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(54) Title: PROTEIN ALLERGEN DERIVATIVES

(57) Abstract: The present invention relates to a method for producing derivatives of wild-type protein allergens with reduced allergenic activity, characterized in by the following steps: providing a wild-type protein allergen with an allergenic activity, splicing said wild-type protein allergen into two parts, said two parts having a reduced allergenic activity or lacking allergenic activity and rejoining said two fragments in inverse orientation; as well as allergen derivatives.

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Protein Allergen Derivatives

The present invention relates to a method for reducing allergenic activity of wild-type protein allergens, novel allergen derivatives and allergy vaccination strategies.

Allergy is the inherited or acquired specific alternation of the reaction capability against foreign (i.e. non-self) substances which are normally harmless ("allergens"). Allergy is connected with inflammatory reactions in the affected organ systems (skin, conjunctiva, nose, pharynx, bronchial mucosa, gastrointestinal tract), immediate disease symptoms, such as allergic rhinitis, conjunctivitis, dermatitis, anaphylactic shock and asthma, and chronic disease manifestations, such as late stage reactions in asthma and atopic dermatitis.

Type I allergy represents a genetically determined hypersensitivity disease which affects about 20 % of the industrialised world population. The pathophysiological hallmark of Type I allergy is the production of immunoglobulin E (IgE) antibodies against otherwise harmless antigens (allergens).

Currently, the only causative form of allergy treatment is an allergen-specific immunotherapy wherein increasing allergen doses are administered to the patient in order to induce allergen-specific unresponsiveness. While several studies have shown clinical effectiveness of allergen-specific immunotherapy, the underlying mechanisms are not fully understood.

The major disadvantage of allergen-specific immunotherapy is the dependency on the use of natural allergen extracts which are difficult, if not impossible to standardise, at least to an industrial production level. Such natural allergen extracts consist of different allergenic and non allergenic compounds and due to this fact it is possible that certain allergens are not present in the administered extract or - even worse - that patients can develop new IgE-specificities to components in the course of the treatment. Another disadvantage of extract-based therapy results from the fact that the administration of biologically active allergen preparations can induce anaphylactic

side effects.

The application of molecular biology techniques in the field of allergen characterisation has allowed to isolate the cDNAs coding for all relevant environmental allergens and allowed the production of recombinant allergens. Using such recombinant allergens has made it possible to determine the individual patient's reactivity profile either by in vitro diagnostic methods (i.e. detection of allergen-specific IgE antibodies in serum) or by in vivo testing. Based on this technology, the possibility to develop novel component-based vaccination strategies against allergy, especially against Type I allergy, which are tailored to the patient's sensitisation profile appeared to be possible. However, due to the similarity of the recombinant allergens to their natural counterparts, also recombinant allergens exhibit significant allergenic activity. Since the recombinant allergens closely mimick the allergenic activity of the wild-type allergens, all the drawbacks connected with this allergenic activity in immunotherapy applying natural allergens are also present for recombinant allergens. In order to improve immunotherapy the allergenic activity of the recombinant allergens has to be reduced so that the dose of the administered allergens can be increased with only a low risk of anaphylactic side effects.

It has been suggested to influence exclusively the activity of allergen-specific T cells by administration of peptides containing T cell epitopes only. T cell epitopes represent small peptides which result from the proteolytic digestion of intact allergens by antigen representing cells. Such T cell epitopes can be produced as synthetic peptides. Tests conducted so far with T cell epitopes, however, only showed poor results and low efficacy. Several explanations for the low efficacy of T cell peptide-based immunotherapy have been considered: first, it may be difficult to administer the optimal dose to achieve T cell tolerance instead of activation. Second, small T cell epitope peptides will have a short half-life in the body. Third, there is considerable evidence that IgE production in atopic individuals represents a memory immune response which does not require de novo class switching and thus cannot be controlled by T cell-derived cytokines. Therapy forms which are based exclusively on

the administration of T cell epitopes may therefore modulate the activity of allergen-specific T cells but may have little influence on the production of allergen-specific IgE antibodies by already switched memory B cells.

It has further been suggested to produce hypoallergenic allergen derivates or fragments by recombinant DNA technology or peptide synthesis. Such derivatives or fragments bear T cell epitopes and can induce IgG antibodies that compete with IgE recognition of the native allergen. It was demonstrated more than 20 years ago that proteolytic digestion of allergens yielded small allergen fragments which in part retained their IgE binding capacity but failed to elicit immediate type reactions. While proteolysis of allergens is difficult to control and standardise, molecular biology has opened up new avenues for the production of IgE binding haptens. Such IgE binding haptens have been suggested to be useful for active immunisation with reduced risks of anaphylactic effects and for passive therapy to saturate effector cell-bound IgE prior to allergen contact and thus block allergen-induced mediator release.

Another suggestion was to produce hypoallergenic allergen versions by genetic engineering based on the observation that allergens can naturally occur as isoforms with differ in only a few amino acid residues and/or in conformations with low IgE binding capacity. For example, oligomerisation of the major birch pollen allergen, Bet v 1, by genetic engineering yielded a recombinant trimer with greatly reduced allergenic activity. Alternatively, introduction of point mutations has been suggested to either lead to conformational changes in the allergen structure and thus disrupt discontinuous IgE epitopes or directly affect the IgE binding capacity (Valenta et al., Biol.Chem.380 (1999), 815-824).

It has also been shown that fragmentation of the allergen into few parts (e.g. into two parts) leads to an almost complete loss of IgE binding capacity and allergenic activity of the allergen due to a loss of their native-like folds (Vrtala et al. (J.Clin.Invest.99 (1997), 1673-1681) for Bet v 1, Twardosz et al. (BBRC 239 (1997), 197-204) for Bet v 4, Hayek et al. (J. Im-

munol.161 (1998), 7031-7039) for Aln g 4, Zeiler et al. (J.Allergy Clin. Immunol.100 (1997), 721-727) for bovine dander allergen, Elfman (Int.Arch.Allergy Immunol.117 (1998), 167-173) for Lep d2), Westritschnig (J.Immunol.172 (2004), 5684-5692) for Phlp 7),...). Fragmentation of proteins containing primarily discontinuous/conformational IgE epitopes leads to a substantial reduction of the allergen's IgE binding capacity. Based on this knowledge, it was investigated in the prior art whether such hypoallergenic allergen fragments can induce protective immune responses in vivo (Westritschnig et al. (Curr. Opinion in Allergy and Clin. Immunol. 3 (2003), 495-500)).

It is an object of the present invention to provide means and methods for improved allergy immunotherapy based on the above mentioned knowledge. Such methods and means should be effective, connected with a low risk for anaphylactic shock, easily applicable and adapted to the needs of an individual patient and easily transformable into industrial scales.

According to a first aspect the present invention provides a method for producing a derivative of a wild-type protein allergen with reduced allergenic activity, comprising the following steps: (a) providing a wild-type protein allergen with an allergenic activity, (b) splicing said wild-type protein allergen into two parts, wherein said two parts have a reduced allergenic activity or lack allergenic activity and (c) rejoining said two fragments in inverse orientation.

According to a second aspect the present invention provides an allergen derivative of a wild-type protein allergen, wherein said wild-type protein allergen comprises an amino

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acid sequence of 1 to Z, and wherein said derivative adjacently comprises, in N-terminal to C-terminal orientation, two wild-type allergen fragments X to Z and 1 to X, wherein said two wild-type allergen fragments have reduced allergenic activity or lack allergenic activity.

According to a third aspect the present invention provides an allergen composition comprising an allergen derivative according to the second aspect, and at least one further allergen.

According to a fourth aspect the present invention provides use of an allergen derivative according to the second aspect, or an allergen composition according to the third aspect, for the preparation of a medicament for allergen specific immunotherapy.

According to a fifth aspect the present invention provides use of an allergen derivative or an allergen composition according to the second and third aspects for the preparation of a medicament for passive immunisation or prophylactic immunisation.

According to a sixth aspect the present invention provides use of a profilin derivative obtainable from a first wild-type profilin molecule by the method according to any one of the above aspects, or an allergen derivative of a first wild-type profilin molecule according to the second aspect or an allergen composition according to the third aspect, for the manufacture of a medicament for prevention and/or treatment of allergic diseases caused by a second wild-type profilin molecule, or for the treatment and/or prevention of

pollen food cross sensitization attributable to profilin allergy.

According to a seventh aspect the present invention provides a method of allergen specific immunotherapy, passive immunisation or prophylactic immunisation comprising administering to a patient in need thereof a therapeutically effective amount of the allergen derivative of the second aspect and/or the allergen composition of the third aspect.

The present invention also provides a method for producing derivatives of wild-type protein allergens with reduced allergenic activity, which is characterized by the following steps:

- providing a wild-type protein allergen with an allergenic activity,
- splicing said wild-type protein allergen into two parts said two parts having a reduced allergenic activity or lacking allergenic activity and
- rejoining said two fragments in inverse orientation.

The present method is based on the fact that fragmentation of proteins containing primarily discontinuous/conformational IgE epitopes leads to a substantial reduction of the allergen's IgE binding capacity. However, fragments of certain allergens were too less immunogenic to induce a protective antibody response (Westritschnig et al., (2004)).

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With the present method, new and defined protein allergen derivatives are provided which combine the advantages of the T cell and B cell epitope-based approaches. At the same time, the dis-

advantages of vaccination with fragments only or sophisticated arrangements of fragments (such as IgE binding haptens and shuffling with three or more fragments) are not present for the allergen derivatives of the present invention.

In fact it could be shown with the present invention that the optimal results can be obtained with the structure which - with respect to completeness of structure elements - most closely resembles the wild-type allergen (i.e. with all amino acids of the wild-type allergen), however, without its allergenic activity (or with a sufficiently reduced allergenic activity). Of course, if only a few amino acid residues are lost (deleted) or added (inserted) in the course of generation of the allergen derivatives or if the parts are combined by a linker instead of a direct combination, the advantages according to the present invention are still present. This reduction or abolishment of allergenic activity is achieved by the known and general principle of dividing the allergen into defined fragments. In addition to this general principle, the present invention rejoins the two parts of the allergen obtained in inverse orientation which leads to allergen derivatives which contain essentially all relevant structural information of the allergen (because the amino acid sequence is contained in full or almost in full in the allergen derivates according to the present invention) but with only low (or no) remaining allergenic activity compared to the wild-type allergen.

These "head-to tail" derivatives according to the present invention enable a suitable, individual and efficient immunotherapy for allergy patients which is easily up-scaleable with routine steps. The derivates according to the present invention induce protective IgG antibodies which can block patient's IgE binding to wild-type allergens and inhibit allergen-induced basophil degranulation.

The present method is specifically suitable for recombinant DNA technology. Once the derivative is constructed by genetic engineering, it can easily be obtained in considerable amounts by transgene expression on an industrial scale in suitable hosts. The allergen derivatives according to the present invention can

preferably be produced in a host with high expression capacity.

Preferred allergens to be modified by the present invention include all major protein allergens available e.g. under www.allergen.org/List.htm. Specifically preferred groups of allergens according to the present invention include profilins, especially Phl p 12, birch allergens, especially Bet v 4, dust mite allergens, especially Der p2, storage mite allergens, especially Lep d 2, timothy grass allergens, especially Phl p 7, and the allergens listed in table A.

