Chapter III

IDENTIFICATION OF MAJOR ALLERGENS OF
Pennisetum typhoides
WHOLE POLLEN EXTRACT
Chapter III  Identification of major allergenic components of *Pennisetum typhoides* pollen

**INTRODUCTION**

Being an important cash crop, *Pennisetum typhoides* (Pearl millet) is cultivated widely in the vast arid and semi-arid areas of Indian sub-continent and elsewhere [9,32]. It is also found growing wildly in the region in and around Delhi [28]. Its peak flowering season varies from June to November depending upon the area of cultivation. It produces large amount of wind borne inhalable pollen grains. Human allergy to *Pennisetum typhoides* pollen is known since 1971 [26]. Since then, aerobiological and clinical studies have implicated its role in triggering immediate type allergic reactions amongst pre-disposed human population. Taking this into consideration, the pollen extract of *Pennisetum typhoides* has been an important component of the grass pollen preparations used for the immunodiagnosis and immunotherapy of type I allergies, although nothing is known about its relevant allergens [30].

Immunotherapy of type I allergic diseases is done with preparations based on crude allergen extracts which are known to be highly heterogeneous in nature as they contain a number of allergenic and non-allergenic components [13,19]. On SDS-PAGE, upto 44 different proteins/glycoproteins from grass pollen extract have been seen. On the other hand, IEF resolved grass pollen extract into 50-60 different proteins/glycoproteins. However, as high as 70 different components have been reported by the combination of IEF and SDS-PAGE (i.e. two dimensional gel electrophoresis) [6,10-11]. It has, however, been demonstrated that of these proteins, only a few are of allergenic importance [13-19]. Immunotherapy with crude extracts carrying large number of non-relevant components often leads to failure of allergen immunotherapy mainly because the allergenic components are less in number and may not be present in the extract in sufficient amount. Possible induction of sensitization of patients, to non-allergenic components to which patients were not sensitive originally, has been put up as one of the reasons of controversy over the use of crude extracts for immunotherapy [1-3,34]. These observations have prompted several studies on the identification of relevant allergens in crude extracts of various grass pollens [6,8,11,24].

Initially, a variety of immunochemical methods such as CIE, CRIE etc. were used for the identification of allergens of clinical importance. However, these methods
were later replaced by immunoblotting technique as earlier methods did not produce a complete profile of allergenic components and were generally found to be time consuming and labour intensive. Immunoblot, on the other hand, proved highly successful in the rapid analysis of complete spectrum of allergenic components from crude extracts and this technique was also found to be more sensitive than previously used methods [33]. So far, a number of allergens from various grass pollen extracts have been identified [6,8,11,24]. Depending upon their physico- and immunochemical properties, these allergens were placed in various groups [37]. Classification as ‘major’ and ‘minor’ allergen has also been proposed for these allergens, based on their frequency of recognition by IgE antibodies from individual patients’ serum samples [15-16,37].

In contrast to the pollen allergens of other grass species, little work has been carried out on the pollen extract of *Pennisetum typhoides*. Recently, Sridhara et al (1995) compared the allergenicity of pollen extract of *Pennisetum typhoides* with that of 4 other grass pollen extracts [30]. The principle aim of the present study is to provide a complete and accurate spectrum of the allergenic components present in pollen extract of *Pennisetum typhoides*. In order to realize this objective, allergen extract of *Pennisetum typhoides* was studied with 26 individual patients’ sera by immunoblotting technique.

**MATERIAL AND METHODS**

**Preparation of whole pollen extract of *Pennisetum typhoides***

Pollen with ≥ 98% pollen and ≤ 2% other floral parts of the same species were defatted with peroxide free diethyl ether. The extract (1:20 w/v) was prepared in 0.05 M ammonium bicarbonate buffer (pH 8.0), by continuous stirring for 18 hours on a magnetic stirrer at 4°C. The extract was centrifuged at 10,000 rpm for 30 minutes at 4°C and the supernatant was brought to 90% saturation with ammonium sulfate. The precipitate was dissolved in minimum volume of distilled water and dialyzed extensively against distilled water at 4°C by using Spectrapore™ dialysis membrane (MW cut off 3000). After passing through 0.22 μm filter (Millipore™), the dialyzed extract was lyophilized in aliquots and stored at -20°C. Protein content in the pollen extract was determined by modified method of Lowry et al. [17] using
Identification of major allergenic components of *Pennisetum typhoides* pollen

Phosphotungstic (PTA) reagent (15% PTA in 10% HCl) was used to precipitate the proteins. Bovine serum albumin (BSA) was used as a standard.

