

Two-dimensional immunoblot analysis of allergens of *Cladosporium herbarum*

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Summary

In the present study, allergens of *Cladosporium herbarum* were analysed by SDS-PAGE, two-dimensional gel electrophoresis and immunoblot. The SDS-PAGE under reduced conditions followed by western blotting analysis revealed 10 human IgE-binding components with molecular masses ranging from 15 to 143 kDa. Within the same molecular mass range, five components were detected under non-reduced conditions. Examination of the specificities of 10 individual sera revealed that two patients were sensitive to almost all the allergens, whereas the majority reacted with only some components. Two-dimensional immunoblot was employed to further analyse the allergens in the extract. A silver-stain of the two-dimensional gel revealed that there were more than 30 different protein components visible in the extract. Immunoblot of the proteins separated under denatured conditions disclosed that 17 spots were reactive with human atopic sera.

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Introduction

Cladosporium herbarum has been reported to be one of the most common sources of inhalant allergens causing fungal allergic diseases in many countries [1–4]. The extracts of *C. herbarum* have been demonstrated to contain complex components, mainly carbohydrate and a small amount of protein moiety. This complexity of the extract was considered to be one of the major hindrances to the improvement of immunotherapy for the mould-induced allergy.

Identification, isolation and purification of the allergens are essential for standardization and characterization of the mould allergen extracts. A number of antigenic and allergenic components were found in the crude extract of *C. herbarum* [5,6]. By using crossed radioimmuno-electrophoresis, Lowenstein *et al.* [5] showed that the extract possessed 57 antigens and at least five allergens; further studies reported by Aukrust [6] revealed that the extract contained four major and 10–20 minor allergens. Although the approximate molecular masses and pIs of a few allergens, such as *Cla h* I and *Cla h* II, have been

reported [7,8], the chemico-physical properties of most of these allergens remain as yet unknown.

It was reported previously from our laboratory [9] that the mycelial extract of the fungus contained nine allergens with molecular masses ranging from 14 to 97 kDa using SDS-PAGE and western blotting analysis. In the present study, we further characterized the allergens separated under either denatured or non-denatured conditions by two-dimensional immunoblot.

Materials and methods

Extract of *C. herbarum*

An isolate, IMI 96220, of *C. herbarum* was cultured on liquid synthetic revised tobacco medium at 22°C for 4 weeks as reported [10]. The pellicles, which had been washed and defatted with acetone and air-dried, were extracted with Tris-HCl buffer, pH 7.8, containing 0.15 mmol/l NaCl for 48 h at 4°C. After extraction, the debris and the suspended pellicles were removed by centrifugation at 1000 *g* for 30 min, then at 30 000 *g* for 20 min, at 4°C. The supernatant was concentrated and diafiltered with water using an Amicon YM-5 membrane. The retentate was filtered through a 0.45 µm membrane, lyophilized and stored at 4°C. The broth of the mould culture was also concentrated and similarly diafiltered

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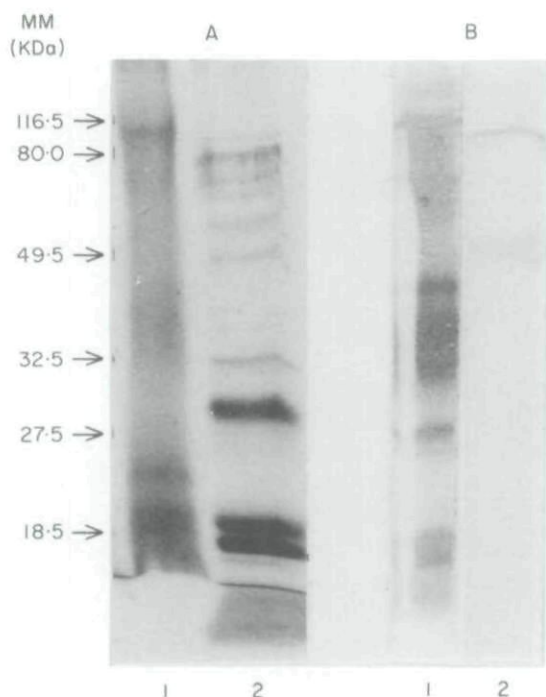


Fig. 1. Immunoblot of the proteins separated by SDS-PAGE under reduced conditions. The proteins in the extract of mycelia (lane 1) and in the broth (lane 2) separated by SDS-PAGE were stained with Coomassie blue (A) and electrotransferred onto a polyvinylidene difluoride membrane and immunoblotted with human IgE antibodies of the atopic human serum pool (B). MM. Molecular mass.

