

Influence of the pH of the extraction medium on the composition of birch (*Betula verrucosa*) pollen extracts

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Extracts from birch (*Betula verrucosa*) pollen were prepared at different pH, with constant pH monitoring and adjustment to preset values in the range 5.5–8.5. The total protein content of these extracts was directly correlated with the pH. Coomassie brilliant blue-stained isoelectric focusing and SDS–PAGE gels and immunoblot analysis demonstrated qualitative differences: some proteins were lost while others appeared when pH was changed. At pH 8.5, formerly unknown birch pollen allergens were detected with pI 9, 9.10, and 9.30 by about 30% of birch pollen-sensitive sera. Birch pollen extracts prepared at a pH close to neutrality, namely, 6.5 and 7.5, showed the greatest protein and different allergen diversity. Thus, extraction pH values are necessary to analyze the whole pattern of allergenic components in an extract.

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It has been demonstrated that the composition of allergenic extracts varies according to extraction conditions, such as temperature, extraction period, extraction buffer, and the presence or absence of protease inhibitors (2, 5, 10, 12, 13, 15). Likewise, the pH of the extraction medium is believed to be important, since proteins are likely to be unstable at certain pH. Consequently, it is possible that some clinically relevant antigens are missing in current extracts because of their instability under the pH conditions used.

However, the influence of pH is not well documented: it has been shown that the amount of AgE (*Amb a I*) released from ragweed pollen is about seven- to eightfold higher in an extract prepared at pH 8.5 than one made at pH 5.5 (11). Another study (6) found some influence of pH on the composition of birch pollen extracts, as evaluated by isoelectric focusing (IEF).

Marsh et al. (11) noticed that the pH of their buffered solution tended to shift to the value imposed by the extracted pollen, and that it is necessary to add an acid or a base to adjust to the desired pH. Before the present study, we found that this applies also to other pollen species, such as birch (*Betula verrucosa*), ryegrass (*Lolium perenne*), and hazel (*Corylus avellana*), even when highly buffered solu-

tions were used (unpublished). Consequently, we decided to evaluate the effect of the pH of extraction under constant monitoring.

Only Marsh et al. (11) have used controlled pH conditions, but only to study the extraction rate of a single ragweed antigen, while others (6) have studied the effect of pH on the composition of birch pollen extracts, using buffer solutions without pH monitoring.

In the present study, we describe the influence of rigorously controlled pH on the protein profile of birch pollen extracts.

Material and methods

Pollen

B. verrucosa pollen was obtained from H.A.L. (Haarlem, The Netherlands) (Allergon batch number 021485101). It was >95% pure and contained 0.32% detectable spores and 0.1% foreign pollen.

Human sera

Sera from birch pollen-allergic patients were selected from the serum bank in our clinical laboratory. All had CAP values >17.5 units to birch pollen. Sera

with IgE titers > 2000 units, as measured by CAP, were discarded.

Extraction

Pollen was extracted 1/10 (w/v) in distilled water at room temperature for 3 h under constant agitation. The natural pH of such extracts was 5.5. We carried out extractions at pH 6.5, 7.5, and 8.5, using a 632 pH-meter with a 665 Dosimat and a 614 Impulso-mat device (Metrohm, Switzerland), which automatically and permanently adjusts to a preset pH (± 0.1) by addition of 1 M NaOH to the medium. The pH was stable from the first minute of extraction.

The extracts were centrifuged (2×30 min, 17500 g, 4°C), sterilized through a 0.45- μ m membrane (Gelman, Michigan, USA), aliquoted, and kept at -20°C until use.

Protein content

Protein content was determined by the Coomassie brilliant blue assay, according to the manufacturer's instructions (BIO-RAD, California, USA). BSA was used as a standard.

SDS-PAGE

SDS-PAGE was performed in $1.5 \times 120 \times 140$ mm slab acrylamide gels (T5C3 in the stacking gel and T14C3 in the separation gel) by the discontinuous buffer system of Laemmli (9). Samples were heated at 100°C for 7 min in sample buffer pH 6.8 containing Tris 125 mM, glycerol 17.5%, SDS 4%, β -mercaptoethanol 1%, and bromophenol blue 0.002%. The electrophoresis was performed at 150 V in the stacking gel and at 350 V in the separation gel. Low molecular weight markers (Pharmacia, Sweden) were included for molecular weight determination, and the gels were stained with Coomassie brilliant blue, or used for immunoblotting as described below.

Isoelectric focusing (IEF)

IEF was performed with the LKB 2117 Multiphor II (LKB Pharmacia, Sweden) in 0.5-mm-thick polyacrylamide gels (T5C3) containing 7.5% pH-3-10 ampholytes (Isolab, Ohio, USA) poured on Gelbond PAG film (Pharmacia, Sweden). The cathodic and anodic solutions were 1 M NaOH and 1 M H_3PO_4 , respectively. The running conditions were 1500 V, 25 mA, 15 W for a total of 1250 Vh. The gels were either stained with Coomassie brilliant blue or used for immunoblotting.

