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Short crystallization paper

Crystallization and preliminary crystallographic studies of the recombinant antitumour lectin from the edible mushroom *Agrocybe aegerita*

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Abstract

The antitumour lectin from *Agrocybe aegerita*, named AAL, shows strong inhibition effects on human and mouse tumour cells via apoptosis induction activity. Recombinant AAL (rAAL) has been expressed and purified. Both rAAL and rAAL–lactose complex have been crystallized and their X-ray diffraction data were collected to resolutions of 1.9 Å and 1.6 Å, respectively. Both crystals belong to space group $P2_1$ with unit cell parameters $a=53.20$ Å, $b=66.01$ Å, $c=57.86$ Å, $\beta=109.38$ and $a=53.38$ Å, $b=66.29$ Å, $c=58.02$ Å, $\beta=109.03$, respectively.

Keywords: Lectin; Mushroom; Antitumour; Apoptosis; Crystallization

Mushrooms are famed for their nutritional and medical values. A wide variety of compounds with important pharmacological properties have been isolated from mushrooms [1,2]. Among them, the study of mushroom lectins with immunomodulatory, antiproliferative, antitumour/cytotoxic and hypotensive activities has recently attracted growing interest [3–5]. Recently, a novel antitumour lectin, named AAL, has been purified from the edible mushroom *Agrocybe aegerita* [6]. AAL possesses potent tumour-suppressing function for several tumour cell lines like human HeLa, SW480, SGC-7901 and mouse sarcoma S-180. The experiments further show that AAL exerts its antitumour effects via apoptosis-inducing activity [6]. Furthermore, AAL exhibits a series of distinct bioactivities, including agglutination of human and animal erythrocytes regardless of blood types or animal species, inhibition activity to tobacco mosaic virus on *Nicotiana glutinosa*, and promotion for the differentiation of fruit body primordial from the mycelia of *Agrocybe aegerita* and *Auricularia polytricha* [7]. As a first step to understand the structural basis of these significant functions, we report here the expression, purification, crystallization and preliminary crystallographic studies of recombinant AAL (rAAL) and the complex of rAAL with its carbohydrate ligand, lactose.

1. Cloning, expression and purification

Total RNA was isolated from the mycelia of *Agrocybe aegerita* following by the recommendations given by TRIZOL. Using this protocol, oligonucleotides for RT-PCR were designed from the available N-terminal peptide sequences QGVNIYNI [7] 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

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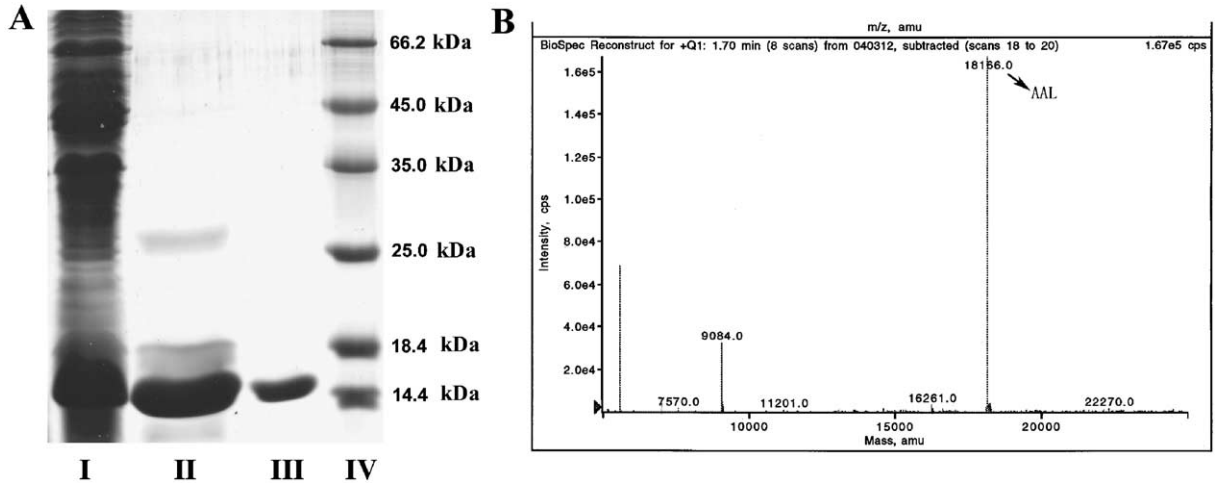

