

Epi p 1, an allergenic glycoprotein of *Epicoccum purpurascens* is a serine protease

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Abstract

Epicoccum purpurascens (EP) is a ubiquitous saprophytic mould, the inhalant spores and mycelia of which are responsible for respiratory allergic disorders in 5–7% of population worldwide. The diagnosis/therapy of these disorders caused by fungi involves the use of standardized and purified fungal extracts. A 33.5 kDa glycoprotein, Epi p 1 released histamine from whole blood cells of EP allergic patients at a concentration of 50-ng protein. The high specific IgE values detected in EP hypersensitive sera indicated that Epi p 1 is capable of mediating type I hypersensitive reaction in predisposed individuals. It also showed protease activity by virtue of its dose dependent cleavage of serine protease specific synthetic substrate, N-benzoyl arginine ethyl ester hydrochloride (BAEE). The serine protease nature of Epi p 1 was confirmed by its N-terminal sequence (ADG/FIVAVELD/STY) homology to a subtilisin like serine protease. The protease activity of Epi p 1 may be responsible for making its way into the system of pre-disposed individuals through epithelial cell detachment and the histamine releasing ability by cross-linking of IgE antibodies on cell surface is the cause of its allergenic nature.

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Keywords: *Epicoccum purpurascens*; Allergen; Histamine; Serine protease

1. Introduction

Fungal spores are universal atmospheric components both indoors and outdoors and are important causative agents of respiratory allergies due to inhalation of spores and fine mycelial components [1–4]. The fungal extracts from spore, mycelial or both components together are the complex mixture of proteins, and glycoproteins. *Epicoccum purpurascens* (EP) is one of the predominant moulds belonging to the class Deuteromycetes is responsible for causing severe allergic disorders including hypersensitive pneumonitis and allergic

fungal sinusitis in 5–7% of different population worldwide [5–7]. The whole mass extracts used for diagnosis/therapy, contains many irrelevant materials in addition to proteins, which hamper diagnostic skin test results and may cause additional sensitization of patients [8]. Thus, purification of allergens from crude extracts is important. Also, the purified allergenic protein can be explored for its biological function such as enzymatic activity, which may be relevant for the induction of allergic disease [9]. The opening of tight junctions of lung epithelium by the allergen due to its protease activity has been suggested which in turn helps in the entrance of allergenic proteins into the system [10,11]. Allergenic proteins from fungi such as *Aspergillus*, *Penicillium* species, *Trichophyton*, basidiomycetes have been reported to possess protease activity [12–15]. This activity of proteins can be exploited to understand the correlation of allergenicity of a protein with its biological

Abbreviations: EP, *Epicoccum purpurascens*; ELISA, Enzyme linked immunosorbent assay; BAEE, N-benzoyl arginine ethyl ester hydrochloride; DEPC, Diethylpyrocarbonate.

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function and subsequently to devise new strategies to tackle fungal allergy in the patients of respiratory allergy [15]. In our earlier study, IgE binding proteins were identified in standardized spore-mycelial and culture filtrate extract of *Epicoccum nigrum* (now *purpurascens*) [7,16]. Also, a 33.5 kDa glycoprotein allergen Epi p 1 (as per IUIS allergen nomenclature subcommittee) was isolated from crude spore-mycelial extract. Both the carbohydrate and protein moieties of this protein were involved in IgE binding indicating their equal importance and it showed allergenic cross-reactivity with other fungi of clinical relevance [17]. In the present study, the biological function of the protein was analyzed. Epi p 1 was checked for its ability to cause in vitro histamine release as the measure of allergenicity, from whole blood cells of EP allergic patients and the presence of protease activity to reveal its biological nature.

2. Materials and methods

2.1. Purification of EP protein

Briefly, the 33.5 kDa glycoprotein allergen from EP, Epi p 1 was isolated from 13th day spore-mycelial extract by Concanavalin (Con) A Sepharose and Sephadex G-75 chromatography followed by electro-elution [17]. The protein obtained was lyophilized and its protein content estimated by dye binding assay from Bio-Rad using bovine serum albumin (BSA) as standard.

