CHAPTER 2

Purification and characterization of lectins from *Agrobacterium radiobacter*

SUMMARY

Many isolates of *Agrobacterium* obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, (India), were screened for extracellular as well as cell bound lectin production. The microorganisms were grown in liquid media containing ($g.L^{-1}$) glucose 20, peptone 5, yeast extract 3 and malt extract 3, pH 7.0 for 72 hours. Lectin activity in an aliquot of the culture broth was checked every 24 hours by haemagglutination of rabbit

erythrocytes. Out of 11 cultures tested, isolate number 7, i.e. *Agrobacterium radiobacter*, showing consistently higher, extracellular as well as cell bound lectin activity than other isolates was chosen for further studies.

The growth medium for the chosen isolate was optimized by varying the carbon source and pH of the medium. Lectin production in synthetic medium was also checked. The optimized medium which supported highest lectin production contained ($g.L^{-1}$) sucrose 20, peptone 5, yeast extract 3 and beef extract 3, pH 6.5.

The time course of lectin production of *Agrobacterium radiobacter* was followed by monitoring the protein content and lectin activity every 24 hours upto 120 hours. The highest lectin (extracellular and cell bound) activity was found at the end of 48 hours of fermentation and therefore the culture was harvested after every 48 hours.

After optimizing the growth conditions and lectin production,

the extracellular lectin (Lectin I) was purified to homogeneity by ion exchange chromatography on DEAE-cellulose followed by hydrophobic chromatography on phenyl Sepharose. Lectin I was a monomer of relative molecular mass 37000 as determined by denaturing gel electrophoresis as well as size exclusion chromatography on Sephacryl S-300. Its isoelectric point was 4.0. Amino acid analysis revealed that acidic amino acids and glycine were predominant amino acids and cysteine was absent. The lectin was stable under acidic environment and at room temperature for 2 hours. It agglutinated rabbit erythrocytes but not human blood group A, B, or O erythrocytes. The lectin was specific for chitobiose as well as to some extent to mannose. Glycoproteins having high mannose type of carbohydrate structure inhibited the lectin activity strongly.The exact sugar structure which it recognized was glcNAc-glcNAc-man3. Tobacco plant tissue extracts also inhibited the lectin activity.

Agrobacterium radiobacter produced another lectin (Lectin II) which was cell bound. It was extracted in 6M urea at 60°C and purified on Sepharose -4B column in 6M urea after 0.1 % sodium deoxycholate treatment. It was a monomer of M_r 40000 as shown by SDS-PAGE. The lectin had a pI of 9.1 and amino acid composition of the lectin showed that it had 44 % of hydrophobic amino acids. The lectin showed specificity towards D-glucosamine. Fetuin as well as desialylated fetuin and tobacco plant tissue extract inhibited the lectin activity strongly. Locust bean polysaccharide was the best inhibitor among plant polysaccharides.

2.1 INTRODUCTION

It is known that several bacteria produce lectins and lectin like proteins (1). These include exotoxins, surface proteins and small appendages like pili which are involved in specific adhesion of the bacterial cell to eukaryotic cell surface (2). Physiological role of some of these lectins is known as they contribute to microbial pathogenicity. However, bacterial lectins, especially those from plant pathogenic microorganisms have not been studied in much detail. *Agrobacterium* species are gram negative, obligate aerobic soil bacteria capable of saprophytic or parasitic growth and are responsible for the crown gall and hairy root diseases of dicotyledonous plants (3).

The disease production by Agrobacteria, is believed to start with a site specific attachment of the pathogen to its host. Specific constituents for attachment are probably located on the surface of both bacteria and host cells. The genus contains two widely studied pathogenic species : *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. The genus includes only one nonpathogenic species *viz. Agrobacterium radiobacter* (4). Agrobacteria infect a wide variety of plants and infect only at wound sites. They infect individual cells at the site of infection and cause these cells to proliferate. This is achieved by the transfer of a discrete fragment of bacterial DNA to the nuclei of plant cells, where it is integrated into plant genomic DNA and directs the overproduction of plant

growth hormones. Understanding how *Agrobacterium* species perceive wounded plants and transfer DNA into them has been aimed at by many workers till date.

Agrobaterium is a peritrichous motile organism and the first and foremost step in the infection is supposed to be site specific attachment of the bacterium to the host cell surface. This attachment might involve lectin carbohydrate interaction (5). Although *Agrobacterium radiobacter* is a nonpathogen, there are several reports available on the successful biocontrol of pathogenic Agrobacteria using nonpathogenic ones (6,7).

In case of *Agrobacterium tumefaciens*, Depierreux *et al.* (5) have shown that the lectin resides on the bacterial surface and host plant carbohydrates act as receptors. The nonpathogenic *Agrobacterium radiobacter* probably competes with pathogenic agrobacteria for the same receptor sites on the host and this could be through lectin mediated specific attachment. Therefore, to see whether the nonpathogenic Agrobacteria also produce any lectins was of interest. The present study was aimed at screening of *Agrobacterium* species for extracellular lectin production and optimization of its growth conditions as well as purification and characterization of these lectins.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Various isolates of Agrobacteria were obtained from National Collection of Industrial Microorganisms (NCIM),

National Chemical Laboratory, Pune, (India). Glucose, sucrose, peptone, yeast extract, malt extract, beef extract and agar were obtained from HiMedia Laboratories, Bombay, (India). Other salts required in synthetic medium, like magnesium sulphate, ammonium sulphate, boric acid, citric acid, calcium carbonate etc. were analytical grade and obtained from Qualigens, (India). DEAE- cellulose DE-52 was purchased from Whatman (UK), phenyl Sepharose and Sephacryl S-300 were obtained from Pharmacia (Sweden). Ampholines, SDS-PAGE molecular weight markers, glycoproteins, sugars and gums were obtained from Sigma (USA). Urea and sodium deoxycholate were from Qualigens, (India).

All other chemicals used in this study were of analytical grade.

2.2.2 Maintenance of the microorganisms

All the cultures of *Agrobacterium* were regularly maintained on agar slants containing ($g.L^{-1}$): glucose 20, peptone 5, malt extract 3 and yeast extract 3, pH 6.8.

2.2.3 Screening

All the cultures were grown in 50 ml liquid medium containing ($g.L^{-1}$): glucose 20, peptone 5, malt extract 3 and yeast extract 3, pH 6.8, in 250 ml flasks at 28°C for 72 hours and the lectin activity was checked after every 24 hours. Inall 11 number of isolates were screened.

2.2.4 Medium optimization

Various conditions for growth were tested and the medium

supporting highest lectin production was selected. Sucrose as carbon source was tested instead of glucose; beef extract was supplemented for malt extract; and pH values of the medium were also varied to see its effect on lectin production and growth of the microorganism. Synthetic medium used for growth contained ($g.L^{-1}$) : glucose 10, KH_2PO_4 5, $MgSO_4$ 0.2, $(NH_4)_2SO_4$ 2, citric acid 0.2, H_3BO_3 0.006, ZnO 0.006, $FeCl_3$ 0.002, $CaCO_3$ 0.002, pH 6.8. To check whether PO_4 ions inhibit the polysaccharide production, the synthetic medium was supplemented with 2.2 % (w/v) KH_2PO_4. The selected *Agrobacterium* spp. was also grown in nutrient broth containing ($g.L^{-1}$) : peptone 5 and beef extract 3, pH 7.0. All these experiments were done in duplicates. The flasks were incubated at 28°C for 48 hours under shaking conditions and cells were separated by centrifugation (9770 X g,20 min.) from liquid medium. The lectin activity was checked for both of the lectins, extracellular as well as cell bound lectin.

2.2.5 Time course of production of lectin by Agrobacterium radiobacter

Time course for the production of lectin by *Agrobacterium radiobacter* was followed by monitoring protein as well as haemagglutination activity every 24 hours upto 120 hours. One ml aliquot was removed aseptically for checking the activity. The cells were separated by centrifugation and 100 µl of the culture filtrate was used for haemagglutination activity and 900 µl of the sample was subjected to 12.5 % trichloroacetic acid precipitation. After 30 min. of incubation in ice, the

protein precipitate was separated by centrifugation, washed twice with acetone and then dissolved in minimum volume of distilled water. This sample was used for protein estimation by the method of Lowry *et al.* (6). For determination of cell bound lectin activity, the cell pellet was washed 3 times with TBS (20 mM Tris-HCl, pH 7.2 with 0.15 M NaCl) and suspended in 100 µl of the same buffer. Then haemagglutination activity of the cells was checked.

