Biochemical and structural properties of a lectin purified from seeds of the legume *Parkia nitida* Miq.

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ABSTRACT

As the main carbohydrate-binding proteins, lectins are responsible for several biological functions, although their specific roles are still being unveiled. In the current work, a legume lectin from the seeds of *Parkia nitida* (*Mimosoideae* subfamily) was isolated by a combination of saline precipitation, mannose affinity chromatography, and gel filtration chromatography. The lectin, henceforth designated as PNL, demonstrated remarkable similarity to other *Parkia* lectins at the biochemical and structural levels. Overall, PNL is a stable lectin with a molecular mass of 48,760.3 Da, composed of 451 amino acid residues that fold into 3 side-by-side β-prism domains, each with its own carbohydrate-recognition domain specific to mannosides. Additionally, PNL displays a degree of toxicity against *Artemia* sp., albeit weaker than similar lectins. In conclusion, a representative of the Mimosoideae subfamily of legumes could be purified and characterized. This represents an advance in the understanding of *Mimosoideae* lectins, a group of unique proteins that receive significantly less focus in the plant lectin field.

Keywords: Lectin, *Parkia nitida*, structural characterization, *Mimosoideae*

1. Introduction

The capacity of specific and reversible binding to carbohydrates gives lectins several biological roles. These proteins can be found in all organisms, although most research focuses on plant lectins. As one of the main groups of studied lectins, the advance of the knowledge about these proteins by researchers is happening in parallel to their use as biotechnological tools in the most diverse areas [1,2]. The applicability of lectins is also being boosted by the discovery of their interaction with non-carbohydrate hydrophobic molecules through a specific region on their structure [3–5].

The Fabaceae family has the greatest richness of tree species in Neotropical forests with many endemic taxa in this region. Some Brazilian ecosystems are centers of diversity for the group with many species being unique to these environments. *Parkia nitida* Miq. (*Leguminosae* family, *Mimosoideae* subfamily) is an example of a plant found mostly in the Amazon rainforest environment. The considerably primitive *Mimosoideae* subfamily is sorely lacking in lectin studies although Parkia lectins distinguish themselves for having a good number of reports in the literature and being one of the rare cases of legume plants that do not contain lectins with properties related to the legume lectin family, instead presenting lectins closely related to the Jacalin-related lectin family [1,6].

Current literature reports that *Parkia* lectins belong to a group of lectins that bind to mannose/glucose, with a preference for mannose and its derivatives. The lectins from this genus share some properties, such as dimeric oligomerization, overall high thermostability, and a monomeric molecular weight of about 45 kDa. Structurally, *Parkia* lectins are made up of three repeated β-prism domains, with each domain composed of about 150 residues. Each domain is structurally related to Jacalin and shares a minimum of 45% identity with each other [7–9].

Parkia lectins are known to elicit several biological activities, including toxicity against *Artemia* sp. [10], inhibition of *L. infantum* promastigotes growth [11], an antiproliferative effect on B-cell hybridoma (HB98) [12], and possess antiparasitic, antinociceptive, and anti-inflammatory properties [8]. These biological activities give them high potential applicability in biotechnology, but further research is required to enable the use of such proteins.

To contribute to the knowledge about the relatively unknown group of *Mimosoideae* lectins, the biological, biochemical, and structural properties of a newly purified lectin from the seeds of *Parkia nitida* have been compared with similar lectins to better understand their underlying properties.

2. Materials and methods

2.1 Material

Parkia nitida Miq. (taxonID:23109) seeds were collected in the Ducke reserve which is located at coordinates 59° 52' 40" to 59° 58' 00" West longitude and 03° 00' 00" to 03° 08' 00" South latitude, close to the city of Manaus in Amazonas. The species was registered in the herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA) under registration code INPA92469.

2.2 Pre-purification steps

Soluble proteins were extracted from ground, and peeled *Parkia nitida* seeds after defatting with n-hexane. The extraction was set up by mixing the seed flour with a 100 mM NaOAc buffer at pH 4.0 + 150 mM NaCl (1:10 w/w) with continuous stirring for 4 hours in an orbital shaker at 25 °C. The protein extract was recovered by centrifugation (10,000 x g - 20 min) and subjected to ammonium sulfate precipitation (0-60% saturation). The precipitated

proteins were resuspended in 100 mM Tris-HCl buffer + 150 mM NaCl (TBS) and then dialyzed against TBS to remove excess salt.

During each step of the purification process, the lectin activity was semi-quantitatively assessed by agglutination assays with a 3% suspension of rabbit red blood cells (RBCs) in 96-well microtiter plates. The hemagglutination activity unit (HU) was defined as the reciprocal of the highest dilution showing positive agglutination, and the specific activity was determined as the hemagglutination units per milligram of protein.

To determine the carbohydrate specificity of the lectin, different concentrations of carbohydrates (50 mM, 25 mM, 12.5 mM, 6.25 mM, and 3.125 mM), namely D-glucose, D-mannose, α-methyl-D-mannoside, D-galactose, α-methyl-D-galactoside, L-fucose, sucrose, D-raffinose, and melibiose, were brought into contact with the extract/lectin solution (4 H.U./mL) prior to the addition of RBCs. The lowest concentration capable of abolishing the agglutination phenomenon was defined as the minimal inhibitory concentration (MIC) for that specific carbohydrate.[13].

2.3 PNL purification

To purify the lectin, the desalted 0-60% fraction was subjected to affinity chromatography using a TBS-equilibrated Sepharose-mannose matrix (8 x 1.5 cm; Sigma-Aldrich - USA). Unretained proteins were removed with the equilibration buffer until the absorbance at 280 nm, checked by spectrophotometry using an Ultrospec2100Pro (Amersham Biosciences - UK), reached the baseline. Proteins adsorbed in the matrix were eluted by pH variation using 100 mM Glycine-HCl buffer + 150 mM NaCl at pH 2.6. The bound proteins were pooled together, dialyzed, freeze-dried, and applied in the next purification step. The chromatography ran at a constant flow rate of 700 µL/min, and fractions of 1 mL were collected and monitored.

For further purification, a solution containing the retained proteins from the affinity chromatography (3 mg/mL in 300 mM NaCl) was applied to a BioSuite 250 size-exclusion matrix (0.78 cm x 30 cm) coupled to an ultra-performance system (Waters, USA). A 300 mM NaCl solution was used for matrix equilibration and protein separation. In this chromatographic step, two peaks, termed AI and AII, were obtained, respectively. The fraction collector was set to 1 mL, and the system was adjusted to perform the process at a flow rate of 600 µL/min. Both fractions were desalted by dialysis and freeze-dried.

The protein content during the purification steps was estimated by the Bradford protocol [14] with bovine serum albumin as a standard protein. The Dubois protocol with glucose as standard was chosen to check for neutral sugar content in the purified lectin [15].

2.4 Thermal stability, optimal pH and cations dependence

The temperature at which there is irreversible denaturation of PNL was accessed by heating a 1 mg/mL PNL solution (in 150 mM NaCl) up to 100 °C, in increments of 10 °C, for 1 hour at each temperature. The treated lectin solution was then allowed to cool down to room temperature and its activity was tested and compared with a room-temperature lectin solution to determine the percentage of activity.

PNL optimal pH was determined by dialyzing the lectin solution against different buffers at 0.1 M containing NaCl 150 mM with varying pH (4 to 10) for 24 h. Acidic buffer solutions were made with sodium acetate (pH 4.0) and sodium citrate (pH 5.0 and 6.0). The neutral buffer solution was made with sodium phosphate (pH 7.0) and the alkaline buffers solutions with Tris-HCl (pH 8.0) and glycine-NaOH (pH 9 and 10). The activity was then measured [16].

As it is common to lectins, mainly of the legume lectin type, the divalent cations dependence was accessed in the current work [17]. Briefly, PNL was demetallized by dialysis

against EDTA (100 mM) for 24h followed by NaCl 150 mM to remove the chelating agent. The addition of $CaCl_2$ and/or $MnCl_2$ to a final concentration of 10 mM, separately or not, was tested to check for a possible recovery in activity. In all conditions, the activity of the lectin was measured by agglutination assays.