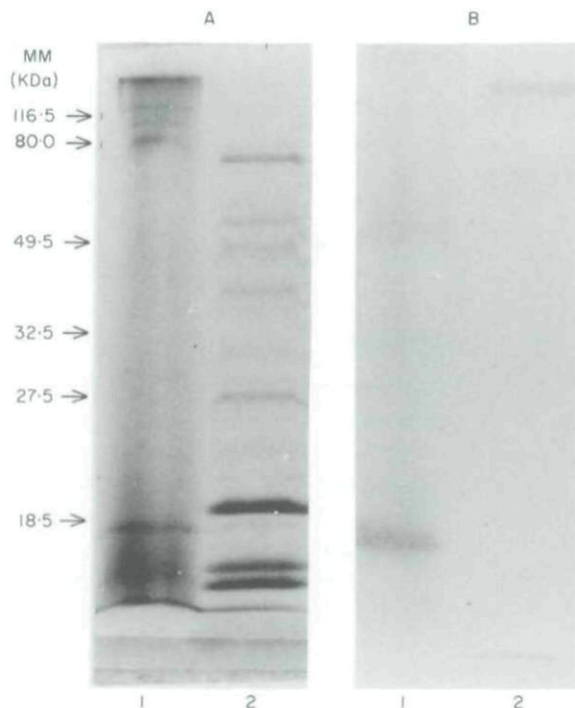


Fig. 2. Immunoblot of the proteins separated by SDS-PAGE under non-reduced conditions. (A) Coomassie blue stain; (B) immunoblot with IgE antibodies of the atopic human serum pool. Lanes 1 and 2 contain mycelial and broth proteins, respectively. MM. Molecular mass.

with water through an Amicon YM-5 membrane. The protein contents of these preparations were determined by a dye-binding assay (Bio-Rad, CA, USA).

Sera from atopic patients

The IgE antibody preparation was a mixture of equal volumes of sera from six patients highly allergic to *Cladosporium*, as determined by a skin-test reaction of 4+ and a direct RAST titre of 2+ to 3+ [9]. Sera from six non-mould-allergic individuals were also pooled and used as a negative control. Ten sera from *Cladosporium*-allergic patients with a RAST titre of 2+ were examined individually. These individual sera and the serum pools were stored at -20°C .

SDS-PAGE and Western blot

SDS-PAGE was performed on a vertical slab gel apparatus using a 12% polyacrylamide gel to separate the proteins of the extract essentially according to the method of Laemmli [11]. The mycelial extract and concentrated broth were treated under either reduced or non-reduced

conditions, i.e. with or without β -mercaptoethanol (Bio-Rad) in the loading buffer before being applied to the gels. Each lane was loaded with 20 μg of either mycelial or broth proteins, followed by electrophoresis for separation of the proteins. The molecular masses of the components were estimated by comparison with the mobilities of prestained protein standards (Bio-Rad). The separated proteins were either stained with Coomassie blue or electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, CA, USA). The membrane was probed with a 1:5 dilution of either the atopic human serum pool or the individual sera after saturation with 3% blocking reagent (Boehringer Mannheim, Quebec, Canada). The bound human IgE antibodies were detected with a conjugate of goat anti-human IgE antibodies and alkaline phosphatase (Sigma, MO, USA) and the substrate, NBT and BCIP, as described by Towbin *et al.* [12].

Two-dimensional immunoblot

Two-dimensional gel electrophoresis was performed as described by O'Farrell [13] and modified by Hochstrasser *et al.* [14] and Robertson *et al.* [15]. For the first dimension, the samples were prepared with 2 $\times$ denatur-

Fig. 3. Recognition of allergens by individual atopic sera. The proteins separated by SDS-PAGE under reduced conditions were electrotransferred onto a polyvinylidene difluoride membrane and probed with atopic serum pool (lane 1), normal serum pool (lane 2) and 10 different individual sera (lanes 3–12). MM. Molecular mass.

