

Purification and characterization of an allergen from celery immunochemically related to an allergen present in several other plant species. Identification as a profilin

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Summary

The purification of a 15 kD allergen from celery was obtained by a four step procedure. Evidence for at least two isoallergenic forms was obtained after analysis by two-dimensional-electrophoresis. A rabbit polyclonal antibody raised against this purified allergen allowed us to confirm our precedent results on the occurrence of allergenically and molecular mass-related components in celery and birch and mugwort pollens. In addition such components were also present in numerous other species like *Cynodon dactylon*, *Sorghum halopense*, *Poa pratensis*, *Ambrosia elatior* and in apple and carrot. The 15 kD allergen was identified as profilin by use of a specific rabbit polyclonal antibody that recognized a recombinant birch profilin. In addition, the purified celery allergen binds IgE from sera of patients allergic to birch profilin. These results reinforce the concept of profilin as a panallergen responsible in some patients for cross-allergic manifestations to various and unrelated species of grasses, weeds, trees, vegetables and fruits.

Clinical and Experimental Allergy, Vol. 22, pp. 774–782. Submitted 10 October 1991; revised 6 February 1992; accepted 11 February 1992.

Introduction

Studies dealing with identification and characterization of allergens shared by various species responsible for allergic symptoms have at least two justifications. The first one belongs to the clinical field: a complete description of a cluster of hypersensitivity is essential for the knowledge of the allergens a patient is likely to be sensitized to. The second, being more basic, deals with the demonstration that a same protein or a similar structure in different or related proteins acts as an allergen in various and sometimes unrelated species, thus reducing the apparent great diversity of allergens. An example is the now well established fact that cytochrome c is an allergen for several species [1]. More recent studies have

shown that related species like grasses [2] or several trees of the Fagales order [3] have similar allergens. In addition, the greater the number of structurally defined allergens in term of B and T epitopes) the more likely the problem of what structural characteristic makes a protein an allergen will be solved.

We recently reported in celery tuber and in pollen from *Betula verrucosa* and from *Artemisia vulgaris* the presence of 15 kD components sharing some homology [4]. This was supported by the fact that IgE from RAST positive sera to celery, mugwort and birch pollens recognized similar molecular mass components and by the results of immunoprint cross inhibition experiments. To confirm this observation by another and more convenient way and to better characterize this allergen, its purification from celery tuber and the preparation of a specific rabbit antibody were undertaken. The results obtained with this antibody are reported here, in particular identification of this allergen as being profilin, an actin regulatory protein.

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Materials and methods

Allergen extracts

Extraction of pollens (*Betula alba*, *Ambrosia elatior*, *Dactylis glomerata*, *Poa pratensis*, *Cynodon dactylon*, *Artemisia absinthium* and *Sorghum halopense* from Sigma, France; *Lolium perenne* and *Phleum pratense* from Allergbio, France; *Artemisia vulgaris* from Allergon, Sweden) was done by overnight agitation of the pollen grains in distilled water, using a magnetic stirrer. The $100\,000 \times g$ for 30 min supernatants were dialysed against 10 mM Tris/HCl pH 6.8 and concentrated with a 60% (w/v) polyethylene glycol 35 000 solution made in 10 mM Tris/HCl pH 6.8. In some experiments, proteins were used after precipitation with 75% saturated $(\text{NH}_4)_2\text{SO}_4$ for 1 hr. Crude extracts from carrot and apple were prepared as for crude celery extract and then dialysed and concentrated as indicated for pollen extracts. Extracts from *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* were from Laboratories des Stalleghènes, Paris, France. Purified recombinant birch profilin was obtained as indicated [5].

Purification of the 15 kD allergen from celery

Extract from raw root celery was made by grinding piece-cut celery in a Waring Blender homogenizer in 1.5 ml/g of buffer H (50 mM Tris/HCl pH 7.6, 1 mM benzamidine, 10 mM Na_2EDTA , 0.2 mM phenylmethylsulphonylfluoride). After filtration on gauze and centrifugation ($6000 \times g$ for 15 min) solid ammonium sulphate was added to the supernatant up to 75% saturation (at 4°C). Proteins were resolubilized in and dialysed extensively against 15 mM Tris/HCl pH 7.6. Extract was further defatted with ethyl ether. This was referred to as crude celery extract.

Purification of the 15 kD allergen from the crude extract was performed following a four step procedure. The crude celery extract was loaded on a prepacked cartridge (DEAE Zetaprep 250 from CUNO Inc, U.S.A.), equilibrated with 15 mM phosphate buffer pH 6.5. Stepwise elution was conducted from 100 mM to 500 mM NaCl in 15 mM phosphate buffer pH 6.5 at a flow rate of 20 ml/min. Protein elution was checked by measuring the absorbance at 280 nm. The presence of the 15 kD allergen was detected by dot immunoblotting using patient sera containing IgE directed solely against the 15 kD allergen. The 500 mM NaCl fraction was dialysed overnight against 15 mM Tris/HCl pH 7.6 containing antiproteases (10 mM benzamidine and 0.2 mM phenylmethylsulphonylfluoride). This fraction was passed on a 40×450 mm Sephacryl S200 superfine column (Pharmacia, Sweden) previously equilibrated with 15 mM Tris/

