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Partial Purification of Lectin from Mycoparasitic Species of *Trichoderma*

Tanuja Singh[1], Ratul Saikia[2] and Dilip K. Arora[2]*

[1]*Glyco-Immunochemistry Research Laboratory, Institute of Molecular and Cellular Biology, College of Medicine, Chang-Gung University, Kwei-san, Tao-yuan, 333, Taiwan*

[2]*National Bureau of Agriculturally Important Microorganisms, Kushmaur, Mau 275101, UP, India*

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***Trichoderma* species/isolates exhibited varied degree of agglutination on sclerotial (Sc) and hyphal (Hy) surface of *Macrophomina phaseolina*. The agglutination efficiencies on Sc and Hy ranged from 11 to 57%. Isolates of *T. harzianum* (*Th*) and *T. viride* (*Tv*) showed greater agglutination on Sc (23-57%) and Hy (16-47%). Different enzymes (trypsin, pepsin, proteinase k, α-chymotrypsin, lyticase and glucosidase) and inhibitors (tunicamycin, cycloheximide, brefeldin A, sodium azide, dithiothreitol and SDS) reduced the agglutination potential of conidia of *Th*-23/98 and *Tv*-25/98; however, the extent of response varied greatly in different treatments. Different fractions of *Th*-23/98 and *Tv*-25/98 exhibited haemagglutinating reaction with human blood group A, B, AB and O. Haemagglutinating activity was inhibited by different sugars and glycoproteins tested. Crude haemagglutinating protein from outer cell wall protein fraction of *Th*-23/98 and *Tv*-25/98 were eluted on Sephadex G-100 column. Initially *Th*-23/98 and *Tv*-25/98 exhibited two peaks showing no agglutination activity; however, lectin activity was detected in the third peak. Similar to crude lectin, the purified lectin also exhibited haemagglutinating activity with different erythrocyte source. SDS-PAGE analysis of partially purified lectin revealed single band with an estimated molecular mass of 55 and 52 kDa in *Th*-23/98 and *Tv*-25/98, respectively. Trypsin, chymotrypsin and b-1,3-glucanase totally inhibited lectin activity. Similarly, various pH also affected the haemagglutinating activity of *Th*-23/98 and *Tv*-25/98. From the present observations, it can be concluded that the recognition/attachment of mycoparasite (*T. harzianum* and *T. viride*) to the host surface (*M. phaseolina*) may be most likely due to lectin-carbohydrate interaction.**

Keywords : agglutination, lectin, *Macrophomina phaseolina*, recognition, *Trichoderma*

In natural environment microorganisms interact with each other to maintain their growth, development and stability within ecosystem. The interaction process can only be revealed by understanding the fundamentals of ecological relationships of diverse microbial population including pathogens and antagonists in the soil environment. In many instances physical attachment of microorganisms is mediated by specific compatible macromolecules i.e. receptors and ligands. The specific interaction between microbial populations may play a key role for successful establishment, persistence and colonization (Glandorf et al., 1994; Kwon and Epstein, 1997; Manocha and Sahai, 1993; Ofek et al., 1996; Srivastava et al., 2001). The role of cell surface macromolecules, potential ligands and receptors of cell surface agglutinins or lectins as recognition factor during attachment of two interacting partners, are well known (Manocha and Sahai, 1993; Sharon and Lis, 1989a; Tunlid et al., 1992). Though biological functions of lectins are not clear, there are numerous evidences that lectins mediate cellular and molecular recognition in a wide range of biological interactions (Rosen et al., 1997; Sharon and Lis, 1989b).

Several investigations have demonstrated the role of lectins as cell-cell recognition molecules in antagonistic interactions (Inbar and Chet, 1994; Lis and Sharon, 1998; Manocha et al., 1997). Roles of lectins in recognition and attachment between plant host and fungal pathogens (Epstein and Nicholson, 1997; Van Damme et al., 1998), in mutualistic relationship between fungi and algae in lichens (Lockhart et al., 1988), mediation of predatory fungi to nematodes (Nordbring-Hertz and Mattiasson, 1979), recognition and attachment of nitrogen fixing bacteria (*Rhizobium* or *Bradyrhizobium*) to roots of leguminous plants (Weisneiwski and Delmotte, 1996), other saprophytic soil bacteria to plant roots (Glandorf et al., 1994), and cell cohesion in slime moulds (Kuo and Hoch, 1996) are well documented in the literature. Extensive work has been done by different workers dealing with cellular interactions in fungi, and the specificity of attachment of fungal parasites to their hosts (Inbar and Chet, 1997; Jones, 1994; Manocha and Chen, 1990; Manocha and Sahai, 1993; Manocha et al., 1997; Tunlid et al., 1992;). The phenomenon such as induction of cell-wall degrading enzymes (e.g. chitin-

*Corresponding author.
Phone) +91-547- 2530358 , FAX) +91-547- 2530358
E-mail) aroradilip@yahoo.co.in

ases) and secretion of adhesins have been found to be directly induced by lectin-carbohydrate interactions. The biomimetic system (Inbar and Chet, 1992) provided experimental proof that the recognition signal triggers differentiation processes that lead to the formation of infection structures concomitant with the induction of the chitinolytic enzyme needed to penetrate the host cell wall (Inbar and Chet, 1995; Omero et al., 1999).

