CHAPTER 4
Purification and characterization of a lectin from *Xanthomonas campestris*

SUMMARY

Several isolates of a typical and well studied bacterial plant pathogen, *Xanthomonas* were screened for extracellular lectin production. Bacteria were grown at 28°C, in a liquid medium containing ($g.L^{-1}$): glucose 20, peptone 5, yeast extract 3 and malt extract 3, at pH 7.0 for 72 hours. Lectin activity was monitored every 12 hours by haemagglutination assay using 3% suspension of freshly prepared rabbit RBC and 100 µl of the culture filtrate (taken out aseptically from the actively growing culture). Out of a total of 25 isolates of *Xanthomonas* cultures, screened for extracellular lectin activity, *Xanthomonas campestris* NCIM 5028 showed the highest and consistent extracellular lectin activity. Hence it was chosen for further studies. The time course of lectin production by *Xanthomonas campestris* NCIM 5028 was followed for 120 hours by monitoring the protein content, viscosity (as a measure of extracellular polysaccharide (EPS) and lectin activity (heamaglutination assay) after every 24 hours of growth. After optimization of the growth conditions for the production of lectin, the lectin was purified to homogeneity using hydrophobic interaction chromatography on a column of phenyl-Sepharose after initial ammonium sulfate precipitation. The lectin was found to be a heterodimer of subunits having relative molecular masses of 30000 and 28000. Gel filtration on Sephacryl S-300 column calibrated with markers, showed its native molecular mass to be approximately 70000. It's isoelectric point was determined to be 7.2. It agglutinated rabbit erythrocytes but did not agglutinate human A, B and O blood group erythro-

cytes. The carbohydrate specificity of the lectin was determined using simple and complex sugars. Among the sugars tested, the agglutination of rabbit erythrocytes was strongly inhibited by glucosamine, galactosamine and mannosamine. Fetuin glycopeptide (O- linked) and host plant (*Brassica oleracea*) polysaccharides were the best inhibitors among oligo and polysaccharides.

4.1 INTRODUCTION

Microbial agglutinins have elicited considerable attention because of the role they are known to play in adherance to surface colonized by the microorganisms (18, 19). Several reports are available on lectins produced by microorganisms pathogenic especially to animal and human beings. They are well characterized from the point of view of their physiological role. For example, *E. coli*, *Vibrio cholerae*, *Bordetella pertussis* produce lectins which are determinants for host specificity and are known to play a key role in infection. There are very few reports available on lectins produced by plant pathogenic microorganisms and their physiological roles. Though there are several studies on the EPS (extracellular polysaccharide) produced by plant pathogens and their roles in infection (1,2), the lectins associated with EPS are not studied from this point of view. Only a few reports suggest that in plant-microbe interaction, recognition of the host surface could be mediated by lectin carbohydrate interaction, in which a lectin could be produced by the pathogen and plant surface carbohydrates act as receptors (3, 7, 17). Therefore it was of interest to know whether a typical plant pathogen like *Xanthomonas campestris*, produces a lectin and whether this lectin has a role in adhesion. Hence the studies were carried out a) to screen several isolates of *Xanthomonas* for lectin production and b) to isolate and characterize the lectin produced.

4.2 MATERIALS AND METHODS

4.2.1 Microorganism and maintenance of culture

Various isolates of *Xanthomonas* were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. They were routinely maintained on agar slants of a medium containing ($g.L^{-1}$) : glucose 20 , peptone 5, yeast extract 3 and malt extract 3 at pH 7.0.

4.2.2 Chemicals

Glucose was purchased from Qualigens, (India), peptone, yeast extract and malt extract were from Hi Media, (India). Sephacryl S300 was from Pharmacia, (Sweden), DEAE cellulose (DE 52) was from Whatman (UK), marker proteins for SDS-PAGE and gel filtration were from Sigma Chemical Company, (USA). All other chemicals used were of analytical grade.

4.2.3 Screening of different Xanthomonas isolates

Xanthomonas cultures were grown in a medium containing ($g.L^{-1}$): glucose 20, peptone 5, yeast extract 3 and malt extract 3. The pH of the medium was adjusted to 7.0 and was not controlled further. The cells were grown in 1000 ml Erlenmeyer flasks containing 200 ml of medium. The inoculum was built in the same medium for 24 hours. A 10 % inoculum was added to the production medium and the flasks were incubated for 72 hours at 28 °C on a rotary shaker with 200 rev. min^{-1}. Aliquotes of 1 ml were taken out aseptically after every 12 hours and centrifuged (9770 X g, 20 min.) to remove cells. The haemagglutination activi-

ty was checked from the supernatant.

