



COMMON IgE-EPITOPES OF RECOMBINANT *Phl p* I, THE MAJOR TIMOTHY GRASS POLLEN ALLERGEN AND NATURAL GROUP I GRASS POLLEN ISOALLERGENS

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Abstract—Grass pollen allergens are potent elicitors of Type I allergy. More than 95% of grass pollen allergic patients display IgE-cross-reactivity to group I grass pollen allergens of different grass species. A cDNA coding for the major timothy grass pollen allergen, *Phl p* I, was isolated previously. To investigate the presence of common IgE-epitopes among naturally occurring group I grass pollen isoallergens, *Phl p* I was expressed in *Escherichia coli* and used for IgE-absorption experiments. Recombinant *Phl p* I was able to inhibit IgE-binding to most of group I isoallergens from seven grass species as identified by two dimensional electrophoresis. When tested in competitive ELISA experiments, recombinant *Phl p* I bound a high percentage of grass pollen specific IgE. The results indicate that recombinant *Phl p* I shares many of the IgE-epitopes with natural group I grass pollen allergens and hence may represent a useful tool for specific diagnosis and therapy of grass pollen allergy. Copyright © 1996 Elsevier Science Ltd.

Key words: IgE, recombinant allergen, *Phl p* I, grass pollen allergy.

INTRODUCTION

Type I allergy to pollen and plant proteins represents a major health problem in industrialized countries (Wüthrich, 1989). In order to characterize allergenic plant proteins, cDNA cloning techniques have been successfully used to determine the sequence of relevant allergens, and recombinant allergens were produced for diagnosis and therapy (Valenta *et al.*, 1994, 1995; Schenker and Kraft, 1995). Among the relevant plant allergens, grass pollen allergens have been identified as potent elicitors of Type I allergy. More than 95% of grass pollen allergic individuals display IgE-reactivity to group I grass pollen allergens which represent glycoproteins of approximately 30 kDa (Freidhoff *et al.*, 1986) that occur in most grass species. IgE-antibodies from grass pollen allergic patients and group I specific antibodies have been shown to cross-react with natural group I grass pollen allergens of different grass species (Esch and Klapper, 1989). We have recently isolated a cDNA coding for the major timothy grass pollen allergen, *Phl p* I (Laffer *et al.*, 1994b). The cDNA coding for *Phl p* I has an average amino acid sequence identity of 90% with the major rye grass isoallergens from *Lolium perenne* (Perez *et al.*, 1990; Griffith *et al.*, 1991).

As other plant allergens (Rafnar *et al.*, 1991; Silvanovich *et al.*, 1991; Larsen *et al.*, 1992; Breiteneder *et al.*, 1993), group I grass pollen allergens are known to occur as isoallergenic variants of a similar molecular weight but different sequence and pI (Rohac *et al.*, 1991). The occurrence of plant allergens is of biomedical interest because isoallergenic variants of the major hazel pollen allergen *Cor a* I have been shown to display different IgE-binding capacity (Breiteneder *et al.*, 1993) and ability to stimulate specific T-cell clones (Schenk *et al.*, 1994). In the present study the presence of common IgE-epitopes of recombinant *Phl p* I and naturally occurring group I isoallergenic variants was investigated to evaluate its potential usefulness for diagnosis and perhaps therapy of grass pollen allergy.

MATERIALS AND METHODS

Biological materials

Pollen from *Phleum pratense* (timothy grass), *Anthoxanthum odoratum* (sweet vernal grass), *Avena sativa* (cultivated oat), *Cynodon dactylon* (Bermuda grass), *Lolium perenne* (ryegrass), *Phragmites communis* (common reed), and *Poa pratensis* (Kentucky bluegrass) were purchased from Allergon AB (Engelholm, Sweden). Recombinant *Phl p* I β -galactosidase fusion protein was produced upon

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infection of lysogenic *E. coli* Y1089 with lambda gt11 phage (Laffer *et al.*, 1994a). Recombinant non-fusion *Phl p I* was expressed in *E. coli* BL21 (DE3) (Studier *et al.*, 1990) (Novagen, Madison, U.S.A.) using plasmid pMW172, a derivative of pRK172 (McLeod *et al.*, 1987).

Preparation of natural grass pollen extracts

Two grams of grass pollen were shaken in 30 ml distilled water for 2 hr at 4°C. Extracts were centrifuged for 1 hr at 4°C and 20,000g. The supernatants were dialysed against distilled water at 4°C and lyophilized. Amount and quality of the protein extracts were estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fling and Gregerson, 1986) and Coomassie Brilliant Blue (Imperial Chemical Industries, Ltd., Macclesfield, U.K.) staining of the gels (Bradford, 1976).

Expression of the cDNA coding for Phl p I as fusion and non-fusion protein in E. coli expression of recombinant Phl p I fusion protein

Lysogenic *E. coli* Y1089 cells infected with recombinant phage, were grown at 32°C to an optical density at 600 nm of 0.5. After 20 min at 42°C the protein synthesis was induced by adding isopropyl- β -D-thiogalactoside (IPTG) to a final concentration of 10 mM. *E. coli* cells were incubated at 37°C for 1 hr, harvested by centrifugation and washed twice with 0.9% sodium chloride.

Expression of recombinant Phl p I non-fusion protein

The cDNA coding for *Phl p I* was PCR amplified so that the signal peptide was replaced by a methionine by placing an NdeI restriction site that contains the ATG at the start of the coding region of the mature protein. After the stop codon an EcoRI site was introduced and the NdeI–EcoRI cut PCR product was ligated into plasmid pMW172 and transformed in *E. coli* BL21 (DE3). *E. coli* in liquid culture were induced with IPTG (0.2–0.4 mM) at an OD₆₀₀ of 0.8–1.2. Recombinant *Phl p I* was purified from inclusion bodies and refolded as described (Vrtala *et al.*, in press).

