Purification, characterization of an entomopathogenic fungal lectin from *Purpureocillium lilacinum* and its involvement in pathogenesis leading to mycotic keratitis

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Abstract

A lectin PCL, from *Purpureocillium lilacinum* a saprophytic, filamentous fungus was purified from the crude extract of the mycelia using 70% ammonium sulphate precipitation followed by affinity chromatography on mucin-Sepharose 4 B column. PCL is a monomer with an apparent molecular mass of 18.5 kDa as revealed by SDS-PAGE under both reducing and non-reducing conditions. PCL is a blood group non-specific lectin and has highest affinity towards chitin, mucin, asialomucin, fetuin with a MIC of 0.15 µg/mL and also recognizes L-fucose, galactose, lactose, N-acetyl galactosamine, hyaluronic acid. PCL is stable up to 60 °C and within the pH range 4–8. To understand its role in pathogenesis, effect of PCL was evaluated on human corneal epithelial cells (HCECs). PCL showed strong glycan mediated binding to HCECs and PCL showed proinflammatory response at lower concentrations by stimulating secretion of IL-6, 8. In contrast PCL at higher concentrations revealed opposite effect of HCECs growth inhibition. All these results collectively support the involvement of PCL in mediating host pathogen interactions possibly leading to pathogenesis. In addition, considering the entomopathogenic effect of *Purpureocillium lilacinum*, PCL may be attributed for this beneficiary effect, which needs to be explored.

Keywords *Purpureocillium lilacinum* lectin · Affinity chromatography · HCECs · Proinflammatory · Interleukins · Host pathogen interactions

Introduction

*Purpureocillium lilacinum* is a saprophytic, filamentous fungus from Ophiocordycipitaceae family which is ubiquitous in its occurrence and has been isolated from soil, vegetable, decaying matter and also from nematodes (Luangs-Ard et al. 2011). Recent studies have reported the beneficial effects of the fungus as a biocontrol agent, as the conidial suspension of the fungus has showed nematocidal activity against leaf-cutting ant Acromyrmex lundii (Goffré and Folgarait 2015). Entomopathogenic effect of *Purpureocillium lilacinum*, formerly also known as *Paecilomyces lilacinus* was also effective against commercial crop evaders like the cotton aphid Aphis gossypii, *Tetranychus urticae*, *Triatoma infestans*, the vector causing Chagas disease (Marti et al. 2006; Fiedler and Sosnowska 2007). In contrast to these beneficial applications *Purpureocillium lilacinum* is an important human pathogen implicated in causing many medically important infectious diseases (Luangs-Ard et al. 2011). *P. lilacinum* is an infective organism in tropical and subtropical regions of the world implicated in severe infections in immunocompromised patients who have undergone liver and kidney transplantation (Castro et al. 1990). *P. lilacinum* is also known for causing upper respiratory tract infections (Ono et al. 1999), cavitary pulmonary disease and invasive fungal rhinitis (Ciecko and Scher 2010) in human beings as well as in other vertebrates.

