CHAPTER : 2

PURIFICATION AND CHARACTERIZATION OF *FUSARIUM* LECTIN
Chapter 2: Purification

SUMMARY
A lectin from the mycelial extract of an endophytic strain of *Fusarium* sp. was purified to homogeneity by successive and repeated chromatography on Phenyl-Sepharose followed by gel filtration with an overall yield of 26%. The molecular mass of the lectin determined by gel filtration is 26 kDa and it is made up of two identical subunits of 13 kDa. It is a basic protein with a pI of 8.7. The purified lectin is a glycoprotein and contains 3.9% carbohydrate. It exhibited high pH stability and temperature stability. The partial N-terminal sequence did not show similarity with any known lectin. Purified lectin agglutinated neuraminidase/pronase treated human erythrocytes (A, B and O), with very low titre values. Its hemagglutinating activity was inhibited by glycoproteins containing N-linked or O-linked glycans. Chemical modification studies suggested the involvement of tyrosine in hemagglutinating activity.

INTRODUCTION
Lectins are non-immunogenic proteins or glycoproteins that selectively bind carbohydrates without processing them enzymatically (1). They are widespread in origin and have been isolated from animals, plants, bacteria, viruses and fungi. Although extensive studies have been carried out on plant and animal lectins (2,3), very little information is available on lectins from fungal sources (4,5). However, over the past decade fungal lectins have attracted increased attention due to their antitumor, antiproliferative and immunomodulatory activities (6-8).

Endophytic fungi, which colonize inside the host plant in an asymptomatic way, are between benign parasites and true symbionts. Screening of several endophytic fungal cultures revealed that the mycelial extract of *Fusarium* sp. (LR11/NCIM1330) produces high levels of hemagglutinating activity with complex sugar specificity. The present Chapter describes the purification and characterization of a lectin from mycelia of *Fusarium*. 
MATERIALS
N-Acetylimidazole, 2,4,6-trinitrobenzenesulphonic acid, phenylglyoxal, diethylpyrocarbonate, phenylmethylsulfonyl fluoride, N-bromosuccinimide, 5,5′ dithiobis-(2-nitrobenzoic acid), sodium borohydride, hydroxylamine hydrochloride, galactose, galactosamine, 2-deoxygalactose, L-fucose, glucose, mannose, methyl-α-D-galactose, methyl-β-D-galactose, mellibiose, lactose, Phenyl-Sepharose CL-4B, pronase-E, all glycoproteins, gums and the molecular weight markers (Sigma Chemical Co. St. Louis, U.S.A); Sephadex G-25 and Sephacryl S-200 (Amersham Bioscience, Uppasala, Sweden); trypsin and neuraminidase (Sisco Research Laboratories, Mumbai, India) and dextrose, peptone, malt extract, agar and yeast extract (HiMedia Laboratories Pvt. Ltd, Mumbai, India), were used. All other reagents were of analytical grade.

METHODS
Erythrocyte preparation
Human erythrocytes of A, B and O blood groups were washed 5 to 6 times with 20 mM Tris-HCl buffer pH 7.2 containing 150 mM NaCl. A 3% (v/v) suspension of the erythrocytes in the above buffer was treated with different enzymes (pronase 0.05%, trypsin 0.05% and neuraminidase 0.1 U/ml) at 37 ºC for 1 h, washed 3 times with the same buffer and used for further studies.

Hemagglutination assays
Hemagglutination assays were performed in standard microtitre plates by the two-fold serial dilution method. A 50 µl aliquot of the erythrocytes suspension was mixed with 50 µl of serially diluted lectin and agglutination was examined visually after incubation for one hour. A unit of hemagglutination activity (U) is expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination. The specific activity of the lectin is defined as the number of hemagglutination units/mg of the protein.
Hemagglutination inhibition assays
Hemagglutination inhibition assays were performed similarly, except that serially diluted sugar solutions (25 µl) were pre-incubated for 15 min at 27 °C with 25 µl of the lectin (8 U). Erythrocyte suspension (50µl) was then added, mixed and the plates read after one hour. The glycoproteins were desialated by incubating in 0.1 M H$_2$SO$_4$ at 60 °C for 1 h followed by dialysis against distilled water.

Protein determination
Protein concentrations were determined according to Bradford (9) using BSA as standard.

Microorganism and growth
The *Fusarium* sp. (LR11/ NCIM 1330) was routinely maintained on PDA slants (potato 20% w/v, dextrose 1% w/v and agar 1.5% w/v). The fermentation was carried out by inoculating a small piece of mycelium from a 7-day old PDA slant, into 100 ml liquid MGYP medium (malt extract 0.3% w/v, yeast extract 0.3% w/v, peptone 0.5% w/v and glucose 1% w/v), in 500 ml Erlenmeyer flasks, followed by incubation under stationary condition at 26±1 °C for 5 days. After the fermentation period, the mycelium was collected by filtration, washed three times with glass distilled water, and used as the source of lectin.

