Role of Glycoproteins Isolated from *Epicoccum purpurascens* in Host-Pathogen Interaction

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**Key Words**
Allergic fungal sinusitis  *Epicoccum purpurascens*  Hemagglutination activity  Hemagglutination inhibition  High-performance anion exchange chromatography  Mannose-binding lectin  Pulsed amperometric detection  Sinusitis, fungal

**Abstract**

**Objective:** Attachment to host matrix is an important provisory step for the institution of any fungal infection. The present study investigates the role of glycoproteins of *Epicoccum purpurascens* in host-fungal adherence. **Methods:** *Epicoccum* spore-mycelial extract was fractionated on a concanavalin A-Sepharose column. Three glycoproteins of 12, 17 and 33 kDa (Epi p 1) were electroeluted and checked for hemagglutination and hemagglutination inhibition. The monosaccharide content of the highly potent protein Epi p 1 was determined by high-performance anion exchange chromatography and pulsed amperometric detection. The interaction of Epi p 1 with mannose-binding lectin (MBL) leading to the activation of the complement system was studied by immunoblot, ELISA and ELISA inhibition techniques. Immunoblot and immunoblot inhibition were carried out with culture filtrate to determine the nature of Epi p 1. **Results:** 33 (Epi p 1)-, 17- and 12-kDa proteins were 58, 46 and 38 times more potent than crude extract in hemagglutination activity (HA). The HA of Epi p 1 was inhibited by N-acetyl glucosamine, glucose and laminin. Epi p 1 had a high mannose content, showed MBL binding in ion-dependent manner and caused complement activation. The protein was detected in culture filtrate and thus seems to play a significant role in fungal invasion. **Conclusion:** Epi p 1, an allergenic glycoprotein of *E. purpurascens*, is involved in host-fungal interactions through MBL.

**Introduction**

Invasive and non-invasive growth of fungi causes debilitating diseases in the upper and lower respiratory tract [1]. Identification of inhabiting or infecting fungi is a prerequisite for appropriate treatment. The primary etiological agents in non-invasive disease, i.e. allergic fungal sinusitis, are the phaeoid fungi such as *Alternaria, Aspergillus, Bipolaris, Cladosporium, Curvularia, Fusarium* and *Rhizomucor* [2]. The inhabiting fungi cause hypersensitivity in paranasal sinuses of atopic, immunocompetent individuals and produce a typical 'allergic mucin'. Only a few fungi are detected in the mucin, but these patients show skin reactivity to many other fungi [3]. In an earlier study, *Epicoccum purpurascens* (nigrum) was consistently recovered from mucin of 4 patients diagnosed with allergic fungal sinusitis and one of them had mycelial mass in nasal sinuses, portions of which were culture positive for *Epicoccum* [2]. However, mucin cultures are frequently negative in case of deep-seated fungal infections. A PCR-based method detected fungal pathogens, viz *Aspergillus*...
fumigatus, Candida albicans, C. krusei, Cryptococcus neo-
formans, C. tropicalis and E. nigrum, in samples which
were culture negative [4].

E. purpurascens is a ubiquitous mold known to cause a
variety of fungal diseases, e.g. allergic asthma, rhinitis,
sinusitis and pneumonitis [2, 5, 6]. Previous studies with
Epicoccum have identified 25–33 IgE-binding proteins in
spore-mycelial extract [7–10]. A few proteins have been
isolated from E. purpurascens and evaluated for allergenic
activity [11]. E. purpurascens is involved in both invasive
and non-invasive fungal diseases, but knowledge on
host-fungal interactions is lacking. Studies with A. fumi-
gatus, C. albicans and Penicillium marneffei have shown
that host-fungal interactions are mediated by lectins/gly-
copolymers through specific recognition of glycoconju-
gates on host epithelial membrane [12–15]. Glycoproteins
of Epicoccum were thus chosen for the present study.

