



Structural characterization and bacterial agglutination of a new OAAH-type lectin from the marine red alga *Amansia multifida* J.V. Lamouroux

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Abstract

Lectins are carbohydrate-binding proteins involved in diverse biological processes and with increasing relevance in biotechnological applications. In this study we report the structural characterization of a lectin from the marine red alga *Amansia multifida* (AML). Mass spectrometry analysis revealed two major isoforms with molecular masses of 28,388 Da and 28,661 Da. The primary structure of a lectin with a molecular weight of 28,661 Da was determined by combination of N-terminal Edman degradation, tandem mass spectrometry, and RACE-PCR, revealing four tandemly repeated domains and high sequence identity with members of the OAAH (*Oscillatoria agardhii* agglutinin homologous) lectin family. Notably, *A. multifida* belongs to the order Ceramiales, suggesting a broader taxonomic distribution for this lectin family, previously restricted to species from the order Gigartinales. Secondary and tertiary structure analyses suggested a predominantly β -sheet conformation and a β -barrel fold, characteristic of OAAH lectins. Molecular docking showed conserved recognition of mannosylated glycans, supported by the presence of the QWGGREGPI motif in all carbohydrate recognition domains. Although AML did not exhibit direct antibacterial or antibiofilm activity, it was able to agglutinate *Escherichia coli* and *Staphylococcus aureus* strains, indicating potential interactions with mannoside-rich surface glycans. These findings enhance our understanding of marine lectin diversity and highlight AML as a new lectin of OAAH family.

Keywords Lectin · Rhodophyta · β -barrel fold · OAAH-family · Bacterial agglutination

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Introduction

Lectins are proteins or glycoproteins that reversibly bind to carbohydrates, acting as key recognition molecules between cells and the extracellular matrix. Due to their carbohydrate-binding properties, lectins are involved in diverse physiological processes across various organisms (Sharon and Lis 2004; Costa Filho et al. 2025). Marine algal lectins, in particular, have demonstrated a wide range of biological activities (Singh and Walia 2018; Romero et al. 2021; Alvarez et al. 2023; de Arruda et al. 2023; Costa Filho et al. 2025). For instance, AML, a 24.5 kDa lectin isolated from marine red alga *Amansia multifida* J.V. Lamouroux (Costa et al. 1999), has been shown to modulate human lymphocyte activation (Lima et al. 1998), induce neutrophil migration (Neves et al. 2001), and exert antinociceptive and anti-inflammatory effects (Neves et al. 2007; Mesquita et al.

2021). Furthermore, AML has shown antiviral potential, effectively inhibiting HIV-1, HIV-2, and certain strains of the influenza virus at nanomolar concentrations (Gondim et al. 2019). However, it should be noted that the lectin preparations used in these studies likely contained multiple isoforms, as suggested by previous biochemical analyses of *A. multifida* lectins (Mesquita et al. 2021).

Despite the broad range of biological activities reported for AML, its primary structure and detailed structural characteristics remain poorly elucidated. In general, only a few lectins from marine red algae have been structurally characterized (Singh and Walia 2018). Among them, a notable group is the *Oscillatoria agardhii* agglutinin homologs (OAAHs), a family of lectins characterized by internal tandem repeats, specificity for high-mannose N-glycans, and occurrence across diverse taxa, including red algae, cyanobacteria, and certain soil bacteria (Sato et al. 2000; Koharudin et al. 2012; Whitley et al. 2013; Chaves et al. 2018a).

The observed inhibition of AML activity by glycoproteins such as avidin, fetuin, egg white, and yeast mannan (Costa et al. 1999), known to contain high-mannose-type N-glycans (Baenziger and Fiete 1979; Bruch and White 1982; Orlean 2012; Utratna et al. 2017), suggests its inclusion in OAAH family, as well as, a possible specificity toward mannosylated glycans. This glycan-binding profile is often associated with antimicrobial and anticancer effects in other lectins (Fukuda et al. 2006; Chaves et al. 2018b; Barre et al. 2019b, 2021; Romero et al. 2021; Alvarez et al. 2023), underscoring the relevance of further investigation into the potential antimicrobial properties of AML.

In this study we determined the primary structure of AML, predicted its three-dimensional structure, and performed molecular docking analyses. Additionally, we evaluated its antibacterial effects. Our findings indicate that AML is a novel member of the OAAH lectin.

Methods

Algae collection

Specimens of the marine red alga *Amansia multifida* were collected from the intertidal zone of Pacheco Beach, Ceará, Brazil. The specimens were brought to the laboratory on ice and kept at $-20\text{ }^{\circ}\text{C}$ until use. A small portion of the alga was stored at $-80\text{ }^{\circ}\text{C}$ for RNA extraction.

All collections and experiments with algae were authorized by environmental agencies through registrations in SISBIO (Sistema de Autorização e Informação em Biodiversidade, ID: 33,913–8) and SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado, ID: AC14AF9).

Purification of lectin

The lectin from *A. multifida* (AML) was purified using ion-exchange chromatography on a DEAE-Sephacel column ($1.5 \times 15.0\text{ cm}$). Extraction and precipitation with ammonium sulfate were performed following the protocol previously described (Costa et al. 1999). The fractions from chromatography were collected and tested for hemagglutinating activity against 3% trypsin-treated rabbit erythrocytes. Fractions showing hemagglutinating activity were pooled, dialyzed against distilled water, and freeze-dried. The hemagglutination activity assay was conducted as previously described, using rabbit erythrocytes treated with the protease trypsin (Sampaio et al. 1998). The hemagglutinating activity inhibition assay was performed with mannan from *Saccharomyces cerevisiae* (yeast mannan). Blood collection from the animals was authorized by the local institutional ethics committee (CEUAP – Ethics Committee for the Use of Production Animals ID: 2,211,202,101).

Molecular mass determination

The isotopic average molecular mass of AML was determined by liquid chromatography coupled with electrospray ionization-mass spectrometry (LC–ESI–MS) using a Synapt HDMS hybrid mass spectrometer (Waters, USA). Twenty-five micrograms of AML were applied to a C-18 nanocolumn ($75\text{ }\mu\text{m} \times 100\text{ mm}$) and eluted with a gradient of 10 to 85% acetonitrile (ACN) containing 0.1% formic acid (FA) at a flow rate of $0.6\text{ }\mu\text{L min}^{-1}$. Eluates were directly infused into the mass spectrometer using a nanoAcquity system connected to a nanoelectrospray source.

The instrument was calibrated with [Glu1] fibrinopeptide-B CID fragments. Mass spectra were acquired by scanning in the m/z range from 500 to 3500 at 2 scans s^{-1} . The mass spectrometer was operated in positive mode, with a source temperature of 363 K and a capillary voltage of 3 kV. Data collection and processing were controlled by MassLynx 4.1 software (Waters). The deconvolution of ESI mass spectra was performed using the MaxEnt 1 algorithm in the MassLynx software.

Primary structure determination

N-terminal amino acid sequencing

The N-terminal amino acid sequence of AML was determined by Edman degradation using a Shimadzu model PPSQ-31A protein and peptide sequencer (Shimadzu, Japan). PTH-amino acids from the N-terminus sequence were separated on a $2.0 \times 250\text{ mm}$ Wakosil ODS column

(Wako Pure Chemical, Japan) connected to a model LC-20AT pump. The absorbance was detected at 269 nm with a UV–vis SPD-20A detector.

The sequence similarity of the N-terminus was evaluated online using the basic local alignment search tool (BLAST) program (<http://www.ncbi.nlm.nih.gov/BLAST/>) from the National Center for Biotechnology Information (NCBI), where homologous sequences were identified using PROTEIN BLAST.