Extraction and purification of lectin
All purification steps were carried out at 10±1 °C. During purification the lectin activity was monitored by hemagglutination assay.

The mycelia (10 gm wet weight) was suspended in 100 ml of 20 mM Tris-HCl buffer, pH 8.0 containing 500 mM NaCl and homogenized. The homogenate was stirred for 5-6 h and then centrifuged (10,000 g, 15 min). The pellet was then reextracted as described above. The supernatants were pooled and used for subsequent steps.
The crude extract, obtained from the above step, was dialyzed extensively against 20 mM Tris-HCl buffer, pH 8.0, containing 1.4 M ammonium sulfate and loaded on a Phenyl-Sepharose column (1.5×9 cm) pre-equilibrated with the same buffer at a flow rate of 10 ml/h. The column was then washed with the same buffer till A$_{280}$ of the fractions was < 0.05. The bound lectin was eluted by double gradient *i.e.* decreasing concentration of ammonium sulfate (1.4 M → 0 M) and increasing concentration of ethylene glycol (0% → 10%) at a flow rate of 10 ml/h. Fractions of 2 ml were collected and checked for hemagglutinating activity and protein. Fractions showing hemagglutinating activity were pooled and dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 1.4 M ammonium sulfate and rechromatographed on the same Phenyl-Sepharose column as described above. The bound lectin was eluted by a decreasing gradient of ammonium sulfate (1.4 M → 0 M) at the flow rate of 10 ml/h. Fractions of 2 ml were collected and those showing hemagglutinating activity were pooled, concentrated by ultrafiltration and loaded on a Sephacryl S-200 column (1.75×108 cm) pre-equilibrated with 20 mM Tris-HCl buffer, pH 8.0 containing 150 mM NaCl and eluted with the same buffer at the flow rate of 10 ml/h. Fractions (1 ml) were collected and those showing hemagglutinating activity were pooled and stored at −20 °C till further use.

**Electrophoresis**

Native PAGE of the purified lectin was carried out in 8% (w/v) polyacrylamide gel, pH 4.3, according to Reisfeld *et al.* (10) and the gels were stained with Coomassie Brilliant Blue R-250. SDS-PAGE was performed in 15% (w/v) polyacrylamide gel at pH 7.2 according to Weber and Osborn (11). After electrophoresis the gels were visualized by silver staining according to Blum *et al.* (12). Isoelectric focusing (IEF) in polyacrylamide gels was done according to Vesterberg (13) over the pH range 3-10.
Chapter 2: Purification

Carbohydrate content
Purified lectin (400µg in 400 µl water) was incubated with 400 µl of 5 % (w/v) phenol for 10 min at room temperature. Two ml of sulphuric acid was then added and the mixture was allowed to cool for 20 min at room temperature. The color developed was then measured spectrophotometrically, at 490 nm by using galactose-mannose (4:3) as standard (14).

Molecular mass determination

Gel filtration: The molecular mass of the purified lectin was determined by HPLC (Waters Corporation) on a gel permeation column (Protein-PAK SW300, 300x7.8 mm) at pH 6.0 and 27 °C using β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa) as reference proteins.

SDS-PAGE: This was carried out in 15 % (w/v) polyacrylamide gels, at pH 7.2, according to Weber and Osborn (11) using glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa).

MALDI-ToF: Molecular mass of the purified lectin was determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry using a Voyager DE-STR (Applied Biosystems) equipped with a 337-nm nitrogen laser.

Amino acid analysis
The amino acid analysis was done using amino acid analysis kit AccQ-Fluor supplied by Waters Corporation. Salt free lyophilized lectin (50 µg) was hydrolysed using 6 N constant boiling HCl, in vacuum sealed hydrolysing tubes for 24 h at 110 °C. The sample was then derivatized by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in borate buffer at pH 9 and 10 picomoles of the hydrolysate were loaded on AccQ-Tag column equipped with a fluorescent detector. Total cysteine was determined
according to Cavallini et al. (15) and total tryptophan according to Spande and Witkop (16).

**N-terminal sequence analysis**

N-terminal sequence was determined by automatic Edman degradation by use of a Procise™ protein sequencer (Applied Biosystem) at the protein sequencing facility of IIT, Mumbai, India. The sample (700 pmol) was applied to a glass-fiber filter and then cycled through a conditioning process of fifteen repetitions of Edman chemistry.

