RESEARCH ARTICLE

Purification and characterization of an antitumor protein with deoxyribonuclease activity from edible mushroom *Agrocybe aegerita*

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Scope: Mushrooms are well known for their nutritional and medicinal value. *Agrocybe aegerita* has been used as a nutritious food around the world and for its herbal medicinal properties in Asia. In recent years, several antitumor proteins have been identified from *A. aegerita*. The objective of this study was to purify a novel antitumor protein from *A. aegerita*.

Methods and results: A novel antitumor protein *A. aegerita* deoxyribonuclease (AAD) was purified through a two-step chromatographic procedure and was shown to possess antitumor activity against different cancer cell lines. Cells treated with AAD produced typical apoptotic morphological changes, which include chromatin condensation, the accumulation of sub-G1 cells and caspase-8 cleavage. Biochemical characterization of AAD showed that it was a member of the DNase I family and that it possessed divalent metal ion-dependent endonuclease activity. The optimal temperature for AAD activity was 50°C and its optimal pH was 8.5. The MS-identified peptides of AAD were found to match to Unigene3821, which has 97% homology with Aa-Pri1, in our *A. aegerita* transcriptome.

Conclusion: We have identified a novel antitumor protein from *A. aegerita*. This fuller understanding of *A. aegerita* would help us to enhance its use in nutritional and medical applications.

Keywords:
Agrocybe aegerita / Antitumor / Characterization / Deoxyribonuclease / Purification

1 Introduction

Mushrooms are well known for their nutritional and medicinal value due to their bioactive substance and pharmacological properties [1, 2]. Numerous mushroom-derived compounds with antitumor, immunomodulatory, antiviral, antimicrobial, and other activities have been isolated and identified, including small molecular compounds, polysaccharides, peptides, and proteins [3]. Experience has shown that mushrooms could play an important role in the prevention and treatment of cancer [3]. As some polysaccharides or polysaccharide–protein complexes from mushrooms are able to stimulate the nonspecific immune system and to exert antitumor activity [4–6], there is an incentive value for exploring and identifying novel antitumor components from medicinal mushrooms.

Mushrooms have a high protein content, which account for 10–40% of dry fruiting body [7]. Our previous research has shown that protein components of edible/medicinal mushrooms possess high antitumor activity [8]. To date, antitumor proteins such as ubiquitin-like proteases [9], ribosome inactivating proteins [10], and lectins [11–15] have been identified. Several deoxyribonucleases (DNases) have also been separated from fungus [16–18], but none were reported to have any antitumor activity. DNases were proposed as attractive candidates for cancer therapy [19]. DNase I is the most

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Abbreviations: β-Me, β-mercaptoethanol; **AAD**, *Agrocybe aegerita* deoxyribonuclease; **CD**, circular dichroism; **DNase**, deoxyribonuclease; **GuHCl**, guanidine hydrochloride; **PARP**, poly (ADP-ribose) polymerase; **p-JNK**, phosphorylated JNK

well known Ca^{2+}/Mg^{2+}-dependent endonuclease and is involved in DNA fragmentation during programmed cell death (PCD) [20, 21]. DNase I treatment has been shown to reduce cell metastasis, enhance tumor cell arrest, and increase tumor cell aggregate dispersion [22–24]. Nawrot et al. showed that exposure to DNases of *C. majus* milky sap can result in the induction of apoptosis in tumor cells [25]. CdtB, a DNase I-like bacterial toxin, was shown to inhibit proliferation in tumor cell lines by inducing chromatin fragmentation and cell cycle arrest [26, 27].

Agrocybe aegerita is a popular and highly nutritional edible mushroom, which has been used as a traditional Chinese herbal medicine. It has an abundant amount of proteins, which accounts for 25–30% of dry fruiting bodies. Previously, we reported that the protein components from the edible/medicinal mushroom *A. aegerita* showed tumor rejection activity [8]. Two antitumor lectins, AAL and AAL-2, were identified from the protein components of *A. aegerita* [28–32]. In this report, we aimed to further identify the active protein components, which resulted in the identification of a novel antitumor protein named *A. aegerita* deoxyribonuclease (AAD). Characterization work showed that AAD is a member of the DNase I family. AAD-treated cells produced typical apoptotic changes, which include DNA fragmentation, chromatin condensation, the dose-dependent accumulation of sub-G1 cells, and caspase-8 cleavage. The MS-identified peptides of AAD were found to match to Unigene3821, which has 97% homology with Aa-Pri1, in our *A. aegerita* transcriptome.

In the current work, we have identified a novel antitumor protein (AAD) with DNase activity from *A. aegerita*. We also reveal that DNase is one of the active protein components in *A. aegerita*. This fuller understanding of *A. aegerita* would help us to enhance its use in nutritional and medical applications.

