PURIFICATION AND CHARACTERIZATION OF AN *N*-ACETYL-D-GLUCOSAMINE SPECIFIC LECTIN FROM THE AUSTRALIAN MUSHROOM *PSATHYRELLA ASPEROSPORA*

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ABSTRACT

Australia has a spectacular biodiversity including animals, plants and fungi. Indigenous Australians have been using higher fungi traditionally as medicines and in religious practice for thousands of years. It has been estimated that there is a large number of different mushroom species present in Australia that are poorly explored and catalogued. Importantly, very little is known about the extent and diversity of lectins from Australian mushroom species [1]. *Psathyrella asperospora* (Family: Psathyrellaceae) (synonym *Lacrymaria asperospora*) is an Australian indigenous mushroom from which we have isolated an *N*-acetyl-D-glucosamine (GlcNAc) specific lectin [1]. *De novo* sequencing of *Psathyrella asperospora* lectin (PAL) using LC-MS/MS, identified 10 tryptic peptides that revealed substantial sequence similarity to the GlcNAc specific lectin from *Psathyrella velutina* (PVL) in both carbohydrate binding and calcium binding sites. Significantly, we also found that PAL has anti-proliferative effect on human colon cancer HT29 cells with an IC_{50} of 0.48 µM that represents one of the most potent mushroom lectin yet reported [2]. Further characterization of PAL's anti-proliferative activity using propidium iodine staining revealed that it induced cell cycle arrest at G_2/M phase in a manner dependent on its ability to bind GlcNAc on the cell surface. Large scale purification of PAL has now been performed in order to fully characterise the carbohydrate binding specificity including its thermodynamic properties and structural determination using glycan arrays, isothermal calorimetry (ITC) and X-ray crystallography.

Keywords: *Psathyrella asperospora*; *N*-Acetyl-D-glucosamine (GlcNAc) specific lectin; mushroom lectin; anti-proliferation; G_2/M cell cycle arrest

INTRODUCTION

Lectins are proteins, non-immunoglobulin in nature, capable of specific recognition and reversible binding to the carbohydrate moiety of glycoconjugates on the cell surface, resulting in cell agglutination and subsequent precipitation in a solution [3]. The specificity of lectins makes them an important tool in glycoproteins purification, identification and glycan analysis [4]. Lectins are ubiquitous in nature, occurring in plants, humans, animals, fungi, bacteria, viruses, and also in all foods, with their abundance being wider in mushrooms compared to plants [5]. Over the past few decades, a number of lectins have been isolated from mushrooms, and they have attracted considerable interest due to their various bioactive properties, including anti-proliferative [6-8], anti-tumour [9-11], mitogenic [6, 10, 12], immunomodulatory [9, 13, 14], hypotensive and vasorelaxing [15], and antiviral [8, 10, 16] activities.

Australia has a spectacular biodiversity including animals, plants and fungi. Indigenous Australians have been using fungi traditionally as medicines and in religious practice for thousands of years [17]. A large number of different mushrooms species are present in Australia that are poorly explored and catalogued. Importantly, very little is known about the extent and diversity of lectins from Australian mushroom species [1]. *Psathyrella asperospora* (Family: Psathyrellaceae) (Syn.: *Lacrymaria asperospora*) is an Australian indigenous mushroom, not used for food, that we recently reported to express an *N*-acetyl-D-glucosamine (GlcNAc) specific lectin [1]. The specie (*P. velutina*) of the same genus has been reported to express a GlcNAc/*N*-acetyl-neuraminic acid (Neu5Ac) specific lectin, referred to as PVL [18, 19]. PVL has now been

well characterized with respect to specificity and interaction kinetics [19, 20], and a 1.5 Å crystal structure of PVL complexed with GlcNAc is also available [21]. However, until now there has been no report of its pharmacological activity including cytotoxic or anti-proliferative activity.

Lectins with high affinity towards GlcNAc have been isolated and characterized from both vertebrates and invertebrates [3]. They have been found to be potent and selective inhibitors of human immunodeficiency virus and cytomegalo virus replication *in vitro* [22] as well as other human pathogens [23]. GlcNAc specific lectins are also known to be cytotoxic towards human hepatocellular carcinoma, human placenta choriocarcinoma and rat osteosarcoma cells [24].

A previous report showed that the GlcNAc specific lectin from the fruiting body of *P. asperospora* named PAL exhibited potent anti-proliferative activity against colon adenocarcinoma (HT29) cells. Further characterization of PAL's anti-proliferative activity showed that HT29 cells are arrested at G_2/M phase of the cell cycle, and that this effect can be halted through the addition of free GlcNAc. Here we report on the large-scale purification of PAL and the preliminary characterisation of the carbohydrate binding specificity using glycan arrays and structure determination by X-ray crystallography.