4.2.4 Time course of the production of lectin by selected Xanthomonas isolate

The time course of lectin production of the selected isolate was followed by monitoring protein content, viscosity (as a measure of EPS production) and haemagglutination activity after every 24 hours of growth upto 120 hours. Protein was measured after TCA (12.5 %) precipitation. Viscosity of the culture filtrate was measured on Ubbelöhde viscometer (14).

4.2.5 Purification and characterization of lectin

For purification of the lectin, 48 h grown cells were removed from the fermentation broth by centrifugation (9770 x g, 30 min) and the culture filtrate was concentrated by ultrafiltration on Amicon ultrafiltration YM3 membrane. The concentrate was then treated at 4°C with ammonium sulphate upto 80% saturation, allowed to stand for 3 hours and the precipitate was removed by centrifugation (9770 x g, 20 min). The supernatant was diluted 1:3 with buffer (Tris-HCl 20 mM, pH 7.2) and subjected to hydrophobic chromatography on phenyl Sepharose (1.5 x 15 cm) preequilibrated with 20 mM Tris-HCl buffer, pH 7.2 containing 25% ammonium sulphate. The column was washed with the same buffer till the A_{280} was below 0.03 and the bound lectin was then eluted by the reverse gradient of ammonium sulphate (25% - 0%) in 20mM Tris-HCl buffer of pH 7.2 containing 5% glycerol. The fractions showing haemagglutination activity were pooled and dialyzed against buffer (20mM Tris-HCl, pH 7.2 containing 5% glycerol) concentrated by ultrafiltration and used for further

studies .

4.2.6 Determination of protein concentration

Protein concentration was determined as described by Lowry *et al* (12), using bovine serum albumin as a standard.

4.2.7 Haemagglutination assays

For haemagglutination assays, two-fold serial dilutions of lectin solution (50 µl) in microtiter plate were mixed thoroughly with 50 µl of 3% rabbit erythrocyte suspension in Tris buffer (10 mM, pH 7.2) containing 0.15 M NaCl, at room temperature for one hour. HA (haemagglutination activity) is expressed as titer, i.e. a reciprocal of the highest dilution of the lectin that gave complete agglutination. Specific activity of the lectin was defined as titer of the lectin per mg of protein. Agglutination of erythrocytes by the cells of *Xanthomonas campestris* was carried out similarly.

For HA inhibition, sugars and glycopeptides were serially diluted two - fold in microtiter plates and were incubated with lectin (of titer four) at room temperature. Rabbit erythrocytes (3% suspension in Tris buffer saline) were added after 15 min. and the microtiter plates were read for HA inhibition after an hour of incubation at room temperature.

4.2.8 Determination of molecular mass

The relative molecular mass of pure lectin was estimated by SDS-PAGE as described by Laemmli (9) using the low range protein markers (M_r 14000 - 66000) and by gel filtration using a column of Sephacryl S-300 (1 x 60 cm) equilibrated with 20 mM

Tris buffer, pH 7.2, containing 10% ethylene glycol. The column was calibrated with alcohol dehydrogenase (153000), bovine serum albumin (66000), ovalbumin (45000) and carbonic anhydrase (29000).

4.2.9 Isoelectric focussing

Isoelectric focussing was carried out according to Sathivel *et al* (16), using ampholines of pH range of 3.0 to 10.0.

4.2.10 Plant tissue extraction

Leaves of *Brassica oleracea* var *botrytis* (host for *Xanthomonas campestris* var *campestris* NCIM 5028) were extracted with buffer (Tris-HCl 20 mM, pH 7.2) and centrifuged to separate the insoluble material. The supernatant was termed as 'buffer extract' and used in inhibition studies of lectin. The precipitate was then subjected to alkali extraction for an hour (kept in boiling water bath with 1 M NaOH) and centrifuged. The supernatant was then neutralized with acetic acid and subjected to ethanol precipitation (1:2 v/v). The precipitate was dissolved in buffer (Tris-HCl,20mM, pH 7.2), dialyzed thoroughly against the same buffer and used as 'alkali extract' in inhibition studies (4). Extractions of *Oryza sativa* (Rice) plant were also carried out in a similar manner and extracts were tested for inhibition. Sugar concentrations were measured by phenol-sulphuric acid method in terms of neutral sugar (5), using mannose as standard. Glycopeptides of fetuin and soybean lectin were prepared according to Townsend (20) and Lis and Sharon (10) respectively.