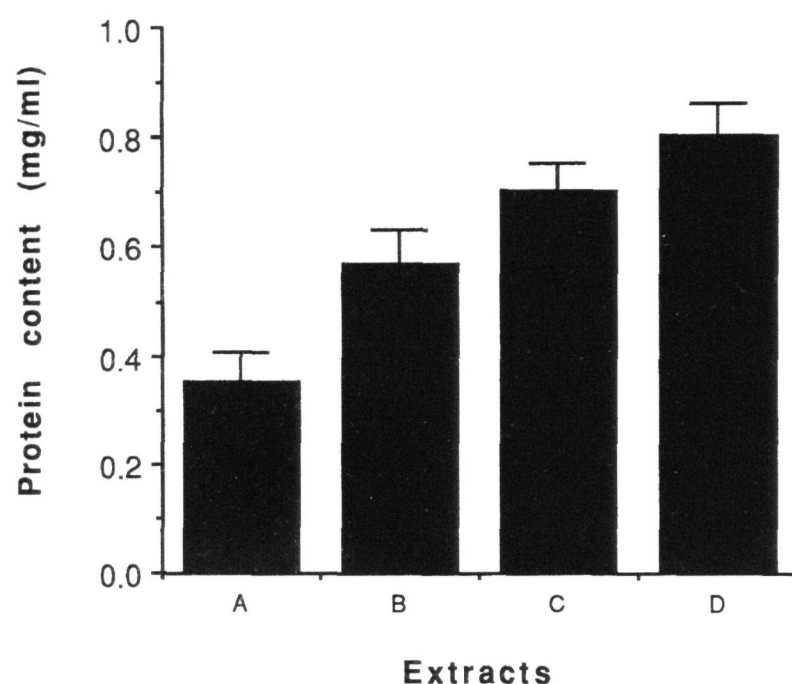


Fig. 1. Determination by Coomassie brilliant blue method of protein content of birch pollen extracts prepared at pH 5.5 (A), 6.5 (B), 7.5 (C), and 8.5 (D). Mean $\pm$ SD of three experiments. Log-lin correlation: $r = 0.96$; $P < 0.001$.

Immunoblotting

Protein bands from the SDS-PAGE gels were electrophoretically transferred to a PVDF membrane

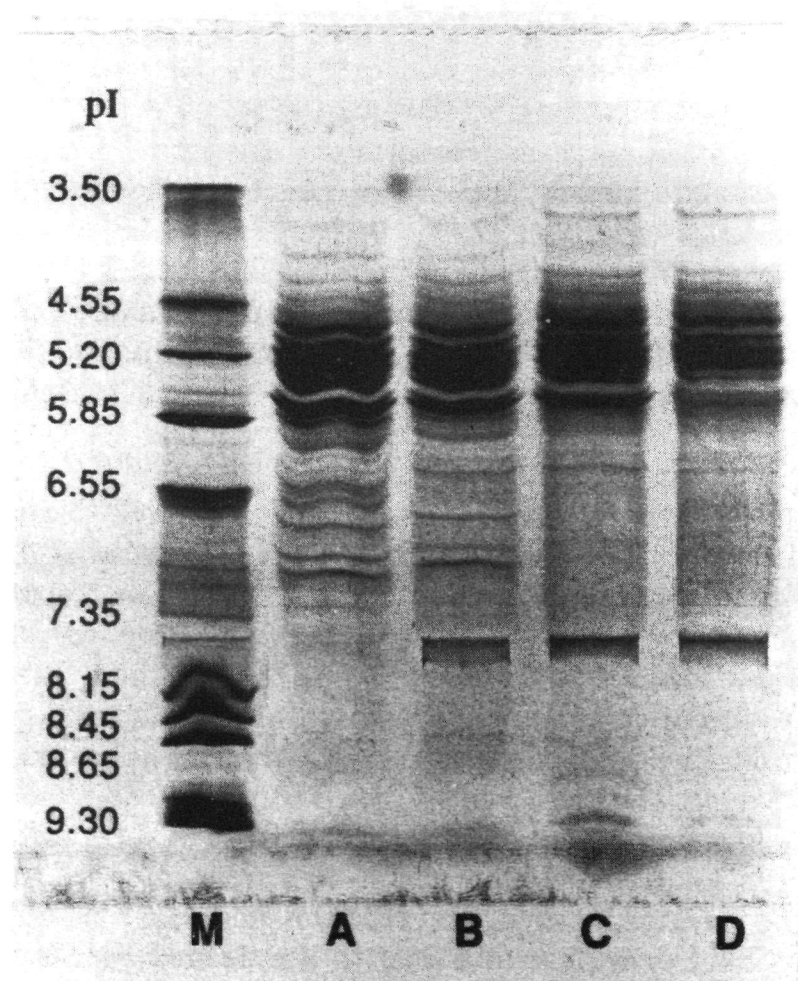


Fig. 2. Coomassie brilliant blue-stained IEF gel showing protein patterns of birch extracts prepared at pH 5.5 (A), 6.5 (B), 7.5 (C), and 8.5 (D). Amount of 20 μ g protein was applied per lane. Markers (M) were included for isoelectric point determination.