Tandem mass spectrometry (MS/MS) of peptides

SDS-PAGE was performed as described by Laemmli (1970). After staining, AML spots were excised following the procedure outlined as previously described (Shevchenko et al. 2006). The excised spots were subjected to digestion with trypsin and chymotrypsin (Promega, USA). Digestion was carried out in 25 mM ammonium bicarbonate at enzyme-to-substrate ratios of 1:50 or 1:100 (w/w). The digestion was maintained at 37 °C for 16 h and then stopped with 2 μ L of 2% formic acid. The peptides were extracted, separated by reverse-phase chromatography on a C-18 column as described above, and directly injected into the mass spectrometer.

LC–MS/MS was performed using data-dependent acquisition (DDA). The selected precursor ions were fragmented by collision-induced dissociation (CID) with argon as the collision gas. The lock mass used during acquisition was the m/z 785.84 ion of [Glu1] fibrinopeptide B. The CID spectra were manually interpreted. Peptide sequence similarity was evaluated online using PROTEIN BLAST from NCBI to identify homologous sequences.

Rapid Amplification of cDNA Ends (RACE)

Total cellular RNA from *A. multifida* was isolated using the AxyPrep Multisource Total RNA Miniprep kit (Axygen Biosciences, USA) following the manufacturer's protocol. Full-length cDNAs were synthesized using the SMARTer RACE cDNA Amplification kit (Clontech, USA).

cDNA was PCR-amplified using one-tenth of the RT reaction as a template. Degenerate primers for 3'RACE (AML_3'RACE; 5'-GTI CAG AAT CARTGG GGI GG-3') and 5'RACE (AML_5'RACE; 5'-CCI CCC CAY TGA TTC TAI AC-3') were designed based on MS/MS sequences: VQNQWGG. PCR was performed with Taq DNA polymerase (Thermo Scientific, USA) in a total volume of 25 μ L, including 2 μ L of cDNA, 0.4 μ M of each primer, 0.4 mM dNTP mix, 1 U of enzyme, and 1X PCR Buffer with 3 mM $MgCl_2$.

3'RACE PCR was performed using primer UPM 10X (Clontech, USA) with AML_3'RACE, and 5'RACE PCR was performed using primer UPM 10X with AML_5'RACE.

Both amplification protocols included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 90 s, and a final extension step at 72 °C for 5 min. The reactions were analyzed by agarose gel electrophoresis.

PCR products were purified using the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, USA), then cloned into the pGEM-T-easy vector and transformed into *E. coli* DH5 α (Novagen, Germany). Screening was performed by blue-white selection on LB agar containing 100 μ g mL⁻¹ ampicillin (Thermo Scientific, USA), 0.5 mM IPTG (Isopropyl β -D-1-thiogalactopyranoside; Thermo Scientific), and 80 μ g mL⁻¹ X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; Thermo Scientific). Cloning was performed in a laboratory with quality certification in biosafety according to national requirements (CQB: R007-2016). Recombinant plasmids were extracted using the illustraTM plasmidPrep Mini Spin kit (GE Healthcare, USA), verified by EcoRI digestion (Promega, USA), and sequenced using a Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, USA) by Helixxa (<http://www.helixxa.com.br/>, Brasil) with primers T7 promoter and SP6. Sequencing data were analyzed with the Phred-Phrap-Consed program (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998).

Full-length AML was amplified using primers AML_5'Fw (5'-CCA TGG GTC GTC GCT ACA CAG-3') and AML_3'Rv (5'-CTC GAG GGA GGA AAG AAT GCC CTT GAA G-3') based on sequences obtained from 3' and 5'RACE. The PCR conditions included an initial denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 5 min. Products were analyzed by agarose gel electrophoresis. Purification, cloning, and sequencing followed previously described protocols. Contigs were translated using the ExPASy translate tool (<http://web.expasy.org/translate/>).

Sequence similarity to the primary structure of AML was evaluated using PROTEIN BLAST tool available on the NCBI website. Amino acid sequences were aligned using MultAlin (Corpet 1988) and visualized with EsPript 2.2 (Gouet et al. 1999). Potential N-glycosylation sites (NXT motifs) were predicted using the NetNGlyc 1.0 server (GUPTA; JUNG; BRUNAK, 2004) (<https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/>).

Phylogenetic analysis was conducted to evaluate the evolutionary relationship between AML and other red algal lectins belonging to the OAAH family. Multiple sequence alignment of the full-length amino acid sequence of AML and other OAAH lectins was performed using MUSCLE (Multiple Sequence Comparison by Log-Expectation). The phylogenetic tree was constructed based on the aligned sequences using the Maximum likelihood algorithm implemented in MEGA version 12.0 (<https://www.megasoftware.net/>).

net/) with evolutionary distances computed using the JTT matrix-based model. Bootstrap analysis with 1000 replicates was performed to assess the robustness of the resulting branches. Lectin from *Oscillatoria agardhii* (OAA) was used as an outgroup to root the tree.

Circular dichroism measurement

Circular dichroism (CD) spectroscopy was performed using a Jasco J-815 spectropolarimeter (Jasco International Co., Japan) equipped with a Peltier system for temperature control. The AML (0.20 mg mL⁻¹ in 20 mM phosphate buffer, pH 7.0) was placed in a rectangular quartz cuvette with 0.05 cm path length. Spectra were acquired at a scan speed of 50 nm min⁻¹ with a bandwidth of 1 nm, covering the wavelength range of 190–240 nm (far UV).

Secondary structure analysis was performed using the DICHROWEB web server (Whitmore and Wallace 2004). The thermodynamic parameters of lectin folding were determined by monitoring changes in ellipticity at 216 nm as a function of temperature (Greenfield 2006). The lectin was subjected to temperatures ranging from 20 to 100 °C.

Dynamic Light Scattering (DLS) analysis

Dynamic Light Scattering (DLS) was performed to evaluate the hydrodynamic behavior and oligomerization profile of the lectin AML under different pH conditions. The theoretical hydrodynamic diameter of AML was first estimated in silico using the HullRad program based on the modeled protein structure.

For experimental measurements, the lectin was solubilized at a final concentration of 0.5 mg mL⁻¹ in three different buffers: 20 mM sodium acetate (pH 5.0), 20 mM sodium phosphate (pH 7.0), and 20 mM glycine–HCl (pH 10.0). Prior to analysis, all samples were centrifuged at 10,000 × g for 20 min at 4 °C to remove insoluble aggregates and particulate matter.

The supernatants were transferred to disposable polystyrene cuvettes, and DLS measurements were performed at 25 °C using a Zetasizer Advance Ultra (Malvern Instruments, UK). Hydrodynamic size distributions were calculated from the intensity autocorrelation functions using the general-purpose analysis model provided by the manufacturer.

Each condition was measured in triplicate, and each replicate represented the average of multiple scans, following procedures adapted from previous reports (Saraiva 2025).

Structural prediction

Structural modeling of the AML lectin was performed using the AlphaFold2 server, utilizing the lectin amino

acid sequence determined (Jumper et al. 2021). The server generated five predicted three-dimensional structures and model analysis was based on sequence coverage and identity graphs. For stereochemical evaluation, criteria such as steric overlaps, C β shift parameters, Ramachandran plots, rotamers, and bond angle quality were evaluated by the MolProbity server (Chen et al. 2010; Williams et al. 2018). The highest quality model was selected and visually inspected in PyMol Molecular Graphics System, Version 1.8 (Schrödinger, LLC).

