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

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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 its candidature for future applicability in lectin research, glycobiology and biomedical applications.

Keywords: Lectin, Marine algae, seaweed, thermo-stable, *Ulva fasciata*.

LECTINS are proteins that bind reversibly to carbohydrates, agglutinate cells and/or precipitate glycoproteins and polysaccharides[1]. 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 applications in various fields such 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.*[2] first identified haemagglutinin from marine algae. Subsequently, different research groups studied the haemagglutinating potential of marine algal extracts[3] and to date, several algal haemagglutinins are reported[4–14].

The survey/study of marine algal lectins has been reported from across the globe ranging from Puerto Rico[2], Britain[15], Japan[16], South-East United States[17], Brazil[6,18], Spain[19] and Vietnam[14]. However, the first and the only

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report of haemagglutinins in marine algae found along the Indian coast was by Barros and Himanshu[20].

We report here the extraction and partial characterization of lectin from *Ulva fasciata*, a species that dominates the shores of Goa, South-West India.

The green alga, *U. 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 h at 4°C. The protocol followed for extraction was a modification of Sampaio *et al.*[10]. Filtering through muslin cloth, insoluble algal material was separated, followed by centrifugation at 12,000 rpm for 25 min 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.

Human blood was withdrawn from healthy donors at the dispensary of Goa University, and also obtained from the Blood Bank of 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.

Lectin-mediated agglutination of human erythrocytes was assessed by a protocol previously reported by Hori *et al.*[21]. The suspension of red blood cells was used directly in this test with serial two-fold dilutions. A 75 µl of algal extract (diluted serially in PBS) was placed in a 96-well V-bottomed microtiter 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 concavalin-A (Con-A), and PBS, respectively.

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% and 60–90% saturation. On centrifuging each fraction at 4°C for 30 min at 12,000 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 l). The buffers were refreshed every 6 h. 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 diethyl-aminoethyl-cellulose (DEAE-cellulose) column (Sigma Chemicals, USA) 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 *Ulva fasciata* haemagglutinin (UFH)) were preserved at –20°C until required for further use.

The molecular weight (MW) of UFH was estimated using SDS-PAGE in a 12% gel as described by Laemmli[22]. Standards and samples were prepared in *Tris*-HCL buffer, pH 6.8, containing 2% SDS and 5% 2-mercaptoethanol and heated at 100°C for 5 min whereas native PAGE was performed without heat treatment and in the absence of 2-mercaptoethanol. Standard protein markers used were ovalbumin (MW 43000), carbonic anhydrase (MW 29000), soyabean trypsin inhibitor (MW 20100), lysozyme (MW 14300), aprotinin (MW 6500) and insulin (MW 3000). Protein bands were identified by the standard silver staining protocol of Blum *et al.*[23].

Protein content was quantified by Lowry's method[24] using bovine serum albumin as the standard. Eluates from the column were monitored spectrophotometrically at 280 nm.

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(+)gulactosamine, D(+)glucoronic acid whereas the glycoproteins tested were avidin from egg white, mannan from *Saccharomyces cerevisae*, trypsin inhibitor, mucin from bovine submaxillary gland and fetuin (Sigma Chemicals, USA). 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. Human erythrocyte suspension (50 µl) was further added and this mixture was kept for 1 h at room temperature. Controls designed to record inhibition was erythrocytes with PBS and with Con-A.

Purified UFH was dialyzed against 50 mM EDTA in 0.15 M PBS (pH 7.4) for 16 h 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 5 mM Ca^{2+} and Mn^{2+}. Each mixture was incubated for 1 h at room temperature and the activity was checked against human erythrocytes.

Effect of pH on haemagglutination was assessed following the method reported by Kawakubo *et al.*[25] using human red blood cells to check the activity. Here, 1 ml 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 h. Further, a thorough dialysis was followed against PBS to nullify the effect of pH on the activity. Buffers utilized 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.

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

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 MHC was highest in protein fractions precipitated with 60–90% ammonium sulphate whereas 0–30% fractions showed moderate activity, and the least haemagglutinating activity was obtained from 30% to 60% fractions (Figure 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 30% to 60% fractions (Figure 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 (Figure 2). This eluted haemagglutinin is designated as UFH and indicates that the proteins in the precipitate consisted exclusively of lectin molecules.

