J. Biochem. **138**, 145–150 (2005)
DOI: 10.1093/jb/mvi109

Molecular Character of the Recombinant Antitumor Lectin from the Edible Mushroom *Agrocybe aegerita*

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Received March 6, 2005; accepted May 2, 2005

The lectin from *Agrocybe aegerita* (AAL) has been found to possess potent tumor-suppressing function and tumor cell apoptosis-inducing activity. In this paper, we report the full sequence, the active expression of the gene encoding AAL at a high level and bioassay of the binding property with lactose, apoptosis-inducing activity and DNase activity of recombinant AAL (rAAL). The results reveal that AAL is a member of the galectin family and the dimeric form is the active unit for the functional performance. The rAAL showed comparable tumor cell apoptosis-inducing activity with the wild AAL but no DNase activity at all. The molecular characters revealed by this study are significant for the in-depth investigation of the functional mechanism of this interesting protein.

Key words: antitumor, apoptosis, galectin, homodimer, mushroom.

Abbreviations: PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonylfluoride; PCR, polymerase chain reaction; NSFC, National Nature Science Foundation of China.

Lectins are a group of proteins that recognize carbohydrate moieties of the cell surface and have diverse physiological functions (*1*, *2*). They are involved in growth regulation, cell adhesion, cell migration, cell apoptosis and immune responses. Among others, the antitumor lectins are the most attractive group due to their pharmaceutical potentials. Mushrooms are well known to possess nutritional and medicine values, and a variety of compounds with important pharmacological properties have been isolated from mushrooms (*3*, *4*), including lectins with immunomodulatory, antiproliferative, antitumor/cytotoxic and hypotensive activities (*5*–*7*). In recent years, some fungal antitumor lectins have also been found in mushroom, which have attracted attention due to their pharmaceutical potentials. *Volvariella volvacea* lectin shows antitumor activities against sarcoma S-180 cells (*8*), *Grifola frondosa* lectin is cytotoxic to HeLa cells (*9*), and *Agaricus bisporus* lectin displays antiproliferation activities against human colon cancer cell line HT29 and breast cancer cell line MCF-7 (*10*). However, their molecular properties and the mechanisms of their antitumor activities are largely unknown. So far, the *kurokawa* lectin is the only mushroom lectin reported to display apoptosis-inducing activity (*7*), but the relationship between the aoptosis-inducing activity and the antitumor property is yet to be identified, and its full-length amino acid sequence and structural information are yet to be reported. Recently, a novel antitumor lectin, named AAL, was purified from the edible mushroom *Agrocybe aegerita* (*11*). A series of cell biological tests identified that AAL possesses potent tumor-suppressing function for several human tumor cell lines like HeLa, SW480, SGC-7901 and tumor cell apoptosis–inducing activity (*11*). Certainly, it would be interesting to elucidate the structure–function relationship between the antitumor effects and apoptosis-inducing activities of AAL. For in-depth study, the molecular character of AAL needs to be understood. Here we report the c-DNA cloning and expression, purification and characterization of recombinant AAL (rAAL), which revealed the main molecular character of rAAL, including the full sequence with the consensus motif of galectin and the active unit as a homologous dimer.

MATERIALS AND METHODS

Cloning and Expression of cDNA—Total RNA was isolated from the mycelia of *A. aegerita* following the recommendations given by TRIZOL. Using this protocol, oligonucleotides for RT-PCR were designed from the available N-terminal peptide sequences QGVNIYNI (*12*) and 3′ polyA. Template RNA was the total RNA extracted above. The purified cDNA product was cloned into pGEM-T vector using the pGEM-T easy system (Promega). Sequencing was done by UnitGene (Shang Hai, China).