Species of *Trichoderma* are antagonistic to several soilborne fungi and serve as potential biocontrol agents (Elad, 1995; Herrera Estrella and Chet, 2002; Inbar and Chet, 1994, 1997). *Macrophomina phaseolina* (Tassi) Goid. is a soilborne plant pathogenic fungus which engenders a major problem for chickpea and other crop plants (Jana et al., 1999; Mihail and Taylor, 1995). We have shown earlier that soil harbours a large number of naturally occurring strains of *Trichoderma* species that can adhere and colonize *M. phaseolina* hyphae and sclerotia to varying degrees (Singh, 2002; Srivastava et al., 1996). It appears that specific (lectin-mediated) or non-specific (cell surface hydrophobicity and charge), or both mechanisms can be implicated in recognition or binding of antagonistic *Trichoderma* species to fungal plant pathogen *M. phaseolina* (Singh, 2002; Singh et al., 2004). In this study, we describe the partial purification of lectin from mycoparasitic fungus *Trichoderma*, and its involvement in binding and recognition of pathogenic fungal host-cell surface.

Materials and Methods

Cultures and cultural conditions. *Trichoderma* species (Singh, 2002) were grown and maintained on acidified potato dextrose agar (APDA, pH 5.5) at 25-28°C. *M. phaseolina* CH-15, pathogenic to chickpea (*Cicer arietinum* L.) var. Radhe, was obtained from the Laboratory of Applied Mycology Culture Collection, Banaras Hindu University and grown on carrot agar plates (pH 5.6) at 25-28°C. After 5 days of incubation, the hyphae, sclerotia and conidia were collected in 100 mM phosphate buffer saline (PBS; pH 7.2) through gentle surface scraping with a sterilized nylon brush, washed 3 times in cold (4°C) PBS and used immediately for agglutination assays.

Agglutination assay. Twenty nine isolates of *Trichoderma* were screened for their agglutination activity on sclerotial (Sc) and hyphal (Hy) surface of *M. phaseolina* (Table 1). The agglutination activity was evaluated by mixing 5 µl of conidial suspension of *Trichoderma* isolate (10^8 conidia/ml) to 5 µl of Sc and Hy (10^5/ml) surfaces on acid-washed glass slides for 30 min. Following exposure, Sc and Hy were washed gently with sterile PBS and agglutination was observed under a phase contrast microscope (Nikon, Japan).

Table 1. Agglutination percent of different *Trichoderma* species on sclerotial and hyphal surface of *Macrophomina phaseolina* CH-15

Trichoderma spp.	Agglutination (%)[a]	
	Sclerotia	Hyphae
T. hamatum		
Tm-2084	33±2.1	29±1.4
Tm-480	27±1.8	24±1.1
Tm-5/98	23±2.4	21±1.3
T. harzianum		
Th-2885	35±3.5	33±2.1
Th-2895	46±2.9	41±2.7
Th-11/98	31±2.5	21±0.7
Th-14/98	25±4.1	16±0.5
Th-21/98	49±3.2	42±1.9
Th-23/98	57±4.2	47±2.8
Th-28/98	23±1.7	19±1.1
T. koningii		
Tk-2/98	23±1.6	19±0.5
Tk-13/98	28±1.8	23±1.2
T. longibrachiatum		
Tl-3/98	15±0.6	11±0.5
T. polyspora		
Tp-33/98	21±0.8	15±0.2
T. pseudokoningii		
Tps-3694	23±1.1	19±1.5
Tps-17/98	19±0.9	12±1.1
T. reesei		
Tr-164	27±1.1	21±1.4
Tr-35/98	22±1.3	14±0.6
T. viride		
Tv-1/98	31±2.5	22±1.3
Tv-3791	37±3.1	25±1.1
Tv-6/98	29±1.5	19±0.7
Tv-9/98	38±2.4	26±0.5
Tv-10/98	31±1.6	20±1.1
Tv-15/98	47±3.2	42±3.4
Tv-19/98	39±1.9	31±2.5
Tv-25/98	55±3.5	47±5.1
Tv-26/98	27±2.1	19±1.2
Tv-31/98	31±2.7	27±1.7
T. virens		
Tvi-30/98	27±2.1	21±1.1

[a] Data are means of 3 replicates ± S.D.