2.2. Histamine release assay

The assay was performed using histamine enzyme immunoassay as per manufacturer's instructions (Immunotech, France). Briefly, whole blood (heparinized) was collected from patients' skin test positive to EP. The consent of patients was obtained to carry out the assay. The total histamine content (basal level) was measured after cell lysis by freeze-thaw method. The whole blood cells were challenged for histamine release using varying concentration of Epi p 1 (1–50 ng). For positive controls, two concentrations (6 and 12 µg) of EP spore-mycelial extract were used for challenging blood cells. The histamine standards (0–100 nM) and released histamine in the supernatants were acylated and assayed by enzyme linked immunosorbent assay (ELISA). The cell challenge at a particular concentration of antigen is considered positive if it induces the release of 10–100% of total histamine.

2.3. Specific IgE estimation by ELISA

Briefly, Epi p 1 (0.1 µg/100 µl/well) and crude extract (1 µg/100 µl/well) were coated on the wells of the

ELISA plate. The ELISA plate was blocked with 3% BSA for 3 h at 37 °C. After washing the plate with PBST (0.1 M Phosphate buffer saline with 0.02% Tween 20), diluted serum from EP positive individual patients selected for histamine assay (1:5 v/v) was added and the plate was incubated overnight at 4 °C. The IgE binding was probed with anti-human IgE horse-radish peroxidase conjugate (1:1000 v/v; Sigma, USA). The color was developed with substrate orthophenylenediamine, hydrogen peroxide in citrate buffer and the absorbance was read at 490 nm on ELISA reader (Spectramax) after stopping the reaction with 5 N sulfuric acid [18]. Sera from healthy subjects were used as control.

2.4. Protease activity of purified protein

The protease activity of purified protein was analyzed qualitatively assessed by agarose plate assay. Briefly, 10 µl each of Epi p 1 and crude EP extract in phosphate buffer saline (PBS; 50 mM, pH 7.0) were incubated in the wells perforated on 1% agarose plate containing 0.1% of defatted milk protein, at 37 °C for 12–16 h. The plates were stained with 0.1% CBB. The lightly stained area around the wells indicated the protease activity of protein. Con A and PBS were used as negative controls.

The protease activity was quantitatively assessed by using azoalbumin, azocollagen (Sigma), casein (Hi Media) and defatted milk protein (Bio-Rad®) as substrate [19]. Briefly, 5 µg of purified protein was added to 1 ml of 1% substrate solutions in 0.01 M Tris-HCl buffer, pH 6.5 and incubated at 37 °C for 30 min. The reaction was stopped with 4% trichloroacetic acid (TCA). After brief centrifugation, absorbance was measured at 520 nm (azocoll) and 440 nm (azoalbumin). For other substrates, the soluble peptides remaining in the supernatant was measured using the Bradford dye binding assay (Bio-Rad®). One unit of protease activity, measured using any of the substrates is defined as an increase per 30 min in 0.001-absorbance unit in a 1 cm light path at indicated wavelength.

The optimum amount of protein required for protease activity was analyzed by incubating varying amounts of Epi p 1 with 1 ml of azoalbumin (10 mg/ml) at 37 °C for 30 min. To determine the optimum pH range for the protease activity, the purified protein was dissolved in Tris-HCl buffer (0.01 M) of pH ranging from 4 to 9 and the activity measured.

2.5. Effect of protease inhibitors

The effect of various protease inhibitors on the activity of the purified protein was analyzed using 11 different inhibitors: EDTA (0.5 mg/ml), PMSF (1 mM), phosphoramidon (330 µg/ml), pepabloc (1 mg/ml), aprotinin (2

$\mu\text{g/ml}$), pepstatin (0.7 $\mu\text{g/ml}$), leupeptin (5 g/ml), chymostatin (60 $\mu\text{g/ml}$), bestatin (40 $\mu\text{g/ml}$), antipain dihydrochloride (50 $\mu\text{g/ml}$) and E-64 (10 $\mu\text{g/ml}$) obtained from Boehringer Mannheim. Five micrograms of purified protein was incubated with different inhibitors for 30 min at 30 °C. The assays were performed using the substrate azoalbumin. An appropriate control without inhibitor was assayed simultaneously. The results were expressed as percentage inhibition of protease activity.