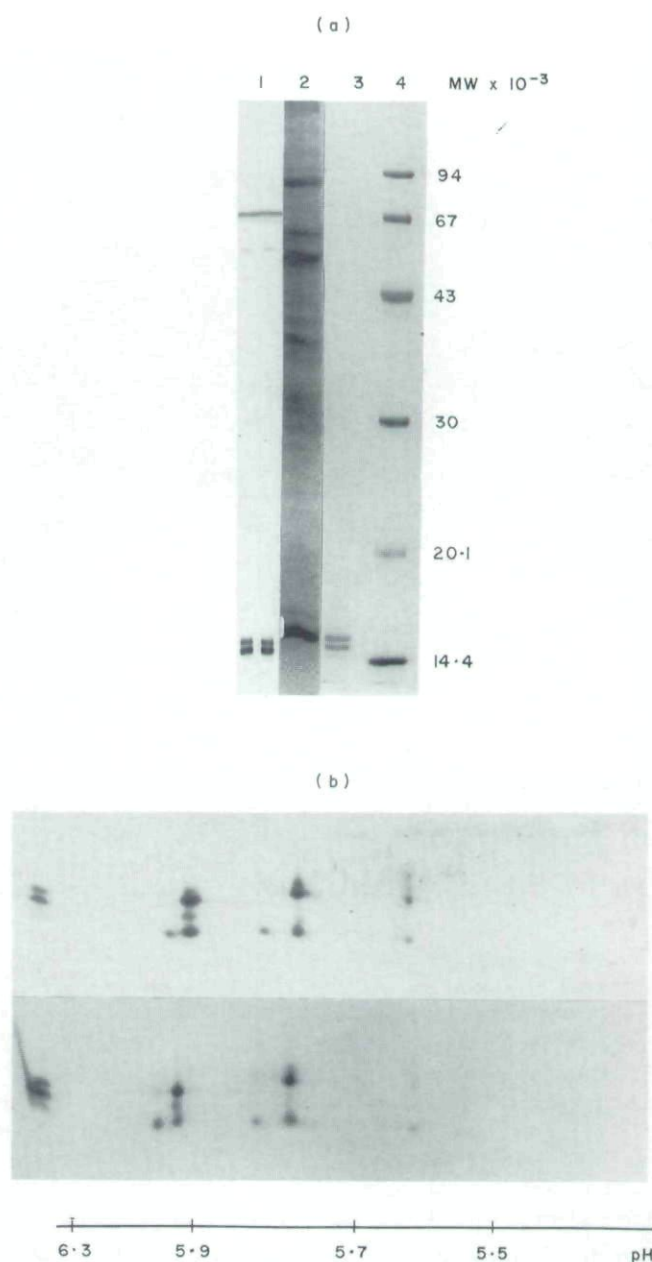


Fig. 1. Characterization of the purified 15 kD allergen from celery. (a) The 15 kD purified preparation (5 μg , lane 3), celery crude extract (lane 2) and molecular mass markers (lane 4) were submitted to electrophoresis on a 12% SDS-PAGE and stained with Coomassie Blue. Lane 1 shows the immunodetection of the celery crude extract blot probed with a 10^{-4} dilution of the anti-15 kD allergen rabbit antiserum. (b) 2D-PAGE analysis of 2.5 μg of the 15 kD purified preparation. Upper panel: silver staining. Lower panel: blot immunodetection with a patient serum containing monospecific IgE against the 15 kD allergen (serum L). On the cathodic side of the gels a sample of the preparation was directly applied to the second dimension gel.

