 Da) of AAL was sequenced as Q(K)PDGPWLVEK(Q)R on mass spectrography (in mass spectrography analysis, Q and K cannot be differentiated because they have the same molecular mass). Similarity with other published sequences in protein databases was not detected as the sequences we obtained were too short.

Antitumour activity of AAL *in vitro* and *in vivo*

AAL showed strong inhibition effects on 7 types of tumour cell lines. In all of the cell lines except SGC-7901, the inhibition ratio of AAL showed concentration-dependence (Table 2). AAL showed no significant cytotoxicity with HeLa, MGC80-3 or BCG-823 cells. At a concentration of 100 µg/ml, the inhibition ratios of AAL for SGC-7901, SW480, MGC80-3, BCG-823, S-180 and HeLa cells were almost the same as, or a little higher than, that of adriamycin (100 µg/ml), but the inhibition ratio of AAL in HL-60 cells was lower than that of adriamycin (100 µg/ml).

Table 2 The inhibition ratio of AAL for tumour cells *in vitro*

Cell line	[AAL] (μg/ml) . .	Inhibition ratio (%)				Adriamycin (100 μg/ml)
		5	10	50	100	
SW480		44.75	51.10	68.78	82.60	80.66
SGC-7901		69.06	71.21	72.23	73.98	65.88
HeLa		34.14	50.00	64.52	67.74	40.86
MGC80-3		35.00	48.40	61.80	67.70	64.40
BGC-823		35.10	48.10	60.80	67.40	64.00
HL-60		10.60	22.10	37.64	42.79	69.12
S-180		19.01	22.17	46.33	68.50	69.44

Table 3 The death ratio and inhibition effect of AAL in S-180 tumour-bearing mice

For both groups, 1.18×10^7 tumour cells were inoculated. The inhibition ratio is defined as $[(C - T)/C] \times 100\%$ (see the Materials and methods section).

Treatment	Death ratio	Inhibition ratio
0.06 ml of 1.67 mg/ml AAL	10 %	36.36 %
0.06 ml of 0.9 % NaCl (control)	50 %	—

Figure 3 Haematoxylin/eosin staining of slides of tumour tissue excised from BALB/c mice treated with physiological salt solution (A and B) or AAL (C and D)

(A) Abnormal mitotic figures (arrowheads) and aggressive tumour cells are shown. (B) Tumour tissues invading into muscle tissues (arrowhead indicates muscle tissue). (C) Tumour tissues surrounded by fibrocytes (arrowhead indicates fibrocyte). (D) Most of the tumour cells are necrotic and lymphocytes (arrowhead) infiltrate tumour tissue.

The effect of AAL on the growth of S-180 cells in BALB/c mice is shown in Table 3. The tumour-bearing mice were injected with 0.06 ml of AAL at a dosage of 0.1 mg/mouse every other day for 20 days. The percentage of tumour growth inhibition caused by AAL was 36.36%; the death ratio of the AAL-treated group was 10%, which was significantly lower than the control group (50%). The results of haematoxylin/eosin staining on the slides of tumour tissues are shown in Figure 3. The tumour tissues of the AAL-treated group were significantly

Figure 4 Apoptosis analysis of tumour tissues by TUNEL analysis

(A) In the control group, the nuclei of tumour cells were completely stained blue. (B) In the AAL-treated group, apoptotic cells were scattered throughout the tissue section and intensely stained brown.

Figure 5 Apoptosis analysis of HeLa cells by Hoechst 33258 staining assay

(A) The entire nuclei of control HeLa cells were diffusely stained pale green. (B) The intense green spots within the nuclei of AAL treated HeLa cells indicated chromatin condensation.

different from that of the salt-solution-treated group. In paraffin sections, the tumour tissue of the control group treated with salt solution exhibited abnormal mitotic figures, tumour cells grew aggressively (Figure 3A) and the tumour tissues invaded into muscle tissue (Figure 3B), whereas in the AAL-treated group, most of the tumour cells were necrotic, and lymphocytes infiltrated tumour tissue (Figure 3D) and tumour tissues were surrounded by fibrocytes (Figure 3C).

