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Cloning, expression, purification and crystallographic studies of galectin-11 from domestic sheep (*Ovis aries*)

Dhanasekaran Sakthivel,[a,b] Dene Littler,[b] Adam Shahine,[b] Sally Troy,[b] Matthew Johnson,[b] Jamie Rossjohn,[b,c] David Piedrafita[a] and Travis Beddoe[d]*

[a]School of Applied Science and Engineering, Federation University, Churchill, Victoria 3842, Australia, [b]Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3800, Australia, [c]Institute of Infection and Immunity, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, Wales, and [d]Department of Animal, Plant and Soil Science and Centre for AgriBioscience (AgriBio), La Trobe University, Melbourne, Victoria 3086, Australia. *Correspondence e-mail: t.beddoe@latrobe.edu.au

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Galectins are an evolutionarily conserved family of proteins that translate glycan recognition into cellular effects. Galectin-11 is a unique member of the galectin family that is only expressed in ruminants such as sheep, goat and cattle and that plays a critical role in several important biological processes, such as reproduction and parasite-mediated innate immune responses. Currently, these two areas are of major importance for the sustainability of ruminant livestock production. Despite the emerging biological significance of galectin-11, no structural information is available. It is expected that structural studies will unravel the functional mechanisms of galectin-11 activity. Here, the expression, purification and crystallization of the ruminant-specific galectin-11 from domestic sheep and the collection of X-ray data to 2.0 Å resolution are reported.

1. Introduction

The sustainability of ruminant livestock production is being threatened by several factors, including climate change, disease control and reproductive success (Singh *et al.*, 2012). Considerable research has led to the identification of several proteins that play important roles in immune responses and reproductive processes. One such protein is termed galectin-11 and belongs to the evolutionarily conserved galectin family that modulates various cellular communications, cell adhesion and pathogen recognition (Vasta, 2012). Galectins are characterized by the presence of a conserved carbohydrate-recognition domain (CRD) and exhibit a preference towards β-galactosides. While other galectins appear to be highly conserved in all higher vertebrates, galectin-11 has only been found in ruminants such as sheep, goats and cattle (Dunphy *et al.*, 2000). Galectin-11 has the predicted CRD as well as LDV and RGD recognition sequences for proposed integrin binding (Farmer *et al.*, 2008). Integrins recognize the RGD and LDV motifs within ligands such as galectin-11 and the binding mediates various cell processes (D'Souza *et al.*, 1991). Integrin binding by galectin-11 in the uterus is important for trophectoderm cell migration, attachment and activation, which are critical for proper blastocyst elongation and implantation (Farmer *et al.*, 2008). Apart from reproduction, galectin-11 also has a proposed role in immune responses against parasites (Meeusen *et al.*, 2005). Expression of galectin-11 in the abomasal mucosa leads to the development of a mucosal microenvironment that discourages parastic larval establishment in the abomasal crypts (Jackson *et al.*, 2004). It has been

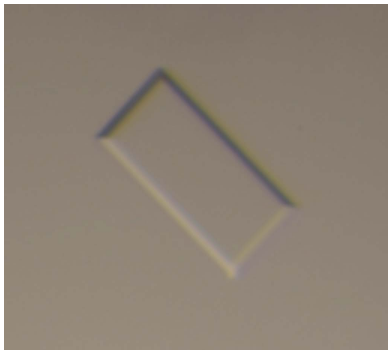

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Table 1
Macromolecule-production information.

Source organism	*O. aries*
DNA source	*O. aries* cDNA
Forward primer	5′-CAGGGACCCGGTATGGACTCCTTGCCG-3′
Reverse primer	5′-CGAGGAGAAGCCCGGTTATAACGTATCCACT-3′
Cloning vector	pET-28 plasmid
Expression vector	pET-28 plasmid
Expression host	*E. coli* BL21 (DE3)
Complete amino-acid sequence of the full-length construct produced (galectin-11$^{1–140}$)	MAHHHHHHSAALEVLFQGPGMDSLPNPYLQSVSL-TVCYMVKIKANLLSPFGKNPELQVDFGTGTGQ-GGDIPFRFWYCDGIVVMNTLKDGSWGKEQKLH-TEAFVPGQPFELQFLVLENEYQVFVNNKPICQ-FAHRLPLQSVKMLDVRGDIVLTSVDTL
Amino-acid sequence of crystallized galectin-11 after HCV-3C protease treatment (galectin-11$^{18–140}$)	GPGMDSLPNPYLQSVSLTVCYMVKIKANLLSPFG-KNPELQVDFGTGTGQGGDIPFRFWYCDGIVVM-NTLKDGSWGKEQKLHTEAFVPGQPFELQFLVL-ENEYQVFVNNKPICQFAHRLPLQSVKMLDVRG-DIVLTSVDTL