To construct the expression vector, the coding region of AAL was amplified by PCR using Pfx DNA polymerase (Invitrogen) with two designed primers (forward primer: acttactcatatgcagggcgtcaacatcta; reverse primer: aatctcgagcgccaaacccgtgtat). The PCR products were purified,

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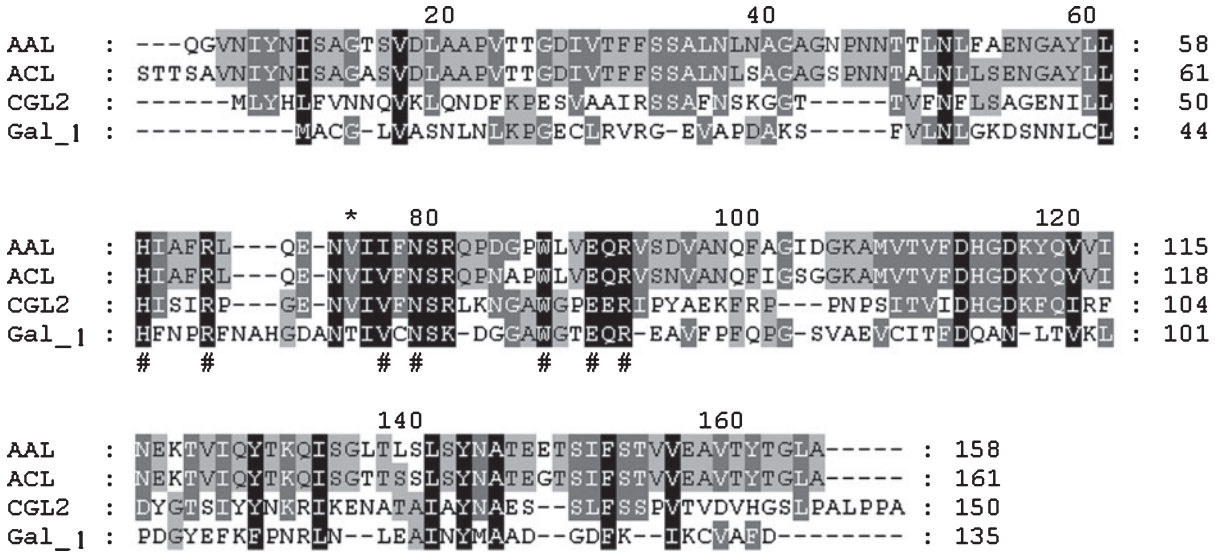

Fig. 1. **Amino acid sequence of AAL in comparison with representative members of the galectin family.** The full sequence of AAL deduced from cDNA contains 158 amino acid residues without any cysteine. The amino acid sequence of AAL shows 13.9, 31 and 88% identity with those of human galectin-1, Coprinus CGL2, and ACL respectively. Dashed lines indicate the gaps inserted for maximizing the match. The consensus β-galactoside-binding motifs in the CRD region of galectins are indicated by , which are strictly conserved in AAL. In AAL, the conserved Val-59 (residue numbered by position in human galectin-1) is replaced by Val-68 (residue numbered by position in AAL) as shown by *. ACL, lectin from *Agrocybe cylindracea* (*15*); CGL2, lectin from *Coprinus cinereus* (*14*); Gal-1, human galectin-1 (*13*).

cleaved with *Nde*I and *Xho*I (Promega) and subcloned into the pT7 expression vector pET22b (+) (Novagen).

Recombinant AAL (rAAL) was expressed in BL21 (DE3) strain of *Escherichia coli* and had a his-tag for purification. Bacteria were induced with 1 mM isopropyl-β-thiodigalactoside for 3 h and collected by centrifugation at 4,000 rpm. The harvested cells were resuspended in 30 ml of lysis buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 10 mM imidazole) with 0.1 mM PMSF, then treated by BugBuster (Novagen) and centrifuged at 17,000 rpm to remove the cell debris. The supernatant was applied on a NTA Ni^+ chelating column (Novagen), and the target proteins were washed off with eluting buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 250 mM imidazole). The eluate was concentrated by ultra-filtration (Millipore), then loaded onto a Hiload Superdex75 16/60 XK column (Amersham Pharmacia) pre-equilibrated with 50 mM NH_4HCO_3 at 20°C. Purified rAAL was pooled and lyophilized, stored at 4°C for further crystallization experiment. The final purified proteins were examined by SDS-PAGE and ESI mass spectrometry using an API 3000 triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Canada).