2 Materials and methods

2.1 Purification of the AAD

AAD protein was directly purified from the fruiting bodies of *A. aegerita*. Fresh fruiting bodies were dried at 50°C and crushed into powder. Approximately 15 g of the powder was extracted twice in 150 mL distilled water at 4°C for 10 h. The extracts were combined and centrifuged at 10 000×g for 20 min. Fraction extracted using 40–80% $(NH_4)_2SO_4$ saturation was resuspended in buffer A (20 mM Tris-HCl, pH 8.5) and dialyzed overnight against this buffer. The resulting solution was applied to a Heparin Sepharose column (Amersham Pharmacia Biotech, USA) (flow rate 1 mL/min) in buffer A and eluted using a linear gradient of 0–1 M NaCl in buffer A. The eluted samples were dialyzed in buffer A and further fractionated on a gel-filtration ZORBAX GF-250 HPLC column (Agilent, USA). The purity of the enzyme after the last purification step was assessed by SDS-PAGE.

2.2 Cell lines and culture

HepG2 (hepatocellular carcinoma), HeLa (cervical cancer), and SH-SY5Y (neuroblastoma) cell lines were obtained from the China Center for Type Culture Collection (CCTCC, China). All cell lines were cultured in DMEM medium (GIBCO, USA) supplemented with 10% fetal bovine serum (GIBCO), 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified (5% CO_2) atmosphere.

2.3 Cell viability

A total of 1×10^3 cells per well were seeded in 96-well culture plates for 12 h. After this time, the culture medium was discarded and the cells were incubated with 0.3125, 0.625, 1.25, 2.5, 5, and 10 μM purified AAD in 0.1 mL of culture medium for 48 h. Cell viability was analyzed with a modified MTT assay using a Cell Counting Kid-8 kit (Dojindo, Japan). The results are representative of three independent experiments.

2.4 Hoechest 33258 staining

HeLa cells were cocultured with or without AAD at 37°C for 48 h. After incubation, the medium was then removed and the cells were fixed at room temperature for 30 min. The cells were washed twice with 0.01 mol/L PBS before staining solution was added and incubated for 20 min. The confining solution was removed and cell apoptosis was determined with a fluorescent microscope.

2.5 Apoptosis activity assays

HeLa cells (2×10^5) cultured with or without AAD at 37°C for 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 for 10 min. Cell cycle analysis was performed with an Epics Altra II Flow Cytometer (Beckman Coulter, USA).

2.6 Western blotting

HeLa cells (2×10^5) were cultured with or without 10 μM AAD at 37°C for 48 h and then harvested. Western blotting was performed as described. The primary antibodies used in this study were raised against cleaved caspase-8, poly (ADP-ribose) polymerase (PARP), phosphorylated JNK (p-JNK) (Cell Signaling Technology, Beverly, MA, USA), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.7 In-gel nuclease assay

In-gel nuclease assay was performed as described with some modifications [33]. Before purified AAD was loaded, salmon sperm DNA at a final concentration of 40 μg/mL was incorporated into SDS-PAGE as the nuclease substrate during gel polymerization. After electrophoresis, the gel was washed with distilled water at room temperature for 2 h to remove excess SDS, and to allow for renaturation of the protein and enzyme activity. The gel was then incubated overnight at 50°C with several changes of incubation buffer (20 mM Tris-HCl,10 mM $MgCl_2$, pH 8.5). The nuclease was visualized with UV and ethidium bromide.

2.8 DNA hydrolysis by the AAD

To determine the substrate specificity of AAD, 1 μg of circular pCMV plasmid dsDNA and salmon sperm ssDNA (Sigma, USA) were incubated with 1 Kunitz U of purified AAD in 10 μL reaction buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5) at 37°C. After 30 min, 5 μL of the sample was analyzed by electrophoresis on a 1.0% agarose gel.

The effect of pH on AAD activity was evaluated over a pH range of 4–10. The effect of temperature of AAD activity was evaluated over a temperature range of 15–80°C. The effect of different metal ions on DNase activity was assessed by incubating the enzyme with different metal ions at 50°C and pH 8.5 for 30 min. The final concentration of metal ions during incubation with the DNase was 10 mM. All conditions were performed in reaction buffer as previously mentioned for 30 min, and analyzed by electrophoresis on a 1.0% agarose gel.

To determine the effects of metal chelators, AAD was preincubated with EDTA at 50°C and pH 8.5 for 30 min., followed by gel electrophoresis. The effects of thiol reagents and detergents on DNase activity were monitored by using the same procedure as described above. The concentrations of DTT, β-mecaptoethanol (2-ME), and Urea were 10 mM in the final DNase reaction buffer. The concentration of guanidine hydrochloride (GuHCl) was 0.6 M in the incubation solution. The concentrations of SDS, Tween-20, and Triton X-100 were 1% (w/v) in the final incubation solution, respectively. All analyses were performed by electrophoresis on a 1.0% agarose gel.