2.6. Protease activity using *N*-benzoyl arginine ethyl ester hydrochloride as substrate

To different concentrations (0.6–7.5 μg , 200 μl) of purified protein, Tris–HCl buffer (0.1 M, pH 7.0; 2.3 ml) and *N*-benzoyl arginine ethyl ester hydrochloride (BAEE) (0.06 M, 0.5 ml) were added and rapidly mixed. The rise in absorbance at 253 nm as the measure of velocity of enzymatic reaction was read every minute over a period of 5 min against a blank sample containing only the buffer and the substrate solution [20].

2.7. Inhibition of protease activity with diethyl pyrocarbonate

The purified protein was checked for the inhibition of its protease activity using different concentration of diethyl pyrocarbonate (DEPC) as inhibitor and azoalbumin (10 mg/ml) as substrate.

2.8. Cyanogen bromide cleavage of purified protein and amino acid sequencing

The purified protein (200 ng) in ammonium bicarbonate buffer (200 μl , 50 mM, pH 7.8) and dithiothreitol (100 mM) was flushed with nitrogen and incubated for 30 min in the dark. Two hundred microlitres of cyanogen bromide (CNBr) solution (2 mg in 0.2 N HCl) was added to the sample and incubated at 25 °C for 16 h. The cleaved protein was applied on HPLC column and the fractions were collected [21].

For N-terminal sequencing, the purified protein/peptide was transferred on polyvinylidene difluoride (PVDF) membrane using CAPS (3-{cyclohexylamino}-1-propane-sulfonic acid) buffer (10 mM) and subjected to amino acid sequencing using automated amino acid sequencer (Applied Biosystems) as per manufacturer's instructions.

3. Results and discussion

EP is one of the important fungi responsible for inducing respiratory allergy disorders. Its spore-mycelial extract is used for allergy diagnosis and immunotherapy in allergy clinics all over the world [2,7,22]. A 33.5 kDa

major glycoprotein allergen from the spore-mycelial extract of this fungus was isolated previously [17]. In the present study, it was further characterized for its biological function.

3.1. *In vitro* histamine release by Epi p 1

The induction of histamine release with Epi p 1 confirmed its allergenic nature. Histamine is known to be an important mediator for the allergic response in skin prick test and is closely related to the affinity of IgE antibody to antigen [23]. In the present study, 50 ng of Epi p 1 induced the release of histamine in 5 out of 8 patients (allergic to EP) tested. Three patients did not show histamine release (less than 10%) with the purified protein (Fig. 1). The fact that these patients were reportedly on the immunotherapy regime and were being administered specific doses of standardized EP spore-mycelial extract may be the reason for no release of histamine in response to Epi p 1 challenge. Immunotherapy/allergen specific therapy aims at the prophylaxis of atopy through gradual induction of tolerance or modification of immune response by switching IgE antibody production to blocking IgG antibody production. This prevents further cross-linking of IgE and subsequent histamine release [1]. The crude EP protein also induced histamine release in 5 out of 8 patients tested (Fig. 1). The specific IgE values were high in all the patients tested as compared to healthy controls

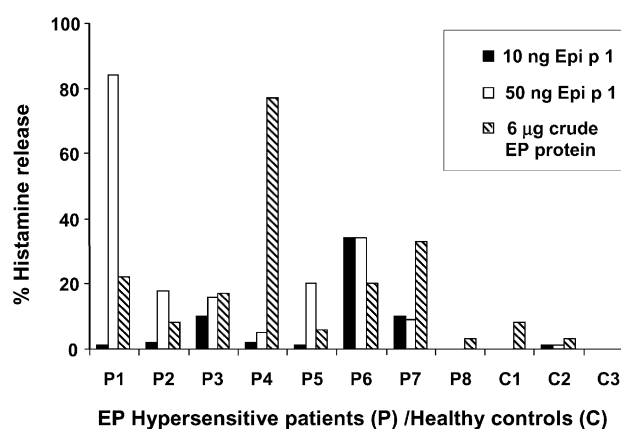


Fig. 1. Percentage histamine release (Y-axis) by whole blood cells of EP allergic patients/normal subjects in response to Epi p 1 (10 and 50 ng) and crude EP protein (6 μg). The heparinized whole blood cells of patients and controls were challenged with different concentration of purified and crude EP antigen for the spontaneous release of histamine. The spontaneous and total histamine released was quantitated by ELISA using monoclonal anti histamine as solid phase and histamine alkaline phosphatase conjugate as the secondary antibody. The color was developed using *p*-nitrophenyl phosphate in diethanolamine-HCl solution. The spontaneous histamine release was calculated as percentage of total histamine released by the blood cells.

