**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 IC₅₀ 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 G₂/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; G₂/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 \cite{19, 20}, and a 1.5 Å crystal structure of PVL complexed with GlcNAc is also available \cite{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 \cite{3}. They have been found to be potent and selective inhibitors of human immunodeficiency virus and cytomegalovirus replication \textit{in vitro} \cite{22} as well as other human pathogens \cite{23}. GlcNAc specific lectins are also known to be cytotoxic towards human hepatocellular carcinoma, human placenta choriocarcinoma and rat osteosarcoma cells \cite{24}.

A previous report showed that the GlcNAc specific lectin from the fruiting body of \textit{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 G2/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 \textit{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**

\textit{P. asperospora} lectin was purified as described by Rouf \textit{et al}. \cite{2}. The extraction, isolation and purification steps were carried out at 4 °C 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 °C 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 (NH4)2SO4 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, (NH4)2SO4 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% (NH4)2SO4 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 Sphacyrl 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 °C 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 \cite{25}, and gels stained with Coomassie brilliant blue R-250. SEC was performed on a Hiprep Sephacyrl 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 µg) was buffer exchanged against PBS, pH 7.4 using centrifugal filter device (<10 kDa; Amicon® Ultra Centrifugal Filters) and labeled with 10 µl of Alexa Fluor® 647 succinimidyl ester (Life Technologies) (10 µl 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. asperspora* 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$_4$)$_2$SO$_4$ precipitation, chitin affinity chromatography and Hiprep Sephacryl S100 size exclusion chromatography (SEC). From 200 g of frozen *P. asperspora* 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 (dynamic light scattering) analysis, with PAL determined to have a molecular mass of 41.8 kDa [2].

![Figure 1](image)

**Figure 1.** (A) SDS-PAGE of purified PAL: lane1 - Molecular weight markers; lane 2 - crude homogenate; lane 3 - 80 % (NH$_4$)$_2$SO$_4$ 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. asperspora* lectin (PAL)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total HA-A (HA-U)</th>
<th>Specific activity (HA-U/mg)</th>
<th>Purification fold</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>1831.5</td>
<td>2475000</td>
<td>1351.4</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>80% (NH$_4$)$_2$SO$_4$ precipitate</td>
<td>367.5</td>
<td>960000</td>
<td>2612.2</td>
<td>2</td>
<td>38.8</td>
</tr>
<tr>
<td>Chitin affinity chromatography</td>
<td>119.9</td>
<td>1080000</td>
<td>9011.3</td>
<td>7</td>
<td>43.6</td>
</tr>
<tr>
<td>Size exclusion chromatography</td>
<td>28.4</td>
<td>336000</td>
<td>11851.9</td>
<td>9</td>
<td>13.6</td>
</tr>
</tbody>
</table>
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, TVALADLVGEGTGGVYLLRGSSLLLQVVKLDNFYNA-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, 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.

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**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 preferentially 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

<table>
<thead>
<tr>
<th>Saccharides</th>
<th>*Saccharide concentration range from 0.2 to 50mM, *Maximum final concentration was 25mM, <strong>Glycoprotein concentration range from 0.0005 to 1.0 mg/ml</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>-</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>-</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>0.78 mM</td>
</tr>
<tr>
<td>GalNAc</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>-</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>-</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>-</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>-</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>-</td>
</tr>
<tr>
<td>D-Maltose</td>
<td>-</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
</tr>
<tr>
<td>Glycoproteins**</td>
<td><strong>Glycoprotein concentration range from 0.0005 to 1.0 mg/ml</strong></td>
</tr>
<tr>
<td>á-acid glycoprotein</td>
<td>0.125 mg/ml</td>
</tr>
<tr>
<td>Fetuin</td>
<td>0.0078 mg/ml</td>
</tr>
<tr>
<td>Asialofetuin</td>
<td>-</td>
</tr>
<tr>
<td>Mucin</td>
<td>0.002 mg/ml</td>
</tr>
<tr>
<td>BSA</td>
<td>0.5 mg/ml</td>
</tr>
<tr>
<td>Conalbumin</td>
<td>1.0 mg/ml</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1.0 mg/ml</td>
</tr>
</tbody>
</table>

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 μg/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, Neu5Acá2-3Galá1-3GlcNAcá1-3Galá1-4Glc, 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