2.9 DNase activity assay

DNase activity was determined by using a modified version of the hyperchromicity assay by Kunitz [34]. A buffer of 20 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5, was used in place of 0.1 M sodium acetate, pH 5.0, employed by Kunitz. The conditions and the definition of units of activity were the same as those described by Price et al. [35]. The concentration of DNase was determined by Bicinchoninic Acid protein assay (Thermo, USA).

2.10 Circular dichroism (CD) spectroscopy

CD measurements of AAD were performed on a Jasco J810 spectrometer (wavelengths set at 190 and 260 nm) at a protein concentration of 30 μM, and using cells with a 0.1 cm path length. AAD was incubated in reaction buffers containing 1% Tween-20, 10 mM EDTA, and 1% Triton X-100, respectively. Each DNase solution was incubated for 30 min at 50°C. All CD measurements were performed at 25°C, and each spectrum was the average of three scans smoothed for further analysis. Data averaging and smoothing operations were performed using spectral analysis software provided with the spectrometer.

2.11 Intrinsic fluorescence spectroscopy

AAD was incubated in reaction buffers containing 1% Tween-20, 10 mM EDTA, and 1% Triton X-100. Each DNase solution, at a final concentration of 10 μM, was incubated for 30 min at 50°C. Intrinsic fluorescence spectroscopic experiments of AAD treated with different buffers were carried out at 25°C using an LS-55 luminescence spectrometer (PerkinElmer Life Sciences, Shelton, CT, USA). The fluorescence spectra were recorded between 300 and 395 nm. The excitation and emission slits were both set at 10 nm, and the scan speed was 1000 nm/min.

2.12 LC-MS/MS

The molecular mass of AAD was determined by LC-MS/MS at Beijing Protein Innovation Co., Ltd. Purified AAD was analyzed by SDS-PAGE and Coomassie blue staining. The protein band was excised from the gel and in-gel tryptic digestion was carried out as described [36]. MS/MS was performed using amaZon ETD. Data analysis was performed by comparing our experimental peptide sequences with "in-silico" translated sequences from the *A. aegerita* transcriptome using MASCOT software.

2.13 Data deposition

Raw IIIumina sequencing data from the *A. aegerita* transcriptome were submitted to the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) under accession number: SRA026731.1.

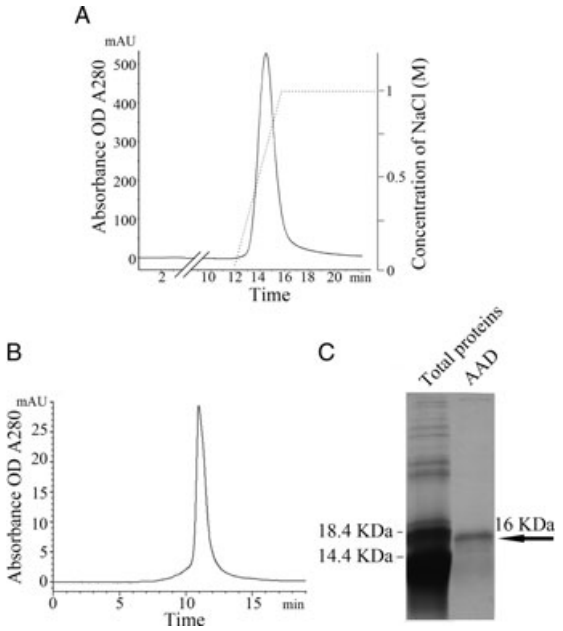

Figure 1. Purification of AAD from *Agrocybe aegerita*. (A) Fractions were eluted from a heparin column over a linear concentration gradient (0–1 M NaCl). (B) Heparin-eluted fractions were further purified by gel filtration HPLC. (C) SDS-PAGE analysis of purified AAD.

3 Results

3.1 Purification of AAD from *A. aegerita*

In previous work, we reported that *A. aegerita* protein components showed tumor rejection activity in a mouse model [8]. To date, we have identified two antitumor lectins, AAL and AAL-2. To further identify the active protein components in *A. aegerita*, a two-step chromatographic procedure was applied. As a first step, crude extract was loaded onto a heparin column. Binding proteins were obtained by eluting over a linear gradient of 0–1 M NaCl (Fig. 1A). Further fractionation was applied by using a gel-filtration column, and a single sharp peak was located on chromatographic elution profile (Fig. 1B). The purified AAD sample produced a single band on coomassie-stained SDS-PAGE, and the apparent molecular mass of AAD subunits was 16 KDa (Fig. 1C).