The percent agglutination was calculated as: number of conidia agglutinated/total number of conidia per microscopic field × 100. In control treatments, PBS was added instead of Sc or Hy fragments.

Scanning electron microscopy. Scanning electron microscopy (SEM) was performed according to the procedures described by Barak et al. (1986). *Trichoderma harzianum* (*Th*-23/98) was incubated in Petri dishes containing water agar covered with cellophane membrane. After 72 h, the cellophane membranes with the conidia were removed and vapour-fixed. The samples were air-dried, coated with gold and examined under SEM (JEOL, Japan).

Effect of enzymes and inhibitors. Since *T. harzianum* (*Th*-23/98) and *T. viride* (*Tv*-25/98) isolates showed strong agglutination activity on Sc and Hy surfaces of *M. phaseolina* (Table 1), these isolates were selected for further study. Conidia (5-day-old) of *Th*-23/98 and *Tv*-25/98 were treated with trypsin, α-chymotrypsin, lyticase, pepsin, proteinase k and glucosidase (50-300 μg/ml; Sigma) or with 10-1000 μM dithiothreitol (DTT) or by 0.1-0.9% SDS for 1 h at 37°C. Prior to incubation with either enzymes or inhibitors the concentration of conidia were adjusted to 10^8/ml. The treated conidia were washed thoroughly in PBS (pH 7.2) and assayed for agglutination as described above. The influence of cycloheximide, brefeldin A, tunicamycin, sodium azide, SDS and DTT (Sigma, USA) on agglutination was also examined. Conidia were obtained from 5-day-old cultures grown in APDA amended with cycloheximide, brefeldin A and tunicamycin (5-25 μg/ml) or sodium azide (10-1000 μM). Conidia that were not treated with any enzymes or inhibitors served as controls. The agglutination assay was performed as described above.

Agglutinin production. The inoculum of *Th*-23/98 and *Tv*-25/98 (ca. 10^5 conidia/ml) was added to 75 ml of synthetic medium (pH 6.8; Okon et al. 1973) and incubated for 10 d at 28-30°C. The cell wall and cytosolic protein fractions were obtained as described by Neethling and Nevalainen (1996). The freeze-dried mycelium was ground with liquid nitrogen, suspended in 10 mL PBS (pH 7.2) containing 1 mM EDTA (pH 8), 0.5 mM phenylmethylsulfonylfluoride and 0.5 mM DTT. The supernatant was decanted and pooled. The pellet was used for further fractionation. The pooled supernatant was dialyzed against 3 × 2000 mL 0.1 M PBS and 1 mM EDTA using a dialysis membrane (molecular mass cut-off 10 kDa; Sigma, USA). The dialyzed supernatant served as "OCP (outer cell wall protein) fraction". The remaining pellets were further placed in 5 ml of PBS to which an additional 0.4 M NaCl has been added and 1 mM PMSF. The pelleted suspension were sonicated in a XL-series sonicator (Ultrasonic, USA), and centrifuged at 10, 000 × *g* for 20 min. The supernatant was pooled and dialyzed as described above. This fraction served as "ICCP (intrinsic cell wall and cytosolic protein) fraction". The remaining pellet was suspended in 20 ml PBS plus 8 M urea, shaken at 200 rpm for 30 min, centrifuged (10,000 × *g*; –4°C) and the supernatant was dialyzed against PBS and pooled. This fraction served as "ICP (inner cell wall protein) fraction". Protein content in OCP, ICCP and ICP fractions of the cultures were estimated using bovine serum albumin as standard (Bradford, 1976). These fractions were stored at –20°C until use.

Haemagglutination assay. Haemagglutinating activity was measured on microtitre plates (Sharon and Lis, 1989). Blood samples were taken from humans. Erythrocyte suspensions of human blood group A, B, AB and O were prepared according to the method of Lis et al. (1994). Trypsin-treated erythrocytes were washed with PBS by four repeated cycles of centrifugation (10 min; 3,000 *g*). OCP, ICCP and ICP fractions of *Th*-23/98 and *Tv*-25/98 isolates (50 ml) were added to each well and mixed with an equal volume of the erythrocyte suspension. The mixture was kept at 28-30°C for 1 h and then examined for haemagglutination. Haemagglutinating activity was defined as the reciprocal of the end-point dilution. Haemagglutination inhibition assay. Hapten inhibition assays were performed by adding 20 mL of sugar or glycoprotein solution (Table 2) in PBS with 20 mL of OCP, ICCP and ICP fractions of *Th*-23/98 and *Tv*-25/98 isolates and 60 ml of 4% suspension of red blood cells.

