Biochimica et Biophysica Acta 1864 (2016) 869–879

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Mass spectrometry-based identification of allergens from *Curvularia pallescens*, a prevalent aerospore in India

Debarati Dey, Bodhisattwa Saha, Gaurab Sircar, Kavita Ghosal, Swati Gupta Bhattacharya *

Division of Plant Biology, Bose Institute, 93/1, Acharya Prafulla Chandra Road, Kolkata-700009, West Bengal, India

ARTICLE INFO

Article history:
Received 2 December 2015
Received in revised form 26 February 2016
Accepted 17 March 2016
Available online 19 March 2016

Keywords:
Allergen
Curvularia pallescens
Immunoblot
MALDI TOF/TOF
Fungal allergic patients

ABSTRACT

The worldwide prevalence of fungal allergy in recent years has augmented mining allergens from yet unexplored ones. *Curvularia pallescens* (*CP*) being a dominant aerospore in India and a major sensitiser on a wide range of allergic population, pose a serious threat to human health. Therefore, we aimed to identify novel allergens from *CP* in our present study. A cohort of 22 *CP*-sensitised patients was selected by positive Skin prick grade. Individual sera exhibited elevated specific IgE level and significant histamine release on a challenge with antigenic extract of *CP*. First gel-based profiling of *CP* proteome was done by 1- and 2-dimensional gel. Parallel 1- and 2-dimensional immunoblot were performed applying individual as well as pooled patient sera. Identification of the sero-reactive spots from the 2-dimensional gel was found to be challenging as *CP* was not previously sequenced. Hence, mass spectrometry-based proteomic workflow consisting of conventional database search was not alone sufficient. Therefore, *de novo* sequencing preceded homology search was implemented for further identification. Altogether 11 allergenic proteins including Brn-1, vacuolar protease, and fructose-bis-phosphate aldolase were identified with high statistical confidence ($p < 0.05$). This is the first study to report on any allergens from *CP*. This kind of proteome-based analysis provided a catalogue of *CP* allergens that would lead an improved way of diagnosis and therapy of *CP*-related allergy.

1. Introduction

About 20% of the global population is found to be affected by IgE-mediated hypersensitivity induced by allergen exposure [1]. Both indoor and outdoor fungal spores elicit different respiratory troubles [2]. The World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee (www.allergen.org) has officially recognised 753 allergens till now. The WHO/IUIS allergen nomenclature database (IUIS; www.allergen.org) has been recently updated a list of 109 fungal allergens including 86 Ascomycota allergens and 23 allergens from Basidiomycota originated from 28 fungal species [3,4].

A substantial number of proteins of *Aspergillus* (Asp f 13, Asp f 18, Asp f 34, Asp n 18, Asp fl 18, Asp o 13) and *Penicillium* (Pen c 13, Pen b 13, Pen ch 13, Pen n 13, Pen n 18, Pen o 18) were identified as major allergens [5–8]. Both major and minor allergens have been accounted from different species of *Candida* [9], *Cladosporium* [10], *Rhizopus* [11], *Alternaria* [12], and *Epicocccum* [13].

In the class Ascomycetes, genus *Curvularia* represents one of the reported airborne fungal allergens worldwide, which affects up to 10–16% of patients with nasobronchial allergy, viz. allergic rhinitis and asthma [14,15]. There are few reports from America [16] and Asian countries [17] demonstrating 18–28% prevalence of *Curvularia* sensitisation. From different parts of eastern India, *Curvularia* spores were found to be present in the air throughout the year [14,18], and *Curvularia lunata* and *Curvularia pallescens* (*CP*) were reported as the most ubiquitous species [14,19]. Though allergens like glyceraldehyde 3-phosphate dehydrogenase [20], serine protease [21] and enolase [22] were identified from *C. lunata*, none has been stated so far for *CP*.