<table>
<thead>
<tr>
<th>Class</th>
<th>Glycan</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal Galactose</td>
<td>Galβ1-3GlcNAc</td>
<td>1A</td>
</tr>
<tr>
<td></td>
<td>Galβ1-4GlcNAc</td>
<td>1B</td>
</tr>
<tr>
<td></td>
<td>Galβ1-4Gal</td>
<td>1C</td>
</tr>
<tr>
<td></td>
<td>Galβ1-6GlcNAc</td>
<td>1D</td>
</tr>
<tr>
<td></td>
<td>Galβ1-3GalNAc</td>
<td>1E</td>
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<tr>
<td></td>
<td>Galβ1-4GlcNAcβ1-3Galβ1-4Glc</td>
<td>1F</td>
</tr>
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<td>Galβ1-4GlcNAcβ1-3Galβ1-4Glc</td>
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<td>Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc</td>
<td>1I</td>
</tr>
<tr>
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<td>Galα1-3Gal</td>
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</tr>
<tr>
<td></td>
<td>GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Man</td>
<td>5B</td>
</tr>
<tr>
<td></td>
<td>Manα1-2Man</td>
<td>5C</td>
</tr>
<tr>
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<td>Manα1-3Man</td>
<td>5D</td>
</tr>
<tr>
<td></td>
<td>Manα1-4Man</td>
<td>5E</td>
</tr>
<tr>
<td></td>
<td>Manα1-6Man</td>
<td>5F</td>
</tr>
<tr>
<td></td>
<td>Manα1-6(Manα1-3)Man</td>
<td>5G</td>
</tr>
<tr>
<td></td>
<td>Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man</td>
<td>5H</td>
</tr>
<tr>
<td>Fucosylated</td>
<td>Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc</td>
<td>7A</td>
</tr>
<tr>
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<td>Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc</td>
<td>7B</td>
</tr>
<tr>
<td></td>
<td>Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc</td>
<td>7C</td>
</tr>
<tr>
<td></td>
<td>Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc</td>
<td>7D</td>
</tr>
<tr>
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<td>Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc</td>
<td>7E</td>
</tr>
<tr>
<td></td>
<td>Fucα1-2Gal</td>
<td>7F</td>
</tr>
<tr>
<td></td>
<td>Fucα1-2Galβ1-4Glc</td>
<td>7G</td>
</tr>
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</table>
Galβ1-4(Fucoα1-3)Glc
Galβ1-4(Fucoα1-3)GlcNAc
Galβ1-3(Fucoα1-4)GlcNAc
GalNAcα1-3(Fucoα1-2)Gal
Fucoα1-2Galβ1-4(Fucoα1-3)Glc
Galβ1-3(Fucoα1-2)Gal
Fucoα1-2Galβ1-4(Fucoα1-3)GlcNAc
Fucoα1-2Galβ1-3GlcNAc
Fucoα1-2Galβ1-3(Fucoα1-4)GlcNAc
SO3-3Galβ1-3(Fucoα1-4)GlcNAc
SO3-3Galβ1-4(Fucoα1-3)GlcNAc
Galβ1-3GlcNAcβ1-3Galβ1-4(Fucoα1-3)GlcNAcβ1-3Galβ1-4Glc
Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc
Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Fucoα1-2Galβ1-3GlcNAcβ1-3)Galβ1-4Glc
Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc
Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Fucoα1-2Galβ1-3GlcNAcβ1-3)Galβ1-4Glc
Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc
Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Galβ1-4(Fucoα1-3)GlcNAcβ1-3)Galβ1-4Glc
8E
Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Fucoα1-2Galβ1-3(Fucoα1-4)GlcNAcβ1-3)Galβ1-4Glc
8F
Galβ1-4GlcNAcβ1-3Galβ1-4(Fucoα1-3)Glc
8G
Fucoα1-2Galβ1-4(Fucoα1-3)GlcNAcβ1-3Galβ1-4Glc
8H
Fucoα1-3Galβ1-4GlcNAcβ1-3Galβ1-4(Fucoα1-3)Glc
8I
Fucoα1-2Galβ1-4(Fucoα1-3)GlcNAcβ1-3(Fucoα1-2)Galβ1-4Glc
8J
Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc
8K
Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Galβ1-4(Fucoα1-3)GlcNAcβ1-3)Galβ1-4Glc
8L
Galβ1-3GlcNAcβ1-3Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc
8M
Galβ1-3GlcNAcβ1-3Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc
8N
Fucoα1-2Galβ1-3GlcNAcβ1-3Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc
8O
GalNAcβ1-3(Fucoα1-2)Galβ1-4Glc
8P
Galβ1-3(Fucoα1-2)Galβ1-4(Fucoα1-3)Glc
9A
Neu5Acα2-3Galβ1-3(Fucoα1-4)GlcNAc
10A
Neu5Acα2-3Galβ1-4(Fucoα1-3)GlcNAc
10B
Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
10C
Galβ1-4(Fucoα1-3)GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc
10D
Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc
10E
Fucoα1-2Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc
10F
Neu5Acα2-3Galβ1-3(Fucoα1-4)GlcNAcβ1-3Galβ1-4Glc
10G
Neu5Acα2-6Galβ1-3GlcNAcβ1-3Galβ1-4(Fucoα1-3)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(Neu5Ac2-6)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