Keratomycotic infection is an vision threatening condition usually caused by opportunistic fungus which leads to the severe inflammatory response often characterized by stromal infiltration by leukocytes (Karsten et al. 2011).
Many fungal species like Aspergillus, Fusarium or Acremonium species have been documented so far mainly implicated in mycotic keratitis (Ono et al. 1999). *Paeclomyces* is a new emerging disease causative organism in recent times, most isolated fungal species from corneal scrapings with sensitivity towards antifungal agents (Pastor and Guarro 2006). Microbial adhesion to the epithelial surfaces has been regarded as an important prerequisite step in the initiation and evasion of the host cell by pathogenic bacteria/fungi and many other diseases causing class of microorganisms (Brouwer et al. 2016; de Groot et al. 2013). Cell surface glycans play an important role in the adhesion, colonization and evasion of these pathogens as they regulate important mechanisms in host-parasite interactions (Hasnain et al. 2013). Host cells have adapted many defense mechanisms such as secretion of highly α-linked secretory oligomerized mucin glycoproteins on many epithelial cell surfaces which form a dense mucus layer preventing the physical attachment and act like physical barriers of host immunity against invading pathogens (Hasnain et al. 2013). Anti-adhesion therapy has been proved to be a promising strategy to prevent the initiation of the pathogenesis and number of targets has been defined in this innovation (Martin et al. 2020). Disruption of *Candida albicans* adhesion to mucosal surfaces of host epithelial cells by using antibodies & biomolecules has been recently elucidated by anti-adhesive therapy in insisting a check point in initiation of infection and colonization (Martin et al. 2020). Pathogenic microorganisms generally have adapted many mechanisms against host immune system to invade the host cells by secreting many virulence factors/components such as carbohydrate binding proteins such as lectins or adhesins which will help the pathogen to interact through cell surface glycan moieties (Sharon 2006). Lectins are carbohydrate binding proteins of non-immune origin which binds to cell surface carbohydrate molecules reversibly and are involved many cellular signaling mechanisms like cell adhesion, cellular trafficking, and glycoprotein synthesis in mediating host pathogen interactions (Lis and Sharon 1998). Pathogenic fungi are known to secrete these molecules to interact with host cells to initiate pathogenesis and have been reported as important components in pathogenesis (Ballal et al. 2017; Jagadeesh et al. 2020).

Considering the significance of lectins from pathogenic fungi, the present study was designed to purify the carbohydrate binding proteins/lectins from a pathogenic fungal strain *Purpureocillium lilacinum*, implicated in causing mycotic keratitis in humans. It describes the purification, characterization of a mucin specific lectin from *Purpureocillium lilacinum* and its interaction with immortalized HCECs to evaluate its physiological role. The findings are of clinical significance as the initiation of infection can be targeted by anti-adhesive and carbohydrate-based therapy.

**Materials and methods**

Mucin (porcine stomach, type III), fetuin (fetal calf serum), Cyanogen bromide, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) were procured from Sigma Chemical Co., St. Louis, USA. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Standard protein molecular weight marker was procured from Merck, India. Amicon Ultrafiltration centrifugation tubes 10 kDa were procured from Millipore, USA. Human blood samples were collected from German hospital, Dharwad. For cell culture, Dulbecco’s Modified Eagle’s Medium F12 (DMEM F12) and fetal bovine serum (FBS) were obtained from Gibco Invitrogen (Paisley, UK), with Costar 96-well plates obtained from Corning Incorporate (Corning NY, USA). Bovine serum albumin (BSA), Fluorescein isothiocyanate (FITC) were obtained from Sigma Chemical Co. (St. Louis MO, USA). ELISA kits for IL-6 (#MBS261259), IL-8(#MBS2886709) were obtained from MyBioSource. All other reagents used were of analytical grade.

Asialofetuin, Asialomucin were prepared from fetuin as described by Spiro and Bhoyroo (1974). Mucin-Sepharose 4B affinity matrix was prepared by coupling fetuin to cyanogen bromide activated Sepharose 4B, according to the method of March et al. (1974).

**Cell culture**

Immortalized HCECs were procured from ATCC and were grown and maintained in DMEM/F-12 media (Invitrogen) containing 10% FCS, at 37 °C with 5% CO₂ and used for the study.

**Media and growth conditions**

*Purpureocillium lilacinum* was procured from Postgraduate Institute of Medical Education and Research, Chandigarh, India. Strains were maintained on potato dextrose agar plates containing agar concentrate 1.7% (w/v), stored at 4 ± 1 °C until further use. A loop full of fungal spores from the mother culture were inoculated on to freshly prepared potato dextrose broth in 500 mL Erlenmeyer’s flask and grown for different time points and were screened for the presence of lectin activity on different days at room temperature. The highest cell-associated hemagglutinating titer was observed with human erythrocytes on 5th and 6th day of post inoculation of the culture. Hence mycelial mat was harvested on 6th day post inoculation and used for lectin purification.
**Lectin purification and characterization**

