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(54) Title: PHL P 5A DERIVATIVES WITH REDUCED ALLERGENICITY AND RETAINED T-CELL REACTIVITY

(54) Bezeichnung: PHL P 5A-DERIVATE MIT REDUZIERTER ALLERGENITÄT UND ERHALTENER T-ZELLREAKTIVITÄT

(57) Abstract: The invention relates to the production and use of variants of the group 5 allergens of *Pooideae*, characterised in displaying a reduced IgE reactivity relative to known wild type allergens and also a substantially retained reactivity with T-lymphocytes. Said hypoallergenic allergen varieties can be used for specific immunotherapy (hyposensitisation) of patients with grass pollen allergies or for preventative immunotherapy of grass pollen allergies.

(57) Zusammenfassung: Die vorliegende Erfindung betrifft die Herstellung und Verwendung von Varianten der Gruppe-5-Allergen der *Pooideae*, welche durch eine gegenüber den bekannten Wildtyp-Allergenen verringerte IgE-Reaktivität und gleichzeitig eine weitgehend erhaltene Reaktivität mit T-Lymphozyten gekennzeichnet sind. Diese hypoallergenen Allergenvarianten können zur spezifischen Immuntherapie (Hyposensibilisierung) von Patienten mit Graspollenallergie oder zur präventiven Immuntherapie von Graspollenallergien eingesetzt werden.

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Phl p 5a derivatives having reduced allergeneity and retained T-cell reactivity

The present invention relates to the preparation and use of variants of the group 5 allergen of the *Pooideae* which are characterised by reduced IgE reactivity compared with the known wild-type allergens and at the same time by substantially retained reactivity with T lymphocytes.
These hypoallergenic allergen variants can be employed for the specific immunotherapy (hyposensitisation) of patients having grass pollen allergy or for the preventative immunotherapy of grass pollen allergies.
A preferred embodiment of the invention relates to variants of the major allergen Phl p 5a from the pollen of timothy grass (*Phleum pratense*).

Background of the invention

Type 1 allergies are of importance worldwide. Up to 20% of the population in industrialised countries suffer from complaints such as allergic rhinitis, conjunctivitis or bronchial asthma. These allergies are caused by allergens present in the air (aeroallergens) which are liberated from sources of various origin, such as plant pollen, mites, cats or dogs. Up to 40% of these type 1 allergy sufferers in turn exhibit specific IgE reactivity with grass pollen allergens (Freidhoff et al., 1986, J. Allergy Clin. Immunol. 78, 1190-2002).

The substances which trigger type 1 allergy are proteins, glycoproteins or polypeptides. After uptake via the mucous membranes, these allergens react with the IgE molecules bonded to the surface of mast cells in sensitised individuals. If two IgE molecules are crosslinked to one another by an allergen, this results in the release of mediators (for example histamine,

prostaglandins) and cytokines by the effector cell and thus in the corresponding clinical symptoms.

A distinction is made between major and minor allergens depending on the relative frequency with which the individual allergen molecules react with the IgE antibodies of allergy sufferers.

In the case of timothy grass (*Phleum pratense*), Phl p 1 (Petersen et al., 1993, J. Allergy Clin. Immunol. 92: 789-796), Phl p 5 (Matthiesen and Löwenstein, 1991, Clin. Exp. Allergy 21: 297-307; Petersen et al., 1992, Int. Arch. Allergy Immunol. 98: 105-109), Phl p 6 (Petersen et al., 1995, Int. Arch. Allergy Immunol. 108, 49-54). Phl p 2/3 (Dolecek et al., 1993, FEBS 335 (3), 299-304), Phl p 4 (Haavik et al., 1985, Int. Arch. Allergy Appl. Immunol. 78: 260-268; Valenta et al., 1992, Int. Arch. Allergy Immunol. 97: 287-294, Fischer et al., 1996, J. Allergy Clin. Immunol. 98: 189-198) and Phl p 13 (Suck et al., 2000, Clin. Exp. Allergy 30: 324-332; Suck et al., 2000, Clin. Exp. Allergy 30: 1395-1402) have hitherto been identified as major allergens.

The dominant major allergens of timothy grass (*Phleum pratense*) are Phl p 1 and Phl p 5, with Phl p 5 occurring in two forms 5a and 5b which differ in respect of their molecular weight and are encoded by independent genes. The deduced amino acid sequences both of Phl p 5a and also of Phl p 5b have been determined by means of the recombinant DNA technique. Phl p 5a is a protein of about 32 kDa and reacts with the IgE antibodies of 85 – 90% of grass pollen allergy sufferers. Phl p 5a exists in a series of homologous variants which differ from one another through point mutations and probably correspond to different allelic forms. The pollen of related grass species, such as, for example, *Lolium perenne*, *Poa pratensis* inter alia, contains allergens which are homologous with that of Phl p 5a and together are known as group 5 allergens. The high structural homology of these group 5 allergens of grass species causes correspondingly high

cross reactivity of the molecules with the IgE antibodies of grass pollen allergy sufferers.

A classical approach to effective therapeutic treatment of allergies is specific immunotherapy or hyposensitisation (Fiebig, 1995, Allergo J. 4 (6): 336-339, Bousquet et al., 1998, J. Allergy Clin. Immunol. 102 (4): 558-562). In this method, the patient is injected subcutaneously with natural allergen extracts in increasing doses. However, there is a risk in this method of allergic reactions or even anaphylactic shock. In order to minimise these risks, innovative preparations in the form of allergoids are employed. These are chemically modified allergen extracts which have significantly reduced IgE reactivity, but identical T-cell reactivity compared with the untreated extract (Fiebig, 1995, Allergo J. 4 (7): 377-382).

Even more substantial therapy optimisation would be possible with allergens prepared by recombinant methods. Defined cocktails of high-purity allergens prepared by recombinant methods, optionally matched to the individual sensitisation patterns of the patients, could replace extracts from natural allergen sources since these, in addition to the various allergens, contain a relatively large number of immunogenic, but non-allergenic secondary proteins.
Realistic perspectives which may result in reliable hyposensitisation with recombinant expression products are offered by specifically mutated recombinant allergens in which IgE epitopes are specifically deleted without impairing the T-cell epitopes which are essential for therapy (Schramm et al., 1999, J. Immunol. 162: 2406-2414).
A further possibility for therapeutic influencing of the disturbed T helper cell equilibrium in allergy sufferers is treatment with expressible DNA which encodes for the relevant allergens (immunotherapeutic DNA vaccination). Initial experimental evidence of allergen-specific influencing of the immune response by a DNA vaccine of this type has been furnished in rodents by

injection of allergen-encoding DNA (Hsu et al., 1996, Nature Medicine 2 (5): 540-544).

One object on which the present invention is based consists of the provision of novel variants of the group 5 allergens of the *Pooideae* at the protein and DNA level which are distinguished by reduced IgE activity at the same time as substantial retention of the T-cell reactivity and are therefore suitable for specific immunotherapy and immunotherapeutic DNA vaccination.

In a one aspect there is provided a variant of a group 5 allergen of the *Pooideae* comprising reduced IgE reactivity compared with the corresponding wild-type allergen and a substantially retained reactivity with T-lymphocytes, wherein one or both regions which correspond to amino-acid sequence regions 94 – 113 and 175 – 198 of Phl p 5a are missing compared with the known wild-type allergen and wherein the group 5 allergens is selected from the group consisting of Phl p 5a, Poa p 5 and Lol p 5.

Figures

Figure 1: Alignment of relevant regions of Phl p 5a-homologous cDNA sequences of *Pooideae* species: *Lolium perenne* (Lol p), *Poa pratensis* (Poa p) *Triticum aestivum* (Tri a) and *Hordeum vulgare* (Hor v)
Numbering: nucleotide positions of the DNA insertions
Phl p 5a, Poa p 5 and Lol p 5 sequences: cDNA sequences from "GenBank" database of the National Center for Biotechnology Information (NCBI), Bethesda, USA
Hor v and Tri a sequences: EST sequences from EST database of the Institute for Genomic Research (TIGR), Rockville, USA
Black borders: sequence identity with Phl p 5a (based on GenBank AJ555152)
Dotted borders: deletion corresponding to amino acids 94 – 113 (based on GenBank AJ555152)
Dashed borders: deletion corresponding to amino acids 175 – 198 (based on GenBank AJ555152)

Figure 2: Alignment of Phl p 5a-homologous amino acid sequences (relevant sequence regions, deduced from DNA sequences) of *Pooideae* species: *Lolium perenne* (Lol p), *Poa pratensis* (Poa p) *Triticum aestivum* (Tri a) and *Hordeum vulgare* (Hor v)

Numbering: nucleotide positions of the DNA insertions

Phl p 5a, Poa p 5 and Lol p 5 sequences: cDNA sequences from "GenBank" database of the National Center for Biotechnology Information (NCBI), Bethesda, USA

Hor v and Tri a sequences: EST sequences from EST database of the Institute for Genomic Research (TIGR), Rockville, USA

Black borders: sequence identity with Phl p 5a (based on GenBank AJ555152)

Dotted borders: deletion corresponding to amino acids 94-113 (based on GenBank AJ555152)

Dashed borders: deletion corresponding to amino acids 175-198 (based on GenBank AJ555152)

Figure 3: SDS-PAGE of purified deletion mutants in the form of histidine fusion proteins

1) Marker
2) rPhl p 5a wt (His)
3) Phl p 5a DM-Δ94-113 (His)
4) Phl p 5a DM-Δ94-113, 175-198 (His)
5) Phl p 5a DM-Δ175-198 (His)
6) Marker

Figure 4: SDS-PAGE of the purified non-fusion proteins Phl p 5a DM-D94-113, 175-198 and rPhl p 5a wt (top) and identity test with αPhl p 5 antibodies (bottom)

αPhl p 5 mAb Apha-1D11 binds region 175-198

(only rPhl p 5a wt is positive)

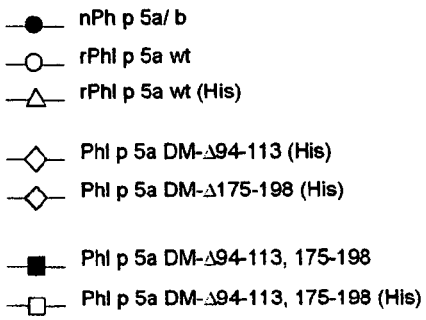
αPhl p 5a mAb Apha-3B2 binds a joint epitope of the two Phl p 5a molecules (both proteins positive)
(mAb: monoclonal antibody)