3.2 Antiproliferative effect of AAD on mammalian cancer cells

The effect of AAD on proliferation of several human cell lines was assessed by CCK-8 assay. Our results showed a dramatic decrease in percentage cell viability when a 2.5 μM concentration of purified protein was used. SH-SY5Y and HeLa cells were more sensitive (70% decrease), while HepG2 cells showed a fall in viability of nearly 35% (Fig. 2A). Purified protein treatment increased the percentage of apoptotic cells. This apoptotic inducing effect was confirmed by microscopic analysis of HeLa cells treated with 10 μM for 48 h. In Fig. 2B, AAD-treated HeLa cells produced apoptotic morphological changes such as cell shrinkage and blebbing (upper panel). DNA condensation was detected by Hoechst staining in AAD-treated cells (lower panel). The sub-G1 fractions of HeLa cells increased dramatically when 5 and 10 μM concentrations of AAD were used for preincubation (Fig. 2C). Significant differences were observed between control and purified protein-treated cells (Fig. 2D).

The classical signal pathway in apoptosis was investigated (Fig. 2E and F). AAD treatment (10 μM) resulted in increased activation of caspase-8, an initiator of apoptosis, and an increase in PARP cleavage. In addition, we show that AAD treatment can also activate proapoptotic kinases through increased JNK phosphorylation. These results show that AAD can evoke tumor cell apoptosis in a caspase-dependent manner. We also show that purified AAD has antiproliferative activity against different cancer cells.

3.3 DNase activity of AAD

Some antitumor proteins have hemagglutinating (lectin), DNase, ribonuclease, and protease inhibitory activities [11, 13–15, 24, 25, 27, 28, 32, 37]. To confirm whether AAD was a DNase, an in-gel DNase activity assay was performed (Fig. 3A). In-gel measurement of DNase activity, following SDS-PAGE produced a single DNase band with a molecular mass of 16 KDa, which is consistent of the same size with the gel band identified from our Coomassie staining gel (Fig. 1C).

DNases can be divided into two classes, DNase I and DNase II. The binding of Mg^{2+} is an essential requirement for the catalytic activity of the DNase I family [26]. To confirm the divalent metal ion requirement for DNase activity of AAD, the DNase activities in the presence of different metal ion solutions were compared. In the absence of divalent metal ions, no activity was observed, whereas in the presence of 10 mM Mg^{2+} or 10 mM Mn^{2+}, AAD was highly active, resulting in complete nicking of the plasmid after 30 min. Metal ions of K^{+}, Ca^{2+}, Zn^{2+}, Ba^{2+} and Cu^{2+} produced slight increases in AAD DNase activity (Fig. 3B).

Members of the DNase I family can actively hydrolyze ssDNA and dsDNA [38]. AAD can utilize and hydrolyze ssDNA and supercoiled plasmid DNA as substrates (Fig. 3C and D). The initial products generated by the enzyme were initially nicked plasmid DNA before plasmid DNA was completely hydrolyzed by AAD.

The optimum pH of AAD DNase activity was determined by incubating DNA with AAD in the presence of 10 mM $MgCl_2$ at different pH values. AAD exhibited optimal activity at pH 8.5 (Fig. 4A and B). Based on the effects of temperature on enzymatic activity, AAD exhibited optimal activity at 50°C (Fig. 4C and D). Our results also show that AAD is a divalent metal ion-dependent, nonspecific endonuclease with DNase