Two-dimensional PAGE of grass pollen extracts from different species—localization of group I isoallergens

Lyophilized protein extracts from the different grass species were separated in the first dimension by isoelectric focussing and subsequently separated by molecular weight in denaturing SDS–PAGE as described (Rohac *et al.*, 1991). Identically prepared gels were either silver-stained (Sammons *et al.*, 1981) to visualize proteins or blotted onto nitrocellulose (Schleicher and Schüll, Dassel, Germany) (Towbin *et al.*, 1979). A rabbit anti-group I antiserum (Laffer *et al.*, 1994b) was used to localize group I isoallergens on nitrocellulose blotted two-dimensionally separated grass pollen extracts. Nitrocellulose blots were incubated twice for 5 min and once for 30 min in buffer A (50 mM sodium phosphate pH

7.5, 0.5% Tween 20, 0.5% BSA, 0.05% NaN₃) to saturate the membrane. The group I antiserum was diluted 1:1000 in buffer A and incubated overnight at 4°C. Membranes were then washed three times in buffer A as above and incubated with a ¹²⁵I labeled donkey anti-rabbit antiserum (Amersham, U.K.) for 2 hr at room temperature. Bound antibodies were visualized by autoradiography using Kodak XOMAT films and intensifying screens (Kodak, Heidelberg, Germany) at –70°C for 48 hr.

IgE-immunoblot inhibition

Serum from a grass pollen allergic patient with exclusive IgE-reactivity to group I and group II grass pollen allergens was diluted 1:10 in buffer A and preincubated overnight at 4°C with nitrocellulose filters (Schleicher and Schüll, Dassel, Germany) containing recombinant β -galactosidase fused *Phl p I* or as a negative control, proteins from *E. coli*. Two identically prepared nitrocellulose sheets containing grass pollen extracts separated by two-dimensional electrophoresis were incubated with the patient's serum that had been pre-adsorbed with recombinant *Phl p I* or proteins from *E. coli* (negative control). Bound IgE was then detected with 1:10 diluted ¹²⁵I labeled anti-human IgE antibodies (Pharmacia, Uppsala, Sweden).

Characterization of sera from grass pollen allergic patients

Grass pollen allergic patients were selected according to case history, RAST and skin prick testing as described (Valenta *et al.*, 1992). Sera from grass pollen allergic patients were tested for IgE-reactivity to nitrocellulose blotted natural timothy grass pollen extract, recombinant *Phl p I* (Laffer *et al.*, 1994b), recombinant *Phl p II* (Dolecek *et al.*, 1993) and recombinant *Phl p V* (Vrtala *et al.*, 1993).

ELISA and ELISA competition assays

ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with 100 μ l/well of *Phleum pratense* pollen extract (300 μ g/ml in PBS) or with 100 μ l (2 μ g/ml) purified recombinant *Phl p I* non-fusion protein overnight at 4°C. The plates were washed twice with 200 μ l PBS, 0.05% (v/v) Tween-20 per well and were incubated with 200 μ l PBS, 0.05% (v/v) Tween-20, 1% BSA for 2.5 hr at room temperature to block non-specific protein binding sites. Patients' sera were preincubated with *Phleum pratense* pollen extract or purified recombinant *Phl p I* non-fusion protein overnight at 4°C; 100 μ l/well of the preincubated patients' sera were then added. After incubation overnight at 4°C the wells were washed five times with 200 μ l PBS, 0.05% (v/v) Tween-20. A monoclonal mouse anti-human IgE antibody (Pharmingen, San Diego, U.S.A.) diluted 1/1000 in PBS, 0.05% (v/v) Tween-20, 0.5% BSA was added overnight at 4°C. After washing the plates as described above they were finally incubated with a sheep anti-mouse antiserum coupled to peroxidase (Amersham, U.K.) that was diluted 1:2000 in PBS, 0.05% (v/v) Tween20, 0.5% BSA for 30 min at 37°C and for 30 min at 4°C. Plates were then washed again.

ABTS solution (Amersham, U.K.) was added and after incubation for 15–30 min in the dark the reaction was stopped by addition of 0.32% NaF. The optical density was determined with an automated ELISA reader (Dynatech, Denkendorf, Germany).

RESULTS

Identification of group I isoallergens in grass pollen extracts separated by two-dimensional electrophoresis

To identify group I isoallergens in pollen extracts from seven different grasses, two-dimensional PAGE was performed. Grass pollen extracts from *Phleum pratense*, *Anthoxanthum odoratum*, *Avena sativa*, *Cynodon dactylon*, *Lolium perenne*, *Phragmites communis* and *Poa pratensis* were separated in the first dimension by isoelectric focussing and subsequently separated by molecular weight in denaturing SDS-PAGE. Identically prepared gels were silver stained or blotted to nitro-

cellulose for the detection of group I isoallergens and IgE-inhibition experiments.

Figure 1 shows a silver stained gel from grass pollen extracts separated by two-dimensional-electrophoresis from seven grass species (Fig. 1, upper). Group I isoallergens were localized on two-dimensional blots (Fig. 1, lower) using a group I specific antibody (Laffer *et al.*, 1994b). Group I grass pollen isoallergens migrate at and slightly above 30 kDa at a neutral to basic pH. In most extracts more than six isovariants could be discriminated. Additional group I isovariants were found at a lower molecular weight in extracts from *Phleum pratense*, *Anthoxanthum odoratum*, *Phragmites communis*, and *Poa pratensis*. Differences in molecular weight among group I allergens may be due to different glycosilation, the occurrence of isoforms with a different amino acid composition or degradation. Comparison of the protein staining and the binding of the group I specific antibody indicated that group I isoallergens did not belong to the most abundant proteins in the grass pollen extracts.