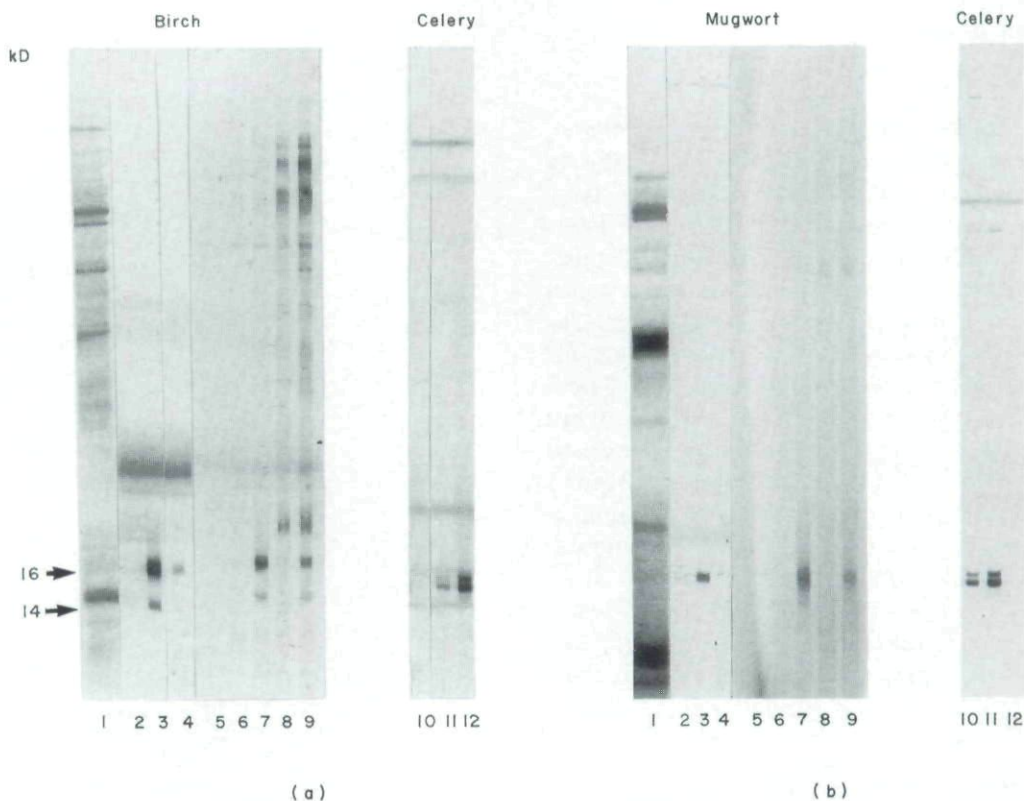


Fig. 2. Immunodetection of blots from celery, mugwort and birch pollen with anti-15 kD allergen rabbit antibody. (a) Lane 1: Coomassie blue staining of birch crude extract after separation by SDS-PAGE. Lanes 2 to 9: immunodetection of birch crude extract with non-immune rabbit serum (lane 2); with a 10^{-4} dilution of rabbit antibody (lane 3); with the same dilution of antibody preincubated for 60 min with 50 μ g of celery crude extract (lane 4); with non-allergic human serum (lane 5); with a twofold dilution of serum M or serum V preincubated with PBS (respectively lanes 7 and 9) or with 2 μ g of purified celery allergen (respectively lanes 6 and 8). Lanes 10 to 12: immunodetection of celery crude extract with non-immune rabbit serum (lane 10); with a 10^{-4} dilution of rabbit antibody (lane 12); with the same dilution of antibody preincubated for 60 min with 50 μ g of birch crude extract (lanes 11). (b) Lane 1: Coomassie Blue staining of mugwort crude extract after separation by SDS-PAGE. Lanes 2 to 9: immunodetection of mugwort crude extract with a 10^{-4} dilution of non-immune rabbit serum (lane 4); with a 10^{-4} dilution of rabbit antibody (lane 3), with the same dilution of antibody preincubated for 60 min with 50 μ g of celery crude extract (lane 2); with non-allergic human serum (lane 5); with a twofold dilution of serum L or serum M preincubated with PBS (respectively lanes 7 and 9) or with 2 μ g of purified celery allergen (respectively lanes 6 and 8). Lanes 10 to 12: immunodetection of celery crude extract with non-immune rabbit serum (lane 12); with a 10^{-4} dilution of rabbit antibody (lane 11) or with the same dilution of antibody preincubated for 60 min with 50 μ g of mugwort crude extract (lane 10).

HCl, pH 7.6. The sample was run in the same buffer at a flow rate of 24 ml/h. The high absorbance fractions of the 15 kD allergen containing peak were pooled and dialysed against 20 mM Tris/HCl pH 7.6, 100 mM NaCl. This fraction was submitted to a preparative anion-exchange high performance liquid chromatography (HPLC) on a Protein PAK DEAE 5PW 21.5 mm $\times$ 15 cm column from Waters. The sample was eluted with a linear NaCl gradient from 100 mM to 400 mM made in 20 mM Tris/HCl pH 7.6 at a flow rate of 0.6 ml/min for 30 min. Fractions eluted between 350 and 400 mM NaCl were pooled, dialysed against 25 mM phosphate buffer pH 6.8, concentrated with a 60% (w/v) polyethylene glycol 35 000 solution made in 25 mM phosphate buffer pH 6.8 and

separated on an exclusion-diffusion HPLC column (Protein PAK SW 300 7.5 mm ID $\times$ 30 cm from Waters). The eluting phase was made of 25 mM phosphate buffer pH 6.8 run at a flow rate of 0.6 ml/min.

Human and rabbit sera reacting against the 15 kD allergen

Sera L and M were human sera with IgE antibodies reacting against 15 kD allergen from celery, mugwort and birch pollens which had been used in a previous study [4]. Serum V was from a patient highly reactive to the 15 kD allergen from carrot and birch pollen.

