Purification and partial characterization of a haemagglutinin from *Ulva fasciata*.

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Abstract

A novel haemagglutinin has been detected from *Ulva fasciata*, a green marine alga. By a combination of ammonium sulphate precipitation and ion-exchange chromatography on DEAE-Cellulose column, we have purified a haemagglutinin and designated it as UFH (*Ulva fasciata* haemagglutinin). It agglutinated native human blood cells without any blood group specificity. Under reducing and non-reducing conditions, this agglutinin was isolated as a monomeric protein with a relatively low molecular weight of 14.5 kDa. The activity of UFH was thermo-stable, expressing functionality at 40-100°C and not inhibited by the inclusion of divalent cations nor pH changes, being stable at pH values 6.0-10.5. None of the tested monosaccharides and glycoproteins inhibited agglutination by UFH. The observations indicate that the properties of UFH are consistent with its recognition as a haemagglutinin isolated from marine algae. The pH stability, high-level activity and thermo-stability thus support it's
candidature for future applicability in lectin research, glycobiology and bio-medical applications.

**Key words:** Marine algae, lectin, seaweed, thermo-stable

**Introduction**

Lectins are proteins that bind reversibly to carbohydrates, agglutinate cells and/or precipitate glycoproteins and polysaccharides. Lectins are widely prevalent in nature and are reported to be present randomly in plants, microorganisms, viruses, fungi, algae and animals (invertebrates and vertebrates). Due to their chemical properties, lectins hold a pivotal role associated with cell-cell recognition and thus consequently find application in various fields as immunology, membrane-structure studies, cell biology and cancer research, to name a few. However, there is limited information/data about algal lectins as compared to that from higher plants and vertebrates.

Boyd *et al.* first identified haemagglutinin from marine algae. Subsequently, different research groups investigated the haemagglutinating potential of marine algal extracts and to date, several algal haemagglutinins are reported.

The survey/study of marine algal lectins has been reported from across the globe ranging from Puerto Rico, Britain, Japan, South East United States, Brazil, Spain and Vietnam. However, the first and the only report of haemagglutinins in marine algae found along the Indian coast was by Barros and Himanshu.
We report here the extraction and partial characterization of the lectin from *U fasciata*, a species that dominates the shores of Goa, South West India.

**Materials and Methods**

*Algal collection and preparation of extract*

The green alga, *Ulva fasciata*, was collected in September/October from the beaches of Goa along the West coast of India [Kakra (15°27′03.5″ N 73°50′14.7″E), Anjuna (15°34′32.5″N 73°44′24.5″E) and Vagator (15°35′59.0″ N 73°43′56.4″E)]. Collected material was washed to remove epiphytes and rinsed in distilled water. Algal material (10 g) was ground by a mortar and pestle to a fine powder and homogenized (1:3 w/v) in phosphate-buffered saline (PBS) 0.15 M at pH 7.4 by stirring for 18 hrs at 4°C. The protocol followed for extraction was a modification of Sampaio et al. 

Filtering through muslin cloth, insoluble algal material was separated, followed by centrifugation at 12000 rpm for 25 minutes at 4°C. The supernatant, a clear “aqueous extract” was separated and tested for haemagglutination and stored at -20°C to be processed for purification.

*Preparation of human red blood cells*

Human blood was withdrawn from healthy donors at the dispensary, Goa University, and obtained from the Blood Bank, Goa Medical College. Approximately 1 ml of human blood was centrifuged at 3000 rpm, for 5 min, at room temperature. The pellet of erythrocytes obtained, was washed thrice in cold PBS at pH 7.4. On centrifuging further, a 2 % v/v of erythrocyte suspension was prepared and tested for haemagglutination.
Assay for haemagglutinating activity

Lectin-mediated agglutination of human erythrocytes was assessed by the protocol previously reported by Hori et al.\textsuperscript{21} The suspension of red blood cells was used directly in this test with serial two-fold dilutions. 75 μl of algal extract (diluted serially in PBS) was placed in a 96-well V-bottomed microtitre plate and 75 μl of red blood cell suspension was added and mixed by gentle shaking. Mixture was incubated in a moist chamber, at room temperature, for one hour. The reciprocal of the highest dilution of extract, causing haemagglutination is denoted as the “haemagglutination titer”. It is also referred to as “the minimal haemagglutinating capacity” (MHC) i.e. the minimum amount of protein-extract tested, that produces agglutination. Controls that recorded agglutination and non-agglutinating activity were respective anti-sera and Con-A, and PBS, respectively.