Docking calculations

For the current purpose, docking calculations were performed with a typical substrate of this lectin family, the pentamannoside, 3 α ,6 α -mannopentaose, which coordinates were obtained from the crystal structure of BOA lectin from *Burkholderia oklahomensis* (ID: 4GK9) (Whitley et al. 2013). The calculations were conducted with AutoDock Vina version 1.5.6 (Trott and Olson 2010), where the receptor structure was treated as rigid, while the ligand remained flexible. The search was carried out within a cubic space of 30 Å × 30 Å × 30 Å positioned over each carbohydrate binding sites, under the following conditions: number of conformations = 20, exhaustiveness = 15, and seed = 2009. The calculations were analyzed based on theoretical binding energy (in kilocalories per mole) and selected according to structural comparisons.

Additionally, the carbohydrate recognition domains (CRD) of AML were analyzed with the DogSiteScorer tool (<https://proteins.plus>), which applies a Gaussian Difference filter to a three-dimensional representation of the protein to locate potential binding sites (Volkamer et al. 2012; Schoning-Stierand et al. 2020).

AutoDock Vina was validated for the presented purpose through redocking calculations with the above mentioned crystal structure of BOA lectin, complexed with the pentamannoside 3 α ,6 α -mannopentaose, whose structure is deposited in the RCSB Protein Data Bank (<https://www.rcsb.org/>). The results were analyzed based on the root-mean-square deviation (RMSD) of atomic positions between the experimentally determined and calculated ligand positions, using the RMSD calculator tool in the software Visual Molecular Dynamics (Humphrey et al. 1996).

Antibacterial activity

Three reference strains from the American Type Culture Collection (ATCC): *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 700698 and *Escherichia coli* ATCC 11303, were used.

Bacterial agglutination

The bacteria were grown overnight in Trypticase Soy Broth (TSB; Himedia, India) at 37 °C and harvested by centrifugation at 2000×g for 10 min. The cells were washed twice with 20 mM Tris–HCl, pH 7.6, containing 150 mM NaCl (TBS), suspended in TBS containing 4% formaldehyde, and incubated at 4 °C for 16 h. Afterward, the cells were washed twice with TBS and resuspended in the same buffer. Prior to use, the cell density of each bacterial suspension was adjusted to a final concentration of 2×10^6 cells mL⁻¹. Agglutination assays were as previously described (de Melo et al. 2014).

Effects of AML on planktonic cells

The effects on planktonic cells of AML was evaluated by the microdilution method according to the Clinical and Laboratory Standards Institute document M07-A10, with some modifications (Vasconcelos et al. 2014; CLSI 2015).

Effect of AML on biofilm formation

The effect on biofilm formation of AML was evaluated by microtiter plate test described by Stepanovic and collaborators, with some modifications (Stepanović et al. 2000; Vasconcelos et al. 2014; Marques et al. 2018).

Results

Purification of lectin AML

AML was purified by combination of ammonium sulfate precipitation and a single chromatographic step on a DEAE-Sephacel column. SDS-PAGE analysis revealed a single band with a relative molecular mass of approximately 29 kDa (data not shown), consistent with the purification protocol and yields previously described by Costa et al. (1999). Hemagglutinating activity was monitored throughout the purification process, and the lectin was able to agglutinate trypsin-treated rabbit erythrocytes at 512 U.H mL⁻¹ at a protein concentration of 1 mg mL⁻¹. The hemagglutination inhibition assay revealed the minimum inhibitory concentration (MIC) of hemagglutination activity with yeast mannan (7.8 µg mL⁻¹).

N-terminal amino acid sequence

The first twelve amino acids of AML were determined by Edman degradation. Residues in positions 1, 2, 3, and 10 could not be identified. The N-terminal sequence of AML was XXXYTVENQXGG. A search for similarity using

PROTEIN BLAST from NCBI revealed a high similarity between AML and members of the OAAH family.

Molecular mass and amino acid sequencing by MS/MS

The LC–MS spectra showed distinct ion series related to AML (Fig. 1). The two main ions, with masses of 28,388 Da and 28,661 Da, were observed, which are close to the apparent molecular mass of 29 kDa observed in SDS-PAGE. These two ion series indicate the presence of at least two isoforms in the AML preparations. The small variations in the ion series suggest the presence of adduct formations or other isoforms.

Manual interpretation of the sequencing data revealed peptides similar to lectins from marine red algae belonging to the OAAH family (ASL-1: BAX08598.1; KAA-2: BAU19431.1; KSA-2: BAR91206.1; ESA-2: P84331.1; SfL-1: C0HL89.1). In some regions of the sequence, more than one peptide with slight amino acid differences was detected, suggesting the presence of isoforms in the AML preparation (Table S1). As a result, it was not possible to overlap the amino acid sequences of the peptides to obtain a complete primary sequence of at least one AML isoform.

Primary structure of AML

The sequencing of the 3'RACE PCR resulted in a lectin protein coding sequence of 911 nucleotides, while the 5'RACE PCR resulted in 442 nucleotides. Specific oligonucleotides designed from these sequences for the open reading frame (ORF) of the lectin resulted in a sequence of 810 nucleotides, equivalent to 270 amino acids. The amino acid sequences determined by MS/MS overlapped and confirmed the sequence obtained from cDNA, thereby establishing the primary structure of one of the AML isoforms (Fig. 2).

The AML ORF consists of 270 amino acids, including an initiating methionine residue and one cysteine residue, with no glycosylation sites. The sequence obtained by overlapping the peptides with the cDNA data contains 269 amino acids, excluding the initiating methionine. The peptide sequences showed some heterogeneities, confirming the existence of isoforms (data not shown). The calculated mass for the AML with 269 amino acids matches the mass determined by LC–MS (28,661 Da).

The primary structure of AML includes four tandemly repeated domains, each consisting of approximately 66 amino acids, with 45.44% sequence identity. AML showed sequence similarity with MPA-2 (BAX08602.1), ASL-1 (BAX08598.1), SfL-1 (C0HL89.1), SfL-2 (C0HL90.1), KAA-2 (BAU19431.1), KSA-2 (BAR91206.1), ESA-2