Table A: preferred allergen to be modified by shuffling according to the present invention (including reference examples)

| ALLERGENS<br>Species Name | Allergen Name | Biochem.ID or Obsolete name | MW | cDNA or protein | Reference, Acc.No. |
|---|---|---|---|---|---|
| Ambrosia artemisiifolia | | | | | |
| short ragweed | | | | | |
| | Amb a 1 | antigen E | 8 | C | 8, 20 |
| | Amb a 2 | antigen K | 38 | C | 8, 21 |
| | Amb a 3 | Ra3 | 11 | C | 22 |
| | Amb a 5 | Ra5 | 5 | C | 11, 23 |
| | Amb a 6 | Ra6 | 10 | C | 24, 25 |
| | Amb a 7 | Ra7 | 12 | P | 26 |
| Ambrosia trifida | | | | | |
| giant ragweed | | | | | |
| | Amb t 5 | Ra5G | 4.4 | C | 9, 10, 27 |
| Artemisia vulgaris | | | | | |
| mugwort | | | | | |
| | Art v 1 | | 27-29 | C | 28 |
| | Art v 2 | | 35 | P | 28A |
| | Art v 3 | lipid transfer protein | 12 | P | 53 |
| | Art v 4 | profilin | 14 | C | 29 |
| Helianthus annuus | | | | | |
| sunflower | | | | | |
| | Hel a 1 | | 34 | | 29A |
| | Hel a 2 | profilin | 15.7 | C | Y15210 |
| Mercurialis annua | | | | | |
| | Mer a 1 | profilin | 14-15 | C | Y13271 |
| Caryophyllales | | | | | |
| Chenopodium album | | | | | |
| lamb's-quarters, pigweed, | | | | | |
| | Che a 1 | | 17 | C | AY049012, 29B |
| white goosefoot | | | | | |
| | Che a 2 | profilin | 14 | C | AY082337 |
| | Che a 3 | polcalcin | 10 | C | AY082338 |
| Salsola kali | | | | | |
| Russian-thistle | | | | | |
| | Sal k 1 | | 43 | P | 29C |
| Rosales | | | | | |
| Humulus japonicus | | | | | |
| Japanese hop | | | | | |
| | Hum j 4w | | C | | AY335187 |
| Parietaria judaica | | | | | |
| | Par j 1 | lipid transfer protein 1 | 15 | C | see list of isoallergens |
| | Par j 2 | lipid transfer protein 2 | | C | see list of isoallergens |
| | Par j 3 | profilin | | C | see list of isoallergens |
| Parietaria officinalis | | | | | |
| | Par o 1 | lipid transfer protein | 15 | | 29D |

| | | | | | |
|---|---|---|---|---|---|
| B. Grasses | | | | | |
| Poales | | | | | |
| Cynodon dactylon | | | | | |
| Bermuda grass | | | | | |
| Cyn d 1 | | 32 | C | | 30, S83343 |
| Cyn d 7 | | | C | | 31, X91256 |
| Cyn d 12 | profilin | 14 | C | | 31a, Y08390 |
| Cyn d 15 | | 9 | C | | AF517686 |
| Cyn d 22w | enolase | data | | pending | |
| Cyn d 23 | Cyn d 14 | 9 | C | | AF517605 |
| Cyn d 24 | Pathogenesis- related p. | 21 | P | | pending |
| Dactylis glomerata | | | | | |
| orchard grass | | | | | |
| Dac g 1 | AgDg1 | 32 | P | | 32 |
| Dac g 2 | | 11 | C | | 33, S45354 |
| Dac g 3 | | | C | | 33A, U25343 |
| Dac g 5 | | 31 | P | | 34 |
| Festuca pratensis | | | | | |
| meadow fescue | | | | | |
| Fes p 4w | | 60 | - | | |
| Holcus lanatus | | | | | |
| velvet grass | | | | | |
| Hol l 1 | | | C | | Z27084 |
| Lolium perenne | | | | | |
| rye grass | | | | | |
| Lol p 1 | group I | 27 | C | | 35, 36 |
| Lol p 2 | group II | 11 | P | | 37, 37A, X73363 |
| Lol p 3 | group III | 11 | P | | 38 |
| Lol p 5 | Lol p IX, Lol p 1b | 31/35 | C | | 34, 39 |
| Lol p 11 hom: | trypsin inhibitor | 16 | | | 39A |
| Phalaris aquatica | | | | | |
| canary grass | | | | | |
| Pha a 1 | | | C | | 40, S80654 |
| Phleum pratense | | | | | |
| timothy | Phl p 1 | 27 | C | | X78813 |
| Phl p 2 | | | C | | X75925, 41 |
| Phl p 4 | | | P | | 41A |
| Phl p 5 | Ag25 | 32 | C | | 42 |
| Phl p 6 | | | C | | Z27082, 43 |
| Phl p 11 | trypsin inhibitor hom. | 20 | C | | AF521563, 43A |
| Phl p 12 | profilin | | C | | X77583, 44 |
| Phl p 13 | polygalacturonase | 55-60 | C | | AJ238848 |
| Poa pratensis | | | | | |
| Kentucky blue grass | | | | | |
| Poa p 1 | group I | 33 | P | | 46 |
| Poa p 5 | | 31/34 | C | | 34, 47 |
| Sorghum halepense | | | | | |
| Johnson grass | | | | | |
| Sor h 1 | | | C | | 48 |
| C. Trees | | | | | |
| Arecales | | | | | |
| Phoenix dactylifera | | | | | |
| date palm | | | | | |
| Pho d 2 | profilin | 14.3 | C | | Asturias p.c. |
| Fagales | | | | | |
| Alnus glutinosa | | | | | |
| alder | | | | | |
| Aln g 1 | | 17 | C | | S50892 |
| Betula verrucosa | | | | | |
| birch Bet v 1 | | 17 | C | | see list of isoallergens |
| Bet v 2 | profilin | 15 | C | | M65179 |
| Bet v 3 | | | C | | X79267 |
| Bet v 4 | | 8 | C | | X87153, S54819 |
| Bet v 6 h: | isoflavone reductase | 33.5 | C | | see list of isoallergens |
| Bet v 7 | cyclophilin | 18 | P | | P81531 |
| Carpinus betulus | | | | | |
| hornbeam | | | | | |
| Car b 1 | | 17 | C | | see list of isoallergens |
| Castanea sativa | | | | | |
| chestnut | | | | | |
| Cas s 1 | | 22 | P | | 52 |
| Cas s 5 | chitinase | | | | |

| | | | | |
|---|---|---|---|---|
| | Cas s 8 lipid transfer protein | 9.7 | P | 53 |
| Corylus avellana | | | | |
| hazel | Cor a 1 | 17 | C | see list of isoallergens |
| | Cor a 2 profilin | 14 | C | |
| | Cor a 8 lipid transfer protein | 9 | C | |
| | Cor a 9 11S globulin-like protein | 40/? | C | Beyer p.c. |
| | Cor a 10 luminal binding prot. | 70 | C | AJ295617 |
| | Cor a 11 7S vicilin-like prot. | 40 | C | AF441864 |
| Quercus alba | | | | |
| White oak | | | | |
| | Que a 1 | 17 | P | 54 |
| Lamiales | | | | |
| Oleaceae | | | | |
| Fraxinus excelsior | | | | |
| ash | Fra e 1 | 20 | P | 58A, AF526295 |
| Ligustrum vulgare | | | | |
| privet | Lig v 1 | 20 | P | 58A |
| Olea europea | | | | |
| olive | Ole e 1 | 16 | C | 59, 60 |
| | Ole e 2 profilin | 15-18 | C | 60A |
| | Ole e 3 | 9.2 | | 60B |
| | Ole e 4 | 32 | P | P80741 |
| | Ole e 5 superoxide dismutase | 16 | P | P80740 |
| | Ole e 6 | 10 | C | 60C, U86342 |
| | Ole e 7 | ? | P | 60D, P81430 |
| | Ole e 8 Ca2+-binding protein | 21 | C | 60E, AF078679 |
| | Ole e 9 beta-1,3-glucanase | 46 | C | AF249675 |
| | Ole e 10 glycosyl hydrolase hom. | 11 | C | 60F, AY082335 |
| Syringa vulgaris | | | | |
| lilac | Syr v 1 | 20 | P | 58A |
| Plantaginaceae | | | | |
| Plantago lanceolata | | | | |
| English plantain | | | | |
| | Pla l 1 | 18 | P | P842242 |
| Pinales | | | | |
| Cryptomeria japonica | | | | |
| sugi | Cry j 1 | 41-45 | C | 55, 56 |
| | Cry j 2 | | C | 57, D29772 |
| Cupressus arizonica | | | | |
| cypress | | | | |
| | Cup a 1 | 43 | C | A1243570 |
| Cupressus sempervirens | | | | |
| common cypress | | | | |
| | Cup s 1 | 43 | C | see list of isoallergens |
| | Cup s 3w | 34 | C | ref pending |
| Juniperus ashei | | | | |
| mountain cedar | | | | |
| | Jun a 1 | 43 | P | P81294 |
| | Jun a 2 | | C | 57A, AJ404653 |
| | Jun a 3 | 30 | P | 57B, P81295 |
| Juniperus oxycedrus | | | | |
| prickly juniper | | | | |
| | Jun o 4 hom: calmodulin | 29 | C | 57C, AF031471 |
| Juniperus sabinoides | | | | |
| mountain cedar | | | | |
| | Jun s 1 | 50 | P | 58 |
| Juniperus virginiana | | | | |
| eastern red cedar | | | | |
| | Jun v 1 | 43 | P | P81825, 58B |
| Platanaceae | | | | |
| Platanus acerifolia | | | | |
| London plane tree | | | | |
| | Pla a 1 | 18 | P | P82817 |
| | Pla a 2 | 43 | P | P82967 |
| | Pla a 3 lipid transfer protein | 10 | P | Iris p.c. |
| D. Mites | | | | |
| Acarus siro | arthropod | | | |
| mite | Aca s 13 fatty acid binding prot. | 14* | C | AJ006774 |
| Blomia tropicalis | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| mite | Blo t 1 cysteine protease | 39 | C | | | AF277840 |
| | Blo t 3 trypsin | 24* | C | | | Cheong p.c. |
| | Blo t 4 alpha amylase | | 56 | C | | Cheong p.c. |
| | Blo t 5 | | C | | | U59102 |
| | Blo t 6 chymotrypsin | 25 | C | | | Cheong p.c. |
| | Blo t 10 tropomyosin | 33 | C | | | 61 |
| | Blo t 11 paramyosin | 110 | C | | | AF525465, 61A |
| | Blo t 12 Bt11a | | C | | | U27479 |
| | Blo t 13 Bt6, fatty acid bind prot. | | C | | | U58106 |
| | Blo t 19 anti-microbial pep. hom. | 7.2 | C | | | Cheong p.c. |
| Dermatophagoides farinae | | | | | | |
| American house dust mite | | | | | | |
| | Der f 1 cysteine protease | 25 | C | | | 69 |
| | Der f 2 | 14 | C | | | 70, 70A, see list of isoallergens |
| | Der f 3 trypsin | 30 | C | | | 63 |
| | Der f 7 | 24-31 | C | | | SW:Q26456, 71 |
| | Der f 10 tropomyosin | | C | | | 72 |
| | Der f 11 paramyosin | 98 | C | | | 72A |
| | Der f 14 mag3, apolipophorin | | C | | | DI7686 |
| | Der f 15 98k chitinase | 98 | C | | | AF178772 |
| | Der f 16 gelsolin/villin | 53 | C | | | 71A |
| | Der f 17 Ca binding EF protein | 53 | C | | | 71A |
| | Der f 18w 60k chitinase | 60 | C | | | Weber p.c. |
| Dermatophagoides microceras | | | | | | |
| house dust mite | | | | | | |
| | Der m 1 cysteine protease | 25 | P | | | 68 |
| Dermatophagoides pteronyssinus | | | | | | |
| European house dust mite | | | | | | |
| | Der p 1 antigen P1, cysteine protease | 25 | C | | | 62, see list of isoallergens |
| | Der p 2 | 14 | C | | | 62A-C, see list of isoallergens |
| | Der p 3 trypsin | 28/30 | C | | | 63 |
| | Der p 4 amylase | 60 | P | | | 64 |
| | Der p 5 | 14 | C | | | 65 |
| | Der p 6 chymotrypsin | 25 | P | | | 66 |
| | Der p 7 | 22/28 | C | | | 67 |
| | Der p 8 glutathione transferase | | C | | | 67A |
| | Der p 9 collagenolytic serine pro. | | P | | | 67B |
| | Der p 10 tropomyosin | 36 | C | | | Y14906 |
| | Der p 14 apolipophorin like prot. | | C | | | Epton p.c. |
| Euroglyphus maynei | | | | | | |
| mite | Eur m 2 | | C | | | see list of isoallergens |
| | Eur m 14 apolipophorin | 177 | C | | | AF149827 |
| Glycyphagus domesticus | | | | | | |
| storage mite | | | | | | |
| | Gly d 2 | | C | | | 72B, see isoallergen list |
| Lepidoglyphus destructor | | | | | | |
| storage mite | | | | | | |
| | Lep d 2 Lep d 1 | 15 | C | | | 73, 74, 74A, see isoallergen list |
| | Lep d 5 | | C | | | 75, AJ250278 |
| | Lep d 7 | | C | | | 75, AJ271058 |
| | Lep d 10 tropomyosin | | C | | | 75A, AJ250096 |
| | Lep d 13 | | C | | | 75, AJ250279 |
| Tyrophagus putrescentiae | | | | | | |
| storage mite | | | | | | |
| | Tyr p 2 | | C | | | 75B, Y12690 |
| E. Animals | | | | | | |
| Bos domesticus | | | | | | |
| domestic cattle | | | | | | |
| | Bos d 2 Ag3, lipocalin | 20 | C | | | 76, see isoallergen list (see also foods) |
| | Bos d 3 Ca-binding S100 hom. | 11 | C | | | L39834 |
| | Bos d 4 alpha-lactalbumin | 14.2 | C | | | M18780 |
| | Bos d 5 beta-lactoglobulin | 18.3 | C | | | X14712 |
| | Bos d 6 serum albumin | 67 | C | | | M73993 |
| | Bos d 7 immunoglobulin | 160 | | | | 77 |
| | Bos d 8 caseins | 20-30 | | | | 77 |
| Canis familiaris | | | | | | |
| (Canis domesticus) | | | | | | |
| | Can f 1 | 25 | C | | | 78, 79 |
| dog | Can f 2 | 27 | C | | | 78, 79 |
| | Can f 3 albumin | | C | | | S72946 |
| | Can f 4 | 18 | P | | | A59491 |