**Effect of metal ions**

The activity of the purified lectin was determined in the presence of 10 mM each of Ca++, Mn++, Mg++ and also after dialysis against 10 mM of EDTA.

**Temperature and pH stability**

Effect of temperature on lectin stability was monitored in the range of 10 °C to 80 °C by incubating 25 µg of the lectin for 15 minutes at the respective temperature, rapidly cooling in ice and assaying for hemagglutinating activity.

The pH stability of the lectin was determined by incubating 25 µg of lectin at different pH (pH 2-12) for 24 h. Buffers used – glycine-HCl (pH 2-3), acetate (pH 4-5), citrate-phosphate (pH 5), phosphate (6-7), Tris-HCl (pH 8-9) and glycine-NaOH (pH 10-12). The hemagglutinating activity was checked after 6, 12 and 24 h.

**Preparation of plant polysaccharides (gums)**

A number of plant gums were tested for hemagglutination inhibition of the lectin. The suspension of plant gums, gum arabic (0.5 g), gum tragacanth (0.5 g), gum guar (0.5 g), gum ghatti (0.5 g), gum karaya (0.5 g), gum locust (0.5 g), gum arabinogalactan (0.5 g), gum rosin (0.5 g) and gum pontianac (0.5 g) in 10 ml of distilled water and gum mastic (0.5 g), gum elemi (0.5 g) and gum storax (0.5 g) in 10 ml of 0.1 M NaOH were boiled for 2 h. The suspensions were centrifuged at 7,800 g for 30 min at 25 °C and the
supernatants were subjected to alcohol precipitation (1:2 v/v). The precipitate formed was re-dissolved in 10 ml of hot water and reprecipitated with alcohol (1:2 v/v). The precipitate obtained was dissolved in distilled water and dialysed against the same. The concentration of sugar solution were determined in terms of neutral sugar by phenol sulphuric acid method (14).

Chemical modification studies
During chemical modification studies, the residual activity of the modified lectin was determined by hemagglutination assay.

Reaction with PMSF
The lectin (100 µg) in 50 mM Tris-HCl buffer, pH 8.0 was incubated with 5 mM PMSF, at 27±1 °C, for 60 min (17). Aliquots were removed at 15 min intervals, the excess reagent removed by dialysis and residual activity determined. Lectin sample incubated in the absence of PMSF served as the control.

Reaction with DTNB
The lectin (100 µg) in 50 mM phosphate buffer, pH 8.0, was incubated with 0.1 mM DTNB at 27±1 °C for 1 h. Aliquots were removed at different time intervals and the residual activity determined. The modification reaction was also followed by monitoring the increase in absorbance at 412 nm and the number of sulphahydryl groups modified were calculated using a molar absorption coefficient of 13,600 M⁻¹cm⁻¹ (18).

Reaction with phenylglyoxal
Arginine residues were modified with phenylglyoxal by the method of Takahashi (19). The reagent was prepared in methanol. The lectin (300 µg), in 50 mM phosphate buffer, pH 8.0, was treated with varying concentrations of phenylglyoxal (0.5-3.0 mM) for 30 min at 25 °C. Excess reagent was then removed by dialysis, and the residual hemagglutination activity determined.
Lectin sample incubated in the absence of phenylglyoxal served as control. The methanol concentration in the reaction mixture did not exceed 2 % (v/v) and had no effect on the activity and stability of the lectin during the incubation period.

**Reaction with WRK**

The modification of carboxylate residues with Woodward’s reagent-K was carried out by incubating 300 µg of the purified lectin, in 50 mM phosphate buffer pH 7.0, with different concentrations (0.01-0.3 mM) of WRK. Aliquots were removed after every 10 min and the reaction stopped by addition of 100 mM acetate buffer, pH 4.5. The reaction mixture was dialysed to remove excess reagent and the residual activity determined. Lectin samples incubated in the absence of WRK served as control. The number of carboxylate groups modified were determined spectrophotometrically, at 340 nm, by assuming a molar absorption coefficient of 7000 M⁻¹cm⁻¹ (20).