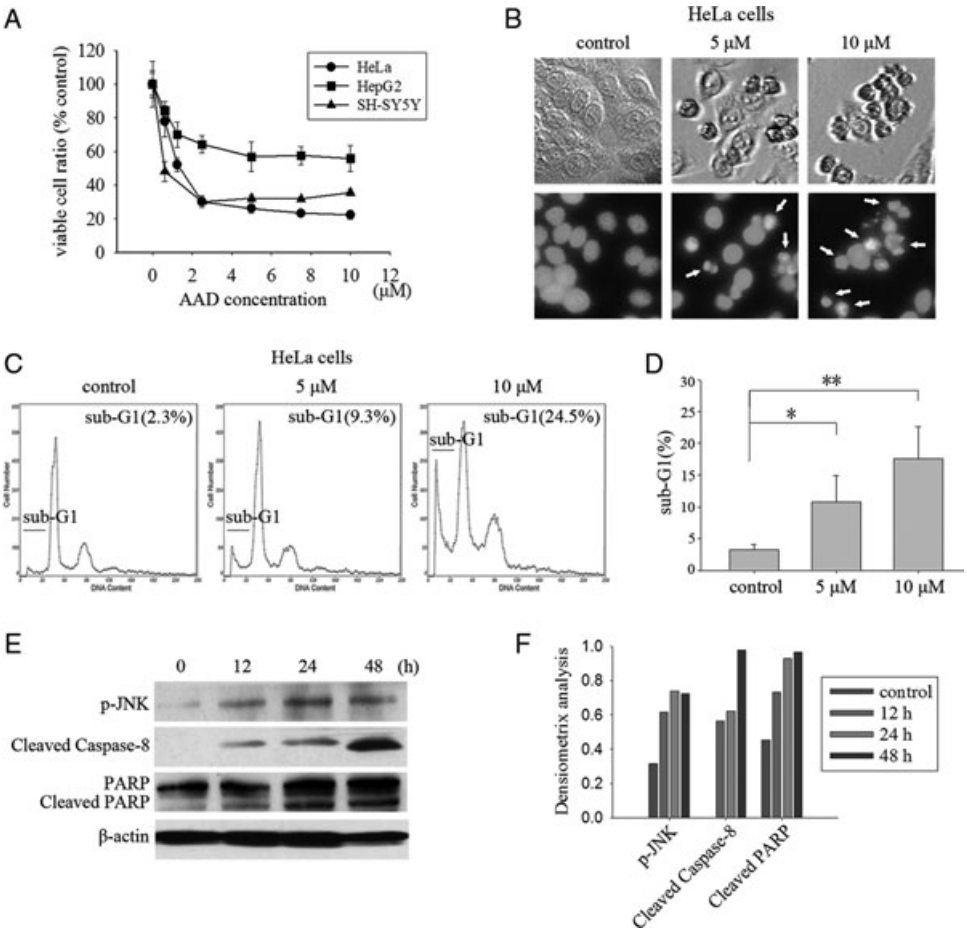

Figure 2. Antiproliferative effect of AAD on mammalian cancer cells. (A) Purified antitumor protein produced growth-inhibitory effects after incubating for 48 h in HeLa, HepG2, and SH-SY5Y. All cell lines were examined with the CCK-8 assay. (B) Bright-field (upper panel) and Hoechst staining (lower panel) of HeLa cells. HeLa cells were treated with control buffer, 5 or 10 μM AAD for 48 h. The arrows indicate apoptotic cells. (C) PI staining for Sub-G1 analysis of AAD-treated HeLa cells. (D) Quantification of Sub-G1 analysis. The experiments were repeated three times, values represent mean ± S.D. $*p < 0.05$; $**p < 0.01$. (E) Western blots of p-JNK, Caspase-8, PARP, and β-actin. HeLa cells were treated with control buffer or 10 μM AAD at 37°C for 48 h. (F) Densitometric data of western blots. All values were normalized by using the housekeeping gene β-actin.

enzymatic activity for single-stranded and double-stranded DNA.

3.4 Effects of metal chelators, thiol reagents, and detergents on *A. aegerita* DNase activity

To determine the effect of possible inhibitors on DNase activity of AAD, AAD was incubated with some common chemical inhibitors (Fig. 5A). Our results show that the purified DNase is active when some thiol reagents and detergents are preincubated with the enzyme. However, enzymatic activity is significantly reduced in the presence of 10 mM EDTA, 0.6 M GuHCl, or 1% Triton X-100.

To investigate the effects of metal chelators, thiol reagents, and detergents on AAD, we used CD and intrinsic fluorescence spectroscopy to study the secondary and tertiary structures of AAD. The metal chelator, EDTA, and the detergents, Tween-20 and Triton X-100 were used as typical reagents in our work. The spectral data of AAD in other detergent solutions are not shown because these agents absorb too strongly to allow reliable CD data to be collected much below 210 nm. The CD spectra of the constructs were almost identical in profile and are indicative of a structure made up of α-helices and β-sheets (Fig. 5B). EDTA, Tween-20, or Triton X-100 did not change the secondary structure whereas Triton X-100 strongly altered the tertiary structure of AAD (Fig. 5C). AAD incubated in reaction buffer had an intrinsic fluorescence emission maximum of about 336 nm when excited at 295 nm. The tryptophan emission maximum of the protein was blue-shifted, reaching 330 nm when AAD was incubated in reaction buffer containing 1% Triton X-100, meanwhile tryptophan fluorescence intensity increased. These results show that Triton X-100 can reduce AAD DNase activity by altering its tertiary structure.

3.5 Identification of AAD peptide sequences by MS/MS

To confirm our AAD protein sequence, the Coomassie-stained band of purified AAD in Fig. 1C was excised and analyzed by LC/MS/MS. Numerous peptides were identified (Table 1). The peptides were then assigned and produced 79% sequence coverage of a protein deduced from the Unigene3821 sequence in our *A. aegerita* transcriptome. Further analysis by BLAST search showed that Unigene3821 had a similar protein, *A. aegerita* Aa-Pri1. Alignment of the