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|---|---|---|---|---|---|
| Equus caballus | | | | | |
| domestic horse | | | | | |
| | Equ c 1 | lipocalin | 25 | C | U70823 |
| | Equ c 2 | lipocalin | 18.5 | P | 79A, 79B |
| | Equ c 3 | Ag3 - albumin | 67 | C | 79C, X74045 |
| | Equ c 4 | | 17 | P | 79D |
| | Equ c 5 | AgX | 17 | P | Goubran Botros p.c. |
| Felis domesticus | | | | | |
| cat (saliva) | | | | | |
| | Fel d 1 | cat-1 | 38 | C | 15 |
| | Fel d 2 | albumin | | C | 79E, X84842 |
| | Fel d 3 | cystatin | 11 | C | 79F, AF238996 |
| | Fel d 4 | lipocalin | 22 | C | AY497902 |
| | Fel d 5w | immunoglobulin A | 400 | | Adedoyin p.c. |
| | Fel d 6w | immunoglobulin M | | | |
| 800- | | | | | |
| 1000 | | | | | |
| | Adedoyin p.c. | | | | |
| | Fel d 7w | immunoglobulin G | 150 | | Adedoyin p.c. |
| Cavia porcellus | | | | | |
| guinea pig | | | | | |
| | Cav p 1 | lipocalin homologue | 20 | P | SW:P83507, 80 |
| | Cav p 2 | | 17 | P | SW:P83508 |
| Mus musculus | | | | | |
| mouse (urine) | | | | | |
| | Mus m 1 | MUP | 19 | C | 81, 81A |
| Rattus norvegius | | | | | |
| rat (urine) | | | | | |
| | Rat n 1 | | 17 | C | 82, 83 |
| F. Fungi (moulds) | | | | | |
| 1. Ascomycota | | | | | |
| 1.1 Dothideales | | | | | |
| Alternaria alternata | | | | | |
| | Alt a 1 | | 28 | C | U82633 |
| | Alt a 2 | | 25 | C | 83A, U62442 |
| | Alt a 3 | heat shock prot. | 70 | C | U87807, U87808 |
| | Alt a 4 | prot. disulfideisomerase | 57 | C | X84217 |
| | Alt a 6 | acid ribosomal prot. P2 | 11 | C | X78222, U87806 |
| | Alt a 7 | YCP4 protein | 22 | C | X78225 |
| | Alt a 10 | aldehyde dehydrogenase | 53 | C | X78227, P42041 |
| | Alt a 11 | enolase | 45 | C | U82437 |
| | Alt a 12 | acid ribosomal prot. P1 | 11 | C | X84216 |
| Cladosporium herbarum | | | | | |
| | Cla h 1 | | 13 | | 83B, 83C |
| | Cla h 2 | | 23 | | 83B, 83C |
| | Cla h 3 | aldehyde dehydrogenase | 53 | C | X78228 |
| | Cla h 4 | acid ribosomal prot. P2 | 11 | C | X78223 |
| | Cla h 5 | YCP4 protein | 22 | C | X78224 |
| | Cla h 6 | enolase | 46 | C | X78226 |
| | Cla h 12 | acid ribosomal prot. P1 | 11 | C | X85180 |
| 1.2 Eurotiales | | | | | |
| Aspergillus flavus | | | | | |
| | Asp fl 13 | alkaline serine protease | 34 | | 84 |
| Aspergillus fumigatus | | | | | |
| | Asp f 1 | | 18 | C | M83781, S39330 |
| | Asp f 2 | | 37 | C | U56938 |
| | Asp f 3 | peroxisomal protein | 19 | C | U20722 |
| | Asp f 4 | | 30 | C | AJ001732 |
| | Asp f 5 | metalloprotease | 40 | C | Z30424 |
| | Asp f 6 | Mn superoxide dismut. | 26.5 | C | U53561 |
| | Asp f 7 | | 12 | C | AJ223315 |
| | Asp f 8 | ribosomal prot. P2 | 11 | C | AJ224333 |
| | Asp f 9 | | 34 | C | AJ223327 |
| | Asp f 10 | aspartic protease | 34 | C | X85092 |
| | Asp f 11 | peptidyl-prolyl isomeras | 24 | | 84A |
| | Asp f 12 | heat shock prot. P90 | 90 | C | 85 |
| | Asp f 13 | alkaline serine protease | 34 | | 84B |
| | Asp f 15 | | 16 | C | AJ002026 |
| | Asp f 16 | | 43 | C | g3643813 |
| | Asp f 17 | | | C | AJ224865 |
| | Asp f 18 | vacuolar serine protease | 34 | | 84C |
| | Asp f 22w | enolase | 46 | C | AF284645 |
| | Asp f 23 | L3 ribosomal protein | 44 | C | 85A, AF464911 |

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|---|---|---|---|---|
| Aspergillus niger | | | | |
| Asp n 14 beta-xylosidase | 105 | C | | AF106944 |
| Asp n 18 vacuolar serine protease | 34 | C | | 84B |
| Asp n 25 3-phytase B | 66-100 | | C | 85B, P34754 |
| Asp n ? | 85 | C | | Z84377 |
| Aspergillus oryzae | | | | |
| Asp o 13 alkaline serine protease | 34 | C | | X17561 |
| Asp o 21 TAKA-amylase A | 53 | C | | D00434, M33218 |
| Penicillium brevicompactum | | | | |
| Pen b 13 alkaline serine protease | 33 | | | 86A |
| Penicillium chrysogenum (formerly P. notatum) | | | | |
| Pen ch 13 alkaline serine protease | 34 | | | 87 |
| Pen ch 18 vacuolar serine protease | 32 | | | 87 |
| Pen ch 20 N-acetyl glucosaminidas | 68 | | | 87A |
| Penicillium citrinum | | | | |
| Pen c 3 peroxisomal mem. prot. | 18 | | | 86B |
| Pen c 13 alkaline serine protease | 33 | | | 86A |
| Pen c 19 heat shock prot. P70 | 70 | C | | U64207 |
| Pen c 22w enolase | 46 | C | | AF254643 |
| Pen c 24 elongation factor 1 beta | | C | | AY363911 |
| Penicillium oxalicum | | | | |
| Pen o 18 vacuolar serine protease | 34 | | | 87B |
| 1.3 Hypocreales | | | | |
| Fusarium culmorum | | | | |
| Fus c 1 ribosomal prot. P2 | 11* | C | | AY077706 |
| Fus c 2 thioredoxin-like prot. | 13* | C | | AY077707 |
| 1.4 Onygenales | | | | |
| Trichophyton rubrum | | | | |
| Tri r 2 | | C | | 88 |
| Tri r 4 serine protease | | C | | 88 |
| Trichophyton tonsurans | | | | |
| Tri t 1 | 30 | P | | 88A |
| Tri t 4 serine protease | 83 | C | | 88 |
| 1.5 Saccharomycetales | | | | |
| Candida albicans | | | | |
| Cand a 1 | 40 | C | | 89 |
| Cand a 3 peroxisomal protein | 29 | C | | AY136739 |
| Candida boidinii | | | | |
| Cand b 2 | 20 | C | | J04984, J04985 |
| 2. Basidiomycotina | | | | |
| 2.1 Hymenomycetes | | | | |
| Psilocybe cubensis | | | | |
| Psi c 1 | | | | |
| Psi c 2 cyclophilin | 16 | | | 89A |
| Coprinus comatus shaggy cap | | | | |
| Cop c 1 leucine zipper protein | 11 | C | | AJ132235 |
| Cop c 2 | | | | AJ242791 |
| Cop c 3 | | | | AJ242792 |
| Cop c 5 | | | | AJ242793 |
| Cop c 7 | | | | AJ242794 |
| 2.2 Urediniomycetes | | | | |
| Rhodotorula mucilaginosa | | | | |
| Rho m 1 enolase | 47 | C | | 89B |
| Rho m 2 vacuolar serine protease | 31 | C | | AY547285 |
| 2.3 Ustilaginomycetes | | | | |
| Malassezia furfur | | | | |
| Mala f 2 MF1, peroxisomal membrane protein | 21 | C | | AB011804, 90 |
| Mala f 3 MF2, peroxisomal membrane protein | 20 | C | | AB011805, 90 |
| Mala f 4 mitochondrial malate dehydrogenase | 35 | | C | AF084828, 90A |
| Malassezia sympodialis | | | | |
| Mala s 1 | | C | | X96486, 91 |

