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# Crystallization and preliminary crystallographic studies of AAL-2, a novel lectin from *Agrocybe aegerita* that binds nonreducing terminal *N*-acetylglucosamine

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AAL-2 is a recently discovered lectin from the mushroom *Agrocybe aegerita* that specifically recognizes nonreducing terminal acetylglucosamine (GlcNAc) and that could be used as a probe in studies of protein O-linked *β*-*N*-acetylglucosamination (O-GlyNAcylation). In order to illustrate the mechanism of how this protein specifically recognizes nonreducing terminal GlcNAc and to evaluate the efficacy of AAL-2 as a macromolecular probe in O-GlyNAcylation studies, expression and crystallization studies of AAL-2 were performed and a diffraction data set was collected to 2.0 Å resolution. Preliminary crystallographic studies revealed that the AAL-2 crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a$ = 52.60, $b$ = 111.70, $c$ = 135.97 Å.

## 1. Introduction

O-GlyNAcylation is a protein post-translational modification in which Thr/Ser residues are glycosylated with O-linked *β*-*N*-acetylglucosamine (O-GlcNAcylated). O-GlyNAcylation is ubiquitous and plays essential roles in processes such as transcription regulation, protein expression, turnover and trafficking, stress survival and the cell cycle by interplaying with protein O-phosphorylation (Wells *et al.*, 2001; Love & Hanover, 2005; Hart *et al.*, 2007, 2011). O-GlyNAcylation may also play roles in the pathogenesis of diabetes and neurology disorders including Parkinson's disease (Marshall *et al.*, 1991; McClain *et al.*, 2002; Liu *et al.*, 2004). More than 500 proteins within the cytoplasm or nucleoplasm have been identified to be O-GlcNAcylated. To date, the best established ways of detecting O-GlyNAcylation utilize GlcNAc-specific lectins (where GlcNAc is *N*-acetylglucosamine). For instance, wheat germ agglutinin WGA (Bains *et al.*, 1992; Muraki *et al.*, 2002; Shen *et al.*, 2011) and fungal lectin GSL-II (*Griffonia simplicifolia* lectin-II) (Nakamura-Tsuruta *et al.*, 2006) with GlcNAc-binding activity have been extensively used as the tools in biochemical and biomedical research, but the affinities and specificities of these lectins toward O-GlcNAc are low, and the lack of highly specific and effective probes has hindered research on O-GlyNAcylation (Zachara *et al.*, 2004).

Recently, a novel lectin, AAL-2 (UniProt accession No. H6CS64), was isolated from the fruiting body of *Agrocybe aegerita* and was found to bind GlcNAc. AAL-2 showed concentration-dependent binding and a very well defined specificity for nonreducing terminal GlcNAc moieties that are either alpha- or beta-linked, with a unique glycan recognition pattern toward GlcNAc-(Gal-GlcNAc)$_{1–3}$ out of 465 tested glycans (Jiang *et al.*, 2012). AAL-2 was found to have a higher affinity for GlcNAc than did WGA and GSL-II; it thus could be used as a probe for detecting terminal nonreducing GlcNAc. To illustrate the structural basis of the glycan specificity of AAL-2 and its unique glycan-recognizing patterns compared with other GlcNAc-binding lectins, we embarked on the crystal structure analysis of AAL-2. As the first step, here we report the cloning, expression, purification, crystallization and preliminary crystallographic analysis of recombinant AAL-2 (rAAL-2). Based on these results, the structural characterizations of AAL-2 and its complexes with GlcNAc will help illustrate the mechanism of AAL-2 in recognizing nonreducing terminal GlcNAc and provide a solid structural basis to apply this novel fungal lectin in studies of O-GlyNAcylation.

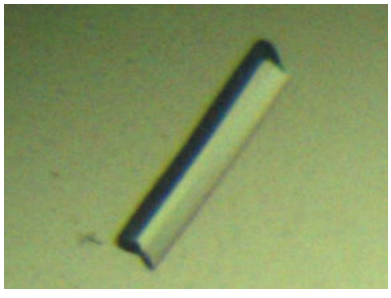

**Table 1**
Data-collection and refinement statistics for rAAL-2.

Values in parentheses are for the outermost resolution shell.

| | |
|---|---|
| Space group | $P2_12_12_1$ |
| Unit-cell parameters | |
| *a* (Å) | 52.60 |
| *b* (Å) | 111.70 |
| *c* (Å) | 135.97 |
| Wavelength (Å) | 1.5418 |
| Subunits in asymmetric unit | 2 |
| Resolution range (Å) | 51.66–2.00 (2.11–2.00) |
| $R_{\rm merge}$† (%) | 5.7 (27.6) |
| Average $I/\sigma(I)$ | 16.8 (3.8) |
| Completeness (%) | 95.7 (87.9) |
| Multiplicity | 5.0 (3.0) |

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th measurement of reflection *hkl* and $\langle I(hkl)\rangle$ is the mean value of $I_i(hkl)$ for all *i* measurements.