Tumour cell apoptosis induced by AAL

The TUNEL assay is a highly sensitive immunostaining procedure designed to detect free 3'-OH single strand DNA breaks produced by DNA fragmentation, typically localized to cells undergoing programmed cell death. The TUNEL assay was used on the slides of the tumour tissues excised from BALB/c mice, and the results are shown in Figure 4. In the control group, the structure of cells was intact and the nucleus was completely stained blue (Figure 4A). However, in the AAL-treated group, a subpopulation of apoptotic cells, scattered throughout the tissue section, were intensely stained (brown) by the TUNEL treatment and subsequent peroxidase immunostaining (Figure 4B).

Hoechst 33258 is a membrane-permeable dye that specifically binds to chromatin and appears green when inspected under UV light. This allows the detection of chromatin condensation, which is typical for cell apoptosis but not for cell necrosis. The results of Hoechst 33258 staining on HeLa cells are shown in Figure 5. Chromatin of control cells was stained throughout the entire nucleus in a dispersed pale green colour (Figure 5A), whereas condensed chromatin of apoptotic HeLa cells appeared as intense green spots within the nucleus (Figure 5B).

Figure 6 Apoptosis analysis of AAL-treated HeLa cells by the MitoSensor kit and flow cytometry

(A) Healthy HeLa cells did not demonstrate intense green fluorescence in the cytoplasm, indicating that MitoSensor aggregated in the mitochondria. (B) In the cytoplasm of AAL-treated HeLa cells, the intense fluorescent green was due to the altered membrane potential of mitochondria. (C) The mean intensity of fluorescence of the control cells was 0.186 in the 525 nm channel. (D) The mean value of the fluorescence intensity of AAL-treated cells was significantly increased to 1.41.

As shown in Figure 6, the ApoAlert™ Mitochondrial Membrane Sensor Kit was used to detect the change of mitochondrial membrane potential in treated and untreated HeLa cells. MitoSensor aggregated in the mitochondria of healthy cells and thus the cells did not demonstrate intense green fluorescence in their cytoplasm (Figure 6A). However, the mitochondrial membrane

potentials of AAL-treated cells were altered, so MitoSensor remained in the cytoplasm and most of the cells demonstrated green fluorescence (Figure 6B). Accordingly, in flow cytometry assays the mean intensity of fluorescence of the control cells was 0.186 in the 525 nm channel (Figure 6C), but that of the fluorescence of the AAL-treated group was significantly increased to 1.41 (Figure 6D).

The result of cell cycle analysis is shown in Figure 7, where it can be seen that the cell cycle of HeLa cells treated with AAL altered significantly. Healthy HeLa cells demonstrated normal cell cycle characteristics (G_1/G_0 and G_2/M phases); the percentage of apoptotic HeLa cells (shown as the Ao peak) in the control group was 3.3%, and 14.3% of the cells were in G_2/M phase (Figure 7A). After treatment with AAL for 24 h, 23.3% of HeLa cells were undergoing apoptosis and the percentage of HeLa cells in G_2/M phase was reduced to 6.54% (Figure 7B). After another 24 h, 31.4% of HeLa cells were undergoing apoptosis and the percentage of HeLa cells in G_2/M phase was reduced to 2.8% (Figure 7C). As the time of treatment increased, the cell cycle behaviour of AAL-treated HeLa cells altered greatly, with the ratio of apoptotic cells increasing gradually and the percentage of HeLa cells in G_2/M phase sharply reduced. However, the percentage of HeLa cells in G_1/G_0 phase did not change accordingly. AAL induced apoptosis in HeLa cells and interfered with the proliferation of tumour cells synchronously.

