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Note

High-performance thin-layer chromatographic analysis of dialysable materials in allergen extracts

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Allergen extracts are used in human medicine for diagnosis and therapy of IgE-mediated allergic diseases. In the crude form they are complex mixtures of proteins, glycoproteins, peptides, carbohydrates and components of low molecular weight. Low-molecular-weight components, *e.g.* free histamine and substances that may liberate histamine or other mediators in humans, are thought to cause non-specific side-reactions in patients. It has therefore been recommended that these substances should be removed from the extracts by appropriate methods[1].

An accepted procedure for the partial purification of allergen extracts is dialysis, which removes components of molecular weight below a certain cut-off limit to a high degree. However, it is necessary to verify that the allergenic activity of an extract is not affected.

This paper reports the analysis of non-dialysed and dialysed allergen extracts of the pollens of birch, six-grass mixture and English plantain and of the mould *Alternaria tenuis* by high-performance thin-layer chromatography (HPTLC). The dyeing reagents used were ninhydrin, iodine and molybdatophosphoric acid. The sensitivities of the dyeing reagents were tested with diluted solutions of histamine hydrochloride, phosphatidylcholine and glucose. In order to show that only substances of low molecular weight migrate in the HPTLC system, a mixture of pure proteins containing histamine hydrochloride was chromatographed. We estimated the carbohydrate and protein contents of the non-dialysed and dialysed extracts. We have also measured the allergenic activity of the extracts by radioallergosorbent test (RAST) inhibition.

EXPERIMENTAL

Allergen extracts

Aqueous, clear filtrated allergen extracts of the pollens of birch (*Betula verrucosa*), six-grass mixture (a mixture of equal weights of *Holcus lanatus*, *Dactylis glomerata*, *Festuca pratensis*, *Lolium perenne*, *Phleum pratense*, and *Poa pratensis*), and English plantain (*Plantago lanceolata*) and of the mould *Alternaria tenuis* were prepared by Allergopharma (Reinbek, F.R.G.). They were investigated in non-dialysed and dialysed form. The non-dialysed extracts contained 0.4% phenol as preservative.

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Dialysis

A 5-ml volume of the allergen extracts was dialysed against four changes of 5 l 0.01 *M* phosphate buffer (pH 7.5) at 4°C. Visking dialysis tubings with a cut-off of 8000–15 000 daltons (Serva, Heidelberg, F.R.G.) were used throughout all experiments. For low-molecular-weight substances, *e.g.* amino acids, the theoretical dilution factor was greater than 100.

High-performance thin-layer chromatography

The extracts were analysed by HPTLC as described by Meyer *et al.*[2], with slight modifications. The procedure was performed as follows: 1–3 μl of an extract were applied two or three times by a fine glass capillary to precoated HPTLC plates (Silica Gel 60 without fluorescent indicator, E. Merck, Darmstadt, F.R.G.). The samples were applied as 1-cm strips at a distance of nearly 1 cm from the bottom of the plate. The plates were placed into a chromatography chamber which contained the resolution system chloroform–methanol–25% ammonia–distilled water (58:32:8:2). The plates were developed for *ca.* 25–30 min. The plates were taken out of the chamber and dried for some minutes at room temperature under a hood, and then treated with iodine, ninhydrin or molybdatophosphoric acid, according to manufacturer's recommendations[3]. To check the sensitivity of the dyeing reagents, six 1:10 dilution steps of a histamine hydrochloride solution (1 mg/ml), L-α-phosphatidylcholine (100 mg/ml) (Sigma, München, F.R.G.) and glucose (1 mg/ml) were prepared and chromatographed by HPTLC. To estimate the separation potency of the method, a mixture of pure proteins of molecular weights of 12 500–67 000 daltons (Serva, Heidelberg, F.R.G.) containing different concentrations of histamine hydrochloride (0.0025–1 mg/ml) was chromatographed.

Dyeing reagents

All chemicals used were of analytical grade (E. Merck). Three different dyeing reagents were used:

(1) Iodine: many organic substances, *e.g.* carbohydrates, can be stained by placing the HPTLC plates in a chamber with some crystals of iodine. After development organic substances show brown spots[3].

(2) Ninhydrin: substances with free amino groups, *e.g.* histamine, can be detected with ninhydrin. The plates must be sprayed with a 0.1% ninhydrin spray reagent under a hood, and then heated at 110°C until visualization of the violet spots reaches a maximum[3].