Figure 5: Analytical SEC of deletion mutant Phl p 5a DM-Δ94-113, 175-198 and of recombinant wild type Phl p 5a (purified non-fusion proteins)
Column: Superdex 75 HR10/ 30 (Amersham Biosciences, Uppsala, Sweden)
Eluent: PBS
Arrow: exclusion volume

Figure 6: Non-denaturing isoelectric focusing of deletion mutant Phl p 5a DM-Δ94-113, 175-198 and of recombinant wild type Phl p 5a (purified non-fusion proteins)
1) IEF marker
2) rPhl p 5a wt
3) Phl p 5a DM-Δ94-113, 175-198
pI rPhl p 5a wt = 8.7
pI rPhl p 5a DM-Δ94-113, 175-198 = 6.4

Figure 7: Strip test for checking the IgE binding ability of Phl p 5a deletion mutants (non-denaturing)
P: sera of clinically defined grass pollen allergy sufferers

Figure 8: Determination of the reduced IgE reactivity of Phl p 5a deletion mutants by means of the EAST inhibition test with two representative single sera (a and b) and a serum pool (c)



P: sera of clinically defined grass pollen allergy sufferers

Figure 9: Determination of the hypoallergeneity of Phl p 5a deletion mutant Phl p 5a DM-Δ94-113, 175-198 by means of the basophil activation test with basophils of six different grass pollen allergy sufferers (P)

Detailed description of the invention

Mutagenesis and cloning of cDNA sequences

The starting point for the – particularly preferred in accordance with the invention – hypoallergenic Phl p 5a variants is the cDNA of an isoform of wild-type Phl p 5a which has been isolated with the aid of specific primers by polymerase chain reaction (PCR) from the total cDNA of pollen of timothy grass (*Phleum pratense*) (NCBI (National Center for Biotechnology Information, Bethesda, USA) GenBank number AJ555152) (SEQ ID NO 1). The amino acid sequence as per SEQ ID NO 2 has been deduced from the cDNA sequence. Phl p 5a, which consists of 284 amino acids, was expressed cytosolically as soluble protein in E. coli and subsequently purified. This recombinant wild-type form of Phl p 5a (rPhl p 5a wt) reacts with monoclonal anti-Phl p 5 antibodies and with IgE antibodies of grass pollen allergy sufferers which have reactivity with natural purified Phl p 5a (nPhl p 5a).

Starting from the described cDNA of rPhl p 5a wt, a series of different deletion variants (deletion mutants) was prepared by restriction/ligation methods and PCR and ligated into the expression vector pProExHTa (Invitrogen, Carlsbad, USA). Sections with a length of 6 to 72 bp distributed over the entire sequence of the cDNA molecule were deleted, causing induction of corresponding deletions in the polypeptide chains of the proteins expressed in *E. coli.*

The deletion variants of Phl p 5a were investigated by immunoblot for their binding ability to IgE antibodies of a representative serum pool of grass pollen allergy sufferers.

In this method, surprisingly, two deletion variants of Phl p 5a (Phl p 5a DM-Δ94-113, deletion of amino acids 94-113 and Phl p 5a DM-Δ175-198, dele-

tion of amino acids 175-198 of rPhl p 5a wt) were found, which have reduced binding of IgE antibodies (representative serum pool).
These two Phl p 5a deletions served as starting point for the construction of a double deletion mutant containing both effective deletions (Phl p 5a DM - Δ94-113, 175-198).

The construction of Phl p 5a DM-Δ94-113, Phl p 5a DM-Δ175-198 and Phl p 5a DM-Δ94-113, 175-198 by genetic engineering methods and the biochemical and immunological characterisation thereof are described below.

For the construction of deletion variant Phl p 5a DM-Δ94-113 (SEQ ID NO 3, cDNA sequence (795 bp), and SEQ ID NO 4, amino acid sequence (264 aa)), firstly two fragments were prepared starting from the cDNA of rPhl p 5a wt. Fragment "F1-93", encoding for amino acids 1-93 of rPhl p 5a wt, was prepared by PCR with the aid of primers 1 and 5, and fragment "F114-284" was prepared with the aid of primers 4 and 6 (primer sequences see Table 1). Fragments "F1-93" and "F114-284" were employed as matrix in a further PCR using primers 1 and 4, which resulted in amplification of the complete cDNA encoding for deletion variant Phl p 5a DM-Δ94-113. The basis of the connection of fragments "F1-93" and "F114-284" by PCR was a sequence region common to both fragments. This sequence region was formed by amplification of fragment "F114-284" by PCR by means of a particular sense oligonucleotide which contained an additional DNA sequence encoding for amino acids 88-93 in the 5' region (Table 1).

The cDNA sequence encoding for deletion variant Phl p 5a DM-Δ175-198 (SEQ ID NO 5, cDNA sequence (783 bp), and SEQ ID NO 6, amino acid sequence (260 aa)) was generated by restriction and subsequent ligation of two separately prepared cDNA fragments. The 5'-terminal fragment

"F1-174" was prepared by PCR with the aid of primers 1 and 2 and the 3'-terminal fragment "F199-284" with the aid of primers 3 and 4. The cDNA fragments were digested with the restriction enzyme SpeI and subsequently ligated (see Table 1). The ligation product was amplified by PCR using primers 1 and 4.

The cDNA of deletion variant Phl p 5a DM-Δ94-113, 175-198 (SEQ ID NO 7, cDNA sequence (723 bp), and SEQ ID NO 8, amino acid sequence (240 aa)) was likewise prepared from two cDNA fragments. The 5'-terminal fragment was generated using primers 1 and 5 and with rPhl p 5a wt-cDNA as matrix, and the 3´-terminal fragment was generated using primers 4 and 6 with Phl p 5a DM-Δ175-198-cDNA as matrix. By means of the common sequence region corresponding to amino acids 88-93 of the rPhl p 5a wt protein, the fragments were connected by a third PCR using primers 1 and 4, and the product was amplified.
The cDNAs encoding for the modified allergens were ligated into the expression vector pProExHT (Invitrogen, Carlsbad, USA) via the *Ehe*I and *Hind*III restriction sites and subsequently sequenced in full.

The immunological cross reactivity of the group 5 allergens of the *Pooideae, such as, for example, Poa pratensis and Lolium perenne,* is based on a very similar amino acid sequence. It can be taken as certain that the corresponding genes go back to a common progenitor gene. Homologous sequence regions in the group 5 allergens of the *Pooideae* exist both for the sequences of deletions Δ94-113 and Δ175-198 of the Phl p 5a wt protein sequence (reference: GenBank AJ555152) and also for the flanking sequence regions thereof. The high homology of the sequence regions in question can be demonstrated both at the DNA level and also at the amino acid sequence level (Fig. 1 and Fig. 2).

Table 1: List of the PCR primers employed for the preparation of deletion variants

Primer	SEQ ID NO	Direction	Sequence (5´→3´)
1	9	sense	gcc gat cta ggc tac ggc ccg gcc
2	10	antisense	aac ata act agt ggc agc gac ctt gaa ggc ggc gtc
3	11	sense	atc ta act agt acg ggc ggc gcc tac gaga
4	12	antisense	aac ata aag ctt tca gac ttt gta gcc acc agt
5	13	antisense	gga gct gga ttc ggc ggc gcc ctt ggg
6	14	sense	gcc gcc gaa tcc agc tcc ggc gcg acg cct gag gcc aag tac gac

The *Spe*I restriction sites are indicated by underlining

Expression and purification of recombinant Phl p 5a molecules

The recombinant proteins were expressed as histidine fusion proteins with integrated protease cleavage site (expression vector pProExHT; Invitrogen, Carlsbad, USA) for optional removal of the histidine fusion component (His) in *Escherichia coli* (strain JM109). rPhl p5a wt and the deletion mutants were firstly purified by specific binding of the N-terminal histidine residues to an Ni2+ chelate matrix (immobilised metal ion affinity chromatography, IMAC) and subsequently by preparative gel filtration (size exclusion chromatography, SEC).

The purity of the eluted proteins was monitored by SDS-PAGE and analytical SEC. The results showed that rPhl p 5a wt (His), Phl p 5a DM-Δ94-113 (His); Phl p 5a DM-Δ175-198 (His) and Phl p 5a DM-Δ94-113, 175-198 (His) could be prepared with high purity and in monomeric form

(Fig. 3). The identity of the proteins was demonstrated by Phl p 5a-specific monoclonal antibodies.

The checking of the IgE reactivity by means of IgE binding techniques (immunoblotting, strip test, EAST inhibition test and basophil activation test) and the investigation of the T-cell reactivity was in addition carried out with test substances without a histidine fusion component.

To this end, the deletion variants was prepared in parallel to the comparative protein rPhl p 5a-wt firstly as fusion proteins. However, the histidine fusion component was subsequently cleaved off enzymatically (TEV protease, Invitrogen, Carlsbad, USA), leaving only a glycine as residue of the protease cleavage sequence on the N terminal of the target protein. Both the cleaved-off histidine component and also the protease used for the cleavage were separated off completely by IMAC. After preparative SEC, the purity and conformation of the eluted proteins was checked by SDS-PAGE and analytical SEC, as shown in Figures 4 and 5 for rPhl p 5a wt and the mutant Phl p 5a DM-Δ94-113, 175-198 respectively. All proteins were prepared in pure and monomeric form. An investigation by non-denaturing isoelectric focusing (IEF) of the non-fusion proteins always showed high homogeneity with respect to the surface charge (see Fig. 6, illustrative for Phl p 5a DM-Δ94-113, 175-198).

The identity of the recombinant proteins was demonstrated by the monoclonal anti-Phl p 5 antibodies (Allergopharma, Reinbek, Germany) Apha-1D11 or Apha-3B2 (see Fig. 4, illustrative for Phl p 5a DM-Δ94-113, 175-198) and N-terminal sequencing.

Determination of reduced IgE binding of the Phl p 5a deletion variants

A simple test method for determination of the IgE reactivity of allergenic molecules is investigation of the binding of specific IgE from the sera from allergy sufferers to membrane-bound test proteins by the strip test.

For this purpose, the test substances are bound in the same concentration and amount alongside one another to a strip of nitrocellulose membrane under non-denaturing conditions. A series of such membrane strips can be incubated in parallel with various sera from allergy sufferers. After a washing step, the specifically bound IgE antibodies are rendered visible on the membrane by a colour reaction promoted by an anti-hIgE/alkaline phosphatase conjugate.