MATERIALS AND METHODS

Mushroom collection and reagents

The fruiting bodies of *P. asperospora* (Accession no. MEL 2061945) were collected in Melbourne, Australia, identified at the Royal Botanic Gardens, Melbourne and immediately frozen at -20 °C. Unless otherwise stated all the reagents were purchased from Sigma. Fructose was obtained from Ajax chemicals, lactose from OXOID Ltd, Neu5Ac from Jülich Chiral Solutions GmbH, chitin affinity sepharose from New England BioLabs, and rabbit erythrocytes from IMVS Veterinary Services Division.

Large-scale PAL purification, molecular mass determination and protein estimation

P. asperospora lectin was purified as described by Rouf *et al*. [2]. The extraction, isolation and purification steps were carried out at 4 ºC except the final size exclusion chromatography step. Thawed fruiting bodies (200 g) was suspended in 550 ml PBS (pH 7.4) homogenized using a Waring blender and left overnight at 4 ºC with gentle shaking. The resulting homogenate was filtered through cotton gauze and centrifuged twice, first for 20 min at 1,500 x g, followed by another 20 min at 10,000 x g. Solid $(NH_4)_2SO_4$ was added to the resulting supernatant (crude homogenate) to a concentration of 40% and allowed to fully dissolve for 45 min. Following centrifugation at 12,000 x g for 25 min, $(NH_4)_2SO_4$ was added to the resulting supernatant to a final concentration of 80%, allowed to dissolve and centrifuged once again to obtain the 80% pellet. This pellet was resuspended in a minimal volume of 20 mM Tris buffered saline (TBS) (pH 8.5) and extensively dialyzed against the same buffer.

Subsequently, the dialyzed 80% $(NH_4)_2SO_4$ precipitate was briefly centrifuged to remove unsuspended/sedimented particles and then it was loaded onto a 10 ml chitin sepharose column equilibrated with TBS (pH 8.4) and the affinity adsorbed PAL eluted with TBS (pH 8.5) containing 50 mM GlcNAc and 10% (v/v) glycerol in 2 cycles. The GlcNAc eluted fraction was extensively dialyzed against TBS (pH 8.5) and applied to a Hiprep Sephacryl S-100 HR column (GE healthcare) (16 × 600 mm; bed volume 120 ml) equilibrated with TBS (pH 8.5). The purification of PAL was monitored at each step using a hemagglutination assay with rabbit erythrocytes. PAL purified in this manner was stored at -20 ºC in TBS (pH 8.5) containing 10% (v/v) glycerol. Protein estimation was performed using the BCA (Bicinchoninic acid) Protein Quantitation Kit as described by the manufacturer (Thermo scientific). Standard curves were prepared using bovine serum albumin (BSA) concentrations between 0 and 2 mg/ml. Samples and standards were read on a Viktor3 1420 Multilabel counter (PerkinElmer) at 595 nm. The molecular mass of purified PAL was determined using SDS-PAGE and size exclusion chromatography (SEC). SDS-PAGE was performed on a 10% (w/v) acrylamide gel as described by Laemmli, 1970 [25], and gels stained with Coomassie brilliant blue R-250. SEC was performed on a Hiprep Sephacryl S-100 HR column (GE healthcare) (16 × 600 mm; bed volume 120 mL) calibrated with Conalbumin (75 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), Ribonuclease-A (13.7 kDa) and Aprotinin (6.5 kDa).

Hemagglutination and Hemagglutination inhibition (HA & HA-I) assays

The hemagglutination assay was modified from that of previously described by Han *et al.* [26]. A serial two fold dilution of the samples was prepared. Each dilution was mixed with an equal volume (25 µl) of PBS and added to 50 µl of neuraminidase treated or normal 2% erythrocytes suspension (rabbit or human) at room temperature in a microtiter U-plate. PBS (50 µl) added to 50 µl of 2% erythrocytes was used as a blank and the results were observed after 1 hr when the blank was fully sedimented (appeared as a dot at the bottom of the well). The hemagglutination titer was defined as the reciprocal of the highest dilution of the lectin solution showing hemagglutination activity (HA-A) and was considered as one hemagglutination unit (HA-U protein). The specific HA activity was defined as the number of HA units per mg of protein (HA-U/mg).