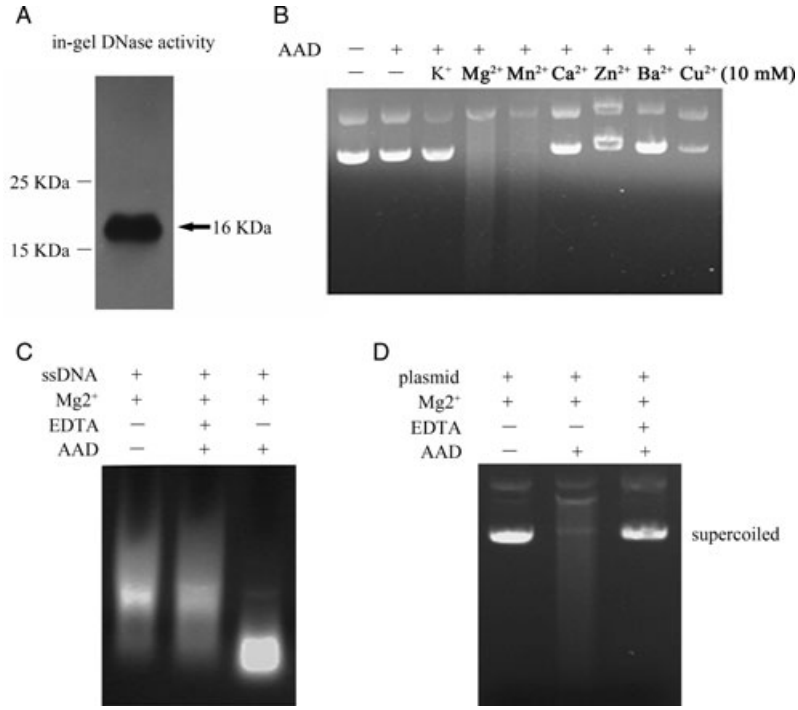

Figure 3. DNase activity of AAD. (A) DNase activity of purified antitumor protein was detected by in-gel DNase assay. Bands corresponding to DNase activity are indicated with arrows. (B) Effects of metal ions on the DNase activity. The pCMV plasmid was digested with AAD in the presence of various metal ions. (C and D) Digestion of various nucleic acids by AAD. The DNase activity toward single-stranded DNA (C) and supercoiled plasmid (D) was analyzed by running the digestion products on 1% agarose gels.

amino acid sequence derived from Unigene3821 with that of Aa-Pri1 showed that Unigene3821 had 97% homology with *A. aegerita* Aa-Pri1 (Fig. 6). The molecular weight of Aa-Pri1 was 16 KDa, which is in agreement with the molecular weight of AAD. These results show that purified AAD was Aa-Pri1.

Amino acid analysis showed that protein AAD was rich in asparagine, glycine, lysine, and serine. AAD contained all essential amino acids in different proportions, which accounted for 38.6% of the overall total amino acid content (Table 2).

4 Discussion

In our earlier work, we showed that protein components from the medicinal mushroom *A. aegerita* have tumor rejection activity [8]. In the current study, we aimed to further identify novel active components from *A. aegerita* protein lysates and isolated an antitumor protein with DNase activity.

DNases can be broadly divided into two classes, DNase I and DNase II [39]. Class II DNases are a group of enzymes that can cleave DNA at acidic pH and have no requirement

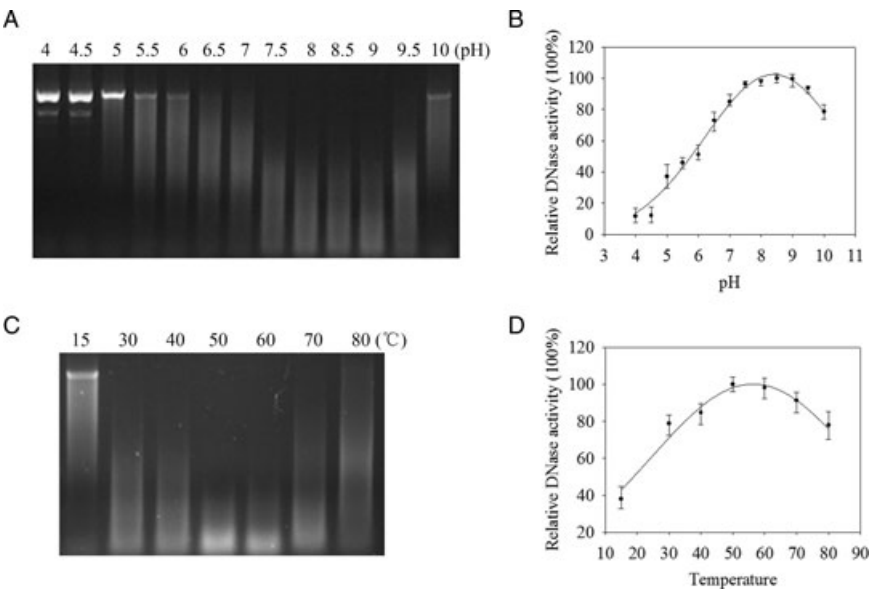

Figure 4. Determining the optimum pH and temperature of AAD. Purified DNase activity was assayed at various pH values ranging from 4 to 10 (A and B) and different temperatures ranging from 15 to 80°C (C and D). The results of A and C were revealed by analyzing the digestion products on 1% agarose gels. The deoxyribonuclease activities in B and D were measured by DNase activity assay.