New Zealand white rabbits were immunized with 60 μ g

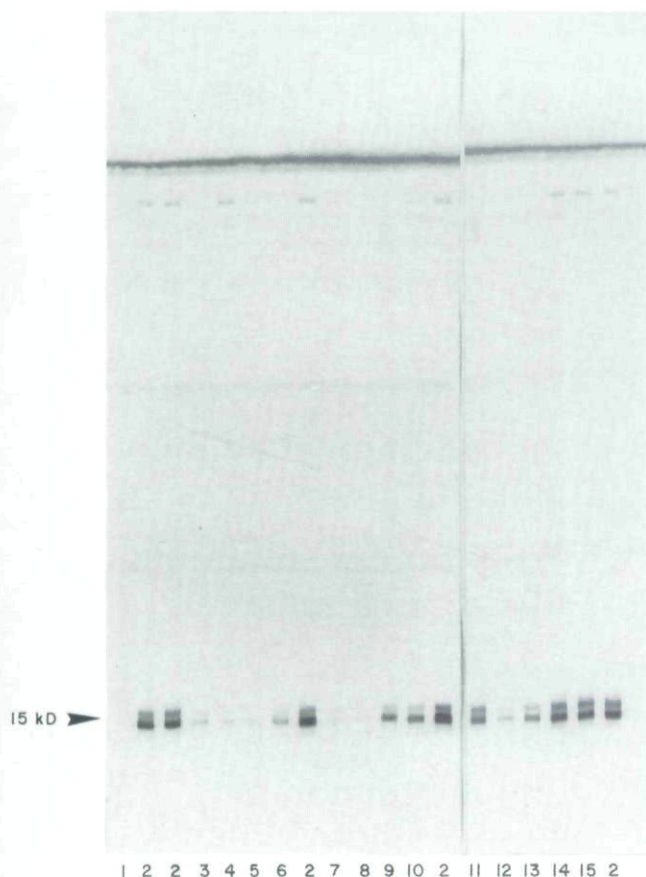


Fig. 3. Effect of preincubation with different allergens on the rabbit antiserum immunodetection of the 15 kD allergen on celery blots. 10^{-4} dilutions of the rabbit antiserum were preincubated with PBS (lanes 2) or with 50 μ g of the following crude extract allergens for 30 min before probing the crude extract celery blot. Lane 3: celery; lane 4: Granny-Smith apple; lane 5: carrot; lane 6: ragweed; lane 7: Johnson grass; lane 8: bermuda grass; lane 9: rye grass; lane 10: timothy grass; lane 11: wormwood; lane 12: Kentucky bluegrass; lane 13: orchard grass; lane 14: *Dermatophagoides pteronyssinus*; lane 15: *Dermatophagoides farinae*. Lane 1: 10^{-4} dilution of a rabbit non immune serum as control.

of the purified 15 kD allergen from celery in Freund's complete adjuvant according to the method of Vaitukaitis [6]. One month later, 15 μ g of allergen in Freund's incomplete adjuvant were injected. This was repeated twice. Serum was taken weekly after the first boost.

Electrophoretic methods

One-dimensional polyacrylamide slab gel electrophoresis was performed in the presence of sodium dodecyl sulphate (SDS-PAGE) according to Laemmli [7]. Two-

dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed according to O'Farrel [8] with the conditions described by Blangarin *et al.* [9]. The second dimension was run in a 12% acrylamide slab gel in the presence of SDS. At the end of the first dimension, a duplicate gel loaded with the protein exactly as the assay gel was cut into 1 cm long pieces. The pH gradient was determined by measuring the pH of each piece of gel after a 1 hr incubation in 1 ml of a 10 mM KCl solution. Silver staining of proteins was according to Oakley [10].

Dot and blot immunodetections

These methods were performed with a multitray apparatus (Sebia, France). For dot immunodetection, proteins diluted in PBS were coated on a nitrocellulose sheet (0.45 μ m, Schleicher and Schüll) overnight at 4°C. For blot immunodetection proteins were transferred from the polyacrylamide gels as previously described [4]. Immunodetection with IgE specific containing sera was done either as previously indicated [4], with the use of a phosphatase labelled streptavidin-biotinylated complex (Amersham, U.K.) or by binding of a rabbit [^{125} I]-labelled anti-human IgE (Pharmacia, Sweden). The binding of the 15 kD a specific rabbit antiserum was detected either by incubation with biotinylated anti-rabbit IgG (Vector supplied by Biosys, France) and the same streptavidin complex as above or directly with donkey [^{125} I]-labelled anti-rabbit IgG antibody (Amersham, U.K.). The substrate was the mixture of 0.40 mM nitroblue tetrazolium and 0.45 mM 5-bromo-4-chloro-3-indolylphosphate disodium salt made in 100 mM Tris/HCl pH 9.5 containing 100 mM NaCl and 5 mM $MgCl_2$.

Poly-L-proline affinity chromatography

Poly-L-proline column (1.5 $\times$ 3 cm, 5 ml bed volume) was prepared by coupling 1.5 g of cyanogen bromide-activated Sepharose 4B to 100 mg of poly-L-proline (Sigma). The chromatography was conducted as described by Haarer *et al.* [11]. The column was washed with 20 ml of column buffer (20 mM imidazole pH 7.5, 150 mM NaCl, 0.5 mM ATP) and loaded with allergen extracts dissolved in the same buffer. After a 1 hr incubation, the column was washed with eight bed volumes of column buffer. The material bound to the column was eluted with 8 ml of a 6 M urea containing column buffer.