**Collection of *Pennisetum typhoides* sensitive patients sera**

Serum samples collected from 26 allergic patients (material and methods of Chapter II) who were found to elicit strong positive reaction in intradermal test (2+ or more) and ELISA (5 times or more the OD observed with NHS) to allergen extract of *Pennisetum typhoides* pollen were selected for the present study. Pooled NHS was also included as a negative control.

**Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE of pollen extract of *Pennisetum typhoides* was carried out on 12% acrylamide gel using the discontinuous buffer system as described in chapter II. Lyophilized extract of *Pennisetum typhoides* was reconstituted in distilled water and 50 μg of it was reduced by mixing equal volume of 2X sample buffer followed by boiling for five minutes in a water bath. Digested samples were then loaded in respective lanes and electrophoresis was carried out at 20 mA in electrode buffer (0.025 M Tris, 0.38 M Glycine and 1% w/v SDS) for 60 minutes. After the completion of run, gel was fixed for 1 hour in fixing solution [methanol: acetic acid: water, 45: 45: 10; (v/v), respectively] and stained for one hour in 0.1% Coomassie Brilliant Blue R-250 (CBB) prepared in destaining solution containing ethanol: acetic acid: water, 45: 10: 45; (v/v). Excess of stain was removed by washing the gel in destaining solution and the gel was preserved in 0.7% acetic acid solution.

**Specific IgE immunoblot analysis**

*Pennisetum typhoides* pollen extract was separated on 12% SDS-PAGE and electrophoretically transferred on to nitrocellulose membrane as described earlier [34]. Free sites on nitrocellulose membrane were blocked with 2% gelatin (prepared in PBS) solution. After washing three times for five minutes each with PBST, the membrane was cut into strips with single individual lane of separated pollen proteins and these individual strips were then incubated with 1:10 (v/v) diluted individual patient’s serum overnight at 4°C with gentle shaking. The nitrocellulose strips were then washed with PBST and again incubated overnight at 4°C with 1:500 (v/v) diluted rabbit anti-human IgE.HRP conjugate (Sigma, USA). After washing the strips
with PBST, the allergenic proteins were detected by keeping them in a substrate solution (12 mg diaminobenzidine and 15 µl 30% (v/v) H₂O₂ in 50 ml sodium acetate buffer, pH 5.0) for few minutes. The colour reaction was stopped by rinsing the strips several times in distilled water.

RESULTS

Allergen extract of *Pennisetum typhoides* resolved into 24-26 CBB stained bands in the molecular weight range of 10-100 kDa on 12 % SDS-PAGE (Fig 1). Upon electrophoretic transfer on to nitrocellulose membrane, probing of allergenic bands was carried out with 26 individual patients serum samples. Out of the 22-26 SDS-PAGE separated bands, only 12 (in the molecular weight range of 14-85 kDa) were found to be allergenic and could bind with specific IgE in the patient sera. Amongst high molecular weight allergens, maximum frequency of IgE binding (96.15% of 26 individual patients sera) was observed with 85 and 70 kDa proteins, followed by 80 and 57 kDa protein bands (88.46% of 26 individual patients sera). Allergenic bands with molecular weights 85 and 80 kDa were recognized by specific IgE antibodies from 96.15% and 88.46% of 26 individual patients serum samples, respectively (fig. 2A and 2B). Among other allergenic bands, 43 kDa protein was recognized by specific IgE antibodies from 96.15% of 26 patient sera tested. Upto 80.76 % of patient sera recognized a protein band with molecular weight 50 kDa as allergenic. On the other hand, 76.92% of patients sera showed IgE antibody binding to 40 and 34 kDa protein bands. Two more allergens with molecular weights 30 and 14 kDa were detected by 34.61% and 19.23% of 26 patients sera, respectively. Figure 3 summarizes the frequencies of IgE antibody recognition for each of the individual allergenic components from the allergen extract of *Pennisetum typhoides* pollen. The IgE binding patterns of individual patients (Table 1) emphasizes the predominance of 85, 70 and 43 kDa proteins, in terms of their allergenicity. It also illustrates the heterogeneity of IgE binding patterns shown by different serum samples. For example, serum from patient numbers 7 and 13 recognized 11 out of 12 allergenic proteins whereas, serum from patient number 8 reacted to only one allergen. Except for the allergenic bands with molecular weights of 14, 18, 25 and 30 kDa, all other allergenic protein bands were recognized by more than 50% of the 26 individual patients sera tested.
FIGURE 1:

DS-PAGE (12%) separated profile of whole pollen extract of *Pennisetum typhoides.*

FIGURE 2A:
Identification of IgE binding components in whole pollen extract of *Pennisetum typhoides* pollen by immunoblot with 26 individual patient serum samples. [Lane 1] Ponceau ‘S’ stained molecular weight marker proteins; [Lane 2] Ponceau ‘S’ stained western blotted profile of whole pollen extract of *Pennisetum typhoides*; [Lanes 3-15] thirteen individual patient’s serum samples (no. 1-13); [Lane 16] Normal human serum.
FIGURE 2B:
Identification of IgE binding components in whole pollen extract of *Pennisetum typhoides* pollen by immunoblot with 26 individual patient serum samples.
[Lane 1] Ponceau ‘S’ stained molecular weight marker proteins; [Lanes 2-14] thirteen individual patient’s serum samples (no. 14-26); [Lane 15] Ponceau ‘S’ stained western blotted profile of whole pollen extract of *Pennisetum typhoides*; [Lane 16] Normal human serum.
Figure 3:
Frequency histogram of IgE antibodies (from 26 individual patient serum samples) binding to each of the SDS-PAGE separated allergenic proteins of *Pennisetum typhoides* pollen extract.
**TABLE 1:**

Patients' sera (n=26) demonstrating specific IgE binding to each of the allergenic proteins on immunoblot, obtained after transfer of SDS-PAGE separated *Pennisetum typhoides* pollen extract.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
<td>kDa</td>
</tr>
<tr>
<td>Allergen No.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>--------------</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Presence of specific IgE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

○ presence of specific IgE
Chapter III Identification of major allergenic components of *Pennisetum typhoides* pollen

DISCUSSION

In case of pollen allergens, the IgE binding epitopes do not seem to be affected by the denaturation and unfolding of the protein. Therefore, immunoblot after SDS-PAGE has been used extensively for the identification of allergenic proteins from several grass pollen extracts [33]. In the present investigation, the same technique has been applied to crude allergen extract of *Pennisetum typhoides* pollen for the identification of relevant allergens. Of the 24-26 CBB stained protein bands, separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membrane, 12 bands (molecular weight range 14-85 kDa) bound IgE antibodies from the individual serum of 26 sensitive patients. This number and the molecular weight distribution of the IgE binding components, is somewhat similar to that observed with other pollen extracts studied so far with similar technique. Pollen of rye grass (*Lolium perenne*) contained fourteen IgE binding proteins (molecular weight range 14-80 kDa). *Parietaria judaica* had nine (molecular weight range 14-80 kDa) and Orchard grass (*Dactylis glomerata*) had 13 (molecular weight range 14-70 kDa) allergenic proteins [5,7-8]. Similarly, Loria et al. (1989) identified 23 allergenic components from the pollen extract of White oak (*Quercus alba*) [14]. From Bermuda grass (*Cynodon dactylon*), Ford and Baldo (1987) reported 17 (molecular weight range 8-94 kDa) allergenic components[6], whereas Shen et al. (1988) found 14 allergenic proteins in the same allergenic pollen extract [25]. Suphioglu et al. (1993) demonstrated the presence of 17 allergenic proteins (molecular weight range 10-100 kDa) in the allergen extract of Canary grass (*Phalaris aquatica*) [31]. It seems, in each kind of allergen extract there are many allergenic proteins differing both in molecular weight and structural complexity. Different patients may develop different IgE responses to the various allergenic proteins.