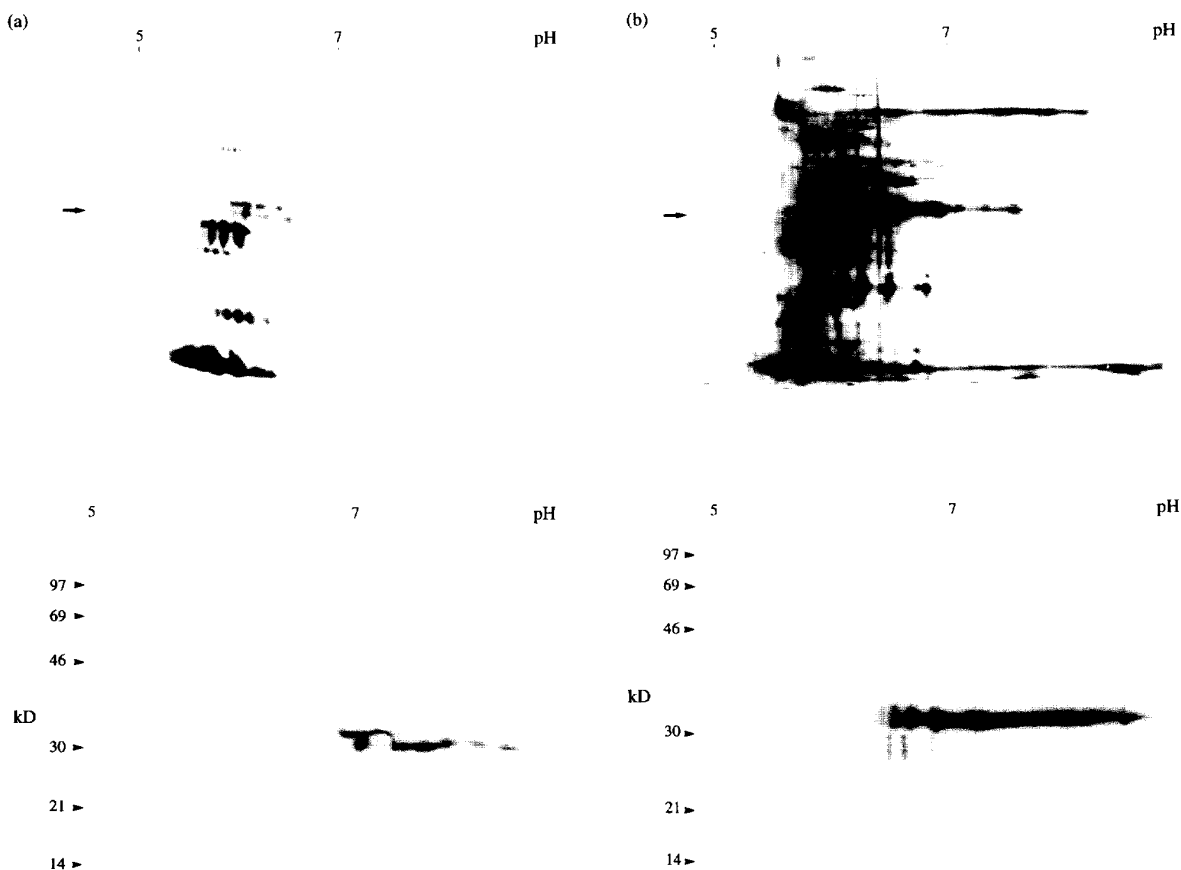


Fig. 1. Identification of group I isoallergens on nitrocellulose blotted two-dimensional separated grass pollen extracts. Protein extracts from pollen of *Phleum pratense* (a), *Anthoxanthum odoratum* (b), *Avena sativa* (c), *Cynodon dactylon* (d), *Lolium perenne* (e), *Phragmites communis* (f) and *Poa pratensis* (g) were separated by two-dimensional electrophoresis. Identically prepared gels were silver stained in the upper part of each Figure. The arrow indicates the position of group I allergens. In the lower part of the figure, proteins were blotted to nitrocellulose and group I isoallergens were detected with a rabbit anti-group I antiserum.