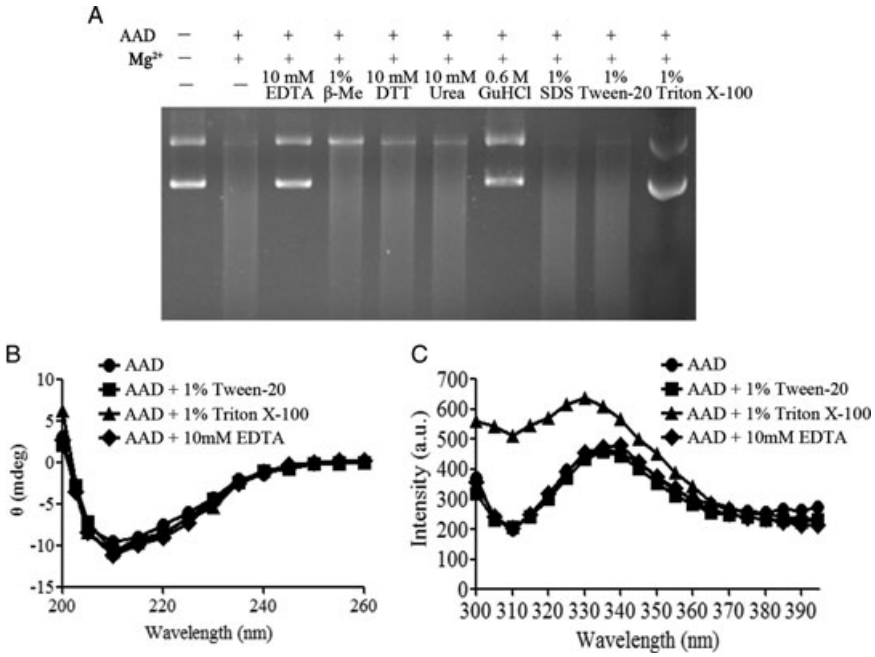

Figure 5. Effects of metal chelators, thiol reagents, and detergents on DNase activity. (A) The plasmid of pCMV was digested with AAD in the presence of metal chelators, thiol reagents, and detergents. (B) Comparison of the circular dichroism spectra of AAD incubated in reaction buffer containing 1% Tween-20 (■), 1% Triton X-100 (▲), and 10 mM EDTA (◆). (C) Comparison of intrinsic fluorescence spectra of AAD incubated in reaction buffer containing 1% Tween-20 (■), 1% Triton X-100 (▲), and 10 mM EDTA (◆).

for divalent cations [40]. In contrast, DNase class I members cleave DNA at an optimal pH range of 6.5–8 and require divalent cations for full activity [41]. Our results show that the catalytic properties of AAD are essentially the same as those well described DNase I family members, e.g., human DNase I [42], DNase γ [43], DNase X [44], DNAS1L2 [42], and bacterial toxin CdtB [26]. Like other DNase I family members, AAD is also a divalent metal ion-dependent endonuclease, which is active against double-stranded DNA and inhibited by EDTA. These results demonstrate that AAD belongs to the DNase I family. However, the activity of AAD slightly differs from some other class I DNases. Unlike DNase γ and DNAS1L2 that require both Ca^{2+} and Mg^{2+} for optimal activity [42], AAD is shown to be activated by Mg^{2+} alone. The optimal pH of AAD is 8.5, which is similar to frog DNase I and fish DNase I [41], while human DNase I, DNase X, and DNase γ

Table 1. Peptide sequences identified by MS/MS

Unigene ID	Protein description	NCBI accession number	Theoretial MW (Da)	Mascot score	Sequence coverage (%)	Peptide sequences identified by MS/MS
Unigene3821	*Agrocybe aegerita* Aa-Pri1	O42717	16086	110	79%	IANLGLSWGK DKEVYPSAYNGK KQNTWTPSGSNTK HFYWECPWGKK TIGPDEKIQINSCGR ENASSGTEGSFDIVDPNDGNKTIR WMVEWSGQNLDSGALGTITVDVLRK

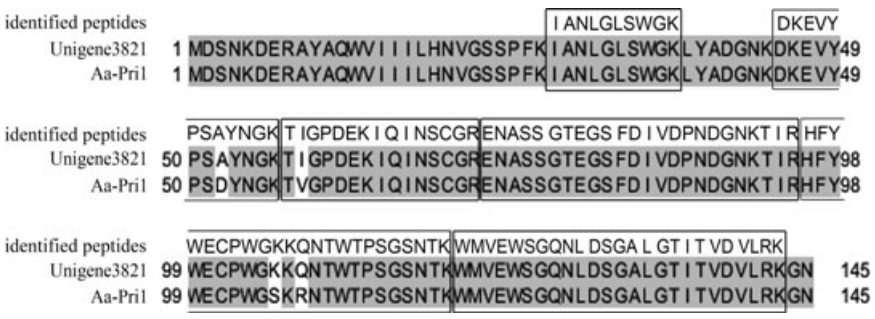

Figure 6. Amino acid sequence alignment of Unigene3821 and Aa-Pri1. Seven peptide sequences (black box) of AAD identified by MS were aligned under the amino acid sequence. The MS results revealed 79% sequence coverage of the full-length Unigene3821. Alignment of the amino acid sequence of Unigene3821 with Aa-Pri1 showed perfect sequence identity.