Mycelial mat was harvested on 6th day of post inoculation and homogenized overnight at 4 °C with phosphate buffered saline (0.05 M, pH 7.2) using mortar and pestle. After extraction, centrifuged at 10,000 rpm for 20 min at 4 °C and supernatant of the crude extract was tested for the presence of lectins/adhesins. The crude extract with lectin activity was then subjected for ammonium sulphate precipitation (70%) and kept for 1–2 h at 4 °C. The precipitates were centrifuged (10,000 rpm, 20 min, 4 °C), dissolved in PBS and dialyzed against the same buffer. The dialysate was loaded on to Mucin Sepharose 4B affinity column pre-equilibrated with PBS. Unbound proteins were washed with PBS till absorbance of eluting fractions read zero at 280 nm. Affinity bound protein was eluted with 100 mM Glycine–HCl buffer pH 2.0, containing 500 mM NaCl. Fractions with high lectin activity were pooled and dialyzed extensively against PBS, pH 7.2. Lectin-positive fractions were concentrated using Amicon Ultrafiltration centrifugation tubes (10 kDa) and stored at −20 °C for further use. All purification steps were carried out in a cold room at 4 °C. At each step of purification, lectin activity was estimated by performing hemagglutination assay.

**Preparation of trypsinized erythrocytes**

Trypsinized human blood erythrocytes required for the determination of lectin activity by hemagglutination assay were prepared as described by Liener and Hill (1953).

**Hemagglutination assay**

Hemagglutination activity of PCL during various stages of purification was determined by the serial two fold dilution method using trypsinized human erythrocytes in a 96 well “U” bottom micro titer plates (Liener and Hill 1953). The highest dilution of the extract causing visible hemagglutination was regarded as the titer and the minimum concentration of the protein was required for agglutination (MCA) as one unit of hemagglutinating activity. The specific hemagglutination activity was expressed as unit mg⁻¹ protein.

**Hapten inhibition studies**

The sugar specificity of the purified lectin was determined by a hapten inhibition assay. Inhibition assays were carried out by incubating the lectin sample showing 4 titer with serially diluted sugar/glycoprotein in a total volume of 50 μL prior to the addition of 50 μL of erythrocytes in PBS and the hemagglutination was visually observed. The minimum concentration of the sugar/glycoprotein required for complete inhibition was taken as the inhibitory titer of the hapten (MIC).

**Estimation of protein**

Protein concentrations in the extract at different stages of purification were determined by Lowry’s method (Lowry et al. 1951).and total sugar content of the lectin was estimated by phenol–sulfuric acid method using d-glucose as standard (Dubois et al. 1956).

**Preparation of FITC conjugated PCL**

Fluorescein isothiocyanate conjugated lectin (FITC-PCL) required for the binding studies was prepared as described by Goldman (1968).

**SDS PAGE**

SDS-PAGE of purified PCL and crude extracts was carried out using 15% gel as described by LaemmLi (1970). Molecular mass of the purified PCL was determined by comparison of its electrophoretic mobility with standard molecular weight marker proteins both under reducing and non-reducing conditions. The gel was stained both with Coomassie brilliant blue and silver staining (Zacharius 1969).

**Periodic acid Schiff staining of PCL**

In order to know the presence of carbohydrate in the lectin the gel after electrophoresis was stained with periodic acid Schiff reagent by the method of Zacharius (1969). Briefly, the gel was immersed in 12.5% TCA for 30 min. After rinsing the gel with water, it was immersed in 1% periodic acid prepared in 3% acetic acid and allowed to stand for 50 min. Then, excess periodic acid was removed by repeated washing of the gel with water. The gel was then transferred to fuchsine sulphite stain and kept in dark for 50 min. The gel was washed with sodium metabisulphite (0.5%) for 30 min with three changes. Finally, the gel was destained by washing it several times with water and stored in acetic acid.

**Effect of temperature and pH on PCL**

To determine the thermo-stability of the lectin, PCL (200 µg/mL) in PBS pH-7.2 was incubated at different temperatures (0 °C to 90 °C) for 20 min in temperature controlled water bath. The stability of the PCL at various pH was determined by evaluating the hemagglutination activity of the lectin incubated in buffers of different pH (2.0–12.0). PCL (200 µg/mL) was incubated with an equal volume of buffers (100 mM) of pH 2.0 (Glycine–HCl), pH 4.3 (Acetate buffer),
pH 7.0–8.0 (Tris–HCl buffer) and pH 9.0–12.0 (Carbonate buffer) for 24 h at 4 °C. After incubation lectin samples were neutralized to pH 7.0 by adding 0.1N NaOH or 0.1N HCl before testing hemagglutination activity.