Characterization of Recombinant AAL—The molecular mass of rAAL was determined by both ESI mass spectrometry and gel filtration. Gel filtration was carried out on a Superdex 75 10/300 GL column (Amersham) in 50 mM phosphate buffer (pH 7.0) with 0.15 M NaCl at room temperature. Standard protein markers including ribonulease A (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa) and bovine serum albumin (67.0 kDa) were used to calculate the molecular standard curve.

Lactose Binding Capacity Test—The eluate of rAAL from NTA Ni^+ chelating column and the wAAL-water solution were loaded onto an alpha-lactose-agarose (Sigma) column pre-equilibrated with PBS (pH 7.2) to confirm the direct lactose-binding capacity of rAAL and wAAL. The column was then washed with PBS, and the lectin was eluted in the same buffer containing 25 mM alpha-lactose.

Hemagglutinating Assays—Mouse erythrocytes were used in this assay. A serial twofold dilution of the native AAL and recombinant AAL solutions in microtiter U-plates (50 μl) was mixed with 50 μl of 2% suspension of red blood cells in PBS (pH 7.2) at 20°C. The initial concentration was 1 mg/ml. The results were read after about 1 h, when the control (no added AAL) had fully precipitated. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit (2^0). Specific activity was the number of hemagglutination units per mg protein.

The Hemagglutinating Inhibition Tests—To investigate inhibition of lectin-induced hemagglutination by lactose, hemagglutinating inhibition tests were performed in a manner analogous to the hemagglutination test. Serial twofold dilutions of lactose were prepared in PBS. Each dilution was mixed with an equal volume (25 μl) of AAL solution at the concentration of 0.015625 mg/ml (2^5 hemaggutination units). The mixture was incubated for 30 minutes at room temperature, then mixed with 50 μl of 1% mouse erythocyte suspension. The minimum concentration of the sugar in the final reaction mixture that completely inhibited 2^5 hemaggutination units of the AAL preparation was calculated.

Apoptosis Activities Assays—The HeLa cells (2×10^5) cultured with or without the samples at 37°C for 24 h were harvested, washed with PBS, and fixed with 75% ethanol at 4°C for 2 h. Cells were then treated with

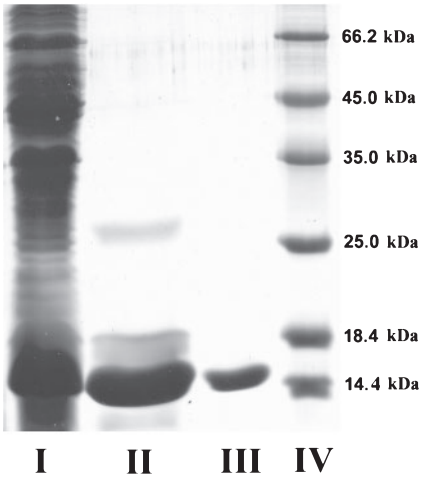

Fig. 2. **SDS-PAGE of recombinant AAL.** Lane I is the supernatant fraction after cell lysis by BugBuster. Lane II is the eluate of NTA Ni^{+} chelating column. Lane III is the eluate of size-exclusion column, the purified rAAL. Lane IV is the protein molecular weight markers. Analyzed by BandScan 4.5 software, the purity of rAAL was about 98% and its molecular mass was around 16–17 kDa.

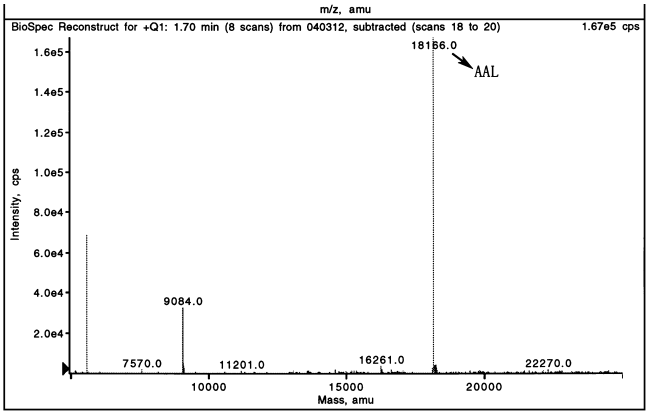

Fig. 3. **Mass spectrometry of recombinant AAL.** The result showed that the molecular weight of rAAL was 18,166.0.