DNase activity assay

The digestion of plasmid DNA by AAL was analysed by agarose gel electrophoresis (Figure 8A). When AAL was used to digest plasmid pCDNA3 in a reaction volume of 20 μ l, 50 μ g/ml AAL could degrade 2 μ g of pCDNA3 completely at 37 °C within 30 min. However, when the concentration was reduced to 5 μ g/ml, the plasmid was only partly digested, but at 0.5 μ g/ml AAL, the integrity of pCDNA3 was not affected. The degraded plasmid appeared as a smear without specific bands in agarose gels, demonstrating that the endonuclease activity of native AAL degraded supercoiled, linear and nicked circular plasmid DNA in the same pattern. The DNase activity of AAL was further tested by visualizing the degradation of salmon sperm DNA on

Figure 7 Propidium iodide staining and flow cytometry assay of AAL-induced apoptosis

(A) No significant apoptosis (Ao) peak appeared in control HeLa cells. (B) In the AAL-treated group after 24 h, the Ao peak represented 23.3% of the HeLa cells that were undergoing apoptosis, and the percentage of cells in G_2/M phase was reduced to 6.54%. (C) After 48 h, 31.4% of AAL-treated HeLa cells were undergoing apoptosis, and the percentage of cells in G_2/M phase reduced to 2.8%.

Figure 8 Agarose gel electrophoresis analysis of AAL DNase activity

(A) Digestion of 2 µg of plasmid pCDNA3 with AAL at varying concentrations: lane 1, 50 µg/ml AAL; lane 2, 5 µg/ml AAL; lane 3, 0.5 µg/ml AAL; lane 4, *Hind*III-digested pCDNA3; lane 5, pCDNA3 without AAL. Reactions were carried out in a volume of 20 µl for 30 min at 37 °C. (B) Agarose gel electrophoresis of salmon testes DNA digested with AAL in 100 mmol/l Tris/HCl, pH 7.1. Lane 1, salmon sperm DNA without AAL; lane 2, 1 mg/ml AAL; lane 3, 200 µg/ml AAL; lane 4, 40 µg/ml AAL.

agarose gels (Figure 8B). The DNase activity of AAL resulted in the loss of high molecular mass DNA, the mean size of the DNA fragments being inversely proportional to the amount of added AAL.

DISCUSSION

Mushroom lectin AAL was first successfully purified from dry fruiting bodies in this work. The purification protocol was simplified by using dry fruiting bodies on account of the fact that lectins are more thermostable than many other proteins that are destroyed during the drying process.

One *A. aegerita* lectin has previously been reported by Ticha et al. [22], who reported an AAL with a molecular mass of 44 kDa, which consisted of two identical subunits, and its haemagglutination activity was blocked by Gal. Obviously, the AAL described in [22] is distinct from the AAL prepared in the present study. Two lectins from *Agaricus edulis* have been purified by ion exchange chromatography and gel filtration [23]. The molecular masses of the two lectins, designated lectin 1 and lectin 2, are 60 and 32 kDa, and their carbohydrate contents are 18% and 2% respectively. Lectins 1 and 2 possess four and two subunits respectively. Their haemagglutination activities remain unabated after heating for 10 min at 75 °C and 85 °C respectively. Incubation of the lectins in 6 mol/l urea for 60 min at room temperature and in buffers with pHs from 2 to 11 does not interfere with their haemagglutination activity. The haemagglutination activity of lectin 2 is not suppressed by any of the common simple sugars [24]. The haemagglutination activity of AAL was preserved after incubation at 50 °C. At 60 and 70 °C, very little activity was retained and at 80 °C or above, it was completely abolished (results not shown); thus AAL is less thermostable than several mushroom *Agaricus edulis* lectins. However, the haemagglutination activity of AAL was largely unaffected by exposure to high concentrations of NaOH and HCl (results not shown). AAL is rich in neutral non-polar and acidic amino acids, but does not possess many basic amino acids or cysteine residues. Its low pI (3.8) is unusual in mushroom lectins, while we know little about other characteristics corresponding to its low pI. There was no carbohydrate content found in AAL (results not shown). Specificity for carbohydrate binding of AAL was

examined by a haemagglutination-inhibition assay (results not shown). The haemagglutinating activities of AAL were not inhibited by the following saccharides at concentrations of up to 200 mM: D-galactose, D-fucose, D-xylose, D-arabinose, D-mannose, D-sorbinose, D-fructose, D-glucose, saccharose, maltose, raffinose, D-galactosamine, dithiothreitol, β-methyl-D-mannoside, β-methyl-D-glucoside, *N*-acetyl-D-glucosamine. Among three tested glycoproteins, hog gastric mucin was the only specific inhibitor, whereas thyroglobulin and ovomucin had no effect. Hog gastric mucin inhibited the haemagglutination activity at the minimum concentration of 7.32 µg/ml. In light of these results, it can be seen that the AAL reported in this study is different from the two lectins from *Agaricus edulis*.