Fig. 1. (A) SDS-PAGE of recombinant AAL. Lane I is the supernatant fraction after cell lysis by BugBuster. Lane II is the eluate of NTA Ni+ chelating column. Lane III is the eluate of size-exclusion column, the purified rAAL. Lane IV is the protein molecular weight markers. Analyzed by BandScan 4.5 software, the purity of rAAL was about 98% and its molecular mass was around 16–17 kDa. (B) Mass spectrometry of recombinant AAL. The result showed that the molecular weight of rAAL was 18166.0.

easy system (Promega). Sequencing was done by UnitGene (Shang Hai, China).

The cDNA sequence of AAL has been deposited in Genebank with accession code AY264782, which shows that the insert is composed of 635 bp. The open reading frame (ORF) is 474 bp long and ends with the stop codon TAG. The deduced amino acid sequence consists of 158 amino acid residues, which has been deposited in the Swiss-Port Protein sequence database with the primary accession code Q6WY08.

To construct the expression vector, the coding region of AAL was amplified by Polymerase Chain Reaction (PCR) using Pfx DNA polymerase (Invitrogen) with two designed primers (forward primer: acttactcatatgcagggcgtcaacatcta; reverse primer: aatctcgagcgccaaacccgtgtat). The PCR products were purified, 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. The harvested cells were resuspended, treated by BugBuster (Novagen) and centrifuged at 17000 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 Superdex75 HR16/60 column (Amersham Pharmacia) pre-equilibrated with 50 mM NH_4HCO_3 at 293 K. The yield of recombinant AAL after purification was 15 mg/L of *E. coli* growth culture. Purified rAAL was examined by SDS-PAGE and mass spectrometry (MS). The results of SDS-PAGE showed the purity of rAAL was about 98% (Fig. 1A). Mass spectrum (Fig. 1B) showed that the molecular weight of rAAL was 18166.0, which is close to the estimated value of 18165.23. Purified rAAL with

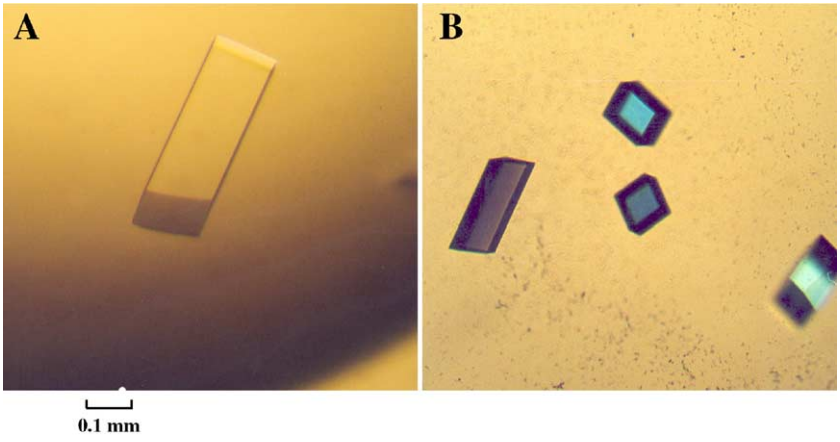

Fig. 2. Crystals of recombinant AAL. (A) Crystal of rAAL. (B) Crystal of rAAL–lactose complex. The length of the line segment is equal to 0.1 mm.

his-tag was pooled and lyophilized, and then stored at 277 K for further crystallization experiments.