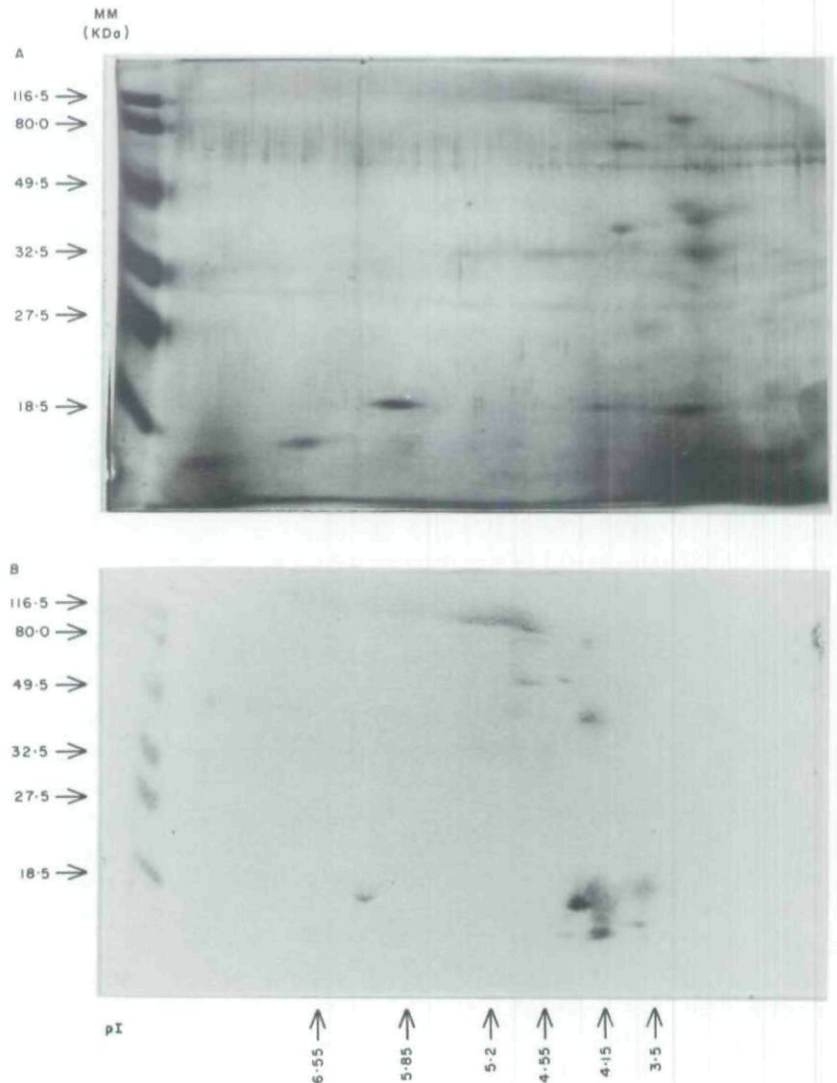
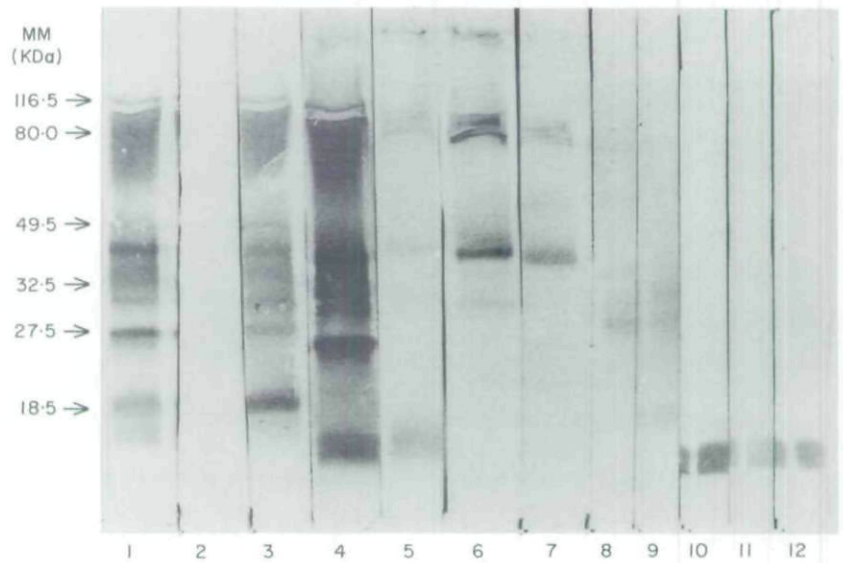