In the present study, we have conducted a two-year aerobiological sampling in Kolkata, a megacity of India to unearth dominant aerospores in the outdoor environment. *CP* was found to be one of the prevalent fungal spores, antigenic extract of which showed high allergenic reactivity amongst the local populace. Till now, to the best of our knowledge, no information is available regarding its allergenicity and its allergens. Hence, we made a first time effort to identify allergens from *CP* through a combinatorial approach consisting of clinical, immunobiochemical and mass spectrometry-based proteomic approaches. Our investigation was initiated with Skin Prick Test (SPT) screening of patients having doctor-diagnosed atopic symptoms using *CP* fungal extract, and further confirmation of *CP* sensitivity was performed with in vitro immunological tests. Immunoproteomic investigations involved with protein profiling of spore mycelia by SDS-PAGE and

* Corresponding author.
E-mail addresses: debaratidey03@gmail.com, debaratidey@jcbose.ac.in (D. Dey), bodhisattwasaha@gmail.com (B. Saha), sircar.gaurab129@gmail.com (G. Sircar), kavita.ghosal@gmail.com (K. Ghosal), swati@jcbose.ac.in, swatigb2929@yahoo.com (S.G. Bhattacharya).

http://dx.doi.org/10.1016/j.bbapap.2016.03.012
1570-9639/

2D-PAGE, followed by corresponding immunoblots to detect IgE-reactive proteins and IgE-binding proteins were identified by mass spectrometry. As the conventional database search precludes identification of peptides not present in the database, *de novo* sequencing combined with homology-driven proteomics became essential to identify proteins from *CP* whose genomes are still not known.

2. Material and methods

2.1. Aerobiological and clinical study

Detailed two-year monitoring (August 2012 to July 2014) of airborne fungal spores has been executed using a Burkard volumetric 7-day sampler (Burkard Manufacturing Co. Ltd., Hertfordshire, UK) in Kolkata, India. Trapped spores were microscopically scanned by a high resolution light microscope (Leitz, Diaplan, Germany) at 400× and counted according to the British Aerobiology Federation guidelines (1995). Several keys, atlases and pictures [23–26] along with their appearance (colour, shape) and morphology (septa) were consulted for spore identification.

Demographic and clinical data of patients who visited a local allergy clinic with seasonal/perennial allergic symptoms were collected retrospectively and analyzed.

2.2. Study subjects

A cohort of 6 non-atopic individuals and 22 atopic individuals are considered as study subjects.

Inclusion criteria — 18–50 years of age; a history of bronchial asthma, allergic rhinitis, urticaria and eye problems (such as angioedema or conjunctivitis) either alone or in different combination; SPT grade >+1 for *CP* extract, total IgE value > 100 International Units, histamine release more than 100 nM and informed consent prior to inclusion, approval from ethical committee of corresponding hospital.

Exclusion criteria — severe asthma, pregnancy/lactation, malignancy or other severe systemic diseases during skin testing. Corticosteroids and antihistamines were prohibited to avoid masking of severe symptoms. Smokers were excluded.

2.3. Preparation of viable fungal culture and antigenic extract of CP

Airborne fungal spores were trapped by an Andersen volumetric air sampler, and identification was verified by Agharkar Research Institute, Pune, India. Then fungi were grown on a Potato Dextrose Agar (PDA) plate followed by subculturing in PD Broth (Himedia, India). 1 g lyophilized spore mycelial mat (14 days old) was crushed in liquid nitrogen and soaked in 5 ml 0.1 M phosphate buffer (pH 7.2) with constant shaking overnight. After centrifugation, the clear extract obtained was filtered through supernatant through a 0.22 μm membrane (Millipore) and used for SPT after the estimation of protein concentration by using Bradford reagent (Bio-Rad Laboratories). The extract was also passed through Sephadex G 25 to remove fungal pigments and other non-protein contaminants that may interfere during skin testing.

2.4. Patient selection by SPT and sera collection

SPTs were performed with 20 μl of crude antigenic extract (1:10 w/v) placed on the ventral side of the arms and pricked with a sterile lancet [27]. The wheal response was measured after 20 min and graded [28]. Histamine diphosphate (1 mg/ml) and phosphate buffer (0.01 M, pH 7.2) was used as positive and negative controls respectively.