2. Crystallization of rAAL and rAAL–lactose complex

Recombinant AAL was dissolved in pure water at a concentration of 6 mg/ml for crystallization. Initial screening was performed using a sparse matrix method [8] with Hampton Research Crystal Screen I and II at 293 K. The hanging-drop vapour-diffusion method was used. Each drop contained equal amounts (1 μl) of protein (6 mg/ml) and reservoir solution equilibrated against 500 μl of reservoir solution in the well. Small rod-like crystals and large plate-like crystals appeared from a few conditions. Through optimization, large crystals suitable for X-ray diffraction were obtained in the crystallization drops formed by mixing equal volumes (1 μl) of protein (6 mg/ml in water) and reservoir solution. The reservoir solution contained 0.1 M Sodium Acetate Trihydrate buffer (pH 4.6), equal concentration (3%–5% w/v) of PEG 8000 and PEG 1000, 5% (v/v) 1.6 Hexanediol and 5% (v/v) glycerol anhydrous. Crystals of rAAL grew to their full size within 2 days. They were inclined columnar in shape with dimensions of $0.15 \times 0.15 \times 0.4$ mm^3 (Fig. 2A). The growth of rAAL crystals was very sensitive to temperature and buffer pH. Very small changes would cause the appearance of many small crystals. Since it has been identified that AAL can specifically bind to lactose, we tried to co-crystallize rAAL with lactose. Crystals of rAAL–lactose were obtained with the same conditions as described above by adding 20 mM lactose in the drop and reservoir solution. These crystals were smaller but less fragile with dimensions of $0.05 \times 0.05 \times 0.1$ mm^3 (Fig. 2B). Of course, whether lactose has really bound to the protein is waiting for identification from the further structural analysis.

3. Data collection and processing

X-ray diffraction data from rAAL crystals were collected on a Rigaku R-Axis IV^{++} image plate using Cu Ká radiation ($\lambda = 1.5418$ Å) from a rotating anode operating at 40 kV and 20 mA with 0.1 mm confocus incident beam diameter. Data were collected at 85 K with a crystal-to-detector distance of 120 mm, $\Delta\varphi = 1°$ and 240 s of exposure time. A total of 200 frames were collected. The data set of the rAAL–latose complex (rAAL–Lac) was collected at the beam line 6A (BL6A) at Photon Factory (Tsukuba, Japan) using an ADSC Quantum-4 CCD detector at 90 K. The wavelength used was 0.98 Å and a total of 180 frames were collected. All of the crystals were briefly soaked in paraffin oil (Hampton Research) used as cryo-protectant and then flash-cooled in a nitrogen-gas stream. All data sets were processed by *mosflm6.2.3* program and scaled using CCP4 program *SCALA* [9].

Statistics of data collection and processing are shown in Table 1. Both Crystals of rAAL and rAAL–Lac belong to space group $P2_1$. The unit cell parameters are $a = 53.20$ Å, $b = 66.01$ Å, $c = 57.86$ Å, $\beta = 109.38$ and $a = 53.38$ Å, $b = 66.29$ Å, $c = 58.02$ Å, $\beta = 109.03$ for rAAL and rAAL–Lac, respectively. These results indicate that lactose exerts little effect on the growth of crystals. Assuming that the Matthews coefficient, V_m [10], is 2.67 Å^3 Da^{-1} for rAAL, there are two molecules in the asymmetric unit with a solvent content of 53.9%. Thus, the dimeric state in one asymmetric unit appears to be the active unit of AAL. The crystals of rAAL and rAAL–lactose can diffract to 1.6 Å and 1.9 Å resolutions, respectively, under the experimental conditions (Table 1). On the in-house X-ray source as used for rAAL crystals, the crystals of rAAL–lactose can also diffract to 1.65 Å resolution (data not shown). This indicates that the inclusion of lactose has considerably improved the crystal quality, which provides a clue to show that lactose may have been bound to rAAL. This paper shows the perfect crystallographic property of recombinant AAL, which has found a sound basis for 3D structure determination and in turn for in-depth study of the structure–function relationship of the antitumour lectin AAL. In fact, the structural determinations with above data are in progress now.