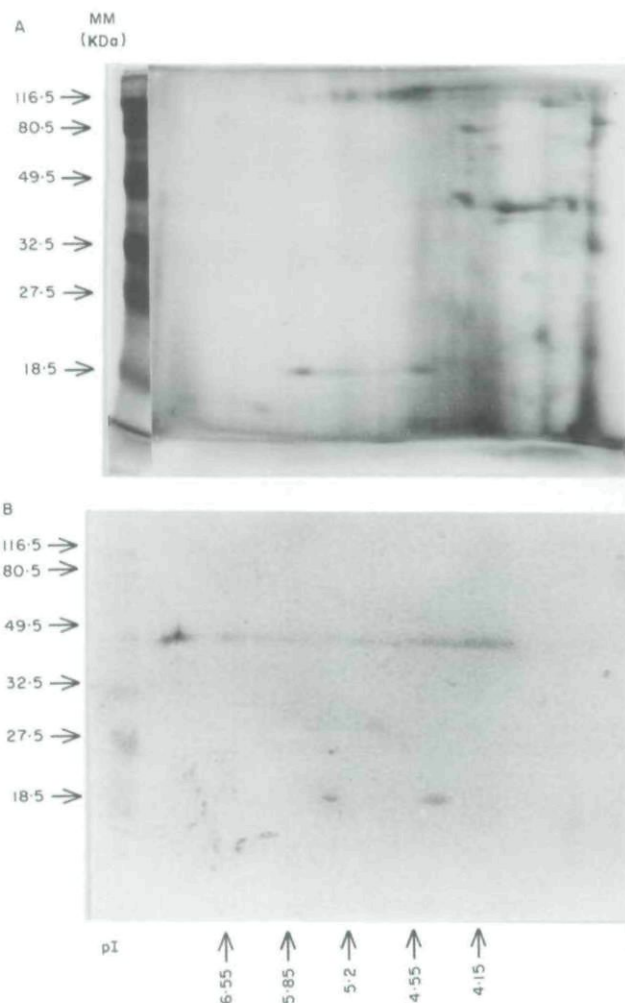
Fig. 4. Two-dimensional immunoblot of the proteins separated under denatured and reduced conditions. The proteins separated by two-dimensional gel electrophoresis were either stained with silver (A) or electrotransferred onto a polyvinylidene difluoride membrane and immunoblotted with IgE antibodies of the atopic human serum pool (B). MM. Molecular mass.

Table 1. Immunodetection of the allergens separated under denatured and reduced conditions

No. of allergens	Molecular mass (kDa)	pI
1	128.23	4.68
2	109.85	5.25
3	105.70	4.93
4	91.75	4.59
5	91.75	3.92
6	58.49	4.28
7	56.62	4.74
8	48.78	6.46
9	47.69	3.88
10	18.65	3.02
11	17.66	5.91
12	17.66	4.07
13	17.35	3.80
14	16.47	3.15
15	15.66	4.24
16	15.66	3.68
17	14.53	3.68

ing gel loading buffer (2.4 g urea, 20 μ l pH 3.5–10 ampholyte solution, 100 μ l pH 4–6 ampholyte solution, 500 μ l 20% Triton X-100, 50 μ l β -mercaptoethanol, 1.7 ml H₂O and 200 μ l 1% bromophenol blue). The denatured proteins were loaded onto a mini-slab gel prepared with 5.4 ml H₂O, 2.0 ml acrylamide solution (30% acrylamide and 1% bis-acrylamide), 48 μ l pH 3.5–10 ampholyte, 240 μ l pH 4–6 ampholyte, 6.0 g urea, 25 μ l 10% APS and 20 μ l TEMED. The pH gradients were made with running buffer of 20 mmol/l NaOH in the upper buffer chamber and 10 mmol/l H₃PO₄ in the lower buffer chamber. The gel was run for 30 min at 150 V and then for 2.5 h at 200 V. The mould proteins were also separated under non-denaturing conditions, using a method similar to the above with the exception that urea was not added to the loading buffer and the gel. The gel was electrophoresed for 1.5 h at 200 V and 1.5 h at 400 V.

