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Glycoprotein Allergens in Pollen of Timothy

III. Immunochemical and Biological Properties of a Basic Glycoprotein

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Abstract. Crossed immunoelectrophoresis (CIE) and tandem CIE indicated that a basic glycoprotein allergen isolated from pollen of timothy is immunochemically identical to the allergen formerly denoted as antigen 30. The glycoprotein gave one band in SDS-PAGE and agarose isoelectric focusing. After transfer to nitrocellulose by electroblotting or by capillary blotting, the allergen specifically bound IgE from persons allergic to timothy pollen. The single allergen inhibited about 15% of the IgE binding to radioallergosorbent test discs of whole timothy pollen extract. Sera from patients investigated also contained detectable amounts of IgG towards the allergen. In quantitative skin prick tests, the mean activity of 2 µg/ml purified allergen in 7 patients was equivalent to that of a timothy pollen extract containing 10 000 BU/ml (assayed relatively to histamine in skin prick test). The ability of the allergen to form a precipitate in rocket immunoelectrophoresis was lost when it was heated to 80°C or incubated with 4 M guanidine hydrochloride.

Introduction

It has been demonstrated by crossed radioimmunoelectrophoresis (CIE) that extracts of timothy pollen contain 11 different allergens [1]. One of the allergens has recently been isolated in our laboratory [2]. It was demonstrated to be a basic glycoprotein containing 9.6% of carbohydrate. Regarding properties as migration in CIE and amino acid composition, the glycoprotein resembled the allergen formerly denoted as antigen 30 [3]. This was later classified as an intermediate allergen [4]. It has been recently proposed that antigen 30 should be named Phl.p. III [5] or Phl.p. VII [6].

The aim of the present investigation has been to assess the biological activity of the isolated glycoprotein and to further characterize it immunochemically. In particular, we wanted to compare the results obtained by immunoelectrophoretic techniques [1-4] with the results of the more recently developed immunoblotting techniques. During the past few years, such techniques have become important also in al-

lergen research: they have been used to study cereal grain allergens [7], mite allergens [8] and the allergenic activity of orchard grass (*Dactylis glomerata*) pollen extract [9].

The IgE binding of a raw and a partly purified timothy pollen extract has been studied after isoelectric focusing and capillary blotting [10]. But as far as we know, immunoblotting techniques have not been used to screen sera from timothy pollen allergics or to study the binding of IgE to one pure timothy pollen allergen. Thus, this technique was not used in the international collaborative study establishing the first international standard for timothy (*Phleum pratense*) grass pollen allergenic extract [5].

The immunoblotting technique involves separation of the allergens by methods as sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis (SDS-PAGE) [7, 8] or isoelectric focusing [9, 10], then transfer to a nitrocellulose membrane by either electroblotting [7, 8] or capillary blotting [9, 10] and finally immunological detection on the nitrocellulose membrane. The isolated glycoprotein is easily dena-

tured [11] and we wanted to study if its IgE-binding ability was maintained after SDS-PAGE and transfer to nitrocellulose. Furthermore, we wanted to investigate if sera from timothy pollen allergics also contained specific IgG to this allergen.

Materials and Methods

Materials

The glycoprotein allergen was isolated from pollen of timothy as described previously [2].

Rabbit antiserum to whole timothy pollen extract (batch 614/12), CNBr-activated paper discs (BK-4/26), serum from patients allergic to timothy pollen [2], timothy pollen extract (Spectralgen® Prikk; 10,000 BE/ml; batch 411532) and histamine reference were obtained from Nycomed (Oslo, Norway).

Agarose immunoelectrophoresis tablets with tricine buffer, pH 8.60, acrylamide, N,N-methylene bisacrylamide, N,N,N,N-tetra-methylethylene diamine and nitrocellulose membrane were obtained from Bio-Rad Laboratories (Richmond, Calif., USA).