Results

Purification and characterization of the 15 kD celery allergen

The four step procedure designed to purify the 15 kD allergen from celery yielded 650 μ g of allergen from 2 kg

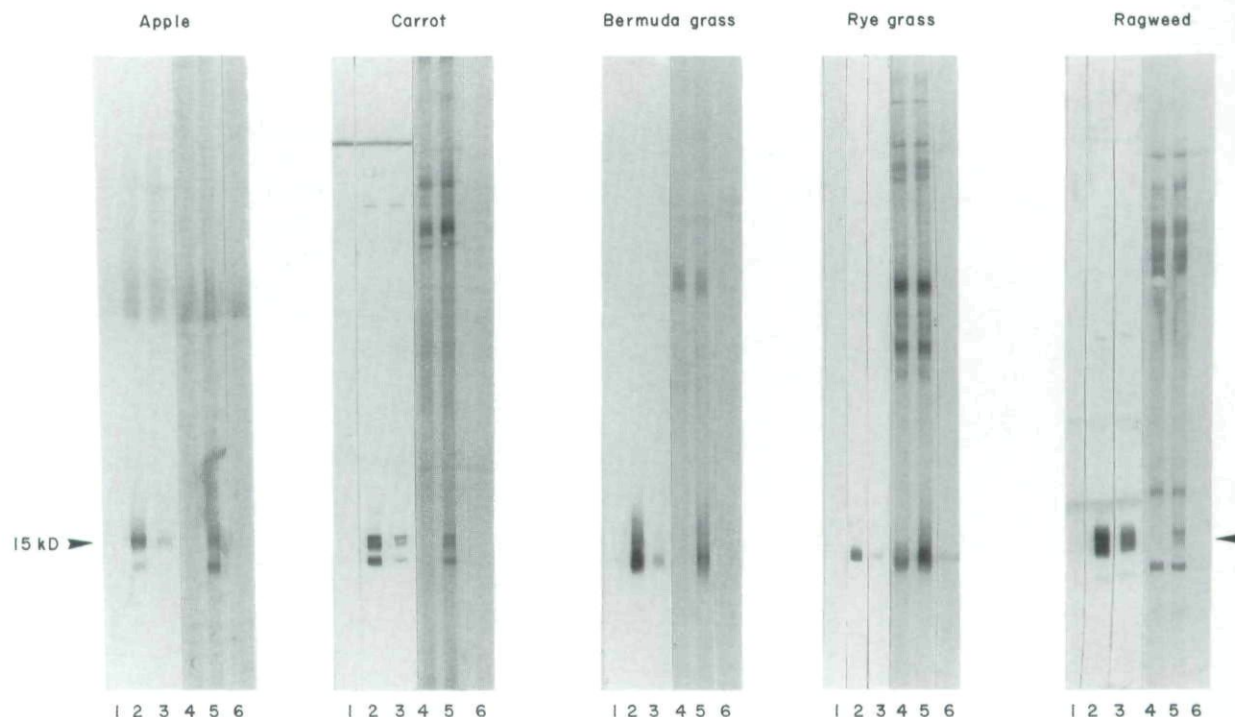


Fig. 4. Immunodetection of blots from different species with the anti-15 kD rabbit antiserum or with sera of allergic patients. Blots from crude extract of indicated species were incubated with non-immune rabbit serum (lanes 1); with a 10^{-4} dilution of rabbit antiserum (lanes 2); with the same dilution of antibody preincubated for 60 min with 50 μ g of celery crude extract (lanes 3); with a twofold dilution of serum L (apple, bermuda grass and rye grass) or serum M (ragweed) or serum V (carrot) preincubated with PBS (lanes 5) or with 2 μ g of celery purified allergen (lanes 4); with non-allergic human serum (lanes 6).

of raw root. The preparation was colourless and exhibited no other maximum absorption but one at 280 nm. After SDS-PAGE in reducing conditions, the allergen appeared as two main close bands of 15 kD molecular mass (Fig. 1a, lane 3). The apparent molecular mass obtained from exclusion diffusion analyses was between 30 and 35 kD on Sephacryl S 200 and between 25 to 32 kD on Protein Pak SW 300. 2D-PAGE analysis allowed to resolve the two bands into two main spots of pI ranging from 5.80 to 5.90 (Fig. 1b, upper panel). The highest molecular mass band gave the more acidic spot. In addition, under each spot another spot of lower intensity with no corresponding band on the 1-D electrophoregram was also visible. This lowest row of spots was believed to be due to the difference in the sample preparation for both methods. Blot immunodetection of the 2D-electrophoregram with a 15 kD monospecific reacting allergic serum revealed that all these spots were able to bind IgE (Fig. 1b, lower panel).

Specificity of the polyclonal rabbit antibody raised against the 15 kD purified preparation was checked on celery crude extract blot (Fig. 1a, lane 1). The antibody preparation appeared to be highly specific for the 15 kD allergen. With the exception of a very faint band around

60 kD, no other band due to the presence of the rabbit antibody was present. However, some primary antibody-independent bands were revealed (i.e. around 70 kD). The routine dilution of the antiserum for immunodetection on blots was 10^{-4} .