Purification of lectin from OCP. All steps of purification were carried at 4°C. The freeze-dried mycelial mats of *Th*-23/98 and *Tv*-25/98 were extracted for OCP fraction by the method described earlier. The crude extract of cultures were dialyzed against PBS (pH 7.2), lyophilized and redissolved in a small volume of 0.1 M PBS containing 1 mM EDTA

Table 2. Carbohydrate-binding specificity of partially purified lectin from OCP fractions of *T. harzianum* (*Th*-23/98) and *T. viride* (*Tv*-25/98)

	Th-23/98	*Tv*-25/98
Sugars (μg/ml)[a]		
D-Glucose	10	25
D-Galactose	25	20
D-Fucose	25	50
N-acetyl-α-D-Galactosamine	20	25
D-Galactosamine	25	–[b]
N-acetyl-α-D-Glucosamine	25	–
Glycoprotein (μg/ml)		
Fetuin	125	150
Mucin	10	10

[a] Concentration needed for 50% inhibition of the agglutination of trypsin-treated erythrocytes.
[b] No inhibition was observed.

(pH 7.2), to give a protein content of ~2 mg/ml. One ml of the supernatant (OCP) of *Th*-23/98 and *Tv*-25/98 were applied to a Sephadex G-100 (60 × 1.5 cm) column and eluted with 0.1 M PBS containing 1 mM EDTA (pH 7.2) at 4°C at a flow rate of 0.5 mL/min. The haemagglutinating fractions were pooled, dialyzed overnight against distilled water at 4°C, further lyophilized and stored at –20°C. The purification procedure was repeated 3 times. Haemagglutinating activity and carbohydrate specificity tests of the purified lyophilized fractions were done as described earlier.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% polyacrylamide vertical slab gels using a Hoefer miniVE vertical electrophoresis system. Electrophoresis was carried out under reducing conditions as described by Laemmli (1970). Following electrophoresis, the gels were stained for protein with Coomassie brilliant blue. The electrophoretic procedure was repeated at least 3 times. Glycoproteins were detected using periodic acid-schiff reagent (PAS) on SDS-PAGE gels according to the method of Dubray and Bezard (1982).

Effect of enzymes on lectin activity. Purified lectins were treated with various enzymes (Sigma; Table 4) by incubating 500 µl of enzyme in the appropriate buffer with 500 µl of agglutinin at the appropriate conditions and assayed for haemagglutination activity. 1,3 β-glucanase (5 mg/ml) and chitinase (62 mg/ml) both in 0.1 M phosphate buffer (pH 5.5), were incubated for 2 h at 37°C. Protease (3 mg/ml), trypsin and chymotrypsin (each at a concentration of 1%, w/v) were mixed in 0.1 M Tris/HCl buffer (pH 7.6) and incubated for 2 h at 37°C. All reactions were stopped by moving the test tubes to an ice-bath and adding the protease inhibitor PMSF (phenylmethylsulfonylfluoride) to the tubes containing proteolytic activity. Control samples were treated with the corresponding buffer only. The titre of lectin activity was determined by haemagglutination assay as described earlier.

Effect of pH on lectin activity. The effect of pH on the lectin activity of the cultures were determined by incubating the lectin at various pH values. Each 500 µl aliquot of the purified lectin was incubated for 1 h with 500 µl of either of the following buffer: 0.05 M sodium acetate buffer (pH 3.5), 0.05 M sodium phosphate buffer (pH 7), 0.05 M Tris-HCl buffer (pH 8) and 0.05 M glycine-NaOH buffer (pH 11). After incubating the samples for 1 h at room temperature the buffers were changed to PBS by ultrafiltration and the haemagglutinating activities were measured as described earlier. All experiments were repeated more than twice and data were subjected to standard error (S.E.) analysis.

Results

Agglutination potential of *Trichoderma* isolates. The agglutination potential of different *Trichoderma* species/isolates varied with respect to the degree of agglutination on Sc and Hy surface of *M. phaseolina* (Table 1). The agglutination efficiencies in Sc and Hy of *M. phaseolina* ranged from 11 to 57%. However, isolates of *T. harzianum* and *T. viride* showed more agglutination on Sc (23-57%) and Hy (16-47%) as compared to other *Trichoderma* species/isolates (Table 1). *Th*-23/98 and *Tv*-25/98 were selected for further study as these isolates exhibited strong agglutination reaction under electromicroscopic observation (Data not shown).