2.2.6 Purification of lectin I

From one liter culture broth of *Agrobacterium radiobacter* (NCIM 2443) grown at 28°C for 48 hours, bacterial cells were separated by centrifugation (9770 X g for 20 min) and the culture filtrate was subjected to 80 % (w/v) saturation with $(NH_4)_2SO_4$. The precipitate formed was dissolved and dialyzed against 20 mM Tris-HCl buffer, pH 7.2 containing 2 mM EDTA (buffer A), sonicated (Ralsonic sonicator, at 10000 Kcyc for 3 min.) to reduce the viscosity and applied onto a column of DEAE- cellulose DE-52 (2 x 40 cm) preequilibrated with buffer A. The column was then washed with the same buffer till all the unbound protein was removed from the column. Subsequently, the bound protein was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A. The fractions showing haemagglutination activity were pooled, dialyzed against buffer A and applied on a phenyl Sepharose column preequilibrated with 20 % $(NH_4)_2SO_4$ in buffer A. Elution was carried out with a gradient of 20 % to 0 % $(NH_4)_2SO_4$ in buffer A. Fractions with haemagglutination activity were pooled and dialyzed. The

purity of the protein was checked by SDS-PAGE. To remove nonproteinaceous material i.e. polysaccharide from the purified protein sample, it was passed through hydroxyapatite column (5 ml) in presence of 5 mM potassium phosphate buffer pH 6.8 containing 1 mM EDTA.

2.2.7 Purification of the lectin II

Cell bound lectin was purified according to de Graaf (25). *Agrobacterium radiobacter* cells were harvested after 48 h, by centrifugation (9770 X g) and washed three times with 20 mM Tris-HCl pH 7.0 buffer containing 0.15 M NaCl (TBS). The cells were incubated at 60°C in tris buffer saline containing 6 M urea for 30 min. in a shaker waterbath. The cells were separated from the extraction medium by centrifugation (9770 X g). The supernatant was subjected to 60 % ammonium sulphate precipitation at 4°C for 3 h and centrifuged (9770 X g, 30 min.) to collect the precipitate. The precipitated protein was dissolved in TBS supplemented with 6 M urea and dialyzed against the same. The ammonium sulphate precipitate was then subjected to 0.1 % sodium deoxycholate treatment i.e. dialysis against buffer without 6 M urea followed by dialysis (for 72 h) against tris buffer having 0.1 % sodium deoxycholate. The lipopolysaccharide precipitate formed was centrifuged and the supernatant was again dialyzed against 6 M urea buffer. This sample was loaded on Sepharose -4B column preequilibrated with urea buffer at room temperature. 2 ml fractions were collected and A_{280} was monitored. The first peak just after void volume showed lectin activity after dialysis.

Its purity was checked by SDS-PAGE.

2.2.8 Protein estimation

Protein concentration was determined by the method of Lowry *et al.* (8) using crystalline bovine serum albumin as standard.

2.2.9 Molecular mass determination

The relative molecular masses of lectin I as well as lectin II were estimated by SDS- PAGE according to Laemmli (11) using molecular weight markers (M_r 14000 - 66000) and by gel filtration using a column of Sephacryl S-300 (1x60 cm) equilibrated with buffer A containing 2 % ethylene glycol. The column was calibrated with alcohol dehydrogenase (M_r 153000), bovine serum albumin (M_r 66000), ovalbumin (M_r 45000) and carbonic anhydrase (M_r 29100) in the above buffer.

2.2.10 Determination of isoelectric point

Isoelectric focussing of lectin I in polyacrylamide gels was carried out according to Vesterberg (12), using ampholines of pH range 2.0 to 6.0. The gels were stained with Coomassie Brilliant Blue R 250. Isoelectric focussing of lectin II was carried out according to Sathivel *et al.* (26).

2.2.11 Amino acid analysis

Amino acid composition of the purified lectin I and lectin II was determined by automated amino acid analyzer (Hewlett Packard series 1050, with HP fluorescence detector). The samples were hydrolyzed in 200 µl of 6N HCl for 20 hours at

110°C and then subjected to analysis. Total cysteine was determined according to Habeeb (13) and total tryptophan was determined according to Spande and Witkop (14).

2.2.12 Haemagglutination assay

For haemagglutination activity determination, two fold serial dilutions of lectin solution (50 µl) in a microtiter plate were incubated at room temperature for one hour with equal volume of 3 % (v/v) suspension of rabbit erythrocytes in buffer A containing 0.15 M NaCl and examined. Activity was expressed as titer, i.e. the reciprocal of the highest dilution of the lectin that gave complete agglutination. Specific activity of the lectin is defined as the titer of the lectin per mg of protein. The haemagglutination activity was also checked using untreated and neuraminidase treated human erythrocytes (A, B and O blood groups). Haemagglutination inhibition studies were performed in the same way as HA, but serial dilutions of monosaccharides, disaccharides and glycoproteins (25 µl) were preincubated for 15 minutes with 25 µl of protein of titer 4 at room temperature. 50 µl of rabbit erythrocyte suspension (3 % v/v) was added and plates were read after one hour of incubation at room temperature.

2.2.13 pH and temperature stability

Effect of pH on stability and haemagglutination activity of lectin I was studied in universal buffer of pH range 3 to 11 (15). Similarly effect of temperature on lectin stability was monitored in the range of 0°C to 60°C.

2.2.14 Plant tissue extraction

Tobacco (*Nicotiana tobaccum*) tissue (25 g wet weight) was blended in presence of buffer (50 mM Tris-HCl, pH 7.2 containing 0.15 M NaCl). The blended tissue was separated by centrifugation and the buffer extract was concentrated by Amicon ultrafiltration, fitted with Amicon YM 3 membrane and used for inhibition studies. The homogenized tissue was then boiled for one hour in presence of 1M NaOH (16). The tissue was separated and extract was subjected to alcohol precipitation after neutralization. The alcohol precipitate was dissolved in buffer A and dialyzed against the same buffer. Its inhibitory effect on haemagglutination activity of lectin I and lectin II was checked after concentration.

2.2.15 Effect of plant polysaccharides (plant gums) on haemagglutination activity of Lectin II

Various plant gums were tested for haemagglutination inhibition of Lectin II. The plant gums (10 % suspension in distilled water) were boiled for 2 hours to inactivate the associated proteins and then subjected to alcohol precipitation (1:2 v/v). The samples were then dialyzed against distilled water and used in inhibition studies after checking their total neutral sugar concentrations.

2.3 RESULTS

2.3.1 Screening

A total of eleven *Agrobacterium* isolates were screened for extracellular lectin production by the microorganism. Table

2.1 shows the time and amount of lectin produced by each isolate. Out of 11 cultures, three isolates were good lectin producers. *Agrobacterium tumefaciens* as well as an isolate of *Agrobacterium radiobacter* produced lectin into the medium extracellularly. Culture number 7 *viz Agrobacterium radiobacter* was chosen for further studies because of its higher and consistent production of lectin.

2.3.2 Optimization of growth conditions for Agrobacterium radiobacter

Initially 2 % (w/v) glucose was used in the medium as a carbon source during screening of the Agrobacterial isolates. When the carbon source was shifted to sucrose 2 % (w/v), the organism produced twice the amount of the lectin as compared to glucose in 48 hours. Figure 2.1 shows the concentration of the carbon source used and the amount of lectin produced. The production of lectin seems to be dependent on the type and concentration of the carbon source used. Table 2.2 shows the media designed and lectin produced at the end of 48 hours of culturing. Control medium was the same as used for screening. When there was no carbon source present in the medium as in the case of nutrient broth, 2 times less activity was produced by the same organism. Growth in this medium also was very low as compared to control. In synthetic medium which had just enough carbon source to support the growth, the microorganism produced same amount of lectin as control. Phosphate ions are known to inhibit the polysaccharide production by the organism (9). *Agrobacterium radiobacter* pro-

Table 2.1 : Screening for production of extracellular and cell bound lectin by Agrobacteria

No.	Culture	Activity		
		24h	48h	72h
1.	*Agrobacterium tumefaciens* NCIM 2145	0	0	0
2.	*Agrobacterium tumefaciens* NCIM 2146	0	0	0
3.	*Agrobacterium tumefaciens* NCIM 2147	0	20	0
4.	*Agrobacterium tumefaciens* NCIM 2148	0	0	0
5.	*Agrobacterium tumefaciens* NCIM 2232	20	80	40
6.	*Agrobacterium tumefaciens* NCIM 2822	80	160	80
7.	*Agrobacterium radiobacter* NCIM 2443	80	160	160
8.	*Agrobacterium tumefaciens* NCIM 2939	40	80	80
9.	*Agrobacterium tumefaciens* NCIM 2940	30	40	20
10.	*Agrobacterium tumefaciens* NCIM 2941	20	40	20
11.	*Agrobacterium tumefaciens* -	80	160	40

Activity is expressed as units (titer)/ ml.