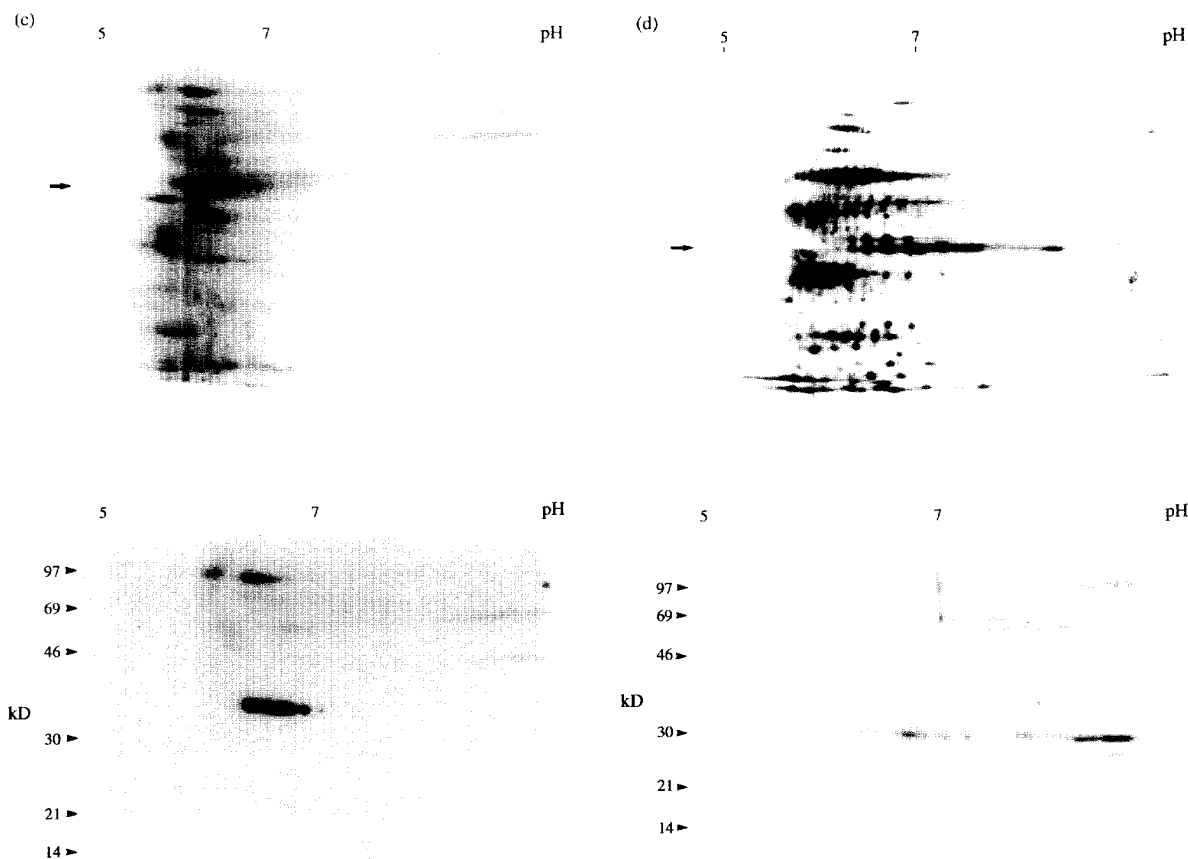


Fig. 1—continued.

IgE-binding to natural and recombinant Phl p I allergens

The major timothy grass pollen allergen *Phl p I* was expressed in *E. coli* as a non-fusion protein without its hydrophobic leader sequence including one methionine at the translation start. Purification of the recombinant allergen from inclusion bodies yielded a more than 95% pure protein (Vrtala *et al.*, in press). Figure 2 shows the IgE-reactivity of a grass pollen allergic patient (left block; a) with nitrocellulose blotted natural timothy grass pollen extract (Fig. 2, lane 1), with recombinant *Phl p I* (lane 2), with recombinant *Phl p II* (lane 3) and recombinant *Phl p V* (lane 4). Serum from a non-allergic control individual (right block; b) showed no IgE reactivity to any of the proteins. The allergic patient displayed IgE-binding exclusively to group I and group II timothy grass pollen allergens whereas no IgE-reactivity to group V allergens was observed. Compared to natural group I and group II allergens, recombinant *Phl p I* and *Phl p II* had a slightly lower molecular weight as determined by SDS-PAGE. Both recombinant and natural group I and II allergens bound IgE in a comparable intensity.

The expression of recombinant *Phl p I* as a β -galactosidase fusion protein and its IgE-binding capacity was reported earlier (Laffer *et al.*, 1994a). No differences regarding IgE-binding were observed when sera were

tested with recombinant *Phl p I* expressed as a β -galactosidase fusion protein and the recombinant non-fusion *Phl p I* (Laffer *et al.*, unpublished data).

Recombinant Phl p I inhibits IgE-binding to group I isoallergens from different grass species

To investigate whether naturally occurring group I isoallergens of different grasses share IgE-epitopes with recombinant *Phl p I*, IgE absorption experiments were performed. Serum from a grass pollen allergic patient with exclusive IgE reactivity against group I and group II isoallergens was selected (Fig. 2) and used to detect two dimensionally separated group I and group II isoallergens from seven grass species (Fig. 3, upper). In the lower part of Fig. 3, the serum was preabsorbed with recombinant β -galactosidase fused *Phl p I*. When the serum was preabsorbed with *E. coli* proteins (negative control) a similar pattern of group I isoallergenic variants was detected as was identified with the group I specific antiserum in the two dimensionally separated grass pollen extracts (Fig. 2). Preincubation of the serum with recombinant *Phl p I* led to an almost complete loss of IgE-reactivity to most group I isoallergens in all seven grass pollen extracts. Binding to group II isoallergens at 10 kD was not affected by the preabsorption.