Effect of enzymes and inhibitors. All six enzymes and inhibitors (trypsin, α-chymotrypsin, pepsin, proteinase k, lyticase, glucosidase, tunicamycin, cycloheximide, brefeldin A, sodium azide, DTT and SDS) reduced the agglutination potential of *Th*-23/98 and *Tv*-25/98; however, the extent of response varied greatly in different treatments (Fig. 1). For instance, the agglutination efficiency of conidia treated for 1 h with 50-300 µg/ml concentration of trypsin exhibited 17 to 58% agglutination. In general, agglutination was 3 to 4 fold less as compared with untreated conidia. Similarly, pretreatment of conidia with α-chymotrypsin and proteinase k also showed marked reduction of agglutination. For example, the agglutination of conidia treated for 1 h with 50-300 µg/ml concentration of α-chymotrypsin and proteinase k, ranged from 21 to 59% and 24 to 63%, respectively (Fig. 1). Pepsin, lyticase and glucosidase treated conidia showed least reduction in agglutination as compared to other enzymes. Conidia treated with tunicamycin, cycloheximide and brefeldin A (5 to 25 µg/ml) and DTT (10-1000 µM) significantly reduced the agglutination efficiency (Fig. 1). For instance, agglutination response of *Th*-23/98 conidia treated with tunicamycin, cycloheximide and brefeldin A was 21, 18 and 20%, and in *Tv*-25/98 was 16, 15 and 18%, respectively (Fig. 1). The agglutination response of conidia supplemented with tunicamycin, cycloheximide and brefeldin A was 3 to 3.5 fold less in comparison to untreated conidia (Fig. 1). The agglutination of conidia treated with DTT was 21-60%, whereas 31-63% agglutination occurred with sodium azide and SDS.

Haemagglutination activity. Haemagglutination reaction was observed with human blood group A, B, AB and O erythrocytes in OCP, ICCP and ICP fractions of *Th*-23/98 and *Tv*-25/98 (data not shown). The agglutination titre in OCP, ICCP and ICP fractions of *Th*-23/98 ranged from 4 to 32, whereas in *Tv*-25/98 the titre increased from 2 to 16. In general, agglutination titre was greater in OCP fraction,

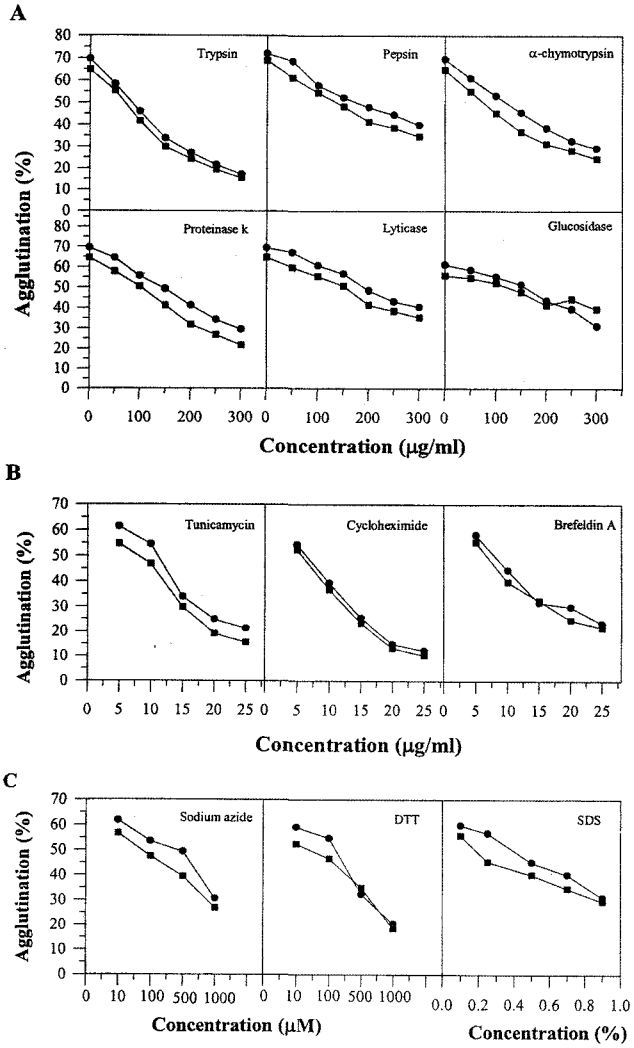

Fig. 1. Effect of different enzymes (A) and inhibitors (B) on agglutination efficiency of *Trichoderma* conidia. ● = *T. harzianum*; ■ = *T.viride*; Data are means of 3 replicates.

followed by ICCP and ICP.

Sugar inhibition of haemagglutination. The carbohydrate binding specificity of agglutinin component in OCP, ICCP and ICP was determined. We have only shown the results obtained with OCP fraction of *Th*-23/98 and *Tv*-25/98 (Table 2). Haemagglutinating activity was inhibited both by saccharides and glycoproteins tested. For instance, OCP fraction of *Th*-23/98 and *Tv*-25/98 were inhibited by sugars at 10-50 mM concentrations, while glycoproteins (mucin and fetuin) inhibited at concentrations of 10-150 μg/ml.