| | | | | | |
|---|---|---|---|---|---|
| Mala s 5 | | 18* | C | | AJ011955 |
| Mala s 6 | | 17* | C | | AJ011956 |
| Mala s 7 | | | C | | AJ011957, 91A |
| Mala s 8 | | 19* | C | | AJ011958, 91A |
| Mala s 9 | | 37* | C | | AJ011959, 91A |
| Mala s 10 heat shock prot. 70 | | 86 | C | | AJ428052 |
| Mala s 11 Mn superoxide dismut. | | 23 | C | | AJ548421 |
| 3. Deuteromycotina | | | | | |
| 3.1 Tuberculariales | | | | | |
| Epicoccum purpurascens | | | | | |
| (formerly E. nigrum) | | | | | |
| Epi p 1 | serine protease | 30 | P | | SW:P83340, 91B |
| G. Insects | | | | | |
| Aedes aegyptii | | | | | |
| mosquito | | | | | |
| Aed a 1 | apyrase | 68 | C | | L12389 |
| Aed a 2 | | 37 | C | | M33157 |
| Apis mellifera | | | | | |
| honey bee | | | | | |
| Api m 1 | phospholipase A2 | 16 | C | | 92 |
| Api m 2 | hyaluronidase | 44 | C | | 93 |
| Api m 4 | melittin | 3 | C | | 94 |
| Api m 6 | | 7-8 | P | | Kettner p.c. |
| Api m 7 | CUB serine protease | 39 | C | | AY127579 |
| Bombus pennsylvanicus | | | | | |
| bumble bee | | | | | |
| Bom p 1 | phospholipase | 16 | P | | 95 |
| Bom p 4 | protease | | P | | 95 |
| Blattella germanica | | | | | |
| German cockroach | | | | | |
| Bla g 1 Bd90k | | | C | | |
| Bla g 2 aspartic protease | | 36 | C | | 96 |
| Bla g 4 calycin | | 21 | C | | 97 |
| Bla g 5 glutathione transferase | | 22 | C | | 98 |
| Bla g 6 troponin C | | 27 | C | | 98 |
| Periplaneta americana | | | | | |
| American cockroach | | | | | |
| Per a 1 | Cr-PII | | | C | |
| Per a 3 | Cr-PI | 72-78 | C | | 98A |
| Per a 7 | tropomyosin | 37 | C | | Y14854 |
| Chironomus kiiensis | | | | | |
| midge Chi k 10 | tropomyosin | 32.5* | C | | AJ012184 |
| Chironomus thummi thummi | | | | | |
| midge Chi t 1-9 | hemoglobin | 16 | C | | 99 |
| Chi t 1.01 | component III | 16 | C | | P02229 |
| Chi t 1.02 | component IV | 16 | C | | P02230 |
| Chi t 2.0101 | component I | 16 | C | | P02221 |
| Chi t 2.0102 | component IA | 16 | C | | P02221 |
| Chi t 3 | component II-beta | 16 | C | | P02222 |
| Chi t 4 | component IIIA | 16 | C | | P02231 |
| Chi t 5 | component VI | 16 | C | | P02224 |
| Chi t 6.01 | component VIIA | 16 | C | | P02226 |
| Chi t 6.02 | component IX | 16 | C | | P02223 |
| Chi t 7 | component VIIB | 16 | C | | P02225 |
| Chi t 8 | component VIII | 16 | C | | P02227 |
| Chi t 9 | component X | 16 | C | | P02228 |
| Ctenocephalides felis felis | | | | | |
| cat flea | | | | | |
| Cte f 1 | | | | | |
| Cte f 2 | M1b | 27 | C | | AF231352 |
| Cte f 3 | | 25 | C | | |
| Thaumetopoea pityocampa | | | | | |
| pine processionary moth | | | | | |
| Tha p 1 | | 15 | P | | PIR:A59396, 99A |
| Lepisma saccharina | | | | | |
| silverfish | | | | | |
| Lep s 1 | tropomyosin | 36 | C | | AJ309202 |
| Dolichovespula maculata | | | | | |
| white face hornet | | | | | |
| Dol m 1 | phospholipase A1 | 35 | C | | 100 |
| Dol m 2 | hyaluronidase | 44 | C | | 101 |
| Dol m 5 | antigen 5 | 23 | C | | 102, 103 |
| Dolichovespula arenaria | | | | | |
| yellow hornet | | | | | |
| Dol a 5 | antigen 5 | 23 | C | | 104 |

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|---|---|---|---|---|---|---|
| Polistes annularies | | | | | | |
| wasp | Pol a 1 | phospholipase A1 | 35 | P | | 105 |
| | Pol a 2 | hyaluronidase | 44 | P | | 105 |
| | Pol a 5 | antigen 5 | 23 | C | | 104 |
| Polistes dominulus | | | | | | |
| Mediterranean paper wasp | | | | | | |
| | Pol d 1 | | | | | Hoffman p.c. |
| | Pol d 4 serine protease | | 32-34 | C | | Hoffman p.c. |
| | Pol d 5 | | | | | P81656 |
| Polistes exclamans | | | | | | |
| wasp | Pol e 1 | phospholipase A1 | 34 | P | | 107 |
| | Pol e 5 | antigen 5 | 23 | C | | 104 |
| Polistes fuscatus | | | | | | |
| wasp | Pol f 5 | antigen 5 | 23 | C | | 106 |
| Polistes gallicus | | | | | | |
| wasp | Pol g 5 | antigen 5 | 24 | C | | P83377 |
| Polistes metricus | | | | | | |
| wasp | Pol m 5 | antigen 5 | 23 | C | | 106 |
| Vespa crabo | | | | | | |
| European hornet | | | | | | |
| | Vesp c 1 phospholipase | | 34 | P | | 107 |
| | Vesp c 5 antigen 5 | | 23 | C | | 106 |
| Vespa mandarina | | | | | | |
| giant asian hornet | | | | | | |
| | Vesp m 1 | | | | | Hoffman p.c. |
| | Vesp m 5 | | | | | P81657 |
| Vespula flavopilosa | | | | | | |
| yellowjacket | Ves f 5 | antigen 5 | 23 | C | | 106 |
| Vespula germanica | | | | | | |
| yellowjacket | Ves g 5 | antigen 5 | 23 | C | | 106 |
| Vespula maculifrons | | | | | | |
| yellowjacket | | | | | | |
| | Ves m 1 phospholipase A1 | | 33.5 | C | | 108 |
| | Ves m 2 hyaluronidase | | | 44 | P | 109 |
| | Ves m 5 antigen 5 | | 23 | C | | 104 |
| Vespula pennsylvanica | | | | | | |
| yellowjacket | | | | | | |
| | Ves p 5 | antigen 5 | 23 | C | | 106 |
| Vespula squamosa | | | | | | |
| yellowjacket | | | | | | |
| | Ves s 5 | antigen 5 | 23 | C | | 106 |
| Vespula vidua | | | | | | |
| wasp | Ves vi 5 | antigen 5 | 23 | C | | 106 |
| Vespula vulgaris | | | | | | |
| yellowjacket | | | | | | |
| | Ves v 1 | phospholipase A1 | 35 | C | | 105A |
| | Ves v 2 | hyaluronidase | 44 | P | | 105A |
| | Ves v 5 | antigen 5 | 23 | C | | 104 |
| Myrmecia pilosula | | | | | | |
| Australian jumper ant | | | | | | |
| | Myr p 1 | | | C | | X70256 |
| | Myr p 2 | | | C | | S81785 |
| Solenopsis geminata | | | | | | |
| tropical fire ant | | | | | | |
| | Sol g 2 | | | | | Hoffman p.c. |
| | Sol g 4 | | | | | Hoffman p.c. |
| Solenopsis invicta | | | | | | |
| fire ant | Sol i 2 | | 13 | C | | 110, 111 |
| | Sol i 3 | | 24 | C | | 110 |
| | Sol i 4 | | 13 | C | | 110 |
| Solenopsis saevissima | | | | | | |
| Brazilian fire ant | | | | | | |
| | Sol s 2 | | | | | Hoffman p.c. |
| Triatoma protracta | | | | | | |
| California kissing bug | | | | | | |
| | Tria p 1 | Procalin | 20 | C | | AF179004, 111A. |
| | | | | | | |
| H. Foods | | | | | | |
| Gadus callarias | | | | | | |
| cod | | | | | | |
| | Gad c 1 | allergen M | 12 | C | | 112, 113 |
| Salmo salar | | | | | | |
| Atlantic salmon | | | | | | |
| | Sal s 1 | parvalbumin | 12 | C | | X97824 |
| Bos domesticus | | | | | | |
| domestic cattle | | | | | | |

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|---|---|---|---|---|---|---|
| | Bos d 4 | alpha-lactalbumin | 14.2 | C | | M18780 |
| (milk) | Bos d 5 | beta-lactoglobulin | 18.3 | C | | X14712 |
| see also animals | | | | | | |
| | Bos d 6 | serum albumin | 67 | C | | M73993 |
| | Bos d 7 | immunoglobulin | 160 | | | 77 |
| | Bos d 8 | caseins | 20-30 | | | 77 |
| Gallus domesticus | | | | | | |
| chicken | | | | | | |
| | Gal d 1 | ovomucoid | 28 | C | | 114, 115 |
| | Gal d 2 | ovalbumin | 44 | C | | 114, 115 |
| | Gal d 3 | Ag22, conalbumin | 78 | C | | 114, 115 |
| | Gal d 4 | lysozyme | 14 | C | | 114, 115 |
| | Gal d 5 | serum albumin | 69 | C | | X60688 |
| Metapenaeus ensis | | | | | | |
| shrimp | Met e 1 | tropomyosin | | C | | U08008 |
| Penaeus aztecus | | | | | | |
| shrimp | Pen a 1 | tropomyosin | 36 | P | | 116 |
| Penaeus indicus | | | | | | |
| shrimp | Pen i 1 | tropomyosin | 34 | C | | 116A |
| Penaeus monodon | | | | | | |
| black tiger shrimp | | | | | | |
| | Pen m 1 | tropomyosin | 38 | C | | |
| | Pen m 2 | arginine kinase | 40 | C | | AF479772, 117 |
| Todarodes pacificus | | | | | | |
| squid | Tod p 1 | tropomyosin | 38 | P | | 117A |
| Helix aspersa | | | | | | |
| brown garden snail | | | | | | |
| | Hel as 1 | tropomyosin | 36 | C | | Y14855, 117B |
| Haliotis midae | | | | | | |
| abalone | | | | | | |
| | Hal m 1 | | 49 | | | 117C |
| Rana esculenta | | | | | | |
| edible frog | | | | | | |
| | Ran e 1 | parvalbumin alpha | 11.9* | C | | AJ315959 |
| | Ran e 2 | parvalbumin beta | 11.7* | C | | AJ414730 |
| Brassica juncea | | | | | | |
| oriental mustard | | | | | | |
| | Bra j 1 | 2S albumin | 14 | C | | 118 |
| Brassica napus | | | | | | |
| rapeseed | | | | | | |
| | Bra n 1 | 2S albumin | 15 | P | | 118A, P80208 |
| Brassica rapa | | | | | | |
| turnip | Bra r 2 | hom: prohevein | 25 | | | P81729 |
| Hordeum vulgare | | | | | | |
| barley | Hor v 15 | BMAI-1 | | 15 | C | 119 |
| | Hor v 16 | alpha-amylase | | | | |
| | Hor v 17 | beta-amylase | | | | |
| | Hor v 21 | gamma-3 hordein | 34 | C | | 119A, |
| SW:P80198 | | | | | | |
| Secale cereale | | | | | | |
| rye | Sec c 20 | secalin | | | | see isoall. list |
| Triticum aestivum | | | | | | |
| wheat | Tri a 18 | agglutinin | | | | |
| | Tri a 19 | omega-5 gliadin | 65 | P | | PIR:A59156 |
| Zea mays | | | | | | |
| maize, corn | | | | | | |
| | Zea m 14 | lipid transfer prot. | 9 | P | | P19656 |
| Oryza sativa | | | | | | |
| rice | Ory s 1 | | | C | | 119B, U31771 |
| Apium graveolens | | | | | | |
| celery | Api g 1 | hom: Bet v 1 | 16* | C | | Z48967 |
| | Api g 4 | profilin | | | | AF129423 |
| | Api g 5 | | 55/58 | P | | P81943 |
| Daucus carota | | | | | | |
| carrot | Dau c 1 | hom: Bet v 1 | 16 | C | | 117D, see isoallergen list |
| | Dau c 4 | profilin | | C | | AF456482 |
| Corylus avellana | | | | | | |
| hazelnut | | | | | | |
| | Cor a 1.04 | hom: Bet v 1 | 17 | C | | see list of isoallergens |
| | Cor a 2 | profilin | 14 | C | | AF327622 |
| | Cor a 8 | lipid transfer protein | 9 | C | | AF329829 |
| Malus domestica | | | | | | |
| apple | Mal d 1 | hom: Bet v 1 | | C | | see list of isoallergens |
| | Mal d 2 | hom: thaumatin | | C | | AJ243427 |
| | Mal d 3 | lipid transfer protein | 9 | C | | Pastorello p.c. |
| | Mal d 4 | profilin | 14.4* | C | | see list of isoallergens |

