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PURIFICATION AND CHARACTERIZATION OF A NEW LECTIN FROM THE RED MARINE ALGA *HYPNEA MUSCIFORMIS*

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ABSTRACT: A lectin from the red marine alga *Hypnea musciformis* (HML) was purified by extraction with 20 mM PBS, precipitation with 70% saturated ammonium sulphate, ion-exchange DEAE-Cellulose chromatography and RP-HPLC. The 9.3 kDa polypeptide agglutinates erythrocytes from various sources and shows oligomerization tendencies under certain MALDI-TOF/MS conditions. Preliminary N-terminal sequencing and biological assays strongly suggest that the HML may belong to a new class of algae lectins.

INTRODUCTION

Lectins from terrestrial plants and animals have been isolated, characterized and exploited extensively in many aspects of biochemistry and biomedicine. Since the first findings on the presence of agglutinins in extracts of marine algae by Boyd and co-workers in 1966 [1], several studies have been published, mainly on haemagglutinating activity. However, few lectins from marine algae have been characterized in detail up to now, one of the reasons for this is related to difficulties in their isolation and obtaining sufficient material for study [2] The first report upon amino acid sequence of a lectin from marine algae has only been recently published [3].

Although marine algal lectins show proteinaceous content similar to lectins from terrestrial plants, they differ in some aspects. Early publications on this issue, reported that in general, lectins from algae have low molecular masses, no affinity for monosaccharides, strong specificity for complex oligosaccharides and/or glycoproteins. Moreover, they appear to have no requirement for metal ions, showing high content of acidic residues and even in high concentrations tend to stay in the monomeric form [2, 4, 5]. However there is a few

reports showing that some of these molecules may be inhibited by simple sugars and are cation dependent as showed for the lectins from the green marine alga genus, *Codium* [6] and red marine alga genus, *Ptilota* [7-10].
Lectins from *Hypnea japonica* have been purified, characterized and their primary structures determined [11, 12]. Our group have also demonstrated that aqueous extract of the red marine algae *Hypnea musciformis* and *H. cervicornis* show haemagglutinating activity [13] and now, the present work deals with the isolation, characterization and partial amino acid sequence of a lectin from *Hypnea musciformis*.

MATERIALS AND METHODS

Specimens of the red alga *H. musciformis* were collected in the Atlantic coast of Brazil (Pacheco beach, Ceará State), rinsed with distilled water, cleaned of epiphyte and kept in plastic bags at -20 ºC. The frozen alga was ground to a fine powder in liquid nitrogen, stirred for 18 h with three volumes of 20 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl, filtered through nylon tissue and centrifuged at 7,000 x *g*, for 30 min, at 4 ºC. Finally, the supernatant (crude extract) was precipitated by addition of solid ammonium sulphate (0/70% saturation) for 18 h. Precipitated proteins were recovered by centrifugation, dialysed against distilled water and applied to a DEAE cellulose-column equilibrated and eluted with 20 mM phosphate buffer, pH 7.0. The unretained fraction containing the haemagglutinating activity was rechromatographed on the same column, dialysed against water Milli-Q and lyophilised. Aliquots of lectin were incubated at the temperatures of 40, 60 and 90 ºC. After 30 min, 200 µL of samples were taken, cooled and assayed for haemagglutinating activity [13]. The sample was submitted to a reverse-phase high-performance liquid chromatography (RP-HPLC) on a BioRad RP-318 SP column (25 x 2.5 cm, 5 µm particle size) eluted at 2.5 mL/min with a mixture of 0.1% (v/v) TFA in water (solvent A) and acetonitrile (solvent B) employing the following chromatography conditions: first, isocratic (0% B) for 5 minutes, followed by gradients of 0-35% B for 15 minutes, 35-45% B for 20 min. and 45-70% B for 25 min. The protein elution was monitored at 216 nm and 280 nm, and fractions were collected manually and freeze-dried in a Speed Vac (Savant, Germany). The protein purity and molecular mass were assessed by polyacrylamide gel electrophoresis (15%) in the presence of SDS and 2-mercaptoethanol [16] and gel filtration chromatography on Sephadex G-50 column (83 x 1.5 cm) using the proteins bacitracin (1,400 Da), ribonuclease (13,700 Da), trypsin inhibitor (21,100 Da), and α-chymotrypsinogen (25,000 Da) as molecular makers. N-Terminal analysis was performed using a Shimadzu PPSQ-23 sequencer or an Applied Biosystems 477A sequencer, following the manufacturer's instructions. Molecular mass was determined by MALDI-TOF mass spectrometry using an Applied Biosystems Voyager DE-STR operating at a 25 kV accelerating voltage in the linear mode. The sample matrix was α-cyano-4 dimetoxicinamic acid.