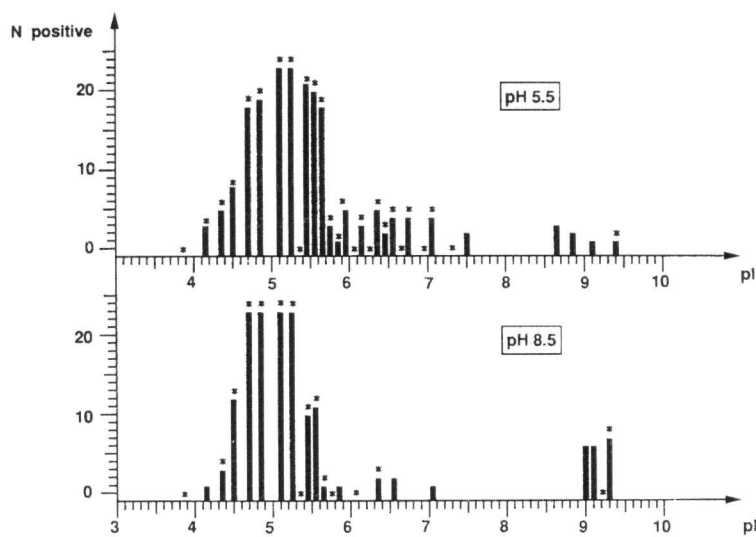


Fig. 3. Number out of 23 patient sera reacting with birch pollen proteins on immunoblots from IEF gels. Asterisk indicates bands revealed by Coomassie brilliant blue staining.

(Immobilon, Millipore, Massachusetts, USA) in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) under 60 V for 5 h at 4°C. For IEF gels, press-blotting was used to transfer the proteins to a PVDF membrane. In both cases, after transfer, the membrane was saturated for 2 h with phosphate-buffered saline (PBS) containing 0.2% nonfat dry milk (PBS-NFDM 0.2%) before 0.5-cm-wide strips were cut and incubated overnight with patients' sera diluted 1/8 in PBS-NFDM 0.1%. Bound IgE was then detected by monoclonal mouse antihuman IgE antibodies (Janssens, Belgium), visualized by peroxidase-conjugated rat antimouse light-chain kappa antibodies (Janssens, Belgium), TMB being used as a substrate.