4.2.11 Preparation of Soybean lectin

Soy beans were macerated in phosphate buffer saline (20 mM, pH 7.2) and centrifuged to remove the insoluble material. The supernatant was then subjected to ammonium sulphate precipitation (80 %) and the precipitate was dialyzed against phosphate buffer saline. The soybean lectin was further purified by affinity chromatography using guar gum matrix. The lectin was eluted with 0.2 M lactose and dialysed thoroughly to remove the sugar. The lectin was dried to powder by lyophilization.

4.2.12 Desialylation of fetuin

Fetuin was treated with 0.2 N H_2 SO_4 at 50°C for 2 hours. After this treatment, it was dialysed against distilled water till free from acid and lyophilized to dryness.

4.2.13 Preparation of glycopeptides from fetuin and soybean lectin

Proteins in powder form were mixed with 0.1N HCl and incubated for 2 hours at 60°C. The pH was then brought to 8.0 with 1M NaOH and 40 mg of pronasewas added to the protein solutions. Proteins with pronase were incubated at 37°C for 24 hours and further treatment with 20 and 10 mg of pronase was given in a similar fashion at 24 hours intervals. The digested protein was lyophilized to dryness. The glycopeptides were extracted with minimum amount of 10 mM acetic acid. Pronase digested extract was passed through G-25 and BioGel P-4 columns to remove small digested peptides and amino acids. The peaks with high sugar content were lyophilized and then those glycopeptides were passed through Dowex column to separate them from the oligosaccharides

generated during pronase digestion.

4.3.RESULTS

4.3.1 Screening

Total 25 isolates of *Xanthomonas* were screened for lectin production. Table 4.1 shows the time taken and amount of lectin produced by each isolate.

Out of twenty five isolates screened, nine isolates were good lectin producers (yielding about 320 units/ml of the medium in 48 hours) The lectin activity was dependent mainly on the carbon source used, 2 % glucose being the optimal; decrease in levels of the carbon source, produced very low levels of lectin activity.

Cultures, mumbers 1, 3, 4, 5, 14, 23 and 25 were promising, but numbers 4, 5, and 14 showed loss of 50 % activity after 48 hours. It could be due to the increased levels of protease, produced extracellularly by the organism. Culture isolates numbered 1, 3, 23 and 25 did not show any loss of activity. However, culture number 25 was selected for further studies since it was the highest producer and could produce upto 512 units of lectin/ml of the culture broth at the end of 48 hours.

4.3.2 Time course of production of lectin

Xanthomonas campestris NCIM 5028 produced polysaccharide (EPS) in large amounts as well as the lectin within 48 hours. Lectin was not produced if production of polysaccharide was hampered due to repeated subculturing. It can be seen from Fig.4.1, that production of EPS and lectin go hand in hand upto 48 hours and

Table 4.1 : Screening for lectin producing *Xanthomonas campestris*

No.	Strain	Activity at				
		12h	24h	36h	48h	72h
		Units/ml				
1.	*Xanthomonas malvacearum* NCIM 2310	160	320	320	160	160
2.	*X.campestris* pv.*oryzae* Px Co35raceI	40	80	80	80	80
3.	*X.campestris* pv.*oryzae* xCo 8	160	320	640	320	160
4.	*X.campestris* pv.*oryzae* 3849 II	160	320	320	320	320
5.	*X.campestris* pv.*oryzae* xCo 9	0	0	20	0	0
6.	*X.campestris* pv.*oryzae* xCo 14	0	40	40	40	40
7.	*X.campestris* pv.*oryzae* xCo II c	10	320	640	640	640
8.	*X.campestris* pv.*oryzae* 3813	20	40	80	80	80
9.	*X.campestris* pv. *oryzae* IxCo15	0	0	0	20	40
10.	*X.campestris* pv. *oryzae* 3821	0	0	0	0	0
11.	*X.campestris* pv. *oryzae* 3822	0	0	0	0	0
12.	*X.campestris* pv. *oryzae* xCo36	20	20	40	40	40
13.	*X.campestris* pv. *oryzae* 3863 Ia	20	20	20	20	20
14.	*X.campestris* pv. *oryzae* 3844	160	320	320	640	640
15.	*X.campestris* pv. *oryzae* 3841	0	20	40	40	40
16.	*X.campestris* pv. *oryzae* xCoIb	40	40	40	80	80
17.	*X.campestris* pv. *oryzae* 3843	0	0	0	0	0
18.	*X.campestris* pv. *oryzae* xCoIa	20	20	20	20	20
19.	*X.campestris* pv. *oryzae* 3863	0	0	0	0	0
20.	*X.campestris* pv. *oryzae* 3856	0	0	0	0	0