Hemagglutination inhibition was performed as previously described by Liu *et al.* [27],with slight modification, with the inclusion of additional sugars including Lac, Gal (Fluka biochemica), Man, Ribose (Rib), Xyl (Aldrich), GalNAc and GlcNAc. A serial two fold dilution of the different sugars to give final concentrations ranging from 50 to 0.20 mM in PBS were pre-incubated with equal volume (25 µl) of sample (diluted to the previously determined hemagglutination titer) in U-plate for 30 min. Neuraminidase treated or normal 2% rabbit and human A, B and O erythrocytes solution (50 µl) was then added, and incubated at room temperature for a further 1 hr. The minimum inhibitory concentration (MIC) was then measured by determination of the minimum sugar concentration that was able to completely inhibit the HA-A, visualised as a clear sharp dot at the bottom of the well.

Preparation of glycan arrays

Glycans sourced from Dextra Laboratories (Reading, UK) and Glycoseparations (Moscow, Russia) were functionalised and printed on activated SuperEpoxy 2 glass slides (Array It Microarray Technologies) as previously described by Day *et al*. [28].

Fluorescence labeling of PAL

Purified PAL (200 ìg) was buffer exchanged against PBS, pH 7.4 using centrifugal filter device (<10 kDa; Amicon® Ultra Centrifugal Filters) and labeled with 10 ìl of Alexa Fluor® 647 succinimidyl ester (Life Technologies) (10 ìl dye is sufficient for about 1 mg protein). The reaction mixture was wrapped in aluminum foil and incubated for 1 hr at room temperature. Subsequently a minimum volume of 10 x TBS, pH 8.5 buffer was added to reconstitute the solution back into 20 mM TBS buffer and deactivate any remaining free dye. PAL activity was examined by HA and HA-I assay.

Application to glycan arrays

Prior to use, all slides were blocked with 20 mM TBS containing 0.1% BSA and dried by centrifugation at 900 rpm for 5 min in an empty 50 ml tube. Labeled PAL (65 µl) was applied to the array contained by a Gene Frame, and the solution was evenly distributed with the aid of a Gene Frame Cover slip. After incubation for 15 min in dark at room temperature, the Gene Frame and coverslip was removed carefully in a bath of buffer solution (20 mM TBS buffer). Slides were then washed twice in a 50 ml tube containing 50 ml of fresh 20 mM TBS buffer and dried in an empty 50 ml tube by centrifugation at 900 rpm for 5 min. The slides were scanned using the Pro Scan Array Microarray 4-laser scanner. Fluorescence intensities of the array spots were measured using the Blue Argon 647 excitation laser set to the FITC setting (647 nm excitation and 517 nm emission). The slides were scanned prior to (prescan) and following each experiment. The data was analyzed using "Scan Array Express" (PerkinElmer) imaging software. The relative binding of each glycan was expressed as mean RFU (relative fluorescence units) of four replicates.

Crystallization of PAL

PAL was co-crystallized in 2.4 M malonate pH 5 with GlcNAc [PAL: 9 mg/ml, GlcNAc: 2.15 mM (10x excess)] by vapour diffusion in 1 µl volume, using a sitting drop format. This condition, as well as others, were prepared and monitored by the EMBL crystallization robot, HTX laboratory, Grenoble. Crystals were observed after 2 weeks.

RESULTS AND DISCUSSION

Large-scale purification of PAL and sequence comparison with PVL

We previously showed that the crude homogenate from *P. asperospora* was able to hemagglutinate both rabbit and human blood types A, B and O erythrocytes. The hemagglutination activity was enhanced following neuraminidase treatment of human blood types A, B and O erythrocytes [1]. Neuraminidase treatment of rabbit blood had no effect on activity. Importantly, regardless of the blood types used and treatment prior to activity assays the only saccharide able to inhibit hemagglutination was GlcNAc, with minimum inhibitory concentrations (MICs) in the low mM range [1]. Therefore, in this study due to its high hemagglutination titer, untreated rabbit blood was used to monitor PAL activity during purification.

PAL was purified to homogeneity in three steps; 80% ammonium sulfate $(NH_4)_2SO_4$ precipitation, chitin affinity chromatography and Hiprep Sephacryl S100 size exclusion chromatography (SEC). From 200 g of frozen *P. asperospora* fruiting bodies 28.4 mg of PAL was purified at a purification fold of 9, and a recovery of 13.6 % (Table 1). After SDS-PAGE analysis (Figure not shown), it was found that the initial rise in UV absorbance (shorter peak) represented the presence of contaminating proteins and the second, shaper peak represented the purified PAL that was collected and pooled. The molecular mass of PAL as determined by SEC (Fig. 1B) was approximately 36.0 kDa, which correlated well with that determined by SDS-PAGE under reducing conditions (Fig. 1A, lane 6) of approximately 40 kDa. Accurate determination of molecular mass was afforded by DLS (d*ynamic light scattering*) analysis, with PAL determined to have a molecular mass of 41.8 kDa [2].