Purification of haemagglutinin from Ulva fasciata

All procedures for purification were conducted at 4°C. A three-step precipitation with solid ammonium sulphate was undertaken to obtain fractions consisting of 0-30%, 30-60% & 60-90% saturation. On centrifuging each fraction at 4°C for 30 minutes at 12000 rpm, the obtained precipitate was re-suspended in PBS. The re-suspended material was extensively dialyzed (cut-off 12 kDa) against three changes of PBS (2 liters). The buffers were refreshed every 6 hrs. Dialyzed samples were then tested for haemagglutinating activity. As maximum activity was observed in the 60-90% fraction, this fraction was subjected to ion-exchange chromatography on a DEAE-Cellulose column (Sigma) equilibrated with the same buffer. The column was packed as per the manufacturer’s instructions and washed by 0.15 M PBS at pH 7.4 until absorbance was reduced to zero. Bound protein was eluted by gradual increase in sodium
chloride concentrations from 0.5 M up to 1.5 M at a flow rate of 200 μl/ min. Absorbance was recorded at 280 nm. The active fractions were pooled and extensively dialyzed against distilled water. On lyophilizing, active eluates now considered to be purified haemagglutinin (designated as UFH) were preserved at -20°C until required for further use.

*Determination of molecular weight*

The MW of UFH was estimated using SDS- PAGE in a 12 % gel as described by Laemmli (1970). Standards and samples were prepared in Tris- HCL buffer, pH 6.8, containing 2% SDS and 5% 2-mercaptoethanol and heated at 100 ºC for 5 minutes whereas native PAGE was performed without heat treatment and in the absence of 2-mercaptoethanol. Standard protein markers used were ovalbumin (MW 43,000), carbonic anhydrase (MW 29,000), soyabean trypsin inhibitor (MW 20,100), lysozyme (MW 14,300), aprotinin (MW 6,500) and insulin (MW 3,000). Protein bands were identified by the standard silver staining protocol of Blum *et al*.  

*Protein Estimation*

Protein content was quantified by Lowry’s method using bovine serum albumin as the standard. Eluates from the column were monitored spectrophotometrically at 280 nm.

*Haemagglutination-Inhibition assay*
UFH was investigated for its binding specificity to monosaccharides and glycoproteins using haemagglutination-inhibition assay. The monosaccharides tested were D-glucose, D-mannose, D-galactose, D-fucose, L-rhamnose, L-fucose N-acetyl-D-glucosamine, N-acetyl neuramic acid, D(+)-galacturonic acid, D(+)-glucosamine, D(+)-galactosamine, D(+)-glucoronic acid whereas the glycoproteins tested were avidin from egg white, mannan from *S. cerevisiae*, trypsin inhibitor, mucin from bovine submaxillary gland and fetuin (Sigma Chemicals). Serial two-fold dilutions of tested monosaccharides and glycoproteins were prepared in PBS, pH 7.4. Each dilution was mixed with an equal volume (50 μl) of the algal extract and kept for 30 min at room temperature to permit binding, if any. 50 μl of human erythrocyte suspension was further added and this mixture was kept for 1 hr at room temperature. Controls designed to record inhibition was erythrocytes with PBS and with Concavalin A.

*Effect of divalent cations (Ca\(^{2+}\) and Mn\(^{2+}\)) and EDTA on the activity of UFH*

Purified UFH was dialyzed against 50 mM EDTA in 0.15 M PBS (pH 7.4) for 16hrs at 4°C and then assessed for haemagglutination. Further, the dialyzed fraction was tested for activity both in the absence and presence of divalent cations. The cations used were 5mM Ca\(^{2+}\) and Mn\(^{2+}\). Each mixture was incubated for 1 hr at room temperature and activity was checked against human erythrocytes.

*Effect of pH on haemagglutinating activity*

This effect was assessed following the method reported by Kawakubo *et al*.\(^{25}\) using human red blood cells to check the activity. 1ml of purified UFH was dialyzed against 500 ml (0.10 M) buffered solutions at pH values ranging from 5.0 to 10.5 at 4°C for 24 hrs. Further, a
thorough dialysis followed against PBS to nullify the effect of pH on the activity. Buffers utilised were sodium acetate at pH 5.0, phosphate buffer at pH 6.0, 7.0 and 8.0 and sodium citrate at pH 9.0 and 10.5.