Table 4.1 continued

No.	Strain	Activity at 12h	24h	36h	48h	72h
		Units/ml				
21.	*X.campestris* pv. *oryzae* pxCo86	0	20	40	40	0
22.	*X.campestris* pv. *oryzae* 3858	0	40	40	20	20
23.	*X.campestris* pv. *oryzae* 2954	160	320	320	640	320
24.	*X.campestris* pv. *oryzae* 2956	160	320	320	320	320
25.	*X.campestris* pv. *campestris* NCIM 28	160	320	640	640	640

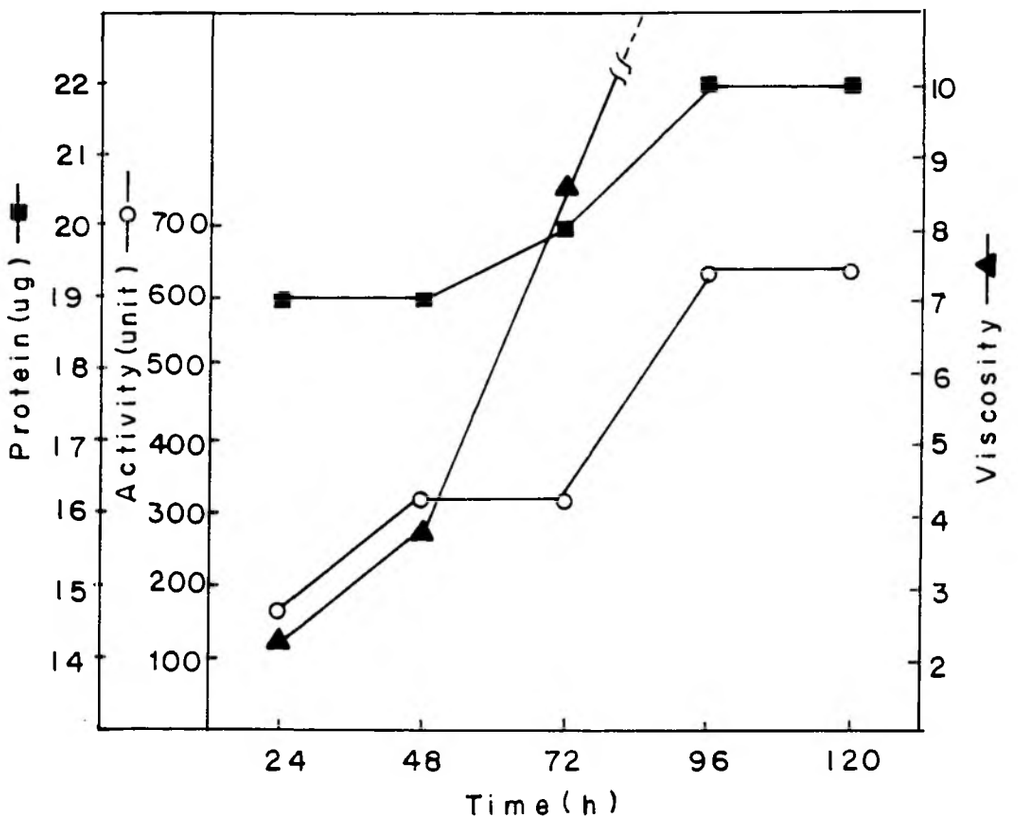
Fig. 4.1 : Time course of production of lectin by *Xanthomonas campestris*

--//-- tremendous viscosity increase after 72 hours of fermentation.

--▲-- viscosity - as a measure of polysaccharide

--■-- protein (TCA precipitable) (µg/ml)

--o-- haemagglutination activity (units/ml)


Protein (ug)
Activity (unit)
Viscosity
Time (h)
22
21
20
19
18
17
16
15
14
700
600
500
400
300
200
100
10
9
8
7
6
5
4
3
2
24
48
72
96
120

there is a tremendous increase in EPS production at 72 hours. For this reason the culture was harvested at the 48th hour, when the viscosity was low and cells could be seperated easily from the broth.