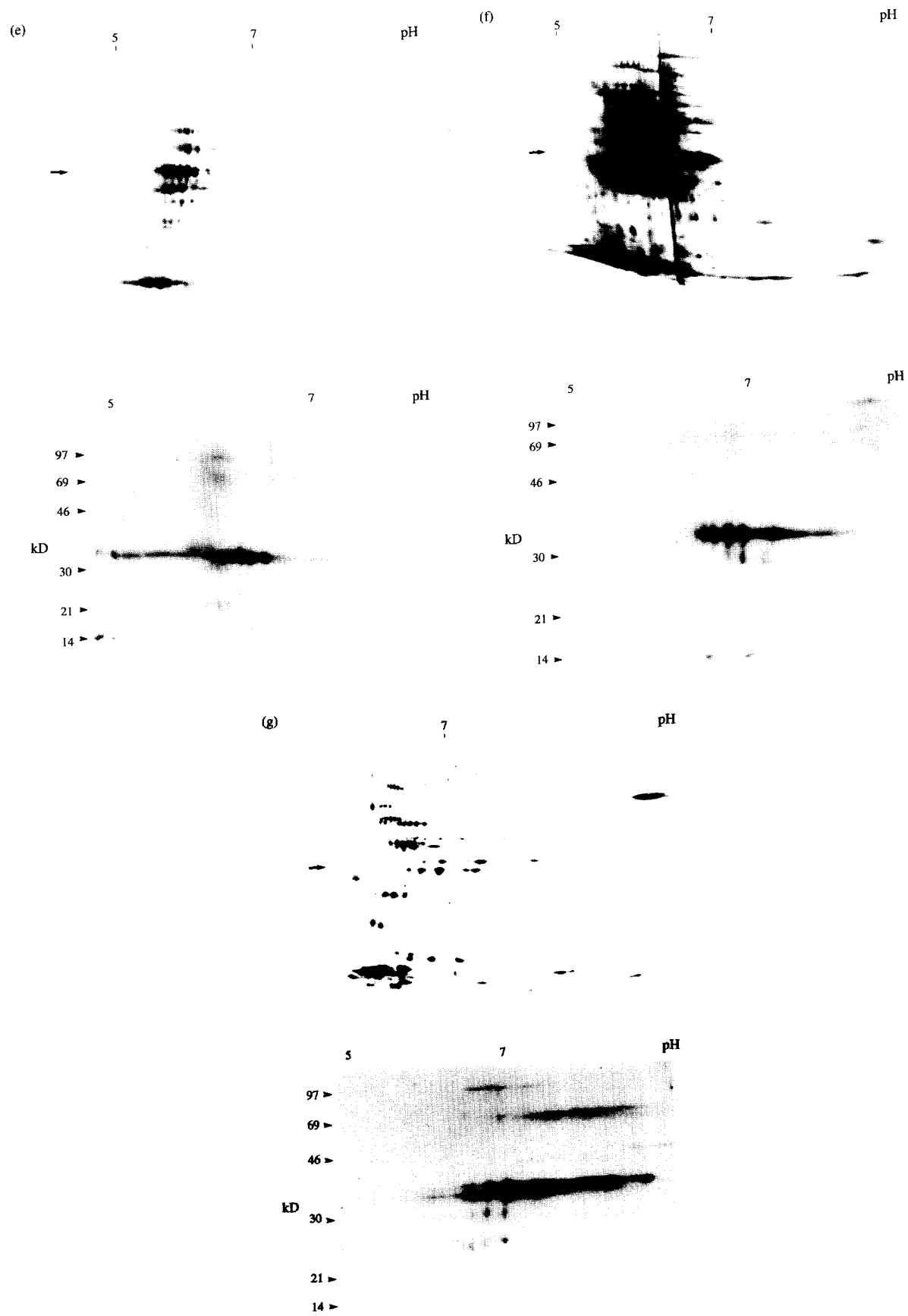
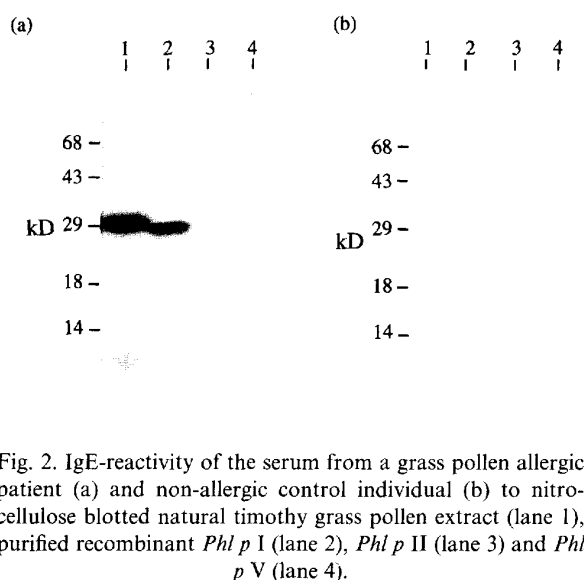


Fig. 1—continued.



Characterization of sera from grass pollen allergic patients with nitrocellulose blotted natural and recombinant timothy grass pollen allergens; IgE-binding capacity of recombinant *Phl p I* determined by ELISA

Sera from grass pollen allergic patients and a non-allergic control person were characterized for the presence of IgE-antibodies specific for recombinant *Phl p I*. Seven out of fifteen patients with high levels of grass pollen specific IgE were further tested in ELISA inhibition studies to estimate the percentage of timothy grass pollen specific IgE antibodies directed against recombinant *Phl p I*. ELISA plates were coated with total timothy grass pollen allergen extract so that immobilized proteins were in excess to IgE-antibodies (determined by serial dilutions of the sera and by using different amounts of proteins for the coating of the plates). Sera from seven grass pollen allergic patients were preincubated with recombinant *Phl p I*, total timothy grass pollen extract and without allergens before IgE was allowed to bind to