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, 9-penta-O-sulphate (Na⁺) 12E
ΔUA-2S→GlcNS-6S Na⁺ (I-S) 12F
ΔUA→GlcNS-6S Na3 (II-S) 12G
ΔUA→2S-GlcNS Na3 (III-S) 12H
ΔUA→2S-GlcNAc-6S Na3 (I-A) 12I
ΔUA→GlcNAc-6S Na2 (II-A) 12J
ΔUA→2S-GlcNAc Na2 (III-A) 12K
ΔUA→GlcNAc Na (IV-A) 12L
ΔUA→GlcNAc-4S Na2 (Δ Di-4S) 12M
ΔUA→GlcNAc-6S Na2 (Δ Di-6S) 12N
ΔUA→GlcNAc-4S,6S Na3 (Δ Di-disE) 12O
ΔUA→GlcNAc-4S,6S Na3 (Δ Di-diS) 12P
ΔUA→2S-GalNAc-4S Na2 (Δ Di-diB) 13A
ΔUA→2S-GalNAc-6S Na3 (Δ Di-diD) 13B
ΔUA→2S-GalNAc-4S-6S Na4 (Δ Di-tisS) 13C
ΔUA→2S-GalNAc-6S Na2 (Δ Di-YA2S) 13D
ΔUA→GlcNAc Na (Δ Di-HA) 13E
(GlcAβ1-3GlcNAcβ1-4)n (n=4) 13F
(GlcAβ1-3GlcNAcβ1-4)n (n=8) 13G
(GlcAβ1-3GlcNAcβ1-4)n (n=10) 13H
(GlcAβ1-3GlcNAcβ1-4)n (n=12) 13I
(GlcA/IdoAβ1-4GlcNAcβ1-4)n (n=200) 13J
(GlcA/IdoAβ1-3(±4S)GalNAcβ1-4)n (n<250) 13K
(±2S)GlcA/IdoAβ1-3(±4S)GalNAcβ1-4)n (n<250) 13L
(GlcA/IdoAβ1-3(±6S)GalNAcβ1-4)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

GAGs and Related Structure

ΔUA-2S→GlcNS-6S Na⁺ (I-S) 12A
ΔUA→GlcNS-6S Na3 (II-S) 12B
ΔUA→2S-GlcNS Na3 (III-S) 12C
ΔUA→2S-GlcNAc-6S Na3 (I-A) 12D
ΔUA→GlcNAc-6S Na2 (II-A) 12E
ΔUA→2S-GlcNAc Na2 (III-A) 12F
ΔUA→GlcNAc Na (IV-A) 12G
ΔUA→GlcNAc-4S Na2 (Δ Di-4S) 12H
ΔUA→GlcNAc-6S Na2 (Δ Di-6S) 12I
ΔUA→GlcNAc-4S,6S Na3 (Δ Di-disE) 12J
ΔUA→GlcNAc-4S,6S Na3 (Δ Di-disD) 12K
ΔUA→2S-GalNAc-4S Na2 (Δ Di-tisS) 12L
ΔUA→2S-GalNAc-6S Na2 (Δ Di-YA2S) 12M
ΔUA→GlcNAc Na (Δ Di-HA) 13A
(GlcAβ1-3GlcNAcβ1-4)n (n=4) 13B
(GlcAβ1-3GlcNAcβ1-4)n (n=8) 13C
(GlcAβ1-3GlcNAcβ1-4)n (n=10) 13D
(GlcAβ1-3GlcNAcβ1-4)n (n=12) 13E
(GlcA/IdoAβ1-4GlcNAcβ1-4) (n=200) 13F
(GlcA/IdoAβ1-3(±4S)GalNAcβ1-4)n (n<250) 13G
(±2S)GlcA/IdoAβ1-3(±4S)GalNAcβ1-4)n (n<250) 13H
(GlcA/IdoAβ1-3(±6S)GalNAcβ1-4)n (n<250) 13I
HA-4 10 mM 13J
HA-6 10 mM 13K
HA-8 9.7 mM 14A
HA-10 7.83 mM 14B
HA-12 6.5 mM 14C
HA-14 5.6 mM 14D
HA-16 4.9 mM 14E
HA 30000 Da 2.5 mg/ml 14F
HA 107000 Da 2.5 mg/ml 14G
HA 190000 Da 2.5 mg/ml 14H
HA 222000 Da 2.5 mg/ml 14I
HA 1600000 Da 2.5 mg/ml 14J

>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.

**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.

**REFERENCES**