## 2. Materials and methods

### 2.1. Cloning, expression and purification of rAAL-2

The *aal-2* gene was amplified from *A. aegerita* and cloned into pET30a expression vector *via* introduced *Nde*I/*Hin*dIII restriction sites, without any tag. *Escherichia coli* host strain BL21 (DE3) competent cells were transformed with the recombined plasmid and grew in 800 ml LB medium at 310 K with 50 µg ml$^{-1}$ kanamycin. When the $OD_{600}$ of the culture reached 0.8, the cells were induced with 1 m*M* IPTG (isopropyl *β*-D-1-thiogalactopyranoside) at 293 K overnight and were then harvested by centrifugation at 4670*g* for 30 min at 277 K. The cell pellets were then resuspended in 15 ml lysis buffer (50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl) and lysed by sonication on ice. The lysed sample was centrifuged at 14 000*g* for 30 min at 277 K; the supernatant was applied onto a GlcNAc-coupled Sepharose 6B affinity column (this column was prepared by coupling GlcNAc with commercial Sepharose 6B resin, as described in detail by Jiang *et al.*, 2012) and then eluted with 200 m*M* GlcNAc in elution buffer (50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl and 200 m*M* GlcNAc). After desalting and concentration using Amicon Ultra-15 centrifugal devices with a 10 kDa cutoff membrane (Millipore), the elution sample from the affinity column was loaded onto a HiTrap SP XL column (GE Healthcare Life Science) at a flow rate of 2 ml min$^{-1}$ for a gradient elution with 0–1 *M* NaCl for further purification and to remove GlcNAc from the protein. The elution peak was at 11.5–29.1 mS cm$^{-1}$. The collected peak fractions were concentrated to about 0.5 ml and applied onto a Superdex 200 10/300 GL gel-filtration column (Amersham Biosciences) equilibrated with 50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl at a flow rate of 0.5 ml min$^{-1}$. The peak fractions from the size-exclusion column corresponding to rAAL-2 were pooled and concentrated prior to crystallization (Fig. 1). The purified rAAL-2 was stored at 277 K for crystallization experiments.

### 2.2. Crystallization

The concentration of rAAL-2 used in crystallization was approximately 15 mg ml$^{-1}$. Initial screening was carried out using the commercial kits Index, Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2, PEGRx1, PEGRx2, SaltRx1 and SaltRx2 (Hampton Research, California, USA). Crystals were obtained by the hanging-drop vapour-diffusion method, in which protein and reservoir solutions were mixed in equal volumes (1 µl) and equilibrated against 400 µl reservoir solution at 293 K. After the crystals were observed, optimization of crystal conditions was performed by varying the precipitant concentrations, salt species and the pH of buffers.

### 2.3. Data collection

X-ray diffraction data of rAAL-2 were collected on an in-house FR-E Bluemax/R-AXIS IV++/Varimax HF (Rigaku) diffractometer. Crystals were directly mounted on cryoloops (Hampton Research) from the crystallization drops without any cryoprotection procedures and flash-cooled in a nitrogen-gas stream at 95 K. A native data set was collected to 2.00 Å resolution with a total of 180 images using an oscillation of 1° per image and a wavelength of 1.5418 Å. *MOSFLM* v.7.0.4 (Leslie, 2006) and *SCALA* v.6.0 from the *CCP*4 v.6.0.2 program suite (Winn *et al.*, 2011) were used for the indexing, integration and scaling of the diffraction data, respectively.

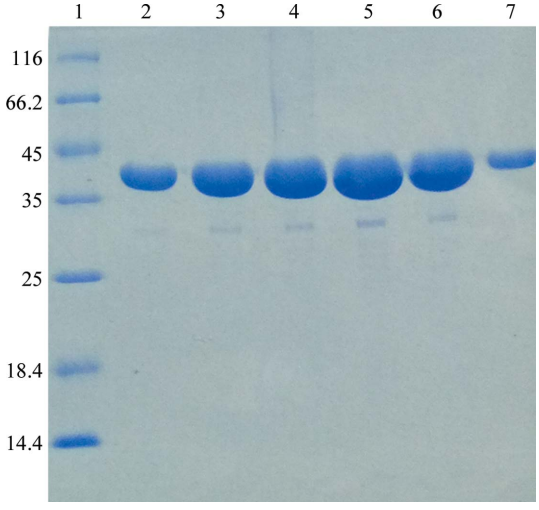


**Figure 1**
Coomassie Blue-stained SDS–PAGE gel (15%) showing the purified rAAL-2 expressed in *E. coli*. Lane 1, molecular-weight (MW) markers (labelled in kDa); lanes 2–7, elution fractions from Superdex 200 10/300 GL gel-filtration column.