Table 2.2 : Optimization of culture conditions for production of extracellular and cell bound lectin by *Agrobacterium radiobacter*

No.	Medium	Ingradients	Activity (U/ml)
1.	Control (MGYP)	glucose 2 %, peptone 0.5 % yeast extract 0.3 %, malt extract 0.3 %	160
2.	MSYP	sucrose 2 %, peptone 0.5 % yeast extract 0.3 %, Malt extract 0.3 %	320
3.	BSYP	sucrose 2 %, peptone 0.5 % yeast extract 0.3 %, beef extract	640
4.	Nutrient broth	peptone 0.5 %, beef extract 0.3 %	80
5.	Synthetic medium	glucose 0.1 %, KH_2PO_4 0.5 % $MgSO_4$ 0.02 %, $(NH_4)_2SO_4$ 0.2 %, citric acid 0.2 %, H_3BO_3 0.0006 %, ZnO 0.0006 % $FeCl_3$ 0.0002 % and $CaCO_3$ 0.0002 %	160
6.	Synthetic medium having 125 mM PO_4^{--}	--"-- , 2.2 % KH_2PO_4	80

Figure 2.1 : Effect of carbon source on production of lectins by *Agrobacterium radiobacter* :

--O-- glucose, --■-- sucrose.

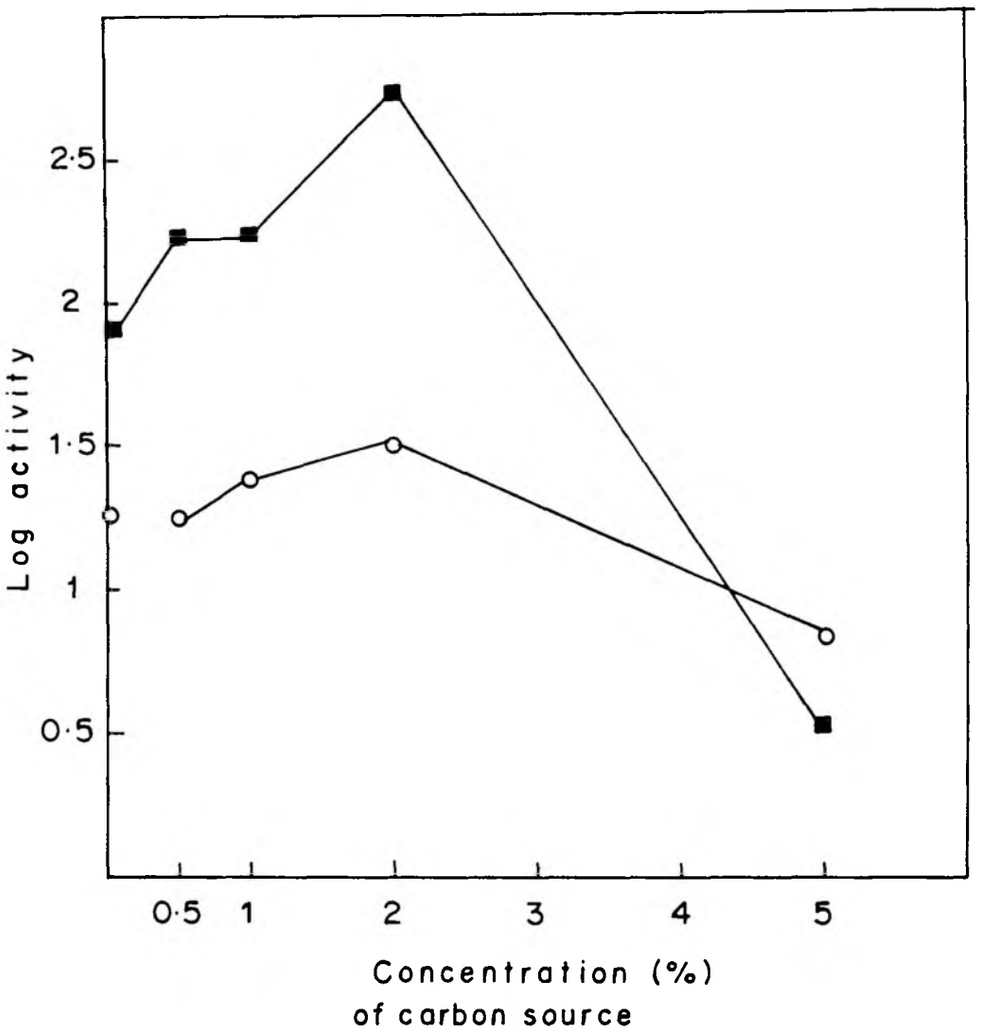
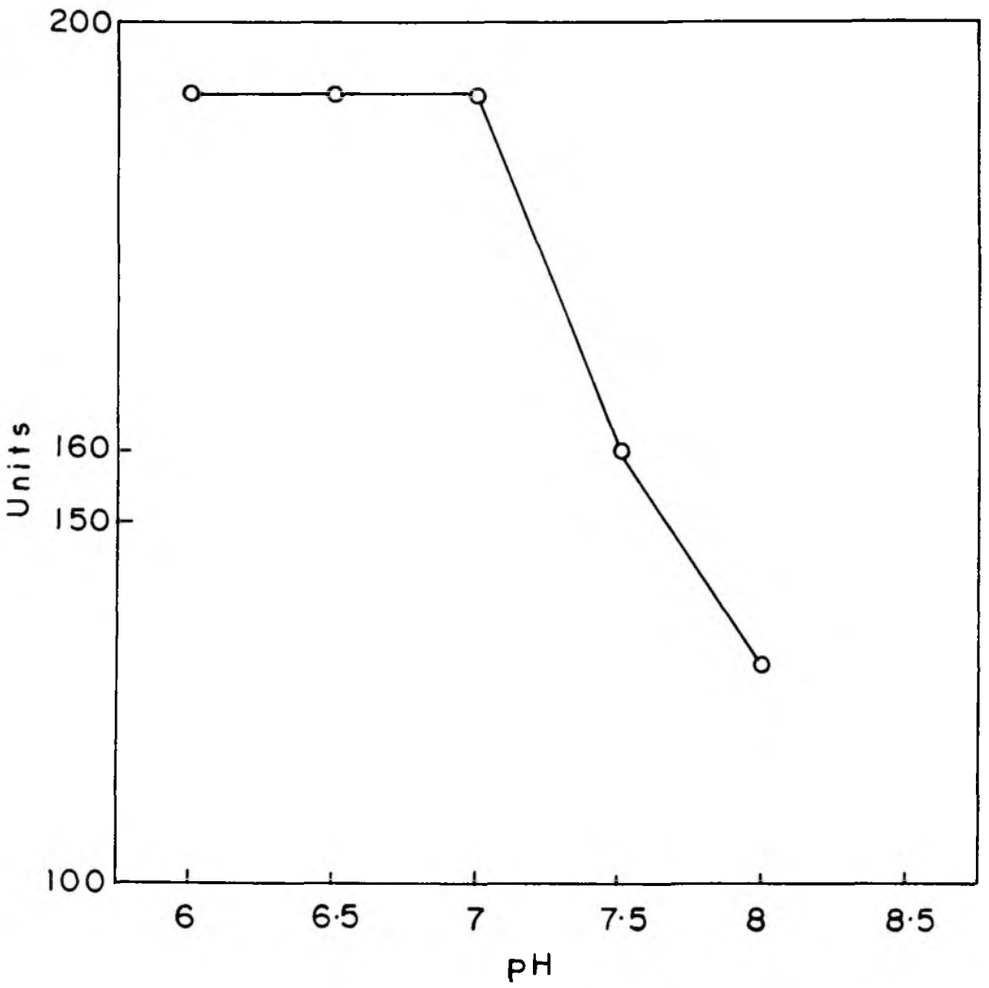