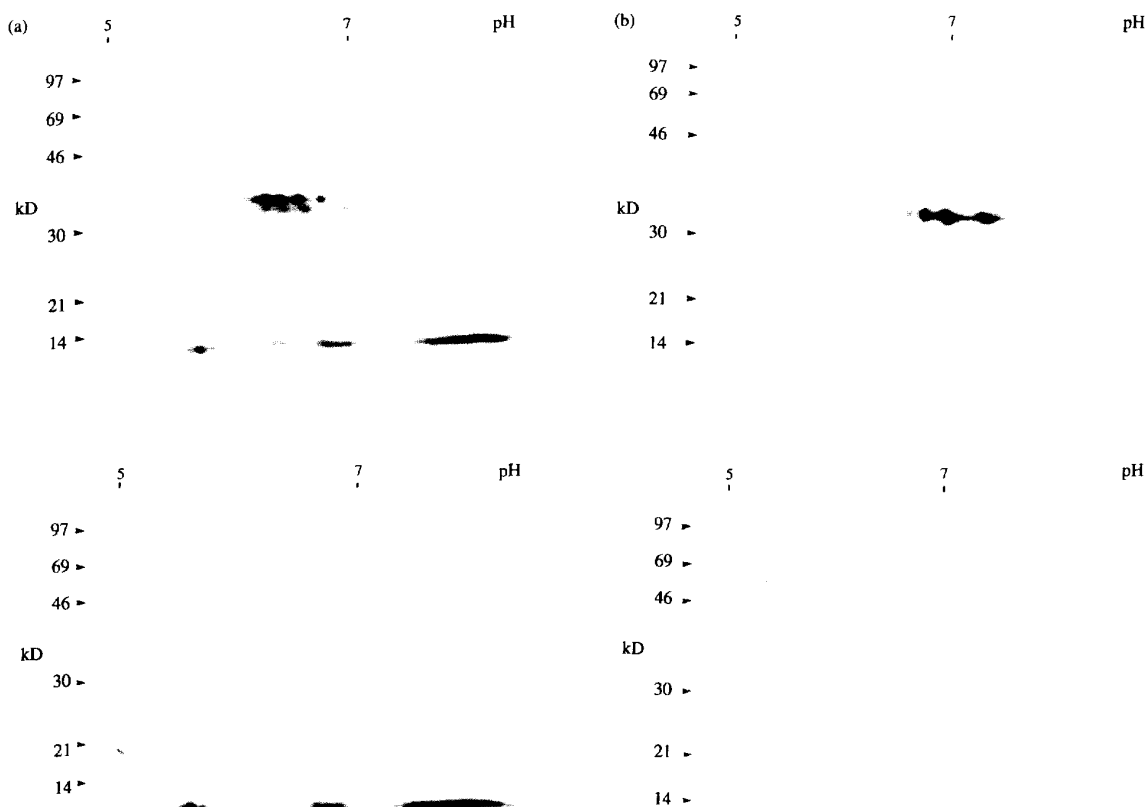


Fig. 3. Recombinant *Phl p I* shares IgE epitopes with two-dimensional separated group I isoallergens in different grass species. Protein extracts from pollen of *Phleum pratense* (a), *Anthoxanthum odoratum* (b), *Avena sativa* (c), *Cynodon dactylon* (d), *Lolium perenne* (e), *Phragmites communis* (f) and *Poa pratensis* (g) were separated by two-dimensional electrophoresis and blotted to nitrocellulose. Group I and group II isoallergens were detected with serum IgE from a grass pollen allergic individual with IgE reactivity to group I and group II allergens. In the upper part of the Figure the serum was preincubated with proteins from *E. coli* (negative control) whereas in the lower part of the figure, recombinant *Phl p I* was used for the preabsorption.

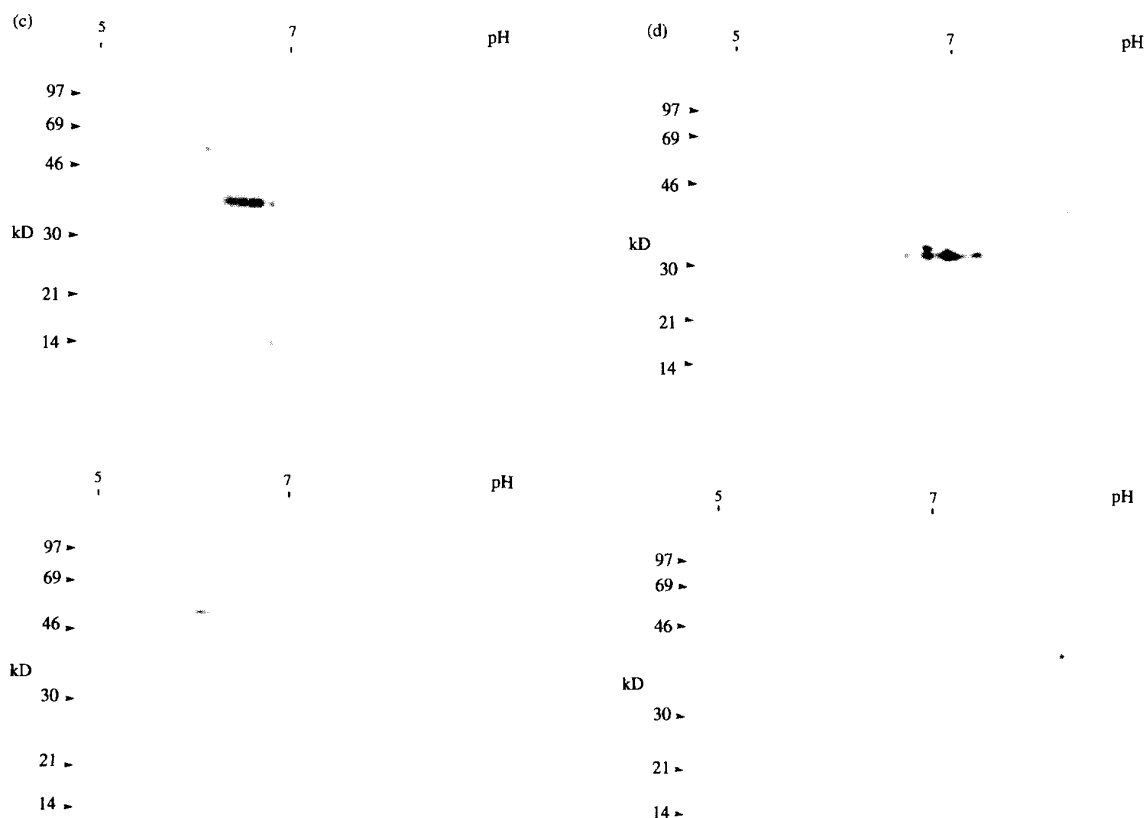


Fig. 3—continued.