The homogeneity of UFH was confirmed by the detection of a single band obtained by PAGE (Figure 3 *a*) and this purified lectin was observed as a single band with MW of 14.5 kDa on SDS-PAGE (Figure 3 *b*), indicating that this lectin, UFH, might be a monomeric protein.

The estimated protein content and the observations from purification are summarized in Table 1. Approximately 39 mg of protein were obtained from 10 g of *U.*
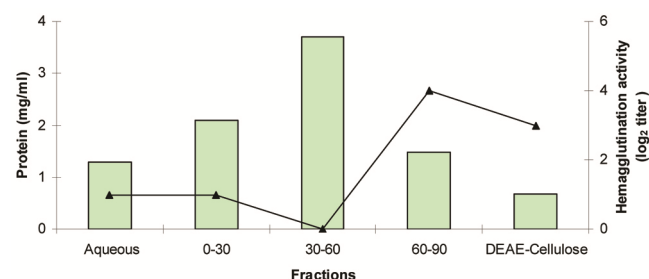

Figure 1. Bar graph of the different steps of purification with the associated yield of protein (bar) and the respective haemagglutination titer (triangle).

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

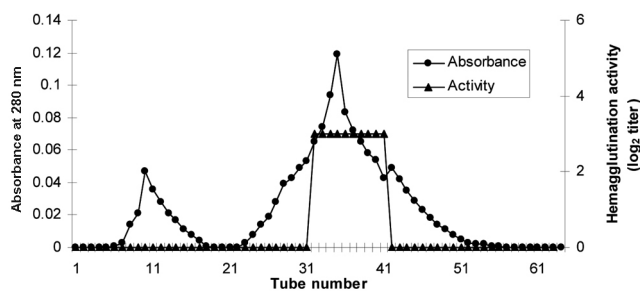| Fraction	Total protein (mg)	Activity (HU)	Specific activity (HU mg⁻¹)	Purification fold	Yield (%)
Aqueous extract	39	60	1.538	1	100
60–90% of $(NH_4)_2SO_4$	5.9	64	10.84	7.1	106
DEAE-cellulose	2.7	32	11.85	7.7	53.3

HU, Haemagglutination unit.

Figure 2. DEAE-cellulose chromatography of the ammonium sulphate-fractionated extract (60–90%). Protein elution was monitored by absorbance at 280 nm (−●−●−) and 75 µl from each fraction was assayed for haemagglutination activity which is represented as $\log_2$ titer (−▲−▲−).
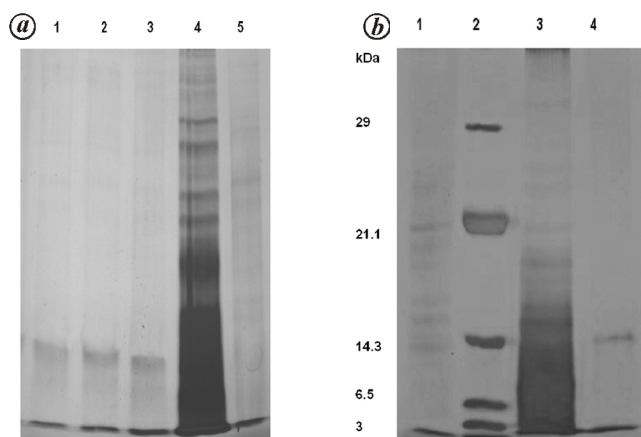

Figure 3. Purified lectin (UFH) from *U. fasciata* subjected to PAGE. *a*, Native PAGE of UFH – Lanes 1 to 3, DEAE-cellulose purified; Lane 4, Aqueous extract; Lane 5, 60–90% ammonium sulphate saturated fraction. *b*, SDS-PAGE of UFH – Lane 1, Aqueous extract; Lane 2, Molecular weight markers; Lane 3, 60–90% ammonium sulphate saturated extract and Lane 4, Column purified.

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.

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.

Observations indicated that the presence of divalent cations may not be a requirement for 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 UFH at the pH range of 6–10.5, but it lost its activity at pH below 6. Haemagglutinating activity of this lectin was unaltered on exposure to temperatures of 40–80°C for 30 min. However, activity was observed to be halved on exposure to 100°C for 10 min.