Table 2. Amino acid percentage composition of AAD

Amino Acid	(%)
Ala	4.83
Arg	2.76
Asn	8.97
Asp	6.90
Cys	1.38
Gln	2.76
Glu	4.83
Gly	11.03
His	1.38
Ile	6.90
Leu	4.83
Lys	8.28
Met	1.38
Phe	2.07
Pro	4.14
Ser	8.97
Thr	5.52
Trp	4.83
Tyr	3.45
Val	4.83
Total	100.00

activities are observed at neutral pH range with maxima at pH 6.8, 6.8, and 7.2, respectively [42].

DNases have been proposed as attractive candidates for cancer therapy due to their physiological importance and recent research interest in the class I DNases [42]. It has been reported that increased DNase activity is associated with improved responses to antitumor therapy in cancer patients [45–47]. Cells deficient in DNase activity have been isolated from over 60 kinds of tumors [48]. Sugihara et al. [49] and Tokita et al. [23] observed inhibition of metastasis and inhibition of tumor formation in the presence of DNase I. Preliminary data of a single-chain antibody (scFv) immunotoxin against bovine pancreatic DNase I showed high cytotoxic activity in vitro [50]. Our results show that purified *A. aegerita* DNase (AAD), which is a member of the DNase I family, also has antiproliferative activity against different cancer cell lines.

Apoptosis is a typical type of cell death by which unwanted or potentially harmful cells are eliminated under a wide variety of physiological and pathological situations [51]. The processes, known as apoptotic DNA fragmentation and chromatin condensation, have been recognized as outstanding features of mammalian apoptosis [52]. In apoptotic cell, the DNase I family members of human DNase I [53] and DNase γ [54] played an important role. DNase I translocated to the nucleus and colocalized with TUNEL positive chromatin aggregates in apoptosis [55], meanwhile, DNase γ was activated and capable of producing DNA fragmentation [42]. CdtB, a nuclease of the DNase I family, was demonstrated to induce chromatin fragmentation and cell death [26]. Our results of PI staining revealed that AAD can increase the fraction of sub-G1 cells and Hoechst staining showed that AAD can also evoke apoptosis in cancer cells (Fig. 2). Previous research showed this antitumor mechanism by DNase I to consist of two steps [24]. The first step involves the entry of a minimum quantity of DNase I into tumor cells to begin the apoptotic process [50, 56]. Second, damaged apoptotic cells or tumor cells have enhanced cell membrane permeability, which may result in eased entry of DNases [57]. AAD may also work through similar mechanism to inhibit the growth of human tumor cells, but this model hypothesis remains to be further verified.

To determine the effect of chemical inhibitors on AAD DNase activity, AAD was incubated with some common chemical reagents. AAD nuclease activity was inhibited by 0.6 M GuHCl and 1% Triton X-100. Incubation with Triton X-100 did not alter the secondary structure of AAD but strongly altered its tertiary structure. The intrinsic fluorescence emission maximum shifted from 336 to 330 nm while tryptophan fluorescence intensity was increased. Triton X-100 treatment may result in the tryptophan residues of AAD to move from an embedded to a more hydrophobic environment. Treatment of EDTA with AAD abolished DNase activity by chelating ions without producing changes in AAD secondary and tertiary protein structure. These results confirm that AAD DNase activity is a divalent metal ion-dependent nuclease.

MS/MS was used to confirm the identity of AAD protein. The MS-identified peptides of AAD were found to match to Unigene3821, which has 97% homology with Aa-Pri1, in our *A. aegerita* transcriptome. The molecular weight of AAD deduced from the amino acid sequence of Unigene3821 was shown to have the same weight as that of Aa-Pri1 [58]. While Aa-Pri1 has been shown to have hemolytic activity [59], there have been no reports on its DNase activity. Based on sequence analysis, Aa-Pri1 has been found to share little homology with any of the known nucleases at the primary structural level, revealing Aa-Pri1 to be a novel member of the DNase I family.

In summary, we show that AAD's antiproliferative activity has the potential for use in antitumor applications. We also reveal that DNase as one of the active ingredients in *A. aegerita* protein lysates. A fuller understanding of *A. aegerita* would help us to understand its use in nutritional and medical applications.

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