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 with a Coulter EpicsXL™ Flow Cytometer.

DNase Activities Assays—DNase activity of rAAL was measured by a plasmid digestion assay, with pUC18 as the substrate. The incubation was performed with 0.2 μg of plasmid in 19 μl digestion buffer (6 mM Tris-HCl, pH 7.0, 6 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol), to which 1 μl of sample was added. After 2 h at 37°C, the integrity of the pUC18 was monitored by gel electrophoresis. Alternatively, the DNase activity was examined by incubation of 0.8 μg salmon sperm DNA together with the sample in 100 mM Tris-HCl, pH 8.0 at room temperature for 2 h. The integrity of the salmon sperm DNA was monitored by gel electrophoresis.

RESULTS AND DISCUSSION

Full Amino Acid Sequence of AAL—The sequence of AAL cDNA has been recorded in Genebank (accession code AY264782), which shows that the insert is composed of 635 bp. and the open reading frame (ORF) is 474 bp long and ends with the stop codon TAG. The deduced amino acid sequence consists 158 amino acid residues as shown in Fig. 1. Two partial amino acids sequences of AAL, the N-terminal peptides QGVNIYNI and the random peptides PDGPWLVEQR previously determined by protein sequencing (*12*) well match the present sequence from 1–8 and 76–85, respectively, which indicates the coincidence between the results from cDNA and protein sequencings.

Based on the deduced full amino acid sequence, the PSI-BLAST search indicated that AAL was homogeneous with the galectin super-family. The amino acid sequence of AAL shows 13.9, 31 and 88% identity with the representative galectins of human galectin-1 (*13*), Coprinus CGL2 (*14*) and ACL (*15*), respectively (Fig. 1). Galectins are a large and widespread family of proteins defined by conserved sequence elements in their carbohydrate-recognition domain (CRD) and their affinity for β-galactosides. They are found in many species from fungi to mammals and involved in a vast and diverse array of processes, including growth regulation, cell adhesion, cell migration and immune responses. To date, 15 members of the galectin family have been isolated from various tissues and cells in mammals (*16*). From the structural information of mammalian galectins (*17–19*), all galectins contain a consensus sequence motif in the conserved CRD region, which consists of His-44, Arg-48, Val-59, Asn-61, Trp-68, Glu-71 and Arg-73 (residues numbered by position in human galectin-1). The sequence alignment shows that this consensus motif clearly occurs in AAL (Fig. 1).

Expression and Purification of Recombinant AAL—The yield of recombinant AAL after purification was 15 mg/liter of *E. coli* growth culture. The purified rAAL was examined by SDS-PAGE and mass spectrometry (MS). The results of SDS-PAGE showed the purity of rAAL reached about 98% (Fig. 2). Mass spectrum (Fig. 3) showed that the molecular weight of rAAL was 18,166.0, close to the estimated value of 18,165.23.

Binding Property of rAAL Identified It as a Galectin—The lactose binding test showed that both rAAL and wAAL could directly bind to the alpha-lactose column and elute with lactose (25 mM) (Fig. 4). The result confirmed that AAL could interact with lactose therefore possesses the binding property for β-galactoside sugars. Members of the galectin family are defined by two properties: a characteristic amino acid sequence and affinity for β-galactoside sugars. The association of the binding property with the distinctive sequence described above identified AAL as a new member of the galectin family.

Most studies on galectins hither to have focused on animal galectins. Prinus isolectins (CGL1 and CGL2) are the first galectins from outside the animal kingdom to be studied, being purified from the inky cap mushroom, *Coprinus cinereus* (*14*). AAL is a novel mushroom galectin with both antitumor abilities and cell apoptosis-inducing effects.