**Effect of metal ions on PCL activity**

The effect of divalent metal ions on lectin activity was assessed by extensive dialysis of PCL against 10 mM EDTA for 48 h at 4 °C followed by deionized water for 48 h (Magnuson et al. 1983). The haemagglutination activity was tested before and after addition of 40 mM Ca²⁺, Mn²⁺, Mg²⁺, Al³⁺ and Fe²⁺ ions. Haemagglutination assay was performed at 0, 2, 4 and 24 h of incubation. Activity was expressed as percentage relative activity as compared to control (lectin incubated with 0.05 M phosphate buffered saline, pH 7.2 at 4 °C).

**Cell surface binding of PCL to HCECs by flow cytometry**

Immortalized HCECs (0.2 × 10⁶) were treated with 3% BSA in PBS for 1 h at 4 °C for blocking the non-specific binding sites. HCECs were then washed and treated with lectins and lectin-hapten mix and allowed to bind for 1 h at 4 °C. Lectin-hapten mix required for inhibition studies was prepared by incubating lectin (2 µg/mL) with mucin (100 µg/mL) and Chitin (200 mM/mL) in PBS for 1 h at 37 °C. After incubation, HCECs were washed twice with PBS and then processed for flow cytometry using Beckman Coulter FC500. Data were acquired for 10,000 events using lectin untreated cells as control and analyzed using CXP Software.

**Effect of PCL on viability of HCECs by MTT assay**

To study the effect of PCL on HCECs, cells were seeded in 96-well plates (density 5 × 10⁴ cells/mL) and grown in complete DMEM F12 medium for 48 h prior to lectin treatment. Medium was replaced with serum-free DMEM F12 after 48 h, then treated with PCL at different concentrations (0.15–10 µg/mL) and maintained in humidified atmosphere (37 °C, 5% CO₂) for 24 h and 48 h. At each time point, 50 µL of MTT (5 mg/mL) was added to each well followed by lysis with 100 µL dimethyl sulfoxide (DMSO). To observe effects of competing glycans, PCL (5 µg/mL) was pre-incubated for 1 h with Chitin (200 mM/mL), before its addition to cells, and processed at 24 and 48 h as mentioned above. Cell viability was quantified by measuring absorbance at 570 nm using a micro-plate reading spectrophotometer. Percentage viable cell number was calculated, by comparing with untreated controls, considered as 100%.

**Quantification of interleukins secreted in PCL treated HCECs by ELISA**

In order to monitor the expression of IL-6 and IL-8 in immortalized HCECs following PCL treatment for 24 and 48 h, the spent media from lectin-treated cells were collected and further used for the quantification of interleukins. ELISA kit (MyBioSource) for detection of interleukins using different dilutions of the spent media was prepared by using the dilution buffer provided in the kit and IL-6 and IL-8 were estimated following the manufacturer’s instructions. Spent media from the lectin untreated cells were used as negative control. Data were represented as picogram of IL-6 and IL-8 per 100 µl of spent media.

**Statistical analysis**

Results were expressed as mean ± SD. Statistical comparisons were performed using the Student’s t-test in order to determine statistical significance. Microsoft Excel was used to perform statistical analysis.

**Results**

**Isolation and purification of PCL**

Purification of PCL, from 70% ammonium sulphate precipitate of the crude extract, performed by affinity chromatography on mucin coupled Sepharose-4B column resulted in purification of PCL by 34.8-fold (Fig. 1a, Table 1) indicating the efficiency of the protocol developed in our laboratory. PCL is a glycoprotein with 5% of carbohydrate content as determined by phenol sulfuric acid method and as confirmed by PAS staining (Fig. 1d).

The fold purification and the percentage recovery of the lectin purified from 10 g of fungal mycelia mat are summarized in Table 1.