Agarose isoelectric focusing, carrier ampholyte for isoelectric focusing (Pharmalyte®; pH 3–10), ¹²⁵I-labeled antihuman IgG and Gel-bond® film were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Fuji RX medical X-ray film was obtained from Fuji Photo Film Co. (Japan). X-ray film cassettes and Titan 2HS intensifying screens were obtained from Siemens (FRG). Kodak LX 24 X-ray developer and Kodak AL 4 X-ray fixer were obtained from Kodak (UK). Rabbit immunoglobulins to sheep immunoglobulins (lot 073A) were obtained from Dako immunoglobulins A/S (Denmark).

Immuno-electrophoretic Techniques

Rocket immunoelectrophoresis (RIE), CIE and tandem CIE were performed essentially as described by Axelsen and Bock [12], Høiby and Axelsen [13] and Krøll [14], respectively. CIE was performed in 25 mM tricine buffer, pH 8.60, with Tris-tricine (9.8 and 4.3 g/l), pH 8.60, as electrode buffer. In RIE, 3 µg of the purified allergen was applied and the cathodic gel contained 4 µl/cm² of the antibody preparation against timothy pollen extract raised in sheep [2]. In the denaturation experiments, samples of the allergen were incubated with 4 M urea or 4 M guanidine hydrochloride for 24 h, heated to 60, 70 or 80°C for 5 min, or frozen and thawed before RIE was performed. In some experiments, 25 µl/cm² of an antiserum to sheep immunoglobulins (Dakopatt Z 138) was incorporated in intermediate gels to prevent the formation of an anodic precipitate in RIE. To investigate if the patient serum contained precipitating antibodies to the glycoprotein, RIE was performed using a cathodic gel containing 20 µl/cm² of the serum pool [2]. RIE with both 4 µl/cm² sheep antibody preparation to timothy pollen extract and 20 µl/cm² of the serum pool from timothy pollen allergics was also performed. In CIE and tandem CIE, 7.5 µg purified allergen and/or 7 µl timothy grass allergenic extract diluted 1:10 (Pharmacia lot CD 9847) were applied. The second-dimension gels in these experiments contained 17.5 µl/cm² of a rabbit antiserum to timothy pollen extract (Nycomed, batch 614/12). Detection of IgE-binding precipitates was performed by the radioimmuno-

electrophoresis technique of Weeke et al. [15], as described previously [2].

Electrophoresis in Agarose Gel

2.5 and 5 µg purified allergen were subjected to electrophoresis at 10 V/cm for 1 h in 1% agarose gel using the same buffer as in immunoelectrophoresis. The glycoprotein was fixed in a solution of trichloroacetic acid (155 g/l) and sulfosalicylic acid (34 g/l), pressed, dried and stained with Coomassie brilliant blue R-250.

SDS-PAGE

SDS-PAGE was performed in a linear 7.5–20% polyacrylamide gradient with a stacking gel of 4% polyacrylamide using the buffer system described by Laemmli [16]. Approximately 300 µg raw timothy pollen extract and approximately 20 µg purified allergen were applied and electrophoresis run at 100 V for 18 h. One part of the gel was stained with Coomassie brilliant blue R-250, one part with the periodic acid-Schiff (PAS) glycoprotein stain [17] and the rest of the gel was subjected to electroblotting.

Electroblotting

The proteins were transferred from the polyacrylamide gel to a nitrocellulose sheet (Trans-blot® nitro-cellulose membrane 0.45 µm, Bio-Rad) using a Trans-blot (Bio-Rad) electroblotting cell. The transfer was performed at 60 V for 5 h in 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.30, at 18°C [18].

Isoelectric Focusing Combined with Capillary Blotting

Raw extract of timothy pollen and purified allergen were subjected to isoelectric focusing on 0.5 mm agarose gel and capillary transfer to a nitrocellulose sheet (Trans-blot nitrocellulose membrane 0.45 µm, Bio-Rad), essentially as described by Peltre et al. [9].