Heat stability of UFH

On incubating 1ml Aliquots of purified UFH at temperatures ranging from 40°C - 100°C for intervals of 10, 20 and 30 minutes of incubation at the respective temperature, heat stability was assayed. 75 μl of extract was cooled to room temperature and tested for haemagglutination.

Results

Isolation of haemagglutinin from Ulva fasciata

The results of haemagglutination assays are reported as the minimum amount of protein-extract tested, that produced agglutination. During assay for activity with each fraction, it was observed that the minimal haemagglutinating capacity (MHC) was highest in protein fractions precipitated with 60-90% ammonium sulphate whereas the 0-30% fractions showed moderate activity and the least haemagglutinating activity was obtained from the 30-60% fractions (Fig 1). Haemagglutinating activity was expressed as a titer i.e. the reciprocal of the highest two fold dilution exhibiting haemagglutination. However, maximum protein was precipitated from the 30-60% fraction (Fig 1).

Fig. 1
The fraction with highest activity (60-90%) was subjected to ion-exchange column chromatography. The active peak coincided with a major protein peak, the highest activity and absorbance being observed in fractions 32-41 from the eluate. (Fig.2). This eluted haemagglutinin is designated as UFH and indicates that the proteins in the precipitate consisted exclusively of lectin molecules.

**Fig. 2**

*Molecular weight determination*

The homogeneity of UFH was confirmed by the detection of a single band obtained by PAGE (Fig 3a) and this purified lectin was observed as a single band with molecular weight of 14.5 kDa on SDS –PAGE (Fig 3b), indicating that this lectin, UFH, may be a monomeric protein.

**Fig. 3**

*Protein Estimation*

The estimated protein content and the observations from purification are summarized in Table1. Approximately 39 mg of protein were obtained from 10g of *U. fasciata* (wet weight) in the aqueous extract. The total protein recovered from the final DEAE ion-exchange step was 2.7 mg and specific activity of UFH i.e. Haemagglutination Unit (HU) was 11.85 (mg of protein)\(^{-1}\). The purified haemagglutinin, UFH, had approximately 7.7 fold more activity than the aqueous extract.

**Table 1**

*Haemagglutination-Inhibition Assay*
By haemagglutination inhibition test, the binding specificity for carbohydrates, of UFH, was observed to be non-specific to any of the tested monosaccharides and glycoproteins. Agglutination produced by Con A was consistently inhibited by N-acetyl –D-glucosamine and mannose.

Effect of EDTA, pH and heat on haemagglutinating potential of UFH

Observations indicate that the presence of divalent cations may not be a requirement for the haemagglutinating function of UFH, as the activity of EDTA- treated UFH was observed to be unaltered.

Altering the pH, was observed to have no effect on the activity of the lectin, UFH, in the range of pH 6-10.5, but lost it’s activity at pH below 6. Haemagglutinating activity of this lectin was unaltered on exposing for 30 min to temperatures of 40-80°C. However, activity was observed to be halved on exposure to 100°C for 10 min.

Discussion

Ulva Species is distributed throughout the world with several researchers studying this as a potential resource of exploitable lectin. A lectin from U. lactuca binding to fucose has been reported before. Other Ulva species have yielded lectins that are inhibited by complex glycoproteins but not by simple sugars. Most of those lectins were analysed in crude or semi-purified extracts of marine alga. The first report of purification of a lectin from the genus Ulva was in 1996. Since then, three lectins have been isolated from the genus Ulva.
Haemagglutinating activity in aqueous extracts of *U. fasciata* was first detected by Barros and Himanshu (2005). The lectin UFH reported here was purified by a combination of ammonium sulphate precipitation and ion-exchange chromatography on DEAE-cellulose column, and observed by SDS-PAGE. Ion-exchange chromatography has proven to be an useful tool in isolating lectins from marine algae. The properties of lectin UFH are largely unvarying with earlier reports of lectins from *Ulva* and other marine algae. Similar to most lectins from *Ulva* species, UFH is a small, single- banded monomeric protein with a relative molecular weight of 14,500 Dalton, stable at pH 6-10.5 and is thermo-stable at temperatures 40-80ºC. Unlike other *Ulva* lectins, activity, albeit reduced, was also observed at 100ºC. This observation of the activity at high temperatures may be attributed to the fact that *Ulva fasciata* has been isolated from tropical waters along the shores of the Arabian Sea. This unusual thermo-stability has also been reported in *Hypnea japonica* and *Hypnea musciformis*. The first report on tropical algae have also reported activity at 100ºC of a few species.