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|---|---|---|---|---|---|---|
| Pyrus communis | | | | | | |
| pear | Pyr c 1 hom: Bet v 1 | | 18 | C | | AF05730 |
| | Pyr c 4 profilin | | 14 | C | | AF129424 |
| | Pyr c 5 hom: isoflavone reductas | | 33.5 | C | | AF071477 |
| Persea americana | | | | | | |
| avocado | Pers a 1 | endochitinase | 32 | C | | Z78202 |
| Prunus armeniaca | | | | | | |
| apricot | | | | | | |
| | Pru ar 1 | hom: Bet v 1 | | C | | U93165 |
| | Pru ar 3 lipid transfer protein | | 9 | P | | |
| Prunus avium | | | | | | |
| sweet cherry | | | | | | |
| | Pru av 1 hom: Bet v 1 | | | | C | U66076 |
| | Pru av 2 hom: thaumatin | | | C | | U32440 |
| | Pru av 3 lipid transfer protein | | 10 | C | | AF221501 |
| | Pru av 4 profilin | | 15 | C | | AF129425 |
| Prunus domestica | | | | | | |
| European plum | | | | | | |
| | Pru d 3 lipid transfer protein | | 9 | P | | 119C |
| Prunus persica | | | | | | |
| peach | Pru p 3 lipid transfer protein | | 10 | P | | P81402 |
| | Pru p 4 profilin | | 14 | C | | see isoallergen list |
| Asparagus officinalis | | | | | | |
| Asparagus | | | | | | |
| | Aspa o 1 lipid transfer protein | | 9 | P | | 119D |
| Crocus sativus | | | | | | |
| saffron crocus | Cro s 1 | | 21 | | | Varasteh A-R p.c. |
| Lactuca sativa | | | | | | |
| lettuce | | | | | | |
| | Lac s 1 | lipid transfer protein | 9 | | | Vieths p.c. |
| Vitis vinifera | | | | | | |
| grape | Vit v 1 | lipid transfer protein | 9 | P | | P80274 |
| Musa x paradisiaca | | | | | | |
| banana | Mus xp 1 | profilin | 15 | C | | AF377948 |
| Ananas comosus | | | | | | |
| pineapple | | | | | | |
| | Ana c 1 profilin | | 15 | C | | AF377949 |
| | Ana c 2 bromelain | | 22.8* | C | | 119E-G, D14059 |
| Citrus limon | | | | | | |
| lemon | Cit l 3 lipid transfer protein | | 9 | P | | Torrejon p.c. |
| Citrus sinensis | | | | | | |
| sweet orange | | | | | | |
| | Cit s 1 germin-like protein | | 23 | P | | Torrejon p.c. |
| | Cit s 2 profilin | | 14 | P | | Torrejon p.c. |
| | Cit s 3 lipid transfer protein | | 9 | P | | Torrejon p.c. |
| Litchi chinensis | | | | | | |
| litchi | Lit c 1 | profilin | 15 | C | | AY049013 |
| Sinapis alba | | | | | | |
| yellow mustard | | | | | | |
| | Sin a 1 | 2S albumin | 14 | C | | 120 |
| Glycine max | | | | | | |
| soybean | Gly m 1 | HPS | 7 | P | | 120A |
| | Gly m 2 | | 8 | P | | A57106 |
| | Gly m 3 | profilin | 14 | C | | see list of isoallergens |
| | Gly m 4 | (SAM22) PR-10 prot. | 17 | C | | X60043, 120B |
| Vigna radiata | | | | | | |
| mung bean | | | | | | |
| | Vig r 1 | PR-10 protein | 15 | C | | AY792956 |
| Arachis hypogaea | | | | | | |
| peanut | Ara h 1 | vicilin | 63.5 | C | | L34402 |
| | Ara h 2 | conglutin | 17 | C | | L77197 |
| | Ara h 3 | glycinin | 60 | C | | AF093541 |
| | Ara h 4 glycinin | | 37 | C | | AF086921 |
| | Ara h 5 profilin | | 15 | C | | AF059616 |
| | Ara h 6 hom: conglutin | | 15 | C | | AF092846 |
| | Ara h 7 hom: conglutin | | 15 | C | | AF091737 |
| | Ara h 8 PR-10 protein | | | 17 | C | AY328088 |
| Lens culinaris | | | | | | |
| lentil | Len c 1 vicilin | | 47 | C | | see list of isoallergens |
| | Len c 2 seed biotinylated prot. | | 66 | P | | 120C |
| Pisum savitum | | | | | | |
| pea | Pis s 1 vicilin | | 44 | C | | see list of isoallergens |
| | Pis s 2 | convicilin | 63 | C | | pending |
| Actinidia chinensis | | | | | | |
| kiwi | Act c 1 cysteine protease | | 30 | P | | P00785 |
| | Act c 2 thaumatin-like protein | | 24 | P | | SW:P81370, 121 |
| Capsicum annuum | | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| bell pepper | | | | | |
| | Cap a 1w osmotin-like protein | 23 | C | | AJ297410 |
| | Cap a 2 profilin | 14 | C | | AJ417552 |
| Lycopersicon esculentum | | | | | |
| tomato | Lyc e 1 profilin | 14 | C | | AJ417553 |
| | Lyc e 2 b-fructofuranosidase | 50 | C | | see isoallergen list |
| | Lyc e 3 lipid transfer prot. | 6 | C | | U81996 |
| Solanum tuberosum | | | | | |
| potato | Sola t 1 patatin | 43 | P | | P15476 |
| | Sola t 2 cathepsin D inhibitor | 21 | P | | P16348 |
| | Sola t 3 cysteine protease inhibitor | 21 | P | | P20347 |
| | Sola t 4 aspartic protease inhibitor | 16+4 | P | | P30941 |
| Bertholletia excelsa | | | | | |
| Brazil nut | | | | | |
| | Ber e 1 2S albumin | 9 | C | | P04403, M17146 |
| | Ber e 2 11S globulin seed storage protein | 29 | C | | AY221641 |
| Juglans nigra | | | | | |
| black walnut | | | | | |
| | Jug n 1 2S albumin | 19* | C | | AY102930 |
| | Jug n 2 vicilin-like prot. | 56* | C | | AY102931 |
| Juglans regia | | | | | |
| English walnut | | | | | |
| | Jug r 1 2S albumin | | C | | U66866 |
| | Jug r 2 vicilin | 44 | C | | AF066055 |
| | Jug r 3 lipid transfer protein | 9 | P | | Pastorello |
| Anacardium occidentale | | | | | |
| Cashew | Ana o 1 vicilin-like protein | 50 | C | | see isoallergen list |
| | Ana o 2 legumin-like protein | 55 | C | | AF453947 |
| | Ana o 3 2S albumin | 14 | C | | AY081853 |
| Ricinus communis | | | | | |
| Castor bean | | | | | |
| | Ric c 1 2S albumin | | C | | P01089 |
| Sesamum indicum | | | | | |
| sesame | Ses i 1 2S albumin | 9 | C | | 121A, AF240005 |
| | Ses i 2 2S albumin | 7 | C | | AF091841 |
| | Ses i 3 7S vicilin-like globulin | 45 | C | | AF240006 |
| | Ses i 4 oleosin | 17 | C | | AAG23840 |
| | Ses i 5 oleosin | 15 | C | | AAD42942 |
| Cucumis melo | | | | | |
| muskmelon | | | | | |
| | Cuc m 1 serine protease | 66 | C | | D32206 |
| | Cuc m 2 profilin | 14 | C | | AY271295 |
| | Cuc m 3 pathogenesis-rel p. PR-1 | 16* | P | | P83834 |
| | | | | | |
| I. Others | | | | | |
| Anisakis simplex | | | | | |
| nematode | | | | | |
| | Ani s 1 | 24 | P | | 121B, A59069 |
| | Ani s 2 paramyosin | 97 | C | | AF173004 |
| | Ani s 3 tropomyosin | 41 | C | | 121C, Y19221 |
| | Ani s 4 | 9 | P | | P83885 |
| Argas reflexus | | | | | |
| pigeon tick | | | | | |
| | Arg r 1 | 17 | C | | AJ697694 |
| Ascaris suum | | | | | |
| worm | Asc s 1 | 10 | P | | 122 |
| Carica papaya | | | | | |
| papaya | Car p 3w papain | | 23.4* | C | 122A, M15203 |
| Dendronephthya nipponica | | | | | |
| soft coral | | | | | |
| | Den n 1 | 53 | P | | 122B |
| Hevea brasiliensis | | | | | |
| rubber (latex) | | | | | |
| | Hev b 1 elongation factor | 58 | P | | 123, 124 |
| | Hev b 2 1,3-glucanase | 34/36 | C | | 125 |
| | Hev b 3 | 24 | P | | 126, 127 |
| | Hev b 4 component of microchelix complex | 100-115 | P | | 128 |
| | Hev b 5 | 16 | C | | U42640 |
| | Hev b 6.01 hevein precursor | 20 | C | | M36986, p02877 |
| | Hev b 6.02 hevein | 5 | C | | M36986, p02877 |
| | Hev b 6.03 C-terminal fragment | 14 | C | | M36986, p02877 |
| | Hev b 7.01 hom: patatin from B-serum | 42 | C | | U80598 |
| | Hev b 7.02 hom: patatin from C-serum | 44 | C | | AJ223038 |
| | Hev b 8 profilin | 14 | C | | see list of isoallergens |
| | Hev b 9 enolase | 51 | C | | AJ132580 |

| | | | | |
|---|---|---|---|---|
| Hev b 10 Mn superoxide dismut. | 26 | C | see list of isoallergens |
| Hev b 11 class 1 chitinase | | C | see list of isoallergens |
| Hev b 12 lipid transfer protein | 9.3 | C | AY057860 |
| Hev b 13 esterase | 42 | P | P83269 |
| Homo sapiens | | | |
| human autoallergens | | | |
| Hom s 1 | 73* | C | Y14314 |
| Hom s 2 | 10.3* | C | X80909 |
| Hom s 3 | 20.1* | C | X89985 |
| Hom s 4 | 36* | C | Y17711 |
| Hom s 5 | 42.6* | C | P02538 |
| Triplochiton scleroxylon | | | |
| obeche Trip s 1 class 1 chitinase | 38.5 | P | Kespchl p.c. |

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With the present splicing/head to tail modification significant reduction in allergenic activity can be obtained. Depending on the method, this activity can mostly be extinguished from the wild-type protein allergen. According to a preferred embodiment of the present invention, reduction in allergenic activity is measured by a reduction of inhibition of IgE binding capacity of at least 10 %, preferably at least 20 %, especially at least 30 %, compared to the wild-type allergen. A preferred method is shown in the example section below.

An alternative, but also preferred way for defining the reduction in allergenic activity uses measurement of IgE binding. Lack of binding of IgE antibodies of allergen sensitised patient's sera to a dot blot of said derivative is taken as an in-

dication of most significant reduction. Also this method is shown in the example section below.

The derivatives obtained according to the present invention may be easily combined with a pharmaceutically acceptable excipient and finished to a pharmaceutical preparation.

Preferably, the derivatives are combined with a suitable vaccine adjuvant and finished to a pharmaceutically acceptable vaccine preparation.

According to a preferred embodiment, the derivatives according to the present invention are combined with further allergens to a combination vaccine. Such allergens are preferably wild-type allergens, especially a mixture of wild-type allergens, recombinant wild-type allergens, derivatives of wild-type protein allergens or mixtures thereof. Such mixtures may be made specifically for the needs (allergen profile) of a certain patient.