proposed that galectin-11 changes the mucosal micro-environment by increasing the viscosity *via* carbohydrate binding (Meeusen *et al.*, 2005). In spite of the emerging biological significance of galectin-11 in livestock production, no structural study on this protein has been reported. This lack of structural knowledge has hampered research efforts in the development of galectin-11 for the treatment of parasitic diseases or for increasing reproductive success. Here, we report our progress on the expression, purification and preliminary crystallographic study of galectin-11.

2. Materials and methods

2.1. Macromolecule production

2.1.1. Cloning. The *Ovis aries* galectin-11-encoding sequence was amplified using cDNA prepared from sheep abomasum tissue by polymerase chain reaction (PCR) with galectin-specific primers (Table 1) using Phusion High-Fidelity DNA polymerase (New England Biolabs). The galectin-11 gene was cloned into a modified pET-28 vector (Luna-Vargas *et al.*, 2011), giving a protein with an HRV 3C-cleavable N-terminal hexahistidine tag using a ligation-independent cloning method as described in Doyle (2005) (Table 1). The ligated plasmid was transformed into *Escherichia coli* XL-1 Blue cells and plated onto a Luria–Bertani (LB) agar plate containing kanamycin (50 µg ml^{-1}). The clone obtained was confirmed by DNA sequencing.

2.1.2. Recombinant protein production of galectin-11. Recombinant protein expression of full-length galectin-11 (encoding amino acids 1–140; galectin-11$^{1–140}$) was performed in the BL21 (DE3) *E. coli* strain (Table 1). A starter culture was grown from a single colony overnight in 100 ml LB medium containing kanamycin (50 µg ml^{-1}). The starter culture was used at 1:100 dilution to inoculate 800 ml fresh LB medium containing 50 µg ml^{-1} kanamycin and grown at 310 K until the OD_{600} reached 0.6. Expression of galectin-11$^{1–140}$ was induced with 0.5 m*M* IPTG and the cells were allowed to grow for a further 16 h at 291 K. The cells were collected following centrifugation at 4000*g* for 30 min at 277 K and were stored at 193 K.

2.1.3. Purification of recombinant protein. The frozen cell pellet was thawed on ice and resuspended in 20 ml lysis buffer [20 m*M* Tris–HCl, 150 m*M* NaCl, 0.1%(*v*/*v*) Triton X-100] per gram in the presence of 0.2 µ*M* phenylmethanesulfonylfluoride. The bacterial suspension was lysed by sonication, the cell debris was removed by centrifugation and fine particles were filtered. The crude *E. coli* extract was applied onto 5 ml nickel Sepharose beads (GE Healthcare) under native conditions. The unbound proteins was washed with three column volumes of wash buffer [50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 5%(*v*/*v*) glycerol, 30 m*M* imidazole] and eluted with 15 ml elution buffer [50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 5%(*v*/*v*) glycerol, 300 m*M* imidazole] and collected as 2 ml fractions. The eluted protein fractions were analysed by SDS–PAGE.