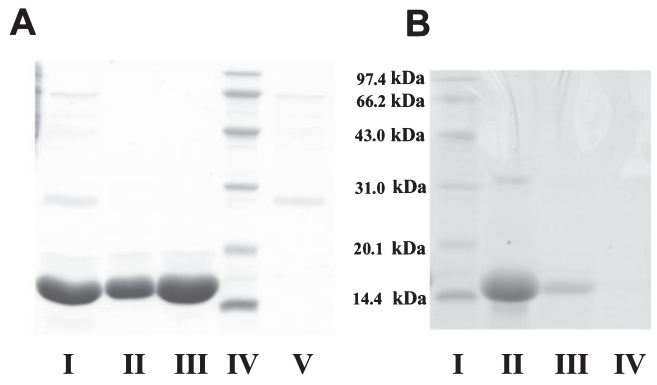

Fig. 4. **SDS-PAGE of recombinant AAL (A) and native AAL (B) on alpha-lactose column.** (A) Lane I is the eluate of NTA Ni^{+} chelating column. Lanes II and III are the eluates of alpha-lactose column taken from different collecting tubes. Lane IV is the protein molecular weight markers. Line V is the flow-through fraction of alpha-lactose column. (B) Lane I is the protein molecular weight markers. Lane II is native AAL dissolved in pure water at the concentration of 4 mg/ml. Lane III is the eluate from alpha-lactose column. Lane IV is the flow-through fraction (almost nothing could be discerned). The assigned molecular weight fits both markers in (A) and (B). The results showed almost all the recombinant lectin could bind to the alpha-lactose column, while the contaminating proteins were in the flow-through fraction. The target lectin could be eluted with PBS containing 25 mM lactose.

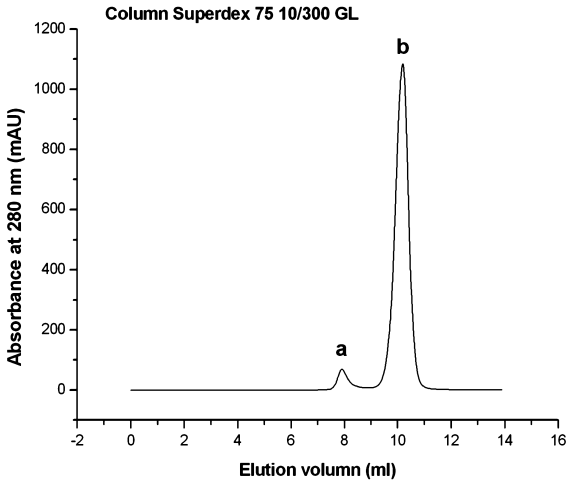

Fig. 5. **Size exclusion chromatography on Superdex 75 10/300 GL column.** A 100 μl sample of rAAL (10 mg/ml in pure water) was loaded on the column.The eluent was 50 mM phosphate buffer (pH 7.0) with 0.15 M NaCl at the flow rate of 0.5 ml/min. Peak a is the flow-through fraction. rAAL appeared in peak b with an elution volume of 10.19 ml. The estimated apparent molecular mass was 34 kDa.