Purification of lectin from OCP. Since agglutination titre was greater in OCP as compared to other fractions, this fraction was further purified and tested. Figure 2 shows the elution pattern of crude lectin on Sephadex G-100 column. OCP fraction of *Th*-23/98 and *Tv*-25/98 revealed two peaks

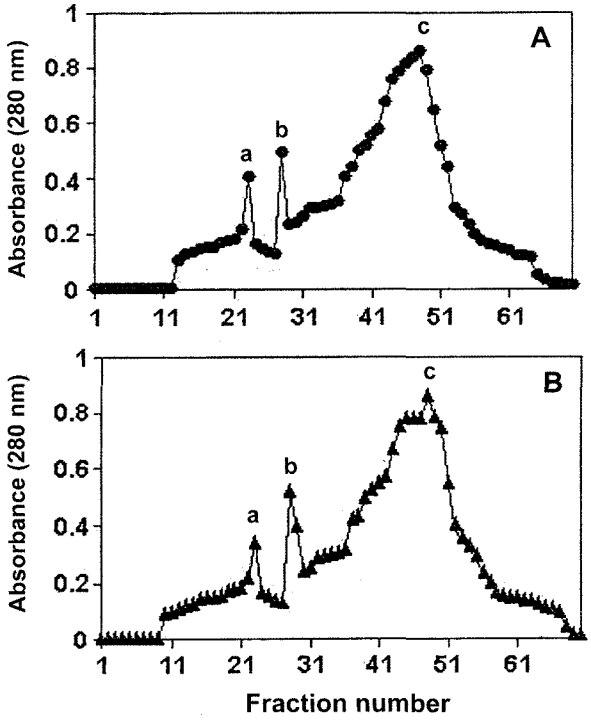

Fig. 2. Separation of OCP fraction from *Th*-23/98 (A) and *Tv*-25/98 (B) using Sephadex G-100 column; a, b = non-agglutinating fraction; c = agglutinating fraction.

(a, b), which emerged first showing absorption at 280 nm with no agglutination activity. However, haemagglutinating activity was detected with blood groups A, B, AB and O of human in the third peak (c) that appeared 30-60 ml beyond the void volume. The haemagglutinating activity was inhibited by different mono- or disaccharides and with glycoproteins (Table 2). Several protein bands were observed in the crude lectin of *T. harzianum* (*Th*-23/98) and *T. viride* (*Tv*-25/98). However, after elution single band of 55 and 52 kDa protein from *Th*-23/98 and *Tv*-25/98 were observed (Fig. 3). The protein was stained by the periodic-acid-schiff reagent on SDS-PAGE gel, indicating that the lectin was a glycoprotein (result not shown). Table 3 provides summary of the eluted fraction of 55 and 52 kDa protein. The titre increased from 32 to 128 and 16 to 64 in partially purified lectin of *Th*-23/98 and *Tv*-25/98, respectively. The specific activity (units/mg) of purified lectin increased from 250 to 1041 and 148 to 446 and purification was 3- to 4- fold with 40% recovery in *Th*-23/98 and *Tv*-25/98.

Effect of enzymes on lectin activity. The effect of various enzymes on lectin activity of *Th*-23/98 and *Tv*-25/98 was determined (Table 4). Treatment with trypsin, chymotrypsin and β-1,3-glucanase totally inhibited the lectin activity, whereas chitinase had no effect (Table 4).

Effect of pH on lectin activity. The effect of various pH

Table 3. Purification of haemagglutinating protein (lectin) from *T. harzianum* (*Th*-23/98) and *T. viride* (*Tv*-25/98)

Fraction	Volume (ml)		Titre		Total Haemagglutinating activity (units)		Protein (mg)		Specific activity (units/mg)		Recovery (%)		Purification (-fold)	
	TTh	*TTv*	*TTh*	*TTv*	*TTh*	*TTv*	*TTh*	*TTv*	*TTh*	*TTv*	*TTh*	*TTv*	*TTh*	*TTv*
Crude Extract[a] (OCP)	50	50	1:32	1:16	3125	1562	12.5	10.5	250	148	100	100	1	1
Purified lectin[b] (peak c)	5	5	1:128	1:64	1250	625	1.2	1.4	1041	446	40	40	4	3

[a] Crude extract obtained after grinding with liquid nitrogen, centrifugation and concentration.
[b] Protein eluted from Sephadex G-100 Gel chromatography; *Th* = *Th*-23/98 and *Tv* = *Tv*-25/98.