2.5 Electrophoretic analysis

The quality of the purification process was monitored by SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) [18]. The SDS-PAGE ran in a 15% acrylamide separation gel using a Mini-Protean II apparatus (BioRad) with electrical current of 25 mA, 10 W and 150 V. Gels were stained with Coomassie Brilliant Blue R-250. The Pierce™ Prestained Protein (Thermo Scientific) consisting of proteins with molecular weight varying from 20 to 120 kDa was applied as a molecular weight marker. The presence of disulfide bridges was verified by adding 2% β–mercaptoethanol to the sample buffer prior to the run.

2.6 Lectin sequencing and secondary structure prediction

To obtain data about its primary structure, PNL was digested with proteolytic enzymes following the protocol previously determined by Shevchenko et al. (2006) [19]. The digested peptides were dried and sent to the Mass Spectrometry facility located at the Laboratório Nacional de Biociências (LNBio) of the Nacional de Pesquisa em Engenharia e Materiais (CNPEM) in Campinas, São Paulo, Brazil. Briefly, peptides (8 µmol/µL) were diluted in 0.1% formic acid and then applied to a C18 column (100 µm × 100 mm) coupled to an ultra-performance system (UPLC). PNL was eluted at a 600 µL/min flow rate with a gradient of 0-90% acetonitrile and formic acid 0.1%. After elution, the peptides were ionized in a nanoESI-Q mass spectrometer operating in continuous MS mode with voltages of 3.5 kV and 30 V with

80°C as source temperature. Data were acquired at 100-2000 m/z range, with 1s as scan rate and 300 scans have been performed to generate the spectra.

The generated data was processed and analyzed with the MassLynx v. 4.1 software (Waters). Multicharged spectra were deconvoluted by applying the maximum entropy protocol and the generated spectra were smoothed and mass centroid values were obtained using 80% of the top of the peak, with a minimum peak width at half the height of 4 channels. Each MS/MS spectrum was manually interpreted using MassLynx to determine the peptides sequence [10]. The protein sequence has been submitted to the UniProt Knowledgebase under the accession number C0HM44. PsiPred v4.0 server was used to predict the secondary structure of PNL [18,20].

2.7 3D structure and binding simulations

To obtain the three-dimensional structure of PNL, homology molecular modeling was employed using the structure of *Parkia biglobosa* lectin (PDB Id: 4mq0) [9] as template, chosen due to its high sequence identity and overall good quality parameters. A total of 200 models were generated using Modeller v10.4 [21] and 1 model was generated using the Phyre2 server in intensive mode [22]. Ten models from Modeller were pre-selected in a first evaluation using the modeler's objective score (molpdf) and the discrete optimized score (DOPE), which are generated by the program itself. These 10 models and the Phyre2 model were then evaluated on the SAVES 6.0 server [23] to obtain ERRAT [24] and Verify3D [25,26] quality scores, and other structural parameters (Ramachandran, steric overlaps, Cβ deviation parameters, rotamers, and bonds angles) using PROCHECK [27]. QMEANDisCo scores were calculated using Protein Structure and Assessment tools [28]. The model that presented the best scores and parameters during this evaluation was chosen to represent PNL 3D structure.

To evaluate the interaction of PNL with α-methyl-D-mannoside (MMA), molecular docking was performed using GOLD v. 2022.2 (CDCC, Cambridge, England). To compare the scores, tests were also performed with the PBL model (PDB Id: 4mq0). The default configuration of the program and the PLANTSPLP scoring function were adequate for the simulations [29]. The docking search was limited to all amino acids in a 7 Å to the CRD cavities of the three PNL domains. Lectin models complexed with MMA were selected by combining hydrogen bonding and nonpolar interaction scores, and ligand geometry penalties [30–33]. The 3D figures were generated by the UCSF Chimera v1.16 [34] and Pymol Molecular Graphics System v 2.5 (Schrödinger, LLC) programs, and 2D representations of the interactions were generated by the LigPlot+ v2.2 program [35].

2.8 Toxicity assay on microcrustaceans

The toxicity test with *Artemia sp*. is widely applied in lethality studies and is a convenient starting point for the assessment of natural products cytotoxicity. This technique is standardized by the Italian Environmental Protection Agency and the Italian Water Research Institute (APAT IRSA-CNR) and the Italian Chemical Sector Standardization Agency (Unichim) [36]. The applied methodology was performed by executing the following steps: (I) cysts eclosion after 24 hours of contact with filtered and sterilized seawater at a temperature of 25± 3 ºC and constant oxygenation and a photoperiod of 12 hours; (II) PNL toxicity test through contact of lectin at concentrations of 100-12.5 μg/mL with instar in stages II-III after 24 h of hatching; (III) Count of nauplii (dead/immobile) 24 hours after the start of the test. The test consisted of four groups: 1st group (no lectin), 2nd group (100-12.5 μg of PNL), 3rd group (100 μg PNL + 100 mM D-mannose) and 4th group (100 μg of heat-denatured PNL), all performed in triplicate. The results obtained were compiled from the means (SEM) and standard deviation with a confidence index of 5% or $p<0.05$ analyzed by ANOVA followed by

the Bonferroni test when using multiple comparisons. LC_{50} values were calculated with probit analysis as described by Finney [37].

3. Results

3.1 PNL isolation and purification

Prior tests showed that proteins extracted from *Parkia nitida* in a sodium acetate buffer have a higher hemagglutination titer, suggesting a more efficient extraction of lectin. The agglutination effect of the protein extract and the pure lectin was inhibited by manno- and glucosides. The minimal inhibitory concentration (MIC) of α-methyl-D-mannoside (3.125 mM) was two times lower compared to that of D-mannose and eight times lower compared to that of D-glucose (Table 1), indicating that PNL is a mannose-binding lectin. PNL was purified through a series of steps, including $(NH_4)_2SO_4$ precipitation and two chromatographic steps: first, a mannose-affinity matrix, and second, a size-exclusion chromatography. The affinity chromatography resulted in two protein pools, named PI and PII (Figure 1A), with only the second pool exhibiting activity. PII was then subjected to size-exclusion chromatography, resulting in two peaks (AI and AII) (Figure 1B), both displaying hemagglutination activity. A combination of sequence analysis and electrophoretic profile allowed for AI to be considered the pure lectin, and as such, was designated as *Parkia nitida* lectin, abbreviated as PNL (Table 2).

3.2 SDS-PAGE and stability assays

The degree of purification after each step and the apparent molecular weight of PNL were defined by SDS-PAGE. The peaks obtained from the size-exclusion chromatography displayed a pure protein profile (Figure 1). Neither AI nor AII reacted with phenol sulfuric acid in the Dubois method. PNL kept its maximum activity until 50 °C and completely lost it from

80 °C onwards (Figure 2A). PNL displayed a maximal activity at pH 6.0 and 7.0 with no activity being detected at pH 10 (Figure 2B). PNL significantly lost its activity after dialysis with the 100 mM EDTA solution, with half of its activity being recovered with the addition of calcium ($CaCl_2$) and manganese ($MnCl_2$) metal ions (Figure 2C) suggesting a metalloprotein nature.

3.3 Determination of intact molecular mass and structure prediction

ESI-MS spectra demonstrated the presence of a predominant ion estimated to weigh 48,760.3008 Da. The spectra profile with several ions of lower intensity and molecular weights are consistent with the presence of multiple isoforms (Figure 3A). The complete primary structure of PNL was resolved by aligning 51 peptides sequenced from 3 different enzymatic digests, which resulted in a sequence of 451 amino acid residues (Figure 3B and Supplementary materials 1, 2 and 3). Alignment of PNL sequence with other *Parkia* lectins (Supplementary material 4) using the ESPript 3.0 software [28] revealed that these lectins share about 90% similarity at primary structure level. The prediction of PNL secondary structure suggests it is composed primarily of β-sheets and coils (Supplementary material 5), a similar profile to closely-related lectins such as PpaL, PPL and PBL, and other legume lectins in general [9,10].