The IgE reactivity of the recombinant proteins Phl p 5a wt (His), Phl p 5a DM-Δ94-113 (His), Phl p 5a DM-Δ175-198 (His) and Phl p 5a DM-Δ94-113, 175-198 (His) was investigated comparatively in the strip test using 43 individual sera from grass pollen allergy sufferers (Fig. 7).
All 43 sera from allergy sufferers contained Phl p 5a-specific IgE antibodies which reacted strongly with the natural Phl p 5a (nPhl p 5a, not shown here) and the recombinant equivalent rPhl p 5a wt (His).
Surprisingly, it became clear that the Phl p 5a-specific IgE antibodies of all 43 patient sera did not bind at all to deletion variant Phl p 5a DM-Δ94-113, 175-198 (His) or only did so to a very greatly reduced extent. The reduced IgE binding is attributable both to the deletion Δ94-113 and also to the deletion Δ175-198. Deletion variant Phl p 5a DM-Δ175-198 (His) shows a clearly recognisably reduced IgE binding capacity in this test in 35 of 43 sera from allergy sufferers. In some tests, the influence of the deletion of amino acids 175-198 was so great that IgE binding was virtually completely prevented (Ex.: P3, P20, P28)

The influence of deletion Δ94-113 on the IgE binding reactivity is less pronounced, but likewise clearly visible. Deletion variant Phl p 5a DM-Δ94-113 (His) was bound significantly more weakly by IgE of 19 of the 43 individual sera from allergy sufferers than the reference rPhl p 5a wt (His) (Ex.: P31, P37, P42). However, the reduction in the IgE binding was less drastically

pronounced in many individual tests than the reduction caused by Δ175-198.

It is thus clear that both deletions contribute to the reduction in the total IgE binding reactivity of the deletion mutant Phl p 5a DM-Δ94-113, 175-198 (His).

In contrast to the strip test, the EAST inhibition test (enzyme allergosorbent test) allows the investigation of allergen/IgE interactions in solution, enabling interfering masking of epitopes of the test substance by immobilisation on the membrane to be fundamentally excluded. The EAST inhibition test is carried out as follows. Microtitre plates are coated with allergens, here natural Phl p 5 (nPhl p 5a/b, mixture of Phl p 5a and Phl p 5b). After removal of the unbound allergen molecules by washing, the plate is blocked with bovine serum albumin in order to prevent later non-specific binding. IgE antibodies of allergy sufferers, as representative pool of individual sera (serum pool) or as single serum, is incubated in suitable dilution with the allergen-coated microtitre plates. The amount of allergen-bound IgE antibodies is quantified photometrically via an enzyme coupled to a second antibody (anti-hIgE/alkaline phosphatase conjugate) through conversion of a substrate into a coloured end product.
The binding of the IgE antibodies is inhibited substance-specifically by a soluble allergen or the substance to be tested (recombinant modified allergen) depending on the concentration. Immunochemically identical substances show identical inhibition curves.

The reference molecules used in this work were nPhl p 5, rPhl p 5a wt, and the histidine fusion protein rPhl p 5a wt (His). Besides other molecules, the IgE binding of the histidine fusion proteins Phl p 5a DM-Δ94-113 (His), Phl p 5a DM-Δ175-198 (His) and Phl p 5a DM-Δ94-113, 175-198 (His) and

that of the non-fusion protein Phl p 5a DM-Δ94-113, 175-198 was investigated by comparison with these references.

Figs. 8 a-c show representatively the specific inhibition curves of test substances raised with two individual sera and a serum pool of grass pollen allergy sufferers. nPhl p 5a/b showed the greatest inhibitory effect in all tests (about 80-95% inhibitory effect at a concentration of 10 µg/ml). The inhibitory effect of rPhl p 5a was significantly lower with a maximum inhibition of 70-80%. This effect is caused by the composition of nPhl p 5a/b, which also contains the isoform Phl p 5b in addition to the isoform Phl p 5a. The specific IgE antibodies against Phl p 5b cannot be inhibited by rPhl p 5a wt.

The histidine fusion component showed no effect on IgE binding. This is clear in all tests through the identical inhibition curves of rPhl p 5a wt (His) and rPhl p 5a wt. This demonstrates the validity of tests with histidine fusion proteins.

In general, two groups of patient sera were distinguished with respect to qualitative IgE binding.

The first group is represented by individual serum P15 (Fig. 8 a). These sera from allergy sufferers contained IgE antibodies whose binding to Phl p 5a was reduced by both deletions, Δ94-113 and Δ175-198. Deletion mutant Phl p 5a DM-Δ94-113 (His) showed only a maximum inhibitory effect of about 50% here, and the deletion mutant Phl p 5a DM-175-198 (His) showed an inhibitory effect of only 20-30%.

The double deletion mutant Phl p 5a DM-Δ94-113, 175-198 (His) was only able to inhibit the binding of IgE antibodies by 0-10% at the highest concentration employed. The use of the non-fusion protein Phl p 5a DM-Δ94-113, 175-198 confirmed this result (0-10% maximum IgE inhibition).

The second group of sera from allergy sufferers, represented by individual serum P44 (Fig. 8 b), differed from the first group through the fact that the IgE antibodies present in the sera reacted equally well with Phl p 5a DM-Δ94-113 (His) as with the reference rPhl p 5a wt (His) (70-80% maximum inhibition), whereas no or non-detectable amounts of IgE antibodies reacted with Phl p 5a DM-Δ175-198 (His) (0-10% maximum inhibition).
The double deletion mutant Phl p 5a DM-Δ94-113, 175-198 likewise showed a greatly reduced inhibitory effect (0-10%) with this group of sera from allergy sufferers, which was shown both for the fusion protein and also for the fusion component-free protein.
The sera of these allergy sufferers apparently contained IgE antibodies directed principally against epitopes of the C-terminal part of the molecule.

The measurement data of the IgE binding reactivity of IgE antibodies of a serum pool of 20 allergy sufferers underline the importance of the deletions Δ94-113 and Δ175-198 for the reduction in the IgE binding of Phl p 5a (Fig. 8 c). Both individual deletion mutants, Phl p 5a DM-Δ94-113 (His) and Phl p 5a DM-Δ175-198 (His) show a lower maximum inhibitory effect, of 40-50% and about 30% respectively, than rPhl p 5a wt (about 70%). The double deletion mutant Phl p 5a DM-Δ94-113, 175-198 was only bound very weakly by the IgE antibodies of the serum pool (10-15% maximum inhibition), which, in agreement with the test of 43 allergy sufferers in the strip test, indicates greatly reduced IgE binding reactivity of this Phl p 5a variant in very many, if not all, grass pollen allergy sufferers.

Determination of the hypoallergeneity of the deletion mutants by basophil activation test

By means of a basophil activation test, the effects of reduced IgE binding ability of the deletion mutants on the functional effect in the crosslinking of

membrane-bound IgE of the effector cells and activation thereof were investigated. The functional reduction in allergeneity was thus measured in a sensitive in-vitro test.

For the basophil activation test, heparinised full blood from grass pollen allergy sufferers is incubated with various concentrations of the test substances. Allergenic substances are able to bind specific IgE antibodies, which are associated with the high-affinity IgE receptors of the basophilic granulocytes.
Crosslinking of the IgE/receptor complexes initiated by the allergen molecules results in signal transduction, which results in degranulation of the effector cells and thus initiation of the allergic reactions in vivo.

In vitro, allergen-induced activation of basophilic immunocytes can be determined by quantification of the expression of a surface protein (CD203c) coupled to signal transduction of the IgE receptor crosslinking (Kahlert et al., Clinical Immunology and Allergy in Medicine Proceedings of the EAACI 2002 (2003) Naples, Italy 739-744). The number of expressed surface proteins on a cell and the percentage of activated cells of a cell pool is measured highly sensitively via the binding of a fluorescence-labelled monoclonal antibody to the surface protein and subsequent analysis by fluorescence-activated flow cytometry.

The reference substances employed here were both purified natural Phl p 5a (nPhl p 5a) and also rPhl p5a wt in parallel with the test substances. The test results of the double deletion mutant Phl p 5a DM Δ94-113, 175-198 with basophils from six test persons are shown as curves in Figure 9. The test results with basophils from a total of 10 clinically defined allergy sufferers are shown in Table 2.
The A50 values (A50: allergen concentration at 50% of the number of basophils activated to the maximum) of the reference molecules were, varying individually, between ~1.3-15 pM for rPhl p 5a wt and ~0.3-10 pM

for nPhl p 5a (Table 2). By contrast, the A50 values of deletion variant Phl p 5a DM Δ94-113, 175-198 were between ~18-8400 pM.

The A50 values determined for the three substances employed were used to determine the allergenic efficacy of deletion variant Phl p 5a DM Δ94-113, 175-198 in relation to the unchanged reference molecules nPhl p 5a and rPhl p5a wt for each test person (Table 2).

The relative allergenic efficacy (Pr, relative potency) of deletion variant Phl p 5a DM Δ94-113, 175-198 was reduced between ~12-5000 fold compared with the reference rPhl p 5a wt or ~16-32000 fold compared with the reference nPhl p 5a (Table 2).

Table 2: Determination of the hypoallergeneity of deletion mutant Phl p 5a DM-Δ94-113, 175-198 by means of basophil activation test

	Test substance A_{50} [pM] [a]			**Pr value [b] Phl p 5a DM-Δ94-113, 175-198**	**Pr value [b] Phl p 5a DM-Δ94-113, 175-198**
Donor [c]	**nPhl p 5a**	**rPhl p 5a wt**	**Phl p 5a DM-Δ94-113, 175-198**	**relative to rPhl p 5a wt [d]**	**relative to nPhl p 5a [e]**
P13	4.08	5.34	477.2	0.0111	0.0085
P17	6.44	2.68	466.6	0.0057	0.0137
P20	0.26	1.68	8433.0	**0.0002 [f]**	**0.00003 [f]**
P23	1.02	1.26	39.2	0.0321	0.0260
P24	1.22	2.57	58.1	0.0442	0.0209
P28	9.43	11.35	198.2	0.0573	0.0476
P29	1.77	2.34	33.7	0.0694	0.0525
P31	10.15	14.66	3967.0	0.0037	0.0026
P34	3.48	2.54	165.1	0.0153	0.0211
P40	1.08	1.45	17.5	**0.0829**	**0.0617**

[a] Allergen concentration at 50% of the number of basophils activated to the maximum
[b] Relative potency
[c] Clinically defined grass pollen allergy sufferers
[d] Calculated from A50 rPhl p 5a wt/ A50 Phl p 5a DM-Δ94-113, 175-198
[e] Calculated from A50 nPhl p 5a/ A50 Phl p 5a DM-Δ94-113, 175-198
[f] Bold: minimum and maximum values

T-cell reactivity

T helper lymphocytes react with peptide fragments of the allergens (approx. 12-25 amino acids) formed by enzymatic degradation in antigen-presenting cells (APCs) and are presented to the T-cells after inclusion of the suitable peptides in the individual MHC class II molecules at the surface of the APCs. This allergen-specific activation of the T helper lymphocytes is the prerequisite for subsequent reactions (proliferation, anergy, apoptosis) and for functional differentiation (TH1 and TH2). The influencing of allergen-specific T-lymphocytes by treatment with an allergen or an allergen variant in hyposensitisation is regarded as the key for the therapeutic efficacy.