**Hemagglutination and hapten inhibition assay**

PCL showed hemagglutination activity towards trypsinized human A, B, AB, O and rabbit erythrocytes. Hapten inhibition studies of PCL showed that the hemagglutinating activity was inhibited strongly by glycoproteins with mucin, asialomucin, fetuin with minimum inhibitory concentration (MIC) of 3.125 µg/50 µL, sugar derivatives like N-acetyl galactosamine with MIC of 12.5 mM/50 µL and sugar derivatives like chitin with MIC of 3.125 mM/50 µL and hyaluronic acid with MIC of 25 mM/50 µL. PCL also showed glycan specificity towards simple sugars like l-Fucose, galactose, lactose with MIC of MIC 6.25 µM/50 µL each (Table 2).
Fig. 1 A.1 Purification and characterization of *Purpureocillium lilacinum* lectin (PCL) by affinity chromatography: a. Purification of PCL by affinity chromatography using Mucin-Sepharose-4B column (1.5×10 cm) equilibrated in PBS for overnight. Redissolved ammonium sulphate precipitate was subjected to affinity chromatography on Mucin-Sepharose-4B column. Column was washed with PBS till absorbance was read zero. Bound lectin was eluted with 100 mM Glycine–HCl buffer, pH 2.0 containing 500 mM NaCl. Fractions of 3.0 mL were collected at flow rate of 15 mL/h. A.2 SDS PAGE of PCL in 15% gel: A Gel Stained with Coomassie brilliant blue, lane 1- Crude, lane 2- Ammonium sulphate Ppt, lane 3- Affinity purified PCL(non-reducing), lane 4- Standard protein molecular weight markers, lane 5- PCL (reducing). A.3 Silver staining of the gel: Lane 1- Standard protein molecular weight marker, lane 2- PCL in non-reducing condition. A.4 PAS staining of PCL: Lane 1- PCL (50 μg). A.5 Calibration curve for the determination of molecular weight of PCL by SDS PAGE: Y-axis relative mobility and X-axis log molecular weight of protein markers; phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29 kDa), soya-bean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa)

Table 1 Purification of *Purpureocillium lilacinum* lectin

| Sample              | Volume (mL) | Total protein (mg) | MCA*a (µg)  | Specific activity*b (units) | Total activity*c (units) | Fold purification | %Recovery of activity |
|---------------------|-------------|--------------------|-------------|-----------------------------|--------------------------|--------------------|------------------------|
| Crude               | 100         | 120                | 2.375       | 0.12×10^3                  | 1.4×10^4                 | –                  | 100                    |
| Ammonium sulphate ppt| 20          | 80                 | 0.48        | 2.1×10^3                   | 1.6×10^5                 | 7.45               | 38                     |
| Affinity purified   | 0.6         | 0.6                | 0.019       | 6.0×10^3                    | 3.6×10^3                 | 34.8               | 28                     |

Wet mycelial weight: 10g

*a*Minimum concentration of protein required to agglutinate human ‘O’ erythrocytes

*b*Specific activity: hemagglutinating activity/mg protein

*c*Total activity: hemagglutinating activity of lectin in total protein
Estimation of molecular mass of PCL by SDS-PAGE

Purified PCL eluted from Mucin Sepharose-4B affinity column was found to be a monomer as observed by single band on SDS-PAGE both under reducing and non-reducing conditions and it has a subunit molecular mass of 18.5 kDa (Fig. A2, 3 and 5). Monomeric nature of PCL was also confirmed by SDS-PAGE under reducing conditions (Fig. A2; lane 5). Silver staining of affinity purified PCL further confirms the homogeneity of the lectin (Fig. A3; lane 2c).

Effect of pH and temperature on PCL activity

Purified PCL exhibited optimum lectin activity at pH 7.2. Lectin activity was stable over a wide range of from pH 4.0 to 8.0 with complete loss of activity at pH 12.0. PCL is thermos-stable up to 0–60 °C. Lectin activity was completely lost at higher temperatures above 80 °C.