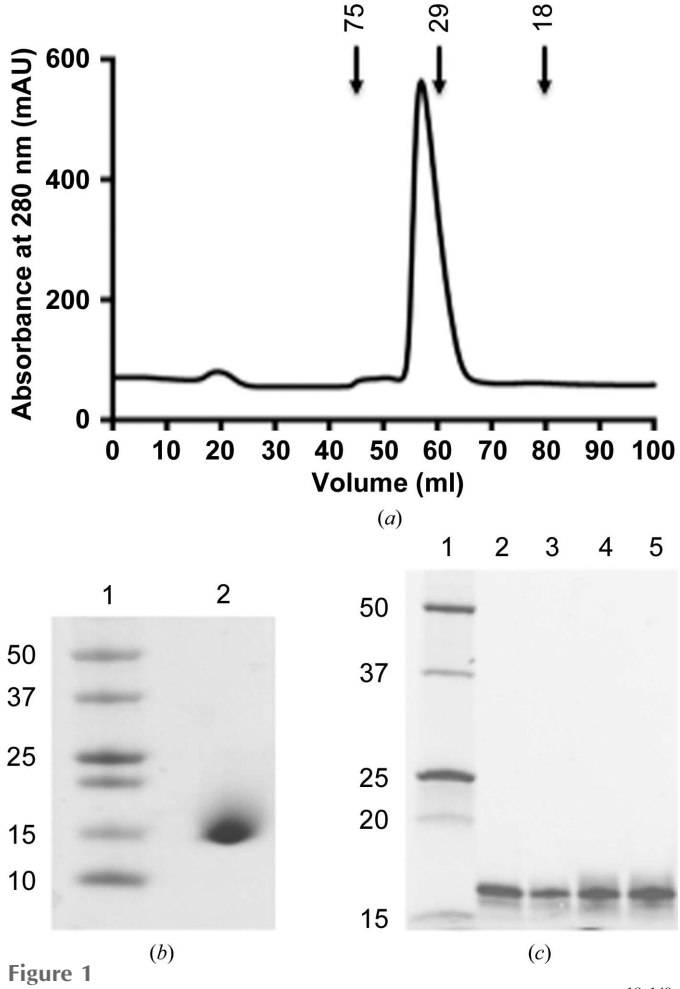

Figure 1
(*a*) Size-exclusion chromatography trace of recombinant galectin-11$^{18–140}$. Arrows indicate the elution volumes of proteins of known molecular weight (labelled in kDa). (*b*) SDS–PAGE analysis of purified protease-treated recombinant galectin-11$^{18–140}$ (lane 2). Molecular weights (lane 1) are indicated on the left in kDa. (*c*) Recombinant galectin-11$^{1–140}$ is shown to be functional by elution from a galactose Sepharose column with different sugars. Lanes 2, 3, 4 and 5 show galectin-11$^{1–140}$ eluted from a column with galactose, lactose, mannose and fructose, respectively. The positions of molecular-weight markers (labelled in kDa) are shown in lane 1.

Fractions containing galectin-11$^{1–140}$ were pooled and concentrated to 5 ml using Amicon ultracentrifugal filters (3000 kDa molecular-weight cutoff; Millipore). After the addition of 50 m*M* arginine and 50 m*M* glutamic acid, the purified protein fractions were concentrated to 5 mg ml^{-1} and the hexahistidine tag was cleaved by incubation at 277 K overnight with HRV-3C protease in a 1:100 ratio (protease: protein). Uncleaved protein was purified using nickel Sepharose beads and the cleaved protein was confirmed by Western blotting with an anti-hexahistidine antibody. The cleaved galectin-11 (containing amino acids 18–140; galectin-11$^{18–140}$; Table 1) was further purified by size-exclusion chromatography with a Superdex S75 16/60 gel-filtration column (GE Healthcare Life Sciences) equilibrated in TBS (10 m*M* Tris–HCl, 125 m*M* NaCl, 5 m*M* TCEP [tris(2-carboxyethyl)-phosphine] and 10%(*v*/*v*) glycerol using an ÄKTA Basic fast protein liquid-chromatography (FPLC) system (Fig. 1*a*). The molecular weight, purity and identity of the protease-treated galectin-11$^{18–140}$ were confirmed by SDS–PAGE (Fig. 1*b*), Western blotting with anti-Gal-11 antibody (Dunphy *et al.*, 2000) and N-terminal sequencing. The purified protein was concentrated to 5.0 mg ml^{-1} and stored at 277 K for use in crystallization trials. The concentration of galectin-11$^{18–140}$ was determined spectrophotometrically (NanoDrop 1000, Thermo Scientific) at 280 nm and calculated using an extinction coefficient of 16 960 M^{-1} cm^{-1} as determined using the *ProtParam* tool (http://web.expasy.org/protparam)

2.1.4. Sugar binding by recombinant galectin-11. To determine whether recombinant galectin-11$^{1–140}$ is functional, the following procedure was followed. 500 µl recombinant galectin-11$^{1–140}$ (2.5 mg ml^{-1}) was incubated with 200 µl galactose Sepharose (Pierce) equilibrated in sugar-binding buffer (20 m*M* Tris–HCl pH 8.2, 200 m*M* NaCl, 5 m*M* β-mercaptoethanol) overnight at 277 K using an end-to-end shaker. Unbound protein was washed with three column volumes of sugar-binding buffer and eluted with 2 ml 1 *M* galactose, lactose, mannose and fructose in sugar-binding buffer. The eluted protein was analyzed by SDS–PAGE and Coomassie Blue staining (Fig. 1*c*).