Detection of Allergens on Nitrocellulose

The nitrocellulose sheets were incubated in 50 mM phosphate buffer, pH 7.40, containing 9 g/l NaCl, 3 g/l dextran 70 and 0.5% Tween 20 for 1 h at 37°C to block unoccupied binding sites on the membrane. The blots were then cut into strips of appropriate size and incubated with patient sera diluted 1:5 in blocking buffer overnight at room temperature. They were then washed with 0.9% NaCl containing 0.5% Tween 20 for 3×10 min and incubated with ¹²⁵I-anti-IgE (Phadebas RAST®) diluted 1:5 in blocking buffer for 4 h at room temperature. The ¹²⁵I-anti-IgE incubated strips were washed with 0.9% NaCl containing 0.5% Tween 20 for 3×10 min and with distilled water for 2×10 min, dried and subjected to autoradiography at -80°C for 1–3 days [2].

To investigate binding of IgG to the allergen the nitrocellulose strips were incubated with patient sera diluted 1:20 in blocking buffer overnight at room temperature. They were then washed as described above and incubated with ¹²⁵I-anti-IgG (Phadebas IgG-RAST®) diluted 1:5 in 0.1 M phosphate buffer, pH 7.40, containing 9 g/l NaCl, 0.5% Tween 20 and 10 g/l bovine serum albumin for 4 h at room temperature. Washing and autoradiography were then performed as in detection of IgE binding. Sera from 47 timothy pollen allergics were tested by this technique.

Radioallergosorbent Test Inhibition

Radioallergosorbent test (RAST) inhibition using RAST discs of whole timothy pollen extract [11] was performed by a modification [19] of the method of Yman et al. [20].