In order to investigate T-cell reactivity, oligoclonal T-cell lines (TCLs) of Graminae pollen allergy sufferers are established by conventional methods with stimulation by nPhl p5 or rPhl p 5 molecules.
In a proliferation test, the various T-cell lines were stimulated with the reference allergens nPhl p5a and rPhl p5a wt and the double deletion mutant Phl p 5a DM Δ94-113, 175-198. The proliferation rate was determined by the incorporation of [^{3}H] thymidine by conventional methods.

Table 3: Determination of the T-cell reactivity of deletion mutant Phl p 5a DM-Δ94-113, 175-198 by means of proliferation tests with Phl p 5-specific T-cell lines (TCLs)

		Stimulation index [a]		
Donor [b]	**TCL**	**nPhl p 5a**	**rPhl p 5a wt**	**Phl p 5a DM-Δ94-113, 175-198**
A	3.2	9.8	4.9	4.4
B	8.2	21.0	15.5	13.3
C	11.2	5.2	4.7	7.2
C	11.3	3.3	2.9	3.5
C	11.43	3.0	3.9	2.6
D	19.1	6.5	4.7	7.5
D	19.2	9.6	3.3	2.6
E	23.22	21.8	29.0	20.8
E	23.50	7.5	8.4	6.6
F	89.23	1.8	3.5	1.8

[a] Calculated from [^{3}H] measurement values. cpm measurement values of allergen-stimulated cell cultures/cpm measurement values of unstimulated cell cultures

[b] Donor: clinically defined grass pollen allergy sufferers

The results with ten TCLs from six allergy sufferers show that these TCLs were stimulated to proliferation by Phl p 5a DM Δ94-113, 175-198 in comparable strength as by the unchanged natural or recombinant wild-type allergen (Table 3).

The present invention thus relates to variants of the group 5 allergens of the *Pooideae* which are characterised by reduced IgE reactivity compared with the known wild-type allergens and by retained reactivity with T-lymphocytes. These group 5 allergens are preferably Phl p 5a, Poa p 5 and Lol p 5, very particularly preferably Phl p 5a.

As it has proven particularly favourable for the purposes of the invention for amino-acid sequence regions which correspond to amino-acid sequence

regions 94 – 113 and 175 – 198 of Phl p 5a to be missing or removed in the group 5 allergens, this invention relates, in particular, to such allergen variants. The first-mentioned or second-mentioned region may be missing individually, but also both said regions may be missing simultaneously, with the latter embodiment being very particularly preferred.

Owing to the high sequence homologies within the group 5 allergens from *Pooideae*, these regions can be unambiguously identified in sequence alignments of the Phl p 5a sequence with sequences from other group 5 allergens. The above-described allergen variants preferably originate from Phl p 5a or correspond to the sequences in accordance with SEQ ID NO 4, 6 or 8.

The allergen variants according to the invention can be prepared starting from the cloned DNA sequence with the aid of genetic engineering methods. In principle, however, chemical modifications of the native allergen extract are also possible (Fiebig, 1995, Allergo J. 4 (7), 377-382).

Naturally, further modifications in other positions – for example in order to increase the hypoallergeneity – are also possible via the variations of group 5 allergens described in the present patent application. These modifications can be, for example, amino acid insertions, deletions and exchanges, cleavage of the protein into fragments and fusion of the protein or fragments thereof with other proteins or peptides.

During preparation of the allergen variants described in more detail here, an His tag was introduced by genetic engineering methods for the purposes of improved purification of the overexpressed proteins.

The invention furthermore relates to a DNA molecule encoding for an allergen variant described above, in particular corresponding to a sequence in accordance with SEQ ID NO 3, 5 or 7, to a recombinant expression vector containing this DNA molecule, and to a host organism transformed with

said DNA molecule or said expression vector. Suitable host organisms may be prokaryotic or eukaryotic, single- or multicelled organisms, such as bacteria or yeasts. A host organism which is preferred in accordance with the invention is *E. coli*.

The invention furthermore relates to a process for the preparation of an allergen variant according to the invention by cultivation of the said host organism and isolation of the corresponding allergen variant from the culture.

The present invention additionally relates to the allergen variants, DNA molecules and expression vectors described above in their property as medicaments.

The present invention furthermore relates to pharmaceutical compositions comprising at least one of these allergen variants or a corresponding DNA molecule or a corresponding expression vector and optionally further active ingredients and/or adjuvants for the treatment of allergies in the triggering of which group 5 allergens of the *Pooideae* are involved, or for the immunotherapeutic vaccination of patients having allergies in the triggering of which group 5 allergens of the *Pooideae* are involved and/or for the prevention of such allergies.
If these are pharmaceutical compositions of the second type (comprising at least one DNA molecule or an expression vector), these compositions preferably furthermore comprise aluminium hydroxide, an immunostimulatory CpG-containing oligonucleotide or a combination of the two as adjuvants.

For the purposes of this invention, pharmaceutical compositions can be used as therapeutic agents in human or veterinary medicine. Suitable excipients are organic or inorganic substances which are suitable for parenteral administration and do not react with group 5 allergen variants according to the invention. Suitable for parenteral administration are, in

particular, solutions, preferably oily or aqueous solutions, furthermore suspensions, emulsions or implants. The allergen variants according to the invention may also be lyophilised and the resultant lyophilisates used, for example, for the preparation of injection preparations. The compositions indicated may be sterilised and/or comprise adjuvants, such as lubricants, preservatives, stabilisers and/or wetting agents, emulsifiers, salts for modifying the osmotic pressure, buffer substances and/or a plurality of further active ingredients.

Furthermore, appropriate formulation of the allergen variants according to the invention enables depot preparations to be obtained, for example by adsorption on aluminium hydroxide.

Finally, the present invention relates to the use of at least one allergen variant according to the invention or a DNA molecule according to the invention or an expression vector according to the invention for the preparation of a medicament for the treatment of allergies in the triggering of which group 5 allergens of the *Pooideae* are involved or for the immunotherapeutic vaccination of patients having allergies in the triggering of which group 5 allergens of the *Pooideae* are involved and/or for the prevention of such allergies.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as, an acknowledgement or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

The Claims defining the invention are as follows:

1. A variant of a group 5 allergen of the *Pooideae* comprising reduced IgE reactivity compared with the corresponding wild-type allergen and a substantially retained reactivity with T-lymphocytes, wherein one or both regions which correspond to amino-acid sequence regions 94 – 113 and 175 – 198 of Phl p 5a are missing compared with the known wild-type allergen and wherein the group 5 allergen is selected from the group consisting of Phl p 5a, Poa p 5 and Lol p 5.

2. The allergen variant according to Claim 1 wherein the variant is derived from Phl p 5a.

3. A variant of allergen Phl p 5a wherein one or both amino-acid sequence regions 94 – 113 and 175 – 198 are missing.

4. The variant of allergen Phl p 5a according to Claim 3 wherein said variant comprises an amino-acid sequence selected from the group consisting of SEQ ID NO 4, SEQ ID NO 6 and SEQ ID NO 8.

5. The allergen variant according to any one of Claims 1 to 4 wherein the variant is obtained by recombinant genetic engineering methods.

6. A DNA molecule encoding for an allergen variant according to any one of Claims 1 to 5.

7. A DNA molecule corresponding to a DNA sequence selected from the group consisting of SEQ ID NO 3, SEQ ID NO 5 and SEQ ID NO 7.

8. A recombinant expression vector containing a DNA molecule according to Claim 6 or Claim 7 wherein the DNA molecule is functionally bonded to an expression control sequence.

9. A host organism transformed with a DNA molecule according to Claim 6 or

Claim 7 or an expression vector according to Claim 8.

10. A process for the preparation of the allergen variant according to any one of Claims 1 to 5 by cultivation of the host organism according to Claim 9 and isolation of the corresponding allergen variant from a culture of the host-organism.

11. The allergen variant according to any one of Claims 1 to 5 when used as a medicament.

12. A pharmaceutical composition comprising at least one allergen variant according to any one of Claims 1 to 5 for the treatment of an allergy wherein a group 5 allergen of the *Pooideae* is involved in triggering the allergy.

13. The pharmaceutical composition of claim 12 comprising one or more further active ingredients and/or adjuvants.

14. Use of at least one allergen variant according to any one of Claims 1 to 5 for the preparation of a medicament for the treatment of an allergy wherein a group 5 allergen of the *Pooideae* is involved in triggering the allergy.

15. The DNA molecule according to Claim 6 or 7 when used as medicament.

16. The recombinant expression vector according to Claim 8 when used as medicament.

17. A method of treatment of an allergy involving a group 5 allergen of the *Pooideae* comprising administration of at least one allergen variant according to any one of Claims 1 to 5, the DNA molecule of Claim 6 or 7, or the expression vector of Claim 8 to a patient in need thereof.

18. A pharmaceutical composition comprising at least one DNA molecule according to Claim 15 or at least one expression vector according to Claim

16 for the immunotherapeutic DNA vaccination of patients in the triggering of which group 5 allergens of the *Pooideae* are involved and/or for the prevention of the allergy.

19. The composition according to Claim 18 further comprising further active ingredients and/or adjuvants.

20. The pharmaceutical composition according to Claim 18 or Claim 19, further comprising aluminium hydroxide, an immunostimulatory CpG-containing oligonucleotide or a combination thereof as adjuvants.