Blood (5 ml) was collected from each of the 22 *CP*-sensitive patients; serum separated and stored at −20 °C. Sera from six non-atopic individuals were also collected to be used as negative control.

2.5. Total IgE and specific IgE estimation

Total IgE in the serum was estimated using 'PATHOZYME® Total IgE Estimation kit.' Indirect ELISA was utilized to determine specific IgE levels in *CP* susceptible patients according to the protocol described [29,30]. 100 ng/μl of crude antigen extract (50 μl/well) was used for coating ELISA plates (Nunc), individual patient sera (1:5) used as primary antibody, enzyme-labelled (alkaline phosphatase) monoclonal anti-human IgE (1:1000)–alkaline phosphatase conjugate produced in mouse as secondary antibody and para-nitro phenyl phosphate (pNPP) (Sigma, St Louis, Mo) as liquid substrate. OD was taken at 405 nm in an ELISA reader (Multiscan-Labsystem, Finland). Tests were performed in triplicate.

CP-specific IgE level in each patient was represented as P (OD_{405} of patient)/N (OD_{405} of normal subject) ratio. In our study P/N ratio ≥2.5 was considered as a significantly elevated level of IgE.

2.6. Stripped basophil histamine release assay

Induced histamine release was performed as previously described [31]. Briefly, the peripheral blood was drawn from non-allergic donors and mixed (1: 5 v/v) with 6% dextran in saline containing 0.01 M EDTA and 2% dextrose. After incubation, the leukocyte-rich upper layer was drawn, centrifuged and washed twice with ice cold phosphate buffer saline. The basophils in suspension were stripped off bound IgE by incubation with lactic acid buffer (13.4 mM lactate, 140 mM NaCl, and 5 mM KCl at pH 3.5) for 3 min. Following incubation, cells were washed in HEPES buffer (pH 7.5). Subsequently, the cells were resensitised (with 150 μl sera with elevated IgE against *CP* allergen plus 4 mM EDTA and 20 mM sodium citrate) and after passive sensitisation, those were stimulated with 150 ng of protein in 50 μl of *CP* extract in HEPES buffer containing 1 mM CaCl2. After 1-h incubation, the reaction was stopped by the addition of ice-cold 0.9% NaCl (w/v). Following centrifugation, the cell-free supernatant containing histamine was estimated according to the manufacturer's instructions using a kit (Immunotech, France). Histamine release was calculated in nM from the dose response curve with standards. Assays were performed in triplicate.

2.7. Protein extraction, solubilisation, and quantification for proteomic study

2 g of lyophilized spore-mycelial mat of *CP* was crushed in liquid N2 and mixed with 1 ml of extraction buffer containing 0.5 M Tris–HCl (pH 8), 2% (v/v) Nonidet P-40, 20 mM MgCl2, 2% (v/v) β-mercaptoethanol, 700 mM sucrose and 1 mM PMSF and was incubated for 1 h at 4 °C with continuous shaking. After centrifugation the supernatant was mixed with equal volume of Tris–HCl (pH 8) buffered phenol. The mixture was homogenized for 30 min at 4 °C and finally centrifuged. The proteins in the upper phenol phase were precipitated with five volumes of 0.1 M ammonium acetate in methanol overnight at −20 °C and pelleted by centrifugation [32]. After repeated washing, the final pellet was dissolved in 100 μl of rehydration (IEF) buffer (without DTT and ampholytes) containing 7 M urea, 2 M thiourea and 4% CHAPS and stored at −80 °C. Protein content was estimated as mentioned previously.

2.8. SDS-PAGE and 2D gel electrophoresis

Total protein was resolved in 12% SDS-PAGE, and protein bands were observed by CBB (Coomassie Brilliant Blue)-R250 staining. For 2DE protein was processed through a Focus Perfect 2D Clean up Kit (G Biosciences, USA) and reconstituted freshly in 125 μl IEF buffer. 1% immobilized pH gradient (IPG) pH 4–7 linear buffer (v/v) (GE Healthcare, Uppsala, Sweden), 25 mM DTT and traces of Bromophenol blue were added. Rehydration loading was done with 7 cm Immobiline