From the 11 preselected PNL homology models. The chosen model presented a Global Quality Factor (ERRAT) of 84.82 and 96.55% of its amino acids was a conformation compatible with its expected three-dimensional structure, a percentage calculated by Verify3D. Local and global structural parameters, as calculated by PROCHECK, were inside acceptable ranges. The Ramachandran plot indicated that 98.4% of the amino acids are in favorable and allowed regions of the graph, while 1.6% are in generously allowed regions. The QMEANDisCo value was 0.86, which is within the range of high-end models. The superposition between the PNL model and the PBL structure (PDB Id: 4mq0) resulted in a

root-mean-square deviation (RMSD) of 0.438 nm (431 Cα aligned), indicating a reliable prediction. The final model representative of PNL monomer can be seen in Figure 4A. Molecular docking simulations suggest a favorable interaction between PNL and α-methyl-D-mannoside (MMA) in its three domains, corroborating with the inhibition assays (Table 1). Compared with PBL, all domains interacted with lower score values (Table 3). From the docking outputs, the main CRD residues interacting with monosaccharides could be identified. Domain 1 (Figure 4B; Supplementary material 6) is made up of residues Gly15, Gly16, Val92, Gly137, Tyr138, Tyr139 and Asp141, domain 2 (Figure 4C; Supplementary material 7) is made up of Ala164, Gly165, Asp166, Phe239, Gly282, Trp283, Tyr284 and Asp286, and finally domain 3 (Figure 4D; Supplementary material 8) which is formed by Gly310, Gly311, Phe389, Gly432, Asp433, Tyr343 and Asp436. These amino acids participate in a network of hydrogen bonds, as well as hydrophobic and van der Waals interactions that stabilize the binding.

3.4 Ecotoxicity assay

PNL is toxic to *Artemia nauplii* in a dose-dependent fashion with an average lethality of 5.7, 4.3, 2 and 1.7 nauplii for PNL at 200 μg/mL, 100 μg/mL, 50 μg/mL and 25 μg/mL respectively (Figure 5). Heat-inactivated PNL toxicity is negligible with an average lethality of 2 nauplii, thus suggesting that the correct folding is essential to the toxic activity. Incubation with mannose had very little effect on the lectin-induced toxicity.

4. Discussion

The crude extract obtained from protein extraction at pH 4 showed significant specific hemagglutinating activity, which means that it extracts a reasonable amount of lectin. PNL was successfully purified by ammonium sulfate fractionation combined with two chromatographic steps: affinity followed by SEC. The latter resulted in two protein peaks, named AI and AII,

with overlapping amino acid sequences but different electrophoretic profiles, which could indicate different stages of post-translational processing. Altogether, AII was considered to be the pure lectin, which resulted in a specific activity 1,723 times higher than the crude extract.. PNL-induced agglutination was abolished in the presence of D-mannose (6.25 mM), D-glucose (25 mM) and their derivatives. The mannose-derivative, α-methyl-D-mannoside (3.125 mM) was the tested carbohydrate with the lowest MIC value. As already described for other *Parkia* lectins and other mannose-specific legume lectins, the methyl group present at C1 favors additional hydrophobic interactions in the lectin CRD which leads to a stronger interaction compared to D-mannose [9]. The preference of PNL for mannose and its derivatives over glucose is an expected result since *Parkia* lectins interact more favorably with the C2 axial hydroxyl of mannose than the equatorial hydroxyl of glucose. In addition, water molecules form bridges between the O2 atom of the mannose and the CRD residues of *Parkia* lectins [10,38]. PNL is a thermostable protein, a property shared with other similar lectins, including *Parkia biglobosa* (PBL), *Parkia panurensis* (PpaL), *Parkia Platycephala* (PPL) and *Parkia pendula* (PpaL). Maximal activity is kept until 50 °C, with gradual reduction at 50 °C (75%) and 60 °C (87.5%) and complete denaturation at 80 °C. PNL optimal pH range is between 6.0 and 7.0, in line with has been observed for other similar lectins. Demetalization induced a dramatic reduction of activity (93%), suggesting the presence of divalent cations in its structure. About 50% of hemagglutinating activity was recovered when the Ca^{2+} and Mn^{2+} were added in combination. However, when the two ions were added separately, only 12.5% of the activity was recovered, which suggests further that PNL is a metalloprotein that requires both divalent cations for its optimal activity. PpaL, unlike PNL, appears to prefer Mn^{2+} ions in comparison to Ca^{2+} ions [10]. On the other hand, activity of PBL is retained after demetallization [8]. We can see here that the three proteins of the same genus that share structural similarity have different profiles with regard to dependence on metal ions for their

activities. However, analyses of the three-dimensional structures of PPL and PBL do not report a metal-binding site [9], which contrasts with the activity data. This contradiction needs to be unveiled with further studies on *Parkia* lectin structure and activity relationships.

PNL displayed a single band of 50 kDa on SDS-PAGE gel, suggesting a successful purification. *Parkia* lectins usually show very heterogeneous profiles when applied in SDS-PAGE, ranging from one band for PBL, PPL, and PPal [7,8,10], two bands for *Parkia javanica* lectin [39], three bands for *Parkia speciosa* lectin [40] up to four bands for *Parkia roxburghii* lectin [12]. The multiple bands are not generated by disulfide bonds as reducing agents have not changed their electrophoretic profile.

SDS-PAGE and MS data about PNL molecular mass are in agreement, with MS reporting 48,760.30 Da. This value is close to the mass value calculated by adding all amino acid residues obtained in the primary structure, which equals 48,205.17 Da, leaving a difference of 555.13 Da. This difference probably belongs to the few blocked *N*-terminal amino acid residues not normally found when the sequence of *Parkia* lectins is elucidated by MS. According to Mann et al. (2001), PPL contains N-terminal blocked by acetylation of certain amino acid residues, and this fragment can contain from 3 to 5 amino acid residues. However, this fragment is usually not found due to its high hydrophobicity and incompatibility with the reverse phase matrix applied in MS systems [7]. As another example, a 326 Da difference is observed for PBL in the same situation which results from the Ser-Leu-Lys amino acid residues that have not been found in its amino acid sequence, as determined by MS/MS [8].

PNL amino acid sequence, determined by overlapping sequenced peptides, consists of a total of 451 amino acid residues. A small difference in the length of amino acid sequences can be observed among *Parkia* lectins (PPL: 447 aa/47951 Da; PBL: 443 aa/47562 Da; PpeL: 447 aa/474100 Da and PNL: 451 aa/48760 Da), as reflected by their differences in intact mass and probably also in the number of amino acids that make up the blocked N-terminal. Structural

studies of PPL, PpaL, PBL and PpeL showed that the lectins of this genus are constituted by multiple isoforms. Thus, twelve heterogeneous sequences were found in the PNL primary structure at positions 69 (H/K), 70 (R/X), 92 (V/X), 96 (Y/X), 152 (T/E), 213 (L/F), 215 (F/E), 294 (K/P), 295 (L/R), 264 (P/D), 298 (Y/X) and 300 (T/E). The MS/MS spectra of P5 and T9 (Supplementary Tables S1 and S3) belonging to ion Y shows the substitutions of amino acids G/F and F/E at positions 213 and 215, respectively. *Parkia* lectins are related to jacalin-type lectins with mannose specificity and show similarities at primary and tertiary structure levels [7]. The alignment of the amino acid sequence of PNL with PBL and PPL showed a high similarity in the amino acid sequence. A cysteine residue was found at position 199 of the PNL primary sequence. This residue is conserved in lectins from *Parkia* genus, such as PBL, PpaL, PPL and PpeL, as well as in the Heltuba lectin (isolated from *Helianthus tuberosus*) [32], which is a lectin related to Jacalin, but without interchain disulfide bonds. PNL has a predicted glycosylation site between positions 104-106, but the negative reaction to the Dubois test suggests an absence of glycans in its structure. *Parkia* lectins are not homogeneous in their glycosylation with some lectins such as PpeL, PPL and PBL not having glycans in their structure and others such as *Parkia biglandulosa*, *Parkia roxburghii* [19] and *Parkia javanica* lectins [30] being glycosylated.