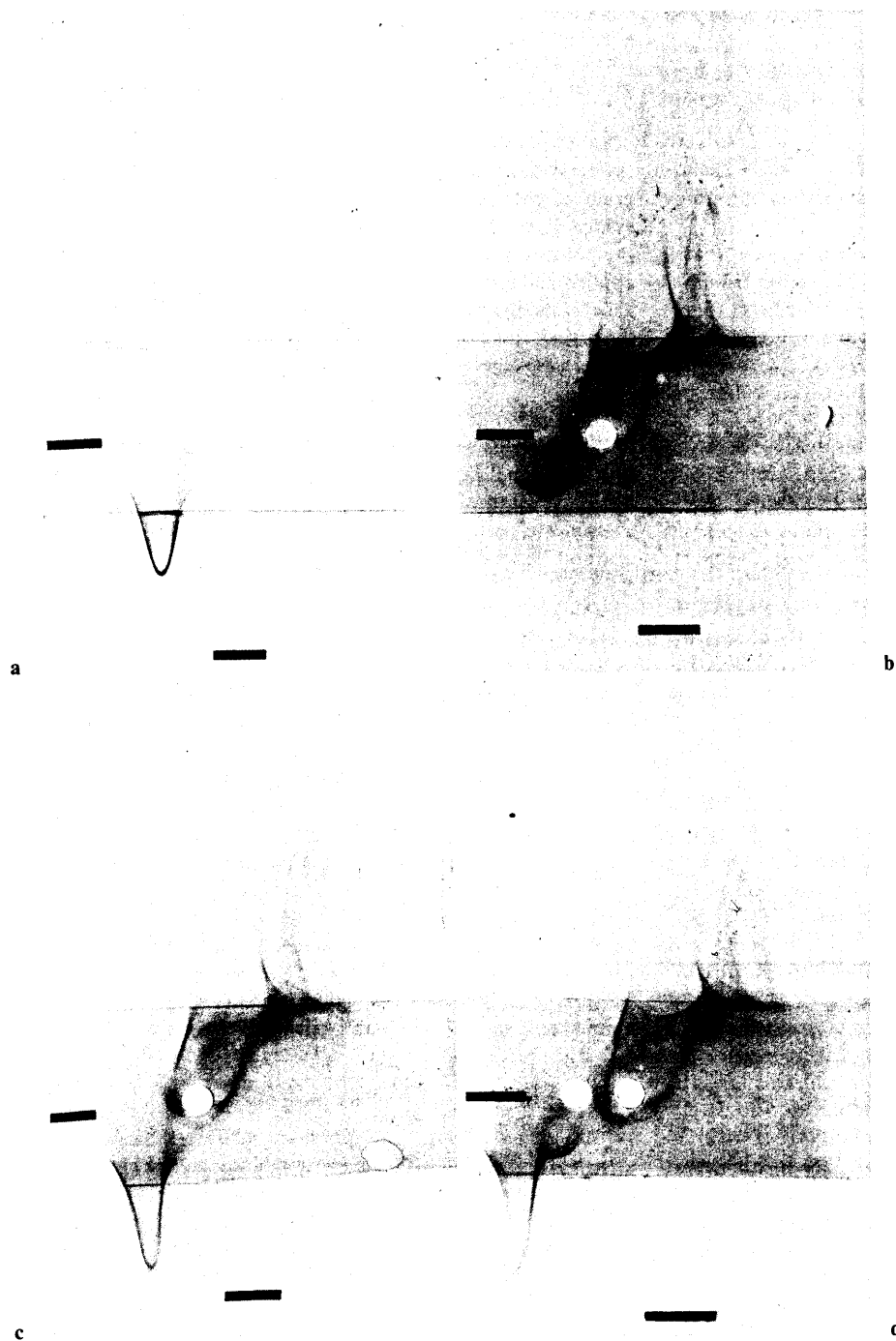


Fig. 1. CIE [13] plates stained with Coomassie brilliant blue. The first-dimension electrophoresis was performed at 10 V/cm for 30 min in 1% agarose with tricine buffer pH 8.60 (Bio-Rad) and the second-dimension electrophoresis at 2 V/cm for 16 h into similar agarose gel containing 17.5 $\mu\text{l}/\text{cm}^2$ rabbit antiserum to timothy pollen extract. **a** Allergen isolated from timothy pollen [2]. **b** Timothy pollen raw extract. **c** Timothy pollen raw extract with purified allergen added. **d** Tandem CIE [14] of timothy pollen raw extract (right) and purified allergen.



Fig. 2. RIE [12] of 3- μ g samples of the purified allergen [2]. The electrophoresis was performed at 2 V/cm for 16 h into 1% agarose pH 8.60 (Bio-Rad) containing 4 μ l/cm² antibody preparation against timothy pollen extract [2]. **a** Stained with Coomassie brilliant blue. **b** Corresponding autoradiograph after incubation with serum pool from timothy pollen allergics and ¹²⁵I-labeled anti-IgE [2]. Prior to the electrophoresis the allergen solution was heated for 5 min at 60°C (1), 70°C (2), 80°C (3) or incubated for 24 h at 4°C with 4 M guanidine hydrochloride (4), 4 M urea (5) or frozen at -20°C and thawed (6).

Skin Prick Test

Skin prick test of 100–0.01 μ g/ml of the purified allergen dissolved in 0.9% NaCl and timothy pollen extract (Spectralgen Prikk Timotei® 10,000 BU/ml [21]) was performed with lancets in duplo on the volar side of both forearms of 7 patients with clinical timothy pollen allergy. Histamine hydrochloride, 1 mg/ml, was used as a positive control. The determination was performed outside the grass pollen season. The mutual perpendicular diameters were measured and the skin reactivity calculated as described by Aas et al. [21].

Results and Discussion

The purified allergen gave rise to one precipitate when subjected to CIE (fig. 1a). This demonstrates