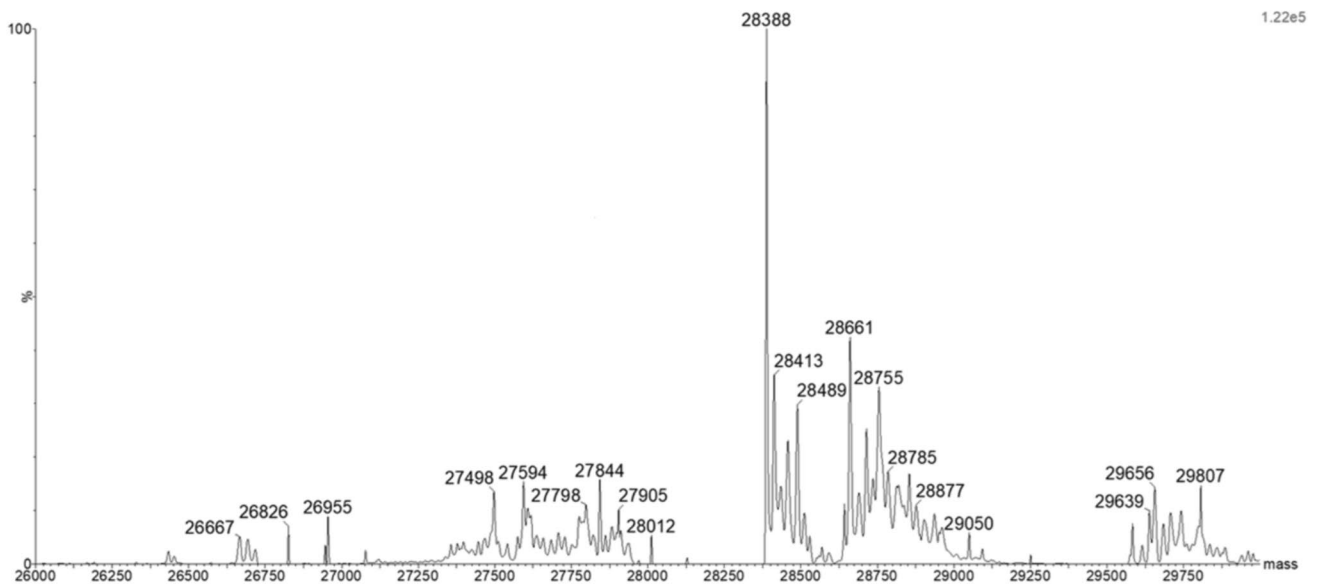
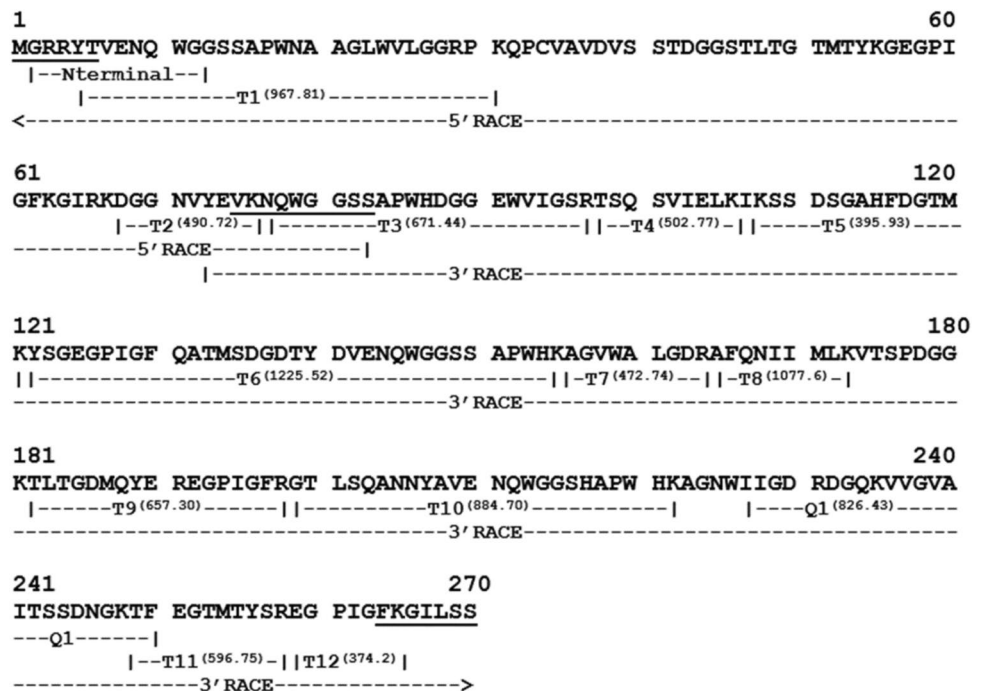


Fig. 1 Molecular mass determination with major ions 28,388 Da and 28,661 Da of AML. ESI–MS deconvoluted mass spectra of LC–MS with zoom of region of mass between 28,000 and 29,000 Da

Fig. 2 Primary structure of one of the AML isoforms. The primary structure was determined by cDNA cloning, N-terminal sequencing, and tandem mass spectrometry of tryptic peptides (T). Peptide molecular masses, as determined by mass spectrometry, are shown in parentheses. Underlined residues indicate primer design locations. Arrows represent the sequencing directions obtained from the 3' RACE and 5' RACE experiments



(P84331.1), EDA-2 (BAR91516.1) and BOA (4GK9_A) (Fig. 3), among others.

The phylogenetic tree constructed using the Maximum likelihood method based on the alignment of red algal OAAH-type lectins revealed that AML, from *Amansia multifida* (order Ceramiales), forms an independent clade clearly separated from most lectins of the Gigartinales (Fig. 4). The topology also showed coherent grouping among closely

related taxa: ESA-2 and EDA-2, both from *Eucheuma* species, clustered within the same branch, while SfL-1 and SfL-2 from *Solieria filiformis* also grouped together. The exclusion of near-identical sequences, such as KAA-2 and KSA-2, improved the tree's resolution and boosted bootstrap support at several internal nodes. These results suggest distinct phylogenetic positions for each lectin consistent with their taxonomic origin.