PNL presents a monomer consisting of three β-prism domains in tandem (Figure 8A), each containing a CRD with specificity to mannose and derivatives, which is very similar to the described domains of other Parkia and Jacalin-like lectins [7,17,41,42]. The first domain from PNL monomer consists of two β-sheets with four antiparallel strands and another one with three antiparallel strands. The second domain has three β-sheets composed of four antiparallel strands. The third domain is similar to the first with two β-sheets composed of four antiparallel strands and one is composed of three antiparallel strands [9]. Even though the 3 CRDs have the same specificity, each has a different amino acid composition (Figure 8B-D;

Supplementary Figures S1-3). The PNL and PBL domains had a similar pattern in their score values, which domain 2 > domain 3 > domain 1. PNL and PBL domains have only one residue different in composition, Trp238 (in PNL, in the case of PBL is Tyr237) present in domain 2. However, despite the change being only in domain 2, all PBL domains interacted more favorably with MMA when compared to PNL (Table 3). The difference in identity in different regions of the two lectin monomers probably impacts how the domains interact with carbohydrates.

PNL exhibits toxicity towards *Artemia* sp., with an LC_{50} value of 177.8 µg/mL. However, when comparing this value with the LC50 of other mannose-specific lectins, ranging from 2.53 µg/mL for *Dioclea guianensis* lectin to approximately 376 µg/mL for ConA, PNL demonstrates moderate cytotoxicity towards this organism [43,44]. Interestingly, PNL displays significantly lower toxicity compared to the closely-related lectin PpaL (LC_{50} of 20 µg/mL). A possible explanation for the difference in the degree of toxicity between PpaL and PNL may be related to the difference in the topology of the binding sites of these lectins which may influence the recognition and fitting of more complex glycans, found on the cell surfaces of these organisms. Another possibility is the stronger binding of PNL to mannose in comparison to PpaL. A study by Santos et al. (2009) evaluated the toxic effects of different mannose-binding lectins against *Biomphalaria glabrata* and *Artemia salina*. Although these lectins have the same binding specificity to common monosaccharides and have biochemical and structural similarities, the authors concluded that their toxic activities are different, which is suggested to result from slight structural differences between lectins [43]. A mechanism for the toxic effect of lectins against *Artemia* sp. is still being researched. Other biological activities besides those of Parkia lectins have been reported in the literature, such as antiproliferative and mitogenic [12], antinociceptive and anti-inflammatory [9], and inhibition of the development of bacterial

strains and *Haemonchus contortus* [45]. These characteristics make lectins a good alternative as biopharmaceuticals for the treatment of various types of diseases.

The incubation of PNL with D-mannose before the toxicity tests did not affect the lethality promoted by the lectin, suggesting that its activity is not dependent on the CRD or the lectin may have a higher affinity for a glycan present in the *Artemia* sp organism, this was different from what has been observed for PpaL [12]. In addition, heat denaturation significantly reduced the lethality promoted by PNL demonstrating that its toxic activity is dependent on its native structure. These results show variability in biological effects between *Parkia* lectins caused by a structural element other than the CRD.

Recent studies on *Parkia* lectins have highlighted their potential against pathogenic organisms. For instance, PPL, a lectin from *Parkia platycephala* with glucose/mannose specificity, has been found to impact the development of *Haemonchus contortus* larvae with an I50 of 0.31 mg/mL. This parasite is known for its detrimental effects on small ruminant production worldwide. Furthermore, when combined with gentamicin, PPL has shown antimicrobial effects against multidrug-resistant strains of *Staphylococcus aureus* and *Escherichia coli*. The synergistic effect of PPL reduced the Minimum Inhibitory Concentration (MIC) of gentamicin from 64 to 25.4 μg/mL for S. aureus and from 32 to 20.2 μg/mL for E. coli [45].

Another biological application of *Parkia* lectins is their orofacial antinociceptive effect, as demonstrated by Leite et al. in zebrafish and adult rodents. PPL reduced nociceptive behavior associated with temporomandibular joint and neuropathic pain in zebrafish [46]. Additionally, the primary structure of another Parkia lectin, named PpeL, was recently determined. Extracted from the seeds of *P. pendula*, PpeL showed high sequence homology with lectins from *P. platycephala* and *P. biglobosa*. In *in silico* and *in vitro* assays, PpeL exhibited antiparasitic effects against *Leishmania infantum*, inhibiting the growth of

promastigote stages by 45.6 ± 1.92% in 48 hours. This effect was found to be dependent on the carbohydrate-binding capacity of the lectin, suggesting an interaction between PpeL and *L. infantum* glycans [11]. Moreover, certain mannose/glucose-specific lectins have shown promising potential in yeast flocculation assays [47].

Overall, the lectin purified in this study represents an advancement in understanding *Mimosoideae* lectins and plant lectins in general. The wide-ranging applicability of *Parkia* lectins makes these proteins highly interesting for biotechnology and the study of the rich biodiversity found in South America.

Acknowledgments

We are grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) for their financial support. To professors K.S.N. and B.S.C., coordinators of the Biologically Active Molecules Laboratory (BioMol-Lab) and senior investigators of CNPq. This research also used facilities of the Brazilian Biosciences National Laboratory (LNBio), part of the Brazilian Center for Research in Energy and Materials (CNPEM), a private non-profit organization under the supervision of the Brazilian Ministry for Science, Technology, and Innovations (MCTI). VJSO is grateful to FWO-Vlaanderen for the postdoctoral fellowship (12T4622N).

References

[1] M. Tsaneva, E.J.M. Van Damme, 130 years of Plant Lectin Research, Glycoconj. J. (2020). https://doi.org/10.1007/s10719-020-09942-y.

[2] V.J.S. Osterne, J. Verduijn, C.F. Lossio, B. Parakhonskiy, M.V. Oliveira, V.R. Pinto-Junior, K.S. Nascimento, A.G. Skirtach, E.J.M. Van Damme, B.S. Cavada,

Antiproliferative activity of Dioclea violacea lectin in CaCO3 particles on cancer cells after controlled release, J. Mater. Sci. 57 (2022) 8854–8868.

[3] S.S. Komath, K. Bhanu, B.G. Maiya, M.J. Swamy, Binding of porphyrins by the tumor-specific lectin, jacalin [Jack fruit (Artocarpus integrifolia) agglutinin], Biosci. Rep. 20 (2000) 265–276.

[4] S.S. Komath, M. Kavitha, M.J. Swamy, Beyond carbohydrate binding: new directions in plant lectin research, Org. Biomol. Chem. 4 (2006) 973–988.

[5] V. Bogoeva, M. Rangelov, N. Todorova, A. Lambert, C. Bridot, A. Yordanova, G. Roos, C. Grandjean, J. Bouckaert, Binding of Gold(III) Porphyrin by the Pro-metastatic Regulatory Protein Human Galectin-3, Molecules. 24 (2019). https://doi.org/10.3390/molecules24244561.

[6] L. Augusto Gomes de Souza, Biodiversity of Fabaceae in the Brazilian Amazon and its timber potential for the future, in: Tropical Forests - Ecology, Diversity and Conservation Status, IntechOpen, 2023.

[7] K. Mann, C.M. Farias, F.G. Del Sol, C.F. Santos, T.B. Grangeiro, C.S. Nagano, B.S. Cavada, J.J. Calvete, The amino-acid sequence of the glucose/mannose-specific lectin isolated from Parkia platycephala seeds reveals three tandemly arranged jacalin-related domains, Eur. J. Biochem. 268 (2001) 4414–4422.

[8] H.C. Silva, A.U. Bari, B.A.M. Rocha, K.S. Nascimento, E.L. Ponte, A.F. Pires, P. Delatorre, E.H. Teixeira, H. Debray, A.M.S. Assreuy, Others, Purification and primary structure of a mannose/glucose-binding lectin from Parkia biglobosa Jacq. seeds with antinociceptive and anti-inflammatory properties, J. Mol. Recognit. 26 (2013) 470–478.