Confirmation of structural relationship between the 15 kD allergens in celery and in mugwort and birch pollens

The specific antibody to the celery 15 kD allergen was used to probe blots from mugwort and birch pollens. In both cases this antibody reacted specifically only with the 15 kD allergens (lanes 3). Preincubation of the antibody with the crude extract of one of the three different allergens at least partially inhibited the immunodetection of the 15 kD allergen of the other two species (Fig. 2a: lanes 4 and 11, Fig. 2b: lanes 4 and 10). Preincubation with purified celery allergen prevented reaction of human sera to the 15 kD allergen in mugwort and birch pollen extracts (lanes 6 to 9).

Crossreactivity studies in other plant species

The rabbit antibody was used to screen other species for the presence of some crossreacting material. In a first step

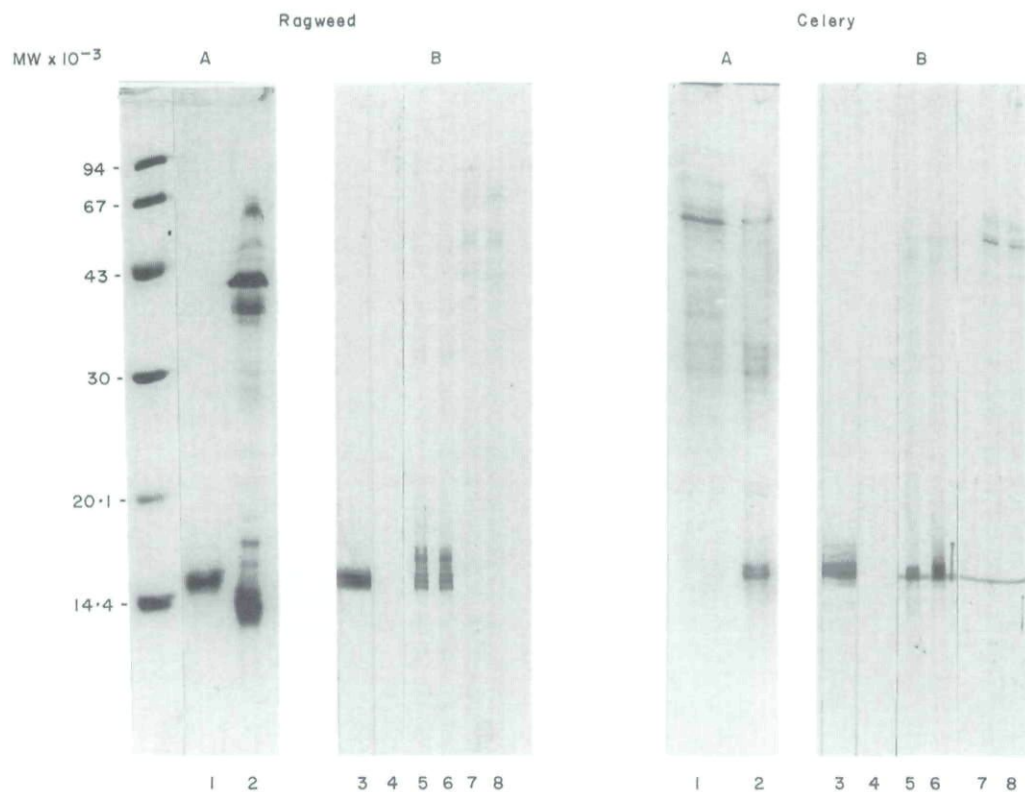


Fig. 5. Poly-L-proline affinity chromatography of celery crude extract and ragweed pollen extract. Affinity column was loaded with 2.5 mg of protein from celery or ragweed crude extracts and processed as described in Methods. The bound fraction was eluted in 6 M urea. About 60 μ g of proteins were dialysed, concentrated and submitted to electrophoresis on a 12% SDS-PAGE. One part of the gel was stained with Coomassie Blue (A). Celery: lane 1, total extract; lane 2, bound fraction-Ragweed; lane 2, total extract; lane 1, bound fraction. On the right are protein markers of indicated molecular weights. The other part of the bound fraction (B) was electrotransferred to nitrocellulose and probed with non-immune rabbit serum (lanes 4); with anti-15 kD rabbit serum (lanes 3) or with M or L human serum (respectively lanes 5 and 6). Celery or ragweed sensitized human sera but without IgE to the 15 kD allergen were used as control (lanes 7 and 8).

this was done by immunodetection of celery blot with rabbit antiserum preincubated with the crude extract of the species to be tested (Fig. 3). On a per mass basis the more potent inhibiting allergens were Granny-Smith apple, carrot, bermuda and Johnson grasses. These latter two allergens were more potent inhibitors than celery itself. Pollen from other common grasses, as rye grass, timothy or cockfoot appeared to be less active. Compared to mugwort, wormwood was far less crossinhibiting. Crude extract from ragweed pollen was not quite as potent as celery. Extracts from two species of mite were devoid of inhibiting activity.