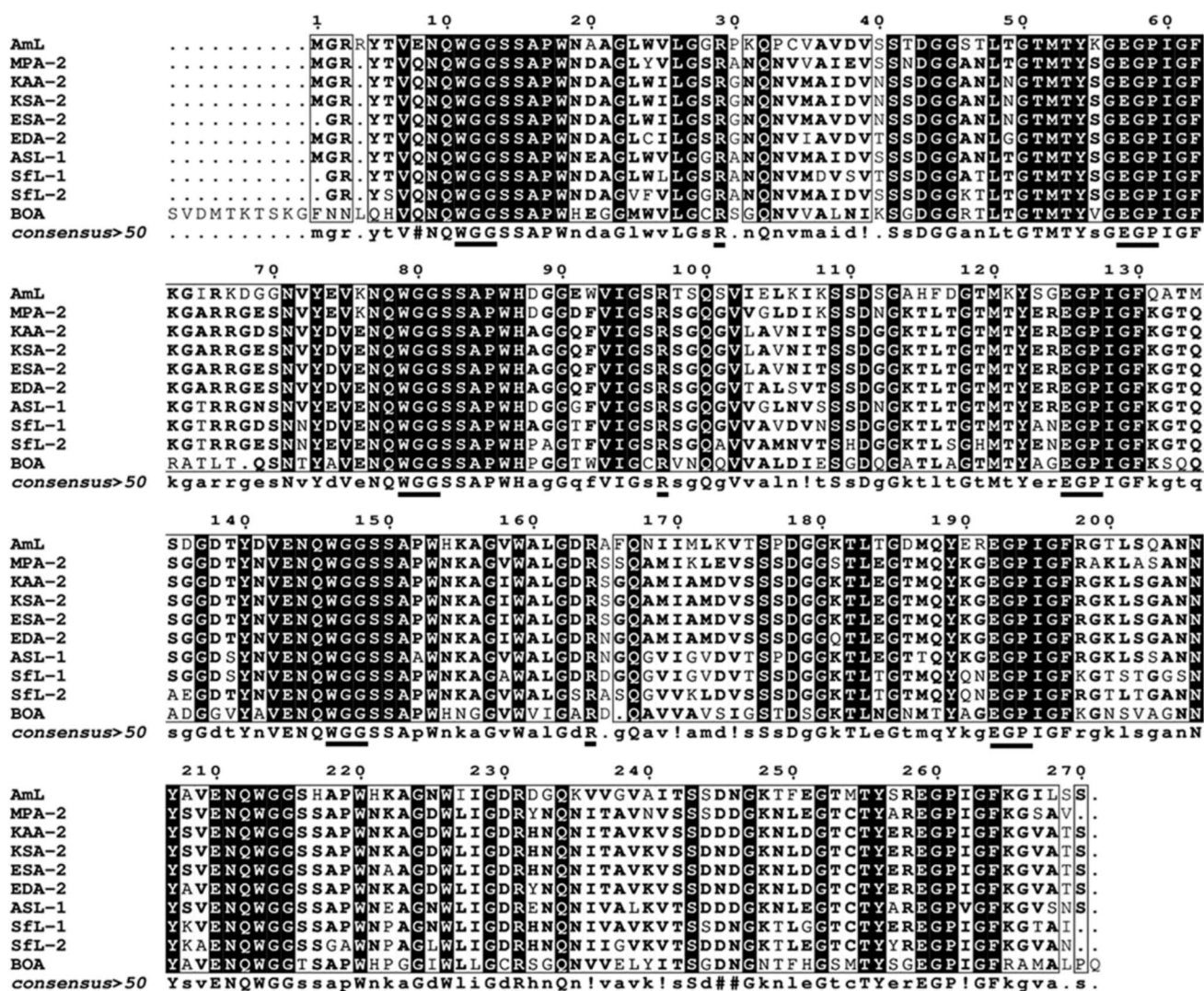


Fig. 3 Alignment of AML with other OAAH-family members. The amino acid sequence of AML was aligned with *Meristotheca papulosa* agglutinin (MPA-2), *Kappaphycus alvarezii* agglutinin (KAA-2), *Kappaphycus striatus* agglutinin (KSA-2), *Euclima serra* agglutinin (ESA-2), *Euclima denticulatum* agglutinin (EDA-2), *Agar-*

dhiella subulata agglutinin (ASL-1), *Solieria filiformis* lectin (Sfl-1 and Sfl-2) and *Burkholderia oklahomensis* EO147 agglutinin (BOA). Underlined residues indicate amino acids involved in carbohydrate binding, as described by Whitley et al. (2013). Black and white boxes represent identical and non-identical amino acids, respectively

Circular dichroism

Native AML exhibited minimum absorption at 215 nm in CD measurements (Fig. 5 A). As determined by the CON-TIN prediction method, a general-purpose constrained regularization method for continuous distributions (van Stokkum et al. 1990), the theoretical secondary structure of AML was composed of 3% α -helix, 40% β -sheet, 19% β -turn, and 38% coil.

Thermal denaturation analysis of AML revealed a non-linear unfolding profile (Fig. 5 B). The midpoint of the thermal transition (T_m), determined from the smoothed ellipticity curve, was estimated at approximately 60.8 °C,

consistent with partial unfolding events occurring over an extended temperature range.

Dynamic Light Scattering (DLS) analysis

The hydrodynamic behavior of AML was evaluated by DLS under three different pH conditions (Fig. S1). The theoretical hydrodynamic radius of the monomer, estimated in silico using HullRad, was 2.4 nm, corresponding to a theoretical hydrodynamic diameter of approximately 4.8 nm. At pH 5.0 (acetate buffer), the experimental DLS profile revealed a monodisperse population with a mean

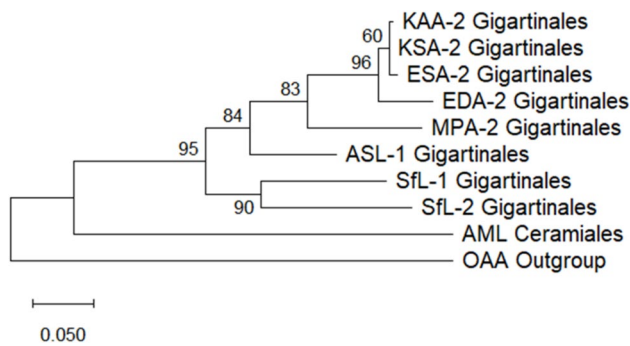


Fig. 4 Phylogenetic relationship among red algal lectins of the OAAH family. Maximum likelihood phylogenetic tree of OAAH-type lectins from red algae, constructed in MEGA 12 using the JTT substitution model. The tree was based on multiple sequence alignment generated by MUSCLE and evaluated with 500 bootstrap replicates. Lectins from *Amansia multifida* (AML), *Meristotheca papulosa* (MPA-2: BAX08602.1), *Kappaphycus alvarezii* agglutinin (KAA-2: BAU19431.1), *Kappaphycus striatus* agglutinin (KSA-2: BAR91206.1), *Euclima serra* (ESA-2: P84331.1), *Euclima denticulatum* (EDA-2: BAR91516.1), *Agardhiella subulata* (ASL-1: BAX08598.1), and *Solieria filiformis* (Sfl-1: C0HL89.1, and Sfl-2: C0HL90.1) were included. The lectin from *Oscillatoria agardhii* (OAA: WP_227350609.1) was used as the outgroup. Bootstrap values greater than 50% are indicated at the respective nodes

hydrodynamic diameter of about 4.5 nm, which is consistent with the monomeric state predicted by the theoretical model.

At pH 7.0 (phosphate buffer), AML displayed a single population centered around 7–8 nm, suggesting the formation of a dimeric species. The observed increase in particle size at this pH indicates the stabilization of interfacial interactions between subunits, likely mediated by changes in surface charge distribution.

At pH 10 (glycine–HCl buffer), AML exhibited a dramatic shift in its hydrodynamic profile, with the appearance of a single, broad peak centered around 1.5–2.0 μm in diameter, indicating extensive aggregation of protein under highly basic conditions.

Structural prediction

Predictive analysis revealed that the AML lectin structure consists of two β -barrel domains arranged transversely and connected by a flexible segment (Fig. 6 A). The carbohydrate binding sites are located on the apical and basal portions of each barrel, totalling four CRDs, being numbered according to the structure of the lectin, suggesting site 1 as the closest to the N-terminus, and site 4 the closest to the C-terminus (Fig. 6 B). The obtained structure was analyzed and validated, showing 98.13% of amino acid residues disposed in favoured regions of the Ramachandran plot.

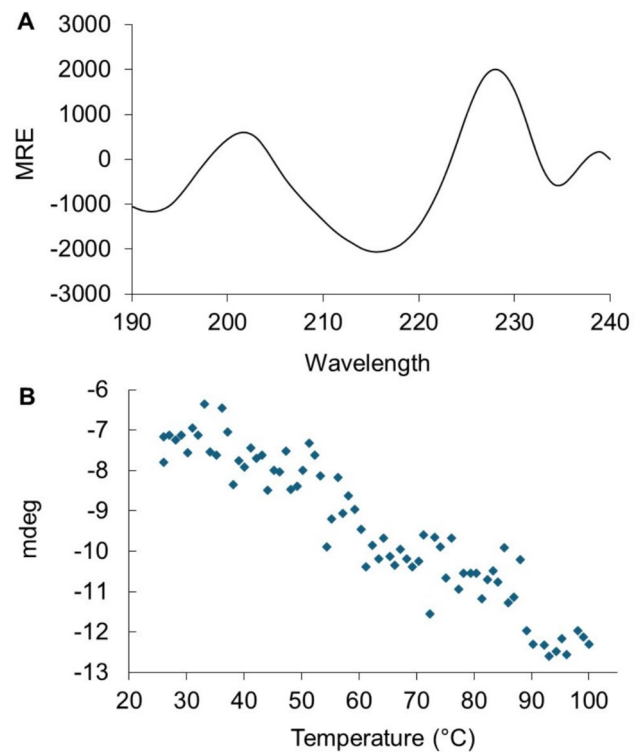


Fig. 5 Far-UV CD spectra of AML. **(A)** Far-UV circular dichroism spectrum of AML recorded from 190 to 240 nm. The measurement was performed using a 0.05 cm path length cuvette at a protein concentration of 0.2 mg mL⁻¹ in PBS, pH 7. **(B)** Thermal denaturation profile of AML monitored at a fixed wavelength. The midpoint of the thermal transition (T_m) was estimated at approximately 60.8 °C, and the curve shows a non-linear unfolding pattern suggestive of multi-state transitions

Docking calculations

The results of the docking calculations revealed similar interaction patterns with pentamannoside in all binding sites (Table 1). Man1–Man5 correspond to the five mannose units of the pentamannoside ligand. The side chains of the residues arginine, three glycines, glutamine, glutamic acid, and proline form polar interactions with different oxygen atoms of the α -D-mannose (Bma1) and α -mannose (Man2 and Man3) units of the ligand (Fig. 7). However, residues such as tryptophan and isoleucine are responsible for hydrophobic contacts, establishing interactions with the Bma1 and Man3 units of the ligand. In this case, the Trp residue forms a hydrophobic interaction with the pyranoside ring of one of the mannose residues in the ligand (Bma1), influencing properties such as binding affinity and the conformational stability of the ligand.