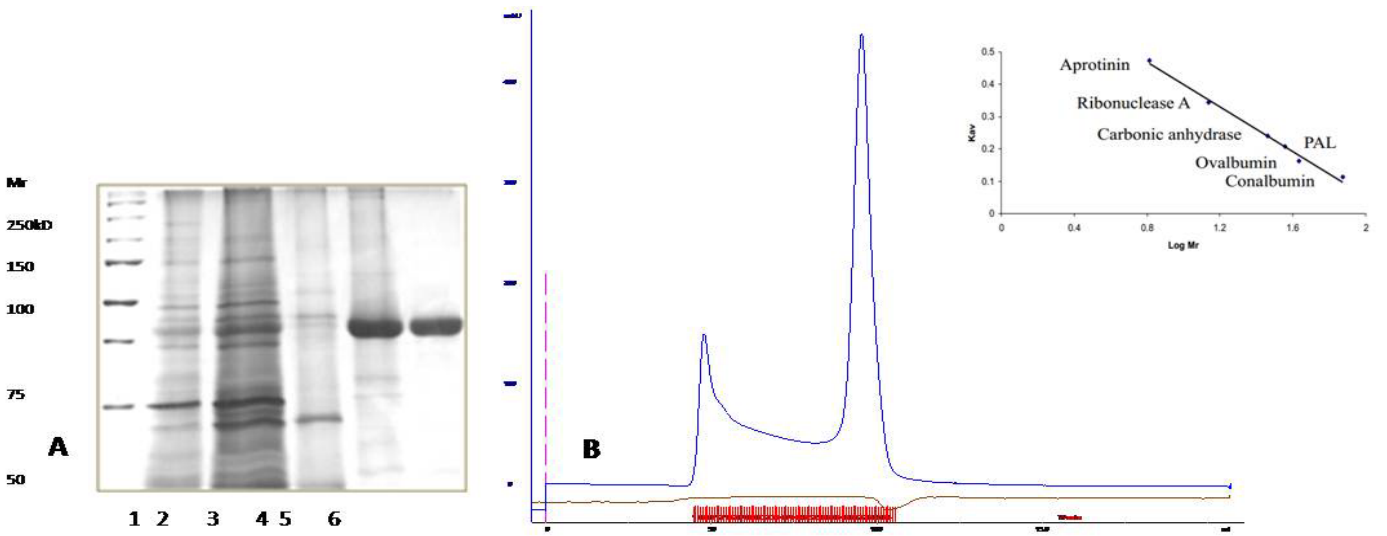

Figure 1. (A) SDS-PAGE of purified PAL: lane1 - Molecular weight markers; lane 2 - crude homogenate; lane 3 - 80 % $(NH4)_2SO_4$ precipitation; lane 4 – flow though of chitin affinity chromatography, lane 5 - Eluate of chitin affinity chromatography and lane 6 - pooled SEC fraction, (B) The chromatogram profile of size exclusion chromatography (SEC) on Hiprep Sephacryl S100 column. The calculated molecular weight by SEC of purified PAL was 35.96 KDa (insert).

Table 1. Purification of *P. asperospora* lectin (PAL)

Fraction	Total protein (mg)	Total HA-A (HA-U)	Specific activity (HA-U/mg)	Purification fold	% Recovery
Crude homogenate	1831.5	2475000	1351.4	1	100
80% $(NH_4)_2SO_4$ precipitate	367.5	960000	2612.2	2	38.8
Chitin affinity chromatography	119.9	1080000	9011.3	7	43.6
Size exclusion chromatography	28.4	336000	11851.9	9	13.6