Haemagglutination activity of UFH was independent of divalent cations as also observed in other lectins. In reference to specificity for carbohydrate-binding, there are reports that many algal lectins have no affinity for monosaccharides but do have for glycoproteins. The inability to confirm sugar-binding specificity of UFH may be attributed to a more complex process of recognition by the agglutinins, one that may involve oligosaccharides rather than simple monosaccharides. However, the activity of Concanavalin A, used as a control in the test, was inhibited by N-acetyl-D-glucosamine and D-mannose.

It has been postulated and reported that erythrocytes of rabbits are an ideal option to screening for agglutination. However, UFH was observed to be active towards human erythrocytes. A higher activity / titer perhaps may have been obtained from this study if other erythrocytes or treated erythrocytes were used. In reference to specificity towards blood groups, UFH did not display any specificity. Similar observations have been reported earlier.
although, some algal lectins are indicated to be highly specific to either A, B and / or O erythrocytes.\textsuperscript{21, 27}

Here, we aimed at purification and characterizing the prospective haemagglutinin by ion-exchange chromatography of ammonium sulphate (60-90\%) precipitated cell extract. We observed that the fraction precipitated with 60-90\% ammonium sulphate has higher haemagglutination activity as compared to the aqueous extract. This indicates the importance of salt precipitation methodology in concentrating the lectin protein from the algal homogenate. However, we observed a decrease in haemagglutination activity following column chromatography implying need for improvisation of the final purification step to attain a higher yield of active UFH.

Thus, UFH similar to other marine algae agglutinins, is a small monomeric glycoprotein. It is intriguing how such a monomeric form can cause the cells to agglutinate. The results imply that \textit{U. fasciata} could be a valuable source of a lectin that works even at high temperature and over a wide pH range. Further characterization studies are currently under way. Based on the results obtained from this study and the earlier studies that have been reported, it is probable that lectins share some important role in the algae. It is therefore also important to establish the physiological properties of this lectin.

Thus, we report herein the purification and characterization of a novel haemagglutinin from \textit{U. fasciata} with pH and temperature stability making it a strong candidate in glycobiology and biomedical applications. It is important to elucidate the complete primary structure of this haemagglutinin and compare it to other \textit{Ulva} lectins. The application and primary sequence analysis of this purified lectin is currently under progress.

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References:


**LEGENDS**

Fig. 1 Bar graph of the different steps of purification with the associated yield of protein (bar) and the respective haemagglutination titer (triangle).
Fig. 2 DEAE-cellulose chromatography of the ammonium sulphate-fractionated extract (60-90 %). Protein elution was monitored by absorbance at 280 nm (-●-●-). 75µl from each fraction was assayed for haemagglutination activity and is represented as log₂ titer (-▲-▲-).

Fig. 3 Purified lectin (UFH) from *U. fasciata* subjected to PAGE

(a) Native PAGE of UFH: Lane 1 to 3: DEAE-cellulose purified, lane 4 aqueous extract; and lane 5: 60-90% ammonium sulphate saturated fraction.

(b) SDS-PAGE of UFH Lane 1: aqueous extract, lane 2: molecular weight markers, lane 3: 60-90% ammonium sulphate saturated extract and lane 4: column purified.

Table 1. Purification and protein estimation of UFH from *U. fasciata*

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### TABLES

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<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein (mg)</th>
<th>Activity (HU)</th>
<th>Specific activity (HUmg⁻¹)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
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<td>Aqueous extract</td>
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<td>1.538</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>60-90% of (NH₄)₂SO₄</td>
<td>5.9</td>
<td>64</td>
<td>10.84</td>
<td>7.1</td>
<td>106</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>2.7</td>
<td>32</td>
<td>11.85</td>
<td>7.7</td>
<td>53.3</td>
</tr>
</tbody>
</table>

HU-Haemagglutination Unit