Table 1

EP specific IgE values (absorbance, OD at 490 nm) in the sera of EP hypersensitive patients using purified Epi p 1 or whole EP extract as coating antigen in ELISA

Patients (P)/healthy controls (C)	Crude EP specific IgE value (absorbance, OD at 490 nm)	Epi p 1 specific IgE value (absorbance, OD at 490 nm)
P1	0.918	0.75
P2	1.032	1.011
P3	0.684	0.6
P4	1.125	0.738
P5	0.9	0.864
P6	1.188	1.023
P7	1.176	0.717
P8	0.912	0.717
C1	0.031	0.019
C2	0.053	0.052
C3	0.079	0.039

(Table 1). The high specific IgE values in patients on immunotherapy indicated that the EP allergen binds IgE (as detected in ELISA) but could not cross link them in vivo on the surface of antigen presenting cells (preferably mast cells/basophils) to trigger histamine release due to the presence of competing IgG antibody. Earlier, Verma et al. [24] reported the release of histamine when the *Fusarium* allergic patients blood cells were chal-

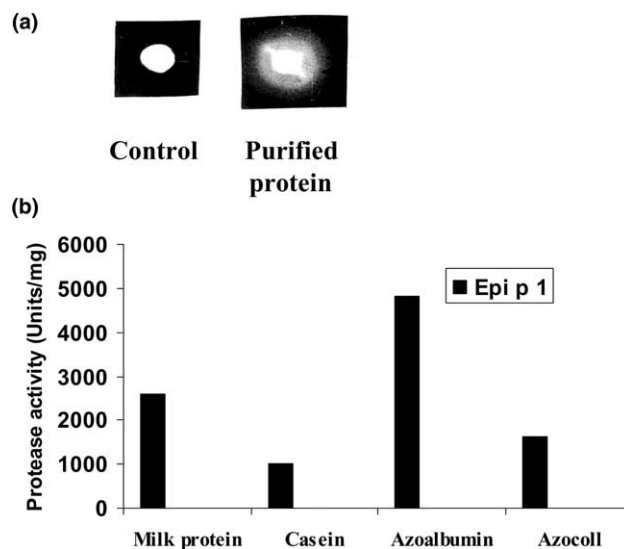


Fig. 2. Protease activity of Epi p 1. (a) Qualitative analysis of protease activity of purified protein, Epi p 1 (5 μ g) on 1% agarose containing 0.1% milk protein showing lightly stained areas around the wells. C – control, Con A. (b) Quantitative analysis of protease activity of Epi p 1 (5 μ g) using azocollagen, azoalbumin, casein and milk protein as substrates. The protein was incubated in different substrate solutions for 30 min at 37 °C. The uncleaved protein substrate was precipitated with 4% TCA and absorbance (440 nm for azoalbumin; 520 nm for azocollagen and dye binding assay for casein and milk protein) of cleaved soluble peptides remaining in the solution was measured with respect to substrate blank.