2·5
2
1·5
1
0·5
Log activity
0·5
1
2
3
4
5
Concentration (%)
of carbon source

Figure 2.2 : Effect of pH on production of lectin I by *Agrobacterium radiobacter*


200
160
150
100
Units
6
6·5
7
7·5
8
8·5
pH

duces acidic polysaccharide of succinoglycan type (10). When the synthetic medium was supplemented with 125mM KH_2PO_4, it produced 50 % less lectin than in synthetic medium. The production of extracellular lectin activity was checked at various pH values since it is an important factor for growth. pH 6.0 to 6.8 was best suited for growth and therefore a complex medium at pH 6.5 was used for growth in all the further studies (Fig. 2.2). Supplementation of beef extract for malt extract at the same concentration produced better results. Table 2.2 shows the production of lectin was 4 times higher as compared to the control when beef extract was used. Thus a medium containing ($g.L^{-1}$): sucrose 20, peptone 5, beef extract 3 and yeast extract 3, pH 6.5 was used in all further studies.

2.3.3 Time course of lectin production

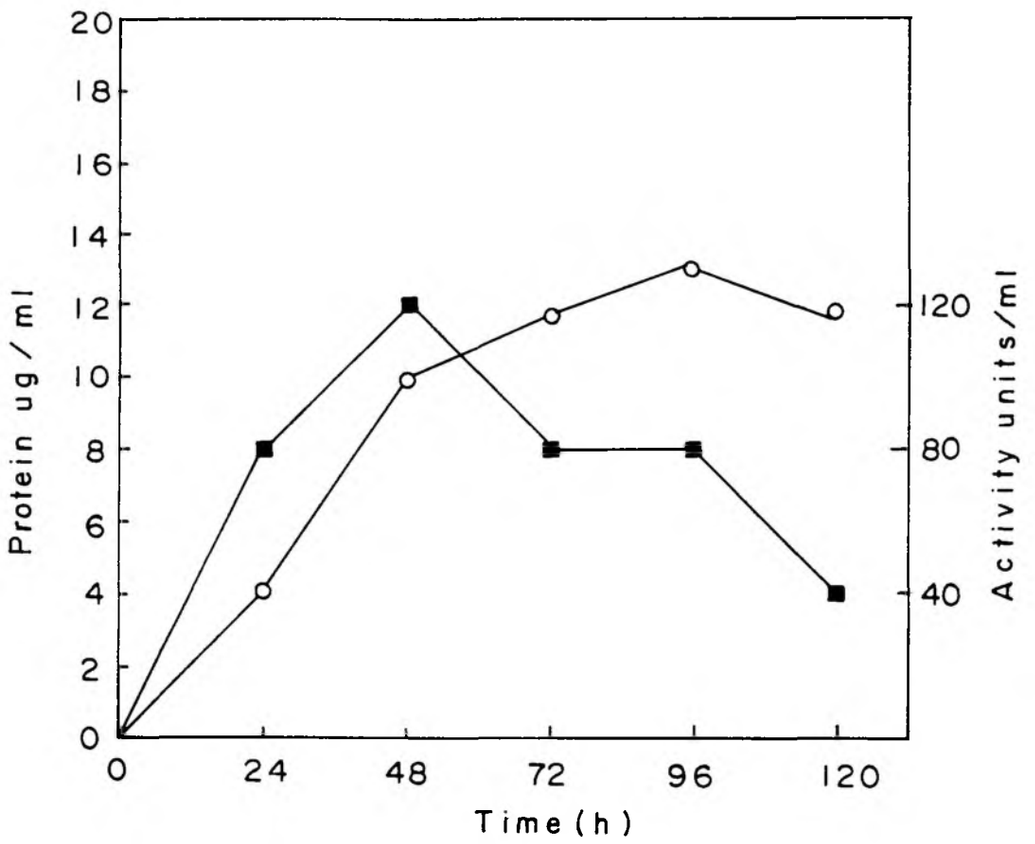
Production of extracellular lectin in the optimized medium by *Agrobacterium radiobacter* NCIM 2443 was monitored for 120 hours. Protein was determined after TCA (12.5 %) precipitation and the activity was checked as haemagglutination with the help of 3 % rabbit erythrocytes. The lectin is produced within 24 hours of fermentation and the highest amount of lectin was produced at 48 hours (Fig. 2.3).

2.3.4 Purification and characterization of Lectin I

Agrobacterium radiobacter NCIM 2443 produced a lectin extracellularly in the culture broth with a titer of 160 units/ml of the culture broth. It was concentrated by ammonium sul-

Figure 2.3 : Time course of production of Lectin I by *Agrobacterium radiobacter* in the optimized growth medium

--o-- protein, --■-- activity.


20
18
16
14
12
10
8
6
4
2
0
Protein ug / ml
120
80
40
Activity units/ml
0
24
48
72
96
120
Time (h)

phate precipitation of the culture filtrate and purified by two sequential chromatographies. The overall summary of the purification process is represented in Table 2.3. Presence of EDTA (1 mM) was necessary during the whole process of purification as well as for storage of the purified protein. The ammonium sulphate precipitate was a very viscous solution due to the polysaccharide produced by the *Agrobacterium radiobacter*. To reduce the viscosity, the ammonium sulphate precipitate was sonicated (17). Ion exchange and hydrophobic chromatographies could remove contaminating proteins as well as excess amounts of polysaccharide and colouring substances, yielding a single protein which had haemagglutination activity. Some nonproteinaceous material was still associated with the purified protein and it was separated fully from the protein on a hydroxyapatite column. The polysaccharide was bound to the matrix whereas the protein came out in column washing.

The relative subunit molecular mass of the purified lectin as determined by SDS-PAGE (Fig. 2.4) and native molecular mass determined by gel filtration (Fig. 2.5) was 37000 indicating that the lectin is a monomeric protein. Its pI was 4.0 suggesting the acidic nature of the lectin (Fig. 2.6). This observation is also supported by the amino acid composition data which shows abundance of acidic amino acids in the protein. The protein has large amount of glycine, two tryptophans and no cysteine (Table 2.4). The lectin was active when incubated at room temperature for 2 h and was stable at

Table 2.3 : Purification table of *Agrobacterium radiobacter* Lectin I

Step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity	Purification % Recovery	fold
Culture filtrate	1000	20	32000	1600	100	0
$(NH_4)2SO_4$ precipitation	70	15.7	25600	1630	80	1
Ion exchange chromatography	50	7	20480	2925.7	64	1.8
Hydrophobic chromatography	2	3	13312	4437.3	41.6	2.7
Hydroxyapatite chromatography	3	2.8	13000	4642.8	40.6	2.9

Figure 2.4 : Native and SDS PAGE of the *Agrobacterium radiobacter* Lectin I

a) Native PAGE

b) SDS- PAGE

Lane 1 : Molecular weight markers
bovine serum albumin (66000),ovalbumin (45000)
glucose 6 Phosphate dehydrogenase (36000)
carbonic anhydrase (29000), soybean trypsin
inhibitor (20000), lactalbumin (14000)

Lane 2 : Purified Lectin I

(b)

(a)

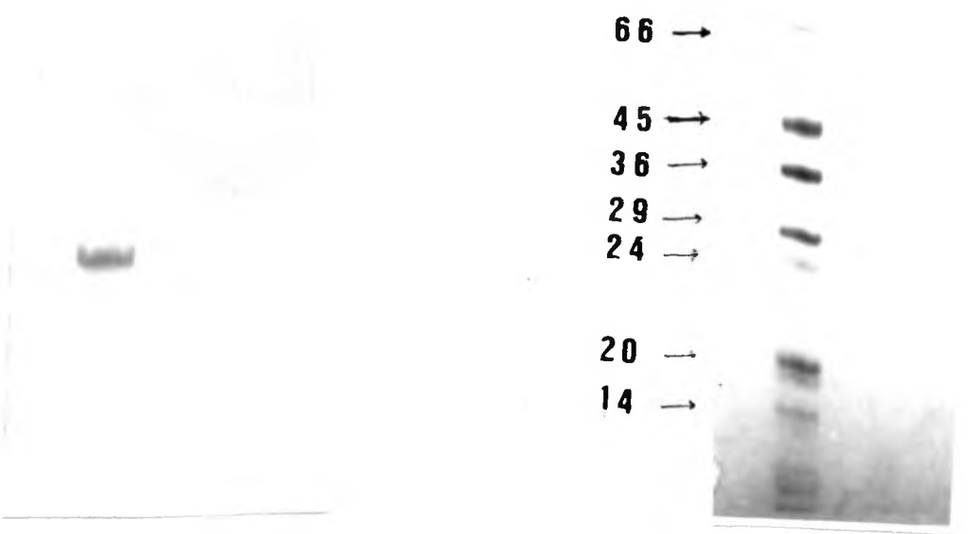


Figure 2.5 : Gel filtration of the *Agrobacterium radiobacter* lectin I

--○-- : Molecular weight markers - alcohol dehydrogenase (153000) bovine serum albumin (66000), ovalbumin (45000), and carbonic anhydrase(29000).