Crude extracts of the more inhibiting allergens were submitted to SDS-PAGE and the resulting blots were directly probed with the rabbit antiserum or with sensitized patient sera (Fig. 4). In all five species the rabbit antiserum reacted strongly with components of molecular mass identical to that of the celery extract. This detection was inhibited by preincubation of the antibody with the

celery crude extract. Sera L, M and V containing IgE directed against the 15 kD celery allergen also gave a positive IgE dependent reaction with the 15 kD component of the different species. This reaction was prevented by preincubation of the sera with the purified celery allergen.

Identification of the 15 kD celery allergen as profilin

Many experiments were carried out unsuccessfully to try to identify the celery 15 kD allergen. Finally, after results obtained by one of us in birch and other plants [5], the hypothesis was made that the 15 kD celery allergen could be profilin, an actin-regulatory protein. The first experiment was to test the ability of the celery allergen to bind to a poly-L-proline affinity column, a well documented property of profilin from different origins. Figure 5 shows that 15 kD proteins were quite specifically retained. These proteins were recognized by the rabbit antiserum and

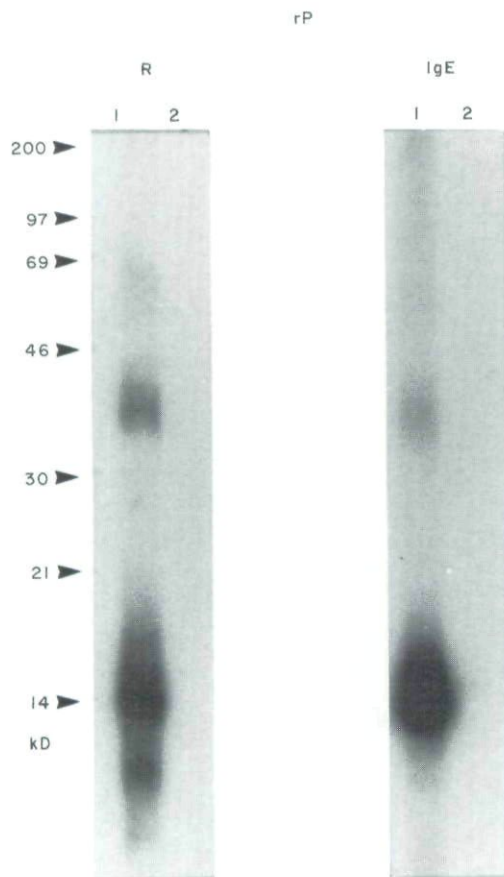


Fig. 6. Immunodetection of purified recombinant birch profilin by the anti-15 kD celery rabbit antiserum. Blot of the recombinant birch profilin (rP) was probed either with a 10^{-4} dilution of the anti-15 kD allergen rabbit antiserum (R) or with the serum of a birch pollen profilin allergic patient (IgE). Lanes 2 represent negative controls done without addition of the rabbit antibody in (R) and in (IgE) without addition of patient serum.

were allergens since they were able to bind serum IgE from the 15 kD allergen sensitized subjects. The same results were obtained with ragweed pollen or birch pollen (result not shown). In the case of ragweed, affinity chromatography led to very pure 15 kD proteins. To obtain further proof that the 15 kD celery allergen was structurally related to profilin the rabbit antibody raised against the celery allergen was tested for reactivity against recombinant birch profilin. The rabbit antibody recognized the recombinant birch profilin as a strong spot around 15 kD (Fig. 6). Some reaction was also apparent around 40 kD. This picture was very similar to the pattern observed when immunodetection was done with anti-birch profilin IgE containing sera. The experiment presented on Fig. 7 demonstrates that IgE from the serum of birch profilin allergic patients bound to the purified 15 kD celery allergen. This binding was prevented by preincuba-

tion of the serum with recombinant birch profilin in a specific manner. In the same conditions, no binding was observed with the sera of patient allergic to the major birch pollen allergen *Bet v* I but not to profilin.