RESULTS

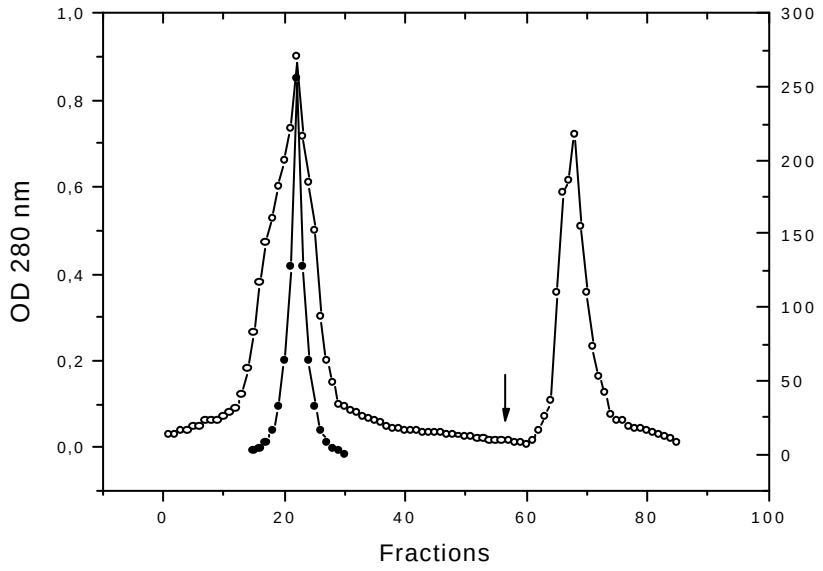
The lectin fraction from DEAE-Cellulose chromatography of saline extracts of *Hypnea musciformis* agglutinated preferentially rabbit erythrocytes. No differences in the haemagglutinating activity were observed upon enzymatic treatment. The lectin of *H. musciformis* was able to agglutinate all enzyme treated human

\erythrocytes to almost the same intensity (Table 1). The haemagglutinating activity present in the DEAE fraction was not affected by exposure to temperatures of 40, 60, 90 ºC for 30 minutes.

Table 1. Hemagglutinating activity of the HML with various erythrocytes. (Haemagglutinating units / mL). (NA) Not assayed.

Erythrocytes	Native	Enzyme treated			
		Trypsin	Papain	Bromelain	Subtilisin
Rabbit	4	64	128	128	128
Goat	-	-	-	-	-
Chicken	-	-	-	-	-
Cow	-	32	NA	NA	NA
Sheep	-	4	NA	NA	NA
Human A	-	2	8	8	2
B	-	2	8	8	2
O	2	4	16	16	16

Figure 1. Ion exchange chromatography on DEAE-Cellulose of the ammonium sulphate fraction (F0/70) from *Hypnea musciformis* extracts. The fraction was dialysed with 2 mM phosphate buffer, pH 7.0 and applied to the column (26 x 1.5 cm). The column was washed with the same buffer at flow rate of 30 mL.h^{-1} and eluted with 1M NaCl in buffer. Fractions of 3 mL were collected and assayed for haemagglutinating activity, using rabbit enzyme-treated erythrocytes. H.U. =Haemagglutinating units. (-· -) haemagglutinating activity. (-●-) absorbance at 280 nm.