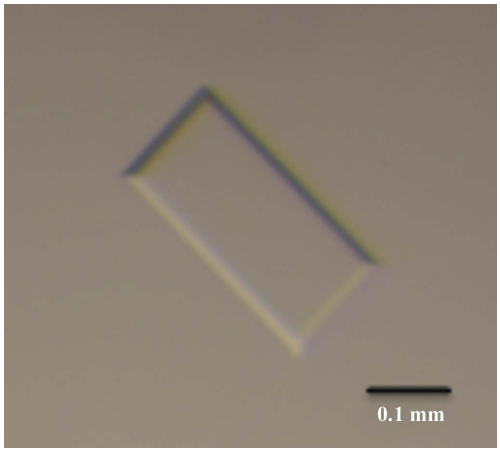

Figure 2
Crystal of galectin-11$^{18–140}$ from *O. aries*. The approximate dimensions of the crystal used for data collection were 1.8 × 0.8 × 0.1 mm. The scale bar represents 0.1 mm.

Table 2
Crystallization.

Method	Hanging drop
Plate type	24-well Linbro plate
Temperature (K)	293
Protein concentration (mg ml^{-1})	5
Buffer composition of protein solution	10 m*M* Tris–HCl, 125 m*M* NaCl, 5 m*M* TCEP, 10%(*v*/*v*) glycerol
Composition of reservoir solution	2%(*v*/*v*) Tacsimate pH 7.0, 0.1 *M* HEPES pH 7.5, 20%(*w*/*v*) PEG 3350
Volume and ratio of drop	1 µl protein solution:1 µl reservoir solution
Volume of reservoir (µl)	500

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	MX1, Australian Synchrotron
Wavelength (Å)	0.9537
Temperature (K)	100
Detector	ADSC Quantum 210r CCD
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	2
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 96.2, b = 127.5, c = 141.4, $\alpha = \beta = \gamma$ = 90.0
Resolution range (Å)	38.86–2.00 (2.03–2.00)
Total No. of reflections	915448 (35018)
No. of unique reflections	115982 (5501)
Completeness (%)	98.6 (95.4)
$\langle I/\sigma(I)\rangle$†	6.6 (1.9)
$R_{\rm p.i.m.}$‡ (%)	10.8 (59.3)
Wilson B factor (Å^2)	14.26

† I is the integrated intensity and $\sigma(I)$ is the estimated standard deviation of that intensity. ‡ $R_{\rm p.i.m.} = \sum_{hkl}\{1/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the scaled intensity of the ith measurement and $\langle I(hkl)\rangle$ is the mean intensity for that reflection.

2.2. Crystallization

The initial crystallization conditions of protease-treated galectin-11$^{18–140}$ (Table 1) were screened using 288 conditions from commercially available kits from Hampton Research (Crystal Screen, Crystal Screen 2 and PEG/Ion) and Qiagen (The JCSG+ Suite) with a CrystalMation integrated robotic workstation (Rigaku). Over 30 different conditions produced crystals of galectin-11$^{18–140}$ with various morphologies within 4 d. The crystals with the best morphology were obtained in 24-well Linbro plates (Hampton Research) with drops consisting of 1 µl protein solution and 1 µl precipitant solution and a reservoir volume of 500 µl (Fig. 2). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

Crystals were soaked for 30 s in cryoprotectant solution consisting of 20%(*v*/*v*) glycerol, 2%(*v*/*v*) Tacsimate pH 7.0, 0.1 *M* HEPES–HCl pH 7.5, 20%(*w*/*v*) PEG 3350 before cooling at 100 K in a stream of nitrogen gas. A complete data