**Reaction with EDC/NTEE**

The lectin solution (200µg), in 1 ml 50 mM MES/HEPES buffer, pH 6.0 was incubated with 50 mM EDC and 30 mM NTEE at 27±1 ºC for 45 min. After the incubation period, a 100 µl aliquot was taken and the excess reagent was removed by gel filtration on Sephadex G-25 column and the residual hemmaglutinating activity was determined. The lectin sample in same buffer incubated for the same time and temperature without EDC and NTEE served as control. Subsequently the reaction was arrested by the addition of 10 % (w/v) TCA and the precipitated protein was collected by centrifugation, washed extensively with chilled acetone, air dried and dissolved in 100 mM sodium hydroxide. The number of nitrotyrosyl groups incorporated was determined spectrophotometrically, at 430 nm, using a molar absorption coefficient of 4600 M⁻¹cm⁻¹ (21).
Modification of lysine

*Estimation of lysine with Trinitrobenzenesulphonic acid. (TNBS):*

The reaction mixture containing 0.25 ml of lectin (50 µg) and 0.25 ml 4% (w/v) sodium bicarbonate was incubated with 25 µl of 0.5% (w/v) TNBS at 37 °C, in the dark, for 2 h. The reaction was terminated by adding 0.125 ml HCl (1 N), and 0.25 ml of 10% SDS was added to dissolve the precipitate formed (22). The number of free amino groups was determined, spectrophotometrically by assuming a molar absorption coefficient of 9950 M⁻¹cm⁻¹ for trinitrophenylated lysine at 335 nm.

*Acetylation:*

Acetylation was performed as described by Fraenkel-Conrat (23). To 500 µg of *Fusarium* lectin, in 1 ml saturated sodium acetate, pH 3, aliquots of acetic anhydride (0.1-1 mM) were added over the course of one hour, after each addition, an aliquot was removed and assayed for hemagglutination activity. The numbers of amino groups modified at the end of the reaction by acetic anhydride were estimated by determining the number of free amino group as determined earlier.

*Succinilation:*

This was carried out by the method of Habeeb (24). Lectin (500 µg), in 100 mM sodium carbonate buffer pH 8.5, was incubated with varying concentration of succinic anhydride (0.5-3.0 mM) prepared in dioxane. The residual activity and the number of amino groups modified were determined as described above.

*Reductive methylation:*

This was carried out as described by Means and Feeney (25). To 1 ml of the lectin (0.3 mg/ml) in 200 mM borate buffer pH 9.0, at 0 °C, 0.1 ml of sodium borohydride solution (0.5 mg/ml) was added, followed by 6 aliquots (5 µl each) of 0.35% (v/v) formaldehyde at 10 min interval. The procedure was repeated using 3.5 (v/v) % formaldehyde. At the end of the reaction, the residual hemagglutinating activity and the number of amino groups modified were determined as
described above. Lectin incubated in the absence of formaldehyde served as control.

Reaction with NBS
The lectin (300 µg), in 100 mM sodium acetate buffer pH 5.0, was titrated with a total of 0.02 mM NBS, prepared in the same buffer. The reagent was added in five installments and the reaction was monitored spectrophotometrically by monitoring the decrease in absorbance at 280 nm. The number of tryptophan residues modified were determined by assuming a molar absorption coefficient of 5500 M⁻¹cm⁻¹ (16). The residual activity was determined by hemagglutination.

Reaction with NAI
This was performed as described by Riordan et al. (26). The lectin (300 µg) in 50 mM phosphate buffer pH 7.5 was incubated with different concentrations of NAI (0.1-10 mM) at 27±1 °C for 60 min followed by estimation of the residual activity. The excess reagent was removed by gel filtration on Sephadex G-25 column (1×10 cm) pre-equilibrated in the 50 mM phosphate buffer, pH 7.5. The lectin incubated in the absence of NAI served as control. The tyrosine residues modified were determined spectrophotometrically, using a molar absorption coefficient of 1160 M⁻¹cm⁻¹ at 278 nm.

Reactivation with hydroxilamine hydrochloride
Excess NAI was removed by dialysis against 20 mM phosphate buffer pH 7.5, from the reaction mixture prior to reactivation. The reactivation of the lectin was carried out by incubating with hydroxilamine (150 mM) in 50 mM phosphate buffer, pH 7.5 for 6 h (27).

Ligand protection
This was carried out by pre-incubating the lectin with Galβ1→3GalNAc (400 fold molar excess) followed by treatment with NAI. Both modified and
unmodified protein samples were dialysed, the residual activity estimated and the number of residues modified determined.

**CD measurements**
CD spectra of the native and chemically modified lectin samples were recorded on a JASCO-715 spectropolarimeter, at 25 °C, in the range of 178-260 nm at scan speed of 200 nm/min with a response time of 1 s and slit width 1 nm. A cylindrical quartz cell of 1 mm path length was used. All measurements were made at a lectin concentration of 0.4 mg/ml. For each spectrum, fifteen successive scans were collected and the averaged spectra were used for further analysis. Measurements were made in 50 mM phosphate buffer pH 7.5 and buffer scans recorded under the same conditions were subtracted from the protein spectra before further analysis.
RESULTS AND DISCUSSION

Lectin characterization

The results of a typical procedure for the purification of *Fusarium* sp. lectin is given in Table 2.1.