Effect of metal ions on PCL activity

To determine the effect of metal ions on lectin activity, purified PCL was dialyzed against EDTA and followed by deionized water. Interestingly no change in lectin activity was noticed upon dialysis against EDTA which results in demetallization of lectin or after addition of divalent metal ions like Fe^{2+}, Mg^{2+}, Mn^{2+} and Ca^{2+} ions indicating that PCL does not require any of the above tested metal ions for its hemagglutination activity.

PCL strongly binds to immortalized HCECs

To study the interaction of PCL with immortalized HCECs, cell surface binding studies using FITC labeled PCL was used followed by flowcytometric analysis. A total of 90.7% HCECs were found to be positive staining for PCL with high mean fluorescence intensity (MFI) of 655, compared to unstained cells with MFI of 1.29 (Fig. 2 A1). Glycan mediated interaction of PCL with HCECs was determined by preincubating the lectin with glycans, before treating them with HCECs, which resulted in significant reduction in cell-surface binding of PCL to HCECs. HCECs stained with FITC-PCL, MFI decreased to 28.6 and 67.3 in presence of competing glycans chitin and mucin respectively (Fig. 2 A2).

PCL showed differential effects on HCECs growth

The results of MTT assay of PCL treated HCECs showed differential effects at different concentrations. At lower concentrations i.e., at 0.6 µg/mL and below, PCL showed proliferative/mitogenic effect i.e., increased the viability of HCECs in a dose- and time-dependent manner. At 0.6 µg/mL concentrations the cell viability was increased by 108.3 ± 0.7% (n = 9, p < 0.001), at 0.3 µg/mL by 119.5 ± 0.8% (n = 9, p < 0.001), 0.15 µg/mL by 130.6 ± 2.2% (n = 9, p < 0.001) and 0.07 µg/mL by 137.1 ± 1.3% (n = 9, p < 0.001), Maximum mitogenic activity on HCECs growth was observed at 0.07 µg/mL over 48 h which resulted in an increase of HCECs population to 137% at 48 h post lectin treatment. In contrary, at higher concentrations of PCL i.e., more than 1.25 µg/mL significantly reduced the growth of HCECs in a dose- and time-dependent manner (Fig. 2 A3). Maximum cell growth inhibition was observed at 10 µg/mL of lectin over 48 h, with PCL inhibiting cellular growth of HCECs at higher concentrations of 2.5 µg/mL by 31.5 ± 1.3% (n = 9, p < 0.001) at 5 µg/mL by 44.0 ± 1.3% (n = 9, p < 0.001) and maximum at 10 µg/mL by 70.3 ± 1.5%. Glycan-mediated effect of PCL on HCECs was observable and could effectively be blocked by 99% in presence of competing glycoprotein Chitin. IC₅₀ value of PCL for HCECs was found to be 4.65 µg/mL at 48 h. These results clearly showed that the effect of PCL on HCECs growth is mediated by lectin binding to carbohydrate moieties present on the cell surface.

PCL induces proinflammatory response in HCECs at lower doses by stimulating interleukin secretion

As PCL showed the mitogenic effect with HCECs at lower concentrations, in order to evaluate its proinflammatory effects on HCECs, the spent media upon PCL treatment was collected at different time intervals and tested for the expression of proinflammatory Interleukins which are responsible for mitogenic activity. Interleukins, IL-6 (Fig. 3. A1)
and IL-8 (Fig. 3. A2) in HCECs spent media were quantified and data are depicted as pg of ILs secreted per 100 µl of lectin treated spent media. HCECs were incubated with FITC-conjugated PCL in absence (blue line) or presence of competing sugar (Chitin; green) or glycoprotein (mucin; pink line), cells untreated with lectin indicated (black line). Binding was analyzed by flow cytometry. The overlays are representative data with X-axis and Y-axis expressed as mean fluorescence intensity (MFI) and cell count, respectively. Panel A.2 represents inhibition of binding (MFI) in presence or absence of competing glycans, Chitin (green bar) or mucin (pink bar) compared to FITC-conjugated lectin alone (blue bar). A.3 Effect of PCL on cellular viability on immortalized HCECs growth: HCECs seeded in 96-well plates (5×10⁴ cells/mL) grown in complete DMEM F12 medium for 48 h prior to lectin treatment. Medium was replaced with serum-free DMEM F12 and then treated with PCL at different concentrations (0.15–20 µg/mL) for 24 h and 48 h and processed for MTT assay. Data are expressed as % cell viability compared to untreated controls (100%). Chitin (M), at 200 mM/mL, effectively blocked PCL (L)-mediated inhibition of growth (5 µg/mL). Data are expressed as mean ± SD of three independent experiments and 694 ± 17.23 pg respectively (n = 6, p < 0.05). These results clearly demonstrate that PCL induces proinflammatory effect in HCECs at a lower concentrations subsequently leading to pathogenesis.