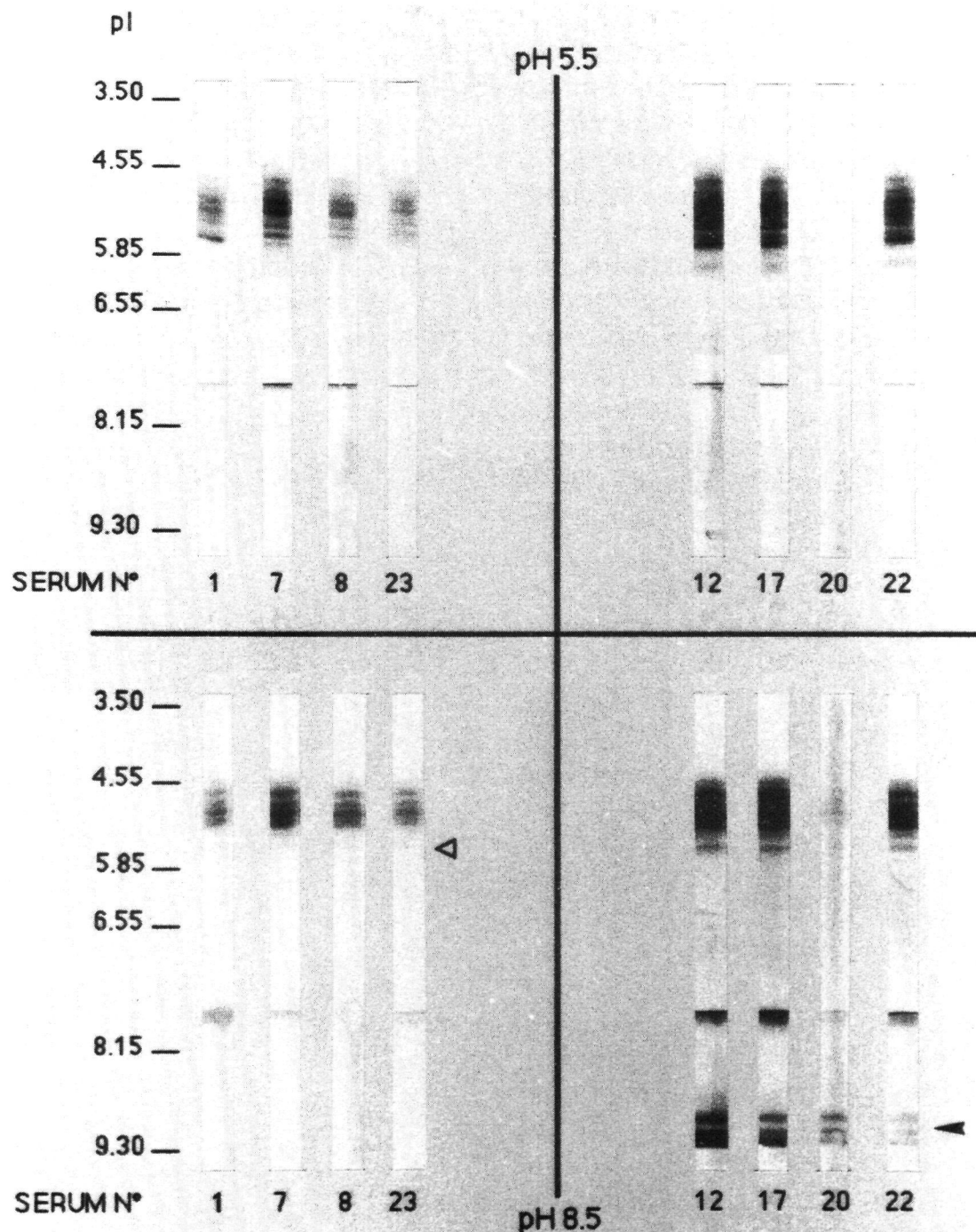


Fig. 4. IgE immunoblots from IEF patterns of birch pollen extracts made at pH 5.5 (upper part) and pH 8.5 (lower part of figure). Strips were selected to show (left) disappearance of bands at pI = 5.45–5.65 (◁) and (right) appearance of bands at pI = 9, 9.10, and 9.30 (◀).

Results

We extracted proteins from birch pollen in distilled water for 3 h at room temperature under several pH conditions. The pH was constantly monitored and adjusted. The extracts were then evaluated by protein content measurement, and by IEF and SDS-PAGE combined with Coomassie brilliant blue staining and immunoblotting.

Protein content

Protein content was measured in four extracts prepared at pH 5.5, 6.5, 7.5, and 8.5. Results are presented in Fig. 1 and indicate that the higher the extracting pH, the better the extraction yield. The relationship of yield and pH was logarithmic ($r = 0.96$) and statistically significant ($P < 0.001$).

Analysis of profiles by IEF

Protein profile. Extracts made at pH 5.5, 6.5, 7.5, and 8.5 were analyzed by IEF. The Coomassie brilliant blue-stained IEF gel of birch pollen extracts (Fig. 2) showed that an increase in the pH of the extraction fluid left bands from pI 4.35 to 5.75 relatively unaffected, while there was progressive fading of bands from pI 5.85 to 9. A basic band (pI 9.30) was visible only at pH 7.5 and 8.5. A total of 27 bands could

be detected at pH 5.5, while extracts made at pH 8.5 displayed only 16 bands.

Allergen profile. Extracts made at pH 5.5 and extracts made at 8.5 were assayed for IgE binding by immunoblotting with 23 birch-sensitive serum samples and one negative control (Fig. 3).

At pH 5.5, seven major allergens (recognition frequency: 78–100%) were detected in the pI region 4.70–5.65. Fourteen proteins from pI 5.75 to 9.40 were minor allergens, recognized by only 4–21% of the samples.

Birch extracts made at pH 8.5 showed a different recognition pattern, since only four of the seven major allergens, in the pI range 4.70–5.25, were detected by most sera. Indeed, the recognition frequency dropped from 91, 87, and 78% to 43, 49, and 4%, respectively, for the bands at pI 5.45, 5.55, and 5.65. At pH 8.5, seven bands between pI 5.75 and 8.85 disappeared, and the recognition frequency decreased for most of the others.

In the basic region, two antigens at pI 9 and 9.30 were detected by 26 and 30% of the sera, respectively, in extracts at pH 8.5 only. A third antigen (pI 9.10) was recognized more frequently in a pH 8.5 extract.

The differences in total immunoblot patterns of individual sera for extracts at pH 5.5 and 8.5 are shown in Fig. 4.