PAL was previously characterized by *de novo* sequencing using LC-MS/MS, with 10 tryptic peptides identified [2]. Mascot analysis revealed the 10 peptides to share sequence similarity to the *P. velutina* lectin (PVL, gi:78057570) which is GlcNAc specific [18, 29]. An NCBI-BLASTP search and ClustalW2 analysis of a 43 amino acid peptide sequence constructed from peptides 4, 6 and 7, TVALADLVGEGTGGVYLLRGGSLLLQVVKVLDNFGYNA-GGSVR, exhibited high identity to PVL (70% identity) (Fig. 2). Similarly, PAL peptides 1, 2, 3, 5, 8, 9 and 10 also showed high identity to PVL. Significantly, a number of these peptides overlapped with known carbohydrate (peptides 2, 3, 5, 7, 8, 9 and 10) and calcium (peptides 3 and 5) binding domains. Fig. 2 shows the multiple sequence alignment of PVL and the PAL tryptic peptides, with the carbohydrate and calcium binding domains as determined through the X-ray crystal structure of PVL being highlighted. Although highly conserved, some differences between PAL and PVL are evident. In particular, a number of PVL residues known to be involved in carbohydrate recognition and binding, Trp54, Glu191, Asn214, Asp270 and Try306 are, based on *de novo* sequencing of tryptic peptides, substituted in PAL with Ser, Val, Pro, Phe and Leu respectively. However, given the high affinity and strict specificity for GlcNAc that PAL exhibits, it would appear that these substitutions have no dramatic effect on the structure of the carbohydrate-binding sites. In addition, Leu183 in the PVL Ca^{2+} binding domain is conservatively substituted with Ala in PAL (Fig 2), and a two-residue insertion (an Arg and Leu) was found in PAL between PVL residues Leu183 and Leu184. However all other residues in the PVL Ca^{2+} binding consensus sequence, Asp-h-Thr-Gly-Asp-Gly-h-h-Asp, are conserved.

PAL and PVL [18] both bind GlcNAc in a divalent cation independent manner, however the crystal structure of PVL revealed two Ca^{2+} binding sites consisting of nine residue loops with the consensus sequence Asp-h-Thr-Gly-Asp-Gly-h-h-Asp (where h is an hydrophobic residue) [21]. It is still unclear as to the exact function of Ca^{2+} in PVL for mushroom metabolism, although it has been postulated that Ca^{2+} binding may play a role in lectin stabilization in a similar way to that seen in integrins [21]. A sequence alignment of PVL and tryptic peptides of PAL revealed a high conservation of residues in Ca^{2+} binding site 1, with only Leu183 substituted with an Ala in PAL, and a His in AAL-II. PAL sequence information was not obtained for Ca^{2+} binding site 2. Therefore it would seem highly probable that the ability to bind Ca^{2+} is a conserved feature of this family of GlcNAc specific lectins.

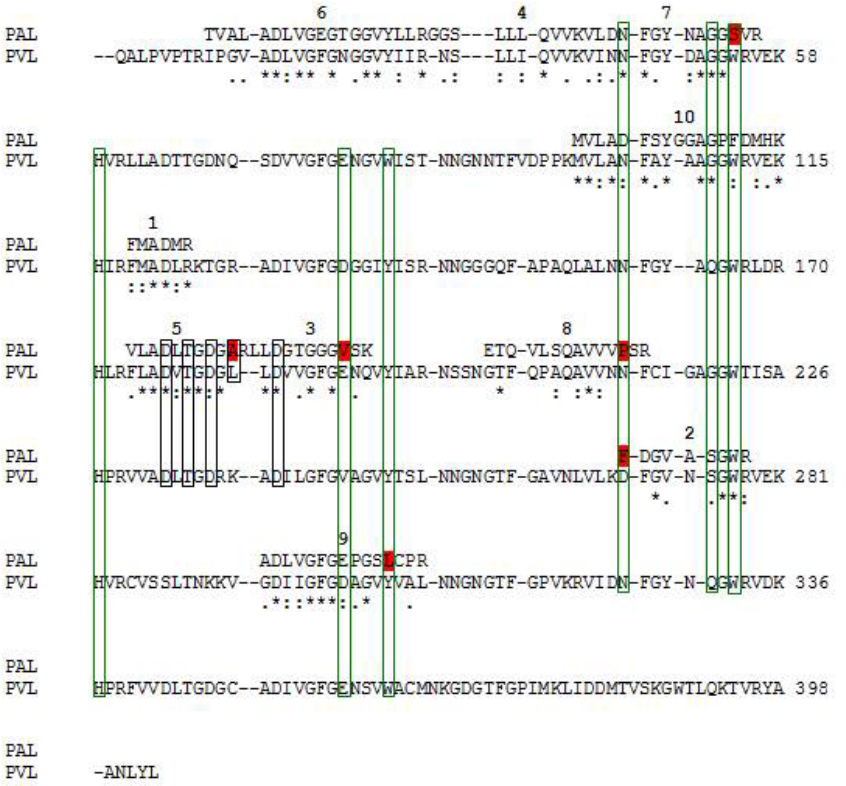

Figure 2. ClusterW2 sequence alignment of 10 PAL tryptic peptide sequences (numbered) and PVL (gi:78057570). Residues involved in carbohydrate and Ca^{2+} binding in PVL are boxed in green and black respectively. Amino acids highlighted in red represent PAL residues not conserved in PVL.