The described interactions occur within a radius of 4.0 Å from the ligand surface. Therefore, longer π - σ type interactions (4.0–5.0 Å), characteristic of an aromatic-single bond system, are detected between a mannose unit and the side

Fig. 6 Overall structure of AML. **(A)** The structure of AML is represented as a pale green cartoon. **(B)** The carbohydrate recognition domain (CRD) of AML is shown as a transparent pale green cartoon, with interactive residues as green lines and the bound carbohydrate molecules as pink sticks

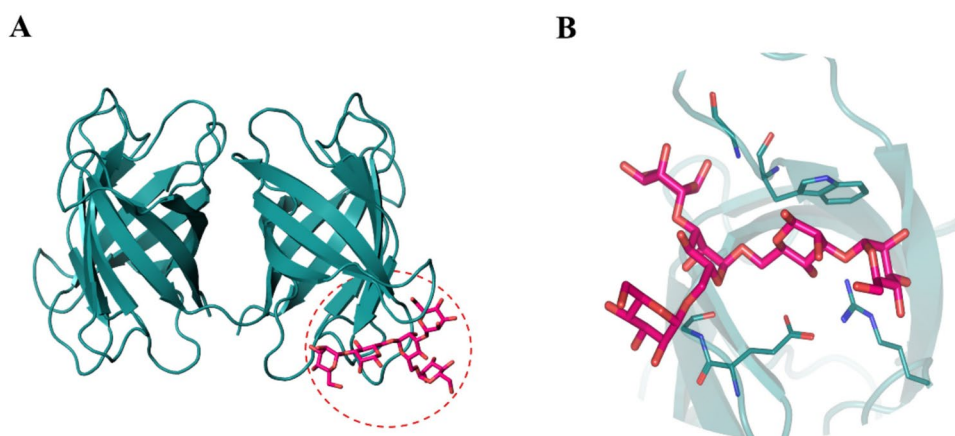


Table 1 Amino acid composition and topological properties of AML carbohydrate binding sites

Sites	Amino acid composition	Depth (Å)	Surface area (Å ²)	Volume (Å ³)
1	GLN ¹⁰ , TRP ¹¹ , GLY ¹² , GLY ¹³ , ARG ⁹⁷ , GLU ¹²⁵ , GLY ¹²⁶ , PRO ¹²⁷ , ILE ¹²⁸	16.49 Å	845.72 Å ²	925.70 Å ³
2	ARG ²⁹ , GLU ⁵⁷ , GLY ⁵⁸ , PRO ⁵⁹ , ILE ⁶⁰ , GLN ⁷⁸ , TRP ⁷⁹ , GLY ⁸⁰ , GLY ⁸¹	18.26 Å	666.45 Å ²	741.38 Å ³
3	ARG ¹⁶⁴ , GLU ¹⁹² , GLY ¹⁹³ , PRO ¹⁹⁴ , ILE ¹⁹⁵ , GLN ²¹² , TRP ²¹³ , GLY ²¹⁴ , GLY ²¹⁵	23.16 Å	863.87 Å ²	1254.40 Å ³
4	GLN ¹⁴⁵ , TRP ¹⁴⁶ , GLY ¹⁴⁷ , GLY ¹⁴⁸ , ARG ²³¹ , GLU ²⁵⁹ , GLY ²⁶⁰ , PRO ²⁶¹ , ILE ²⁶²	18.21 Å	916.43 Å ²	884.22 Å ³

chains of Trp residues. Additionally, the Man4 and Man5 units do not establish detectable interactions with site residues within the expected radius.

Bacterial aggregation

The AML was able to agglutinate cells of *E. coli* and two strains (ATCC 25923 and ATCC 700698) of *S. aureus* at 500 µg mL⁻¹ (Fig. 8). However, AML did not show inhibition of planktonic growth and had no effect on biofilm formation (data not shown).

Discussion

In this study, we successfully purified and characterized the lectin from *Amansia multifida* (AML). The purified protein exhibited an apparent molecular mass of approximately 29 kDa in SDS–PAGE and its hemagglutinating activity was inhibited by yeast mannan, as previously reported for the lectin from this species (Costa et al. 1999). Differences between SDS–PAGE estimations reported in different studies may reflect variations in electrophoretic migration and calibration with distinct molecular weight standards. Mass spectrometry analysis provided further insights into the molecular mass and sequence of AML, with two major ions detected at 28,388 Da and 28,661 Da, suggesting the existence of at least two isoforms. This heterogeneity was corroborated

by peptide sequencing, which indicated sequence variations. The calculated molecular mass of 28,661 Da, based on the determined primary structure, closely matched the observed mass, validating the accuracy of our structural characterization.

The amino acid sequence of AML revealed strong similarity to other lectins of the OAAH family, presenting tandemly repeated domains characteristic of this group. Sequence identity was observed with MPA-2 (70.72%), ASL-1 (69.66%), Sfl-1 (68.06%), Sfl-2 (67.80%), KAA-2 (66.67%), KSA-2 (66.67%), ESA-2 (66.67%), EDA-2 (66.67%), and BOA (60.92%). Interestingly, all macroalgal lectins previously characterized as members of the OAAH family have been isolated from species within the order Gigartinales (Agardh 1847, 1872; Kraft and Wynne 1979; Gabrielson 1985; Gabrielson and Cheney 1987; Liao 1996). In contrast, *A. multifida* belongs to the order Ceramiales (Lamouroux 1809), suggesting that AML may represent the first OAAH lectin identified in a macroalga outside Gigartinales, and indicating a possible broader taxonomic distribution for this lectin family.

In fact, phylogenetic distinction of AML from other OAAH-type lectins highlights a potentially unique evolutionary path within this lectin family. While all previously characterized red algal OAAH lectins have been derived from species in the Gigartinales, AML originates from *A. multifida*, a member of the Ceramiales. This phylogenetic separation, supported by bootstrap analysis, suggests