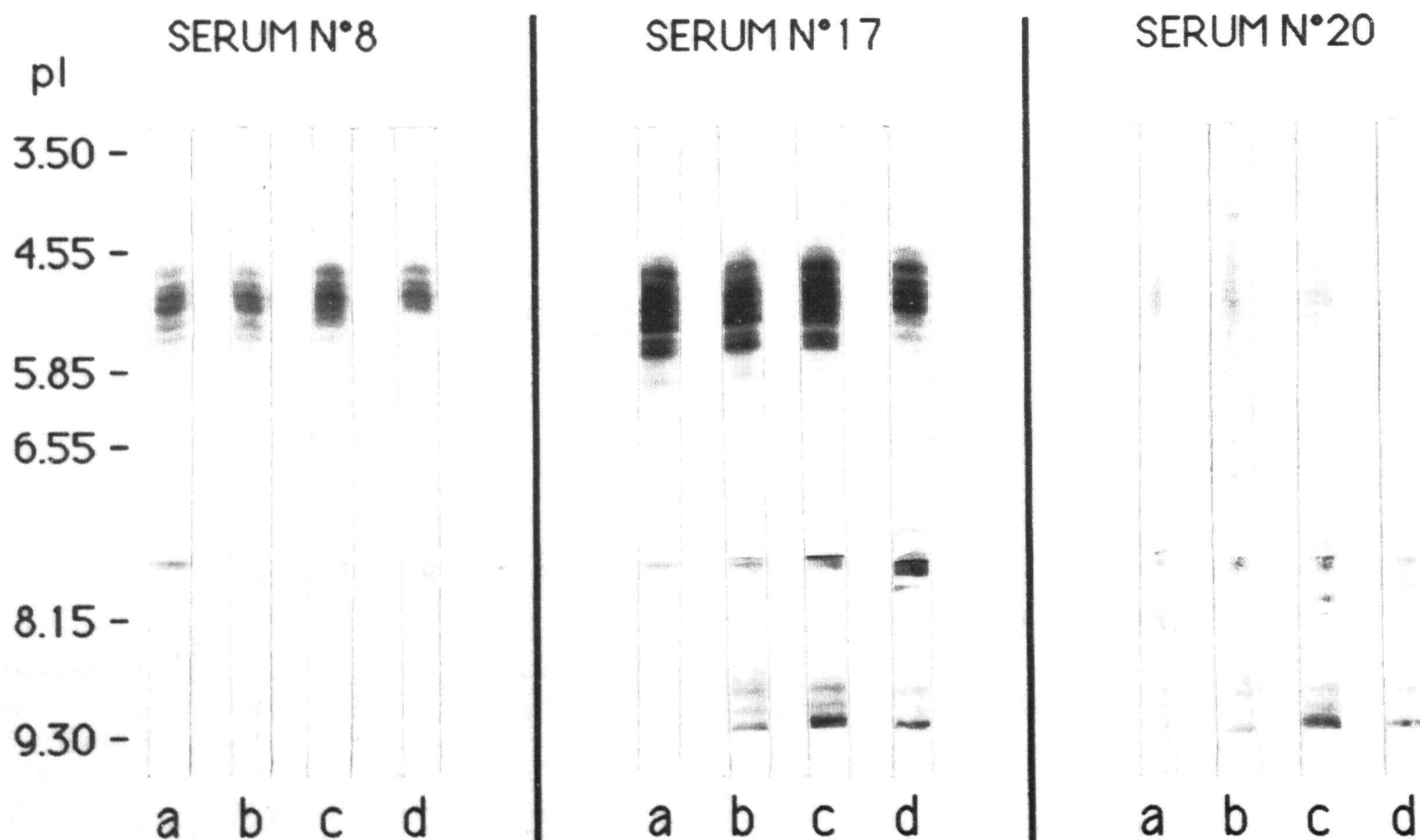


Fig. 5. IgE immunoblots from IEF patterns of birch pollen extracts made at pH 5.5 (a), 6.5 (b), 7.5 (c), and 8.5 (d). Three representative sera were used to probe strips; pI values are indicated on left.

For demonstration of the possibility of selection of an appropriate extraction pH, extracts made at pH 5.5, 6.5, 7.5, and 8.5 were probed with three sera (Fig. 5). Fig. 5 shows that, in extracts at pH 6.5 and 7.5, all allergens present in extracts made at higher and lower pH were visualized.

Analysis of profiles by SDS-PAGE

Protein profile. Extracts made at pH 5.5, 6.5, 7.5, and 8.5 were analyzed by SDS-PAGE (Fig. 6). Some of the bands (18 and 37 kDa) became more intense with increasing pH, while others (15, 17, 20, 29, 62, 67, and 72 kDa) tended to fade. One band (28 kDa) remained apparently unaffected. The band containing the major allergen *Bet v I* (17 kDa) was present in every extract, although the size of the band decreased at increasing pH.

Allergen profile. The proteins on blots from SDS-PAGE gels (Fig. 7) were probed with the same 24-serum samples that were used for the immunoblots from IEF gels.