In a preferred embodiment, such a pharmaceutical preparation further contains an allergen extract.

According to another aspect of the present invention, an allergen derivative of a wild-type protein allergen is provided, said wild-type protein allergen having an amino acid sequence of 1 to Z, characterized in that said derivative adjacently contains - in N-terminus to C-terminus orientation - the two wild-type allergen fragments X to Z and 1 to X, said two wild-type allergen fragments having reduced allergenic activity or lacking allergenic activity.

Preferably, the allergen derivative according to the present invention is characterized in that X to Z and 1 to X are at least 30 amino acid residues long, preferably at least 50 amino acid residues, especially at least 60 amino acid residues.

It is even more preferred, if X to Z and 1 to X differ in length by 50 % or less, preferably by 30 % or less, especially by 20 % or less.

Specifically preferred allergen derivatives according to the present invention are selected from a type I allergen, preferably from an allergen of table A, more preferred of timothy grass (Phelum pratense) pollen, especially Phl p 12, birch (Betula verrucosa) pollen, especially Bet v 2 and Bet v 4, yellow jacket (Vespula vulgaris) venom, paper wasp (Polistes annularis) venom, Parietaria judaica pollen, ryegrass pollen, dustmite allergens, especially Der p 2, etc..

Preferably, the derivatives according to the present invention are provided as a allergen composition wherein not only one allergen is present, but two or more. The present derivatives may also be mixed with allergen extracts which are supplemented by the derivatives of the present invention to substitute for the lack of sufficient amounts of specific allergens in the natural extracts. Mixtures of allergens are specifically needed in patients which have allergenic reactions to not only one allergen. It is therefore preferred to provide the present derivatives as in combination with further (other) allergens to a combination vaccine.

The allergen derivatives according to the present invention may therefore be preferably combined with wild-type allergen to an allergen composition, especially a mixture of a wild-type allergens, recombinant wild-type allergens, derivatives of wild-type protein allergens or mixtures thereof (each of the same and/or different allergen and/or isoforms or mutants thereof; as long as an overall reduction of allergenic activity, compared to the wild-type protein or recombinant allergen is given in the preparation as a whole).

Preferably, the present preparation further contains an allergen extract.

The allergen or allergen composition according to the present invention preferably contains a pharmaceutically acceptable excipient.

Another aspect of the present invention relates to the use of an allergen derivative according to the present invention for the

preparation of an allergen specific immunotherapy medicament.

Yet another aspect of the present invention relates to the use of an allergen derivative or an allergen composition according to the present invention for the preparation of a medicament for the passive immunisation.

Another aspect of the present invention relates to the use of an allergen derivative or an allergen composition according to the present invention for the preparation of a medicament for the prophylactic immunisation.

The allergen derivatives and compositions according to the present invention can be used for the prophylactic immunisation of individuals leading to an effective prevention of allergy. Since the allergen derivatives and compositions according to the present invention, like Der p 2 allergen derivatives, show a reduced allergic immune response compared to the wild-type allergen, they do not lead to undesired side effects. Advantageously such a medicament may be administered to children at the age of 1 to 3 years. Such a vaccination before said child will get in contact with allergens prevents the formation of allergen specific IgE antibodies in said child.

Preferably, the medicament further contains other suitable ingredients, such as adjuvants, diluents, preservatives, etc..

According to a preferred embodiment of the present invention the medicament comprises 10 ng to 1 g, preferably 100 ng to 10 mg, especially 0,5 µg to 200 µg of said recombinant allergen derivative per application dose. Preferred ways of administration include all standard administration regimes described and suggested for vaccination in general and allergy immunotherapy specifically (orally, transdermally, intraveneously, intranasally, via mucosa, etc). The present invention includes a method for treating and preventing allergy by administering an effective amount of the pharmaceutical preparations according to the present invention.

Another aspect of the present invention relates to a method for

producing an allergen derivative according to the present invention which is characterized in by the following steps:

- providing a DNA molecule encoding an allergen derivative according to the present invention,
- transforming a host cell with said DNA molecule and
- expressing said derivative in said host cell and isolating said derivative.

Preferably, said host is a host with high expression capacity.

As used herein, a "host with high expression capacity" is a host which expresses a protein of interest in an amount of at least 10mg/l culture, preferably of at least 15mg/l, more preferably of at least 20mg/l. Of course, the expression capacity depends also on the selected host and expression system (e.g. vector). Preferred hosts according to the present invention are E.coli, Pichia pastoris, Baciullus subtilis, pant cells (e.g. derived form tabacco) etc..

Of course, the allergen derivatives according to the present invention can also be produced by any other suitable method, especially chemical synthesis or semi-chemical synthesis.

Another aspect of the present invention relates to the use of a profilin derivative obtainable from a first wild-type profilin molecule by a method according to the present invention or an allergen derivative of a first wild-type profilin molecule according to the present invention for the manufacture of a medicament for the prevention or the treatment of allergic diseases caused by a second wild-type profilin molecule.

It turned surprisingly out that antibodies induced by an directed to profilin derivatives of a first wild-type profilin molecule according to the present invention bind also to other wild-type profilin molecules. Therefore said derivatives can be employed for the treatment or prevention of a number of allergic diseases. Such profilin derivatives may be used as broad spectrum vaccines which allow to immunize individuals with only one or two immunogenic molecules. Profilin represents an allergen that is expressed in all eukaryotic cells and thus represents a

pan-allergen that might induce inhalative allergies (e.g. rhino-conjunctivits, asthma) as well as oral allergy syndromes after oral ingestion (itching and swelling of lips and the tounge) in sensitized patients.

For instance, the reshuffled Phl p 12-derivative, MP12, induces IgG antibodies after immunization that recognize profilins from both pollens as well as form plant-derived food. MP 12-induced antibodies inhibit patients' serum IgE binding to profilins from pollens and also to plant food-derived profilin. Thus, the MP12 as well as other reshuffled profilin molecules are suitable for the treatment of pollen-food cross-sensitization attributable to profilin allergy.

According to a preferred embodiment said first and said second profilin molecules are selected from the group consisting of Phl p 12, Bet v 2, Art v 4, Ana c, Api g 4, Mus xp 1, Cor a 2, and Dau c 4.

Especially these allergens are suited to be used according to the present invention because of their structural similarities. However, it is obvious that also other allergens which share structural similarities among each other can be used accordingly.

Said first profilin molecule is preferably Phl p 12 and said second profilin molecule is preferably selected from the group consisting of Bet v 2, Art v 4, Ana c, Api g 4, Mus xp 1, Cor a 2, and Dau c 4.

Experiments revealed that especially derivatives of Phl p 12 can be used as broad spectrum vaccines. A particular preferred derivative consists of a fusion protein, wherein amino acids 1 to 77 of the wild-type Phl p 12 are N-terminally fused to amino acids 78 to 131 (see Fig. 1).

Profilin derivatives of Bet v 2, Art v 4, Ana c, Api g 4, Mus xp 1, Cor a 2, and Dau c 4 as disclosed herein and obtainable by a method according to the present invention are preferably used for the treatment and/or prevention of pollen-food sensitization at-

tributable to profilin allergy.
The present invention is further described by the following examples and the drawing figures, yet without being restricted thereto.

**Fig. 1** shows a schematic representation of the primary structure of MP12 (a reshuffled Phl p 12 allergen according to the present invention) compared to Phl p 12 wild-type;

**Fig. 2** shows CD spectra of Phl p 12 wild-type and MP12. The mean residue ellipticity [Θ] (y-axis) of Phl p 12 and the derivative MP12 is shown for a range of wavelengths (x-axis);

**Fig. 3** shows Coomassie staining of a 14% SDS PAGE loaded with fractions of recombinant MP12 that was exposed to a polyproline column. Lane M represents the molecular weight marker, lane 1 represents the flow-through fraction, lanes 2-4 wash fractions, lanes 5-6 elution fractions. Molecular weights (kDa) are indicated on the left margin;

**Fig. 4** shows IgE reactivity of nitrocellulose-dotted Phl p 12 and MP12. Dotted proteins, as well as human serum albumin (HSA) for negative control purposes, were exposed to sera from 24 Phl p 12-allergic patients (lanes 1-24). Lane N represents serum from a non-allergic control individual. Bound IgE antibodies were detected with anti-human IgE antibodies;

**Fig. 5** shows induction of basophil histamine release in two Phl p 12-allergic patients. Patients' granulocytes were incubated with various concentrations (x-axis) of Phl p 12 (squares) and MP12 (circles). The percentage of total histamine released into the supernatant is displayed on the y-axis;

**Fig. 6** shows reactivity of rabbit antisera with profilins from timothy grass, birch and mugwort pollen. Rabbit antisera raised against Phl p 12 (diamonds) and MP12 (squares) were tested for reactivity to Phl p 12 (A), Bet v 2 (B), and mugwort profilin (C) by ELISA. Dilutions of sera are shown on the x-axis, the corresponding OD values on the y-axis. The corresponding preimmune sera did not display any reactivity;

**Fig. 7** shows inhibition of rPhl p 12-induced basophil degranulation by anti-rPhl p 12 (P12) and anti-MP12-induced IgG. Rat basophils had been loaded with Phl p 12-specific mouse IgE;

**Fig. 8** shows a schematic representation of the primary structure and generation of Der p 2 Hybrid (a reshuffled Der p 2 allergen according to the present invention) compared to Der p 2 wild-type;

**Fig. 9** shows Coomassie-stained SDS-PAGE containing protein extracts of BL21 (DE3) expressing rDer p 2 and rDer p 2 derivatives as his-tagged proteins (lanes 1), purified rDer p 2, rDer p 2 fragments and rDer p 2 hybrid (lanes 2), and a molecular marker (lanes M).

**Fig. 10** shows a mass spectroscopical analysis of purified rDer p 2 and rDer p 2 derivatives. The x-axes show the mass/charge ratios and the signal intensities are displayed on the y-axes as percentages of the most intensive signals.

**Fig. 11** shows far ultraviolet CD spectra of purified recombinant Der p 2, rDer p 2 fragments and rDer p 2 hybrid. The spectra of the proteins are expressed as mean residue ellipticities (y-axis) at given wavelengths (x-axis).

**Fig. 12** shows IgE-recognition of recombinant Der p 2 and recombinant Der p 2 derivatives. Sera from 17 mite allergic individuals (lanes 1-17), a non-allergic individual (lane 18) and buffer without serum (lane 19) were tested for IgE reactivity with dot-blotted recombinant Der p 2, rDer p 2 fragments, rDer p 2 hybrid and BSA. Bound IgE was detected with 125I-labeled anti-human IgE antibodies and visualized by autoradiography.

**Fig. 13** shows basophil activation by recombinant Der p 2 and rDer p 2 derivatives as measured by CD203c expression. Blood samples from 10 mite-allergic patients were exposed to 10µg/ml recombinant rDer p 2, each of the Der p 2 fragments, a mixture of the fragments, αIgE or buffer. The results of three representative patients are shown. CD203c expression was determined by

FACS analysis and is displayed as mean fluorescence index (MFI).

**Fig. 14** shows basophil activation by recombinant Der p 2 and rDer p 2 derivatives as measured by CD203c expression. Blood samples from the same 10 mite allergic patients were exposed to several concentrations of rDer p 2 and rDer p 2 hybrid, αIgE or buffer (x-axes). The results of six representative patients are shown. CD203c expression was determined by FACS analysis and is displayed as stimulation index (SI).