4. Genebank and Swiss-port accession number

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.

Table 1
X-ray data-collection statistics

Data set	rAAL	rAAL–Lac
X-ray source	Cu Kα	PF (BL6A)
Wavelength (Å)	1.54	0.98
Temperature	85 K	90 K
Space group	P 2_1	P 2_1
Unit cell parameters	$a = 53.20$ Å	$a = 53.38$ Å
	$b = 66.01$ Å	$b = 66.29$ Å
	$c = 57.86$ Å	$c = 58.02$ Å
	$\beta = 109.38°$	$\beta = 109.03°$
Resolution range (Å)	24–1.9	21–1.6
Highest resolution range (Å)	2.00–1.90	1.66–1.60
Observed reflections	117,508	186,906
Unique reflections	29,879	50,373
Completeness (%)	100 (100)	100 (100)
Mean I/σ (I)	10.7 (3.6)	8.0 (3.2)
R_{sym} (%)[a]	5.5 (20.3)	5.1 (22.6)

Values in parentheses are for outer (highest) resolution shell.
[a] $R_{sym} = \sum (| I | - | < I > |) / \sum | I |$.

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References

[1] H.X. Wang, T.B. Ng, W.K. Liu, V.E.C. Ooi, S.T. Chang, Polysaccharide–Peptide complexes from the cultured mycelia of the mushroom coriolus versicolor and their culture medium activate mouse lymphocytes and macrophages, Int. J. Biochem. Cell Biol. 28 (1996) 601–607.

[2] T. Mizuno, M. Ando, R. Sugie, H. Ito, K. Shimura, T. Sumiya, A. Matsuura, Antitumor activity of some polysaccharides isolated from an edible mushroom, Ningyotake, the fruiting body and the cultured mycelium of *Polyporus confluens*, Biosci. Biotechnol. Biochem. 56 (1992) 34–41.

[3] L.G. Yu, D.G. Fernig, M.R. White, D.G. Spiller, P. Appleton, R.C. Evans, I. Grierson, J.A. Smith, H. Davies, O.V. Gerasimenko, O.H. Petersen, J.D. Milton, J.M. Rhodes, Edible mushroom (*Agaricus bisporus*) lectin, which reversibly inhibits epithelial cell proliferation, blocks nuclear localization sequence-dependent nuclear protein import, J. Biol. Chem. 274 (1999) 4890–4899.

[4] T. Kazutaka, A. Yutaka, O. Shoji, M. Takashi, I. Mamoru, Isolation of a novel collagen-binding protein from the mushroom, hypsizigus marmoreus, which inhibits the Lewis lung carcinoma cell adhesion to type IV collagen, J. Biol. Chem. 270 (1995) 1481–1484.

[5] Y. Koyama, Y. Katsuno, N. Miyoshi, S. Hayakawa, T. Mita, H. Muto, S. Isemura, Y. Aoyagi, M. Isemura, Apoptosis induction by lectin isolated from the mushroom *Boletopsis leucomelas* in U937 cells, Biotechnol. Biochem. 66 (2002) 784–789.

[6] C. Zhao, H. Sun, X. Tong, Y. Qi, An antitumour lectin from the edible mushroom *Agrocybe aegerita*, Biochem. J. 374 (2003) 321–327.

[7] H. Sun, C. Zhao, X. Tong, Y. Qi, A lectin with mycelia differentiation and antiphytovirus activities from the edible mushroom *Agrocybe aegerita*, J. Biochem. Mol. Biol. 36 (2003) 214–222.

[8] J. Jancarik, S.-H. Kim, Sparse matrix sampling: a screening method for crystallization of proteins, J. Appl. Crystallogr. 24 (1991) 409–411.

[9] Collaborative Computational Project Number 4, The CCP4 suite: programs for protein crystallography, Acta Crystallogr., D 50 (1994) 760–763.

[10] B.W. Matthews, Solvent content of protein crystals, J. Mol. Biol. 33 (1968) 491–497.