4.3.3 Purification and characterization of lectin

The ammonium sulphate precipitation step removed most of the contaminating proteins as well as a considerable amount of EPS. However, the supernatant of the 80% saturated culture filtrate retained almost 84% of the lectin activity. Hydrophobic chromatography of this supernatant yielded a single protein peak with agglutination activity. The lectin was purified 25 fold with 24% recovery (Table 4.2). It showed two closely placed bands on SDS - PAGE, with relative molecular masses of 28000 and 30000 (Fig. 4.2). The gel filtration of the lectin on Sephacryl S-300 in the presence of 10% ethylene glycol showed a single peak with a relative molecular mass of 70000 (Fig. 4.3). The lectin, therefore, appears to be a heterodimer. The presence of ethylene glycol was necessary because this lectin had a tendency to be retarded on any gel filtration matrix; perhaps because of its hydrophobic nature. The difference in the molecular mass observed for gel filtration and SDS-PAGE cumulative mass of the subunits, could be due to ethylene glycol being used to remove the interaction of the lectin with the gel filtration matrix. On isoelectric focussing, the lectin showed a single peak of activity, and the pI calculated was 7.2 (Fig. 4.4).

The agglutination of rabbit erythrocytes by the purified lectin was inhibited only by aminated sugars among the mono and

Table 4.2 : Purification of lectin from *Xanthomonas campestris* NCIM 5028

Step	Volume ml	Total activity units	Total protein mg	Specific activity units/mg	Purification yield %	fold
Culture filtrate	1000	320000	210[a] (3000*)	1524	100	1
$(NH_4)_2SO_4$ supernatant	150	256000	19.7 (1750*)	13000	84	8.5
phenyl Sepharose	60	76800	2.3	38400	24	25.1

* = protein determined before TCA precipitation.

a = Protein content determined after precipitation with TCA. The sterile medium contains 90 µg/ml TCA precipitable protein.

Fig. 4.2 : Native and SDS-PAGE of the purified *Xanthomonas campestris* lectin

a) Native PAGE

b) SDS PAGE

Lane 1. Purified *Xanthomonas campestris* lectin

Lane 2. Molecular weight markers, from the top:
bovine serum albumin(66000), ovalbumin(45000),
glyceraldehyde-3-phosphate dehydrogenase(36000),
carbonic anhydrase(29000),
soybean trypsin inhibitor(20100),
lactalbumin(14200).

3. *Xanthomonas campestris* lectin after IEF.

(a)

(b)

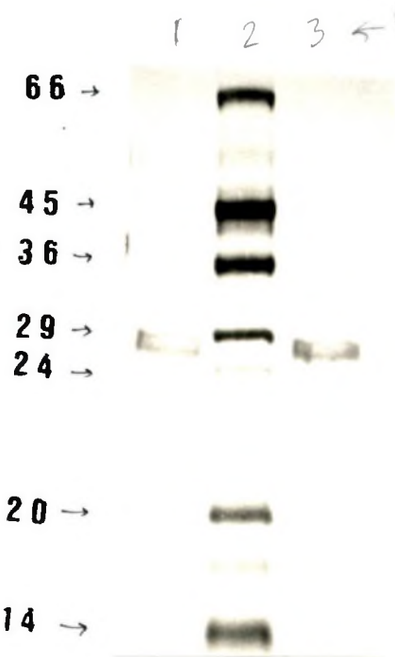


Fig. 4.3 : Gel filtration of lectin on Sephacryl S-300-
Markers used are : alcohol dehydrogenase (153000),
bovine serum albumin (66000), ovalbumin (45000),
carbonic anhydrase (29000) (-○-);
Xanthomonas campestris lectin (-■-).