the ELISA plate coupled timothy grass pollen extract. Figure 4 shows the comparison of bound IgE after preincubation of the sera. An average of 60% of timothy grass pollen specific IgE antibodies could be preabsorbed by preincubation of the sera with recombinant *Phl p I*.

DISCUSSION

cDNAs coding for a number of relevant allergens have been isolated and expressed as functional allergens in *E. coli*. Recombinant allergens are presently considered as potent tools for diagnosis and perhaps also therapy of Type I allergic diseases (Scheiner and Kraft, 1995; Valenta *et al.*, 1995). However, many of the relevant plant allergens, although conserved in sequence and immunologically related among several plant species, have been shown to occur as isoforms by two-dimensional electrophoresis or DNA sequencing (Perez *et al.*, 1990; Rafnar *et al.*, 1991; Silvanovich *et al.*, 1991; Larsen *et al.*, 1992; Breiteneder *et al.*, 1993; Swoboda *et al.*, 1995). From the biological point of view the occurrence of major plant allergens as isoforms indicates that these proteins may fulfill essential functions in plant metabolism. This is evident for the family of profilins which have been described as relevant plant allergens and which also represent essential components of the plant cytoskeleton (Valenta *et al.*, 1993; Staiger *et al.*, 1993). A similar diversity has been shown for the major birch pollen allergen, *Bet v I* (Swoboda *et al.*, 1995) and its homologues present

in pollen of trees belonging to the order fagales (Larsen *et al.*, 1992; Breiteneder *et al.*, 1993), vegetables and fruits (Hirschwehr *et al.*, 1992). These allergens most likely represent pathogenesis related proteins (Breiteneder *et al.*, 1989) that are perhaps needed to defend plants against microbial attack.

The occurrence of plant allergens as isoforms has however also implications for biomedical research. If isoallergenic variants differ too much in sequence and hence in their immunological properties, it may be difficult to use certain defined recombinant isoallergenic variants for diagnosis and therapy. In fact, differences regarding the IgE-binding capacity and ability to stimulate allergic patients T-cells have been shown for isoallergenic variants of the major hazel pollen allergen *Cor a I* (Breiteneder *et al.*, 1993; Schenk *et al.*, 1994).

Recently the isolation and expression of a cDNA coding for the major timothy grass pollen allergen, *Phl p I*, was reported. *Phl p I* was selected as a model allergen because it represents a target for IgE antibodies of more than 95% of grass pollen allergic patients. In the present study we have investigated whether bacterially expressed *Phl p I* has common IgE epitopes with group I isoallergens from seven grass species. For this purpose group I isoallergens were identified in two-dimensional separated pollen extracts from seven grass species with a group I specific antibody. Because group I and group V migrate with a comparable molecular weight in SDS-PAGE, serum IgE from a patient without IgE-reactivity to group