Table 2.1: Purification of *Fusarium* lectin

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (^a) (U)</th>
<th>Total Protein (mg)</th>
<th>Specific activity (^b) (U/mg)</th>
<th>Fold Purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2.1 (x) 10^5</td>
<td>3.2</td>
<td>7.0 (x) 10^4</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Phenyl Sepharose chromatography I</td>
<td>9.5 (x) 10^4</td>
<td>1.11</td>
<td>8.5 (x) 10^3</td>
<td>1.22</td>
<td>44</td>
</tr>
<tr>
<td>Phenyl Sepharose chromatography II</td>
<td>5.8 (x) 10^4</td>
<td>0.28</td>
<td>2.1 (x) 10^3</td>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>5.6 (x) 10^4</td>
<td>0.25</td>
<td>2.3 (x) 10^3</td>
<td>3.32</td>
<td>26</td>
</tr>
</tbody>
</table>

\(^a\) The reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination was expressed as a unit of hemagglutinating activity.

\(^b\) The specific activity of the lectin is defined as units of the hemagglutinating activity per milligram of lectin.

*Fusarium* lectin was purified approximately 3-fold with an overall recovery of 26%. The purified lectin moved as a single band in native PAGE indicating its homogeneity (Fig. 2.1A). The molecular mass of the lectin determined by gel filtration was 26 kDa (Fig. 2.2). The molecular mass determined by SDS-PAGE, in the absence and presence of β-mercaptoethanol, showed a single band corresponding to 12.8 kDa (Fig. 2.2A) and it also corresponded to a MALDI-ToF peak at \(m/z\) 12.7 kDa (Fig. 2.3B). The results of SDS-PAGE, gel filtration, and MALDI-ToF mass analysis indicated that this lectin is a homodimer of 13 kDa subunits with no disulphide linkages. The lectin showed resemblance in the molecular mass with other lectins reported from same group of fungi: Deuteromycetes. Lectins isolated from *Sclerotina minor*, *S. trifoliorum*, *S. miyabeana*, *Botrytis cinerea* are
homodimers of 17 kDa subunits (28). Lectin of *Rhizoctonia solani* is a dimer of 13 kDa subunits, whereas lectin from *Rhizoctonia crocorum* is a tetramer of 11 kDa subunit (29).

**Fig. 2.1. Electrophoresis of purified lectin.**

(A) Native electrophoresis of *Fusarium* lectin at pH 4.3.

(B) IEF-PAGE of *Fusarium* lectin. A 50 µg of purified lectin was loaded and carrier ampholines of the range 3-10 were used.
Fig. 2.2: Molecular mass determination of *Fusarium* lectin by gel-filtration.

(a) β-amylase (200 kDa), (b) alcohol dehydrogenase (150 kDa), (c) bovine serum albumin (66 kDa), (d) carbonic anhydrase (29 kDa) and (e) cytochrome-C (12.4 kDa).

*Fusarium* sp. lectin was a glycoprotein and contained 3.9% neutral sugar. The amino acid composition of *Fusarium* lectin showed that it contained high amount of tyrosine, lysine and phenylalanine; and two residues of tryptophan and cysteine per dimer (Table 2.2). It was a basic protein with a pI of 8.7 (Fig. 2.1B). Partial N-terminal sequence (first fifteen residues) of the purified lectin was VDVRQIVNLGGLNIV and did not show similarity to any known lectin.
Fig. 2.3 : Subunit molecular mass determination of the lectin

A. SDS-PAGE : Lane 1 : (1) glutamic dehydrogenase (55 kDa), (2) ovalbumin (45 kDa), (3) glyceraldehyde 3-phosphate dehydrogenase (36 kDa), (4) carbonic anhydrase (29 kDa), (5) trypsin inhibitor (20 kDa), (6) α-lactalbumin (14.2 kDa). Lane 2: purified *Fusarium* lectin.