Discussion

Fungal lectins are implicated in different physiological functions and some are known for their involvement in host pathogenic interactions eventually leading to pathogenesis. A novel mucin/chitin specific lectin, PCL from

![Graph](image)

**Fig. 2** A.1 PCL strongly binds to immortalized HCECs by flow-cytometry. FITC conjugated PCL was used to study the interaction of PCL to immortalized HCECs. HCECs were incubated with FITC-conjugated PCL in absence (blue line) or presence of competing sugar (Chitin; green) or glycoprotein (mucin; pink line), cells untreated with lectin indicated (black line). Binding was analyzed by flow cytometry. The overlays are representative data with X-axis and Y-axis expressed as mean fluorescence intensity (MFI) and cell count, respectively. Panel A.2 represents inhibition of binding (MFI) in presence or absence of competing glycans, Chitin (green bar) or mucin (pink bar) compared to FITC-conjugated lectin alone (blue bar). A.3 Effect of PCL on cellular viability on immortalized HCECs growth: HCECs seeded in 96-well plates (5×10⁴ cells/mL) grown in complete DMEM F12 medium for 48 h prior to lectin treatment. Medium was replaced with serum-free DMEM F12 and then treated with PCL at different concentrations (0.15–20 µg/mL) for 24 h and 48 h and processed for MTT assay. Data are expressed as % cell viability compared to untreated controls (100%). Chitin (M), at 200 mM/mL, effectively blocked PCL (L)-mediated inhibition of growth (5 µg/mL). Data are expressed as mean ± SD of three independent experiments and 694 ± 17.23 pg respectively (n = 6, p < 0.05). These results clearly demonstrate that PCL induces proinflammatory effect in HCECs at a lower concentrations subsequently leading to pathogenesis.

**Discussion**

Fungal lectins are implicated in different physiological functions and some are known for their involvement in host pathogenic interactions eventually leading to pathogenesis. A novel mucin/chitin specific lectin, PCL from...
**Purpureocillium lilacinum** a pathogenic fungus implicated in mycotic keratitis was purified, characterized and its involvement in host pathogen interactions was demonstrated by studying its interaction with HCECs. PCL is purified in a single step by affinity chromatography using Mucin Sepharose 4B column by 34.8-folds with an overall recovery of 31%. PCL is a monomer with apparent molecular weight of 18.5 kDa under reducing and non-reducing conditions and lectin is blood group non-specific. PCL has wide range of pH stability between 4.0 and 8.0 and thermal stability up to 60 °C. PCL strongly binds to immortalized HCECs and showed proliferative/mitogenic effect at lower concentration and growth inhibitory or anti proliferative effect at higher concentration. PCL at lower concentration induced the expression of proinflammatory cytokines IL-6 and -8. In contrast at higher concentration, PCL exerted growth inhibitory effect in HCECs.

