Expression, Purification, and Applications of the Recombinant Lectin PVL from *Psathyrella velutina* Specific for Terminal N-Acetyl-Glucosamine

Oriane Machon, Annabelle Varrot

► To cite this version:

Oriane Machon, Annabelle Varrot. Expression, Purification, and Applications of the Recombinant Lectin PVL from *Psathyrella velutina* Specific for Terminal N-Acetyl-Glucosamine. *Lectin Purification and Analysis. Methods in Molecular Biology*, pp.421-436, 2020, 10.1007/978-1-0716-0430-4_41 . hal-02928046

HAL Id: hal-02928046

<https://hal.science/hal-02928046>

Submitted on 30 Sep 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Chapter 41

Expression, Purification and Applications of the Recombinant Lectin PVL from *Psathyrella velutina* Specific for Terminal *N*-Acetyl-Glucosamine.

Oriane Machon and Annabelle Varrot*

Univ. Grenoble Alpes, CNRS, CERMAV, 38000 Grenoble, France

*Corresponding author: Annabelle Varrot, annabelle.varrot@cermav.cnrs.fr

Running Head: Expression, purification and applications of the PVL lectin

Abstract

The lectin PVL from the mushroom *Psathyrella velutina* is the founding member of novel family of fungal lectins. It adopts a seven bladed β -propeller presenting six binding sites specific for the recognition of non-reducing terminal *N*-acetyl-glucosamine (GlcNAc). The latest can be mainly found in glycoconjugates presenting truncated glycans where aberrant β -GlcNAc terminated glycans represent tumor markers. It can also be found in *O*-GlcNAcylated proteins where disruption of the *O*-GlcNAcylation homeostasis is associated with many physiopathological states. The recombinant PVL lectin proved to be a very powerful tool for labelling terminal GlcNAc antigens displayed by extracellular glycoconjugates but also by *O*-GlcNAcylated proteins found in the cytoplasm and nucleus. This chapter will describe how to produce and purify recombinant PVL and several applications for rPVL as probe for the detection of terminal *O*-GlcNAc.

Keywords: Terminal *O*-GlcNAc, tumor markers, *O*-GlcNAcylation detection, lectin probe, ELLA.

1 Introduction

Glycosylation is one of the most prominent and diverse form of post-translational modification (PTM) of proteins and lipids. It is performed by an incredibly complex and dynamic biosynthetic machinery affected by scores of genetic and environmental factors. It results in a non-templated glycome, where glycans have a key role on mediating or modulating the function of their carriers. The glycome carries cell signatures of health and disease as glycosylation defects or alterations are commonly connected with physiopathological states such as cancers, chronic inflammatory diseases or infections [1].

N-Acetyl-glucosamine (GlcNAc) is one of the major glycan moieties found in glycoconjugates of the extracellular matrix where it is usually β -linked and rarely exposed in terminal position on the surface of healthy

tissues except in gastric mucosa where α 1,4-GlcNAc capped mucins, secreted by gland mucous cells, are exposed [2]. Truncated *N*- and *O*-glycans and glycosphingolipids displaying aberrant non reducing terminal β -GlcNAc are considered as tumor markers: i.e. truncated core 3 *O*-glycans (GlcNAc β 1-3GalNAc α -*O*-Ser/Thr) in colorectal cancers [3]. A simple and distinct *O*-glycosylation called *O*-GlcNAcylation has been uncovered in the early 1980s [4]. It consists of the addition of a single GlcNAc to specific serine or threonine residues of cytoplasmic, nuclear and mitochondrial proteins. *O*-GlcNAcylation is ubiquitous in metazoans and widespread in living beings, where it plays a key role in the regulation of multiple cellular processes from epigenetics and stress response to cell signaling. It is a highly dynamic modification interplaying extensively with phosphorylation. Disturbance of *O*-GlcNAcylation homeostasis can be associated with the pathogenesis of many diseases such as diabetes, cancers, or Alzheimer disease (reviewed in [5, 6]). GlcNAc is a small, non-charged, and labile moiety to be readily detectable by conventional mass spectrometry and gel electrophoresis. The detection of *O*-GlcNAcylation remains challenging, and the lack of efficient tools for this has hampered both the discovery and the study of this essential modification.