B. MALDI-ToF : Lectin (0.5 μg in 0.5 μl) was applied to a polished stainless steel target and a solution (0.5 μl) of sinapic acid (10 mg/ml) in 30% (v/v) acetonitrile containing 0.1 % TFA, was added. The mixture was allowed to air-dry at ambient temperature. Spectra were acquired in the range of 10 kDa to 100 kDa, on linear mode with delayed ion extraction and with accelerating voltage of 25kV. Low mass ion gate was set to 4500 Da.
Table 2.2: Amino acid composition of the *Fusarium* sp. lectin

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>No. of residues/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid and asparagine</td>
<td>20</td>
</tr>
<tr>
<td>Threonine</td>
<td>24</td>
</tr>
<tr>
<td>Serine</td>
<td>14</td>
</tr>
<tr>
<td>Glutamic acid and glutamine</td>
<td>16</td>
</tr>
<tr>
<td>Proline</td>
<td>10</td>
</tr>
<tr>
<td>Glycine</td>
<td>42</td>
</tr>
<tr>
<td>Alanine</td>
<td>14</td>
</tr>
<tr>
<td>Valine</td>
<td>10</td>
</tr>
<tr>
<td>Methionine</td>
<td>6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8</td>
</tr>
<tr>
<td>Leucine</td>
<td>14</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>18</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>10</td>
</tr>
<tr>
<td>Lysine</td>
<td>30</td>
</tr>
<tr>
<td>Histidine</td>
<td>8</td>
</tr>
<tr>
<td>Arginine</td>
<td>14</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cysteine</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>262</strong></td>
</tr>
</tbody>
</table>


*Fusarium* sp. lectin showed high pH and thermal stability. It was active between pH 2-12, and temperature upto 70 °C. It lost 25% activity at 80 °C after 15 min; and 90% activity after 30 min. Moreover, it did not show the requirement of divalent cations for its activity since extensive dialysis of the lectin against 10 mM EDTA in 20 mM Tris-HCL buffer, pH 8.0 containing 150 mM NaCl followed by dialysis against plain buffer (to remove EDTA) did not affect the hemagglutinating activity. The far-UV CD spectra was analyzed to derive more quantitative information regarding secondary structural elements of the lectin by using three different methods, viz. CDSSTR (30,31),
CONTINLL (32,33) and SELCON3 (34,35) available at http://lamar.colostate.edu/~sreeram/cdpro/main.html. A basis set containing 43 proteins was used as reference for fitting the experimental spectrum. The results obtained from this analysis are given in Table 2.3. CDSSTR yielded the best fit values and indicated that the *Fusarium* lectin is a predominantly β-sheet protein with a relatively small α-helical content. Moreover, CLUSTER program (36) used for determination of tertiary class of protein showed that lectin belongs to ‘All-Beta’ class.

**Table 2.3 : Results of CD spectral analysis**

<table>
<thead>
<tr>
<th>Method</th>
<th>α_R</th>
<th>α_D</th>
<th>Total α</th>
<th>β_R</th>
<th>β_D</th>
<th>Total β</th>
<th>Turn</th>
<th>Unordered</th>
<th>NRMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDSSTR</td>
<td>0.07</td>
<td>0.09</td>
<td>0.16</td>
<td>0.17</td>
<td>0.09</td>
<td>0.26</td>
<td>0.22</td>
<td>0.36</td>
<td>0.176</td>
</tr>
<tr>
<td>SELCON3</td>
<td>0.057</td>
<td>0.078</td>
<td>0.135</td>
<td>0.236</td>
<td>0.117</td>
<td>0.353</td>
<td>0.216</td>
<td>0.216</td>
<td>0.626</td>
</tr>
<tr>
<td>CONTINLL</td>
<td>0.080</td>
<td>0.024</td>
<td>0.104</td>
<td>0.208</td>
<td>0.130</td>
<td>0.338</td>
<td>0.336</td>
<td>0.220</td>
<td>0.277</td>
</tr>
<tr>
<td>Average</td>
<td>0.133</td>
<td></td>
<td>0.317</td>
<td>0.257</td>
<td></td>
<td>0.265</td>
<td></td>
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</tr>
</tbody>
</table>

α_R and α_D corresponds to regular and distorted α helical structures; β_R and β_D corresponds to regular and distorted β-sheet structures. NRMSD, normalized root mean square deviation.

The lectin did not agglutinate normal or trypsinized erythrocytes but did neuraminidase or pronase-E treated erythrocytes (Table 2.4). The specific activity was approximately 250 times higher with pronase treated erythrocytes than neuraminidase treated erythrocytes. The affinity of lectin for desialated glycoconjugates was also reflected in its better inhibition with desialated glycoproteins. Pronase-E, a cocktail of different proteolytic enzymes, removes all protruding polypeptide from the erythrocyte membrane exposing GPI-anchors and glycolipids, which may serve as better ligands for *Fusarium* lectin than asialo-glycoconjugates on the erythrocyte membrane.
Table 2.4: Specific activity of *Fusarium* sp. lectin with different blood group erythrocytes treated with different enzymes

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Hemagglutinating activity with different erythrocytes (U/mg)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>$1.16 \times 10^5$</td>
</tr>
<tr>
<td>Pronase</td>
<td>$3 \times 10^7$</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0</td>
</tr>
</tbody>
</table>

For details refer to Methods.

