CHAPTER IV

Isolation, Purification and Characterization of Polysaccharide from *Ulva fasciata*

4.1 INTRODUCTION

Polysaccharides are a class of carbohydrates consist of a number of monosaccharides joined by glycosidic bonds in branched or unbranched chains. They are considered as one of the active compounds in plants and fungi. In the last decades, the polysaccharides have attracted a great deal of attention in the biomedical area because of their broad spectrum of therapeutic properties and relatively low toxicity. Among the polysaccharides extracted from natural sources, water soluble sulphated polysaccharides are more important for their significant pharmacological activities such as anticancer, anti inflammation, immunomodulatory, antithrombotic and antidiabetic. However the activities of polysaccharides are strongly related to their monosaccharide composition, molecular mass, configuration, position of glycosidic linkage etc. Therefore quality control of polysaccharides is necessary for ensuring their efficacy and safety.

Selection of an extraction method is the most important process for quality control. In this, hot water extraction is found to be a popular approach. The extraction method involves elimination of low molecular substances from sample material with certain organic solvent, followed by the extraction with water near boiling temperature at certain time. This extraction yields water soluble polysaccharides. The parameters such as extraction time, temperature, solid liquid ratio, immersing time are optimized (Wang *et al.*, 2007). Purity is
crucial for determination of polysaccharide’s properties. Viscosity (Peng et al., 2005), refractive index (Yua et al., 2007), Diffusion ordered spectroscopy (Politi et al., 2006) are rapid method to verify purity. Nuclear magnetic spectroscopy can provide the structural characteristics of polysaccharides, which is sensitive for the purity test.

The molecular size of polysaccharides is an important physicochemical parameter which correlates with its biological activity. It was reported that the antitumor activities of the polysaccharide Levan depend on the molecular weight and that a specific class of molecular weight may be responsible for this effect (Calazans et al., 2007).

The structural analysis of polysaccharides requires specialized techniques, which differ significantly from those methods used for small molecules and other biopolymers. Since structural analysis of polysaccharides is a complex and demanding task, a good strategy is necessary before starting any experiments. A polysaccharide extracted from plant materials or food products is usually purified before being subjected to structural analysis. Sequencing of polysaccharides is difficult to achieve because of the heterogeneous nature of the polysaccharide structure, high molecular weight and polydispersity of the polymer chains.

The methods for the structural analysis of polysaccharide include GC, HPLC, FTIR, MALDI-TOF-MS and NMR. Among these, NMR method is a powerful analysis for the structural analysis of polysaccharides and is a fast, reliable and non-destructive technique.
4.2 RESULTS

4.2.1 Extraction and purification

The polysaccharide was extracted with water and the yield was found to be 23% of algal dry weight. Partially purified polysaccharide was further purified by gel filtration chromatography with water as the mobile phase. Three fractions obtained were pooled separately and carbohydrate content was analysed by Phenol-Sulphuric acid method. Fraction with high polysaccharide content was recovered and was used for further studies. The gel filtration chromatogram of the polysaccharide is shown in Figure IV-1.

**Figure IV-1**: Gel filtration chromatogram of the polysaccharide isolated from *Ulva fasciata*. 
4.2.2 Molecular weight determination

The Debye plot obtained from a sample of polysaccharide in water is shown in Figure IV-2. The weight-average molecular weight obtained from the measurement is found to be $1.31 \times 10^6 \pm 0.00$ Da.

Figure IV-2: Debye plot for Polysaccharide from *Ulva fasciata*:

![Debye Plot](image)

4.2.3 Composition analysis

4.2.3.1 Chemical parameters:

Average chemical composition of the polysaccharide isolated from *Ulva fasciata* is represented in Table IV-1.
Table IV-1: Chemical composition of polysaccharide (expressed in % weight of the polysaccharide)

<table>
<thead>
<tr>
<th>Composition</th>
<th>Weight %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>17.3</td>
</tr>
<tr>
<td>Ash</td>
<td>20.40</td>
</tr>
<tr>
<td>Total carbohydrates</td>
<td>48.60</td>
</tr>
<tr>
<td>Total proteins</td>
<td>3.41</td>
</tr>
<tr>
<td>Sulphate</td>
<td>17.50</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>16.40</td>
</tr>
</tbody>
</table>

4.2.3.2 Monosaccharide analysis

The monosaccharide analysis of the isolated polysaccharide was carried out. HPLC of the polysaccharide hydrolysate was taken and the results are given in figure IV-3

Figure IV-3: HPLC of polysaccharide hydrolysate
HPLC for monosaccharide standards are taken and results are given in figure IV-4 (a b c d).