Several fungal lectins highly specific for non-reducing terminal GlcNAc epitope have been isolated from mushrooms. The first one, PVL (*Psathyrella velutina* lectin), has been isolated from *Lacrymaria velutina* formally known as *P. velutina* [7]. It is also able to recognize non-reducing terminal *N*-acetylneuraminic acid (Neu5Ac) residues but with lesser affinity [8]. The resolution of PVL structure uncovered a novel family of lectins characterized by a seven bladed β -propeller fold [9]. Only three fungal members have been structurally characterized to date: PVL, AANL (*Agrocybe aegerita* lectin 2 formerly called AAL2) and PAL (*P. asperospora* lectin) [10-12]. Their structures in complex with GlcNAc gave the molecular basis for the recognition of terminal GlcNAc as well as those for terminal Neu5Ac in the case of PVL. Six binding sites in the form of a shallow pocket were identified at the interface between blades apart on the one involved in the Velcro closure of the propeller (Fig 1).

A signature motif was identified for this family (PropLec7B) that can now be used to predict new members to this family in translated genomes and sequence databases [13]. PVL and AANL can be produced in recombinant form without impairing their structure and recognition aptitudes [10, 11]. A clear avidity effect could be observed for rPVL where its affinity increases from 132 μ M for single binding event as observed for GlcNAc on PVL-coated sensor chip by surface plasmon resonance or in solution by isothermal microcalorimetry [10] to 60 nM in the case of multivalent presentation of rPVL on GlcNAc-coated SPR chips [14]. Lectins from this family present therefore great potential in biomedical applications as reagent for the detection of terminal *O*-GlcNAc and for glycoconjugates

Fig 1: Surface and cartoon representation of the seven-bladed β -propeller of recombinant PVL in complex with GlcNAc- β 1,3Gal disaccharide depicted in balls and sticks (PDB 4UP4).

separation [8, 15]. rPVL can be conjugated with different molecules (fluorophore, biotin, or peroxidation) and proved to be a very powerful probe to label both truncated glycans on human cancer cells and tissues by FACS, immunofluorescence, or histochemistry [10] and GlcNAcylated proteins using western blot [14]. rPVL can also be used in enzyme-linked lectin assay (ELLA) to detect terminal GlcNAc in glycoconjugates. In this chapter, we will detail protocols used for the expression, purification of rPVL, as well as possible conjugation and its use in different detection methods.

2 Materials

Prepare all solutions using analytical grade reagents and ultrapure water and filter on 0.22 μ M membrane before usage. Follow waste disposal regulations.

2.1 Production of rPVL

1. pET25b-rPVL plasmid encoding for the PVL protein obtained according to previously published protocol [10].
2. *Escherichia coli* TunerTM(DE3) cells (Novagen-Merck).
3. Lennox L Broth Base (LB, Invitrogen). Dissolve 20 g in 1 L of distilled water and autoclave 20 min at 121 °C.
4. 100 mg/mL of ampicillin (Amp, culture grade sodium salt) in water.
5. 1 M isopropyl- β -D-thiogalactopyranoside (IPTG).
6. 1 L polycarbonate centrifuge bottle (Nalgene) and 50 ml sterile conical centrifuge tubes.
7. Centrifuge with adapted rotors.

2.2 Purification of rPVL

1. 0.45 µm polyethersulfone (PES) syringe filter.
2. C10/10 column with flow adapter (GE Healthcare Life Sciences) or empty Econo-Pac® Chromatography Columns (Bio-Rad).
3. LNT2 affinity resin, (GLY011-Gel, Elicityl, Crolles, France).
4. Endonuclease such as Benzonase or Denarase at 250 units/µL.
5. One Shot table top cell disruptor (Constant Systems Ltd).
6. Binding buffer: 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 100 µM CaCl₂.
7. Washing buffer: 20 mM Tris-HCl pH 7.5, 1 M NaCl, and 100 µM CaCl₂.
8. Elution buffer: 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 100 µM CaCl₂ and 500 mM GlcNAc (Carbosynth).
9. 12 % SDS-PAGE gel with vertical gel electrophoresis unit and universal power supply.
10. Precision Plus Protein™ Unstained Standards (Bio-Rad Ltd).
11. Instant Blue™ (Expedeon).
12. Snakeskin dialysis tubing 3500 MWCO (Thermo Fisher Scientific).

2.3 Hemagglutination Assays (HA)

1. U-shaped 96-well plates (Nunc, Rochester NY).
2. 4% rabbit erythrocytes in 150 mM NaCl.

2.4 Affinity Measurements by Surface Plasmon Resonance (SPR)

1. Biacore X100 (GE Healthcare Life Sciences).
2. CM5 sensor chips (GE Healthcare Life Sciences).
1. Running buffer: PBS-Ca (phosphate buffered saline: 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl pH 7.4 supplemented with 100 µM CaCl₂) as running buffer. Dissolve one tablet (Sigma-Aldrich, ref P4417) in 200 mL of water.
3. Amine coupling kit (GE Healthcare Life Sciences).
4. Streptavidin from *Streptomyces avidinii* (Sigma-Aldrich) at 100 µg/mL in 10 mM sodium acetate pH 5.0 for chip immobilization.
5. 1 M ethanolamine for blocking the sensor surface.
6. β-D-GlcNAc-PAA-biotin and α-L-fucose-PAA-biotin (Lectinity, Moscow).
7. GlcNAc (Carbosynth, UK).