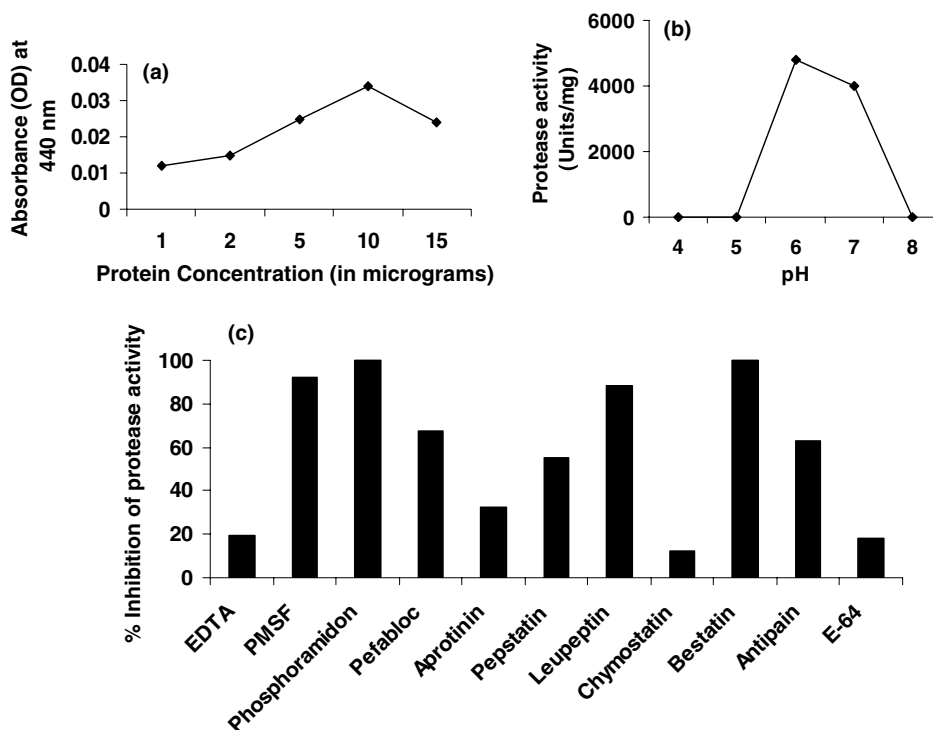


Fig. 3. Protease activity of Epi p 1 using azoalbumin as substrate. (a) Effect of different concentrations of Epi p 1 on protease activity. (b) Effect of different pH (4–9) buffer on protease activity of Epi p 1. (c) Percentage inhibition of protease activity of Epi p 1 using 11 different protease inhibitors.

lenged with 65 kDa purified protein isolated from *F. solani* culture filtrate extract.

3.2. Protease activity of the purified protein

The protease activity of purified protein was analyzed. In a qualitative agarose plate assay, both the crude EP extract and Epi p 1 showed protease activity that appeared as lightly stained area around the wells (Fig. 2(a)). Quantitatively, the protease activity was higher with azoalbumin than with the other substrates used (Fig. 2(b)). Epi p 1 showed dose dependent increase in protease activity, which was maximum with 10 μ g of protein (Fig. 3(a)). The further decrease in the protease activity at higher protein concentration may be due to the feedback inhibition during the reaction. The pH optima required for protease activity was observed to be 6.5 using azoalbumin as substrate (Fig. 3(b)). The inhibition of protease activity of this protein was checked with 11 different protease inhibitors. As evident in Fig. 3(c), >80% inhibition of protease activity was observed with PMSF, Phosphoramidon, Leupeptin and Bestatin, indicating that it may be a serine protease. This was confirmed by performing further experiment with a serine protease specific synthetic substrate, BAEE. Dose dependent hydrolysis of BAEE by purified protein (Epi p 1) was

seen, suggesting that the protein has a similar catalytic site as in other serine proteases (Fig. 4(a)). Also, an initial increase followed by a substantial decrease in the reaction speed (as depicted by rise in absorbance at 5th minute) at Epi p 1 concentration of more than 4 μ g that get almost equilibrated at higher concentrations was observed (Fig. 4(a)). This might be due to feedback inhibition during the reaction at a higher enzyme concentration commonly observed in biological reactions. Further, the effect of DEPC, a histidine-modifying agent in the disruption of catalytic triad of serine proteases was observed by treating Epi p 1 with varying concentrations of DEPC as inhibitor. Epi p 1 showed dose dependent inhibition of its protease activity with DEPC, confirming it to be a serine protease (Fig. 4(b)). Other fungal extracts have also been shown to have serine protease activity. The purified 33–34 kDa serine protease allergen of *Aspergillus fumigatus*, *A. flavus*, *Penicillium chrysogenum* showed inhibition of protease activity with DEPC [14,15,25,26]. EP like *A. fumigatus* is also an allergen responsible for causing hypersensitive pneumonitis and allergic fungal sinusitis in pre-disposed population. The protease nature of Epi p 1 isolated from surface cultures of EP may thus be important factor in making its way into the human system through nasal epithelium and thus causing pathogenesis. Previous studies have reported that the proteases present in fungal extracts not only overcome airway tolerance and elicit allergic lung diseases, but also interact with epithelial cells leading to morphological changes and induction of pro-inflammatory cytokines [11,14,18,27–29].