**Fig. 15** shows the evolution of Der p 2-specific $IgG_1$ induced by immunisation of mice with rDer p 2 and rDer p 2 derivatives. Groups of five mice each were immunized with purified rDer p 2 or rDer p 2 derivatives and induced $IgG_1$ antibodies were determined by ELISA. The optical density values (OD 405nm) displayed on the y-axis correspond to the level of $IgG_1$ antibodies in the mouse sera. The results are shown as box plots where 50% of the values are within the boxes and non-outliers between the bars. Lines within the boxes indicate the median values. Open circles and stars indicate outliers and extremes of each mouse group.

**Fig. 16** shows the low in vivo allergenic activity of rDer p 2 derivatives visualized by β-hexosaminidase release from RBL cells. Rat basophil leukemia (RBL) cells were loaded with mouse sera obtained before (Preimmunesera) and after (Immunesera) immunization with rDer p 2 wild-type allergen and rDer p 2 derivatives. Release of β-hexosaminidase was induced with rDer p 2 and is displayed as percentage of total β-hexosaminidase release (mean values ±SD for the five sera from each mouse group) (y-axis).

**Fig. 17** shows reactivity of rabbit antisera with profilins from timothy grass pollen (Phl p 12), birch pollen (Bet v 2), mugwort pollen (Art v 4), cashew nut (Ana c ), celery (Api g 4), banana (Mus xp 1), hazelnut (Cor a 2), and carrot (Dau c 4). Rabbit antisera raised against Phl p 12 (diamonds) and MP12 (squares) were tested for reactivity to said profilins by ELISA. Dilutions of sera are shown on the x-axis, the corresponding OD values on the y-axis. The corresponding preimmune sera did not display any reactivity.

**EXAMPLES:**

In examples 1 to 5 the principles of the present invention are exemplified by a profilin allergen, timothy grass pollen profilin Phl p 12. Examples 6 to 11 relate to the main mite (Dermatophagoides pteronyssinus) allergen, Der p 2. Examples 12 and 13 show the cross reactivity of Phl p 12 with profilins of other sources than timothy grass pollen, demonstrating consequently the suitability for using Phl p 12 derivatives as vaccines for allergic diseases caused by other profilins.

**Example 1: Characterisation of a hypoallergenic derivative from timothy grass pollen profilin**

*a) Generation, expression and purification of a hypoallergenic variant from timothy grass pollen profilin, Phl p 12*

Overlapping PCR technique was used for engineering a reshuffled Phl p 12-derivative. PCR template was the cDNA coding for timothy grass pollen profilin, Phl p 12, subcloned in pet17b expression vector. The following primers were used to generate two PCR fragments containing overlapping sequences as well as NdeI and EcoRI restriction sites and a sequence coding for a C-terminal 6x Histidin residue for protein purification. For fragment 1 primer MDE-1: 5'CATATGAGGCCCGGCGCGGTCATC3' and primer MDE-2: 5'GTACGTCTGCCACGCCATCATGCCTTGTTCAAC3' were used,
for fragment 2, primer MABC-1: 5'GTTGAACAAGGCATGATGTCGTGGCAGACG3' and primer MABC-2: 5'GAATTCTTAATGGTGATGGTGATGGTGACCCTGGATGACCATGTA3' were used. In the next step, both PCR products obtained as described were used as templates for the overlapping PCR reaction using primer MDE-1 and MABC-2 to generate the DNA coding for the Phl p 12 derivative (i.e., MP12) (schematically represented in Fig. 1). The MP-12 encoding DNA was cloned into pBluescript vector system (Stratagene) and DNA sequence was confirmed by double-strand sequencing (MWG Biotech, Germany).

For protein purification, MP12-encoding cDNA had to be subcloned into an pet17b expression vector system using NdeI and EcoRI restricition enzymes and the DNA sequence was again confirmed by

double-strand sequencing (MWG Biotech).

For protein purification MP-12 was expressed in Escherichia coli BL21 (DE3) (Stratagene, East Kew, Australia) in liquid culture. E.coli were grown to an $OD_{600}$ of 0.4 in LB-medium containing 100 mg/l ampicillin. The expression of recombinant proteins was induced by adding isopropyl-b-thiogalactopyranoside to a final concentration of 1 mM and further culturing for additional 4 hours at 37°C. E.coli cells from a 500 ml culture were harvested by centrifugation, resuspended in buffer A (100mM $NaH_2PO_4$, 10mM Tris, 8M Urea, pH 7.5). After centrifugation at 20.000rpm, 30 min, the supernatant was transferred to a Ni-NTA agarose column (Quiagen, Hilden, Germany) and elution of the 6x His-tagged MP12 protein was performed using buffer A with decreasing pH values. The protein eluted at a pH of 4.9 and was subsequently refolded by stepwise dialysis against buffer A, pH 7.5, containing 6 - 0 M Urea. The final dialysis step was done against phosphate buffered saline (PBS), where MP12 was soluble as shown by centrifugation experiments.

Protein purity was confirmed by SDS PAGE and quantification was performed using a Micro BCA kit (Pierce, USA).

*b) Secondary structure analysis*

Circular dichroism (CD) measurements were carried out on a Jasco J-715 spectropolarimeter using a 0.1 cm pathlength cell equilibrated at 20°C. Spectra were recorded with 0.5 nm resolution at a scan speed of 100nm/min and resulted from averaging 3 scans. The final spectra were baseline-corrected by substracting the corresponding MilliQ spectra obtained under identical conditions. Results were fitted with the secondary structure estimation program J-700.

The results indicate a considerable amount of secondary structure of the derivative. The spectrum of Phl p 12 is characterized with a minimum at 218nm and a strong maximum below 200nm, whereas the minimum of the derivative is shifted to a smaller wavelength and the zero-crossing of the curve is below 200nm (Fig. 2). These findings are indicative for an increasing por-

tion of random-coil secondary structure within the derivative.

*c) Hypoallergenic Phl p 12 derivative lacks affinity for polyproline*

Affinity to polyproline is a feature common to profilins from various organisms. It was demonstrated that the hypoallergenic Phl p 12 derivative, MP12, does not bind polyproline and thus exhibits altered biochemical properties.

Approximately 5 µg of purified recombinant MP12 in PBS was subjected to a polyproline-loaded CnBr-activated agarose column (Amersham Bioscience, Uppsala, Sweden) equilibrated with PBS. After collecting the flow-through, the column was washed with 3 volumes (PBS) and elution was performed with 5x 1ml PBS containg 2M or 6M Urea, respectively. Ten µl aliquots of the flow-through, the wash fractions and elution fractions were subjected to a 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and proteins were visualized by Commassie staining (Fig. 3). The results indicate a loss of the polyproline binding site due to reorganisation of the primary structure of Phl p 12.

**Example 2: Reduction of IgE binding capacity of MP12**

*a) MP12 shows strongly reduced IgE binding capacity*

The IgE binding capacity of recombinant MP12 was compared to that of recombinant Phl p 12 wild-type by dot blot analysis using sera from 24 profilin sensitised patients (Fig. 4). Phl p 12 and MP12 as well as human serum albumin (HSA) for control purposes, were dotted onto nitrocellulose and probed with sera from 24 profilin-sensitised patients. Bound IgE antibodies were detected using $^{125}$I-labeled anti-human-IgE antibodies. All patients showed IgE reactivity with Phl p 12 wild-type, whereas none of the 24 patients reacted with MP12 or with the control protein HSA (Fig. 4).

To quantify the reduction of IgE binding capacity of MP12, fluid phase inhibitions were performed. For this purpose serum from

six profilin-sensitised patients were preincubated with either 10µg of Phl p 12 and of MP12 and subconsequently incubated with ELISA plate-bound Phl p 12 (5µg/ml). Bound IgE antibodies were detected with an alkaline phosphatase-labeled anti-human IgE antibody (Pharmingen). Inhibition of IgE binding was calculated with the following formula: Inhibition%= 100x[(A-B)/A]; A representing OD values obtained after incubation of serum with BSA, B representing OD values after incubation of serum with Phl p 12 or MP12, respectively.

The ability of MP12 to inhibit binding of IgE to Phl p 12 is shown as percentage inhibition in Table 2, ranging from 20-40% with mean inhibition of 31.2% for MP12, whereas inhibition achieved with Phl p 12 ranged from 76-91% (mean 86%).

Table 2: Inhibition of antibody binding to immobilised Phl 12 using Phl p 12 and MP12. IgE antibody binding was inhibited by preincubation of sera from 6 profilin-sensitised patients with Phl p 12 wild-type or MP12. Mean inhibition of antibody binding was calculated and is displayed.

| Protein name | Amino acid sequence | number of amino acids | calculated Pi | MW (kDa) | structural integrity |
|---|---|---|---|---|---|
| Phl p 12 | MSWQTYVDEHLMCEIEGHHLASAAILGHDGTVWAQS ADFPQFKPEEITGIMKDFDEPGHLAPTGMFVAGAKYM VIQGEPGAVIRGKKGAGGITIKKTGQALVVGIYDEPM TPGQCNMVVERLGDYLVEQGM | 131 | 4.92 | 14.1 | + |
| MP 12 | MEPGAVIRGKKGAGGITIKKTGQALVGIYDEPMTPGQ CNMVVERLGDYLVEQGMMSWQTYVDEHLMCEIEGH HLASAAILGHDGTVWAQSADFPQFKPEEITGIMKDFD EPGHLAPTGMFVAGAKYMVIQGHHHHHH | 137 | 5.68 | 15 | + |

*b) MP12 exhibits reduced allergenic activity*

Next, the reshuffled Phl p 12 was compared with Phl p 12 wild-type for its capacity to induce histamine release from basophils from profilin allergic patients.

Granulocytes were isolated from heparinised blood samples of timothy grass pollen allergic patients by Dextran sedimentation. After isolation, cells were incubated with various concentra-

tions of Phl p 12, MP12 or, for control purposes, with a monoclonal anti-human IgE antibody (Immunotech, Marseille, France). Histamine released into the supernatant was measured by radioimmunoassay (Immunotech). Total histamine was determined after freeze thawing of cells. Results are expressed as mean values of duplicate determinations, and represent the percentage of total histamine.

As exemplified in Fig. 5, Phl p 12 induced strong and dose-dependent histamine release in basophils from both patients, yielding maximal histamine release at concentrations between $10^{-5}$–$10^{-4}$ µg/ml, whereas no histamine release was observed with MP12 at concentrations up to $10^{-2}$ µg/ml indicating more than 1000-fold reduction of allergenic activity. Moreover, the maximum histamine release from basophils after adding MP12 was considerable lower than that achieved with Phl p 12 wild-type.

**Example 3: Immunization with MP12 induces IgG antibodies that recognize Phl p 12 wild-type as well as profilins from other pollens.**

In order to test, whether immunisation with reshuffled Phl p 12 will induce IgG antibodies that react with Phl p 12 wild-type and profilins from other pollens, rabbits were immunized three times with Phl p 12 or MP12 using Freund's complete and incomplete adjuvants (200µg/injection) (Charles River, Kisslegg, Germany). Serum samples were obtained in four weeks intervals. Sera were stored at -20°C until analysis.

Reactivity of MP12 and Phl p 12-induced IgG antibodies was studied by ELISA (Fig. 6). Phl p 12 as well as profilins from birch (Bet v 2) and mugwort were coated onto ELISA plates (5 µg/ml) and incubated with serial dilutions of rabbit antisera (1:2000-1:64000). Bound rabbit antibodies were detected with a 1:1000 diluted peroxidase-labeled donkey anti-rabbit antiserum (Amersham Pharmacia Biotech).

MP12 induced an IgG anti-Phl p 12 antibody response, that was comparable to that induced with Phl p 12 wild-type (Fig. 6A). Moreover, both, Phl p 12- and MP12-induced IgG antibodies,

cross-reacted with profilins from birch and mugwort (Fig. 6B, C).