--●-- : Purified lectin

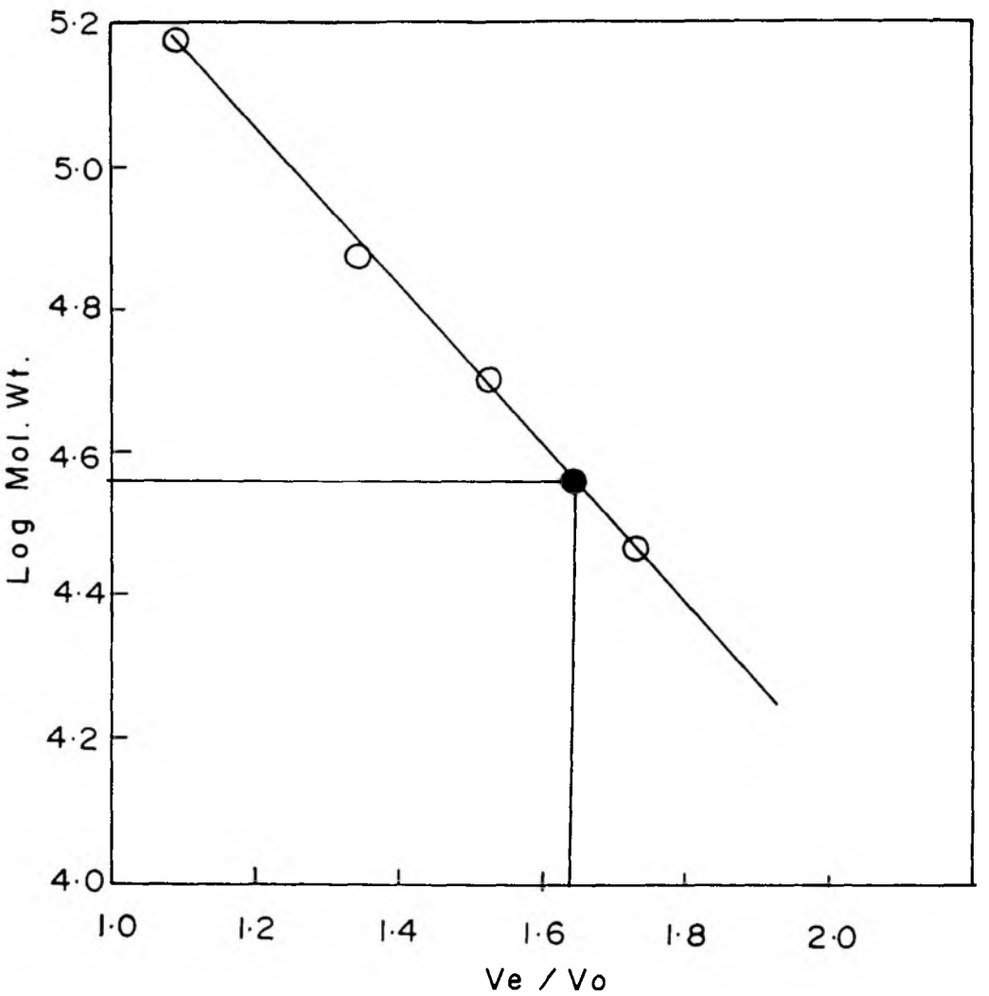
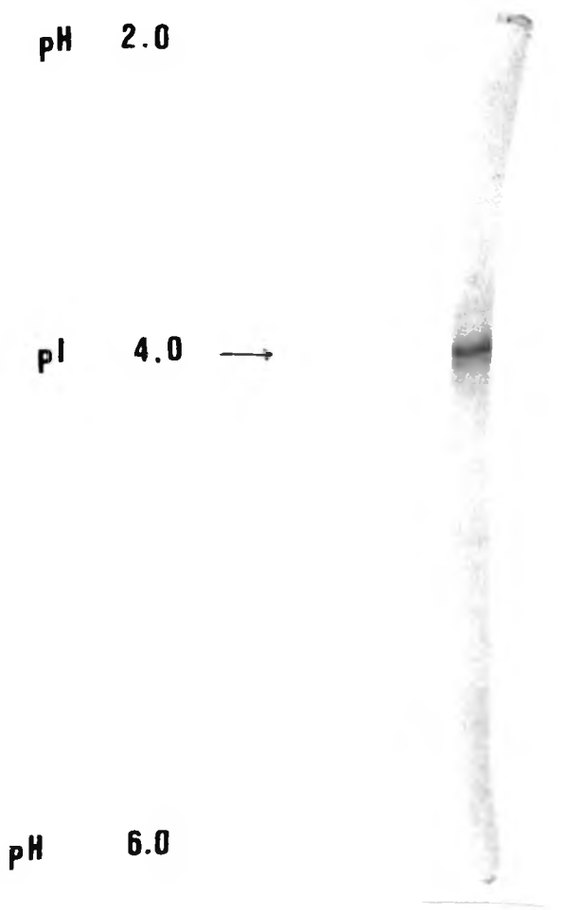

5·2
5·0
4·8
4·6
4·4
4·2
4·0
Log Mol. Wt.
1·0
1·2
1·4
1·6
1·8
2·0
Ve / Vo

Figure 2.6 : Isoelectric focussing of Lectin I


pH 2.0
pI 4.0
pH 6.0

pH 5.0 (4°C). It lost all its activity when stored above pH 7.8 (Fig. 2.7, 2.8). The lectin showed no haemagglutination activity with untreated or neuraminidase treated human RBCs. The HA activity of the lectin was inhibited by N-acetyl glucosamine and chitobiose strongly (Table 2.5); Chitotriose inhibited to a lesser extent whereas chitotetraose and chitopentaose did not inhibit the lectin activity. Lectin I was also inhibited by mannose and its β anomers. The best inhibitor was the trimannoside derivative. The man5-glcNAc oligosaccharide was the best inhibitor among all the mono, di- and oligosaccharides tested. Among glycoproteins tested for haemagglutination inhibition, fibrinogen could inhibit the lectin activity at a concentration of 15 µg whereas fetuin inhibited the lectin activity at slightly higher concentration (30 µg) (Table 2.6). Its sialic acid residues did not have any effect on inhibition as desialylated fetuin also inhibited the lectin activity to the same extent. Orosomucoid inhibited the activity to a still lesser extent than fetuin (50 µg). The lectin showed some affinity towards mannose but very high affinity for also Mannose 9 glycopeptide, yeast invertase,ribonuclease and ovalbumin. Tobacco tissue extracts (buffer and alkali) inhibited the lectin activity. The alkali extract containing polysaccharides from Tobacco plant tissue, strongly inhibited the lectin activity as compared to the buffer extract (Table 2.6).

2.3.5 Purification and characterization of Lectin II

The washed cells of *Agrobacterium radiobacter* (5 g) were

Table 2.4 : Amino acid composition of the *Agrobacterium radiobacter* lectin I

Amino acid	res/mol
Asx	42
Thr	12
Ser	19
Glx	51
Pro	23
Gly	50
Ala	26
Cys*	0
Val	15
Met	8
Ile	17
Leu	24
Tyr	13
Phe	15
His	4
Lys	12
Trp*	2
Arg	11

* = Total cysteine and total tryptophan were determined according to Habeeb (13), Spande and Witkop (14).