PAL carbohydrate specificity and preliminary X-ray crystallography

Mushroom lectins exhibit a broad specificity varying from simple sugars to complex saccharides and glycoproteins [6]. PAL showed strict specificity for GlcNAc among the saccharides tested, but also exhibited high binding affinity towards fetuin and mucin, which possess terminal Neu5Ac (Table 2). Similarly, the closely related PVL preferential binds free and oligosaccharides bearing non-reducing terminal GlcNAc structure [18] as well as terminal Neu5Ac residues on glycoproteins and oligosaccharides [19, 30].

As previously observed for the crude homogenate from *P. asperospora* [1], among the saccharides tested GlcNAc was the only capable of inhibiting the hemagglutination activity of purified PAL, with a MIC of 0.78 mM. Of particular interest was the lack of PAL hemagglutination inhibition exhibited by free Neu5Ac. The closely related PVL is known to bind free Neu5Ac in addition to GlcNAc, but only very weakly ($K_d < 10^{-3}$ M) [21]. However, PAL did show high binding affinity for mucin (MIC 0.002 mg/ml) and fetuin (MIC 0.0078 mg/ml), but not for asialofetuin even at 1 mg/ml concentration (Table 2), suggesting that sialoglycoconjugates may be a ligand for PAL.

Table 2. Inhibition of hemagglutination activity associated with purified PAL

	Minimum inhibitory concentration (MIC)
Saccharides*	
Galactose	-
D-Glucose	-
GlcNAc	0.78 mM
GalNAc	-
Lactose	-
D-Mannose	-
L-Arabinose	-
D-Ribose	-
D-Xylose	-
L-Fucose	-
D-Maltose	-
Neu5Ac#	-
Sucrose	-
Glycoproteins**	
á-acid glycoprotein	0.125 mg/ml
Fetuin	0.0078 mg/ ml
Asialofetuin	-
Mucin	0.002 mg/ml
BSA	0.5 mg/ml
Conalbumin	1.0 mg/ml
Fibrinogen	1.0 mg/ml

* Saccharide concentration range from 0.2 to 50mM, # Maximum final concentration was 25mM, ** Glycoprotein concentration range from0.0005 to 1.0 mg/ml

To explore PAL's carbohydrate specificity in greater detail glycan array experiments were performed. PAL was labeled with Alexa Fluor® 647 succinimidyl ester and two different concentrations of PAL (50 and 100 ug/ml) directly applied to a glycan array slide that were printed as previously described [28]. Table 3 shows PAL binding to glycan structure present on the array, with strong binding (RFU of greater the 2500 coloured dark red) through to no binding (RFU less than 500 coloured white) indicated. Glycans shown in Table 3 are grouped as the function of their terminal residue, Gal, GlcNAc, Mannose, Fucose, Neu5Ac, and also include glycosaminoglycans (GAGs) and related structure. As expected, high binding of PAL to the glycans containing terminal non-reducing GlcNAc was observed. We showed previously that free Neu5Ac does not inhibit PAL hemagglutination activity but Neu5Ac-containing glycoconjugates (fetuin and mucin, see Table 2) do. The ability of PAL to bind glycosidically linked Neu5Ac was confirmed by glycan array analysis with strong binding observed to oligosaccharides containing Neu5Ac (Table 3). PAL binding was also found on GlcNAc containing terminal galactose structures. Among the 120 glycans, the GlcNAc and Neu5Ac containing sialylated oligosaccharide, Neu5Acá2-3Galâ1-3GlcNAcâ1-3Galâ1-4Glc, exhibited the highest binding with about 5000 RFUs. In addition, weak binding interactions of PAL can be observed in a number of GlcNAc containing mannosylated, fucosylated glycans, GAGs and other related structures. Similarly, another GlcNAc specific lectin, *Agrocybe aegerita* lectin-2 (AAL-2) showed high binding selectivity towards almost 30 glycans that possessed terminal non-reducing GlcNAc [29]. AAL-2 was not reported to bind to any sialic acid containing glycans, however, with no test performed on glycoproteins.