At least eight out of 12 allergenic proteins from the allergen extract of *Pennisetum typhoides* pollen have been identified as 'major' allergens, since these were recognized by IgE antibodies from more than 50% of 26 individual patients sera (Fig. 3). Allergens with molecular weight 30, 25, 18 and 14 kDa were identified by less than 50% of these individual patients sera therefore, these allergens were classified as 'minor' allergens [21,37]. Allergens of *Pennisetum typhoides* pollen, by
Chapter III
Identification of major allergenic components of *Pennisetum typhoides* pollen

Virtue of their molecular weights, indicates their affiliation to at least four well known grass pollen allergen groups. A 34 kDa major allergen may be a member of group I allergen family which has a broad molecular weight range (28-34 kDa) due to the presence of isoallergenic forms [12, 20-21]. Lol p 1, Phl p 1, Poa p 1, Dac g 1, Cyn d 1, Pha a 1 and Hol l 1 are some of the well known group I allergens identified in various grass pollen extracts [23]. These are known to be the major allergenic components in terms of both, their relative content in the pollen extract and their frequency of IgE recognition in the patients serum samples tested. A great deal of similarity at the level of antibody recognition and N-terminal amino-acid/cDNA sequence has been reported among various group I allergens [23-24]. Similarly, group V allergens have been identified in different grass pollens and 30 kDa allergic protein of *Pennisetum typhoides* pollen possibly represents this particular group of grass pollen allergens. However, in this study, this allergen has been identified as a ‘minor’ allergen in contrast to other reports from timothy rye and Kentucky blue grass pollens where it has been described as a ‘major’ allergen [23-24].

Another allergen of *Pennisetum typhoides* pollen extract with molecular weight 14 kDa could represent pan-allergen profilin (group XI) family. Only 19.23% of 26 individual patients sera reacted to this allergen on immunoblot (Fig. 3). This is particularly true for the profilins since they are present in almost all living systems (thus ubiquitous) and are generally reported as minor allergen from a number of allergen sources including grass pollens [23, 36]. Group IV allergen [23, 36] is probably represented by a 57 kDa allergic protein of *Pennisetum typhoides* pollen, which was recognized by 24 out of 26 different patients sera. These group representations however, need to be confirmed either by the use of monoclonal antibodies and/or N-terminal and cDNA sequence homology studies.

Major allergenic proteins of *Pennisetum typhoides* pollen have molecular weight 85, 80, 70, 57, 50, 43, 40 and 34 kDa. From another member (*Cynodon dactylon*) of the same sub-family (i.e. Panicoideae), Shen et al. (1988) described allergen with similar molecular weights (88, 78, 75, 46, 40, 35, etc.) from the pollen extract of *Cynodon dactylon* [25]. At the same time, Ford et al. (1987) reported allergens with molecular weight like 68, 60, 50, 46, 43, 34, 30, 25 etc. [6] from the
same allergen source. Allergens with molecular weight 70, 43, 40, 30 kDa are also reported from the allergen extract of *Dactylis glomerata* pollen [8]. Existence of slight molecular weight difference among allergens from different grass pollens could be attributed to several factors such as quality and the place of collection of the source material, extraction protocol, the detection methods applied and the patient population tested [18].

Pollen of Kikuyu grass, a close member of the same genus (*Pennisetum clandestinum*), has also been demonstrated as allergenic by Potter et al. (1993), who described the presence of 70 kDa major allergen along with other allergic proteins (32, 28 and 48 kDa) in its allergen extract [32]. There are at least two possible ways to explain the presence of many allergens in a single grass pollen extract. Existence of different allergenic epitopes on different proteins is one of the possibility. Second explanation could be the presence of similar IgE binding epitopes on different proteins of grass pollen extracts. Supportive evidences in the favour of second explanation are provided by the work of two independent groups who have described monoclonal antibodies which recognize similar epitopes on different proteins from the same pollen extracts. Haas et al. (1986) described a monoclonal antibody recognizing 7 different allergens from the same source [11]. A similar situation was also described by Smart et al. (1983), who showed that monoclonal antibodies FMC A7 and FMC A9 identify similar epitopes on more than one allergen from the same pollen extract [29].

In conclusion, a full spectrum of allergenic proteins of *Pennisetum typhoides* pollen extract has been established by means of immunoblotting technique. Proteins with molecular weight 85, 80, 70, 57, 50, 43, 40 and 34 kDa have been identified as major allergenic proteins of *Pennisetum typhoides* pollen.
Chapter III  Identification of major allergenic components of *Pennisetum typhoides* pollen

BIBLIOGRAPHY


Chapter III  Identification of major allergenic components of *Pennisetum typhoides* pollen


Chapter III Identification of major allergenic components of Pennisetum typhoides pollen


Identification of major allergenic components of *Pennisetum typhoides* pollen