2.5 rPVL Labelling

1. N,N-Dimethylformamide (DMF).
2. D-salt polyacrylamide desalting column 6K (Pierce).

3. Biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester (Sigma-Aldrich) for biotinylation.
4. EZ-Link™ Plus Activated Peroxidase kit (Thermo Fisher Scientific) for coupling with horseradish peroxidase.
5. Alexa Fluor™ 488 NHS Ester (ref A20000) (Thermo Fisher Scientific) for coupling with fluorophore.

2.6 Enzyme-Linked Lectin Assays (ELLA)

1. 96-well plates Microlon® (Greiner Bio-One).
2. Biotinylated rPVL.
3. PBS buffer pH 7.4.
4. PBS-BSA: PBS supplemented with 0.1 % (w/v) Grade V bovine serum albumin (BSA, Sigma-Aldrich, ref A7030).
5. Tween 20 (Sigma-Aldrich).
6. PAA-β-N-acetylglucosamine (Lectinity, Moscow).
7. Streptavidin-peroxidase (Sigma-Aldrich, ref S5512) and 3,3',5,5'-tetramethylbenzidine (TMB, Thermo Fisher Scientific) for detection of biotinylated lectins.
8. TECAN SparkM10 plate reader.

2.7 Western Blotting of O-GlcNAcylated Proteins

1. Cell lines are obtained according to published protocol [14].
2. Lysis buffer: 10 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and protease inhibitors.
3. 10 % SDS-PAGE gel with Mini-PROTEAN® Tetra electrophoresis system, Mini Trans-Blot® Cell, and universal power supply (Bio-Rad Ltd).
4. Nitrocellulose membrane (GE Healthcare Life Sciences).
5. Ponceau S (Sigma-Aldrich).
6. Grade V BSA for membrane saturation.
7. Tris buffered saline (TBS)-Tween buffer: 15 mM Tris/HCl, 140 mM NaCl and 0.05% (v/v) of Tween 20 (Sigma Aldrich), pH 8.0.
8. HRP-labelled rPVL at 1 mg/mL in TBS.
9. Chemiluminescence imaging (Fusion Solo system, Vilber Lourmat Collegien).

2.8 Immunofluorescence Analysis on Whole Cells

1. Cells lines cultured according to recommended protocols.
2. Four-chamber culture slides.
3. Ice-cold PBS buffer pH 7.4.

4. Paraformaldehyde and BSA.
5. Alexa Fluor 488 labelled rPVL at 5 $\mu\text{g/mL}$ in PBS-BSA 2%.
6. BX41 microscope equipped with a DP-70 digital camera system (Olympus, Tokyo, Japan).
7. Pseudo-confocal microscope ApoTome equipped with AxioCam MRm (N/B).

2.9 Immuno-histochemistry

1. Fixed tissue sections.
2. Biotinylated rPVL at 0.7 $\mu\text{g/mL}$.
3. 3% (v/v) hydrogen peroxide or 5% (w/v) bovine serum albumin in PBS buffer.
4. Streptavidin at 0.1 mg/mL and biotin at 0.5 mg/mL.
5. Streptavidin-peroxidase.
6. HRP detection kit (DAB, Ventana Medical Systems or AEC, Vector Laboratories).
7. Mayer's hematoxylin solution (Merck, Whitehouse Station, NJ).
8. Sialidase (New England Biolabs) and β -D-N-acetylhexosaminidase.
9. BX41 microscope equipped with a DP-70 digital camera system (Olympus, Tokyo, Japan) or NanoZoomer slide scanner (Hamamatsu, Hamamatsu City).

3 Methods

All measurements are done at room temperature (22 °C) unless otherwise stated. The protein concentration was verified by measuring optical density at 280 nm by using a theoretical extinction coefficient of 65,890 $\text{M}^{-1}\text{cm}^{-1}$ and a molecular weight of 42974 Da and a NanoDrop 2000 (Thermo Fisher Scientific).