Table 3. PAL binding to glycan structures present on array

Class	Glycan	ID
Terminal Galactose	Galβ1-3GlcNAc	1A
	Galβ1-4GlcNAc	1B
	Galβ1-4Gal	1C
	Galβ1-6GlcNAc	1D
	Galβ1-3GalNAc	1E
	Galβ1-3GalNAcβ1-4Galβ1-4Glc	1F
	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	1G
	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	1H
	Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	1I
	Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	1J
	Galα1-4Galβ1-4Glc	1K
	GalNAcα1-O-Ser	1L
	Galβ1-3GalNAcα1-O-Ser	1M
	Galα1-3Gal	1N
	Galα1-3Galβ1-4GlcNAc	1O
	Galα1-3Galβ1-4Glc	1P
	Galα1-3Galβ1-4Galα1-3Gal	2A
	Galβ1-6Gal	2B
	GalNAcβ1-3Gal	2C
	GalNAcβ1-4Gal	2D
	Galα1-4Galβ1-4GlcNAc	2E
	GalNAcα1-3Galβ1-4Glc	2F
	Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	2G
	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	2H
GlcNAc	GlcNAcβ1-4GlcNAc	4A
	GlcNAcβ1-4GlcNAcβ1-4GlcNAc	4B
	GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAc	4C
	GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAc	4D
	GlcNAcβ1-4MurNAc	4E
Mannosylated	GlcNAcβ1-2Man	5A
	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Man	5B
	Manα1-2Man	5C
	Manα1-3Man	5D
	Manα1-4Man	5E
	Manα1-6Man	5F
	Manα1-6(Manα1-3)Man	5G
	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man	5H
Fucosylated	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	7A
	Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc	7B
	Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	7C
	Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc	7D
	Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc	7E
	Fucα1-2Gal	7F
	Fucα1-2Galβ1-4Glc	7G

	Galβ1-4(Fucα1-3)Glc	7H	
	Galβ1-4(Fucα1-3)GlcNAc	7I	
	Galβ1-3(Fucα1-4)GlcNAc	7J	
	GalNAcα1-3(Fucα1-2)Gal	7K	
	Fucα1-2Galβ1-4(Fucα1-3)Glc	7L	
	Galβ1-3(Fucα1-2)Gal	7M	
	Fucα1-2Galβ1-4(Fucα1-3)GlcNAc	7N	
	Fucα1-2Galβ1-3GlcNAc	7O	
	Fucα1-2Galβ1-3(Fucα1-4)GlcNAc	7P	
	SO3-3Galβ1-3(Fucα1-4)GlcNAc	8A	
	SO3-3Galβ1-4(Fucα1-3)GlcNAc	8B	
	Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	8C	
	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	8D	
Fucosylated	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Fucα1-2Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	8E	
	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3)Galβ1-4Glc	8F	
	Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc	8G	
	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	8H	
	Fucα1-3Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc	8I	
	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3(Fucα1-2)Galβ1-4Glc	8J	
	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	8K	
	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-4(Fucα1-3)GlcNAcβ1-3)Galβ1-4Glc	8L	
	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	8M	
	Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	8N	
	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	8O	
	GalNAcβ1-3(Fucα1-2)Galβ1-4Glc	8P	
	Galβ1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc	9A	
	Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc	10A	
	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc	10B	
	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	10C	
	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	10D	
	Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc	10E	
Sialylated	Fucα1-2Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc	10F	
	Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc	10G	
	Neu5Acα2-6Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc	10H	
	Neu5Acα2-6Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	10I	
	Galβ1-4GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	10J	
	Neu5Acα2-3Galβ1-4GlcNAc	10K	
	Neu5Acα2-6Galβ1-4GlcNAc	10L	
	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	10M	
	Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc	10N	
	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	10O	