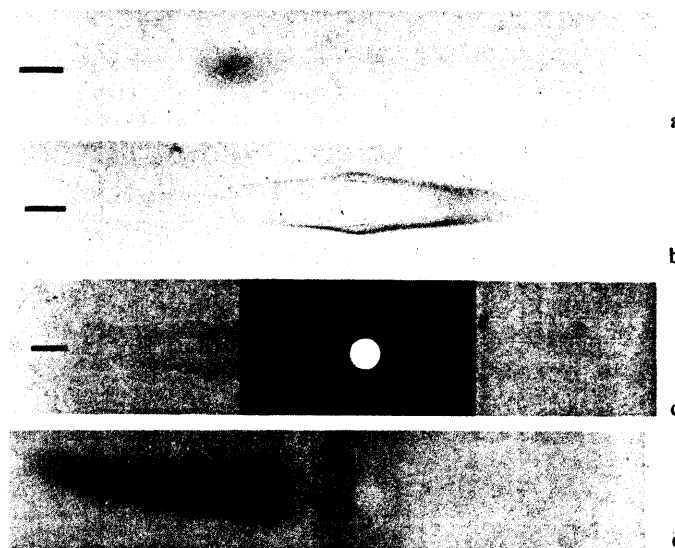


Fig. 3. Electrophoresis of 5 μ g purified allergen [2] in 1% agarose gel pH 8.60 (Bio-Rad). **a** Electrophoresis performed at 10 V/cm for 1 h in gel without antibodies. After the electrophoresis the gel was fixed in a solution containing trichloroacetic acid and sulfo-salicylic acid, pressed, dried and stained with Coomassie brilliant blue. **b** Electrophoresis performed at 2 V/cm for 16 h in gel containing 4 μ l/cm² of an antibody preparation against timothy pollen extract [2]. The gel was then rinsed, pressed, dried and stained with Coomassie brilliant blue. **c, d** As b but with intermediate gels (1-cm wide) containing 25 μ l/cm² of an antiserum against sheep immunoglobulins (Dakopatt Z 138). **c** Stained with Coomassie brilliant blue. **d** Corresponding autoradiograph after incubation with a serum pool from timothy pollen allergics and ¹²⁵I-anti-IgE [2].

that the glycoprotein is immunologically homogeneous with respect to the antiserum used. When CIE was performed on a mixture of whole timothy pollen extract and purified allergen, the size of the precipitate which resembles that of antigen 30 [3–5] was specifically increased (fig. 1b, c). Similarly, tandem CIE of whole timothy pollen extract and single allergen indicated that the isolated allergen is immunologically identical to antigen 30 (fig. 1d).

The ability of the allergen to form a precipitate in RIE was lost when it was incubated with 4 M guanidine hydrochloride or heated to above 70°C for 5 min (fig. 2). Incubation with 4 M urea or freezing and thawing of the allergen solution had no influence on the IgG binding of the allergen in RIE (fig. 2). From this experiment, it is evident that the native IgG-binding conformation of the allergen is easily destroyed. This is in accordance with earlier observations which have indicated that the allergen is prone to denaturation during certain purification methods [11]. The ob-