[9] A.U. Bari, M.Q. Santiago, V.J.S. Osterne, V.R. Pinto-Junior, L.P. Pereira, J.C. Silva-Filho, H. Debray, B.A.M. Rocha, P. Delatorre, C.S. Teixeira, C.C. Neto, A.M.S. Assreuy, K.S. Nascimento, B.S. Cavada, Lectins from Parkia biglobosa and Parkia platycephala: A comparative study of structure and biological effects, Int. J. Biol. Macromol. 92 (2016) 194–201.

[10] B.S. Cavada, A.U. Bari, V.R. Pinto-Junior, C.F. Lossio, M.T.L. Silva, L.A.G. Souza, M.V. Oliveira, C.H.D. Souza-Filho, S.E.G. Correia, Ana Paula Moreira, L.D. Lima, V.J.S. Osterne, K.S. Nascimento, Purification and partial characterization of a new lectin from Parkia panurensis Benth. ex H.C. Hopkins seeds (Leguminosae family; Mimosoideae subfamily) and evaluation of its biological effects, International Journal of Biological Macromolecules. (2019). https://doi.org/10.1016/j.ijbiomac.2019.10.102.

[11] R.F. Carneiro, E.S. Aguiar, V.F. Santos, A.L.E. Santos, M.H.C. Santos, R.R. Roma,

R.R.S. Silva, M.L.M.B. Leal, L.T. Silva, B.A.M. Rocha, C.G.L. Silva, C.S. Nagano, A.H. Sampaio, R.O.S. Souza, C.S. Teixeira, Elucidation of the primary structure and molecular modeling of Parkia pendula lectin and in vitro evaluation of the leishmanicidal activity, Process Biochem. 101 (2021) 1–10.

[12] N. Kaur, J. Singh, S.S. Kamboj, J.N. Agrewala, M. Kaur, Two novel lectins from Parkia biglandulosa and Parkia roxburghii: isolation, physicochemical characterization, mitogenicity and anti-proliferative activity, Protein Pept. Lett. 12 (2005) 585–595.

[13] V.R. Pinto-Júnior, M.Q. de Santiago, V.J. da S. Osterne, J.L.A. Correia, F.N. Pereira-Júnior, J.B. Cajazeiras, M.A. de Vasconcelos, E.H. Teixeira, A.S.F. do Nascimento, T.B.A.R. Miguel, E. de C. Miguel, A.H. Sampaio, K.S. do Nascimento, C.S. Nagano, B.S. Cavada, Purification, partial characterization and immobilization of a mannose-specific lectin from seeds of Dioclea lasiophylla mart, Molecules. 18 (2013) 10857–10869.

[14] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[15] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric Method for Determination of Sugars and Related Substances, Anal. Chem. 28 (1956) 350–356.

[16] M.V. Oliveira, V.J.S. Osterne, C.F. Lossio, S. Serna, N.C. Reichardt, K.S. Nascimento, E.J.M. Van Damme, B.S. Cavada, Purification and characterization of a highly thermostable GlcNAc-binding lectin from Collaea speciosa seeds, Int. J. Biol. Macromol. 193 (2021) 1562–1571.

[17] B.S. Cavada, V.J.S. Osterne, M.V. Oliveira, V.R. Pinto-Junior, M.T.L. Silva, A.U. Bari, L.D. Lima, C.F. Lossio, K.S. Nascimento, Reviewing Mimosoideae lectins: A group of under explored legume lectins, Int. J. Biol. Macromol. 154 (2020) 159–165.

[18] D.T. Jones, Protein secondary structure prediction based on position-specific scoring matrices, J. Mol. Biol. 292 (1999) 195–202.

[19] A. Shevchenko, H. Tomas, J. Havlis, J.V. Olsen, M. Mann, In-gel digestion for mass spectrometric characterization of proteins and proteomes, Nat. Protoc. 1 (2006) 2856–2860.

[20] D.W.A. Buchan, D.T. Jones, The PSIPRED Protein Analysis Workbench: 20 years on, Nucleic Acids Res. 47 (2019) W402–W407.

[21] B. Webb, A. Sali, Comparative Protein Structure Modeling Using MODELLER, Curr. Protoc. Protein Sci. 86 (2016) 2.9.1–2.9.37.

[22] L.A. Kelley, S. Mezulis, C.M. Yates, M.N. Wass, M.J.E. Sternberg, The Phyre2 web portal for protein modeling, prediction and analysis, Nat. Protoc. 10 (2015) 845–858.

[23] SAVESv6.0 - structure validation server, (n.d.). https://saves.mbi.ucla.edu/ (accessed July 6, 2023).

[24] C. Colovos, T.O. Yeates, Verification of protein structures: patterns of nonbonded atomic interactions, Protein Sci. 2 (1993) 1511–1519.

[25] J.U. Bowie, R. Lüthy, D. Eisenberg, A method to identify protein sequences that fold into a known three-dimensional structure, Science. 253 (1991) 164–170.

[26] R. Lüthy, J.U. Bowie, D. Eisenberg, Assessment of protein models with three-dimensional profiles, Nature. 356 (1992) 83–85.

[27] R.A. Laskowski, M.W. MacArthur, D.S. Moss, J.M. Thornton, PROCHECK: a program to check the stereochemical quality of protein structures, J. Appl. Crystallogr. 26 (1993) 283–291.

[28] G. Studer, C. Rempfer, A.M. Waterhouse, R. Gumienny, J. Haas, T. Schwede, QMEANDisCo-distance constraints applied on model quality estimation, Bioinformatics. 36 (2020) 1765–1771.

[29] O. Korb, T. Stützle, T.E. Exner, Empirical scoring functions for advanced protein-ligand docking with PLANTS, J. Chem. Inf. Model. 49 (2009) 84–96.

[30] M.B. de Ávila, M.M. Xavier, V.O. Pintro, W.F. de Azevedo Jr, Supervised machine learning techniques to predict binding affinity. A study for cyclin-dependent kinase 2, Biochem. Biophys. Res. Commun. 494 (2017) 305–310.

[31] G.S. Heck, V.O. Pintro, R.R. Pereira, M.B. de Ávila, N.M.B. Levin, W.F. de Azevedo, Supervised Machine Learning Methods Applied to Predict Ligand- Binding Affinity, Curr. Med. Chem. 24 (2017) 2459–2470.

[32] R.B. Leal, V.R. Pinto-Junior, V.J.S. Osterne, I.A.V. Wolin, A.P.M. Nascimento, A.H.B. Neco, D.A. Araripe, P.G. Welter, C.C. Neto, J.L.A. Correia, C.R.C. Rocha, K.S. Nascimento, B.S. Cavada, Crystal structure of DlyL, a mannose-specific lectin from Dioclea lasiophylla Mart. Ex Benth seeds that display cytotoxic effects against C6 glioma cells, Int. J. Biol. Macromol. 114 (2018) 64–76.

[33] B.S. Cavada, V.R. Pinto-Junior, V.J.S. Osterne, M.V. Oliveira, I.B. Silva, E.P.P. Laranjeira, A.F. Pires, J.L.C. Domingos, W.P. Ferreira, J.S. Sousa, A.M.S. Assreuy, K.S. Nascimento, In depth analysis on the carbohydrate-binding properties of a vasorelaxant lectin from Dioclea lasiophylla Mart Ex. Benth seeds, J. Biomol. Struct. Dyn. 40 (2022) 6817–6830.

[34] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, UCSF Chimera--a visualization system for exploratory research and analysis, J. Comput. Chem. 25 (2004) 1605–1612.

[35] R.A. Laskowski, M.B. Swindells, LigPlot+: multiple ligand-protein interaction diagrams for drug discovery, J. Chem. Inf. Model. 51 (2011) 2778–2786.

[36] G. Libralato, E. Prato, L. Migliore, A.M. Cicero, L. Manfra, A review of toxicity testing protocols and endpoints with Artemia spp, Ecol. Indic. 69 (2016) 35–49.

[37] A.L. Gould, Technometrics. 15 (1973) 197–199.

[38] F. Gallego del Sol, C. Nagano, B.S. Cavada, J.J. Calvete, The first crystal structure of a Mimosoideae lectin reveals a novel quaternary arrangement of a widespread domain, J. Mol. Biol. 353 (2005) 574–583.