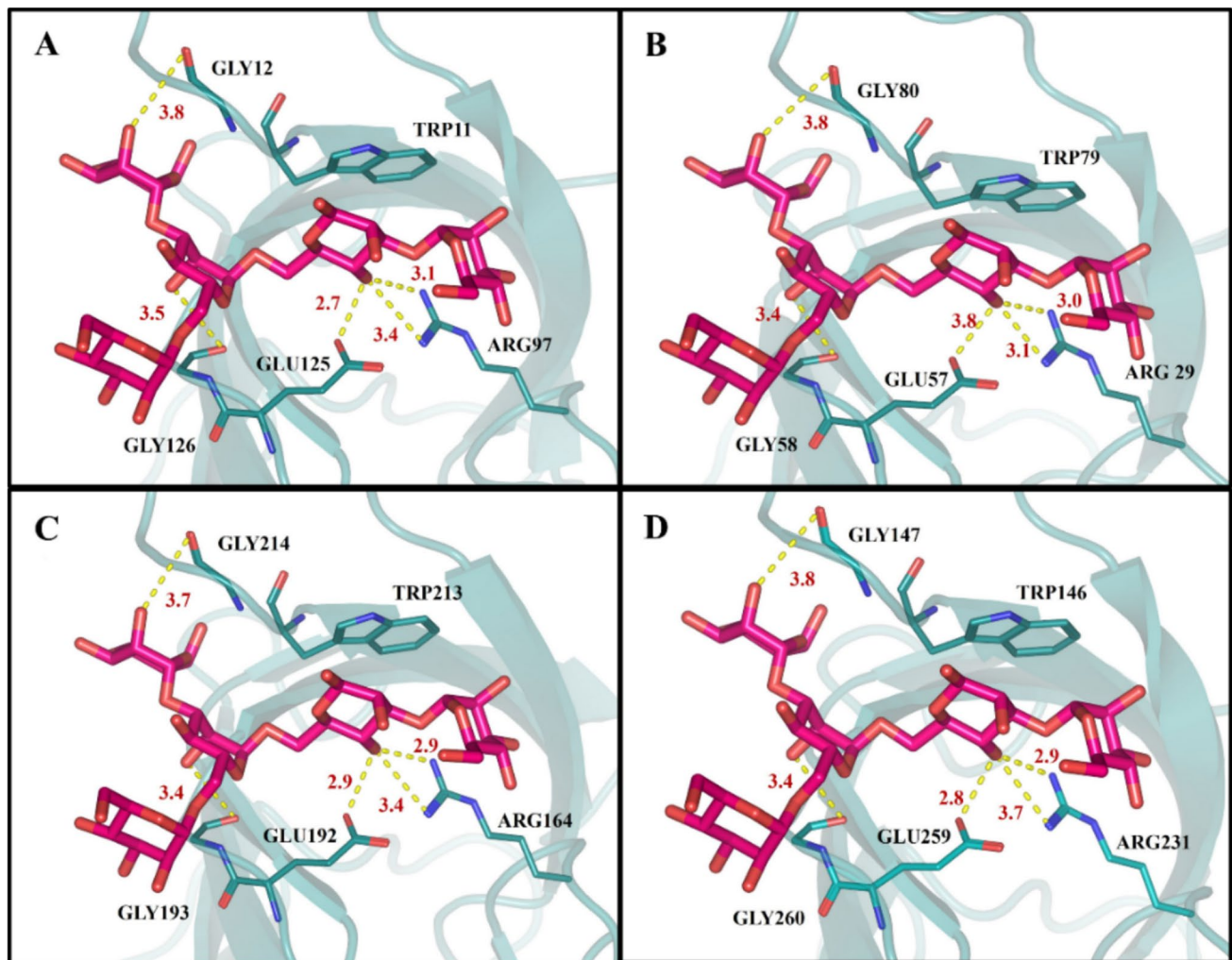


Fig. 7 Carbohydrate binding sites of AML and docking results. Coordination of 3 α ,6 α -mannopentaose at the four CRDs of AML, represented by pink sticks (A, B, C, and D). The protein structure is shown as a pale light green cartoon, while the AML residues and key inter-

actions are represented as light green sticks and yellow dashed lines, respectively. The cutoff distance for interactions considered in the analysis was 4.0 Å. For clarity, some interactions and binding residues are omitted

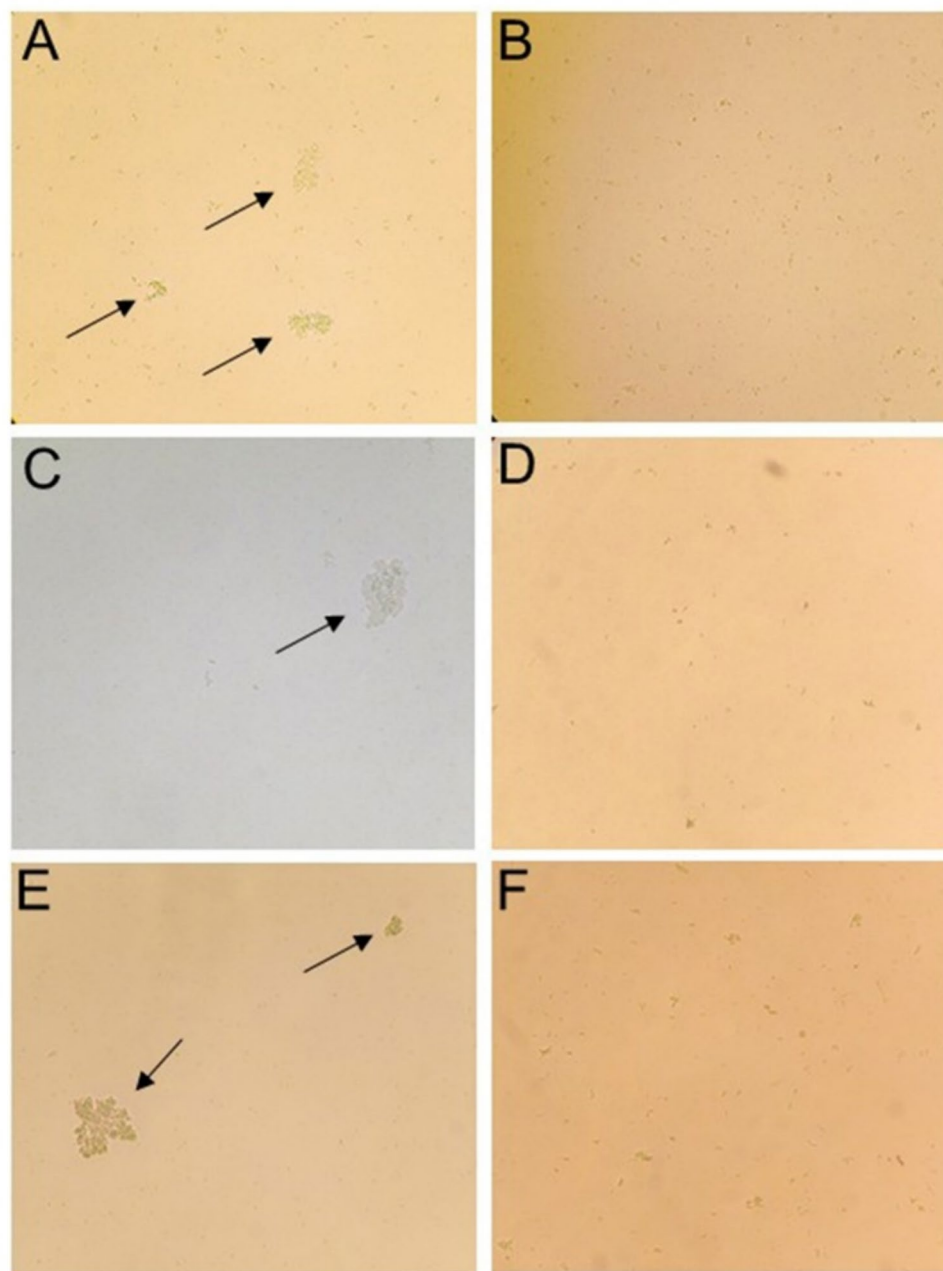
either an early divergence from a common ancestral lectin or a lineage-specific adaptation that may reflect functional or structural shifts within Ceramiales.