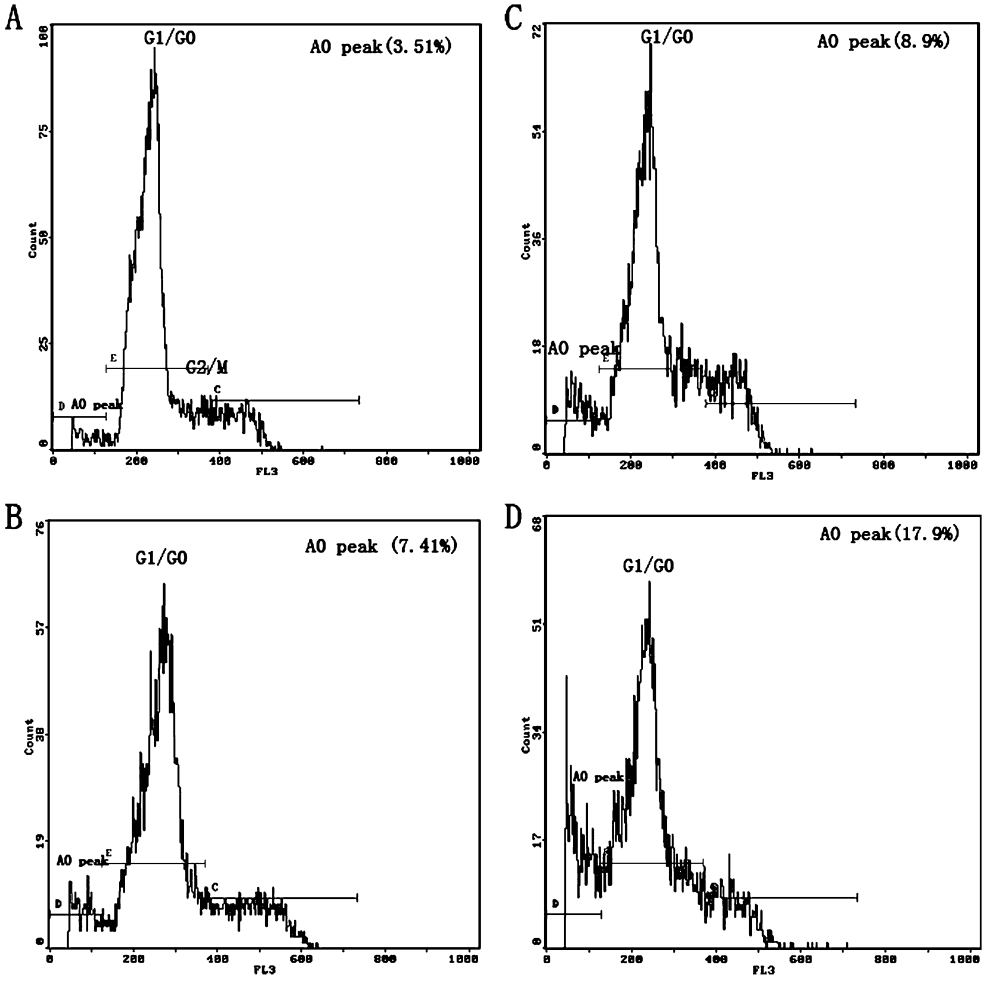

Fig. 6. **Propidium iodide staining and flow cytometric assay of rAAL-induced apoptosis.** (A) No significant apotosis (A^0 peak) appeared in control HeLa cells. (B) In the 50 μg/ml rAAL-treated group after 24 h, the A^0 peak represented 7.41% of the HeLa cells that were undergoing apoptosis. (C), (D) When the concentration of rAAL was increased to 100 μg/ml and 150 μg/ml, the percentage of the HeLa cells undergoing apoptosis increased to 8.9 and 17.9%, respectively.

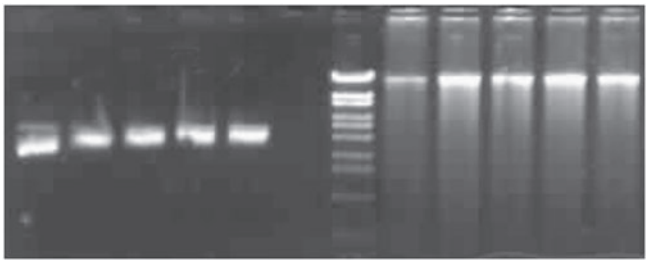

Fig. 7. **Agarose gel electrophoresis analysis of rAAL DNase activity.** Reactions were carried out in a volume of 20 μl for 2 h at 37°C. (Lanes 1–5): Digestion of 0.2 μg of plasmid pUC18 with varying concentrations of rAAL. Lane 1, pUC18 without rAAL; lane 2, 5 μg/ml rAAL; lane 3, 50 μg/ml rAAL; lane 4, 125 μg/ml rAAL; lane 5, 250 μg/ml rAAL. (Lane 7): DNA ladder. (Lanes 8–12): Digestion of salmon sperm DNA with rAAL in 100 mM Tris-HCl, pH 7.0. Lane 8, salmon sperm DNA without rAAL; lane 9, 10 μg/ml rAAL; lane 10, 100 μg/ml rAAL; lane 11, 500 μg/ml rAAL; lane 12, 1,000 μg/ml rAAL.

Bioassay of rAAL Showing the Homodimer of AAL Is the Active Unit—The hamagglutinating titers of wAAL and rAAL against mouse erythrocytes were both 2^9, so the corresponding minimum concentration required to yield hemagglutination was 1.95 μg/ml in both cases. The hamagglutination activities of wAAL and rAAL were inhibitied by lactose at the concentrations of 25 mM and 12.5 mM, respectively. These results shown that rAAL had almost the same properties as wAAL in hamagglutinating activity and carbohydrate binding specificity.