[39] P. Utarabhand, P. Akkayanont, Purification of a lectin from Parkia javanica beans, Phytochemistry. 38 (1995) 281–285.

[40] W. Suvachittanont, A. Peutpaiboon, Lectin from Parkia speciosa seeds, Phytochemistry. 31 (1992) 4065–4070.

[41] R. Sankaranarayanan, K. Sekar, R. Banerjee, V. Sharma, A. Surolia, M. Vijayan, A novel mode of carbohydrate recognition in jacalin, a Moraceae plant lectin with a beta-prism fold, Nat. Struct. Biol. 3 (1996) 596–603.

[42] J.V. Pratap, A.A. Jeyaprakash, P.G. Rani, K. Sekar, A. Surolia, M. Vijayan, Crystal structures of artocarpin, a Moraceae lectin with mannose specificity, and its complex with methyl-alpha-D-mannose: implications to the generation of carbohydrate specificity, J. Mol. Biol. 317 (2002) 237–247.

[43] A.F. dos Santos, B.S. Cavada, B.A.M. da Rocha, K.S. do Nascimento, A.E.G. Sant'Ana, Toxicity of some glucose/mannose-binding lectins to Biomphalaria glabrata and Artemia salina, Bioresour. Technol. 101 (2010) 794–798.

[44] F.V.S. Arruda, A.A. Melo, M.A. Vasconcelos, R.F. Carneiro, I.L. Barroso-Neto, S.R. Silva, F.N. Pereira-Junior, C.S. Nagano, K.S. Nascimento, E.H. Teixeira, S. Saker-Sampaio, B. Sousa Cavada, A.H. Sampaio, Toxicity and binding profile of lectins from the Genus canavalia on brine shrimp, Biomed Res. Int. 2013 (2013) 154542.

[45] R.R.S. Silva, C.R. Silva, V.F. Santos, C.R.S. Barbosa, D.F. Muniz, A.L.E. Santos, M.H.C. Santos, B.A.M. Rocha, K.L.R. Batista, L.M. Costa-Júnior, H.D.M. Coutinho, C.S. Teixeira, Parkia platycephala lectin enhances the antibiotic activity against multi-resistant bacterial strains and inhibits the development of Haemonchus contortus, Microb. Pathog. 135 (2019) 103629.

[46] G. de Oliveira Leite, S.A.A.R. Santos, R.R. Dos Santos Silva, C.S. Teixeira, A.R. Campos, Parkia platycephala Lectin (PPL) Inhibits Orofacial Nociception Responses via TRPV1 Modulation, Molecules. 27 (2022). https://doi.org/10.3390/molecules27217506.

[47] M. Bellal, J. Boudrant, L. Elfoul, R. Bonaly, Flocculation dispersion in Kluyveromyces lactis, Process Biochem. 30 (1995) 641–648.

Figure Legends

Figure 1. *Parkia nitida* lectin purification. A) Chromatogram and SDS-PAGE of the crude extract of Sepharose-mannose matrix (15 × 50 mm). PI and PII correspond to the unbound and bound fractions respectively. B) Chromatogram and SDS-PAGE of the size-exclusion chromatography in a BioSuite 250 matrix (0.78 cm x 30 cm). M – Molecular marker.

Figure 2. Lectin characterization. A) Thermal stability. B) pH stability and C) Divalent cations dependence.

Figure 3. *Parkia nitida* lectin (PNL) mass spectrometry analysis (electrospray ionization). A) Deconvoluted mass spectrum showing the isotopic mass of PNL; B) Overlay of sequenced peptides. Peptides derived from trypsin (T) digestion are in green, while chymotrypsin (Q) derivatives are in blue, and pepsin (P) derivatives are in yellow. Leucine/Isoleucin are represented as a X.

Figure 4. Structural analysis of *Parkia nitida* lectin (PNL); A) Superposition of the modeled structure of PNL (in yellow) with the structure of *Parkia biglobosa* lectin (PBL, PDB Id: 4mq0, in blue); B) PNL domain 1 interacting with α-methyl-D-mannoside (MMA) ; C) PNL domain 2 interacting with MMA; D) PNL domain 3 interacting with MMA. In B to D, the protein residues and the ligands are represented as sticks with carbons in yellow for the protein and in purple for the ligand, hydrogen bonds are represented as dashes in blue.

Figure 5. Dose response graph depicting the toxic effect of *Parkia nitida* lectin against Artemia nauplii. *$p < 0.05$ compared to control; #$p < 0.05$ compared to PNL (200 μg/ml).

Figure 1

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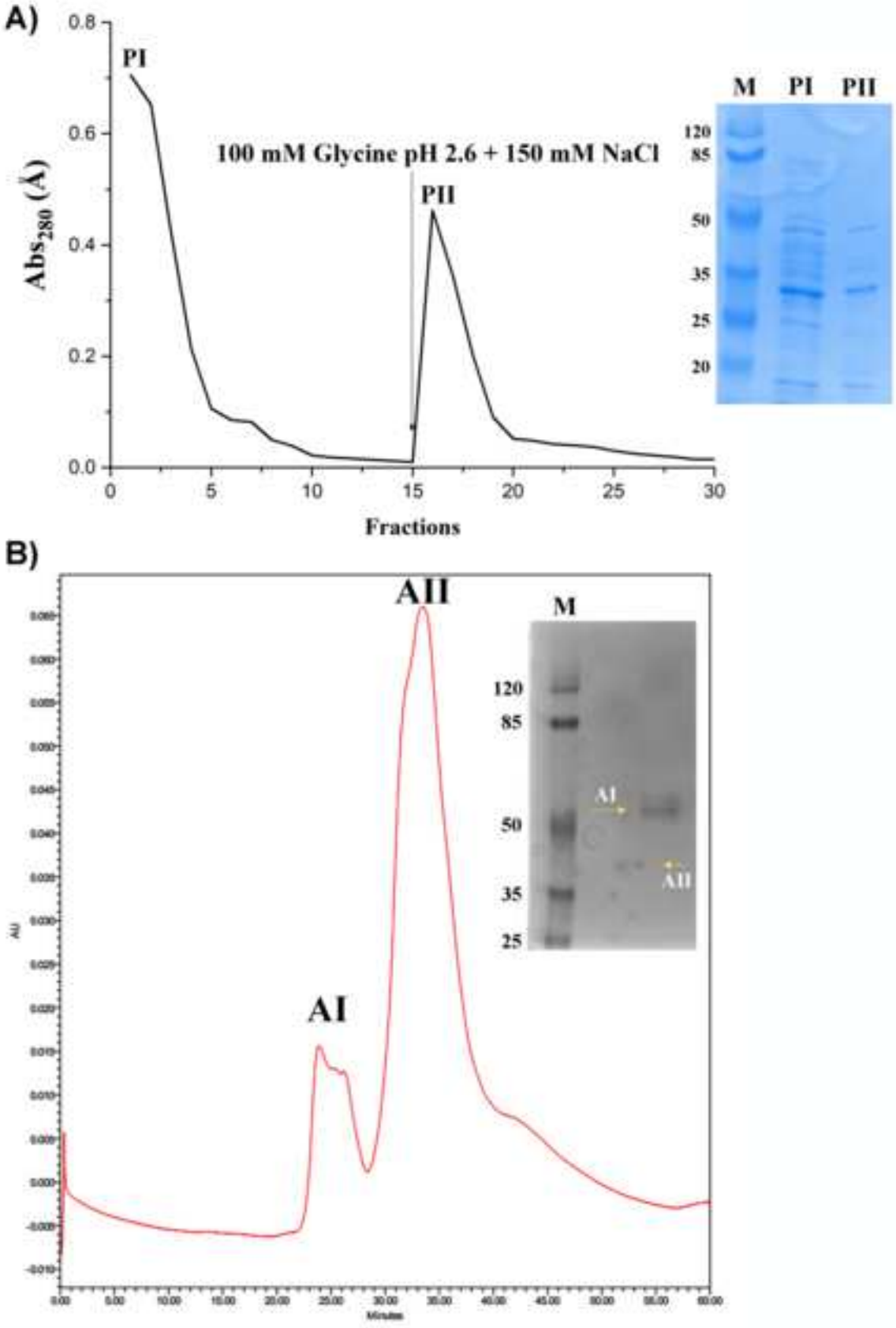