The overall coherence of the tree, including genus-level clustering among *Eucheuma* (ESA-2 and EDA-2) and species-level grouping in *Solieria filiformis* (SfL-1 and SfL-2), reinforces the robustness of the phylogenetic reconstruction and supports the hypothesis that OAAH lectins preserve lineage-specific molecular signatures. The basal placement of AML relative to Gigartinales-derived lectins may indicate that Ceramiales represent a previously unrecognized reservoir of lectin diversity within Rhodophyta. These findings underscore the importance of expanding taxonomic sampling to better understand the distribution and diversification of OAAH-type lectins across red algal lineages.

Members of this family also exhibit conservation in carbohydrate-binding residues (WGGREGPI), which was also observed for the *A. multifida* lectin (Whitley et al. 2013). Combined with its previously reported affinity for glycoproteins containing high-mannose N-glycans, these findings reinforce the classification of AML within the OAAH family. The binding specificity toward mannose-rich N-glycans has attracted significant attention, given the prevalence of these structures on the surfaces of cancer cells and viral capsids (Ferir et al. 2014; Barre et al. 2019b, 2021; Sato et al. 2020).

CD analysis showed that native AML predominantly consists of β -sheet and random coil structures, with minor contributions from α -helix and β -turn, consistent with other OAAH lectins and aligns with the modeling data obtained for AML (Whitley et al. 2013; Chaves et al. 2023). The

Fig. 8 Agglutination of Gram-positive and Gram-negative bacteria by AML. (A) *E. coli* ATCC 11303, (C) *S. aureus* ATCC 25923, and (E) *S. aureus* ATCC 700698 cells incubated with AML (500 $\mu\text{g mL}^{-1}$); (B) *E. coli* ATCC 11303, (D) *S. aureus* ATCC 25923, and (F) *S. aureus* ATCC 700698 cells incubated with TBS (control). Arrows indicate bacterial agglutination. All images were acquired under 120 $\times$ magnification



predicted tertiary structure of AML revealed a conformation composed of two β -barrel domains arranged transversely and connected by a flexible linker. This structural organization is commonly found among OAAH lectins, which are typically monomeric proteins composed of one or two β -barrel domains arising from two or four tandem repeats of homologous sequences, respectively (Koharudin et al. 2011, 2012; Whitley et al. 2013; Chaves et al. 2023). While several mannose-binding lectins exhibit β -prism, β -sandwich or β -trefoil folds, the β -barrel architecture predicted for AML suggests a different structural organization that may influence its carbohydrate-binding properties (Barre et al. 2019a, b).

Moreover, the gradual loss of ellipticity across the temperature ramp, marked by multiple inflection points, is consistent with a multi-domain architecture. Each domain may exhibit varying thermal stabilities, contributing independently to the overall unfolding signature. Furthermore, the presence of multiple isoforms in the protein preparation, two of which are predominant, with at least five isoforms reported in prior studies (Costa et al. 1999; Mesquita et al. 2021), may also contribute to the broad and non-cooperative denaturation behavior. These isoforms likely differ subtly in their structural features, further diversifying the thermal response.

We also evaluated the hydrodynamic behavior of AML at different pH values. The aggregation pattern observed appears to be pH-dependent and closely related to the isoelectric point (*pI*) range of AML isoforms. The most abundant isoforms exhibit *pI* values around 6.05 and 6.33 (Mesquita et al. 2021), and the isoform characterized in the present study has a calculated *pI* of 6.26. Therefore, we suggest that near-neutral pH may reduce electrostatic repulsion, favoring dimer formation, whereas more alkaline conditions may promote non-specific aggregation due to protein destabilization.

The carbohydrate recognition domains (CRDs) of AML exhibit cavities on the protein surface that resemble those observed in other OAAH lectins, including BOA from *B. oklahomensis* and SfL from *S. filiformis* (Koharudin et al. 2011; Whitley et al. 2013; Chaves et al. 2018b). These CRDs share a conserved amino acid composition across binding sites, particularly the QWGGREGPI motif, which is implicated in carbohydrate recognition. Despite this conservation, variations in topological features such as cavity depth, surface area, and volume, were observed. These structural differences may influence ligand accommodation and binding site modularity.

The calculations performed revealed conserved interaction patterns involving the residues QWGGREGPI, which are known to mediate polar interactions with mannose units. In particular, the QGGREGP segment consistently formed hydrogen bonds with various oxygen atoms on the ligand, suggesting these residues play a key role in ligand recognition. Additionally, hydrophobic contacts involving tryptophan and isoleucine contributed to the stabilization of the ligand–protein complex. The ligand conformations observed across all four CRDs were highly similar to those reported in crystallographic complexes of OAAH lectins, supporting the hypothesis of a conserved recognition mechanism (Whitley et al. 2013; Chaves et al. 2023).

The high level of conservation in CRD architecture suggests that OAAH lectins have undergone evolutionary pressure to maintain specificity for similar glycan structures. Nonetheless, subtle differences in intermolecular distances and CRD topology indicate that variations in spatial arrangement of amino acid residues may contribute to binding affinity modulation and selectivity.

Members of the OAAH lectin family are known for their antimicrobial properties (Liao et al. 2003; Ferir et al. 2014; Hung et al. 2015a, b; Hirayama et al. 2016), with some representatives exhibiting bacterial agglutination activity. For example, MEL and SfL were shown to agglutinate *Vibrio alginolyticus* and multidrug-resistant strains of *Salmonella*, *E. coli*, and *S. aureus*, respectively (Chaves et al. 2018a, 2023).

AML has previously demonstrated several biological activities described in the literature, including anti-HIV and anti-Influenza effects (Gondim et al. 2019). In the

present study, although AML did not exhibit direct antibacterial or antibiofilm activities, it was able to agglutinate *E. coli* ATCC 11303, *S. aureus* ATCC 25923 and *S. aureus* ATCC 700698, suggesting the presence of mannoside or mannoside-like structures on the bacterial surface. All biological assays performed in this study were conducted using the purified lectin preparation containing the pool of isoforms, therefore it was not possible to attribute the observed activities to a specific isoform.

Among macroalgal OAAH lectins, antibacterial activity has so far been reported primarily against marine bacterial, particularly species of the genus *Vibrio*, with ESA-2 and EDA-2 being able to inhibit growth of *V. vulnificus* and *V. alginolyticus*, respectively (Liao et al. 2003; Hung et al. 2015b). These findings may indicate that the antimicrobial potential of macroalgal OAAH lectins is more closely associated with marine bacterial targets. However, broader screening against a wider range of bacterial strain, both marine and terrestrial, would be necessary to fully assess the antibacterial potential of lectins from marine macroalgae.

In conclusion, we characterized the structure of a new lectin from the OAAH family 26 years after its initial report by Costa et al. (1999). AML exhibited agglutination activity against Gram-positive and Gram-negative bacteria and shares key structural features with other OAAH lectins, including β -barrel architecture and conserved carbohydrate-binding motifs. These findings expand the taxonomic range of the OAAH family to the order Ceramiales and contribute to the broader understanding of marine algal lectins and their potential biotechnological applications.

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Author contributions The study was conceived and designed by multiple authors, who contributed to data acquisition, analysis, and interpretation to varying degrees and were provided by R.P.C., G.S.D.S., S.R.S., V.P.P.M., A.R.O.S., E.A.M., M.A.V. and H.C.S.. R.P.C. and G.S.D.S. led the original draft preparation, while B.L.S., M.A.V., R.F.C., and C.S.N. contributed significantly to the review and editing process. Supervision and project administration were provided by C.S.N., with additional supervision and funding acquisition by E.H.T., R.F.C., A.H.S., and A.L.C.S.. All authors read and approved the final version of the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethical statement All practices pertaining to the handling and blood collection from the rabbits was authorized by the local institutional ethics committee (CEUAP – Ethics Committee for the Use of Production Animals ID: 2211202101).

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