Earlier, purification of lectins from pathogenic fungi implicated in mycotic keratitis has been reported from our laboratory. A core fucose specific, mitogenic lectin, CSL from *Cephalosporium curvulum* isolated from mycotic keratitis patient was purified in a single step by affinity purification technique using asialofetuin coupled Sepharose 4B column. CSL is a homotetramer with monomeric mass of 14.3 kDa (Nagre et al. 2010). Another fucose specific lectin ANL from *Aspergillus niger* was purified by using mucin coupled Sepharose 4B column (Jagadeesh et al. 2019). ANL is a monomer with of 31.6 kDa. Hence affinity chromatography based on carbohydrate specificity of lectins is generally used for purification. An acidic, sialic acid specific lectin from mushroom *Paecilomyces japonica* (PJA) has been purified using fetuin-agarose column that shows an apparent molecular weight of 16 kDa (Park et al. 2004). In comparison with these reported lectins, in the current study PCL was also purified using an affinity column of mucin Sepharose 4B, showed molecular weight that also falls in range with these fungal lectins. Stability towards high pH and temperatures has been regarded as an important characteristic feature of lectins in general and fungal lectins in particular which could be correlated to the glycoprotein nature of the lectins (Jagadeesh et al. 2019). PCL is glycoprotein containing 5% of carbohydrate and thermo-stable up to 60 °C and stable within the pH range between 4.0 and 8.0. PCL has a broader glycan specificity recognizing fucose, which is expressed on HCECs, glycoproteins comparable with CSL, ANL and PJA in having sugar binding to fetuin (Nagre et al. 2010; Jagadeesh et al. 2019; Park et al. 2004) and polysaccharides like chitin a component of insect cuticle, possibly attributing to reported entomopathogenic effect of the fungus.

The surface epithelium at the human cornea and the conjunctiva interface serves as an important physical barrier against invading opportunistic microbial pathogens. Corneal epithelial cells protect ocular surfaces by secreting inflammatory cytokines as preliminary immune responses against pathogenic fungi (Ballal et al. 2017; Jagadeesh et al. 2020). When tested in-vitro, PCL showed cell surface glycan mediated mitogenic/proinflammatory effect at lower concentrations and growth inhibitory effect at higher concentrations.

The role of lectins in pathogenesis has been well established in many pathological conditions. Recently, we reported an L-fucose specific lectin ANL isolated from the corneal smears of a mycotic keratitis patient (Jagadeesh et al. 2019). ANL showed glycan mediated binding to HCECs and opposite effects at different concentrations i.e., at higher concentration a significant growth inhibitory effect and mitogenic activity at lower concentration. ANL is also shown to be involved in mediating host pathogen interactions (Jagadeesh et al. 2020). A core fucose specific
lectin CSL from Cephalosporium curvulum isolated from corneal smears of a mycotic keratitis patient also exerted the similar effect with HCECs when tested in vitro (Ballal et al. 2017). In the present study to understand the possible role of PCL in mycotic keratitis cell surface binding studies were carried out by flowcytometry. PCL showed strong glycan mediated binding to corneal epithelial cells. In comparison with ANL and CSL, PCL showed similar effect i.e., a mitogenic activity at lower concentration and growth inhibitory effect at higher concentration.

Proinflammatory cytokines like IL-6 and IL-8 are known to be secreted in the host cells against many fungal infections which are known to play a significant role in eliciting host immune response against the pathogen (Wu et al. 2016). A fucose specific lectin CSL from corneal smears of mycotic keratitis patient which was recently reported from our laboratory showed a significant dose and time dependent expression of proinflammatory cytokines IL-6 and IL-8 in HCECs (Jagadeesh et al. 2021). ANL, a lectin from Aspergillus niger from fungal keratitis patient showed similar effect i.e., time dependent expression of proinflammatory cytokines IL-6 and IL-8 in HCECs (Jagadeesh et al. 2020). These results support the significance of interleukins in pathogenesis. Interestingly PCL at higher concentrations exerted opposite effect on HCECs that is growth inhibitory. All these results indicate that PCL may be involved in causing mycotic keratitis and the present study in understanding its role in initiating pathogenesis is of clinical significance to develop therapeutic strategy in due course.

In conclusion PCL, a novel mucin/chitin specific lectin from pathogenic fungus is purified and characterized and it is indeed involved in mediating host pathogen interaction leading to pathogenesis. As the interaction of PCL with HCECs is glycan mediated, carbohydrate based therapy or strategy can be an effective way in controlling initiation of the infection by the fungus eventually leading to the pathogenesis.

Author contributions All authors contributed to the study. NJ: Performed experiments and draft of the manuscript; SSK and NJ: purification of lectin; VBC: procuring the fungal culture and manuscript; SR: formation experiments and data interpretation, final manuscript and laboratory facilities. All authors have read and approved the final manuscript.

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Availability of data and material Data will be made available on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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