Lectins are important candidates for the detection of
cell surface carbohydrates and are implicated in molecu-
lar recognition. Oligosaccharides containing mannose or
N-acetyl glucosamine interact specifically with the C-
terminal carbohydrate recognition domain of mannose-
binding lectin (MBL) [16]. Binding of MBL to its ligands
carbohydrates) activates MBL-associated serine prote-
as, which are homologous to C1q-associated proteases
C1r and C1s, achieving a complement cascade in an an-
tibody- and C1-independent manner. The present study
investigates the role of glycoproteins of E. purpurascens
in host-pathogen interactions and elucidates the method
of infection.

Materials and Methods

Organisms and Culture Conditions

E. purpurascens (Microbial Type Culture Collection, 2,129)
was grown in Sabouraud’s yeast extract medium at 25°C in sta-
tionary condition. The spore-mycelial mass was harvested on
day 13, freeze-dried and powdered. The extraction of antigens
was carried out in 125 m M NH₄HCO₃ buffer, pH 8.0 (1:20 w/v powder:
13, freeze-dried and powdered. The extraction of antigens was
buffer) containing 5 m M ethylenediamine tetraacetic acid (EDTA)
standard.

proteins were removed by washing with 4 M NaCl and 0.25% Triton
plied onto a concanavalin A-Sepharose column. The nonspecific
scribed previously [11]. Briefly, protein extract (100 mg) was ap-
different temperatures (30–80 °C) with 10 °C increments for 30 min.
layer containing red blood cells (RBCs) was washed thrice in 100
inflammatory activity and elucidates the method

of infection.

Hemagglutination Inhibition Assay

Human RBCs were used as a model to simulate host epithelia
that encounter fungal proteins. Fifty microliters of fungal extract
or purified protein in serial twofold dilutions was mixed with an
equal amount of 2% erythrocyte suspension per well of U-shaped
microtiter plates and incubated for 1 h at room temperature. PBS
was used as a negative control. The hemagglutination titer was de-
defined as the reciprocal of the highest dilution of fungal extract that
yielded visible hemagglutination activity (HA). In this study, one
hemagglutination unit (1 HAU) was defined as the amount of fun-
gal extract that causes complete agglutination under the aforemen-
tioned conditions [15]. Specific HA was recorded as the number of
HAUs per microgram of protein.

Effect of Temperature and pH on HA

Epi p 1 (32 HAU; 50 µl) in 100 m M PBS was incubated at dif-
ferent temperatures (30–80 °C) with 10 °C increments for 30 min.
It was then cooled on ice and HA was titrated. The effect of pH on
HA was examined by incubating Epi p 1 with human erythrocytes
in the following buffers: 0.02 M sodium acetate buffer (pH 3.0, 4.0
and 5.0), 0.02 M sodium phosphate buffer (pH 6.0 and 7.0), 0.02 M
Tris-HCl (pH 8.0 and 9.0) and glycine-NaOH (pH 9.0, 10.0 and
11.0) for 24 h at 4°C.
Deglycosylation
Enzymatic deglycosylation was performed as described elsewhere [19]. Briefly, the protein was immobilized on polyvinylidene difluoride membrane. Immobilized protein was reduced using dithiothreitol and was carboxymethylated with iodoacetic acid. Thereafter, residual sites on the membrane were blocked with PVP-360 to prevent adsorption of endoglycosidase. Excess of reagents was removed by washing with RCM buffer (8 mM urea, 360 mM Tris, 3.2 mM EDTA, pH 8.6) after each step. The immobilized protein was treated with PNGase F (Roche, Basel, Switzerland) in 10 mM Tris acetate buffer (pH 8.3) at a concentration of 4 U/ml for 3 h at 37°C. The released oligosaccharides were subjected to hydrolysis with 4 M HCl for 6 h at 100°C for quantitative analysis of amino and neutral sugars. After removal of acid by rotary evaporation, the sample was filtered and subjected to high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [20].