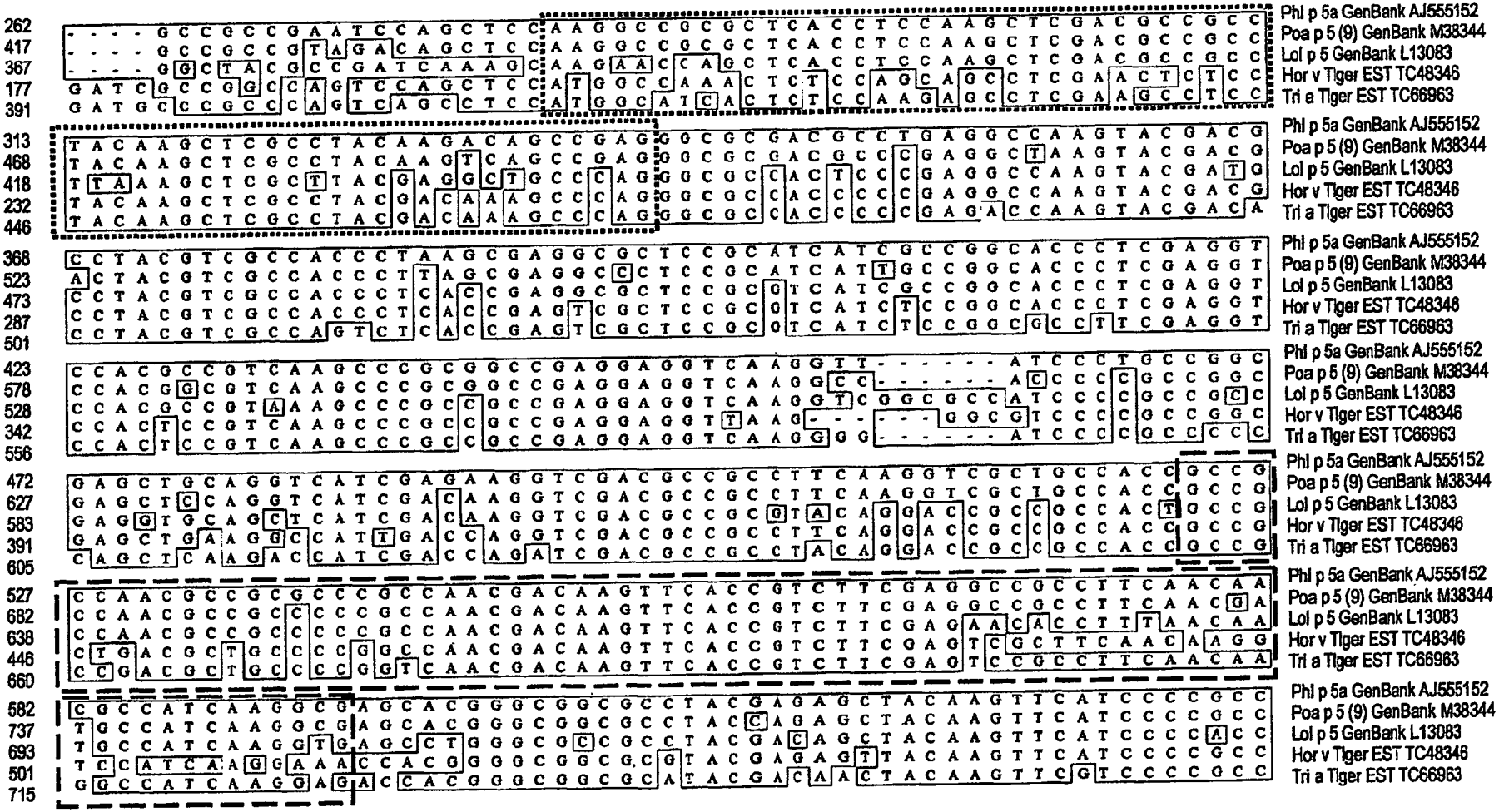
21. Use of at least one DNA molecule according to Claim 6 or Claim 7 or at least one expression vector according to Claim 8 for the preparation of a medicament for the immunotherapeutic DNA vaccination of patients having an allergy wherein a group 5 allergen of the *Pooideae* is involved in triggering the allergy and/or for the prevention of the allergy.

22. A method for the immunotherapeutic DNA vaccination of a patient having an allergy wherein a group 5 allergen of the *Pooideae* is involved in triggering the allergy, the method comprising administering to the patient at least one DNA molecule according to Claim 6 or Claim 7 or at least one expression vector according to Claim 8.

23. A variant of a group 5 allergen of the *Pooideae* comprising reduced IgE reactivity compared with the known wild-type allergen and a substantially reduced reactivity with T-lymphocytes wherein one or both regions corresponding to amino-acid sequence regions 94 – 113 and 175 – 198 of Phl p 5a are missing compared with the known wild-type allergen and substantially as hereinbefore described with reference to the examples.

Alignment of relevant regions of Phl p 5a-homologous cDNA sequences of *Pooideae* species: *Lolium perenne* (Lol p), *Poa pratensis* (Poa p) *Triticum aestivum* (Tri a) and *Hordeum vulgare* (Hor v)

Fig. 1



Numbering: nucleotide positions of the DNA insertions
Phl p 5a, Poa p 5 and Lol p 5 sequences: cDNA sequences from "GenBank" database of the ***National Center for Biotechnology Information (NCBI)***, Bethesda, USA
Hor v and Tri a sequences: EST sequences from EST database of the ***Institute for Genomic Research (TIGER)***, Rockville, USA
Black borders: sequence identity with Phl p 5a (based on GenBank AJ555152)
Dotted borders: deletion corresponding to amino acids 94-113 (based on GenBank AJ555152)
Dashed borders: deletion corresponding to amino acids 175-198 (based on GenBank AJ555152)