Figure 2.7 : Temperature stability of purified Lectin I

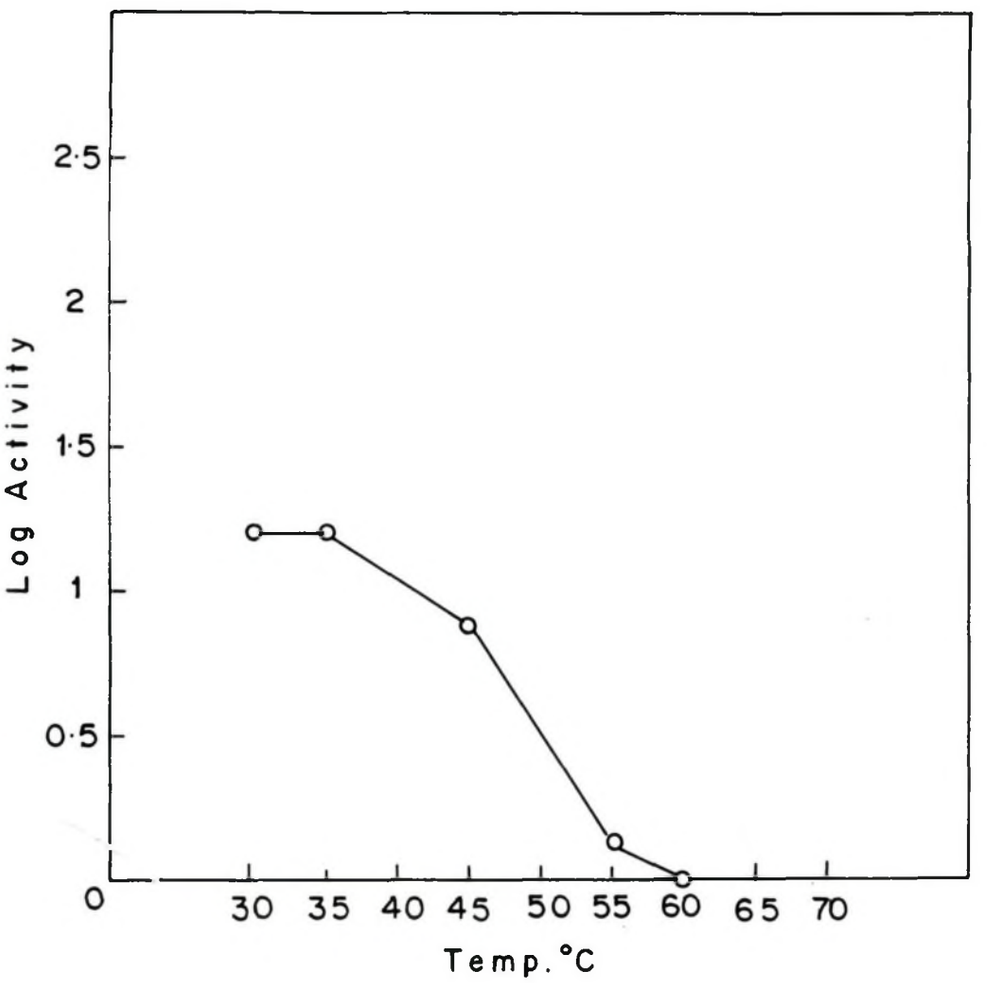

2·5
2
1·5
1
0·5
0
Log Activity
30 35 40 45 50 55 60 65 70
Temp. °C

Figure 2.8 : pH stability of purified Lectin I

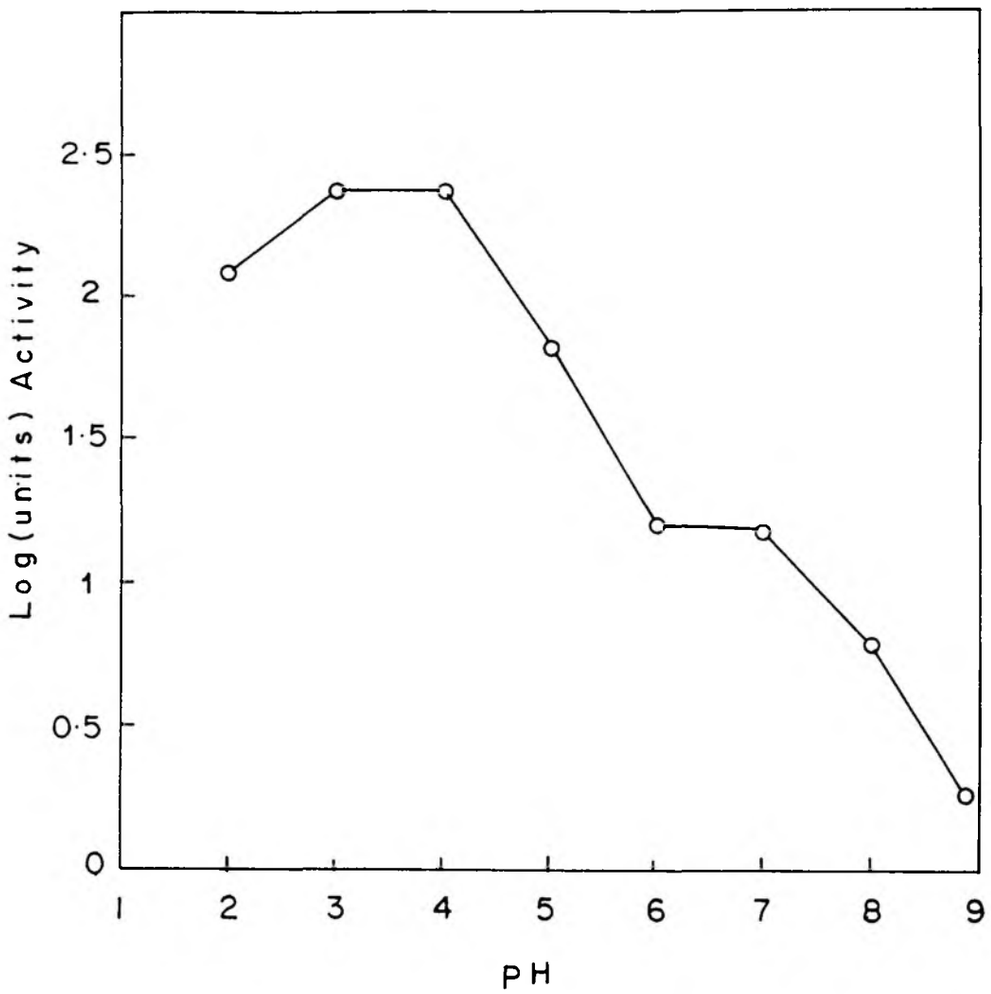

Log (units) Activity
2·5
2
1·5
1
0·5
0
1
2
3
4
5
6
7
8
9
PH

Table 2.5 : Sugar specificity of *Agrobacterium radiobacter* lectin I

Sugar	Inhibitory Concentration (mM)
1. Glucose	--
2. D-glucosamine	--
3. GlcNAc	15.6
4. Me α glc	--
5. Me β glc	--
6. Chitobiose	7.15
7. Chitotriose	12.5
8. Chitotetraose	--
9. Mannose	15.0
10. D-mannosamine	30.0
11. Me α man	30.0
12. p-nitrophenyl α man	12.5
13. p-nitrophenyl β man	6.25
14. Galactose	--
15. D-galactosamine	125.0
16. GalNAc	--
17. Me α gal	--
18. Me β gal	--
19. LacNAc	--
20. L(-)Fucose	--
21. L-Arabinose	125.0

-- = No inhibitory effect at highest (250 mM) concentration.

Table 2.6 : Inhibitory effect of oligosaccharides and glycoproteins on *Agrobacterium radiobacter* Lectin I

Glycoprotein	Inhibitory Concentration (in terms of neutral sugar)
1. Trimannoside	34.3 µg
2. Man5-glcNAc	0.22 µg
3. Man (9) glycopeptide	0.4 µg
4. Fibrinogen	15.0 µg
5. Fetuin	31.0 µg
6. Desialated Fetuin	31.0 µg
7. Orsomucoid	50.0 µg
8. Ovalbumin	7.5 µg
9. Ribonuclease A	0.75 µg
10. Yeast Invertase	0.05 µg
11. Tobacco plant tissue (buffer) extract	25.0 µg
12. Tobacco plant tissue (alkali) extract	3.5 µg

Sugar concentration was determined by phenol-sulphuric acid method.

suspended in 100 ml of urea buffer and the surface lectin was extracted. The deoxycholate treated ammonium sulphate dialysate when passed through sepharose 4B column, yielded a peak after void volume containing lectin activity. Summary of lectin purification is given in Table 2.7. The protein obtained after Sepharose 4B chromatography was homogeneous on SDS-PAGE (Fig. 2.9).