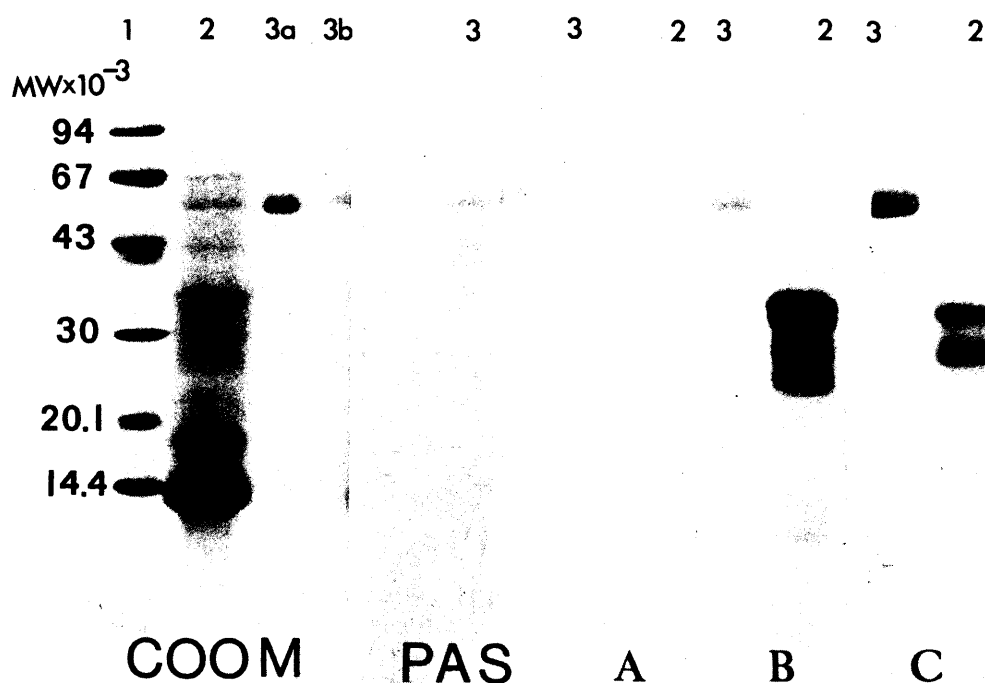


Fig. 4. SDS-slab gel electrophoresis in 7.5–20% PAGE [16] run at 100 V for 18 h. 1 = Phosphorylase b (MW 94,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), carbonic anhydrase (MW 30,000), trypsin inhibitor (MW 14,400); 2 = 300 μ g whole timothy pollen extract [11]; 3 = 20 μ g purified timothy pollen allergen [2]. COOM = Stained with Coomassie brilliant blue; PAS = stained by the PAS method [17]; A–C = autoradiograph of nitrocellulose blots from the polyacrylamide gel incubated with sera and ^{125} I-anti-IgE; incubated with serum with RAST class 0 against timothy pollen extract (A), serum pool from 250 timothy pollen allergics [2] (B) and serum from a patient with RAST class 4 to timothy pollen (C).

served thermal stability of this allergen may be considered as low in comparison to the remarkably high stability of timothy pollen extracts previously reported by several workers. Augustin [22], for instance, found that the skin reactivities of cocksfoot and timothy pollen extracts were not destroyed even after vigorous boiling for 1 min. The heat stability of certain glycoproteins is frequently thought to be due to their carbohydrate content [23]. Obviously, the carbohydrate moiety of this allergen does not protect it from denaturation by heat.

No precipitate was formed in RIE of the allergen when the serum pool from timothy pollen allergics was used in the antibody-containing gel. Neither did this serum pool inhibit precipitation of the allergen by the sheep antibody preparation against timothy pollen extract.

In RIE of the purified allergen using the antibody preparation raised in sheep [2], a precipitate was formed towards both electrodes (fig. 2). This could indicate that the allergen is heterogeneous with respect

to electrophoretic migration in agarose gel at pH 8.60. Electrophoresis of the allergen in agarose gel at pH 8.60 with and without antibody preparation in the gel was, therefore, performed. When no antibody was present in the gel all the allergen moved towards the cathode (fig. 3a). However, in the presence of antibody in the gel a precipitate also was formed towards the anode (fig. 3b). Formation of the anodic precipitate could be prevented by incorporation of antibodies against sheep immunoglobulins in intermediate gels in RIE (fig. 3c, d). This indicates that the anodic precipitate is due to formation of complexes between the allergen and immunoglobulins which migrate towards the anode before they are precipitated.

Some other confusing results obtained in CIE may also be due to reversion of antigen migration by the antibodies. Some of the antigens of bee venom extract, for example, move towards the cathode in the first dimension of CIE and towards the anode in the second dimension [24]. The anodic as well as cathodic migration of antigen 19/20 in *Dermatophagoides fari-*