**Figure IV-4a: HPLC for rhamnose**

![Figure IV-4a: HPLC for rhamnose](image)

**Figure IV-4b: HPLC of xylose**

![Figure IV-4b: HPLC of xylose](image)
Figure IV-4d: HPLC of dextrose

Figure IV-4e: HPLC of galactose
4.2.4. Structural study

4.2.4.1 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectrum of sulphated polysaccharide was analysed and the result is given in figure IV-5. The signal at 1230 cm\(^{-1}\) is due to sulphate ester substitution. Signal at 848 cm\(^{-1}\) is due to bending vibration of C-O-S and 1253 cm\(^{-1}\) is due to stretching vibration of S-O. Two other bands assigned at 1639 and 1056 cm\(^{-1}\) correspond to the stretching of C=O of uronic acid and the vibration of the C-O-C bridge of glucosides.

Figure IV-5: FTIR spectra of *Ulva fasciata* polysaccharide

4.2.4.2 Matrix assisted laser desorption/ionisation spectroscopy (MALDI-TOF)

MALDI-TOF analysis result of acid hydrolysed oligosaccharide fractions of polysaccharide of *Ulva* are given in Table IV-2. MALDI-TOF spectrum of polysaccharide from *Ulva fasciata* was taken which is given in Figure IV-6.
Table IV-2. MALDI-TOF of oligosaccharides

<table>
<thead>
<tr>
<th>m/z</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>945</td>
<td>(Hex)$_4$ (Deoxyhexose)$_1$ (Pent)$_1$</td>
</tr>
<tr>
<td>959</td>
<td>(Hex)$_4$ (Pent)$_1$ (Sulph)$_2$</td>
</tr>
<tr>
<td>1006</td>
<td>(Hex)$_1$ (Deoxyhexose)$_3$ (Pent)$_1$ (Sulph)$_1$ (HexA)$_1$</td>
</tr>
<tr>
<td>1294</td>
<td>(Hex)$_2$(Deoxyhexose)$_1$(Pent)$_1$ (Sulph)$_4$ (HexA)$_2$</td>
</tr>
</tbody>
</table>

The m/z value of acid generated oligosaccharide fragments were analysed with the help of ExPasy Database

Figure IV-6: MALDI-TOF spectra of polysaccharide

4.2.4.3 SEM-EDX (Scanning Electron Microscope/Energy Dispersive X-ray Spectroscopy)

Scanning electron micrograph of the polysaccharide was taken and is given in Figure IV-7 (a, b, c).
4.2.4.4 EDX analysis

Weight percentage and atomic percentage of elements in polysaccharide were analysed by EDX microanalysis and the results are given in Table IV-3 and the spectrum is given in Figure IV-8.
Table IV-3: Elemental EDX micro analysis of polysaccharide (Data are expressed with weight and atomic percents)

<table>
<thead>
<tr>
<th>Element</th>
<th>Weight %</th>
<th>σ Atomic %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>39.285</td>
<td>50.051</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>1.406</td>
<td>1.536</td>
</tr>
<tr>
<td>Oxygen</td>
<td>41.960</td>
<td>40.133</td>
</tr>
<tr>
<td>Sulphur</td>
<td>17.350</td>
<td>8.280</td>
</tr>
</tbody>
</table>

Figure IV-8: EDX spectrum of polysaccharide from Ulva fasciata

4.2.4.5 Particle size distribution study

Particle size distribution of the polysaccharide was analysed by Malvern zetasizer and the result is given in Figure IV-9.
Figure IV-9: Intensity size distribution of polysaccharide in water (10mg/mL)

The polysaccharide was found to have a Z-Average of 448 (d.nm) and the refractive index was found to be 1.52 with viscosity (cP) 0.8872.

4.2.4.6 $^1$HNMR spectrum

$^1$HNMR spectrum of the polysaccharide was taken and is given in figure IV-10.

NMR spectrum is presented in chemical shifts (δ, ppm) relative to internal references (e.g., TMS, tetramethylsilane). In the proton spectrum, all chemical shifts derived from carbohydrates, including mono-, oligo- and polysaccharides, are in the range of 1 to 6 ppm. So the results obtained between 1.039 and 4.704 supports the polysaccharide structure. the signals at 4.3 to 4.8 assigned for the 1H of β glycan.

As the sample is dissolved in D$_2$O all the –OH protons will be exchanged with D, so –OH protons will not appear in the $^1$H NMR spectrum. Only the encircled protons will appear in the $^1$H NMR spectrum. The CH
protons on the ring (encircled) come between 3 and 4 ppm in the $^1$H NMR spectrum. The CH$_2$ protons on the side-chain –CH$_2$OH group will also come between 3 and 4 ppm in the $^1$H NMR spectrum. The side chain –CH$_3$ group in rhamnose will appear at around 1.0 ppm.