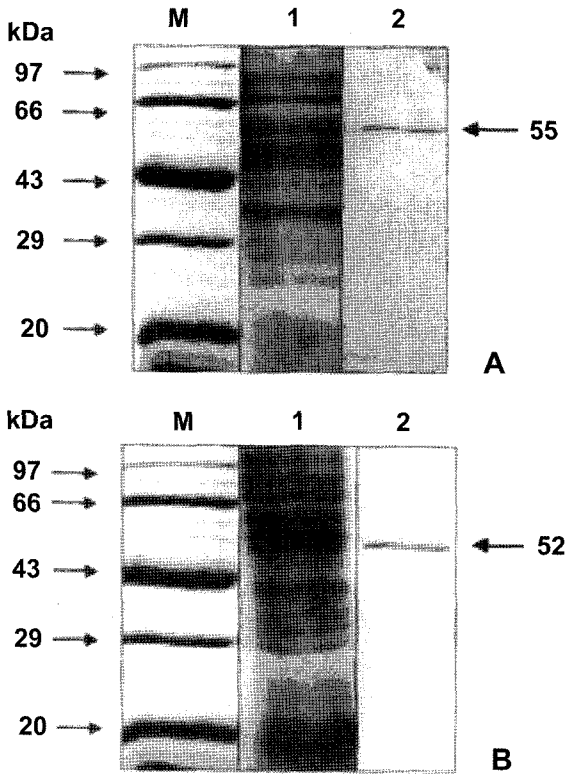

Fig. 3. SDS-PAGE of crude and sephadex eluted proteins of OCP fractions of *T. harzianum* (*Th*-23/98; peak c; A) and *T. viride* (*Tv*-25/98; peak c; B); the gel exhibits one band of 55 and 52 kDa (arrow marked); lanes: M, molecular mass markers; 1 = crude OCP fraction; 2 = sephadex eluted protein.

Table 4. Effect of various enzymes on *Th*-23/98 and *Tv*-25/98 lectin activity

Enzymes	Concentration	Titre[a]	
		Th-23/98	*Tv*-25/98
Trypsin	1%	0	0
Chymotrypsin	1%	0	0
Protease	3 mg/ml	16	8
1,3 β-Glucanase	5 mg/ml	0	0
Chitinase	62 mg/ml	128	64
PBS[b]	0.1 M	128	64

[a] 500 µl of the purified lectin was incubated with 500 µl of each enzyme at conditions described in materials and methods. Lectin titre was determined by haemagglutination assay.
[b] PBS = phosphate buffer saline.

Table 5. Effect of various pH on *Th*-23/98 and *Tv*-25/98 lectin activity

Buffer	pH	Titre[a]	
		Th-23/98	*Tv*-25/98
Sodium acetate buffer	3.5	0	0
Sodium phosphate buffer	7	8	4
Tris-HCl buffer	8	16	32
Glycine-NaOH buffer	11	64	64

[a] 500 µl of the purified lectin was incubated with 500 µl of each buffer at conditions described in materials and methods; Lectin titre was determined by haemagglutination assay.

on lectin activity of *Th*-23/98 and *Tv*-25/98 was also evaluated (Table 5). By incubating the purified lectin at pH 3.5 no activity was detected; however, at pH 7, 8 and 11 greater activity of lectin was observed. Maximum lectin activity was observed at pH 11 (Table 5).

Discussion

Attachment/recognition between the fungal parasite and its host appears to be an essential step for successful continuation of the parasitic process (Herrera-Estrella and Chet, 2002; Inbar and Chet, 1997; Ofek et al., 1996; Tunlid et al., 1992). In such interactions specific recognition/attachment appears to play a key role in the successful establishment and colonization of fungal host by antagonists (Jana et al., 2000). Inbar and Chet (1997) demonstrated that recognition/attachment of the fungal parasite to the fungal host-cell surface is mediated by lectin-carbohydrate interaction, which provides the basis for the specificity of interaction. Lectins associated with the cell walls and extracellular polymers have been found in several fungi including sclerotium-forming pathogens such as *Sclerotium rolfsii* and *Rhizoctonia* (Barak and Chet, 1990; Barak et al., 1986;

Inbar and Chet, 1994, 1996), *Arthrobotrys oligospora* (Rosen et al., 1996), *Neurospora sitophila* (Ishikawa and Oishi, 1989), *Kluyveromyces bulgaricus* (Al-Mahmood et al., 1991), *Agaricus bisporus* (Sueyoshi et al., 1985) and a biotrophic mycoparasite *Piptocephalis virginiana* (Manocha et al., 1997). Considerable researches have been done for demonstrating the role of specific recognition/attachment in different interacting microbes/plants; however, virtually very less is known about the role of specific cell surface mechanisms of recognition between fungal antagonist-fungal pathogen interactions. In most of the earlier studies lectins were isolated from the plant pathogenic fungi and not from the antagonists which parasitize the pathogenic host. The involvement of lectin from mycoparasitic fungus *Trichoderma*, in binding and recognition of pathogenic fungal host-cell has not been investigated. Therefore, to the best of our knowledge the present study provides preliminary data on the biochemical basis of recognition and specificity of interaction (mediated by lectin-carbohydrate interaction) between necrotrophic mycoparasitic *Trichoderma* species and its potential plant pathogenic susceptible host *M. phaseolina*.