(3) Molybdatophosphoric acid: phospholipids and phosphatidic acids can be detected by spraying the plates with a 3.5% molybdatophosphoric acid spray reagent under a hood. Then the plates must be heated at 120°C until visualization of the blue spots reaches a maximum. Additional treatment of the plates with ammonia vapour produces a colourless background[3].

Protein and carbohydrate measurements

Protein measurements of the extracts were done before and after dialysis by a modified Lowry assay[4]. Carbohydrates were assayed by the Orcinol method for the determination of pentoses[5] and by the Anthrone method for the determination of hexoses[6].

RAST inhibition

The allergenic potencies of the extracts were determined *in vitro* before and after dialysis by RAST inhibition[7].

RESULTS

Fig. 1 shows HPTLC patterns of non-dialysed (nd) and dialysed (d) allergen extracts of birch, six-grass mixture, and English plantain pollens and the mould *Alternaria tenuis*. Migrating substances were detected only in non-dialysed extracts: four to five individual bands could be detected with iodine, eight to ten separate bands could be detected with ninhydrin and one to three separate bands could be detected with molybdatophosphoric acid. No migrating substances were detected in dialysed allergen extracts with all three dyeing reagents.

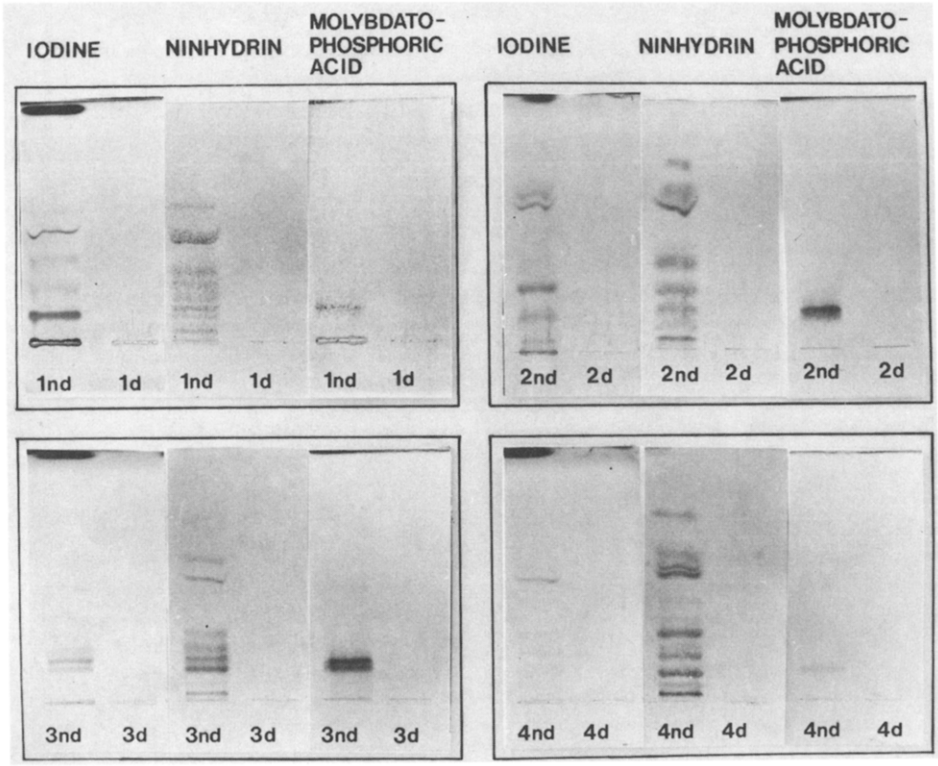

Fig. 1. HPTLC of non-dialysed (nd) and dialysed (d) allergen extracts of birch pollen (1), six-grass mixture pollen (2), English plantain pollen (3) and *Alternaria tenuis* (4), with iodine, ninhydrin and molybdatophosphoric acid used as dyeing reagents.

Histamine was clearly separated from a mixture of proteins with molecular weights of 12 500–67 000 daltons (Fig. 2). Histamine was detected with ninhydrin and had an R_F value of 0.65. All proteins remained on the application point. As can be seen in Fig. 2, the non-dialysed extracts showed similar HPTLC patterns.