3.1 Production of rPVL

1. Inoculate 1 L of LB media supplemented with 100 $\mu\text{g/mL}$ of ampicillin with 30 mL of an overnight preculture of *Escherichia coli* TunerTM (DE3) cells harboring the pET25b-rPVL, plasmid and place in an incubation shaker at 160 rpm and 37 °C.
2. Grow the cells until an OD_{600} of 0.6 and switch the incubator temperature to 16 °C.
3. When the culture reached an OD_{600} of 0.8, induce rPVL expression by adding IPTG at a final concentration of 0.1 mM.
4. Culture the cells overnight for at least 16 h at 160 rpm.
5. Harvest the cells by 10 min centrifugation at 5,000 $\times g$.
6. Transfer the cells in a sterile 50 mL plastic tube and weight the cell pellet (*see Note 1*).

3.2 Purification of rPVL

1. Resuspend the cell pellet with cold binding buffer at the rate of 5 mL of binding buffer per gram of wet cells using a vortex.
2. Add 1 μ L of endonuclease and incubate on a rotating wheel for 30 min.
3. Lyse the cells using the One Shot cell disruptor at a pressure of 1.9 kbars (*see Note 2*).
4. Clarify the sample by centrifugation at 24,000 $\times$ g and 4 °C for 30 min.
5. Filter the supernatant using a 0.45 μ M PES syringe filter.
6. Load the filtered supernatant on 2 mL Toyopearl-LnT2 resin (Elicityl, Crolles France, *see Note 3*) equilibrated with binding buffer for purification by affinity chromatography and apply binding buffer until OD_{280nm} reaches a baseline. Collect fractions of 15 mL.
7. Wash unspecifically bound proteins with 10 column volumes (CVs) of washing buffer and then 5 CVs of binding buffer.
8. Elute with 10 CVs of elution buffer and collect fractions of 1 mL.
9. Analyze fractions of each step by gel electrophoresis on 12% SDS-PAGE gel after adding denaturing loading buffer to each fraction aliquots and heating at 100 °C for 5 min. Load 10 μ L of fraction as well as protein standards prior running the gel at 200 V in Tris-glycine buffer. Unpack the gel and rinse it with water prior coloration with 10 mL of Instant BlueTM for 1h under low shaking. Rinse and store the gel in water (Fig. 2).
10. Pool fractions containing >95 % pure protein and dialyze extensively against PBS buffer supplemented with 100 μ M CaCl₂ for 7 days prior storage at 4 °C (*see Note 4*).

Fig 2: 12 % SDS-PAGE gel electrophoresis of rPVL purification. I: Insoluble fraction, S: soluble fraction, FT: unbound fraction, W1: wash with equilibration buffer, W2: fraction for washing step with 1M NaCl, L: ladder, 1-10: eluted fractions. Ladder is 250, 150, 100, **75**, **50**, 37, **25**, 20 and 15 kDa from top to bottom.

3.3 Hemagglutination Assays (HA)

Hemagglutination permits to check rapidly the binding activity of rPVL and its ability to attach to surface glycoconjugates of the red blood cells. Non-agglutinating cells will sediment at the bottom of the well and form a red button, while agglutinating cells will form a diffuse network preventing the cells' sedimentation.

1. Dilute rabbit erythrocytes (RBC) to a 4% solution in 150 mM NaCl.
2. Deposit 25 μ L of 150 mM NaCl in a 96-well plate.
3. Deposit 25 μ L of rPVL at 1mg/mL in the first well and perform a two-fold dilution series.
4. Add 50 μ L of 4% RBCs and incubate 1h.
5. Determine hemagglutination unit (HAU) as the minimum concentration of lectin required to observe hemagglutination (*see Note 5*).
6. Use 150 mM NaCl as negative control and lectin known to hemagglutinate as positive control.

3.4 Affinity Measurements by SPR

Surface plasmon resonance (SPR) experiments allow to monitor the interaction between two molecules in real time. In a flow channel, one partner (ligand) is immobilized on a biosensor chip and the other (analyte) is passing over the ligand surface. All experiments were performed on a Biacore X100 (GE Healthcare Life Sciences) at 25 °C in PBS-Ca as running buffer at a flow rate of 30 μ L/min.

1. Activate two channels by injecting 340 μ L of a fresh mixture of EDC/NHS for 400 s using a classical amine coupling procedure.
2. Inject streptavidin for 500 s.
3. Inactivate remaining reacting species by injecting 240 μ L of 1 M ethanolamine for 400 s.
4. Immobilize biotinylated PAA- β -GlcNAc at 200 μ g/mL for 10 min on channel 2 (*see Note 6*).
5. Inject rPVL on both channels in series of two-fold dilutions between 0 and 2 μ M (140 μ L, dissociation: 400 s).
6. Regenerate the chip after each injection with 0.5 M GlcNAc in water for 30 s.
7. Evaluate the data by using the Biacore X100 evaluation software and measure binding as resonance units over time after blank subtraction. Determine the dissociation constant by plotting response at equilibrium against analyte concentration (Fig. 3).
8. Perform at least duplicates.