Our study demonstrates that diversity exists among the isolates/species of *Trichoderma* in response to agglutination of hyphal and sclerotial surface of *M. phaseolina* (Table 1). Out of nine *Trichoderma* species, isolates of *T. harzianum* and *T. viride* showed significant agglutination potential. Specific enzymes and inhibitors affected the agglutination reaction by blocking the receptor sites (Jana et al., 2000; Manocha and Sahai, 1993; Mercure et al., 1994; Tronchin et al., 1995). In this study, agglutination efficiency of conidia of *Th*-23/98 and *Tv*-25/98, treated with different enzymes or inhibitors reduced significantly (Fig. 2). These observations indirectly indicate that agglutinin component is proteinaceous in nature (Tronchin et al., 1995).

The haemagglutination experiments showed that mycelium-associated agglutinin components of OCP, ICCP and ICP fractions of *Th*-23/98 and *Tv*-25/98 are composed of possibly lectin (Table not shown). The results are in agreement with the preliminary work of Neethling and Nevalainen (1996) who also demonstrated that the agglutination activity was present in mycelial extracts of *T. harzianum* and *T. viride*. The haemagglutinating activity was inhibited by D-glucose, D-galactose, D-fucose, N-acetylgalactosamine, N-acetylglucosamine, fetuin and mucin. These sugars belong to a group that Makela (1957) has designated as group II sugars, based on the configurations of the hydroxyl groups on C3 and C4 of the monosaccharide molecule but the activity of inhibition was generally greater after the removal of terminal residues from the glycoproteins of the red blood cells (e.g., by treating RBC with trypsin) with exposure of the subterminal galactose residue (Kellens et al., 1989). Rosen et al. (1997) reported the binding specificities of *Arthrobotrys oligospora* lectin (designated as AOL) and *Agaricus bisporus* lectin (ABL) as multispecific lectins that bound to glycoproteins containing O-linked sugar chains (sequence Galβ1-3GalNAc-Ser/Thr), to certain complex N-linked sugars, to sulfated glycoconjugates (e.g. sulfatide) and to two phospholipids. Similarly, in *Trichoderma*, it appears to be a multispecific lectin, although the binding specificity in this case is not known and detail investigation is needed. The predominance of lectins showing affinity for molecules with sugars and glycoprotein residues in several phytopathogenic and sclerotia forming fungi was also earlier demonstrated by different investigators (Candy et al., 2001; Kellens et al., 1992; Neethling and Nevalainen, 1996; Wu et al., 2001).

In this study, accumulation of lectin was greater in OCP fraction of *Th*-23/98 and *Tv*-25/98. This variation may be due to the different amount of agglutinin production in different fractions (Neethling and Nevalainen, 1996). OCP fraction of *Th*-23/98 and *Tv*-25/98 was isolated and purified by gel filtration on Sephadex G-100 column. The eluted fraction exhibited agglutination of blood group A, B, AB and O erythrocytes, indicating that the specificity of the agglutinin is not disturbed. SDS-PAGE analysis of partially purified lectin of *Th*-23/98 and *Tv*-25/98 revealed a single band of 55 and 52 kDa, respectively (Fig. 4).

Result demonstrates that both protein and 1,3-β-glucan are necessary for agglutination activity of the lectin (Table 4; Inbar and Chet, 1994). Proteolysis of the lectin caused reduction in its activity, suggesting a protein moiety of the lectin. Inhibition of lectin activity by β-1,3-glucanase suggests participation of carbohydrate groups in the binding site of the lectin (Barak and Chet, 1990; Inbar and Chet, 1994).

The pH stability of several fungal lectins has been examined (e.g., Kawagishi and Mori, 1991; Tunlid et al., 1992; Zhuang et al., 1996) by incubating and titrating the haemagglutinating activity of the lectin in buffers of different pH and the buffers were then replaced with PBS before the haemagglutinating activity was measured. The highest activity was obtained after incubating the protein with Glycine-NaOH buffer (pH 11; Table 5). The results indicate that high pH induced durable changes in the physical and/or chemical properties of the lectin that led to a change in the haemagglutinating activity.

From the present investigation it can be deduced that the recognition/attachment of mycoparasite (*T. harzianum* and *T. viride*) to the host surface (*M. phaseolina*) may be most likely due to lectin-carbohydrate interaction.

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