**Figure IV-10: $^1$HNMR spectrum of Polysaccharide**

![HNMR spectrum of Polysaccharide](image)

4.2.4.7 COSY analysis

The COSY spectrum of the polysaccharide was taken and is shown in figure IV-11. The intensity of –CH$_3$ peak and the CH proton (to which –CH$_3$ is attached in rhamnose) are high. This means that the major component of the polysaccharide is rhamnose sugar. Even if the other sugars are present, they are present only in minor proportions.
4.2.4.8 $^{13}$C NMR spectrum

$^{13}$C NMR analysis of the polysaccharide was carried out and the spectrum is given in Figure IV-12. The signals from 60 to 85ppm showed the presence of nonanomeric carbon. Signals appeared in a much higher field of 16.98 showed the presence of de-oxygen sugars. The signals of carbon atoms with secondary hydroxyl groups (C2, 3, 4 in pyranoses and C2, 3 in furanoses) appeared in the region of 65 to 85 ppm.
4.3 DISCUSSION

The water soluble polysaccharides obtained from *Ulva fasciata* were purified by gel filtration chromatography and the purified fraction was isolated and characterized with the help of advanced analytical methods. The polysaccharides with different molecular weights and sizes are gradually separated using the precipitant ethanol.

Molecular weight of the isolated fraction was determined by static light scattering technique with the help of Malvern Zetasizer Nano System. This technique enabled for the measurement of particle size, Zeta potential and molecular weight of the purified polysaccharide. The molecular weight of the polysaccharide was found to be 1310 kD. This is high when compared to the molecular weight reported from Ulval polysaccharides. So the polysaccharide isolated in our study would be a novel polysaccharide. The
molecular weight of the polysaccharide is important since one of the factors that support the bioactivity of the polysaccharide is its molecular weight. Suarez et al., (2006) reported that the immunostimulatory activity of arabinogalactans extracted from *chlorella pyrenoidosa* cells depended on their molecular weights, the higher molecular weight arabinogalactans exhibited immunostimulatory activity, but the lower molecular weight fractions did not. Different molecular weights and molecular weight distributions of ulvan have been reported. Sedimentation measurements gave molecular weights ranging from $5.3 \times 10^5$ to $3.6 \times 10^6$ g/mol for *U. pertusa*, *U. conglobata*, and *E. prolifera* ulvans (Yamamoto, 1980). The polysaccharide extracted at 90 °C showed high yield with high molecular weight.

In this study, an attempt was made to extract, purify and characterize the polysaccharide isolated from green algae *Ulva fasciata*. Among the polymers synthesized by *Ulva sps.* water soluble polysaccharide commonly known as ulvan represents the major one. The importance of the study on structural characterisation and biological property of water soluble polysaccharide from *Ulva fasciata* relies on the fact that the ecophysiological growth conditions could affect biosynthesis of ulvan and, thus, its chemistry. Reports indicate that there is a variation in carbohydrate contents with seasons (Lahaye, 1999) and the algal species.

The chemical composition of the polysaccharide was analysed and the presence of sulphate and uronic acids were detected. The sulphate content of the polysaccharide was found to be 17.5% and that of uronic acid was 19.1%. Uronic acid (6.5-19.0%), and sulphate (16.0-23.2%) have since then been reported in ulvan from several species (De Reviers and Leproux, 1993;
Ray and Lahaye, 1995; Quemener et al., 1997). The monosaccharide composition of the polysaccharide was found to be rhamnose, xylose, glucose and galactose. Numerous works have proved the priority of sulphate groups in polysaccharides for their anticoagulant activity (Paradossi et al., 2002; Blomster et al., 1998; Hayden et al., 2003). Sulphate, rhamnose, xylose, glucose, galactose and uronic acid were found to be the major constituents of the isolated polysaccharide. The variation in sugar composition would be due to varying methodological, taxonomical and ecophysiological conditions.

FTIR spectroscopy was used to investigate the vibrations of molecules and polar bonds between the different atoms. Structures of polysaccharides, such as monosaccharide types, glucosidic bonds and functional groups were analyzed using FTIR spectroscopy (Mathlouthi and Koenig, 1986; Zhang, 1994). The FTIR spectrum of the polysaccharide revealed a strong absorption peak at 1056 cm\(^{-1}\) represented by vibration of the C-O-C bridge of glucosides. From the other peaks obtained it is clear that the polysaccharide constituted sulphur ester substitution and uronic acid residues. The purified polysaccharide was imaged by the SEM. Magnifications at x1600, x10000 and x15000 were taken. SEM analysis of ulvan polysaccharide is scanty and no work is available on the study of structure of algal polysaccharides of Kerala Coast and perhaps this is the first report.

Most of the difficulties for the structural studies arise from the fact that these compounds are very heterogeneous polysaccharides, which would give complex \(^{13}\)C-NMR spectra with broad signals hampering resolution (Mulloy et al., 1994). The \(^1\)H NMR spectra of polysaccharide showed clear resolution as it is very sensitive than the \(^{13}\)C-NMR.
It is clearly established that the bioactivities of polysaccharides should depend on their detailed structures. The elucidation of the actual structure of macromolecular polysaccharide is very complicate. So the structure-function relationship could not be clearly elucidated, so far there is no ideal method available for assessing the full structures of polysaccharide.

In our study the structure of the polysaccharide has been elucidated to some extent and the function of the polysaccharide could be established on the basis of the structure of the molecule.