Figure 2

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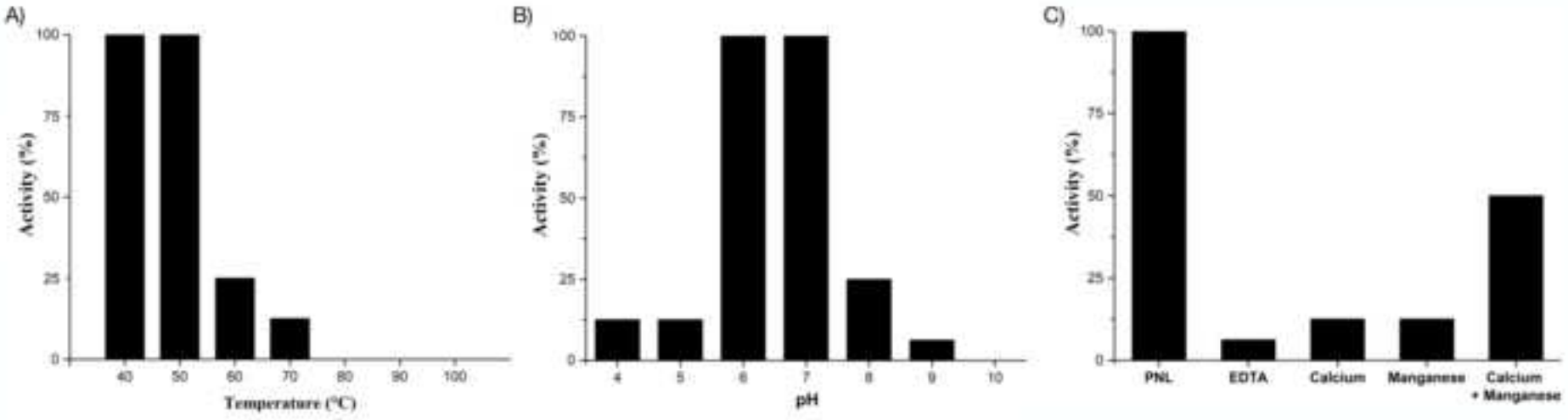

Figure 3

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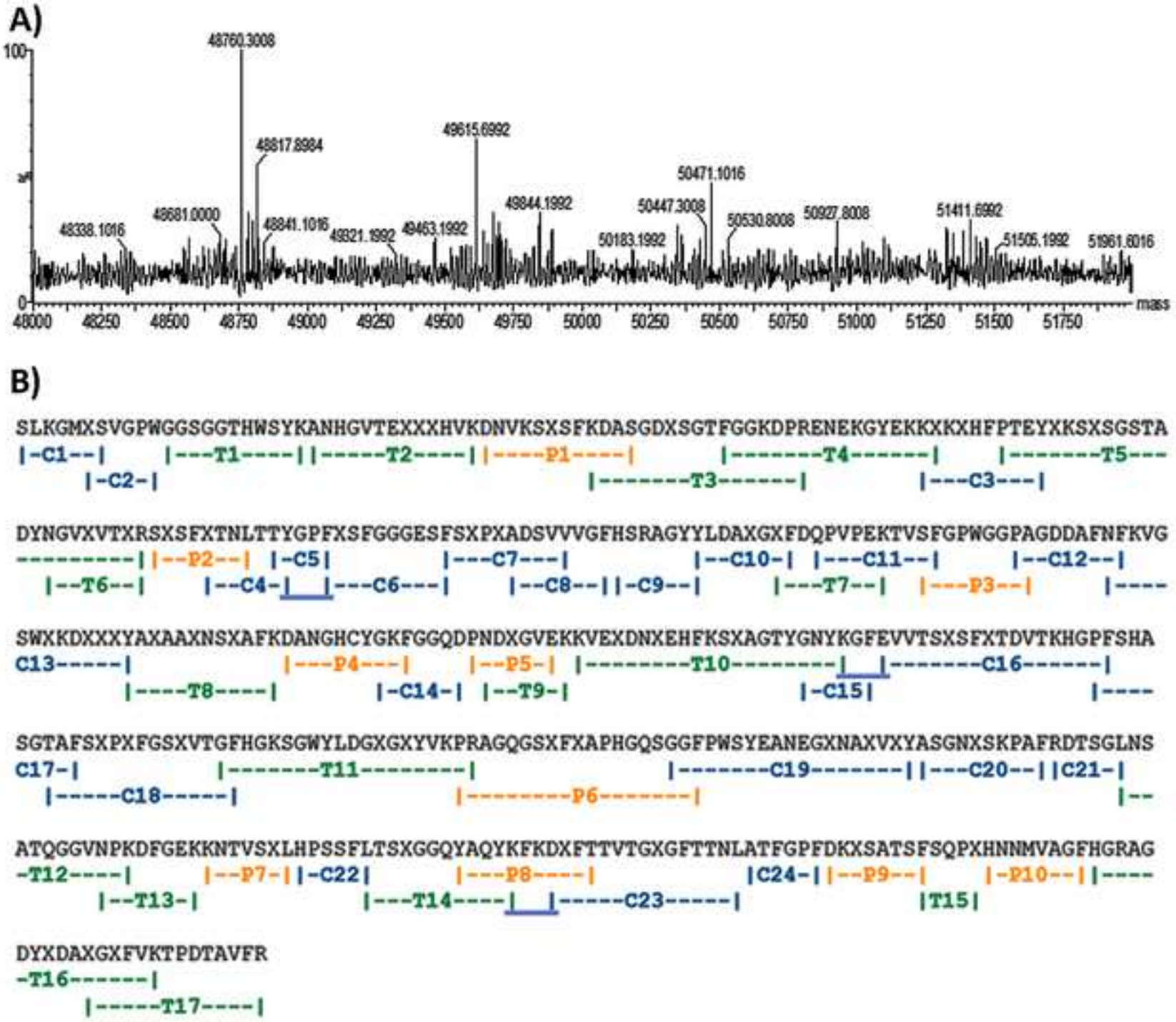