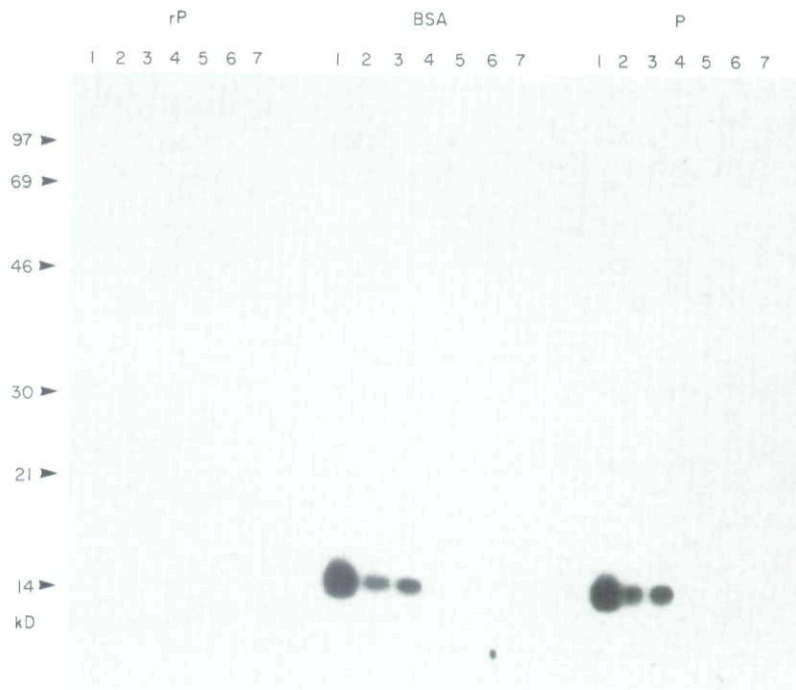
Discussion

Although clinical manifestations due to allergy to celery were recognized to be rather frequent and serious [12], hitherto there have been few attempts to identify allergens in celery. To our knowledge, the allergen from celery this paper deals with is the first one to be purified, characterized and identified in this species. The identification of the 15 kD allergen of celery to profilin was supported by three experimental results: the specific recognition by a rabbit antiserum raised against the 15 kD celery allergen of birch recombinant profilin, the reaction of IgE containing sera against birch profilin with the celery allergen indicating immunological relationship and finally, the specific binding to poly-L-proline. Very recently, profilin was considered to be an allergen after the demonstration that recombinant birch profilin was able to bind IgE from the serum of birch allergic patients [5]. This observation has been extended to profilin of numerous other pollens from grasses, weeds and trees [13,14]. We have previously shown [4] that 21 out of 36 sera (58%) with IgE directed against celery and mugwort components reacted with a 15 kD protein in celery. In addition these sera also reacted with birch and mugwort pollen components of similar molecular mass and structurally similar to the celery 15 kD allergen. In the present study, we found these proteins to be recognized in a very specific manner in birch and mugwort pollen extracts by the specific polyclonal antibody raised against the celery 15 kD allergen. The use of this antibody to probe directly components separated by electrophoresis leads to the conclusion of the presence of immunologically and molecular mass-related structures in numerous other species. Thus the present report confirms the wide occurrence of profilin as allergen and demonstrates that this category of allergens is also present in vegetables like celery and carrot and fruits like apple. Existence of allergens with widespread occurrence in numerous species is not a new concept. The best documented example is cytochrome c which has been shown to be an allergen in many grasses and weed species [1,15].

These observations lead to the questioning of the structural relationship between profilins from such different sources. For example, from the results of the inhibition studies the allergen appears to be more abundant in the Panicoideae than in the Festuceae subfamily of grasses. Another or additional explanation could be that the allergen is immunologically closer to the celery

Fig. 7. Immunodetection of the 15 kD celery allergen by sera from birch pollen profilin allergic patients. Purified celery allergen was submitted to SDS-PAGE (1 μ g/cm of a 1 mm thick gel) and transferred to nitrocellulose.

Nitrocellulose strips were incubated with tenfold dilutions of sera from patients allergic to birch pollen profilin (lanes 1–3); from patients allergic to the major birch pollen allergen *Bet v I* but not to profilin (lanes 4–5). As controls, strips were probed with a pool of serum of non-allergic individuals (lane 6) and with buffer without addition of serum (lane 7). After dilution, sera were preincubated with 5 μ g of purified recombinant birch profilin each (rP); with 5 μ g of BSA (BSA); with the serum dilution buffer alone (P).



allergen in the Panicoideae than in the Festuceae. Comparison of the number and the structure of epitopes of the different profilins will enable progression in the resolution of this problem. Primary structures are known only for a limited number of profilins [5,16]. There is a great percentage of homology in the N-terminal end of these profilins [5]. It is tempting to speculate that at least one B epitope shared by the different profilins is localized in this portion of the molecules.

Heterogeneity in celery profilin is revealed by a two close band pattern on SDS-PAGE. By 2-D PAGE, each of these bands resolves into two main spots, indicating multiple molecular forms of the same allergen with slight differences in molecular mass and/or isoelectric points and so-called isoallergens. The lower molecular mass band on SDS-PAGE could represent either proteolysed isoallergens or some lesser glycosylated forms. In reviewing the data on purified allergens, isoallergens are not an exception but a rather usual feature in grass [17] and weed pollens like short ragweed [18]. Moreover, three different isoforms of profilin have been described in *Acanthamoeba* [16].

This study emphasizes profilin as a so far non-identified allergen of short ragweed. This opens the question of the role of profilin in the frequent association of mugwort and ragweed allergy [19], both pollens being responsible for clinical manifestations at the same period of the year. More generally, profilin is likely to be one of the allergens

responsible for allergic manifestations to a well-defined cluster of hypersensitivity including at least celery and birch and mugwort pollens [12]. However, studies based on cutaneous test and allergic inquiries are necessary to evaluate the actual role of profilin in clinical allergic manifestations in these patients. Finally, profilin is a very easy allergen to purify and appears as a tool of potential interest for investigations on genetic aspects of the IgE response and on the relationship existing between structure and allergenicity.

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