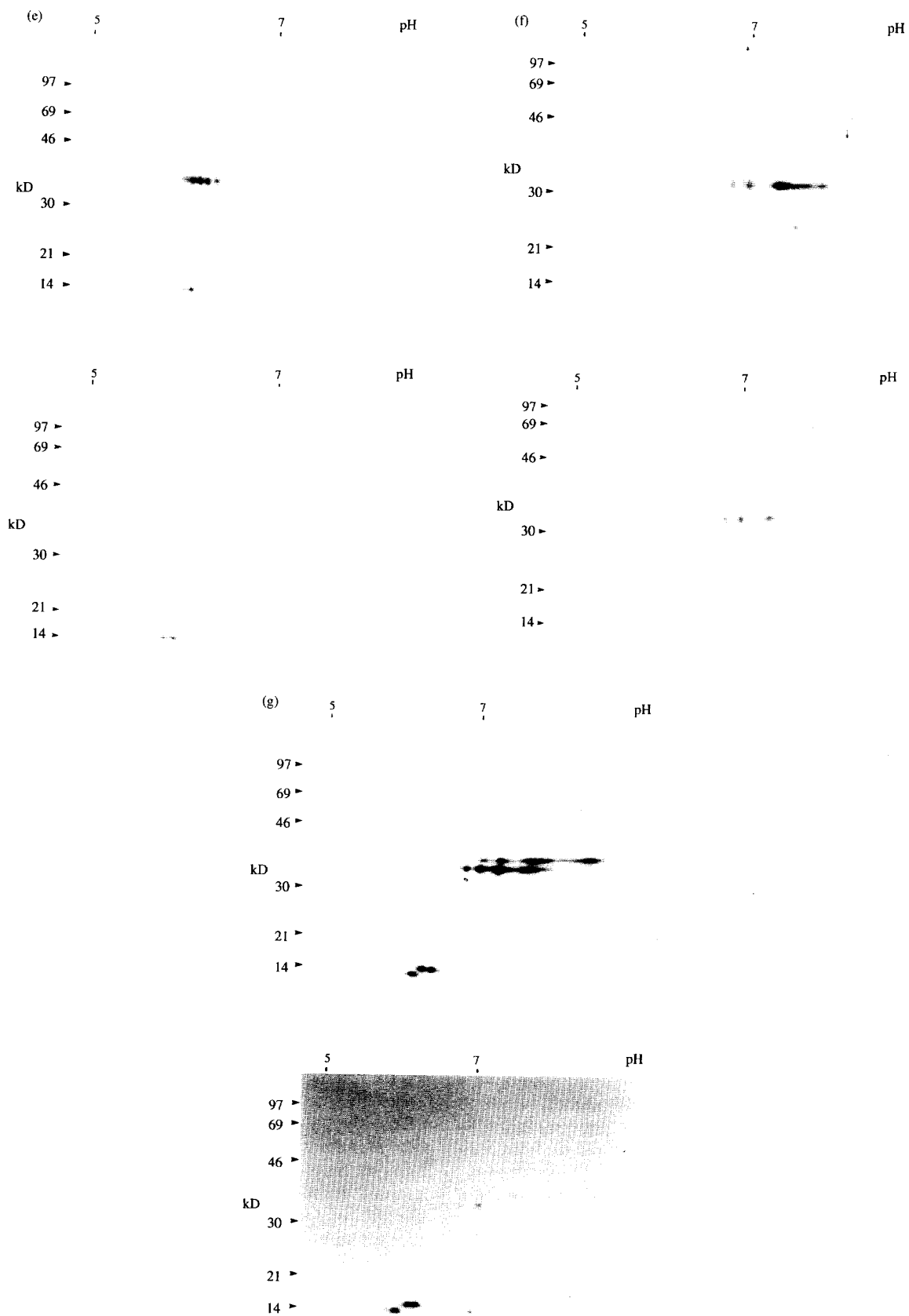


Fig. 3—continued.

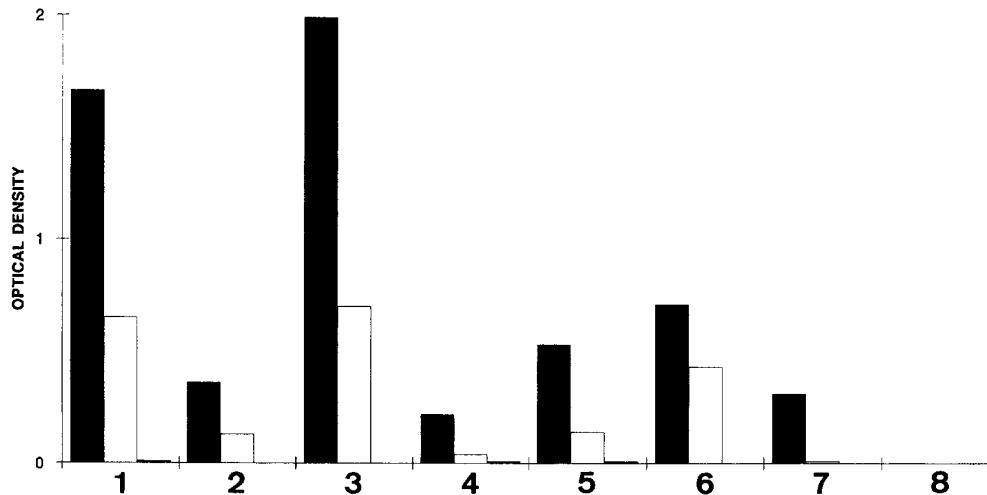


Fig. 4. ELISA binding and competition studies. Sera from seven grass pollen allergic individuals (1–7) and a non-allergic control person (8) were used to bind to ELISA plate coupled natural timothy grass pollen extract. The bars show the OD values as means of duplicate determinations. Closed bars display the values without preincubation of the sera, open bars reflect the binding after preincubation with recombinant *Phl p I* and hatched bars show the absorbence after preincubation with natural timothy grass pollen extract.

V allergens was used for IgE inhibition experiments with two-dimensional separated grass pollen extracts. A large variety of group I isoallergenic variants could be detected in two-dimensional separated grass pollen extracts. In some extracts different group I isoallergens were detected with specific antibodies or serum IgE. However, the preincubation of the allergic patients' serum with recombinant *Phl p I* inhibited IgE binding to almost all group I isoallergens from all seven grass species. Although group I and group II allergens share significant sequence similarities (Dolecek *et al.*, 1993), preincubation of the serum with recombinant *Phl p I* did not affect IgE binding to group II allergens.

An average of 60% of grass pollen specific IgE could be absorbed out of sera from seven grass pollen allergic individuals in ELISA inhibition experiments using recombinant *Phl p I*. The reason for the high IgE-binding capacity of recombinant *Phl p I* is attributed to the selection procedure which has been used to isolate the corresponding cDNA. It is likely that immunological screening procedures particularly those based on the use of IgE antibodies are suited to deliver functional recombinant allergens that are also active in cellular assays. In fact, the biological activity of recombinant *Phl p I* was demonstrated in T-cell proliferation assays (Schenk *et al.*, 1995) and it could be shown that purified recombinant *Phl p I* was able to induce specific histamine release from grass pollen allergic patients' basophils (Vrtala *et al.*, in press).

Because of the high IgE-binding capacity of recombinant *Phl p I* and its immunological similarity with natural group I grass pollen isoallergens of seven different grass species, it is suggested that recombinant *Phl p I* may represent a useful candidate for *in vitro* and *in vivo* diagnosis of grass pollen allergy as well as for specific therapy of grass pollen allergy.

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