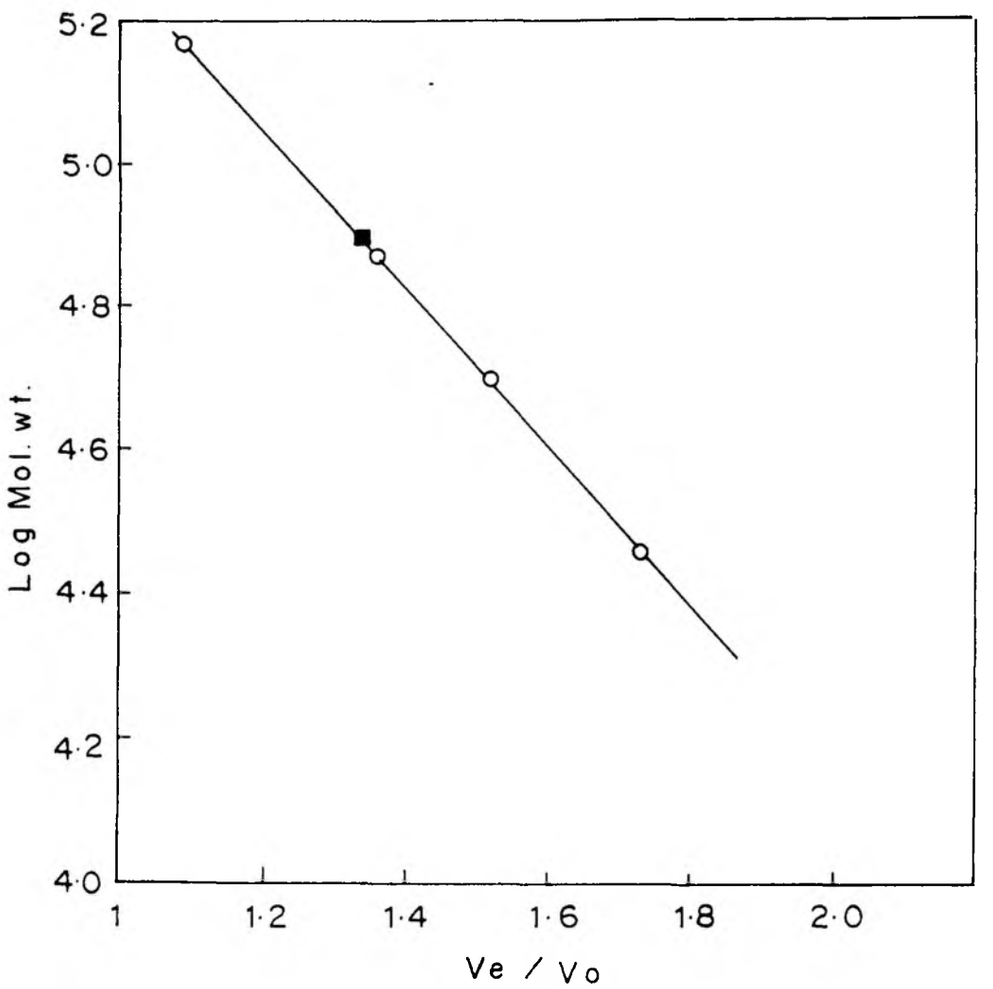

5·2
5·0
4·8
4·6
4·4
4·2
4·0
Log Mol. wt.
1
1·2
1·4
1·6
1·8
2·0
Ve / Vo

Figure 4.4 : Isoelectric Focussing of Purified Lectin Produced by *Xanthomonas campestris* NCIM 5028