The *Hypnea musciformis* lectin (HML) isolated by combination of ion-exchange chromatography on DEAE-Cellulose (Figure 1) and reverse-phase C-18 high performance liquid chromatography (HPLC) (data not shown) is composed by a single protein band in SDS-PAGE under denaturing conditions of around 9,000 Da The homogeneity of HML was also observed by a single symmetrical peak obtained by size exclusion chromatography on Sephadex G-50 (not shown), with apparent molecular mass of about 11,000 Da. When the purified lectin was subjected to MALDI-TOF/MS it showed major ions at 9356.43 (Figure 2) and 18749.40 Da (Figure 3). Partial amino acid sequence of HML revealed a single polypeptide chain: EAQIPASESERPGLVIVGGKMA. No significant hit was found when this data was checked against GENINFOâ BLAST Network-SwissProt sequences.

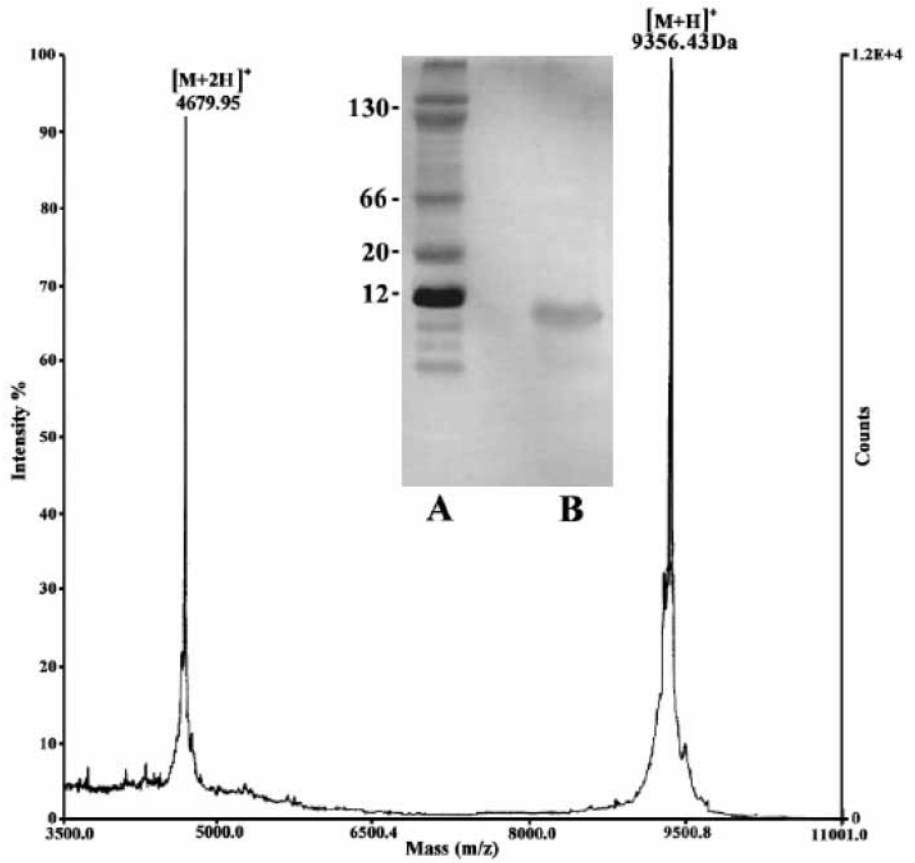


Figure 2. MALDI-TOF/MS spectrum of the purified HML showing a major quasimolecular ion at m/z 9356.43. Insert, lane A, molecular mass standards, from top to bottom, γ globulin (130 kDa), bovine serum albumin (66 kDa), trypsin inhibitor (20 kDa) and acidic seminal fluid protein (12 kDa). Lane B, (15%)- polyacrylamide gel electrophoresis of reduced HML.