On calibrated column of Sephacryl S300 (1 X 75 cm), the dialyzed protein came out with the void volume but SDS-PAGE showed its molecular mass to be 40000. During isoelectric focussing, the lectin focussed as a single band and showed a pI of 9.1 (Fig. 2.10). The amino acid composition of the lectin showed that it had 44 % of hydrophobic amino acids, one tryptophan and no cysteine (Table 2.8)

The purified lectin agglutinated rabbit erythrocytes but not untreated or neuraminidase treated human erythrocytes of blood group A, B, or O. The lectin was inhibited by D-glucosamine and by methylated derivatives of D-glucose at 10 times higher concentration than D-glucosamine. All other monosaccharides failed to inhibit the activity of Lectin II. Among glycoproteins, fetuin and desialated fetuin inhibited lectin activity at 0.6 µg while fibrinogen inhibited it at 50 times higher concentration and ovalbumin failed to inhibit it. Among the plant polysaccharides, the lectin was inhibited by Gum Ghatti, Gum Guar, Gum Arabic and Locust gum, but the best inhibitor was Locust gum which was 100 fold better than the other gums. Tobacco plant extracts, both buffer as

Table 2.7 : Purification of lectin II

Step	Volume (ml)	Total protein (mg)	Total Activity (units)	Specific Activity	Purification fold	Purification recovery %
Cell Extract	100	125	3000	24	0	100
$(NH_4)_2SO_4$ precipitate	14	99.1	2640	26.6	1.1	88
Sepharose 4B column	8	2.97	2560	861.9	32.4	85

Table 2.8 : Amino acid composition of lectin II

Amino acid	res/mol
Asx	45
Thr	17
Ser	13
Glx	35
Gly	46
Ala	43
Cys*	0
Val	26
Met	9
Ile	21
Leu	31
Tyr	10
Phe	16
His	4
Lys	18
Arg	19
Pro	15
Trp*	1

* = Total cysteine and total tryptophan were determined according to Habeeb (13) and Spande and Witkop (14).

Figure 2.9 : SDS-PAGE of Lectin II

Lane 1 : Molecular weight markers;
bovine serum albumin (66000),ovalbumin (45000)
glucose 6 Phosphate dehydrogenase (36000)
carbonic anhydrase (29000), soybean trypsin
inhibitor (20000), lactalbumin (14000)

Lane 2 : Purified Lectin II

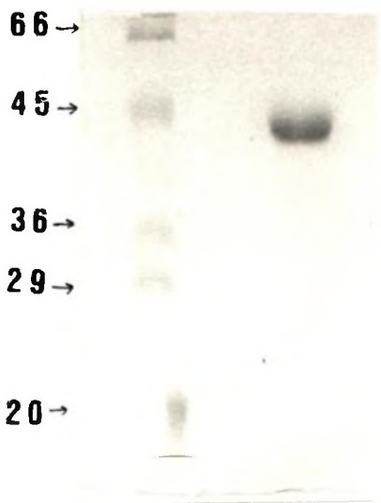


Figure 2.10 : Isoelectric focussing of Lectin II

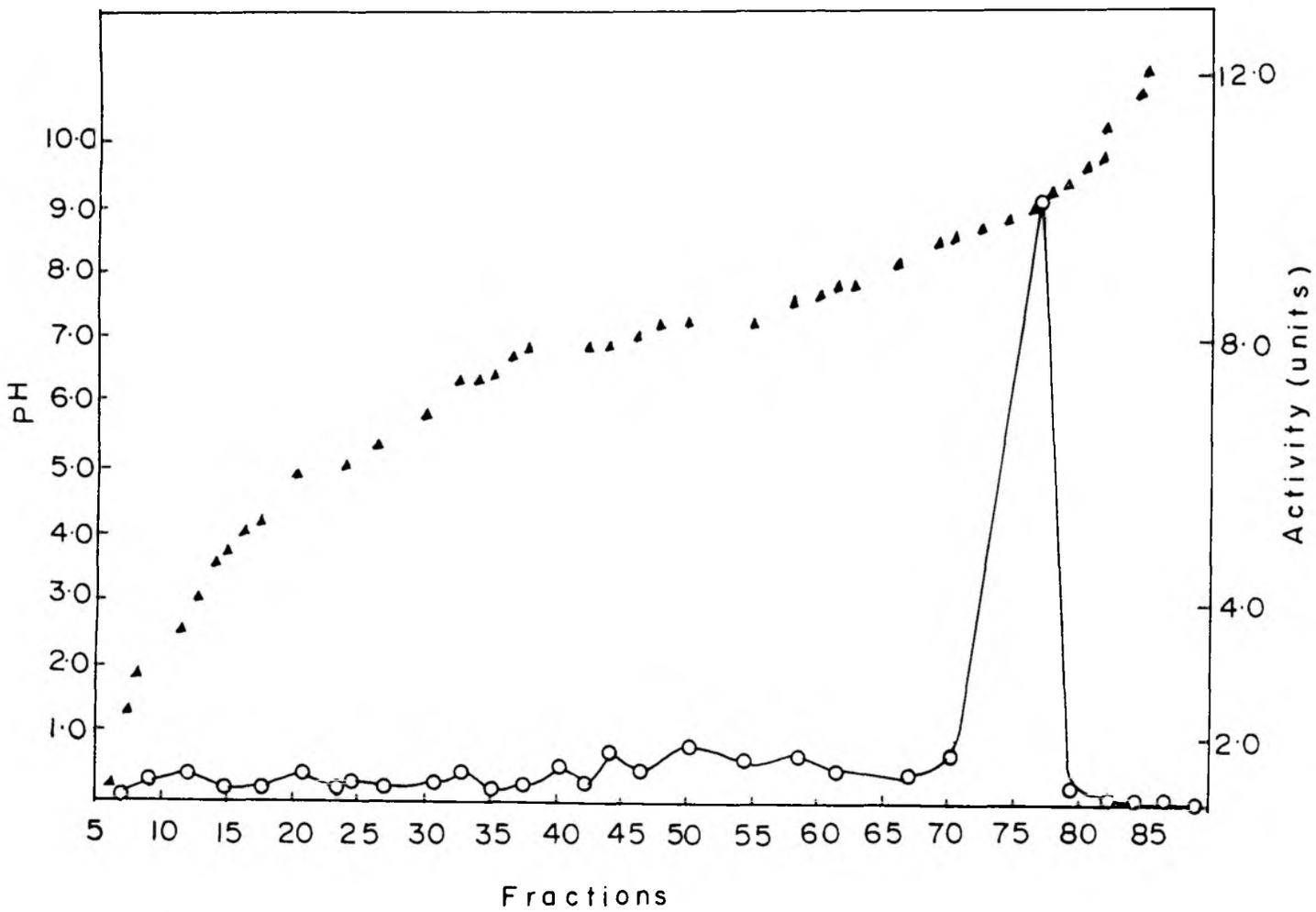

pH
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
12·0
8·0
4·0
2·0
Activity (units)
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85
Fractions

Table 2.9 : Sugar Specificity of Lectin II

Inhibitory Sugar	concentration mM
1. Glucose	--
2. D glucosamine	7.8
3. GlcNAc	250
4. Me α glc	62.5
5. Me β glc	62.5
6. Galactose	--
7. D galactosamine	--
8. GalNAc	--
9. Mannose	--
10.D mannosamine	--
11.Met α man	--
12.Fucose	125
13.Lactose	--
14.Arabinose	125
Glycoproteins	**Concentration**
15.Fetuin	0.6 µg
16.Desialylated fetuin	0.6 µg
17.Fibrinogen	30 µg
18.Ovalbumin	--

-- = No inhibition at highest concentration.