Sialylated	Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc	10P	
	Neu5Acα2-3Galβ1-4Glc	11A	
	Neu5Acα2-6Galβ1-4Glc	11B	
	(Neu5Acα2-8Neu5Ac)n	11C	
	(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)2Manβ1-4GlcNAcβ1-4GlcNAc-Asn	11D	
GAGs and Related Structure	Neocarratetraose-41, 3-di-*O*-sulphate (Na^+)	12A	
	Neocarratetraose-41-*O*-sulphate (Na^+)	12B	
	Neocarrahexaose-24,41, 3, 5-tetra-*O*-sulphate (Na^+)	12C	
	Neocarrahexaose-41, 3, 5-tri-*O*-sulphate (Na^+)	12D	
	Neocarraoctaose-41, 3, 5, 7-tetra-*O*-sulphate (Na^+)	12E	
	Neocarradecaose-41, 3, 5, 7, 9-penta-*O*-sulphate (Na^+)	12F	
	ΔUA-2S→GlcNS-6S Na_4 (I-S)	12G	
	ΔUA→GlucNS-6S Na3 (II-S)	12H	
	ΔUA→2S-GlcNS Na_3 (III-S)	12I	
	ΔUA→2S-GlcNAc-6S Na_3 (I-A)	12J	
	ΔUA→GlcNAc-6S Na_2 (II-A)	12K	
	ΔUA→2S-GlcNAc Na_2 (III-A)	12L	
	ΔUA→GlcNAc Na (IV-A)	12M	
	ΔUA→GalNAc-4S Na_2 (Δ Di-4S)	12N	
	ΔUA→GalNAc-6S Na_2 (Δ Di-6S)	12O	
	ΔUA→GalNAc-4S,6S Na_3 (Δ Di-disE)	12P	
	ΔUA→2S-GalNAc-4S Na_2 (Δ Di-disB	13A	
	ΔUA→2S-GalNAc-6S Na_3 (Δ Di-disD)	13B	
	ΔUA→2S-GalNAc-4S-6S Na_4 (Δ Di-tisS)	13C	
	ΔUA→2S-GalNAc-6S Na_2 (Δ Di-YA2S)	13D	
	ΔUA→GlcNAc Na (Δ Di-HA)	13E	
	$(GlcAβ1\text{-}3GlcNAcb1\text{-}4)_n$ (n=4)	13F	
	$(GlcAβ1\text{-}3GlcNAcβ1\text{-}4)_n$ (n=8)	13G	
	$(GlcAβ1\text{-}3GlcNAcβ1\text{-}4)_n$ (n=10)	13H	
	$(GlcAβ1\text{-}3GlcNAcβ1\text{-}4)_n$ (n=12)	13I	
	$(GlcA/IdoAa/β1\text{-}4GlcNAcα1\text{-}4)_n$ (n=200)	13J	
	$(GlcA/IdoAa/β1\text{-}3(±4/6S)GalNAcβ1\text{-}4)_n$ (n<250)	13K	
	$((±2S)GlcA/IdoAa/β1\text{-}3(±4S)GalNAcβ1\text{-}4)_n$ (n<250)	13L	
	$(GlcA/IdoAβ1\text{-}3(±6S)GalNAcβ1\text{-}4)_n$ (n<250)	13M	
	HA-4 10 mM	13N	
	HA-6 10 mM	13O	
	HA-8 9.7 mM	13P	
	HA-10 7.83 mM	14A	
	HA-12 6.5 mM	14B	
	HA-14 5.6 mM	14C	
	HA-16 4.9 mM	14D	
	HA 30000 Da 2.5 mg/ml	14E	
	HA 107000 Da 2.5 mg/ml	14F	
	HA 190000 Da 2.5 mg/ml	14G	
	HA 222000 Da 2.5 mg/ml	14H	
	HA 1600000 Da 2.5 mg/ml	14I	

>2500 RFU
1500-2500 RFU
500-1500 RFU
<500 RFU

Interestingly, PAL did not bind a number of GlcNAc-containing glycans, for instance the terminal galactose containing glycan, Galâ1-4GlcNAcâ1-6(Galâ1-4GlcNAcâ1-3)Galâ1-4Glc (Table 3). On the other hand, most mannosylated glycans on the array were bound by PAL (although at relatively low levels), this was not observed in our previous studies using free mannose in hemagglutination inhibition assay [1].

In preliminary crystallography experiments, PAL was co-crystallized in 2.4 M malonate, pH 5 with GlcNAc, with crystals being observed after 2 weeks. Fig 3 shows the PAL crystal that was used to obtain preliminary X-ray diffraction data to a resolution of 2.1 Å. Structure determination of PAL by molecular replacement using PVL as the search probe is currently underway.

Figure 3. Microscopic image of droplets of PAL crystal. Crystallization condition: 9 mg/ml PAL in 2.4 M malonate, pH 5 with 2.15mM GlcNAc

CONCLUSION

Lectins are well known to possess cytotoxicity and/or anti-proliferative activity against cultured cells [6-8]. Our previous report described PAL as the first GlcNAc-specific mushroom lectin with potent (IC_{50} 0.43 µM) anti-proliferative activity [2]. Given GlcNAc is known to be aberrantly expressed on the surface of cancer cells, our cytostatic highly selective GlcNAc specific PAL might have a potent application in cancer diagnosis or therapy. We have now successfully purified PAL on a large scale and have obtained more extensive carbohydrate specificity data using glycan array as well as generating PAL crystals that have diffracted to 2.1 Å.

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