HPAEC-PAD for Monosaccharide Analysis
A Dionex DX 500 system equipped with a gradient pump (GP 40), an anion exchange column (Carbopac PA-1, 4 × 250 mm) and an eluant degas module (EDM-2) for pressurizing the eluants with argon was employed for monosaccharide separation under isocratic condition [21]. A 15-min column wash with 200 mM NaOH followed by a 15-min equilibration with starting eluant (18 mM NaOH) was required to obtain highly reproducible retention time. The flow rate was maintained at 1 ml/min at ambient temperature.

The separated monosaccharides were detected by PAD using a gold electrode. Triple-pulsed amperometry was used and the pulse detection potentials applied were E1 = +0.10 V (T1 = 500 ms), E2 = +0.60 V (T2 = 100 ms) and E3 = +0.60 V (T2 = 50 ms) and the integration was done from 300–500 ms. The response time of the PAD was set to 5 s. Standard sugars (Glc, Gal, Man, Fuc, Rha, Rib, GlcN, GalN and GalNAc) and HCl were obtained from Sigma and NaOH pellets were obtained from Merck, Darmstadt, Germany. The standard solutions were injected at a concentration of 50 μM. The standard mixture of sugars was run before and after the analysis of each sample. An internal standard (6-O-methyl mannopyranoside) was also included during analysis. The amount of sugars in the sample was calculated using these standards.

SDS-PAGE and Immunoblot
SDS-PAGE of crude extract and concanavalin A-Sepharose-eluted proteins was carried out as described by Bisht et al. [11]. To study the interaction of Epi p 1 with MBL, 10 μg of Epi p 1 was used for electrophoresis under denaturing conditions. The separated protein was transferred to nitrocellulose membrane as described previously [11]. The unoccupied sites were blocked as described above. After washing with TBS/Tween-20/Ca2+, pooled plasma of healthy volunteers diluted 1:15 v/v (in 20 mM Tris-HCl,10 mM CaCl2,1 M NaCl, 0.05% Triton X-100 and 0.1% BSA, pH 7.4) was added (100 μl/well) and incubated overnight at 4°C [22]. Wells incubated with buffer alone were taken as negative controls. C4b was detected by the addition of rabbit anti-human C4 (Sigma) followed by the incubation with anti-rabbit IgG HRP (Sigma) for 2 h. The reaction was developed as described above.

Results
HA of the Fungal Extract
Day 13 spore-mycelial extract of Epicoccum yielded 25 mg protein/g fungal powder. The extract showed high carbohydrate content and the carbohydrate/protein ratio was 2:1. As little as 6 μg of protein demonstrated HA. A negative control (PBS) did not show HA. This indicated the presence of agglutinins/lectins in *E. purpurascens* extract.
Glycoprotein Purification

Concanavalin-A-bound proteins of *Epicoccum* purpurascens were fractionated on a Sephadex G-75 column. SDS-PAGE of the eluted fraction showed three prominent protein bands of 33, 17 and 12 kDa while the crude spore-mycelial extract fractionated into ∼30 protein bands (fig. 1, lanes 1 and 2). The proteins were immunoreactive to *E.-purpurascens*-hypersensitive patient sera (fig. 1; lane 3). These proteins were isolated by electroelution (0.5–1% yield) and used for the present study. Based upon matrix-assisted laser desorption/ionization-time of flight mass spectrometry, the band above 29 kDa in the eluted fraction has been referred as 33 kDa [11]. Besides 33 kDa, the other two proteins of 17 and 12 kDa were also used in hemagglutination.

HA of the Purified Proteins

The purified proteins exhibited high agglutination values (table 1). Specific HA of the proteins (33, 17 and 12 kDa) was 58, 46 and 38 times higher than crude *Epicoccum* extract. As the 33-kDa protein showed highest HA, it was used for further investigation. It agglutinated RBCs in a temperature range of 30–50°C. The activity was however lost at higher temperatures. The protein retained HA in a pH range of 6–11, but the activity was lost at lower pH (pH 5.0).