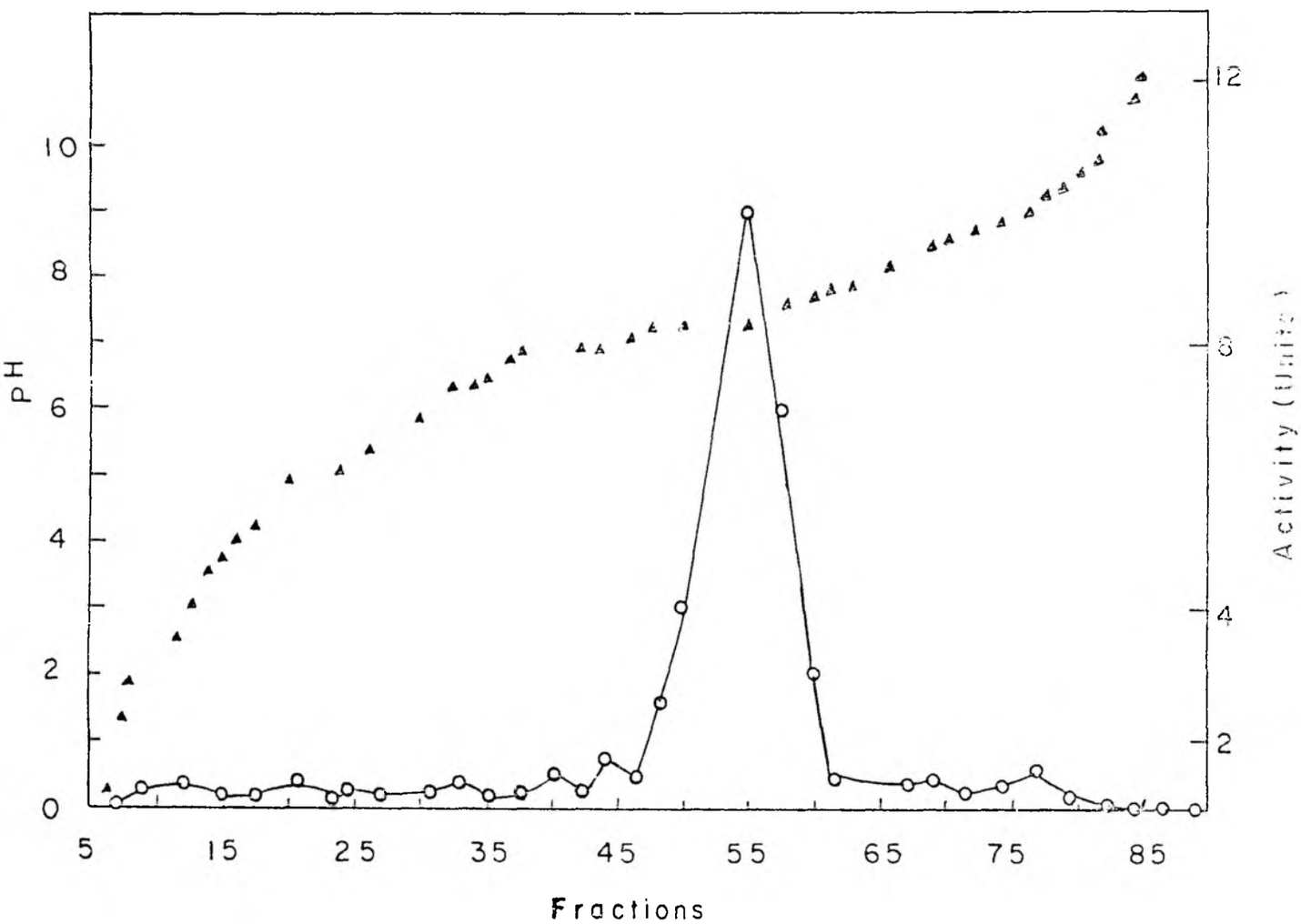

pH
Activity (Units)
Fractions
0
2
4
6
8
10
12
5
15
25
35
45
55
65
75
85

Table 4.3 : Sugar Specificity of *Xanhomonas campestria* lectin

	Sugars	Inhibitory concentration
1.	Glucose	-
2.	D-glucosamine	125 mM
3.	N acetyl glucosamine	-
4.	Me α glc	-
5.	Me β glc	-
6.	Mannose	-
7.	D-mannosamine	125 mM
8.	Me α man	-
9.	Galactose	-
10.	D-galactosamine	125 mM
11.	N acetyl galactosamine	250 mM
12.	Me α gal	-
13.	Me β gal	-
14.	L(-)Fucose	-
15.	Arabinose	-
16.	Rhamnose	-
17.	Melibiose	-
18.	Stachyose	-
19.	Lactose	-
20.	N acetyl lactosamine(50mM)	-

- = No inhibitory effect even at 250 mM concentration.

Table 4.4 : Sugar specificity of *Xanthomonas campestris* lectin

	Glycoprotein	Inhibitory concentration
1.	Fetuin	7.5 µg
2.	Desialylated fetuin	3.9 µg
3.	N - linked glycopeptide of fetuin[a]	6.5 µg
4.	O - linked glycopeptide of fetuin[a]	1.87µg
5	Soybean lectin glycopeptide[b]	NI* (3mg)
6.	*Brassica oleracea* var*botrytis* buffer extract	5.5µg
7.	*Brassica oleracea* var*botrytis* alkali extract	2.3µg
8.	Fibrinogen	200µg

NI = noninhibitory at

a = Fetuin glycopeptides were prepared according to Townsend *et al.*(20).

b = Soybean lectin glycopeptides were prepared according to Lis and Sharon,(10).

disaccharides tested i.e. N acetyl galactosamine (250 mM) D-mannosamine, D-galactosamine and D-glucosamine (125 mM). (Table 4.3). Fetuin and glycopeptides of fetuin were the most potent inhibitors of the HA activity of the lectin. Fibrinogen also inhibited the agglutination but was less potent than fetuin. Soybean lectin glycopeptide failed to inhibit the agglutination. N- linked complex type of glycopeptide of fetuin showed much less inhibitory activity compared to O- linked glycopeptide of fetuin,which are mucin like structures. The O- linked glycopeptide was twice as efficient as desialylated fetuin. Host (*Brassica oleracea* var *botrytis*) extracts (buffer as well as alkali) inhibited the agglutination activity of the lectin (Table 4.4),indicating that the lectin recognizes carbohydrate structures on the host surface. The high concentration of simple sugars and the inhibition pattern shown by the glycoproteins and glycopeptides indicate that this lectin probably recognizes a complex sugar structure.