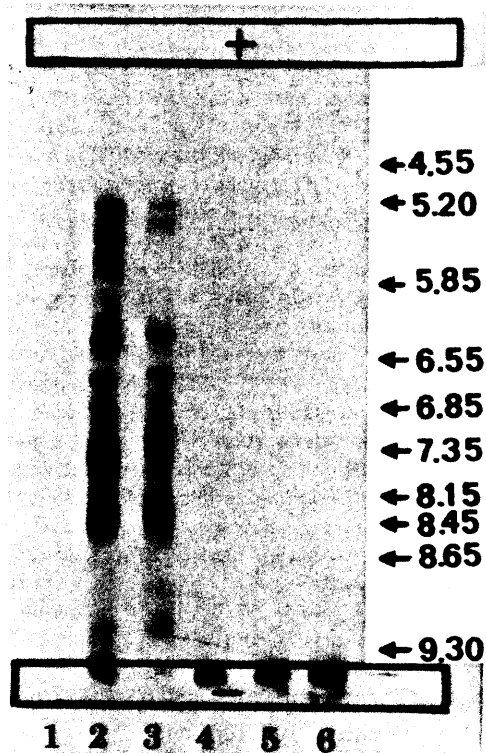


Fig. 5. Autoradiograph of nitrocellulose blots from agarose isoelectric focusing gel, pH 3–10 [9,10]. The nitrocellulose membrane was blocked, cut into strips, incubated with patient sera and ^{125}I -anti-IgE, as described in the experimental section. Isoelectric focusing of raw timothy pollen extract 1–4 and purified timothy pollen allergen (5, 6). Strip 1 was incubated with serum with RAST class 0 against timothy pollen extract; strip 2, with serum pool from 250 timothy pollen allergics; strips 3–6 with sera from 4 persons having class 4 to timothy pollen extract.

nae may also be due to the same effect [25]. Generally, the effect may lead to an overestimation of the number of antigens in CIE. Consequently, if CIE is used in characterization of allergens, the results should always be compared to those obtained by techniques not depending on animal antibodies to the allergens [7–10].

In SDS-PAGE of the allergen, one band staining with Coomassie brilliant blue and the PAS reagent was obtained, demonstrating that it is a homogeneous glycoprotein (fig. 4; COOM and PAS). The apparent molecular weight (MW) in SDS-PAGE (54,000) is higher than the MW estimated after gel filtration (38,000) [2]. Electrophoresis of freeze-dried (fig. 4, 3a) and not freeze-dried allergen (fig. 4, 3b) demonstrated that freeze-drying did not affect its migration in SDS-

PAGE. The abnormally high MW estimate may partly be due to a lower overall negative charge of the SDS-basic allergen complex than of the complexes between the acidic proteins used as standards and SDS. Similarly, abnormally high MW were estimated for lysine-rich histones utilizing the nonbasic protein standard curve [26]. SDS-PAGE has also been reported to give abnormally high MW estimates of many glycoproteins. The MW determined by SDS-PAGE of a membrane glycoprotein, for instance, was considerably higher than the value supposed to be correct [27]. The anomaly is thought to be due to decreased binding of SDS to polypeptide chains containing covalently bound carbohydrate. This effect may also contribute to the abnormally high MW obtained by SDS-PAGE of the glycoprotein allergen. Consequently, we still reckon 38,000 as the most correct MW estimate of the allergen [2].

After SDS-PAGE and transfer to a nitrocellulose membrane, the glycoprotein still bound IgE from timothy pollen allergics (fig. 4 B, C). Consequently, the IgE-binding ability of this allergen is not irreversibly destroyed by the heating with SDS and mercaptoethanol prior to the SDS-PAGE [16]. Previously, the binding of IgE to some wheat flour allergens has been demonstrated to be significantly reduced by incubation with SDS and mercaptoethanol. This occurred despite careful washing to remove the SDS before incubation with serum [7]. If such irreversible denaturation is commonly encountered it limits the applicability of SDS-PAGE in allergen characterization.

The immunoblotting procedure also revealed the presence of IgG to the allergen in 38 of the 47 sera from timothy pollen allergics tested. But as specific IgE to the allergen was detected in all these sera this IgG does not efficiently prevent the binding of IgE to the allergen.

From figure 4 B it is evident that most of the timothy pollen allergens have MW in the region 25,000–35,000. This is somewhat surprising, as antigen 25 having MW 15,000 has been reported to be quantitatively the most important allergen of timothy pollen [1, 3–5].

As the pI of the allergen is 9.45 [2], nitrocellulose was placed on both sides of the gel during the electroblotting at pH 8.30 [18]. By examining the membranes after electroblotting it became evident that almost all the allergens were transferred to the anodal membrane. This demonstrates that SDS was still bound to the glycoprotein during the electrophoretic transfer.