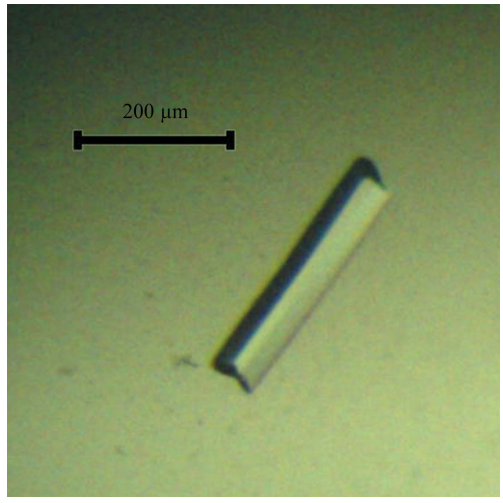


**Figure 2**
An optimized crystal of rAAL-2 obtained under the condition 30%(*w*/*v*) PEG 8000, 0.2 *M* sodium acetate, 0.1 *M* sodium cacodylate pH 6.4 at 295 K.

**Table 2**
Matthews coefficient and solvent content of the crystal calculated using the program *MATTHEWS_COEF* in the *CCP*4 suite.

The estimated molecular weight was 43 176 Da.

| Molecules in asymmetric unit | Matthews coefficient (Å$^3$ Da$^{-1}$) | Solvent content (%) | *P* (2.00) | *P* (tot) |
|---|---|---|---|---|
| 1 | 4.54 | 72.92 | 0.00 | 0.02 |
| 2 | 2.27 | 45.84 | 0.99 | 0.98 |
| 3 | 1.51 | 18.76 | 0.00 | 0.00 |

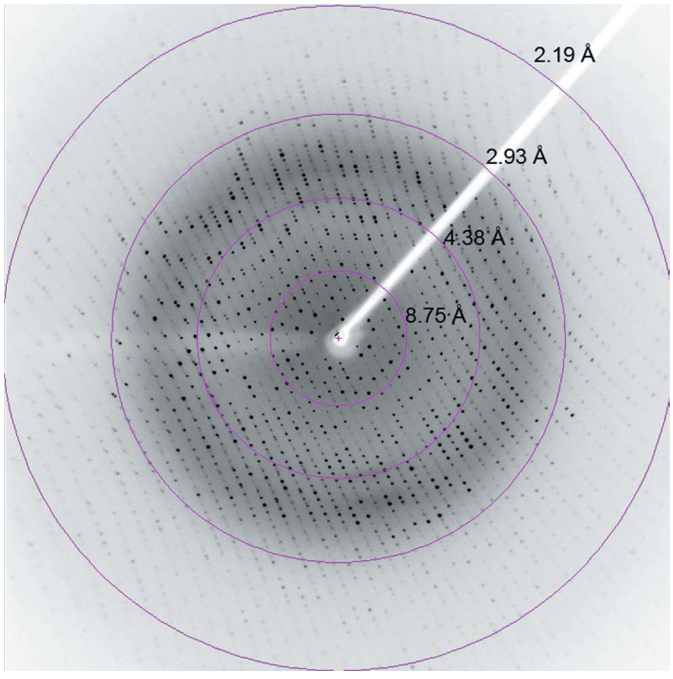


**Figure 3**
A diffraction image of a crystal of rAAL-2 obtained on an in-house Rigaku R-AXIS IV$^{++}$ image plate.

## 3. Results

The crystals of rAAL-2 were obtained in a condition consisting of 30%(*w*/*v*) PEG 8000, 0.2 *M* sodium acetate, 0.1 *M* sodium cacodylate pH 6.4 at 295 K (Fig. 2). Crystals appeared in the drops within 48 h with dimensions of about 0.05 × 0.05 × 0.3 mm.

The statistics of data collection and preliminary crystallographic analysis are summarized in Table 1. The crystal diffracted to a resolution of 2.0 Å (Fig. 3) and belonged to space group $P2_12_12_1$, with unit-cell parameters $a$ = 52.60, $b$ = 111.70, $c$ = 135.97 Å. The asymmetric unit was estimated to contain two rAAL-2 molecules, with a Matthews coefficient of 2.27 Å$^3$ Da$^{-1}$ and a solvent content of 45.84% (Matthews, 1968; Table 2). No noncrystallographic symmetry was observed in the self-rotation function analysis (figure not shown), even though we cannot exclude the possibility that there is a noncrystallographic twofold axis obscured by a parallel crystallographic twofold axis.

The successful crystallization of rAAL-2 and the collection of high-quality diffraction data established a sound basis for the determination of the crystal structures of rAAL-2 and its complex with GlcNAc in future work. We performed a sequence-homology *BLAST* search at NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and identified a protein PVL with 60% sequence identity that has been characterized by X-ray crystallography (Cioci *et al.*, 2006). The structure of PVL (PDB entry 2bwr; Cioci *et al.*, 2006) may serve as a model for the structure determination of rAAL-2 using the molecular-replacement method. We are in the process of solving the crystal structures of AAL-2 and its complexes with various GlcNAcs. These studies will hopefully reveal the binding specificity of rAAL-2 for nonreducing terminal *N*-acetylglucosamine and provide a structural basis for the development of novel and efficient macromolecule probes targeting the distinctive carbohydrate markers with terminal nonreducing GlcNAc and contribute to the research on protein O-GlyNAcylation.

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