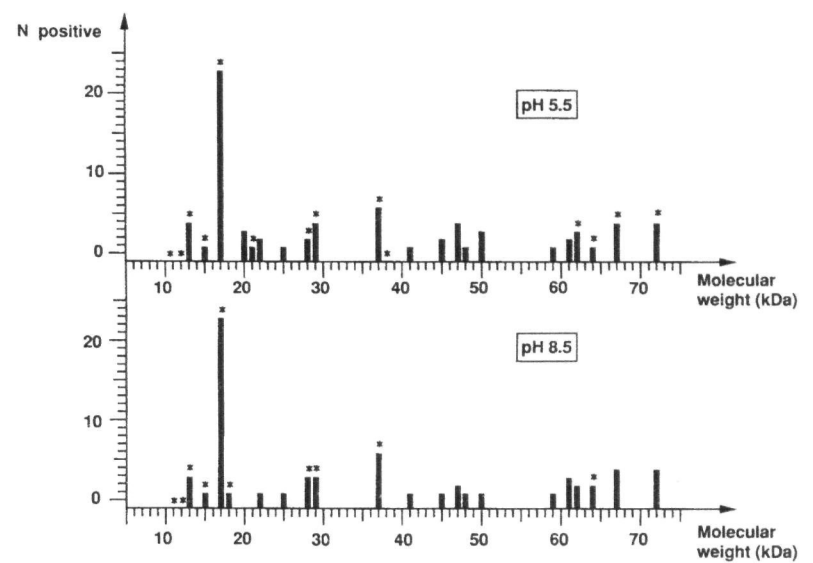


Fig. 7. Number out of 23 patient sera reacting with birch pollen proteins on immunoblots from SDS-PAGE gels. Asterisk indicates bands revealed by Coomassie brilliant blue staining.

The *Bet v I* band (17 kDa) was recognized by all the patients' sera in both extraction conditions. The other bands were minor allergens, revealed by only 4–17% of the sera. A band at 13 kDa, which may correspond to profilin, an allergen involved in cross-reactivities among plant species (18), was detected by 17% of the samples.

Discussion

We have compared extracts from birch (*B. verrucosa*) pollen made at different pH. pH was monitored during extraction and continuously adjusted to preset values in the range 5.5–8.5. Such continuous monitoring is mandatory in studies on extraction pH, because a stable pH, other than the pH imposed by the pollen components, cannot be achieved by any buffer solution. Indeed, in preliminary experiments, we have found that buffers of different composition (acetate, phosphate, Tris, bis-Tris, HEPES, lysine, and biphosphate) and of different molarity (0.01–0.3 M) did not stabilize the pH during extraction of ryegrass (*L. perenne*) pollen. The same applies to birch pollen (unpublished). Consequently, stabilization by controlled addition of a base or an acid seems to be the only means to stabilize the pH during pollen extraction.

We found one earlier study done under constant pH monitoring (11). In this study, the amount of ragweed AgE (*Amb a I*) extracted within the first 15 min was shown to be seven- to eightfold higher at pH 8.5 than at pH 5.5. Similarly, our present results demonstrate that the total protein content was significantly increased in alkaline birch pollen extracts. Furthermore, we found that pH influences not only the extraction yield, but also the relative quantities of individual antigens in birch pollen extracts. Indeed, Coomassie brilliant blue-stained IEF