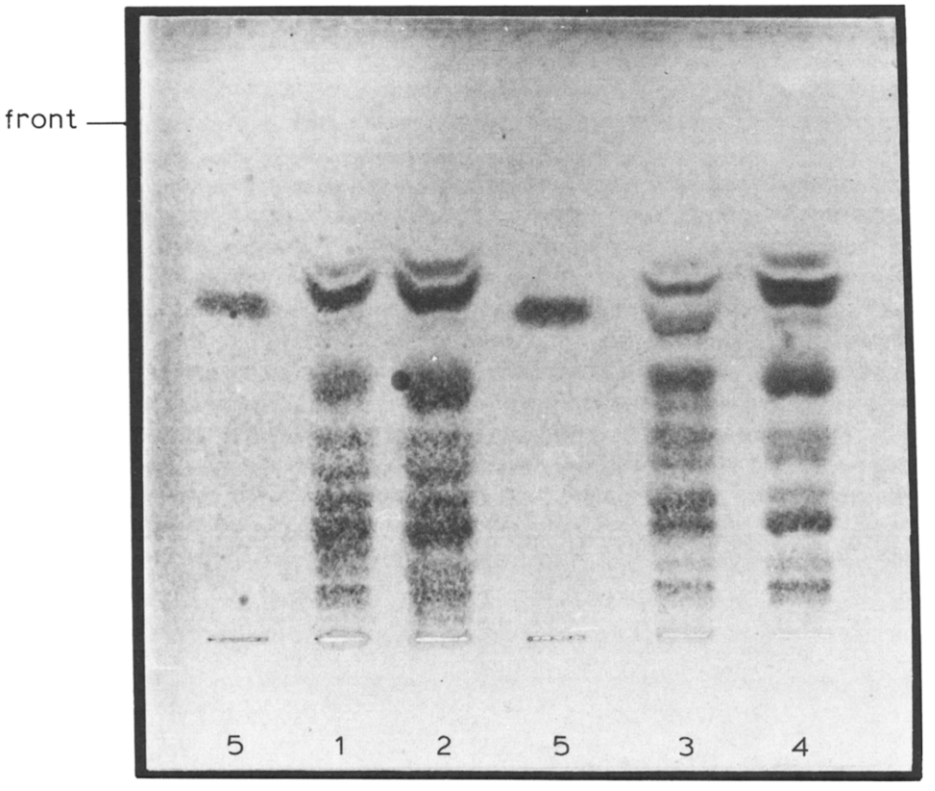

Fig. 2. Detection of NH_2-containing substances in non-dialysed allergen extracts and of histamine in a mixture of pure proteins with ninhydrin. (1) Birch pollen, (2) six-grass mixture pollen, (3) English plantain pollen, (4) *Alternaria tenuis*, (5) mixture of pure proteins (12 500–67 000 daltons) containing histamine. Only histamine could be separated (R_F = 0.65).

The lowest detection limits were 0.01 μg for phosphatidylcholine with molybdatophosphoric acid, 0.02 μg for histamine with ninhydrin and 0.2 μg for glucose with iodine.

Dialysis of crude allergen extracts also resulted in clear-cut decreases of proteins and carbohydrate concentrations in all tested samples. However, dialysis af-

TABLE I

ESTIMATION OF PROTEINS IN NON-DIALYSED AND DIALYSED ALLERGEN EXTRACTS

Allergen extracts	*Non-dialysed extracts (mg/ml)*	*Dialysed extracts (mg/ml)*	*Ratio of proteins in non-dialysed and dialysed extracts*
Birch pollen	0.810	0.651	1:0.80
Six-grass mixture pollen	1.275	0.626	1:0.49
English plantain pollen	0.842	0.581	1:0.69
Alternaria tenuis	0.440	0.135	1:0.31