After the first-dimensional electrophoresis, the gels were sliced into strips and equilibrated for 30 min with equilibration buffer containing 5% β -mercaptoethanol (not added for the non-reduced gels), 62.5 mmol/l Tris-HCl pH 6.8, 2.3% SDS and 10% glycerol. The strips of the gel were then placed on top of 12% SDS-PAGE slab gels. The gels were electrophoresed for 1.5 h at 180 V and then silver-stained as described elsewhere [14,16]. Immunoblot using human IgE antibodies was also performed after the proteins in the gels were transferred onto a PVDF membrane [12].

**Fig. 5.** Two-dimensional immunoblot of the proteins separated under native and non-reduced conditions. (A) silver stain; (B) immunoblot with IgE antibodies of the atopic human serum pool MM. Molecular mass.

Results

Identification of *C. herbarum* allergens by SDS-PAGE and Western blot

The proteins separated by SDS-PAGE under reduced conditions were analysed. From the results of Coomassie blue staining (Fig. 1A), protein bands were clearly visualized from the broth preparation (lane 2) but not from the mycelial extract (lane 1) because of a large amount of carbohydrate in the extract. Immunoblot (Fig. 1B, lane 1) revealed that the mycelial extract contained 10 bands which reacted with human IgE antibodies. Their molecular masses, calculated according to the prestained protein markers, were 128.3, 90.2, 58.3, 46.6, 41.7, 35.8, 25.6, 18.1, 17.8 and 15.6 kDa. The bands at 90.2, 46.6, 41.7, 25.6, 18.1 and 17.8 kDa showed relatively stronger

Table 2. Immunodetection of the allergens separated under non-denatured and non-reduced conditions

No. of allergens	Molecular mass (kDa)	pI
1	45.64	> 6.55
2	45.46	> 6.55
3	45.46	> 6.55
4	45.46	6.25
5	45.46	5.33
6	45.46	4.81
7	45.46	4.53
8	45.46	4.28
9	21.52	4.78
10	21.52	5.77

reactivity than the others with the antibodies. On the other hand, the broth (Fig. 1B, lane 2) contained fewer allergens. Two components, with molecular masses of 103.9 and 46.6 kDa respectively, were weakly reactive with IgE antibodies.

Coomassie blue stain of the proteins separated by SDS-PAGE under non-reduced conditions seemed to be compatible with the above results (Fig. 2A), whereas immunoblot analysis revealed different IgE-binding patterns. Five weakly IgE-reactive bands with molecular masses of 148.5, 51.7, 25.2, 17.52 and 14.9 kDa respectively were found in the mycelial extract (Fig. 2B, lane 1). Since the reactivities of these allergens were low, only two bands, at 51.7 and 17.52 kDa, with relatively stronger reactivities, were visualized by the photography. One IgE-reactive band at 148.5 kDa was observed in the broth preparation (Fig. 2B, lane 2).

Specificity of individual sera to the allergens

Ten sera showing a RAST of 2+ from *Cladosporium*-allergic patients were examined for their specificities against different allergens of the mycelial extract. Of these individual sera two, applied in lanes 3 and 4 in Fig. 3, were used to make the serum pool and the others were newly tested. The extract was treated under reduced conditions. The proteins separated by SDS-PAGE were transferred onto PVDF membranes and probed with individual sera. The results, shown in Fig. 3, demonstrated various reaction patterns of these individual sera. The positive serum pool (lane 1) and two individual sera (lanes 3 and 4) reacted with most of the allergens, while the other individual sera reacted with only some allergens: one (lane 5) recognized three bands, two (lanes 6 and 7) reacted with the middle molecular mass allergens, two

(lanes 8 and 9) reacted with relatively higher molecular mass bands and three (lanes 10–12) seemed to react exclusively with the low molecular mass allergens. The negative control serum pool (lane 2) showed no reaction to the allergens. Of the above individual sera, two (lanes 3 and 4) were also the source of the atopic serum pool, and the other eight were selected randomly from the patients allergic to *C. herbarum*.

Definition of molecular masses and pIs of the allergens

Two-dimensional immunoblot was used to determine simultaneously molecular masses and pIs of the mould allergens.

The proteins separated under denatured and reduced conditions were examined for their antibody-binding activities after they were transferred onto PVDF membranes. Seventeen spots were found to react with human IgE antibodies, while silver-staining of the second-dimensional gel disclosed at least 30 individual proteins in the extract (Fig. 4).