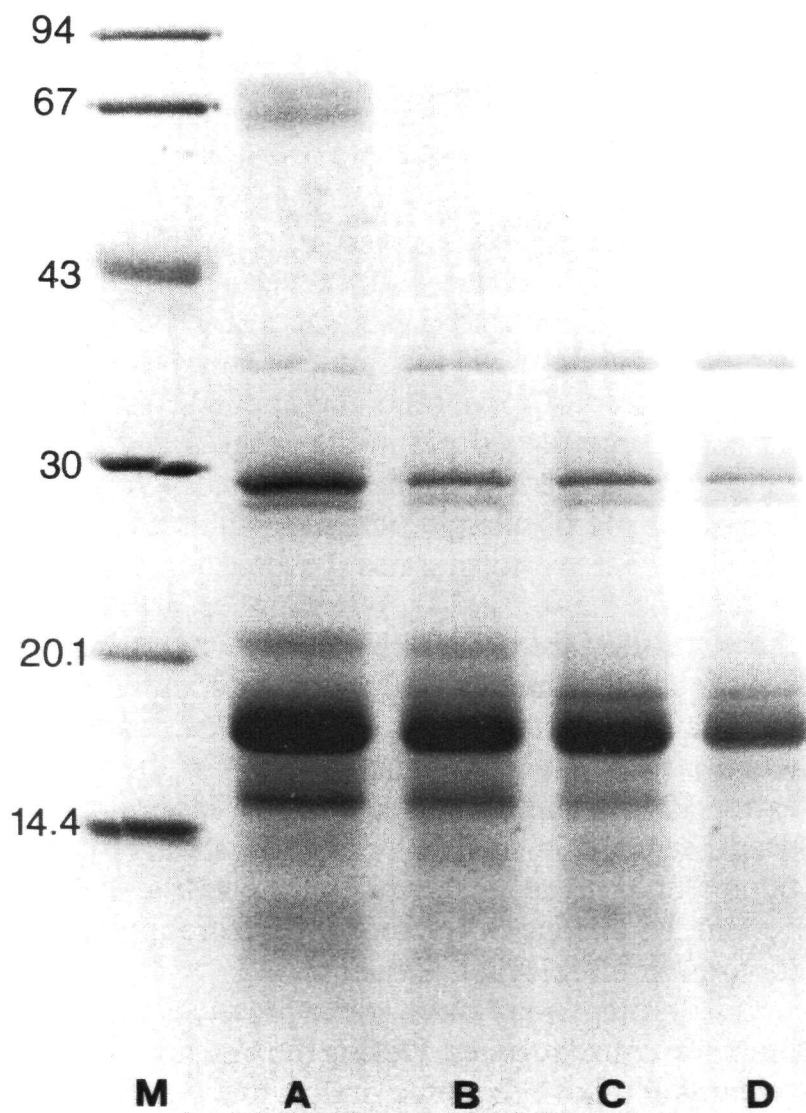


Fig. 6. Coomassie brilliant blue-stained SDS-PAGE gel showing protein patterns of extracts made at pH 5.5 (A), 6.5 (B), 7.5 (C), and 8.5 (D). Amount of 20 µg protein was applied per lane. Molecular markers (in kDa) are represented in lane M.

and SDS-PAGE gels show dissimilar patterns for extracts prepared at different pH (Figs. 2 and 3). Although Coomassie brilliant blue stainings can be evaluated only semiquantitatively, their results strongly suggest that proteins from pI 6 to 7 are selectively decreased in an extract at pH 8.5 compared to an extract at pH 5.5, while proteins in the terminal regions of the pI range tend to become more prominent. Accordingly, some bands disappear with increasing pH in SDS-PAGE patterns, while others become visible or more intense.

The total number of bands decreases in the IEF pattern of alkaline extracts, while it remains about the same in SDS-PAGE. This may be explained by the fact that a cluster of bands in IEF gels may represent isoforms of the same protein. The disappearance of such a protein may thus result in the disappearance of several bands. Isoforms have the same molecular weight. Thus, they are present in an SDS-PAGE pattern as a single band. Conversely, proteins of different molecular weight may share the same pI, and this may account for the persistence of a band in an IEF analysis although one of its components has disappeared from the SDS-PAGE pattern.

At this point, it should be emphasized that for every sample we applied the same quantity of protein in comparative electrophoresis experiments. This requires dilution up to threefold for the high protein content extracts made at pH 8.5. This dilution might result in the disappearance of some bands in Coomassie brilliant blue-stained gels or immunoblots, compared to the original, nondiluted extract. Consequently, in our experiments, the absence at pH 8.5 of bands that are present in a pH 5.5 extract may be the consequence of dilution.

For extracts made at pH 8.5, the IgE immunoblots from IEF gels demonstrated an important decrease in IgE binding to allergens at pI 5.45, 5.55, and 5.65, which probably represent isotypes of *Bet v I* (7, 8, 16). Indeed, detection was lost for most sera. Conversely, three bands at pI 9, 9.10, and 9.30 were detected at pH 8.5 by six, six, and seven out of 23 sera, respectively, whereas two of these three bands were absent in immunoblots of pH 5.5 extracts. For extracts at pH 5.5, only two out of 23 patients reacted with one antigen in the basic terminal region of the pI range. The importance of the three basic allergens at pH 8.5 is strengthened by the fact that they intensely reacted with some sera which react only weakly with other components (e.g., serum 20 in Fig. 4). Membrane filtration experiments indicate that the molecular mass of these proteins is higher than 10 kDa (data not shown), indicating that they are not small split products. On IEF immunoblots, their IgE binding was not inhibited by dilution of the sera in 10 mM EGTA (data not shown). Thus,

we may assume that they are not related to the recently described *Bet v III* (17).

Until now, only one (1) of several authors (1, 4, 7) has mentioned the existence of an IgE-binding component in the basic pI range of birch pollen patterns. As there was no mention of pH monitoring during the extraction, the extracts used in these studies were probably prepared at the pH 5.5 imposed by the pollen components. This may account for the loss of the basic proteins.

We did not find important differences in SDS-PAGE immunoblot patterns between extracts of different pH. Particularly, sera showing reactivity in the basic region with IEF did not show any new band on immunoblots from SDS-PAGE (data not shown). However, it is possible that the differences observed with immunoblots from IEF relate to allergens with only conformational epitopes that are denatured before analysis by SDS-PAGE. Proteins that are allergenic only by virtue of conformational epitopes have already been described for birch (17) and ryegrass pollen (3).

The pH-dependent differences we have described are not restricted to birch pollen extracts. We have observed similar effects on ryegrass and hazel (*C. avellana*) pollen: some proteins are lost and others appear, depending on the extraction pH (unpublished results).

To demonstrate clearly the pH influence, we first compared extracts made at two very distinct pH values, namely, 5.5 and 8.5. Additional immunoblotting experiments showed that at intermediate pH, 6.5 and 7.5, extracts contain the compilation of all allergens present in the extracts at pH 5.5 and at pH 8.5. Therefore, we recommend that birch pollen extracts for diagnostic and therapeutic use be prepared at a monitored pH in the range 6.5–7.5. This range is consistent with the neutral to slightly alkaline nasal pH of allergic patients, which may reach pH 8.0, whereas normal nasal secretions are rather acidic (pH 5.5–6.5) (14). When pollen grains enter the nose, they are likely to be locally extracted at the pH of the nasal mucus. Indeed, it is difficult to imagine that the minute amount of pollen that reaches the nasal airway could influence the pH of the natural extraction medium. Consequently, a laboratory extract prepared in neutral or slightly alkaline conditions would more closely match the extraction process in the allergic nose, and is therefore more likely to contain all relevant antigens.