Figure 4

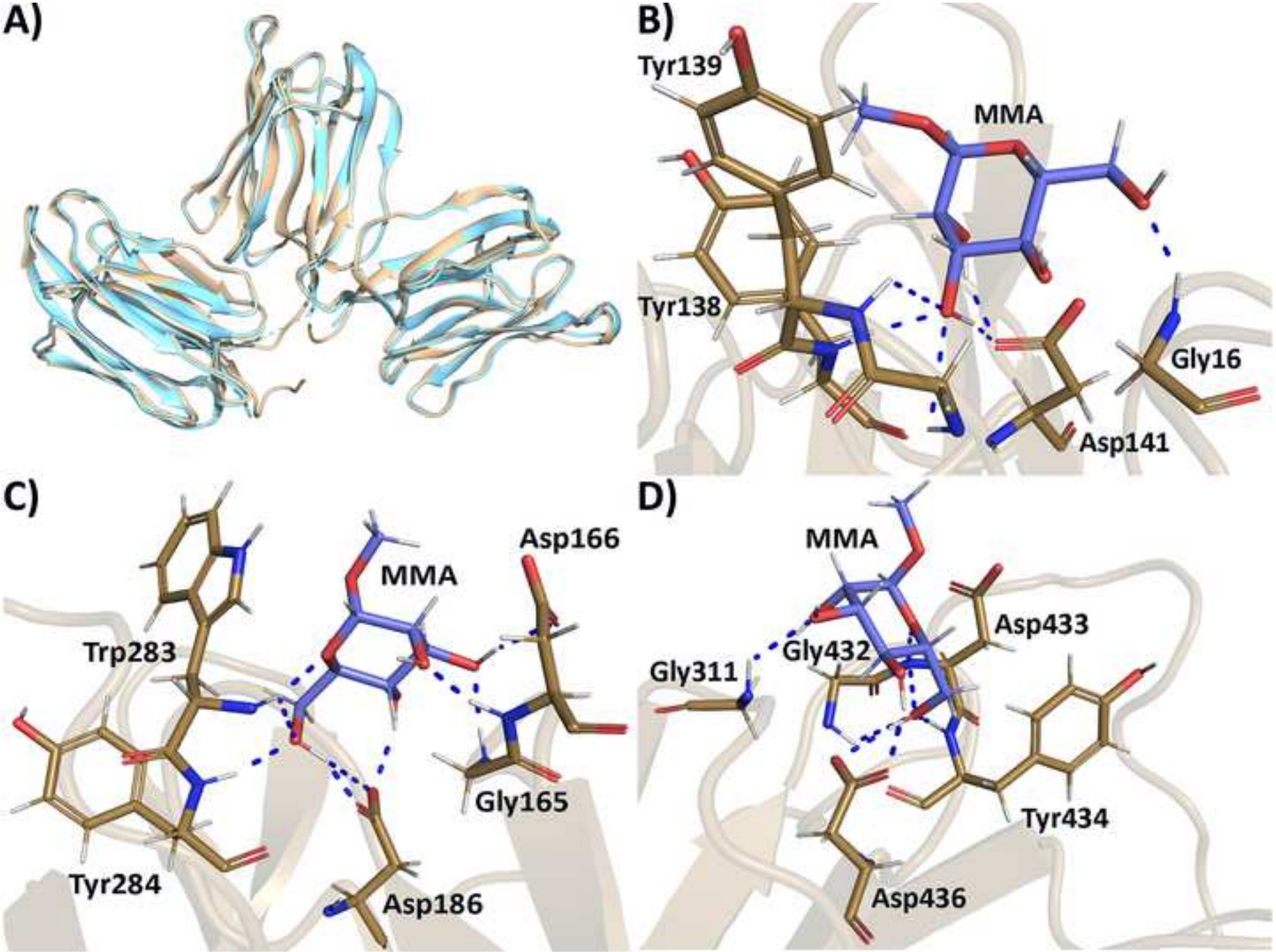

Figure 5

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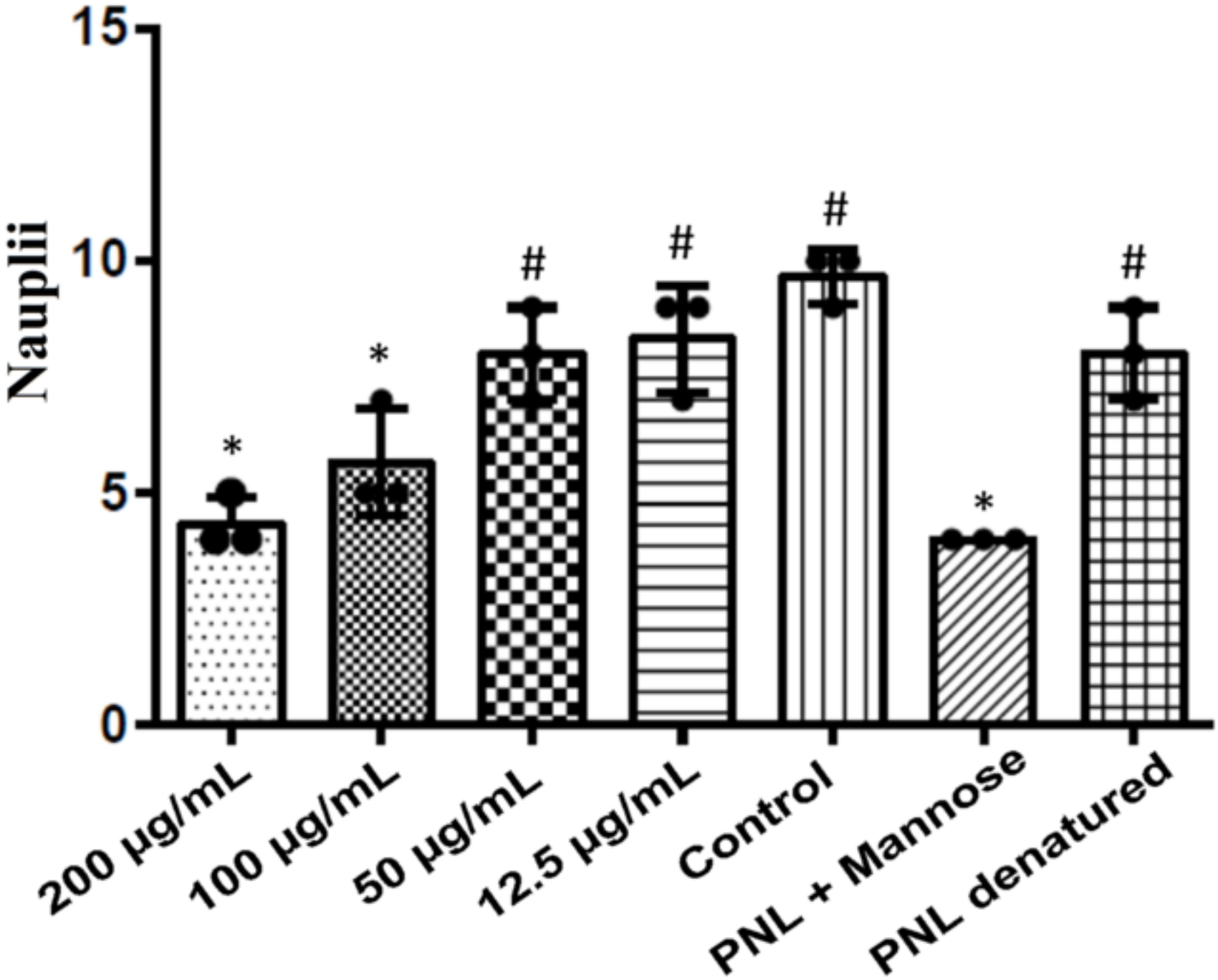

Table 1. Inhibition of hemagglutinating activity of *Parkia nitida* seed lectin by different carbohydrates.

Carbohydrate	**Minimum inhibitory concentration (MIC) (mM)**
D-glucose	25
D-mannose	6.25
α-methyl-D-mannoside	3.125
D-galactose	NI*
α-lactose	NI
L-fucose	NI
Sucrose	NI
D-raffinose	NI
α-methyl-D-galactoside	NI
Melibiose	NI

*NI, sugar not inhibitory until a concentration of 100 mM (sugars).

Table 2. Purification of lectin from *Parkia nitida* seeds (PNL).

Fractions	**[a] Total protein (mg/mL)**	**[b] Total H.A. (U.H./mL)**	**[c] Specific activity (U.H./mg)**	**[d] Purification (fold)**
Crude extract	13.1	128	9.77	1
F-0-60%	3.03	512	169	17,30
Sepharose-mannose affinity chromatography				
PI	0.04	-	-	-
PII	0.01	16	1,231	126
Molecular exclusion chromatography				
AI (PNL)	0.0019	32	16,842	1,723
AII	0.0006	4	6,666	682.3

[a] Protein content.

[b] Hemagglutinating activity expressed in hemagglutinating units (H.U.).

[c] Specific activity calculated as the ratio between the hemagglutinating activity and the protein content.

[d] Purification, calculated as the ratio between the Specific Hemagglutinating Activity of the fractions with the crude extract.

Table 3. Docking results of *Parkia nitida* lectin (PNL, modeled model) and *Parkia biglobosa* lectin (PBL, PDB Id: 4mq0) domains interacting with α-methyl-D-mannoside (in arbitrary units).

PNL	**Final score**	**Hbond score**	**Nonpolar score**	**Buried score**	**Ligand torsion**
Domain 1	-29.37	-7.63	-21.66	-0.19	0.11
Domain 2	-37.75	-9.32	-25.90	-2.63	0.10
Domain 3	-32.42	-7.15	-19.35	-5.60	0.03
PBL	**Final score**	**Hbond score**	**Nonpolar score**	**Buried score**	**Ligand torsion**
Domain 1	-33.25	-7.20	-22.26	-4.16	0.00
Domain 2	-40,63	-8.75	-29.01	-2.87	0.00
Domain 3	-34.18	-7.80	-21.09	-5.68	0.39