Hemagglutination Inhibition by Carbohydrates and Glycoconjugates

To identify the interacting molecules, hemagglutination inhibition assays were carried out. The proteins (protein extract/purified) were incubated with serial dilutions of various sugars/glycoproteins separately and then used for the assay. HA of crude *Epicoccum* extract was inhibited by N-acetyl neuraminic acid at a concentration of 150 mM. Laminin and fetuin inhibited HA at concentrations 120 and 75 g, respectively. N-acetyl glucosamine, glucose and laminin inhibited hemagglutination activity of Epi p 1 at concentrations of 60 and 200 mM and 20 g, respectively (table 2).

Epi p 1 in Culture Filtrate

Immunoblotting of culture filtrate (proteins) with *Epicoccum*-hypersensitive pooled patient sera identified 12 IgE-reactive bands, including a 33-kDa band (fig. 1; Table 1. Specific HA of crude extract and purified proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>1 HAU</th>
<th>Specific HA per mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract protein</td>
<td>5.8 μg</td>
<td>172</td>
</tr>
<tr>
<td>Epi p 1 33 kDa</td>
<td>100 ng</td>
<td>10,000</td>
</tr>
<tr>
<td>17 kDa</td>
<td>120 ng</td>
<td>8,000</td>
</tr>
<tr>
<td>12 kDa</td>
<td>150 ng</td>
<td>6,666</td>
</tr>
</tbody>
</table>

Table 2. Hemagglutination inhibition of Epi p 1 by various sugars and glycoproteins

<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>MIC mM</th>
<th>Proteins</th>
<th>MIC μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetyl neuraminic</td>
<td>–</td>
<td>BSA</td>
<td>–</td>
</tr>
<tr>
<td>D-Galactosamine</td>
<td>–</td>
<td>bovine fetuin</td>
<td>–</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>200</td>
<td>laminin</td>
<td>20</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>–</td>
<td>human fibrinogen</td>
<td>–</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>–</td>
<td>bovine mucin</td>
<td>–</td>
</tr>
<tr>
<td>D-Lactose</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trehalose</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melibiose</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetyl glucosamine</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetyl galactosamine</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MIC = Minimum inhibitory concentration; – = corresponding substances were not inhibitory up to their highest concentration used.
The polyclonal antibody reacted to several fractions (7) on immunoblot. This may be due to the presence of variants of the same protein with slight differences/or due to the shared IgG epitopes of these proteins with Epi p 1 (fig. 1; lane 5). The strips probed with rabbit antibody preincubated with the purified Epi p 1 showed reduced IgG reactivity towards a 33-kDa protein (fig. 1; lane 6).

**Monosaccharide Analysis of Epi p 1**

Epi p 1 showed ~70% carbohydrates estimated by the orcinol-sulfuric acid method. N-linked sugars of this protein were released by digestion with N-glycosidase (PNGase F). Analysis of the released sugars was carried out after hydrolysis with HCl. Four major and two small peaks were detected by HPAEC-PAD (fig. 2). The peaks were identified using standard and the amount was determined by relating the injected amount with the peak area of the corresponding standard. Mannose, ribose, fucose and galactose were detected in approximate molar proportions of 4:2.3:1:4:1. Galactosamine and glucosamine were also identified as small peaks.

**MBL Binding and Complement Activation by Epi p 1**

The 33-kDa (Epi p 1) band showed immunoreactivity to anti-MBL antibody after incubation with MBL. Binding was abolished when MBL was preincubated with EDTA or mannose (fig. 3). This was confirmed by ELISA (table 3). The C4b deposition assay was carried out to check whether MBL binding initiates C4 cleavage. It showed more activation by crude extract (optical density,
Discussion