Fig 3: Sensorgrams for the interaction of rPVL (various concentrations) injected on a CM5 chip coated with streptavidin/biotin-PAA- β -D-GlcNAc leading here to an K_d of 42 nM after steady state analysis.

3.5 rPVL Labelling

All reactions are done at room temperature using essentially manufacturer instructions. Unbound label is separated from labelled rPVL and buffer is exchanged to PBS + 100 μ M CaCl_2 using a D-salt polyacrylamide desalting column 6K (see **Note 7**). Fractions of 500 μ L are collected and their optical density is measured at 280 nm. Labelled rPVL is stored at -20 $^\circ\text{C}$ in aliquots of 250 μ L.

3.5.1 Biotinylation

1. Prepare a solution of biotinamidohehexanoyl-6-aminohehexanoic acid *N*-hydroxysuccinimide ester at 100 mM in DMF.
2. Add 6 mmol of biotin-NHS-ester to a solution of 10 mg/mL of rPVL in PBS buffer pH 7.4 (see **Note 8**). The volume added (μ L) is calculated according to the following equation:

$$V = \frac{m_{\text{protein}}(\text{mg}) \times 6 \text{ mmol}}{M_{\text{protein}}(\frac{\text{g}}{\text{mol}})} \times \frac{1000000}{100 \text{ mM}}$$

3. Incubate 30 min under agitation.

3.5.2 HorseRadish Peroxidase Coupling

1. Reconstitute 1 mg of lyophilized EZ-Link Plus Activated Peroxidase with 100 μ L of ultrapure water to obtain a solution at 10 mg/mL.
2. Prepare rPVL at 1 mg/mL in PBS buffer pH 7.4 (see **Note 9**).
3. Incubate 1 mL of rPVL with 100 μ L of HRP solution for 1 h.
4. Add 10 μ L of sodium cyanoborohydride to the reaction in the fume hood, and incubate at room temperature for 15 min.
5. Add 20 μ L of quenching buffer and incubate for 15 min.

3.5.3 *Alexa Fluor 488* *Coupling*

1. Prepare a solution of Alexa Fluor 488 NHS Ester at 10 mg/mL in DMF (*see Note 10*).
2. Prepare a solution of rPVL at 10 mg/mL in 50 mM sodium bicarbonate buffer pH 9.7.
3. Add 100 µL of reactive compound to 1 mL of rPVL drop by drop while stirring.
4. Incubate for 1 hour under constant stirring.
5. Calculate the degree of labelling (DOL) according to the following formula after correcting the protein concentration at 280 nm for the contribution of the dye ($A_{\text{prot}} = A_{280} - A_{495} \times 0.11$):

$$DOL = \frac{A_{495nm} \times MW_{\text{protein}}}{[\text{protein}] \times 71000}$$

3.6 *Enzyme-Linked Lectin Assay*

1. Prepare 96-well plates by incubating 100 µL of 5 µg/mL solution of β-GlcNAc-PAA diluted in PBS buffer pH 7.4 overnight at room temperature (*see Note 11*).
2. Wash thrice the plate with 200 µL/well of PBS.
3. Saturate wells by 200 µL/well of 2% (w/v) BSA in PBS for 1 h at 37 °C.
4. Wash five times the plate with 200 µL/well PBS-Tween 20 0.05% and washes are repeated between each step until the end of the assay.
5. Dilute biotinylated rPVL in PBS-BSA in a range from 0 to 1000 ng/well.
6. Deposit 100 µL/well and incubate it for 2h at room temperature.
7. Add 100 µL/well of 0.25 µg/mL of streptavidin-peroxidase diluted in PBS-BSA.
8. Incubate for 25 min at room temperature.
9. Wash the plate and reveal with 100 µL/well of 3,3',5,5'-tetramethylbenzidine.
10. Stop the revelation by addition of 100 µL/well of 0.1 M of hydrogen chloride.
11. Read the optical density at 450 nm with a plate reader.

3.7 *Western Blotting of O-GlcNAcylated Proteins*

1. Lyse cell lines with lysis buffer.
2. Deposit equal amounts of protein on 10% SDS-PAGE under reducing conditions.
3. Electroblot the gel on nitrocellulose using Mini Trans-Blot® Cell for 1 h at 100 V in cold transfer buffer (*see Note 12*).