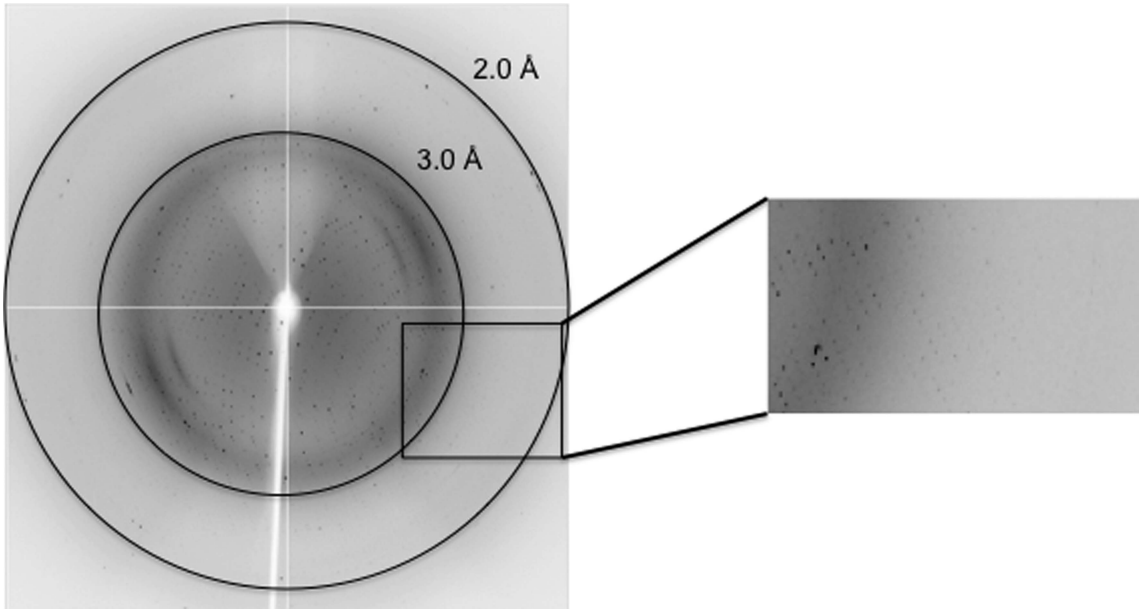

Figure 3
Example of a diffraction image from a galectin-11$^{18–140}$ crystal obtained on beamline MX1 at the Australian Synchrotron. The outer black circle corresponds to a resolution of 2.0 Å.

set was collected from a single crystal on the MX1 beamline at the Australian Synchrotron (Cowieson *et al.*, 2015) using an ADSC Quantum 210r CCD detector (Fig. 3). The data were processed with *MOSFLM* (Leslie & Powell, 2007) and various programs from the *CCP*4 program suite (Winn *et al.*, 2011). The final statistics of data collection and processing are summarized in Table 3.

3. Results and discussion

The coding region of sheep galectin-11 was successfully cloned by ligation-independent cloning and expressed in *E. coli* strain BL21 (DE3). The recombinant galectin-11 was purified purified to homogeneity by two chromatographic steps: nickel-affinity chromatography and His-tag removal by HRV-3C protease followed by size-exclusion chromatography. The major elution volume peak in the gel-filtration column corresponds to a dimer in solution with an approximate molecular weight of 30 kDa (Fig. 1*a*). Purified protein was analysed by SDS–PAGE (Fig. 1*b*) and Western blotting to confirm its identity and purity. This expression and purification strategy routinely produced yields of 9 mg per litre of culture. Recombinant galectin-11$^{1–140}$ was successfully eluted from galactose Sepharose beads by various sugars, suggesting that recombinant galectin-11 is functional and that the glycan specificity of galectin-11 may be very broad (Fig. 1*b*). Crystals of galectin-11$^{18–140}$ were initially obtained by the sitting-drop vapour-diffusion method and were optimized manually using the hanging-drop vapour-diffusion method. Crystals of galectin-11$^{18–140}$ were obtained in many crystallization conditions; however, a complete diffraction data set was collected at 100 K from crystals grown in 2%(*v*/*v*) Tacsimate pH 7.0, 0.1 *M* HEPES–HCl pH 7.3, 14%(*w*/*v*) PEG 3350 (Fig. 3). The diffraction quality of the crystals was strictly dependent on maintaining the recombinant galectin-11 in a reduced state. Preliminary crystallographic analysis indicated that the crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 96.2, b = 127.5, c = 141.4 Å, α = 90.0, β = 90.0, γ = 90.0°. Based on Matthews coefficient calculations, 12 molecules of galectin-11 (52% solvent content) could be accommodated in the asymmetric unit, with an acceptable V_M of around 2.58 Å^3 Da^{-1} (Matthews, 1968). The data-collection and processing statistics are summarized in Table 3. Structure determination by means of molecular replacement was successful using the known structure of human galectin-10 (Charcot–Leyden crystal protein; PDB entry 1g86; Ackerman *et al.*, 2002), which shares 40% sequence identity with galectin-11. Structure verification and model rebuilding are currently in progress, as well as co-crystallization experiments with the identified sugars.

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