The majority of the allergens defined by this assay were observed to be closely related to one another in terms of their molecular masses and pIs. Three major groups of allergens, according to the molecular masses and pIs, could be classified (Fig. 4B): (i) 90–130 kDa, pI 3.9–5.3; (ii) 46–60 kDa, pI 3.8–4.8 and (iii) 14–19 kDa, pI 3.0–4.3. One allergenic protein which had a molecular weight of 17.66 and a pI of 5.91 was well isolated from the other proteins. Since it was in a minute amount, as determined by silver-staining (Fig. 4A), it was not visible by Coomassie blue stain of either the gel or membrane. In fact, most of the allergens, although detected by immunoblot, could not be visualized by Coomassie blue stain after being separated by two-dimensional gel electrophoresis (unpublished results). In general, the majority of these allergens as well as the non-allergenic proteins were localized between pI 3.5 and 5.5. The relationships of the approximate molecular masses and pIs of the allergens are summarized in Table 1.

The proteins separated under non-denatured conditions were also immunoblotted. By using silver-staining, at least 40 different proteins were visualized (Fig. 5A). Immunoblot analysis revealed at least 10 IgE binding spots, with relatively weak reactivities (Fig. 5B). The molecular masses of these allergens were found around either 46 or 20 kDa. The molecular mass and the pI values of these IgE-reactive components are presented in Table 2.

Discussion

The progress in characterization and standardization of these allergens has been slow, mainly due to (i) the limited

amount of protein moiety in the extract, (ii) batch-to-batch variation of the allergens in the cultures and (iii) limitations of the methods used to characterize the allergens. The aim of the present study was to employ a high-resolution separation method, two-dimensional electrophoresis, in conjunction with immunoblot to identify the allergens and to characterize their molecular masses and isoelectric points.

The presence of only a few allergenic components in the broth indicated that most of the allergens were membrane-bound or cytosol proteins and not secreted. The allergens separated under reduced conditions showed strong reactivity with human IgE antibodies, which was consistent with our previous report [9]. However, allergens separated under non-reduced conditions reacted weakly with the antibodies and showed different patterns of reaction from their reduced forms. It is expected that migration of a protein treated with or without reducing agents would be different if there is (are) a disulphide bond(s) involved in the polypeptides of the protein. Since there was no clear correlation between the disappearance and appearance of allergenic bands or spots before and after treatment with β -mercaptoethanol in this report, the disulphide bonds involved were suggested to be mainly intra-molecule in the *C. herbarum* allergens.

At present, the mechanisms underlying different reactivities of the allergens treated under reduced and non-reduced conditions are not fully understood. However, the consistent results from both the one-dimensional and the two-dimensional immunoblots suggested that the unfolding of the proteins was necessary for most of the IgE-binding epitopes to be exposed and accessible to the antibodies. Since denaturation of the allergens with urea, β -mercaptoethanol and SDS did not destroy their antibody-binding activities, the epitopes of these allergens were probably sequential in nature as reported on some other allergens [17–19]. Nevertheless, this study demonstrated that denaturation and reduction of the allergens of *C. herbarum* enhanced their antibody-binding activities and were therefore essential for their immunological identification.

Slight differences of molecular masses of the allergens were observed between the one- and two-dimensional analysis, which might be due to the effect of urea on the proteins. Although at least 17 allergens were defined from this mould, some of these closely related allergens, in terms of their molecular weights and pIs, might be isoforms as observed from studies of the other allergens [20,21]. The major allergens of this mould were indicated in three groups since strong reactive components were found in these regions. However, definition of the major allergens requires the examination of a large number of

individual sera to determine the frequency of reaction of each component.

The limited number of individual sera used in the present report revealed that only a small portion of atopic patients were allergic to the majority of the mould allergens. Most individuals were found to be sensitive to only a few of the allergens. Thus, for the severe atopic patients, who are reactive to almost all the allergens, the mould extract may be suitable for the purpose of specific diagnosis and immunotherapy, whereas those individuals who are allergic only to certain components will require the isolated and purified allergens.

In summary, the approximate molecular masses and pIs of *C. herbarum* allergens were established in this study. However, it should be pointed out that the precise physicochemical values for these allergens can only be established following the determination of the amino acid sequences and post-translational modifications of these allergens. Furthermore, characterization of these allergens would be very difficult by conventional methodologies because of the minute amounts present in the extract.

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