DISCUSSION

The hemagglutinating activity present in aqueous extracts of the red marine alga *Hypnea musciformis* was first detected by Ainouz and co-workers [13]. The aqueous extract exhibited strong agglutination towards trypsin-treated rabbit erythrocytes and at less degree against enzyme treated ABO blood group cells. The results

obtained in the present work have shown the same agglutinating pattern with preference for rabbit enzyme-treated erythrocytes. These finding are in complete accordance to the published results for the *H. cervicornis* [13] and *H. japonica* [11], where the haemagglutinating activity investigated for both lectins revealed that they were able to agglutinate preferentially rabbit erythrocytes and the titration value was markedly increased after cell treatment with proteolytic enzymes.

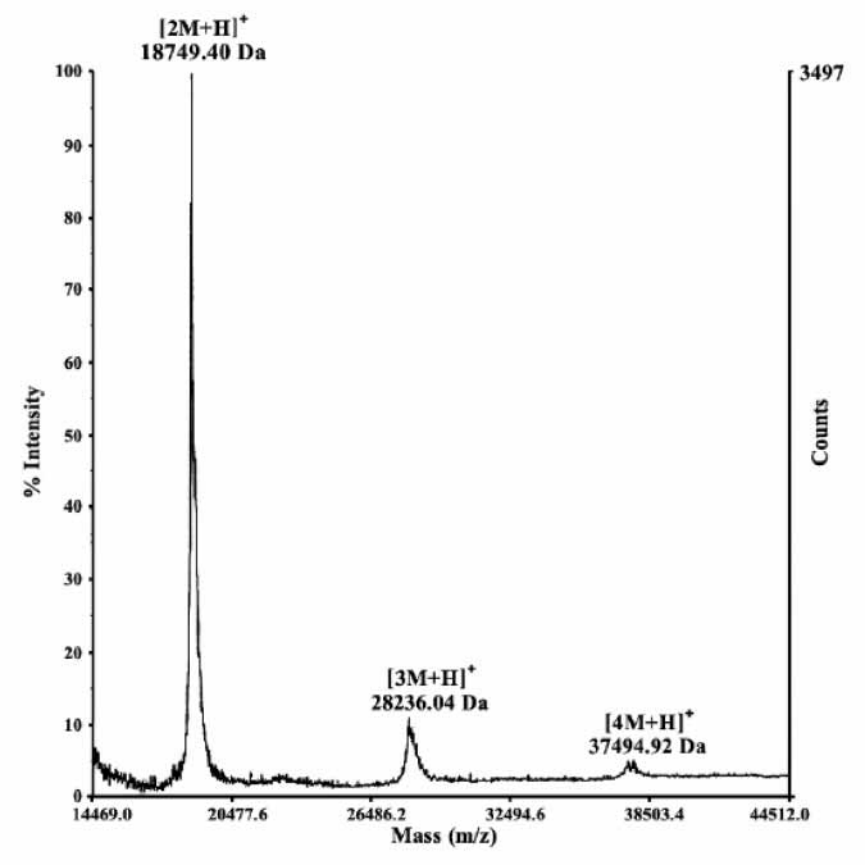


Figure 3: MALDI-TOF/MS spectrum of the purified HML showing multimeric forms of HML. Dimer (18.7 kDa), trimer (28.2 kDa) and tetramer (37.4 kDa).