Fig. 2a

Fig. 2 { Fig. 2a / Fig. 2b }

Alignment of Phl p 5a-homologous amino acid sequences (relevant sequence regions, deduced from DNA sequences) of Pooideae species: *Lolium perenne* (Lol p), *Poa pratensis* (Poa p) *Triticum aestivum* (Tri a) and *Hordeum vulgare* (Hor v)

```
169  P P A D K Y R T F V A T F G A A S N K A F A E G L S G E P K   Phl p 5a GenBank AJ555152
324  P A A N K Y K T F V A T F G A A S N K A F A E A L S T E P K   Poa p 5 (9) GenBank M38344
286  P P A D K Y K T F V E T F G T A T N K A F V E G L A S - - -   Lol p 5 GenBank L13083
82   P P A D K Y K T F E A T F A A A S N K A F A E V L K G A A T   Hor v Tiger EST TC48346
296  P P A D K Y K T F E A T F S A A S N X A F A D V L K A A A S   Tri a Tiger EST TC66963

259  G - - A A E S S S K A A L T S K L D A A Y K L A Y K T A E G   Phl p 5a GenBank AJ555152
414  G - - A A V D S S K A A L T S K L D A A Y K L A Y K S A E G   Poa p 5 (9) GenBank M38344
367  - - - G Y A D Q S K N Q L T S K L D A A L K L A Y E A A Q G   Lol p 5 GenBank L13083
172  G Q I A G Q S S S M A K L S S S L E L S Y K L A Y D K A Q G   Hor v Tiger EST TC48346
386  G Q M P A Q S A S M A S L S K S L E A S Y K L A Y D K A Q G   Tri a Tiger EST TC66963

343  A T P E A K Y D A Y V A T L S E A L R I I A G T L E V H A V   Phl p 5a GenBank AJ555152
498  A T P E A K Y D D Y V A T L S E A L R I I A G T L E V H G V   Poa p 5 (9) GenBank M38344
448  A T P E A K Y D A Y V A T L T E A L R V I A G T L E V H A V   Lol p 5 GenBank L13083
262  A T P E A K Y D A Y V A T L T E S L R V I S G T L E V H S V   Hor v Tiger EST TC48346
476  A T P E T K Y D T Y V A S L T E S L R V I S G A F E V H S V   Tri a Tiger EST TC66963

433  K P A A E E V K V - - I P A G E L Q V I E K V D A A F K V A   Phl p 5a GenBank AJ555152
588  K P A A E E V K A - - T P A G E L Q V I D K V D A A F K V A   Poa p 5 (9) GenBank M38344
538  K P A A E E V K V G A I P A A E V Q L I D K V D A A Y R T A   Lol p 5 GenBank L13083
352  K P A A E E V K - - G V P A G E L K A I D Q V D A A F R T A   Hor v Tiger EST TC48346
566  K P A A E E V K G X X I P A P Q L K T I D Q I D A A Y R T A   Tri a Tiger EST TC66963
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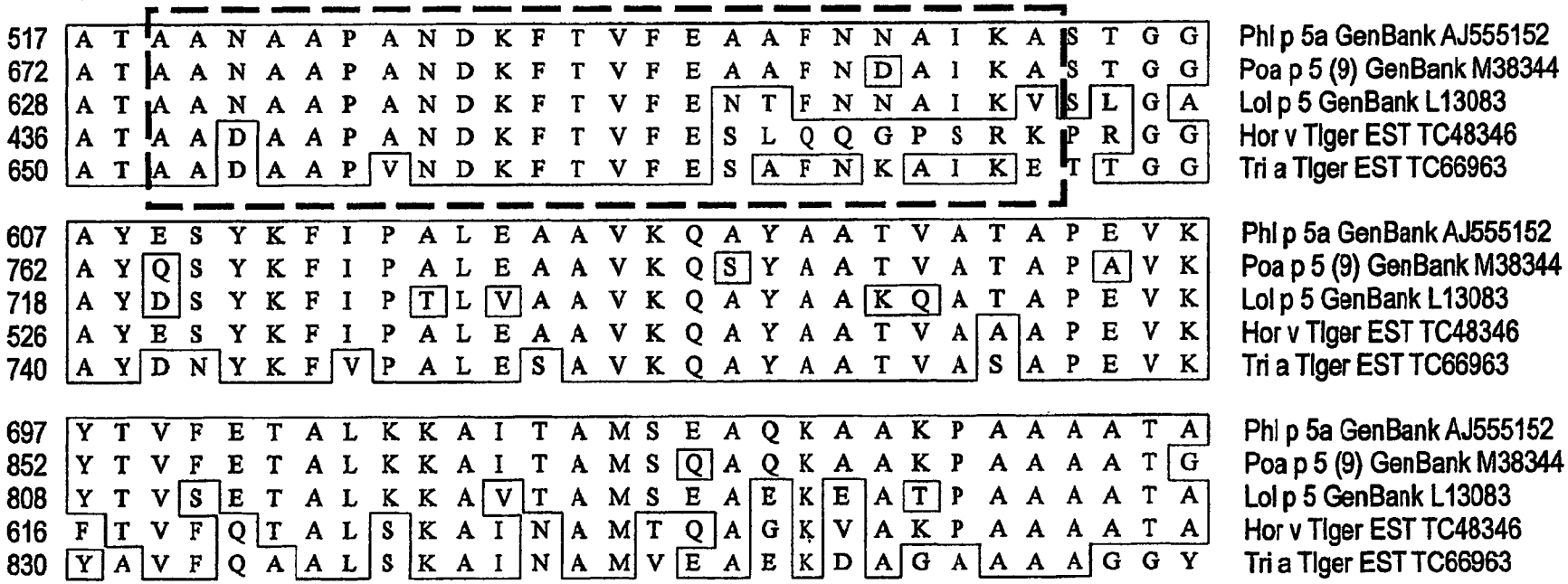
Fig. 2b



Numbering: nucleotide positions of the DNA insertions

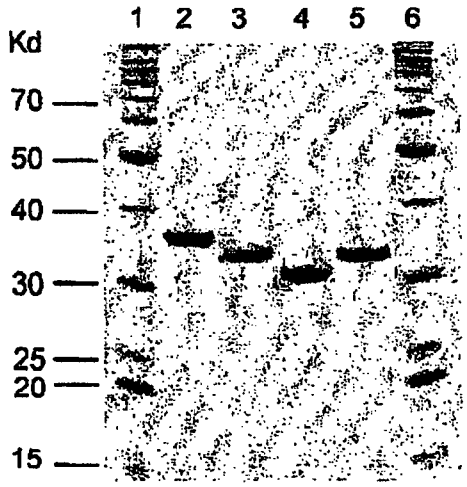
Phl p 5a, Poa p 5 and Lol p 5 sequences: cDNA sequences from "GenBank" database of the *National Center for Biotechnologly Information (NCBI)*, Bethesda, USA

Hor v and Tri a sequences: EST sequences from EST database of the *Institute for Genomic Research (TIGER)*, Rockville, USA

Black borders: sequence identity with Phl p 5a (based on GenBank AJ555152)
Dotted borders: deletion corresponding to amino acids 94-113 (based on GenBank AJ555152)
Dashed borders: deletion corresponding to amino acids 175-198 (based on GenBank AJ555152)

Fig. 3

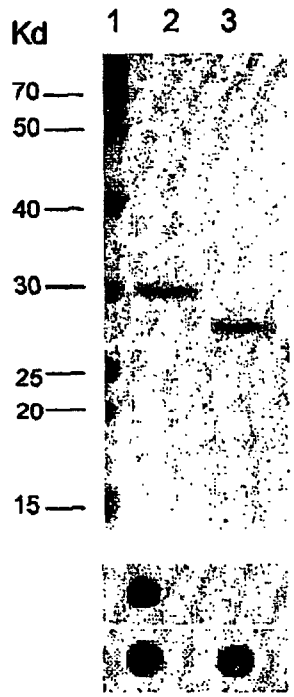
SDS-PAGE of purified deletion mutants in the form of histidine fusion proteins



1) Marker
2) rPhl p 5a wt (His)
3) Phl p 5a DM-Δ94-113 (His)
4) Phl p 5a DM-Δ94-113, 175-198 (His)
5) Phl p 5a DM-Δ175-198 (His)
6) Marker

Fig. 4

SDS-PAGE of the purified non-fusion proteins Phl p 5a DM-Δ94-113, 175-198 and rPhl p 5a wt (top) and identity test with αPhl p 5 antibodies (bottom)



1) Marker
2) rPhl p5a wt
3) Phl p5a DM-Δ94-113, 175-198

mAb 1D11

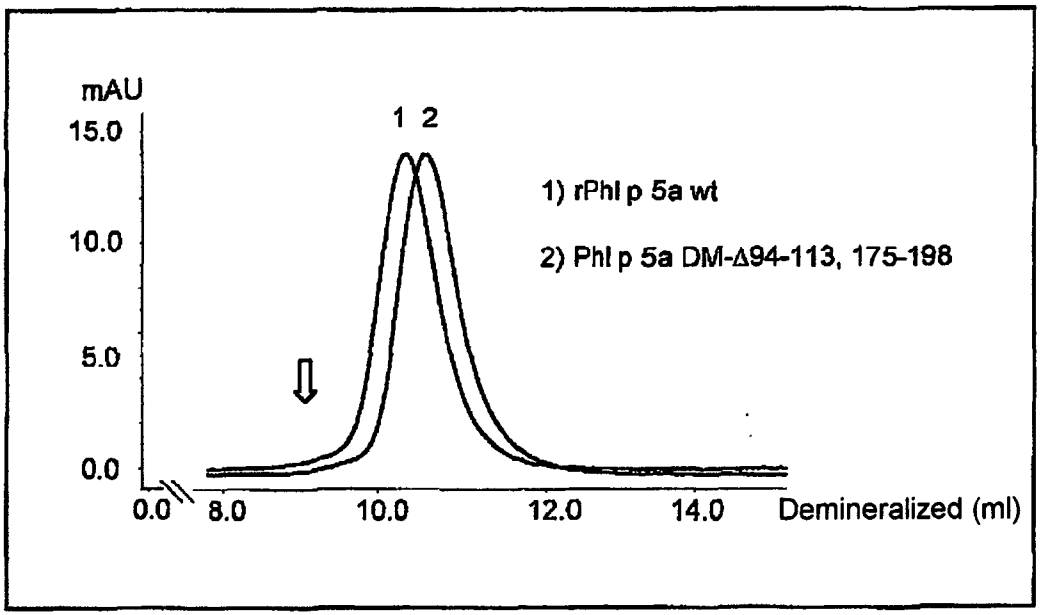
mAb 3B2

αPhl p 5 mAb Apha-1D11 binds region 175-198 (only rPhl p 5a wt is positive)

αPhl p 5a mAb Apha-3B2 binds a joint epitope of the two Phl p 5a molecules (both proteins positive)

(mAb: monoclonal antibody)

Analytical SEC of deletion mutant Phl p 5a DM-Δ94-113, 175-198 and of recombinant wild type Phl p 5a (purified non-fusion proteins)

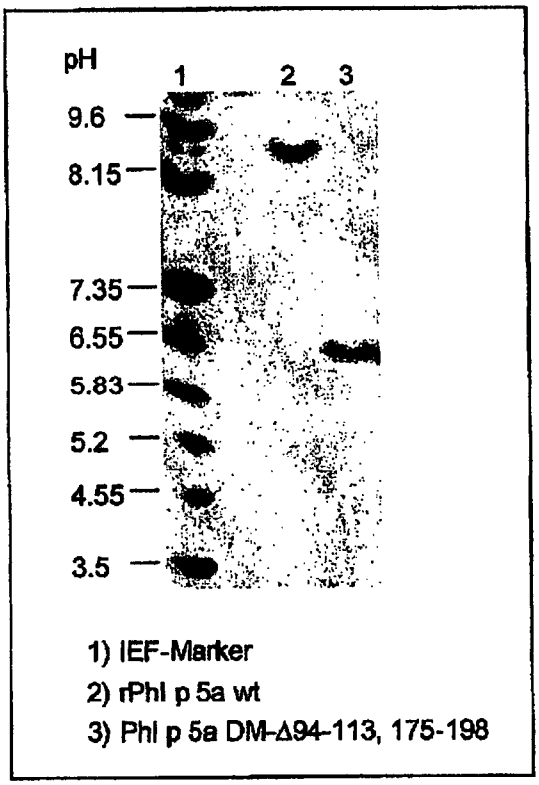


Column: Superdex 75 HR10/ 30 (Amersham Biosciences, Uppsala, Sweden)
Eluent: PBS
Arrow: exclusion volume

Fig. 5

Fig. 6

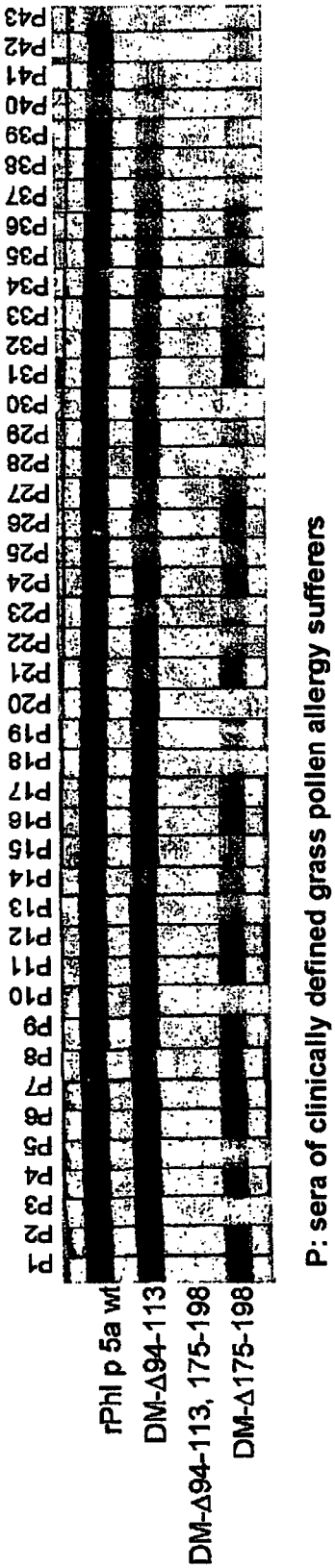
Non-denaturing isoelectric focusing of deletion mutant Phl p 5a DM-Δ94-113, 175-198 and of recombinant wild type Phl p 5a (purified non-fusion proteins)



pI rPhl p 5a wt = 8.7
pI rPhl p 5a DM-Δ94-113, 175-198 = 6.4

Fig. 7

Strip test for checking the IgE binding ability of Phl p 5a deletion mutants
(non-denaturing)



P: sera of clinically defined grass pollen allergy sufferers