TABLE II

CARBOHYDRATE MEASUREMENTS IN NON-DIALYSED AND DIALYSED ALLERGEN EXTRACTS

Allergen extracts	*Hexoses in non-dialysed extracts (mg/ml)*	*Hexoses in dialysed extracts (mg/ml)*	*Pentoses in non-dialysed extracts (mg/ml)*	*Pentoses in dialysed extracts (mg/ml)*	*Ratio of hexoses in non-dialysed and dialysed extracts*	*Ratio of pentoses in non-dialysed and dialysed extracts*
Birch pollen	9.125	0.175	0.881	0.255	1:0.019	1:0.29
Six-grass mixture pollen	5.654	0.230	1.816	0.456	1:0.041	1:0.25
English plantain pollen	7.534	0.172	1.205	0.255	1:0.023	1:0.21
Alternaria tenuis	1.339	1.132	0.201	0.093	1:0.85	1:0.46

fected the concentrations of proteins and carbohydrates to different extents, as can be concluded from the ratios of these substances before and after dialysis. The decrease of protein and carbohydrate after dialysis depends on the different raw materials used for the preparation of allergen extracts (see Tables I and II).

In contrast to this, allergenic potencies as established by RAST inhibition were virtually unaffected by dialysis. Table III shows the 50% inhibition values of the extracts and the relative potency (P_{rel}), which is calculated by forming the quotient of the 50% inhibition values of a reference and the samples.

TABLE III

INHIBITION VALUES AND P_{rel} VALUES (RELATIVE POTENCY) OF NON-DIALYSED AND DIALYSED BIRCH, SIX-GRASS MIXTURE AND ENGLISH PLANTAIN POLLEN AND *ALTERNARIA TENUIS* ALLERGEN EXTRACTS

Allergen extracts	*50% inhibition (µl)*		P_{rel}
	Non-dialysed	*Dialysed*	
Birch pollen	0.8	0.5	1.6
Six-grass mixture pollen	0.031	0.042	0.74
English plantain pollen	0.874	0.625	1.40
Alternaria tenuis	0.052	0.039	1.33

DISCUSSION

Aas[1] found that free histamine and substances that may liberate histamine or other mediators in humans are largely responsible for false positive reactions to allergen extracts in the skin, mucosa or the bronchi. Therefore, irritant materials in allergen extracts should be removed as completely as possible, *e.g.* by dialysis. However, the allergenic activity should not be affected by this purification step.

The effect of dialysis can be controlled by HPTLC in a quick, sensitive and easy way. The results indicate that two of three dyeing reagents tested have sufficiently low detection limits for assessing the efficacy of dialysis. For reasons of practicability ninhydrin is the reagent of choice, particularly because in aqueous allergen extracts low-molecular-weight substances with free amino groups are more common than phospholipids. Substances of low molecular weight, *e.g.* substances with free amino groups, could be detected in non-dialysed allergen extracts. As can be seen in Fig. 2, non-dialysed extracts of different allergen raw materials showed almost the same HPTLC patterns for substances with free amino groups. They also contained carbohydrates, phospholipids[8] and phosphatidic acids (Fig. 1). For further analysis of these substances a preparative HPTLC must be performed, and they should be analysed by different chemical bonds.

Low-molecular-weight components are removed by dialysis. These substances can no longer be detected, even if the dialysed extracts were applied to the HPTLC plates in higher concentrations than the non-dialysed extracts. The protein and carbohydrate contents of the allergen extracts were simultaneously reduced. Four to five separate migrating bands could be detected with iodine, *e.g.* carbohydrates in non-

dialysed extracts. In dialysed extracts no migrating bands could be detected. The disappearance of these bands from dialysed allergen extracts can be correlated with the decrease in carbohydrate concentration, which was very large, especially for hexoses, in the cases of the pollen allergen extracts, but not as pronounced in the case of the extract from the dialysed mould *Alternaria tenuis*. It can be assumed that the concentration of glycoproteins in the *A. tenuis* extract is higher than that in the pollen extracts. These glycoproteins do not migrate by HPTLC but are measured by the Anthrone method.

The amount by which the protein concentration decreases depends on the cut-off limits of the dialysis tubings used and on the raw materials used for the preparation of the allergen extracts. It appears as if mould extracts lose more protein during dialysis than pollen extracts. In contrast to these results, the allergenic potencies of the four allergen extracts measured by RAST inhibition were virtually unaffected by dialysis. The measured changes of the relative potency (P_{rel}: 0.74–1.6) were within the limits of acceptance for the applied method (P_{rel}: 0.47–2.13)[9].

In conclussion, our investigations show that HPTLC is a quick, cheap, easy to perform, and sensitive method for the determination of low-molecular-weight substances in allergen extracts. Dialysis can be monitored by measuring the concentration decrease of low-molecular-weight components.

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