4. Color the nitrocellulose with Ponceau red staining to verify efficiency of the transfer and equal loading (*see Note 12*).
5. Saturate the membrane for 45 min with 4% (w/v) Grade V BSA in TBS-T (*see Note 12*).
6. Deposit HRP-rPVL 1:5,000 for 1 h at 4 °C (*see Note 12*).
7. Wash the membrane three times with TBS-T for 10 min each.
8. Use chemiluminescence imaging to reveal the membrane.

3.8 Immunofluorescence Analysis on Whole Cells

1. Inoculate four-chamber culture slides with 4×10^4 cells per chamber for 24 h.
2. Wash the cells with ice-cold PBS.
3. Fix the cells with 2% paraformaldehyde for 10 min at 4°C.
4. Wash three times with cold PBS for 5 min each.
5. Saturate slides with 1% (w/v) BSA in PBS.
6. Wash again three times with cold PBS for 5 min each.
7. Deposit rPVL-Alexa488 on the cells to stain them (Fig. 4, *see Note 13*).
8. Examine cells with BX41 microscope and a pseudo-confocal microscope ApoTome.

Fig. 4: Merge microscopy images of cancer cells treated for 30 min at 37°C with 5 µg/mL rPVL labelled with Alexa Fluor 488. Blue channel shows nuclei labeled with DAPI staining and green channel shows rPVL-Alexa Fluor 488. Human cancer cell lines: MDA-MB-231: derived from metastatic site of mammary gland/breast, H358: derived from bronchioalveolar carcinoma and non-small cell lung cancer, and H441: derived from papillary adenocarcinoma.

3.9 Immunohistochemistry

1. Deparaffinize tissue sections (*see Note 14*).
2. Block endogenous peroxidases by adding 3% (v/v) hydrogen peroxide in PBS for 5 min (*see Note 15*).
3. Saturate sections with 5% (w/v) BSA in PBS for 30 min.
4. Deposit biotinylated rPVL at 0.7-1 µg/mL for 1h at room temperature for ethanol-fixed sections or 2 µg/mL for formalin-fixed sections and wash it twice with PBS.

5. Add indirect biotin-streptavidin system, and use the corresponding detection kit according to the manufacturer's instructions (*see Note 16*).
6. Wash the developed slides twice with PBS.
7. Perform counterstaining using hematoxylin.
8. Wash with water, dehydrate, and mount the sections.
9. Observe the sections with BX41 microscope or imaged with a NanoZoomer slide-scanner (Fig. 5).

Fig. 5: Labelling of tumoral tissues with rPVL. Colon sections of tumoral or adjacent tissue obtained from tumor surgical resections before the law 88-138 of December 20, 1988 from patients # 5345 stained with 1 μgml^{-1} rPVL-biot in the presence of 0.1 M fucose or 0.1 M GlcNAc followed by streptavidin-HRP. Slides were imaged using a NanoZoomer slide scanner with a 20x magnification. Image partly reproduced from [10].

4 Notes

1. Cells can be resuspended in some LB prior transfer in the preweighted 50 ml sterile conical centrifuge tube and centrifugation for 10 min at 5,000 $\times g$. Remove the supernatant and weight the cell pellet. The cell pellet can be stored at -20 $^{\circ}\text{C}$ or -80 $^{\circ}\text{C}$ if the purification cannot be done immediately.
2. Cell lysis can also be performed with a French press or by sonication on ice. In the latter case, 0.5 pulses are recommended for six periods of 30 s at 250 W spaced with a rest time of 1 minute on ice in between to avoid overheating and protein denaturation.
3. Pack resin in empty chromatography column and use an automated purification system to improve purification yield by allowing a longer contact time with the matrix (flowrate of 0.5 to 1 mL/min). 9 mg of rPVL can be purified for 1 ml of resin with automated system compare to only 3 mg when gravity is used. If gravity is used, reapply the sample three times to the column prior elution. Most GlcNAc affinity matrix tested was not recognized by rPVL apart from the GlcNAc Gel

(EY, USA, ref CG-003-5) but it has been unavailable for many months. The yield of purification was however much lower and we usually purified only 1mg of rPVL per mL of gel. Chitin matrix has been originally used for the native protein [7], but binding of rPVL proved to be very poor on that matrix that led to very big and dilute sample. The LNT2 matrix has been developed especially for the purification of rPVL.