**Hemagglutination inhibition**

Simple sugars like glucose, mannose, lactose, galactose, rhamnose, xylose, fucose, raffinose, glucosamine, mannosamine, galactosamine, N-acetyl-mannosamine, N-acetyl-galactosamine, N-acetyl-glucosamine failed to inhibit the hemagglutinating activity of the *Fusarium* lectin whereas glycoproteins viz. fetuin, asialofetuin, fibrinogen, asialo-fibrinogen, thyroglobulin, holotransferrin, BSM and asialo-BSM; and plant polysaccharide like, gum karaya and gum ghatti were inhibitory. Among the glycoproteins asialofetuin and asialo-BSM were the best inhibitors with minimum inhibitory concentration of approximately 0.1 µg. It was noted that the minimum inhibitory concentration of desialated glycoproteins was 150-300 times lower than the sialated one (Table 2.5).

Glycoproteins possess different N-linked and O-linked glycans, as ligands for lectins to interact. Asialofetuin has three triantennary N-linked core structure with a terminal Galβ1→4GlcNAc and three O-linked structure Galβ1→3GalNAcα1→OSer/Thr (Tα Antigen), while the asialofibrinogen has a biantennary N-linked structure with terminal Galβ1→4GlcNAc residues (37). On the other hand, the Asialo-BSM has several
Galβ1→3GalNAcα1→OSer/Thr and GalNAcα1→OSer/Thr (Tn antigen) structures (38,39). In the present case, the inhibition data suggested that the lectin might be recognizing the complex N-linked as well as O-linked structure.

Table 2.5: Inhibition of hemagglutination activity of *Fusarium* sp. lectin with different glycoproteins and polysaccharides

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Minimum inhibitory amount* (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuin</td>
<td>15.6</td>
</tr>
<tr>
<td>Asialofetuin</td>
<td>0.1</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>30.2</td>
</tr>
<tr>
<td>Asialofibrinogen</td>
<td>0.4</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>0.9</td>
</tr>
<tr>
<td>Holotransferrin</td>
<td>62.5</td>
</tr>
<tr>
<td>BSM</td>
<td>5.0</td>
</tr>
<tr>
<td>Asialo-BSM</td>
<td>0.15</td>
</tr>
<tr>
<td>Gum karaya$^\dagger$</td>
<td>3.06</td>
</tr>
<tr>
<td>Gum ghatti$^\dagger$</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Simple sugars, gum elemi, gum arabinogalactan, gum rosin, gum mastic, gum arabic, gum tragacanth, gum locust bean and gum xanthan were not inhibitory.

* Amount of inhibitor that can cause inhibition of 8 U hemagglutinating activity of neuraminidase treated type O human erythrocytes.

$^\dagger$ In terms of neutral sugar content.

The fungal lectin isolated from *Sclerotium rolfsii* showed similar observations, its activity was inhibited by glycoproteins containing Tα and Tn
structures as asialo-PSM, asialo-BSM and asialofetuin, whereas the sialated glycoproteins showed low inhibitory effect (40). The lectin from *Beauveria bassiana* was found to interact with glycoproteins containing only O-linked structures but not N-linked (41). Contrary, the lectin from opportunistic fungus *Aspergillus fumigatus* (42) showed inhibition with sialated glycoproteins whereas hemagglutinating activity of *Rhizoctonia solani* (43) lectin was inhibited only by simple sugars as N-acetylgalactosamine, galactose, mellibiose, raffinose and others, but not by glycoproteins as fetuin, asialofetuin, ovomucoid and thyroglobulin.

The lectin activity was inhibited by plant polysaccharides such as gum ghatti and gum karaya. Gum ghatti has a backbone chain of (1-6)-linked β-D-galactopyranosyl units with some (1-4)-D-glucopyranosyluronic acid units, some joining (1-2)-D-mannopyranosyl units, and some L-arabinofuranose units. Gum karaya consists of D-galactose, D-glucoronic acid and L-rhamnose but the details of molecular structures are still not known completely (44).