The *Hypnea musciformis* lectin (HML) was purified by a combination of ammonium sulphate precipitation, ion-exchange chromatography on DEAE-cellulose and reverse-phase HPLC chromatography that yielded a purified lectin as observed by SDS-PAGE, size-exclusion chromatography on Sephadex G-50, MALDI-TOF/MS and N-terminal amino acid sequencing. Ion-exchange chromatography proved to be a useful procedure to isolate lectins from marine algae [5, 14, 15]. In this work, the column was effective to retain all pigments and other contaminants present in the fraction, after ammonium sulphate precipitation while the fraction containing all the lectin activity remained unbounded to the resin. Like other marine algal lectins [5, 11,

16-18], HML is a small, single banded protein with a relative molecular mass of about 9,000 Da, shown by SDS-PAGE. The native molecular mass of 11,000 Da obtained by size exclusion chromatography on Sephadex G-50, suggested that HML was separated as a monomer. However, MALDI-TOF/MS results yielded a major component at m/z 9356.43 and an intense ion at m/z 18749.40 (Figures 2 and 3), indicating the formation of a HML dimer $[2M+H]^+$. Since HML was purified to homogeneity and its first 20 N-terminal residues were unambiguously identified, excluding the presence of any contamination artefacts, one could point out that an oligomerization process involving the HML polypeptide chains is possible, at least under the experimental conditions used. Thus, HML could be assembled as a dimer composed by two identical polypeptide chains. Dimeric forms have been reported in some lectins from marine algae [3, 12, 17, 19] and from terrestrial plants [20]. Although monomeric lectins have been one of the characteristics of many agglutinins isolated from marine algae [15,16, 18, 21-23], it is still unknown how algal monomeric proteins would perform cell agglutination. It has been proposed that these proteins have two distinct binding sites as suggested for the lectin from *Hypnea japonica*: carbohydrate-recognition site(s) (probably containing a C-type CRD motif) and protein-recognition site(s) (phospholipase A_2-binding site) [12]. The dimeric form observed in HML, with at least one binding site per monomer, would explain the strong cell agglutination. When the N-terminal sequencing result of HML was compared to other sequences deposited in public databases, no significant homology was obtained, suggesting that HML could not be evolutionary related to any other known lectin structures, and may belong to a new class of agglutinins. Recently, the N-terminal sequences of isolectins (molecular mass of 29 kDa) from three species of the red marine alga *Eucheuma* (*E. serrata*, *E. amakusaensis* and *E. cottonii*) have been published [24]. All reported sequences were almost identical and very similar to a 30-kDa lectin isolated by our group from the Brazilian red marine alga *Meristiela echinocarpa* [unpublished results]. Furthermore, Calvete *et al*. [3] published for the first time the complete amino acid sequence of a marine algal lectin from the red alga *Bryothamnion triquetrum*, which did not show any sequence similarity with the previous one and none with other sequences deposited in public databases. Also, we have determined the partial (70%) amino acid sequence of another red marine alga (*Ptilota serrata*) [unpublished results] and its closely related species *P. filicina* [9]. From all these data, no discernible amino acid sequence similarity was observed with HML. This strongly suggested the existence of structurally different lectins in red marine algae, which in turn do not resemble known lectins structures of higher plants or animals [3]. Differently from the isolectins from *Eucheuma* species [24], HML did not show sequence similarity to the lectin from the closely related species *Hypnea japonica* [11]. This evidence may suggest some evolutionary differences in these algal species.

HML is extremely thermostable since its agglutinating activity was unaffected under thermal treatment at 40, 60 and 90 ºC for 30 minutes. These results are broadly compared to most of the other marine algal lectins and mainly for those that exhibited isoforms. Hori *et al* [11] reported that the lectin of *H. japonica* was stable to heating for 30 min to 100 ºC. The nature of the small-sized double-chain polypeptides, including interchain disulphide bonds, may contribute to the extreme thermostability of the HML and other algal lectins.

Quite clearly there is much work still to be done on the structure of algal lectins since only two primary structures have been determined so far. Further structural studies will contribute to understanding the differences in their biochemical characteristics as well as to the evolutionary aspects upon lectin presence in land plants and marine algae.

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