4. rPVL is stable in PBS+CaCl₂ for several weeks at 4 °C but it is recommended to centrifuge the sample prior use. For long term storage, dialyze the protein against ultrapure water prior freeze-drying and storage at -20 °C. To avoid precipitation due to the brutal change from buffer to water, rPVL should be dialyzed first against a buffer containing 50-100 mM NaCl and 100 µM CaCl₂ for 3 days and then against ultrapure water for another 3 days. CaCl₂ seems to have a structural role that is why it is added to all buffers, but it is not implicated in the binding of ligands. It should not be removed immediately from the dialysis buffer.
5. Hemagglutination inhibition assays (HIA) with GlcNAcylated ligands can also be performed. 25µl of two-fold diluted ligand in 150 mM NaCl was incubated for 60 min at room temperature (RT) with 25 µL of rPVL at a concentration equal to two HAU prior addition of 50 µL of 4 % RBC. Reading was performed after 1 h at room temperature and the HA inhibitory titer is defined as the concentration of inhibitor necessary for complete inhibition of hemagglutination.
6. Use a low density chip to avoid mass transport. A low density chip is obtained by immobilizing a mixture of 10% recognized carbohydrate (β-D-GlcNAc-PAA-biotin) and 90% non-bound carbohydrate (α-L-Fuc-PAA-biotin) to 270 resonance unit (RU). A reference surface with non-bound carbohydrate is present in flow channel 1, thus allowing for subtraction of bulk effects and non-specific interactions with unbound carbohydrate (same as previously, α-L-Fuc-PAA-biotin). This chip will permit to measure avidity (multivalent binding) of rPVL. The chip can also be prepared with other oligosaccharides such as sialylated one but a weaker binding will be observed [14]. To get monovalent affinity, a rPVL chip should be made where 3500 RU of rPVL (100 µg/mL in 10 mM sodium acetate pH 6.2) is immobilized using standard procedure for amine coupling on flow channel 2 and where flow channel 1 has only been activated and deactivated. In this case, two fold cascade dilutions of oligosaccharides are then injected on both channels. Similar procedure has been used for PAL and AANL [11, 12]. Affinity can also be measured in solution by isothermal microcalorimetry as described in [10].
7. The use of polyacrylamide based desalting gel is favored to classical Sephadex G-25 such as PD-10 based on dextran (GE Healthcare Life

Sciences). It will limit weak recognition or unspecific interactions with the sugar based matrix as observed for some lectins.

8. For coupling using primary amine, avoid buffers containing primary amines such as Tris, glycine, and ammonium salts.
9. The use of carbonate-bicarbonate buffer pH 9.4 is usually recommended as it improves the efficacy of HRP conjugation, but the reaction worked well in PBS for rPVL.
10. Resuspend the Alexa Fluor 488 in DMF just before use and use immediately since the compound is not stable in solution. A ratio of 1 mg of compound for 10 mg of protein has been used. Other fluorophores such as Alexa Fluor 680 can also be used.
11. 500 ng/well of antibodies or glycosylated protein to be analyzed can also be immobilized overnight. Use a commercial antibody first such as human IgG1 kappa (Sigma-Aldrich) to do a dilution range of rPVL from 0 to 1000 ng/well and determine which concentration of rPVL to use for further experiments. The concentration giving half signal is chosen since it gives enough signal to differentiate the quantity of terminal GlcNAc on glycosylated proteins without saturation (65 ng/well has often been used to perform ELLA on antibodies).
12. Semi-dry transfer can also be performed. During wet transfer, keeping the transfer buffer cold is essential and could be done by adding a cooling unit or running it in a cold room. Preparing the SDS-PAGE gel the day before usually leads to better transfer. The use of a prestained protein ladder such as the Dual Color Standards (Bio-Rad Ltd) could replace the Ponceau red staining step. The membrane can be blocked either by BSA or milk. Biotinylated-rPVL (starting concentration of 0.6 mg/mL) followed by an incubation with HRP-avidin (dilution of 1:10,000) can also be used but the labelling is less specific due to the recognition of other biotinylated proteins. A mouse monoclonal anti-*O*-GlcNAc (R2, VWR, Fontenay-sous-Bois) can be used according to published protocol but labelling with HRP-rPVL is more efficient [14].
13. rPVL labelled with Alexa Fluor 488 or other fluorophores can also be used for flow cytometry experiments on cancer cell lines where the glycosylation is or not inhibited as described in [10].
14. Follow ethics statements on tissue sections. Required consent or certification should be obtained as well as their use approval by a Research Ethics Committee when necessary.
15. At this point, tissues sections can also be treated with 50 U of sialidase (New England Biolab) for 2 h at 37 °C to limit noise from recognition of sialylated oligosaccharides. 25 U of β -D-N-acetylhexosaminidases can also be used in similar conditions to confirm that the labelling is dependent of GlcNAc recognition. Add then fresh enzymes prior overnight incubation at 37°C. Slides treated with the enzyme buffer

were used as control. Inhibition of binding can be done by adding 100 mM GlcNAc at the same time than the labelled protein.