In gel filtration using a Superdex 7510/300 GL column, the elution volume of rAAL was 10.19 ml, from which the apparent molecular weight of rAAL was calculated as 34 kDa from the molecular standard curve (Fig. 5). This result indicated that the recombinant AAL was probably a homodimeric protein.

The results of cell cycle analysis of rAAL are shown in Fig. 6, which reveals that the cell cycle of HeLa cells treated with AAL altered significantly. Healthy HeLa cells demonstrated normal cell cycle characteristics (G1/G0 and G2/M phases) (Fig. 6A). The percentage of apoptotic HeLa cells (shown as the Ao peak) was 3.51% in the control group. After treatment with rAAL for 24 h at the concentrations of 50, 100 and 150 μg/ml, HeLa cells underwent apoptosis and the percentage of apoptotic HeLa cells rose to 7.41, 8.9 and 17.9%, respectively (Fig. 6, B–D). As the concentration of rAAL increased, the ratio of apoptotic cells increased gradually. Compared with wAAL (Table 1), rAAL retained substantial apoptosis-inducing activity. These results indicated that rAAL could induce apoptosis in HeLa cells and interfere with the proliferation of tumor cells synchronously, as does the wild lectin.

rAAL Lacks DNase Activity—The digests of plasmid pUC18 and salmon sperm DNA with rAAL were analyzed by agarose gel electrophoresis as shown in Fig. 7. The results revealed that rAAL can digest neither the plasmid pUC18 nor the salmon sperm DNA at very high concentrations (250 μg/ml and 1,000 μg/ml, respectively). Previously, however, it was reported that the wild AAL possesses DNase activities against plasmid pCDNA3 and salmon sperm DNA (*11*). The present test definitely showed that rAAL has no DNase activity. Carefully rechecking of the earlier experiment revealed that the wild sample used in the previous assay contained small amount of a subcomponent, which shows a strong DNase activity (data not shown). Evidently, the DNase activity of the wild AAL reported before is due to contamination. This subcomponent also shows stronger apoptosis-inducing ability than the highly purified wAAL. Thus, this should be one of the reasons for the lower activity of rAAL in comparison with wAAL (see Table 1). In addition, it was noticed that, compared with wAAL, the solubility of rAAL was reduced, which could also cause the lower activity of rAAL at the concentration apparently equal to wAAL (Table 1). In fact, a similar case has been observed in the recombinant human galectin-1 (*20*).

Table 1. **The percentage of HeLa cells that were undergoing apoptosis when treated with different concentrations of wAAL and rAAL for 24 h.**

Concentration of lectin (μg/ml)	wAAL (%)	rAAL (%)
0	3.31%	3.51%
50	10.30%	7.41%
100	19.50%	8.90%

This study showed that AAL exerts its antitumor effects mainly *via* apoptosis-inducing activity, and that the dimeric organization is essential for the function. As in the case of other galectins, the first step in the functional performance should be binding to certain glycoconjugates on the cell surface or extracelluar matrix. So far, a series of galectin structures have been determined, which are variable from multi-domain (*e.g.*, galectin-3 and -4) (*21*, *22*), dimer (*e.g.*, galectin-1 and -2) (*18*, *19*) to tetramer (*e.g.*, galactin CGL2) (*23*). This implies that the quaternary organization may correlate with the distinct bioactivities of galectins. In the case of AAL, it should be bivalent in order to cross-link the carbohydrates on cell surface. Probably this is just the initial event, which may promote AAL to be cytoplasmatic and further induce a series of intracellular process, including binding to a unique target protein. The details are largely unknown at present. At this stage AAL may have one or more active sites different from the β-galactoside–binding site, which we are looking for now.

The cDNA sequence of AAL has been deposited in Genebank with the accession code AY264782. The amino acid sequence of AAL has been deposited in the Swiss-Port protein sequence database with the primary accession code Q6WY08. The work was supported by the NSFC (30370320, 30100236), the MOST of China (2002BA711A13, G1999075063, 2004CB520801), and the Chinese Academy of Sciences (KSCX2-SW-322, KSCX1-SW-17).

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