Table 2.10 : Effect of plant polysaccharides on haemagglutination activity of Lectin II

Plant Polysaccharides (Gums)	Inhibitory concentration; µg of neutral sugar
1. Ghatti	17
2. Arabic	--
3. Guar	51
4. Karaya	47
5. Locust	0.5
6. Tobacoco (buffer) extract	25
7. Tobacco (alkali) extract	1.5

-- = No inhibition at 2.5 mg/ml neutral sugar concentration.

well as alkali extract strongly inhibited the lectin activity, the alkali extract being 16 times better than the buffer extract (Table 2.10).

2.4 DISCUSSION

Molecular details of binding of the pathogenic bacteria to the target host cell surface are known only in a few bacterial systems like *E. coli*. The colonization of small intestine by enteropathogenic *E. coli* or of urinary tract by uropathogenic *E. coli* strains, is mediated by cell surface antigens (pili or fimbriae) which enable the bacteria to adhere to the specific site on the host tissue (23). The adhesins involved in this attachment process i.e. pili are often lectins and they are synthesized by large proportion of bacteria in apparently all kinds of environments. Among plant pathogenic bacteria, *Pseudomonas syringae*, *Pseudomonas savastanoi*, *Bradyrhizobium japonicum*, and *Agrobacterium tumefaciens* like organisms are known to produce lectins which are located on the cell surface (24,5).

Agrobacterium radiobacter NCIM 2443 produced two lectins, one which was extracellular (Lectin I) while the other was cell bound (Lectin II). The extracellular lectin was purified by 3 successive chromatographies. Lectin I was associated with the polysaccharide. As a consequence, the polysaccharide protected the protein and it was stable at room temperature. The interaction between polysaccharide and protein did not involve covalent bonding, as hydroxyapatite column step removed most of the associated polysaccharide

from the protein. Presence of EDTA was very essential during the purification process as well as for storage of the protein, otherwise it got fragmented accompanied with loss of activity within 48 hours. The relative inhibitory pattern of the lectin I (Table 2.5) shows that the lectin not only recognizes chitobiose but also the trimannoside with similar affinity. The higher affinity shown for the high mannose oligosaccharide glycoproteins as compared to complex oligosaccharide glycoproteins indicates that antennary structure of complex oligosaccharides sterically interferes in the binding process. The gradation shown in the decrease in affinity from bi-antennary to tetraantennary structures (Tables 2.6 and 2.11) supports this conclusion. Lectin I recognizes a complex carbohydrate structure rather than a simple monosaccharide and the probable structure that can be deduced from inhibition data is glcNAc-glcNAc-man3 core oligosaccharide of Asn linked glycoconjugates.

Tobacco tissue extracts (buffer and alkali) inhibited the lectin I and II activity. Alkali treatment which releases plant polysaccharides from plant tissue, strongly inhibited the lectin activity. This inhibition probably shows a sign of recognition of the receptor molecule on plant surface by lectin.

Lectin II is different from Lectin I which is aparant from their sugar specificity, isoelectric point and amino acid composition. The cell bound lectin (Lectin II) is more hydrophobic than the extracellular lectin (Lectin I). It forms

Table 2.11: **Relative inhibition of the lectin**

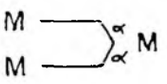
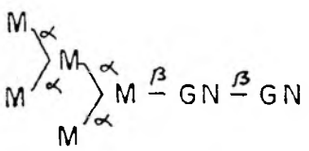
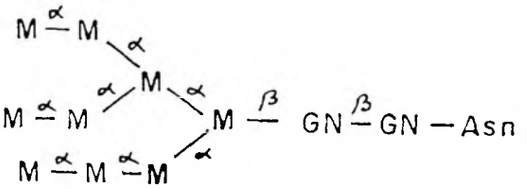
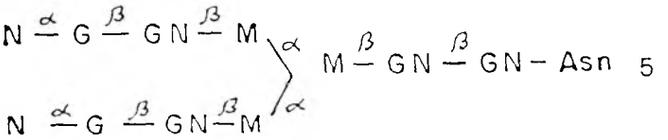
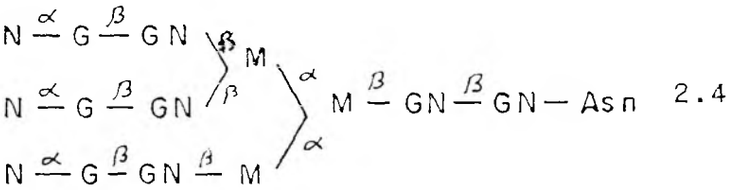
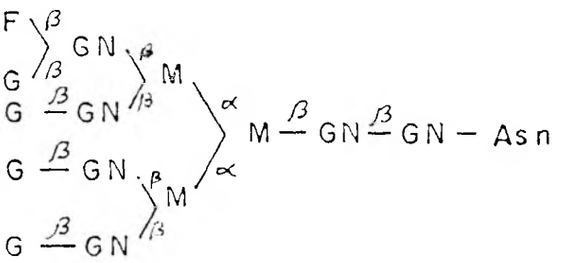
No.	Inhibitory Compound	Structure	Relative inhibition
1.	Chitobiose	GN $\overset{\beta}{-}$ GN	1 (75.7µg)
2.	Trimannoside		2.2
3.	(Man 5-glcNAc)		10^2
4.	Man(9) glycopeptide		10^2
5.	Ribonuclease A	(High mannose type)*	10^2
6.	Yeast invertase	(High mannose type)*	10^3
7.	Ovalbumin	(High mannose type)*	10

Table 2.11 continued

8. Fibrinogen[1]		5
9. Fetuin[2]		2.4
10. Orosomucoid[3]**		1.5

1,2 - Townsend *et al* [25]; 3 - Fournet *et al* [25]

* - Ribonuclease A has man(9)structure; Yeast invertase has more than one man (9) structures and ovalbumin has mixed type of bisected man(5) to man(8) structures.

** - Orosomucoid contains 50 % tetraantennary 30 % triantennary and 20 % biantennary complex oligosaccharides.

aggregates once it is dialysed against plain buffer (without urea). This may be the reason for elution of the protein in void volume of Sephacryl S-300 column. Lectin II shows high affinity for D-glucosamine although it does get inhibited by Me α glc and Me β glc at much higher concentrations. Lectin II has totally different monosaccharide specificity than Lectin I, which is specific for glcNAc and Man. Lectin II is inhibited by fetuin, to lesser extent by fibrinogen but not by ovalbumin. The glycoprotein specificity of Lectin II is reverse of Lectin I, which shows higher specificity for high mannose glycoproteins such as ovalbumin (Table 2.10). The inhibition of Lectin II at very low concentrations of plant polysaccharides indicates that it is specific for complex oligosaccharide structures.

Agrobacterium tumefaciens lectin (5) as well as *Agrobacterium radiobacter* Lectin I are most active at pH 5.0 . But *Agrobacterium tumefaciens* lectin is specific for L(-) fucose and Lectin I shows a complex sugar specificity. Poplar plant polysaccharides inhibited the *Agrobacterium tumefaciens* lectin activity similar to the inhibition of Lectin I by Tobacco plant polysaccharides. The recognition of plant polysaccharide by these lectins has prompted researchers to put forward protein carbohydrate interaction as a basis for microbial recognition and adhesion. However, other workers have reported several types of molecules to be responsible for this early event of infection. It is known that the disease induction takes place when bacteria attach themselves to wounded plant cells at a specific site (18). It is

selves to wounded plant cells at a specific site (18). It is shown by Shaw et al (19) that bacteria are attracted to the wounds by chemotaxis, where sugars like glucose, galactose, and arabinose, or amino acids like valine and arginine act as chemoattractants. According to Gurlitz (20) a protein may be a part of plant receptor site. It was claimed that bacterial lipopolysaccharides (21) and 2 linked β- D glucan also play an important role in recognition and attachment (22).

Although all members of genus *Agrobacterium* with the exception of *Agrobacterium radiobacter* induce tumors in many plants, successful biological control of this disease has been reported using *Agrobacterium radiobacter* cells (6,7). It is shown that the biological control is achieved as *Agrobacterium radiobacter* attaches itself to the same site as *Agrobacterium tumefaciens*, i.e. the pathogenic bacteria, by competing for the same receptor site. Production of lectins by *Agrobacterium radiobacter*, the affinity shown by the lectin towards plant tissue extracts and plant polysaccharides indicate the possibility of these lectins recognizing the same receptor on the plant cell.

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