16. HRP-conjugated avidin D (Vector Laboratories, Burlingame, CA) diluted in 1% BSA in PBS can be used with detection, thanks to the addition of 3-amino-ethyl-carbazole (AEC kit; Vector Laboratories, Burlingame, CA) to the slides.

Acknowledgments

This work has been supported by CDP GLYCO@ALPS (ANR-15-IDEX-02). Thanks to Jacques Le Pendu for permission to use the histochemistry figures.

5 References

1. Reily C, Stewart TJ, Renfrow MB, & Novak J (2019) Glycosylation in health and disease. *Nat Rev Nephrol*, 15:346.
2. Ishihara K, Kurihara M, Goso Y, Urata T, Ota H, Katsuyama T, & Hotta K (1996) Peripheral alpha-linked N-acetylglucosamine on the carbohydrate moiety of mucin derived from mammalian gastric gland mucous cells: epitope recognized by a newly characterized monoclonal antibody. *Biochem J* 318(Pt 2):409-416.
3. Pedersen JW, Blixt O, Bennett EP, Tarp MA, Dar I, Mandel U, Poulsen SS, Pedersen AE, Rasmussen S, Jess P, Clausen H, & Wandall HH (2011) Seromic profiling of colorectal cancer patients with novel glycopeptide microarray. *Int J Cancer* 128(8):1860-1871.
4. Torres CR & Hart GW (1984) Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. *J Biol Chem* 259(5):3308-3317.
5. Yang X & Qian K (2017) Protein O-GlcNAcylation: emerging mechanisms and functions. *Nat Rev Mol Cell Biol* 18(7):452-465.
6. Zachara N, Akimoto Y, & Hart GW (2017) The O-GlcNAc Modification. In: Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, Darvill AG, Kinoshita T, Packer NH et al (eds) *Essentials of glycobiology*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 239–251
7. Kochibe N & Matta KL (1989) Purification and properties of an N-acetylglucosamine-specific lectin from *Psathyrella velutina* mushroom. *J Biol Chem* 264(1):173-177.
8. Ueda H, Kojima K, Saitoh T, & Ogawa H (1999) Interaction of a lectin from *Psathyrella velutina* mushroom with N-acetylneuraminic acid. *FEBS Lett* 448(1):75-80.
9. Cioci G, Mitchell EP, Chazalet V, Debray H, Oscarson S, Lahmann M, Gautier C, Breton C, Perez S, & Imberty A (2006) Beta-propeller crystal structure of *Psathyrella velutina* lectin: an integrin-like fungal protein interacting with monosaccharides and calcium. *J Mol Biol* 357(5):1575-1591.
10. Audfray A, Beldjoudi M, Breiman A, Hurbin A, Boos I, Unverzagt C, Bouras M, Lantuejoul S, Coll JL, Varrot A, Le Pendu J, Busser B, & Imberty A (2015) A recombinant fungal lectin for labeling truncated glycans on human cancer cells. *PLoS ONE* 10(6):e0128190.
11. Ren XM, Li DF, Jiang S, Lan XQ, Hu Y, Sun H, & Wang DC (2015) Structural basis of specific recognition of non-reducing terminal N-acetylglucosamine by an *Agrocybe aegerita* lectin. *PLoS ONE* 10(6):e0129608.

12. Ribeiro JP, Ali Abol Hassan M, Rouf R, Tiralongo E, May TW, Day CJ, Imberty A, Tiralongo J, & Varrot A (2017) Biophysical characterization and structural determination of the potent cytotoxic *Psathyrella asperospora* lectin. *Proteins* 85(5):969-975.
13. Bonnardel F, Kumar A, Wimmerova M, Lahmann M, Perez S, Varrot A, Lisacek F, & Imberty A (2019) Architecture and evolution of blade assembly in beta-propeller lectins. *Structure* 27(5):764–775.
14. Machon O, Baldini SF, Ribeiro JP, Steenackers A, Varrot A, Lefebvre T, & Imberty A (2017) Recombinant fungal lectin as a new tool to investigate O-GlcNAcylation processes. *Glycobiology* 27(2):123-128.
15. Liu W, Han G, Yin Y, Jiang S, Yu G, Yang Q, Yu W, Ye X, Su Y, Yang Y, Hart GW, & Sun H (2018) AANL (*Agrocybe aegerita* lectin 2) is a new facile tool to probe for O-GlcNAcylation. *Glycobiology* 28(6):363-373.