3.3. Amino acid sequencing and homology with other proteins

The N-terminal amino acid sequence of Epi p 1 was ADG/FIVAVELD/STY (Accession # P83340). The sequence showed homology to N-terminal sequence of subtilisin like serine protease from *Pneumocystis carinii* sp. *ratti*, Accession # AAD39924 using BLAST Search homology program and Swiss Prot database. Out of 4 peptides obtained by CNBr cleavage, one peptide showed high IgE binding by ELISA using pooled sera from EP hypersensitive patients (data not shown). The amino acid sequence of this peptide, RGN/SFXK/L also showed homology in the internal sequence of subtilisin like cell surface serine protease from *Pneumocystis carinii* and chitinase 2 precursor from *Candida albicans*, Accession # P40953. Earlier, a novel subtilisin related serine protease from cell wall fraction of *A. fumigatus* is reported [30]. N-terminal amino acid residues of Epi p 1 also showed significant homology with the glycoprotein Con A from various plant lectins. The Con A like glycoproteins from yeast have been re-

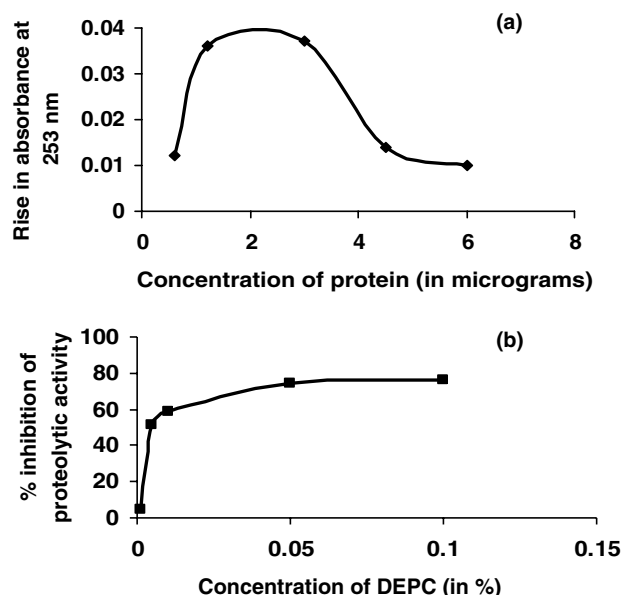


Fig. 4. Protease activity of Epi p 1 (a) Protease activity using serine protease specific synthetic substrate, BAEE. Varying concentrations of purified protein in Tris-HCl buffer (pH 7.0) were rapidly mixed with the substrate (0.06 M) and the rise in absorbance due to products formed was monitored at 253 nm. (b) Dose dependent inhibition of protease activity of Epi p 1 using DEPC (0.001–0.1%) as inhibitor.

ported to bind IgE and play important role in expression of IgE mediated allergic responses. However the purified protein is not Con A as it showed enzymatic properties. Epi p 1 digested milk protein while Con A did not. N-terminal and internal peptide sequences obtained after CNBr cleavage of Epi p 1 also showed homology to subtilisin like serine protease from *Pneumocystis carinii* sp. *ratti*. However, there was no N-terminal sequence identity with 33–34 kDa serine proteases from *Aspergillus* and *Penicillium* species.

To summarize, this is the first report demonstrating the protease activity of Epi p 1 isolated from EP. Epi p 1 also induced histamine release in EP allergic patients. The enzymatic activity of this 33.5-kDa allergenic glycoprotein plays a role in gaining entrance into the system of predisposed individuals and cause pathogenesis of allergy related disorders. N-terminal homology of Epi p 1 with serine protease may thus pave way for further research on this allergenic mould towards cDNA cloning to deduce its complete amino acid sequence and study the allergen-epithelial cell interaction studies in vivo.

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