Another important feature of the lectin was its instability without polysaccharide (xanthan gum). Although the lectin was not a glycoprotein itself, it required association with polysaccharide to remain stable on storage. Other additives such as glycerol did not stabilize the lectin. Addition of xanthan gum stabilized the lectin for a longer time.

4.4 DISCUSSION

Xanthomonas campestris cells (prewashed three times with Tris buffer,20 mM,pH 7.2, saline) agglutinated rabbit erythrocytes, therefore the lectin could be the fimbrial lectin which

must have been shed in the medium during growth. It was observed that the production of lectin was always associated with that of the EPS. Production of polysaccharides is supposed to be a major determinant in the ability of a bacterium to colonize a given niche (24). As capsular polysaccharides are highly hydrated, they protect the bacterium from dessication. Additionally, acidic EPSs produced by bacteria are anionic in nature, which may help bacteria to adhere to biological surfaces. EPS^- mutants of *Erwinia*, *Pseudomonas* and *Xanthomonas* have been shown to have reduced virulence.EPS may have multiple functions in pathogenesis. The acidic polysaccharide produced by *Xanthomonas campestris* is composed of D-glucose, D-mannose, D-glucuronic acid, acetic acid and pyruvic acid. Glucose:mannose:glucuronic acid ratio is 2:2:1.

There are similar reports in literature about other microorganisms, either pathogenic or symbiotic to plants, producing lectins which are associated with the EPS secreted by that organism. For example the fungal plant pathogen - *Sclerotium rolfsii* produces a lectin of M_r 45000 , that is specific for mucin and it is strongly associated with the polysaccharide (1,3-ß-glucan) it produces. The lectin as well as the polysaccharide have a role in pathogenesis (8). Gould and Northcote (6) stated that an integrated fungal molecule that contained both carbohydrate and protein, was responsible for adhesion of *Phialophora radicicola* to the host.

A plant lectin has been shown to be a host range determinant in *Rhizobium* (13); 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 (11). *Agrobacterium tumefaciens* produced a lectin, specific for L(-) fucose, and polysaccharides of Poplar leaves (one of its hosts),inhibits its activity. Depierreux (3) has speculated on its role in specific binding of *Agrobacterium tumefaciens* to the plant host.

The lectin produced by *Xanthomonas campestris* NCIM 5028, is inhibited at high concentrations of aminated sugars and by mucin like structures of fetuin as well as polysaccharides produced by the host plant. It indicates that this lectin has a complex sugar specificity. The agglutination of rabbit erythrocytes by intact cells and identical sugar specificity indicates that this lectin is in all probability a fimbrial lectin.

Bacterial pili or fimbriae which are produced by large proportion of plant pathogenic bacteria also, are mostly sugar binding proteins i.e. lectins. *Erwinia caratovora* produces mannose sensitive as well as mannose resistant lectins (21). The mannose sensitive type of lectin has subunit molecular mass of 16500 whereas the mannose resistant type of fimbrial lectin subunit has the molecular mass of 18000. *Erwinia rhapontici* produces a lectin specific for gal-β 1,4 glcNAc. Other plant pathogens like *Klebsiella* (22) produced fimbriae of type 1 as well as type 3 and nonfimbriated mutants failed to adhere to the host plant surfaces. Plant pathogenic pseudomonads also produce fimbriae but they are mostly useful for epiphytic colonization. *Pseudomonas solanacearum* produced a lectin of subunit molecular mass 9500 which mediated both bacterial binding to tobacco leaf cell walls and bacterial autoagglutination (23). *Xanthomonas*

campestris pv. hyacinthi as well as *Xanthomonas campestris pv. vesicatoria* also produced fimbriae. *Xanthomonas campestris pv. hyacinthi* produced pili type 4 and the sugar specificity of these pili is speculated to be glcNAc β- 1,4 gal (15).

The physiological role played by *Xanthomonas* lectin reported here, is yet unknown although inhibition of the lectin activity by host plant extracts indicate a possible role in adhesion.

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