Fig. 8

Determination of the reduced IgE reactivity of Phl p 5a deletion mutants by means of the EAST inhibition test with two representative single sera (a and b) and a serum pool (c)

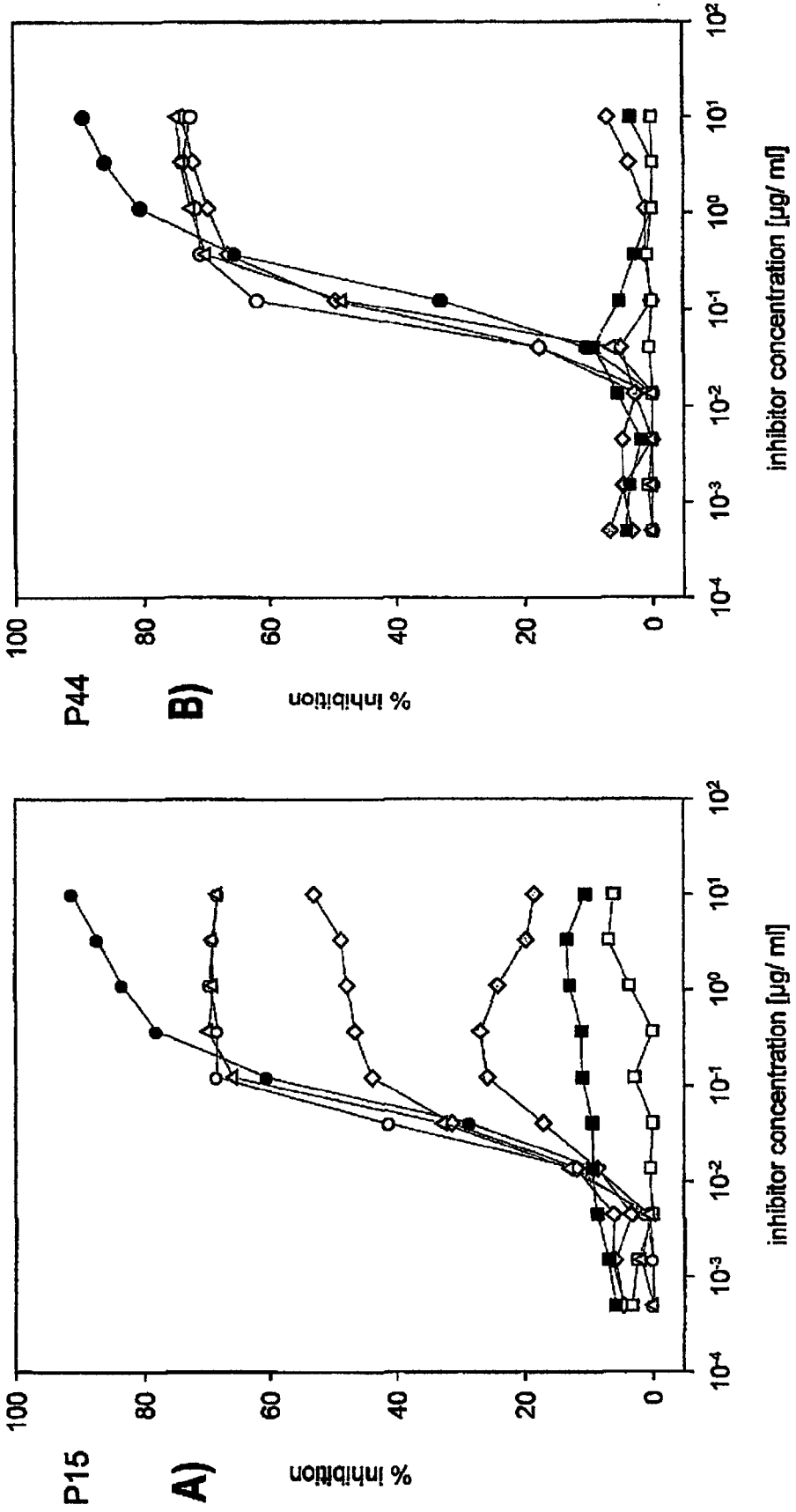


Fig. 8

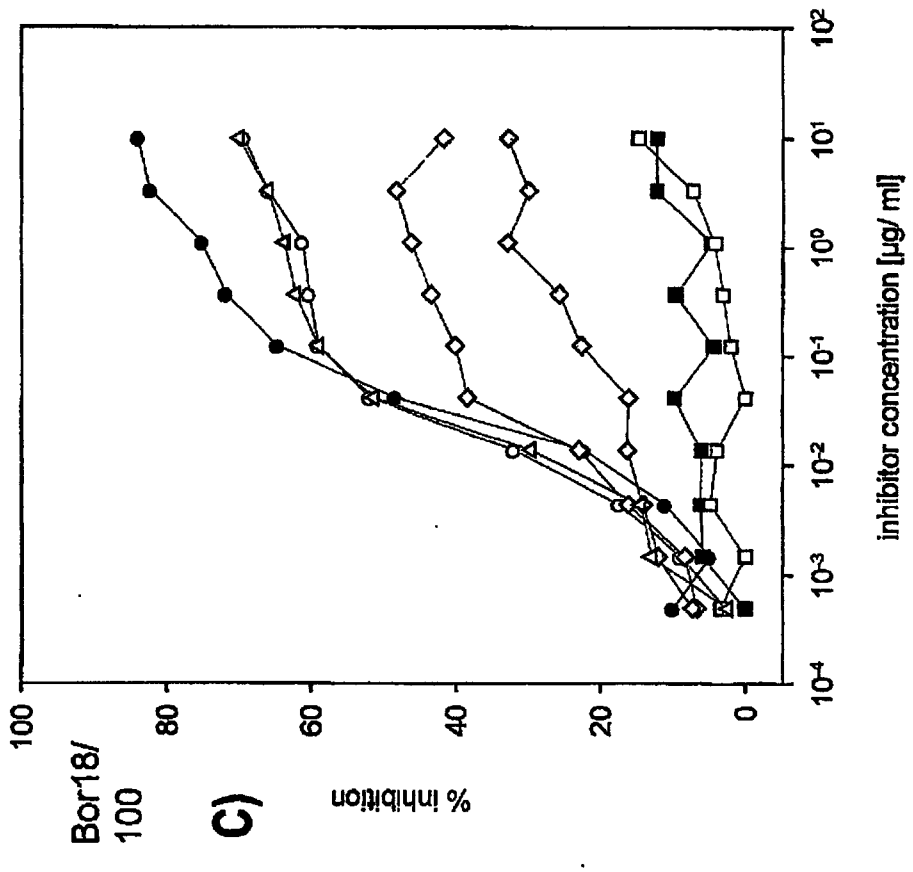
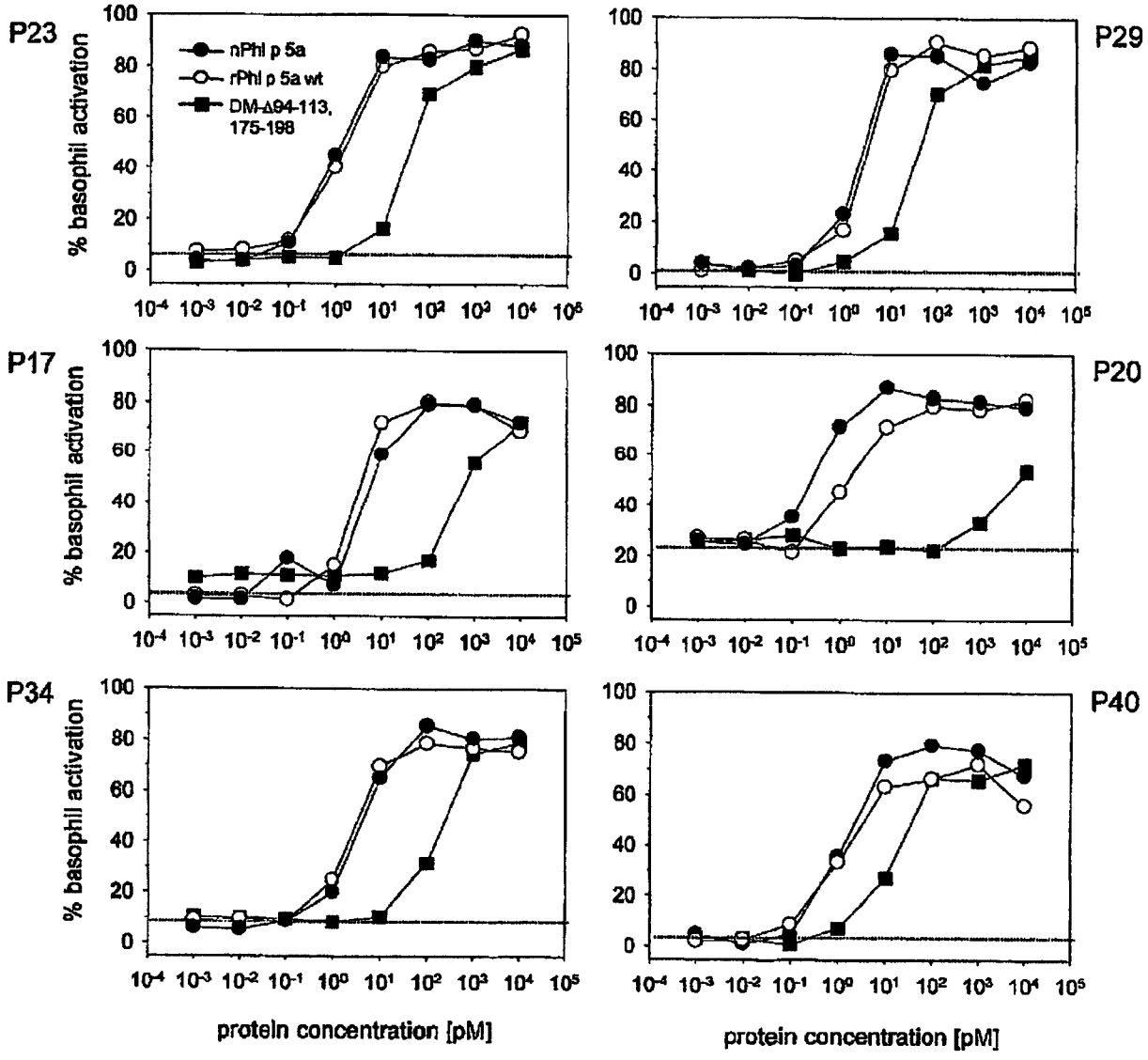

C)
Bor18/100
% inhibition
inhibitor concentration [µg/ ml]
nPh p 5a/ b
rPhl p 5a wt
rPhl p 5a wt (His)
Phl p 5a DM-Δ94-113 (His)
Phl p 5a DM-Δ175-198 (His)
Phl p 5a DM-Δ94-113, 175-198
Phl p 5a DM-Δ94-113, 175-198 (His)
P: sera of clinically defined grass pollen allergy sufferers

Fig. 9

Determination of the hypoallergeneity of Phl p 5a deletion mutant Phl p 5a DM-Δ94-113, 175-198 by means of the basophil activation test with basophils of six different grass pollen allergy sufferers (P)



Sequence Listing

<110> Merck Patent GmbH

<120> Phl p 5a derivatives having reduced allergeneity and retained T-cell reactivity

<130> P 03/109

<140> DE 10325508.7

<141> 2003-06-04

<160> 14

<170> PatentIn version 3.1

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aagatcaacg ccggcttcaa ggcggccttg gccgctgccg ccggcgtccc gccagcggac 180

aagtacagga cgttcgtcgc aaccttcggc gcggcctcca acaaggcctt cgcggagggc 240

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ctcgacgccg cctacaagct cgcctacaag acagccgagg gcgcgacgcc tgaggccaag 360

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ggcggcgcct acgagagcta caagttcatc cccgccctgg aggccgccgt caagcaggcc 660

tacgccgcca ccgtcgccac cgcgccggag gtcaagtaca ccgtctttga gaccgcgctg 720

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<213> Phleum pratense

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Thr Glu Glu Gln Lys Leu Ile Glu Lys Ile Asn Ala Gly Phe Lys Ala
35 40 45

Ala Leu Ala Ala Ala Ala Gly Val Pro Pro Ala Asp Lys Tyr Arg Thr
50 55 60

Phe Val Ala Thr Phe Gly Ala Ala Ser Asn Lys Ala Phe Ala Glu Gly
65 70 75 80

Leu Ser Gly Glu Pro Lys Gly Ala Ala Glu Ser Ser Ser Lys Ala Ala
85 90 95

Leu Thr Ser Lys Leu Asp Ala Ala Tyr Lys Leu Ala Tyr Lys Thr Ala
100 105 110

Glu Gly Ala Thr Pro Glu Ala Lys Tyr Asp Ala Tyr Val Ala Thr Leu
115 120 125

Ser Glu Ala Leu Arg Ile Ile Ala Gly Thr Leu Glu Val His Ala Val
130 135 140

Lys Pro Ala Ala Glu Glu Val Lys Val Ile Pro Ala Gly Glu Leu Gln

145 150 155 160

Val Ile Glu Lys Val Asp Ala Ala Phe Lys Val Ala Ala Thr Ala Ala
165 170 175

Asn Ala Ala Pro Ala Asn Asp Lys Phe Thr Val Phe Glu Ala Ala Phe
180 185 190

Asn Asn Ala Ile Lys Ala Ser Thr Gly Gly Ala Tyr Glu Ser Tyr Lys
195 200 205

Phe Ile Pro Ala Leu Glu Ala Ala Val Lys Gln Ala Tyr Ala Ala Thr
210 215 220

Val Ala Thr Ala Pro Glu Val Lys Tyr Thr Val Phe Glu Thr Ala Leu
225 230 235 240

Lys Lys Ala Ile Thr Ala Met Ser Glu Ala Gln Lys Ala Ala Lys Pro
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Ala Ala Ala Ala Thr Ala Thr Ala Thr Ser Ala Val Gly Ala Ala Thr
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Gly Ala Ala Thr Ala Ala Thr Gly Gly Tyr Lys Val
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aagatcaacg ccggcttcaa ggcggccttg gccgctgccg ccggcgtccc gccagcggac 180

aagtacagga cgttcgtcgc aaccttcggc gcggcctcca acaaggcctt cgcggagggc 240

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tacgacgcct acgtcgccac cctaagcgag gcgctccgca tcatcgccgg caccctcgag 360

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<213> Phleum pratense

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Tyr Thr Pro Ala Ala Pro Ala Gly Ala Glu Pro Ala Gly Lys Ala Thr
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Thr Glu Glu Gln Lys Leu Ile Glu Lys Ile Asn Ala Gly Phe Lys Ala
35 40 45

Ala Leu Ala Ala Ala Ala Gly Val Pro Pro Ala Asp Lys Tyr Arg Thr
50 55 60

Phe Val Ala Thr Phe Gly Ala Ala Ser Asn Lys Ala Phe Ala Glu Gly
65 70 75 80

Leu Ser Gly Glu Pro Lys Gly Ala Ala Glu Ser Ser Ser Gly Ala Thr
85 90 95

Pro Glu Ala Lys Tyr Asp Ala Tyr Val Ala Thr Leu Ser Glu Ala Leu
100 105 110

Arg Ile Ile Ala Gly Thr Leu Glu Val His Ala Val Lys Pro Ala Ala
115 120 125

Glu Glu Val Lys Val Ile Pro Ala Gly Glu Leu Gln Val Ile Glu Lys
130 135 140

Val Asp Ala Ala Phe Lys Val Ala Ala Thr Ala Ala Asn Ala Ala Pro

145 150 155 160

Ala Asn Asp Lys Phe Thr Val Phe Glu Ala Ala Phe Asn Asn Ala Ile
165 170 175

Lys Ala Ser Thr Gly Gly Ala Tyr Glu Ser Tyr Lys Phe Ile Pro Ala
180 185 190

Leu Glu Ala Ala Val Lys Gln Ala Tyr Ala Ala Thr Val Ala Thr Ala