The MTT assays with mice sarcoma S-180 cells and human tumour cell lines demonstrated that AAL exhibited higher inhibition activity than adriamycin with several tumour cell lines. However, the inhibition ratio did not rise sharply at higher concentrations (100 µg/ml) and this may be related to the sugar-binding activity of lectins. The specific carbohydrate chain, which was limited on the surface of tumour cells, acted as a receptor of lectins [25], and when the concentration of AAL was high enough to occupy all of the receptors, the inhibition ratio reached a relatively constant value. There are several kinds of mushroom lectins which have been reported to have anti-proliferative effects on tumour cell lines, but the antitumour action of lectins in tumour-bearing mice have only been demonstrated using *Agaricus bisporus* lectin, *Tricholoma mongolicum* lectin, *Volvariella volvacea* lectin and *Pleurotus ostreatus* lectin [2,20,26]. In the present study, AAL not only exhibited strong inhibition activity with several tumour cells, but also showed significant inhibition of the growth of S-180 cells in BALB/c mice. The survival ratio of AAL-treated tumour-bearing mice was increased. The analysis of the slides of tumour tissue showed that AAL caused great changes in the structure of tumour tissues, and the number of tumour cells growing aggressively decreased remarkably.

The analyses with Hoechst 33258 staining, flow cytometry and ApoAlert™ Mitochondrial Membrane Sensor Kit on HeLa cells showed that AAL possessed apoptosis induction activity; TUNEL assay of AAL-treated tumour tissues gave further evidence of the activity. However, the signal transduction of apoptosis induced by AAL still has to be explored in more detail.

There are only a few proteins with DNase activity that have been isolated from edible mushrooms. For example, several different types of *Coprinus meiotic* nucleases have been reported previously [27], and these are believed to be involved in meiotic chromosome recombination; a type I ribosome-inactivating protein purified from fruiting bodies of the edible mushroom *Volvariella volvacea* exerted a deoxyribonuclease activity on supercoiled simian-virus-40 DNA and demonstrated a strong abortifacient effect in mice [28]. In the present study, a mushroom lectin possessing endonuclease activity has been identified. The DNase assays may suggest another possible mechanism of antitumour activity of AAL. It has been reported that DNase I, a compact, monomeric enzyme, may serve as a very attractive candidate for targeting to tumour cells. Only a small amount of enzyme targeted to a cell needs to enter the nucleus in order to degrade the chromosomal DNA, making a cell incapable of further replication [29]. In cell cycle analysis, the percentage of AAL-treated HeLa cells in G₂/M phase was greatly reduced, indicating that AAL possessed anti-proliferating activity and might cause damage to DNA in tumour cells.

In summary, we have isolated and partially characterized the anticancer activity of a novel lectin from the edible mushroom *A. aegerita*. The lectin is active against several kinds of tumour

cell lines and significantly inhibits the growth of S-180 cells *in vivo*. It is well known that the antitumour mechanism of lectins may come from their immunomodulatory activity [30], but our work proposes different mechanisms: apoptosis induction and DNase activity. Determining the detailed mechanism of action of AAL and exploring its toxicity and pharmacokinetics may lead to the development of a novel antitumour drug.

The technical assistance of Professor Mingqiu Liu is gratefully acknowledged. This work was supported by Funds from National Natural Science Foundation of P. R. China, No. 30100236. We are also grateful to Honggang Li, Honglei Chen, Zuyu Zou and Zhijiao Tang for assistance with animal tests.

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Received 24 February 2003/9 April 2003; accepted 21 May 2003

Published as BJ Immediate Publication 21 May 2003, DOI 10.1042/BJ20030300