Ulva species is distributed throughout the world with researchers studying this as a potential resource of exploitable lectin. A lectin from *U. lactuca* binding to fucose has been reported before[26]. Other *Ulva* species have yielded lectins that are inhibited by complex glycoproteins but not by simple sugars[27]. 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 (ref. 28). Since then, three lectins have been isolated from this genus[11,29,30].

Haemagglutinating activity in aqueous extracts of *U. fasciata* was first detected by Barros and Himanshu[20]. 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[8,31]. The properties of the 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 MW of 14,500 Da, stable at pH 6–10.5 and is thermo-stable at 40–80°C. Unlike other *Ulva* lectins, activity, albeit reduced, was also observed at 100°C. This observation of activity at high temperatures may be attributed to the fact that *U. 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*[32] and *Hypnea musciformis*[33]. The first report on tropical algae have also reported activity at 100°C of a few species[14].

Haemagglutination activity of UFH was independent of divalent cations as also observed in other lectins[32]. In reference to specificity for carbohydrate-binding, there have been reports that many algal lectins have no affinity for monosaccharides, but do have for glycoproteins[10,32]. The inability to confirm sugar-binding specificity of UFH may be attributed to a more complex process of recognition by

agglutinins, one that may involve oligosaccharides rather than simple monosaccharides[4,8,12]. However, the activity of Con-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[5,21,27,32,34]. 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[11,14,21,29], although, some algal lectins are indicated to be highly specific to either A, B and/or O erythrocytes[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 had 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 a need for improvisation of the final purification step to attain a higher yield of active UFH.

Thus, UFH similar to other marine algal agglutinins, is a small monomeric glycoprotein. It is intriguing how such a monomeric form can cause the cells to agglutinate. The results imply that *U. fasciata* could be a valuable source of 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 significant to establish the physiological properties of this lectin.

Thus, we report herein the purification and characterization of a novel haemagglutinin from *U. fasciata* with better 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 Ulva lectins. The application and primary sequence analysis of this purified lectin is currently under progress.

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Marker validation and sequencing in aromatic landrace Mushk Budji

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Aroma trait imparts specialty to rice and enhances its economic value in the market. Most aromatic genotypes are known to possess a truncated version of betaine aldehyde dehydrogenase gene, imparting aroma. Temperate rice varieties of Kashmir, India, have not been assessed for allelic variants of this fragrance-imparting gene. Herein we report allelic variations present in exon 7 of this gene in the popular scented rice Mushk Budji. Unlike basmati-type genotypes, Mushk Budji is a short and bold japonica rice which grows at high altitudes and is cold-tolerant. Moreover, aroma retention after cooking is better in Mushk Budji compared to Pusa Sugandh 3, a long and thin basmati-type rice adapted to low-altitude areas of the Kashmir valley. Analysis showed the presence of a deletion of 8 bps 'GATTATGG' and three single nucleotide polymorphism in exon 7 of aromatic rice genotypes, including Mushk Budji. No such deletion was found in non-aromatic rice varieties. Additionally, one functional marker for badh2 allele was validated in Mushk Budji. These findings can facilitate the development of short and bold fragrant rice varieties through marker assisted selection, especially for high-altitude cold regions and the temperate valley conditions of Kashmir and the rest of India.

Keywords Aromatic landrace, functional markers, sequencing.

GRAIN aroma is the single-most attractive character for rice consumers. There is an increased global demand for aromatic rice varieties because of their pleasant aroma and superior nutritional quality, owing to better amino acid profiles[1]. 2-Acetyl-1-pyrroline [2AP; IUPAC name 5-acetyl-3,4-dihydro-2*H*-pyrrole] is a major chemical compound responsible for the fragrance of aromatic rice[2]. Aroma characteristics are found within three of the distinguished genetic subpopulations of rice: Group V (Sadri and Basmati), indica (Jasmine) and tropical japonica[3]. Mushk Budji, a temperate japonica variety is the most prominent aromatic indigenous rice cultivar of Kashmir, India[4,5]. However, this cultivar was mostly grown as admixtures of Mushk Budji false dublicates by the farmers and there were complaints from consumers regarding the aroma quality in local markets. Keeping this in view, a massive genetic purification programme was conducted over a period of four years (2008–12) at the

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