Protein carbohydrate interactions between microbial lectin and plant cell wall polysaccharides have been put forward as the basis of the host pathogen recognition process. The lectin produced by *Xanthomonas campestris* is inhibited by host tissue extract (45); similarly tobacco tissue extract inhibited the Lectin I activity of *Agrobacterium radiobacter* (46). A bacterial lectin from *Agrobacterium tumefacience* (47), specific for L (−) fucose, has been shown to be inhibited by poplar plant (specific host) polysaccharides, and the lectin from phytopathogen *Aspergillus niger* (48) is also inhibited by plant polysaccharides, thus substantiating the above contention. However, several other types of molecules have also been found responsible during early event of infection. It has also been suggested, in case of bacterial infection, that sugars like glucose, galactose and arabinose, or amino acids like valine and arginine may act as chemoattractants (49). Moreover, polysaccharides associated with lectins have also been found to be involved in host-pathogen interactions. For example, the fungal plant pathogen—*Sclerotium rolfsii* produces a lectin with molecular mass of 45 kDa, that is specific for mucin and it is strongly associated with the
polysaccharide (1,3-β-glucan) it produces. The lectin as well as the polysaccharide have a role in pathogenesis (50). It was observed by Gould and Northcote that an integrated fungal molecule containing both carbohydrate and protein, was responsible for adhesion of *Phialophora radicicola* to the host (51).

Lectins have also been found in symbiotic association of microorganisms and host plant. In symbiotic association of *Rhizobium*, a plant lectin has been shown to be a host range determinant (52); but the symbiotic relationship between *Bradyrhizobium japonicum* and soybean plant has been shown to involve a lectin-carbohydrate interaction in which the lectin is produced by the microorganism (53).

**Chemical modification**

Arginine, cysteine, histidine, lysine, aspartate, glutamate and tryptophan were not found to be involved in hemagglutinating activity of the lectin (Table 2.6). Purified lectin when incubated with 10 mM NAI lost 90% of its initial activity and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in the control samples. Based on a molar absorption coefficient of 1160 M⁻¹ cm⁻¹ for tyrosine at 278 nm (26) and the molecular mass of 26 kDa for *Fusarium* lectin, the total number of tyrosine residues modified were found to be 8.0. However, the plot of percent residual activity against the number of tyrosine residues modified revealed that the loss of hemagglutination activity occurred due to the modification of 2.3 residues suggesting probable involvement of 2 residues/mol in the hemagglutination activity of *Fusarium* sp. lectin (Fig. 2.4).
Table 2.6: Effect of different modifying reagents on the activity of the lectin.

<table>
<thead>
<tr>
<th>Modification reaction</th>
<th>No of residues modified per molecule</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Serine (PMSF)</td>
<td>ND</td>
<td>100</td>
</tr>
<tr>
<td>Cysteine (DTNB)</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Arginine (Pheny]glyoxal)</td>
<td>ND</td>
<td>100</td>
</tr>
<tr>
<td>Tryptophan (NBS)</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Carboxylate (EDAC + NTEE)</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Lysine (Acetic anhydride)</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>Lysine (Succinic anhydride)</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>Lysine (Methylation)</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>Histidine (DEP)</td>
<td>ND</td>
<td>100</td>
</tr>
<tr>
<td>Tyrosine (NAI)</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Ligand protection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Lectin + Galβ1→3GalNAc + NAI)</td>
<td>5.8</td>
<td>25</td>
</tr>
<tr>
<td>Reactivation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NAI modified lectin + Hydroxilamine)</td>
<td>--</td>
<td>100</td>
</tr>
</tbody>
</table>

ND- Not determined
Fig 2.4: Plot of percent residual activity versus number of tyrosine residues modified: The number of tyrosine residues modified were estimated as described in Methods.

Treatment of tyrosine modified lectin with 0.15 M hydroxylamine for 6 h (which reverse the modification) led to a complete recovery of the hemagglutinating activity of the lectin, indicating that the phenolic group of the tyrosine is primarily involved in the activity of the lectin, and the loss of the hemagglutinating activity is due to the O-acetylation of Tyr residues. NAI mediated inactivation was reduced by 25% on incubation of lectin with excess of Galβ1→3GalNAc (400 fold molar excess) prior to modification (Table 2.6). Moreover, the CD spectra of both native and modified lectin were almost identical showing that the loss of activity is due to the modification of tyrosine residues rather than structural changes (Fig. 2.5).
Tyrosine has been implicated in the sugar-binding activity of a number of lectins as from *Artocarpus hirsuta* (54), *Erythrina indica* (55) and *Trichosanthes dioica* (56).

In conclusion, the present studies showed a lectin is produced by *Fusarium* sp. LR11 having complex sugar specificity. It agglutinates pronase or neuraminidase treated human erythrocytes, and the hemagglutination activity does not require any metal ion. Its hemagglutinating activity can be inhibited by glycoproteins and plant polysaccharides. Chemical modification studies, on *Fusarium* lectin, revealed that tyrosine is present at the sugar binding site of the lectin.
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