Our findings may have some practical and some theoretic consequences. During the last several years, researchers have become more interested in the analysis of peptide sequences of antigens and epitopes than in the extraction conditions for crude extracts. However, variables that determine the outcome of extraction procedures appear to be of pri-

mary importance in detecting all molecules with allergenic activity. This is essential to establish the complete set of epitopes that are important for allergic sensitization. Furthermore, any sequence analysis starts from a purified single molecule. The preparation of an appropriate quantity of such a molecule may be greatly facilitated when adequate extraction conditions, such as optimal pH, are determined.

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References

1. ARNTZEN FC, WILHELMSSEN TW, LØWENSTEIN H, et al. The international collaborative study on the first international standard of birch (*Betula verrucosa*)-pollen extract. *J Allergy Clin Immunol* 1989; 83: 66–82.
2. BARANIUK JN, BOLICK M, ESCH R, BUCKLEY CE III. Quantification of pollen solute release using pollen grain column chromatography. *Allergy* 1992; 47: 411–17.
3. EKRAMODDOULLAH EKM, KISIL FT, SEHON AH. Immunochemical characterization of a high molecular weight basic allergen (HMBA) of ryegrass (*Lolium perenne*) pollen. *Mol Immunol* 1983; 20: 465–73.
4. FLORVAAG E, HOLEN E, VIK H, ELSAYED S. Comparative studies on tree pollen allergens. XIV. Characterization of the birch (*Betula verrucosa*) and hazel (*Corylus avellana*) pollen extracts by horizontal 2-D SDS-PAGE combined with electrophoretic transfer and IgE immunoautoradiograph. *Ann Allergy* 1988; 61: 392–400.
5. GEISSLER W, MAASCH HJ, WINTER G, WAHL R. Kinetics of allergen release from house dust mite *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* 1986; 77: 24–31.
6. HJELMROOS M, EGEN N, SCHUMACHER M. Optimizing the extraction of allergens in *Betula* pollen as determined by isoelectric focusing in polyacrylamide gels. *Allerg Immunol* 1993; 25: 137–40.
7. IPSEN H, LØWENSTEIN H. Isolation and immunological characterization of the major allergen of birch pollen (*Betula verrucosa*). *J Allergy Clin Immunol* 1983; 72: 150–9.
8. JAROLIM E, RUMPOLD H, ENDLER AT, et al. Specificities of IgE and IgG antibodies in patients with birch pollen allergy. *Int Arch Allergy Appl Immunol* 1989; 88: 180–2.
9. LAEMMLI UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680–5.
10. LARSEN LV, COPELAND TA, WEBER RW, VAUGHN TR. Allergen extraction methods: effects of time, temperature, buffer, and protease inhibitors [Abstract]. *J Allergy Clin Immunol* 1991; 87: 186.
11. MARSH DG, BELIN L, BRUCE CA, LICHTENSTEIN LM, HUSSAIN R. Rapidly released allergens from short ragweed pollen. I. Kinetics of release of known allergens in relation to biologic activity. *J Allergy Clin Immunol* 1981; 67: 206–16.
12. MONTERO MT, ALONSO E, SAINZ T. Allergens from rye pollen (*Secale cereale*). I. Study of protein release by rye pollen during a 19-hour extraction process. Allergen identification. *Allergy* 1992; 47: 22–5.
13. PARIS S, FITTING C, RAMIREZ E, LATGE JP, DAVID B. Comparison of different extraction methods of *Alternaria* allergens. *J Allergy Clin Immunol* 1990; 85: 941–8.
14. PODOSHIN L, GERTNER R, FRADIS M. Treatment of perennial allergic rhinitis with ascorbic acid solution [Letter]. *Ear Nose Throat J* 1991; 70: 54–5.
15. PORTNOY J, PACHECO F, BALLAM Y, BARNES C. The effects of time and extraction buffers on residual protein and allergen content of extracts derived from four strains of *Alternaria*. *J Allergy Clin Immunol* 1993; 91: 930–8.
16. ROHAC M, BIRKNER T, REIMITZER I, et al. The immunological relationship of epitopes on major tree pollen allergens. *Mol Immunol* 1991; 28: 897–906.
17. SEIBERLER S, SCHEINER O, KRAFT D, LONSDALE D, VALENTA R. Characterization of a birch pollen allergen, *Bet v* III, representing a novel class of Ca^{2+} binding proteins; specific expression in mature pollen and dependence of patient's IgE binding on protein-bound Ca^{2+} . *EMBO J* 1994; 13: 3481–6.
18. VALENTA R, DUCHENE M, EBNER C, et al. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992; 175: 377–85.

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