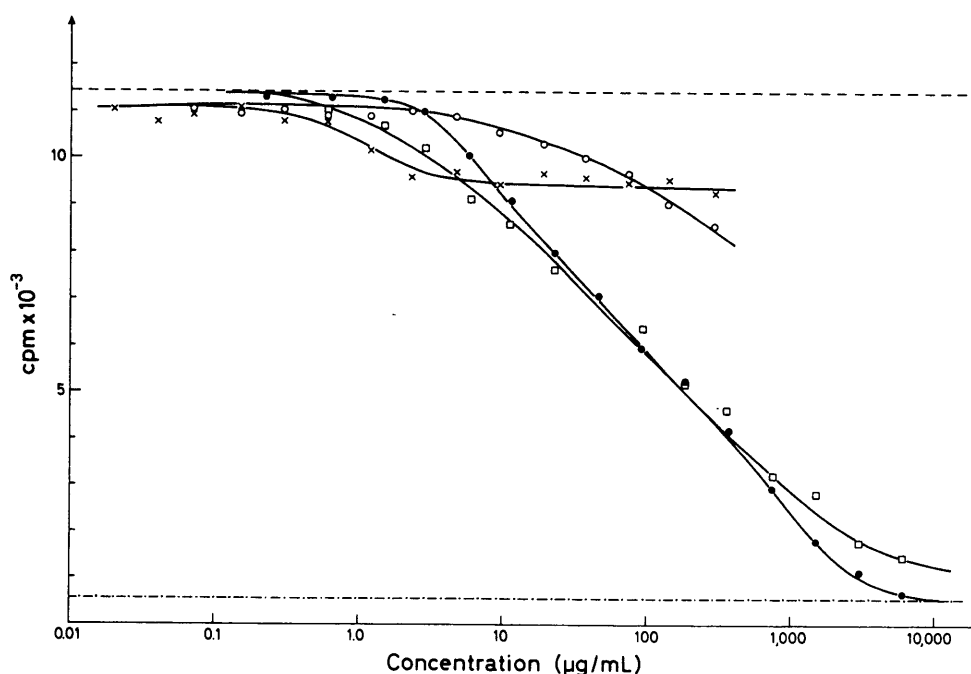


Fig. 6. Inhibition of IgE binding to Rast discs of whole timothy pollen extract [20]. ● = Whole timothy pollen extract; □ = whole timothy pollen extract heated to 80°C for 5 min; × = purified allergen [2]; ○ = purified allergen heated to 80°C for 5 min; -- = no inhibitor added; -.-.- = CNBr-activated paper discs without allergen.

After having been subjected to isoelectric focusing and transfer to nitrocellulose, the allergen bound IgE from the same sera that showed IgE binding to the allergen in RRIE [2] (fig. 5). Most of the sera tested contained IgE to all the main isoelectric focusing bands. One serum, however, contained IgE almost solely against the allergen with pI about 9.4 (fig. 5, strip 4). In the RAST inhibition experiment, the purified allergen maximally inhibited about 15% of the IgE binding to RAST discs of whole timothy pollen extract (fig. 6). This demonstrates that the allergen could account for a considerable portion of the IgE binding in RAST of timothy pollen extracts, but that it only contains a fraction of the different IgE-binding epitopes present on timothy pollen allergens. For this reason, the allergen should be present in allergen extracts and on RAST discs of timothy pollen. The single allergen, however, is unsuited in diagnosis and therapy of timothy pollen allergy.

Heating of the whole extract to 80°C for 5 min did not significantly affect its IgE-binding ability (fig. 6). This is in agreement with previous findings indicating that timothy pollen allergens are remarkably thermostable [22]. Heating of the purified allergen, however,

markedly reduced its IgE binding ability (fig. 6). This indicates that the change of conformation which occurs upon heating affects the binding both of sheep IgG (fig. 2) and of human IgE to this allergen.

In the skin prick test, all the patients tested reacted positively against the isolated allergen. The minimal concentration which was needed to give a positive reaction ranged from 0.1 to 1 µg/ml of the allergen. The mean reactivity of a solution containing 2 µg/ml allergen was equivalent to that of a timothy pollen extract containing 10,000 BU/ml.

This may indicate that a considerable portion of the skin reactivity to timothy pollen extracts is due to this allergen, and the clinical importance of the allergen is possibly greater than reflected by CIE and RAST.

Acknowledgements

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