Adhesion of pathogenic microorganisms to the host tissue is a crucial step for the establishment of infection. It is mediated by recognition of the host components by complementary moieties of the microorganism. Such interaction in molds, viz *A. fumigatus*, *Beauveria bassiana*, *C. albicans* and *Volvariella volvacea*, is mediated by carbohydrate-specific proteins/lectins [13, 14, 23–25]. *E. purpurascens*, a saprophytic mold has been implicated in asthma, rhinitis, allergic fungal sinusitis and hypersensitivity pneumonitis [2, 5, 10]. *Epicoccum* was also recovered from patients with chronic rhinosinusitis and these patients had eosinophilic infiltration in the edematous submucosa and peripheral blood eosinophilia [26]. However, the potential role of this mold in infections such as polyposis, for example, is still underestimated due to the lack of sensitive and specific diagnostic techniques.

The initial interaction that leads to infection occurs at the fungal cell wall and host cell surfaces [25]. Thus, it is of interest to study the interacting components of the mold and the host. The cell wall of most fungi is composed of glycoproteins embedded within a polysaccharide matrix or scaffolding. Glycoproteins on the fungal cell surface are the entities most likely to be exposed to the surrounding environment. The fungus-host interaction begins with carbohydrates before protein, DNA or RNA interaction [25]. The fungal components that mediate adherence to the host tissue have been identified in conidia as well as in mycelia. A 32-kDa fucose-specific lectin that mediates attachment of conidia to the host extracellular matrix components was identified from *A. fumigatus* [15]. Agglutinin/lectin was purified from ‘mycelia’ of mold *B. bassiana* [24]. The present study was undertaken to identify the components of spore-mycelial *E. purpurascens* proteins responsible for host-pathogen interaction. Besides, the role of MBL in host-pathogen interaction was also explored to understand the method of infection.

Studies with RBC hemagglutination as a model system revealed that *Epicoccum* extract interacts with molecules on the host surface. Three glycoproteins of *Epicoccum* were checked for the interaction. A 33-kDa glycoprotein (Epi p 1) showed 58 times greater HA than crude extract. N-acetyl glucosamine and glucose inhibited HA of Epi p 1 indicating involvement of these moieties. However, for mediating such interaction with host, the protein should be secreted out. Immunoblot and immunoblot inhibition of culture filtrate showed that Epi p 1 is a secretory protein. Monosaccharide analysis of Epi p 1 demonstrated high amounts of N-linked mannose, fucose, ribose and galactose (fig. 2). Such mannose-rich surface antigen from *Pneumocystis carinii* has been implicated in mediating attachment to phagocytes [27].

Glycosylated allergens play a role in mediating interaction with host components. Interaction of allergen extract/purified allergens with lung collectins/surfactant proteins, namely SP-A and SP-D, has been shown previously [28, 29]. Earlier, SP-A showed binding to pollens from *Populus nigra italica*, *Poa pratensis*, *Secale cereale* and *Ambrosia elatior* in a concentration-dependent manner [28]. The interaction was mediated by glycoproteins, Ca2+-dependent and inhibited by preincubation with mannose. Further, both SP-A and SP-D showed binding to purified Der p 1 and to crude dust mite extract. Enzymatically deglycosylated mite extract and deglycosylated Der p 1 did not bind to SP-A or SP-D [29]. In the present study, Epi p 1 interacted with MBL in Ca2+-ion-dependent and mannose-inhibitable manner. MBL binding to its ligands activates MBL-associated serine proteases which proteolytically cleave complement components C2 and C4 leading to the formation of the protease complex C4b2a that activates the complement cascade. The role of C4b, the major cleavage product of C4, was assessed here, by ELISA. Deposition took place in wells coated with Epi p 1 implicating that MBL binding to Epi p 1 leads to comple-
The byproducts of complement activation, C3a and C5a, lead to leukocyte activation, smooth muscle contraction, increased vascular permeability and contribute to the severity of inflammation [30].

In conclusion, the study demonstrates the presence of three lectins in *E. purpurascens* that contribute to sustained interaction with the host. Epip1 plays a major role in mediating host-fungal interactions in association with MBL.

**References**