195 200 205

Pro Glu Val Lys Tyr Thr Val Phe Glu Thr Ala Leu Lys Lys Ala Ile
210 215 220

Thr Ala Met Ser Glu Ala Gln Lys Ala Ala Lys Pro Ala Ala Ala Ala
225 230 235 240

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Ala Ala Thr Gly Gly Tyr Lys Val
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<212> DNA

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aagatcaacg ccggcttcaa ggcggccttg gccgctgccg ccggcgtccc gccagcggac 180

aagtacagga cgttcgtcgc aaccttcggc gcggcctcca acaaggcctt cgcggagggc 240

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ctcgacgccg cctacaagct cgcctacaag acagccgagg gcgcgacgcc tgaggccaag 360

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gtccacgccg tcaagcccgc ggccgaggag gtcaaggtta tccctgccgg cgagctgcag 480

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Tyr Thr Pro Ala Ala Pro Ala Gly Ala Glu Pro Ala Gly Lys Ala Thr
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Thr Glu Glu Gln Lys Leu Ile Glu Lys Ile Asn Ala Gly Phe Lys Ala
35 40 45

Ala Leu Ala Ala Ala Ala Gly Val Pro Pro Ala Asp Lys Tyr Arg Thr
50 55 60

Phe Val Ala Thr Phe Gly Ala Ala Ser Asn Lys Ala Phe Ala Glu Gly
65 70 75 80

Leu Ser Gly Glu Pro Lys Gly Ala Ala Glu Ser Ser Ser Lys Ala Ala
85 90 95

Leu Thr Ser Lys Leu Asp Ala Ala Tyr Lys Leu Ala Tyr Lys Thr Ala
100 105 110

Glu Gly Ala Thr Pro Glu Ala Lys Tyr Asp Ala Tyr Val Ala Thr Leu
115 120 125

Ser Glu Ala Leu Arg Ile Ile Ala Gly Thr Leu Glu Val His Ala Val
130 135 140

Lys Pro Ala Ala Glu Glu Val Lys Val Ile Pro Ala Gly Glu Leu Gln
145 150 155 160

Val Ile Glu Lys Val Asp Ala Ala Phe Lys Val Ala Ala Thr Ser Thr

165 170 175

Gly Gly Ala Tyr Glu Ser Tyr Lys Phe Ile Pro Ala Leu Glu Ala Ala
180 185 190

Val Lys Gln Ala Tyr Ala Ala Thr Val Ala Thr Ala Pro Glu Val Lys
195 200 205

Tyr Thr Val Phe Glu Thr Ala Leu Lys Lys Ala Ile Thr Ala Met Ser
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Glu Ala Gln Lys Ala Ala Lys Pro Ala Ala Ala Ala Thr Ala Thr Ala
225 230 235 240

Thr Ser Ala Val Gly Ala Ala Thr Gly Ala Ala Thr Ala Ala Thr Gly
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Gly Tyr Lys Val
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aagatcaacg ccggcttcaa ggcggccttg gccgctgccg ccggcgtccc gccagcggac 180

aagtacagga cgttcgtcgc aaccttcggc gcggcctcca acaaggcctt cgcggagggc 240

ctctcgggcg agcccaaggg cgccgccgaa tccagctccg gcgcgacgcc tgaggccaag 300

tacgacgcct acgtcgccac cctaagcgag gcgctccgca tcatcgccgg caccctcgag 360

gtccacgccg tcaagcccgc ggccgaggag gtcaaggtta tccctgccgg cgagctgcag 420

gtcatcgaga aggtcgacgc cgccttcaag gtcgctgcca ccagcacggg cggcgcctac 480

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gtcgccaccg cgccggaggt caagtacacc gtctttgaga ccgcgctgaa aaaggccatc 600

accgccatgt ccgaggccca gaaggctgcc aagcccgctg ccgctgccac cgccaccgca 660

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<211> 240

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<213> Phleum pratense

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Tyr Thr Pro Ala Ala Pro Ala Gly Ala Glu Pro Ala Gly Lys Ala Thr
20 25 30

Thr Glu Glu Gln Lys Leu Ile Glu Lys Ile Asn Ala Gly Phe Lys Ala
35 40 45

Ala Leu Ala Ala Ala Ala Gly Val Pro Pro Ala Asp Lys Tyr Arg Thr
50 55 60

Phe Val Ala Thr Phe Gly Ala Ala Ser Asn Lys Ala Phe Ala Glu Gly
65 70 75 80

Leu Ser Gly Glu Pro Lys Gly Ala Ala Glu Ser Ser Ser Gly Ala Thr
85 90 95

Pro Glu Ala Lys Tyr Asp Ala Tyr Val Ala Thr Leu Ser Glu Ala Leu
100 105 110

Arg Ile Ile Ala Gly Thr Leu Glu Val His Ala Val Lys Pro Ala Ala
115 120 125

Glu Glu Val Lys Val Ile Pro Ala Gly Glu Leu Gln Val Ile Glu Lys
130 135 140

Val Asp Ala Ala Phe Lys Val Ala Ala Thr Ser Thr Gly Gly Ala Tyr
145 150 155 160

Glu Ser Tyr Lys Phe Ile Pro Ala Leu Glu Ala Ala Val Lys Gln Ala
165 170 175

Tyr Ala Ala Thr Val Ala Thr Ala Pro Glu Val Lys Tyr Thr Val Phe
180 185 190

Glu Thr Ala Leu Lys Lys Ala Ile Thr Ala Met Ser Glu Ala Gln Lys
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Ala Ala Lys Pro Ala Ala Ala Ala Thr Ala Thr Ala Thr Ser Ala Val
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Gly Ala Ala Thr Gly Ala Ala Thr Ala Ala Thr Gly Gly Tyr Lys Val
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<211> 30

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<210> 14

<211> 45

<212> DNA

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