**Example 4: Anti-MP12 antibodies inhibit the binding of serum IgE from grass pollen allergic patients to complete Phl p 12**

The ability of MP12-induced rabbit IgG to inhibit the binding of allergic patients' IgE to Phl p 12 was investigated by ELISA competition assay. ELISA plates (Nunc Maxisorp, Rosklide, Denmark) were coated with Phl p 12 (1µg/ml) and preincubated either with a 1:250 dilution of each of the anti-MP12 antiserum or the Phl p 12-antiserum and, for control purposes, with the corresponding preimmune sera. After washing, plates were incubated with 1:3 diluted sera from seven Phl p 12-sensitised grass pollen allergic patients and bound IgE antibodies were detected with a monoclonal rat anti-human IgE antibody (Pharmingen, San Diego, CA), diluted 1:1000, followed by a 1:2000 diluted HRP-coupled sheep anti-rat Ig antiserum (Amersham). The percentage inhibition of IgE binding achieved by preincubation with the anti-peptide or anti-mutant antisera was calculated as follows: % inhibition of IgE binding= $100-OD_I/OD_P x100$. $OD_I$ and $OD_P$ represent the extinctions after preincubation with the rabbits' immune sera and the corresponding preimmune sera, respectively.
As shown in Table 3, inhibition of patients' IgE binding to Phl p 12 achieved with anti-Phl p 12 antibodies was between 30.2-66.7% (49.8% mean inhibition). Likewise, considerable reduction of anti-Phl p 12 IgE reactivity was observed, ranging from 10.8-27.6% (20.8% mean inhibition) with antibodies raised against MP12 (Table 3).

Table 3: Inhibition of allergic patients' IgE binding to rPhl p 12 by rabbit antibodies. The percentage inhibition of IgE binding to rPhl p 12 achieved by preincubation with rabbit antisera (rabbit anti-Phl p 12, anti-MP12) for seven Phl p 12-allergic patients and the calculated mean inhibition are displayed.

| Patient | % inhibition | |
|---|---|---|
| | anti-Phl p 12 | anti-MP12 |
| 1 | 66.7 | 27.6 |

| | | |
|---|---|---|
| 2 | 53.8 | 18.8 |
| 3 | 46.0 | 16.2 |
| 4 | 43.2 | 18.9 |
| 5 | 45.7 | 27.0 |
| 6 | 30.2 | 10.8 |
| 7 | 63.0 | 26.1 |
| mean | 49.8 | 20.8 |

**Example 5: Anti-MP12 antiserum inhibits basophil degranulation The biological relevance and possible protective activity of peptide-induced IgG antibodies was investigated in a defined cellular model system using rat basophil leukaemia (RBL) cells which were loaded with allergen-specific IgE.**

RBL-2H3 cells were plated in 96 well tissue culture plates ($4x10^4$ cells/well), incubated for 24 h at 37°C using 7% $CO_2$. Passive sensitisation was performed with mouse sera containing profilin-reactive IgE at a final dilution of 1:30 for 2 h. Unbound antibodies were removed by washing the cell layer 2 times in Tyrode´s buffer (137mM NaCl, 2.7mM KCl, 0.5mM $MgCl_2$, 1.8mM $CaCl_2$, 0.4mM $NaH_2PO_4$, 5.6mM D-glucose, 12mM $NaHCO_3$, 10mM HEPES and 0.1% w/v BSA, pH 7.2). RBL cells, preloaded with Phl p 12-specific mouse IgE were exposed to rPhl p 12 (0.005 µg/ml). Phl p 12 was preincubated in Tyrode´s buffer with 0, 2, 5, 7.5 or 10% v/v of rabbit antiserum from a Phl p 12-immunized rabbit, a MP12-immunized rabbit or the corresponding preimmune sera for 2h at 37°C.

Preincubated Phl p 12 was added to the RBL cells for 30 min in a humidified atmosphere at 37°C and their supernatants were analyzed for ß-hexosaminidase activity by incubation with 80 µM 4-methylumbelliferyl-N-acetyl-ß-D-glucosamide (Sigma-Aldrich, Vienna, Austria) in citrate buffer (0.1M, pH 4.5) for 1 h at 37°C. The reaction was stopped by addition of 100 µl glycine buffer (0.2M glycine, 0.2M NaCl, pH 10.7) and the fluorescence was measured at $\lambda_{ex}$: 360/$\lambda_{em}$: 465 nm using a fluorescence microplate reader (Spectrafluor, Tecan, Austria). Results are reported as fluorescence units and percentage of total ß-hexosaminidase released after lysis of cells with 1% Triton X-100.

As exemplified in Fig. 7, both, preincubation of Phl p 12 with increasing concentrations (2-10% v/v) of rabbit anti-MP12 antibodies and with rabbit anti-Phl p 12 antibodies led to a dose-dependent inhibition of rPhl p 12-induced mediator release from RBLs that had been preloaded with Phl p 12-specific mouse IgE. No inhibition of basophil degranulation was observed when the allergen was preincubated with the same concentrations of preimmune Ig.

**Example 6: Expression, purification and characterization of a hypoallergenic derivative from Dermatophagoides pteronyssinus allergen Der p 2 (Der p 2 Hybrid)**

House dust mite (HDM) allergy belongs to the most common allergies worldwide which affects more than 50% of all allergic patients. *Dermatophogoides pteronyssinus* was identified as the most important source of allergens in house dust in Europe.

Twenty groups of mite allergens have been characterized so far, and group 2 allergens were identified as the major mite allergens, against which more than 80% of mite allergic patients are sensitized and they are mainly localized in mite faeces. Group 2 allergens were first characterized as 14000-18000 Da allergens with a high IgE-binding activity. Isolation and analysis of cDNA clones coding for Der p 2, revealed then that Der p 2 comprises an allergen with 129 amino acid residues, a calculated molecular weight of 14000 Da and without N-glycosylation sites. Group 2 allergens contain three disulfide bonds and are composed of two anti-parallel β-sheets. T-cell epitopes of Der p 2 are located in all regions of the protein and IgE-epitopes were shown to be conformational.

Immunotherapy studies with crude mite extracts have demonstrated that dangerous systemic side effects may occur during immunotherapy with HDM-extracts (Akcakaya, N., et al. (2000) Ann Allergy Asthma Immunol 85:317) as well as the induction of new IgE reactivities to sea-foods (van Ree, R., et al. (1996) Allergy 51:108).

To overcome the disadvantages of extract-based immunotherapy,

several strategies have been applied to develop hypcallergenic allergen derivatives. In case of Der p 2, variants were developed with reduced IgE reactivity by destroying disulfide bonds by site-directed mutagenesis, by destroying the disulfide bonds through N- and C-terminal deletion, or by introducing mutations. However, their biological activity is questionable.

In the following examples two recombinant fragments of the group 2 allergen of *Dermatophagoides pteronyssinusm* (Der p 2) comprising aa 1-53 and aa 54-129, to destroy conformational B-cell epitopes and to retain the major T-cell epitopes, were produced. Additionally, a recombinant Der p 2 hybrid molecule (aa 54-129 + 1-53), in which the two rDer p 2 fragments were recombined in inverse order by PCR-based gene-SOEing, was constructed.

Two recombinant fragments of Der p 2 comprising amino acids (aa) 1-53 and aa 54-129 were constructed by PCR-amplification as outlined in example 1 (see Fig. 8). A Der p 2 Hybrid molecule was generated in inverse order (aa 54-129 + 1-53) by PCR-based gene-SOEing (Linhart et al., FASEB J.16 (2002), 1301-1303).

*a) Expression in E. coli and purification of Der p 2, Der p 2 fragments and Der p 2 hybrid*

cDNAs coding for His-tagged Der p 2, Der p 2 fragments (aa 1-53 and aa 54-129) and Der p 2 hybrid (aa 54-129+1-53) were generated by PCR amplification using primers (MWG, Ebersberg, Germany) as indicated in Table 4 and a Der p 2 cDNA was obtained by reverse transcription from Der p RNA.

Table 4:

| Primer | Sequence |
|---|---|
| 1 (F) | 5'-GGAATTCCATATGGATCAAGTCGATGTC-3' |
| 2 (R) | 5'-GGAATTCCTTA***GTGATGGTGATGGTGATG***TTCAATTTTAGCGGT-3' |
| 3 (F) | 5'-GGAATTCCATATGATCAAAGCCTCAAT-3' |
| 4 (R) | 5'-GGAATTCCTTA***GTGATGGTGATGGTGATG***ATCGCGGATTTTA-3' |
| 5 (overlapping) | 5'-CTTTGACATCGACTTGATCATCGCGGATTTTAGCAT-3' |
| 6 (overlapping) | 5'-CATGCTAAAATCCGCGATGATCAAGTCGATGTCAAA-3' |

Forward (F), reverse (R) and overlapping primers are indicated. The *EcoRI* sites and *NdeI* sites are underlined. Nucleotides coding for the His-tags are shown in bold/italic letters.

Primers 1 and 4 were used for the amplification of the rDer p 2 cDNA, primers 1 and 2 for the cDNA coding for rDer p 2 fragment 1 (aa 1-53) and primers 3 and 4 for the cDNA of the rDer p 2 fragment 2 (aa 54-129). rDer p 2 hybrid was generated by PCR-based gene-SOEing using primers 2 and 3 and the two overlapping primers 5 and 6. Upstream primers contained an NdeI and EcoRI site and downstream primers contained an EcoRI site as well as six His codons. PCR products were cut with NdeI/EcoRI, gel-purified and subcloned into the NdeI/EcoRI sites of plasmid pET17b. Calcium chloride method was used for the transformation of the plasmids into E.coli strain XL-1 Blue. Plasmid DNA was isolated by NuceloBond AX kit - maxi-prep (Macherey-Nagel, Germany) and the sequence of the cDNA inserts was confirmed by sequencing of both DNA strands on an automated sequencing system (MWG, Germany).

Recombinant proteins containing C-terminal Hexahistidine-tails were expressed in E.coli strain BL21 (DE3) in liquid culture by induction with 0.5mM isopropyl-β-thiogalactopyranoside (IPTG) at an OD600 of 1 for 5h at 37°C. Cells were harvested by centrifugation at 4,000 x g for 15 minutes at 4°C.

The bacterial pellets obtained from 1l liquid culture were resuspended in 10ml 25mM imidazol, pH 7.4, 0.1% v/v Triton X-100 and treated with 100µg lysozyme for 30 minutes at room temperature. Cells were lysed by 3 freeze/thawing cycles (-70°C/+50°C), DNA was degraded by incubation with 1 µg DNase I for 10 minutes at room temperature and cell debris were removed by centrifugation at 10,000 x g for 30 minutes at 4°C. rDer p 2 fragment 1 was found in the soluble fraction and purified under native conditions over Ni-NTA resin affinity columns (QIAGEN, Germany).

rDer p 2, rDer p 2 fragment 2 and rDer p 2 hybrid were found in the pellet in the inclusion body fraction, which was solubilized with 8M urea, 100mM $NaH_2PO_4$, 10mM Tris-Cl, pH 8 for 60 minutes at room temperature. Insoluble residues were removed by centrifuga-

tion (10,000 x g, 15min, 4°C) and rDer p 2, rDer p 2 fragment 2 and rDer p 2 hybrid were purified under denaturating conditions over Ni-NTA resin affinity columns (QIAGEN).

Fractions, containing recombinant proteins of more than 90% purity were dialysed against 50mM $NaH_2PO_4$ pH 7 and the final protein concentrations were determined by Micro BCA Protein Assay Kit (Pierce, USA).

The construction of a hybrid molecule as outlined above disrupted at least one of the two β-sheets of Der p 2 and the disulfide bond between C8 and C119 and thus the conformational IgE epitopes of Der p 2 destroyed and major T-cell epitopes preserved. The rDer p 2 derivatives were overexpressed as visible bands in E. coli yielded a distinct accumulation (Fig. 9, lanes 1). rDer p 2 fragment 1 was found in the soluble fraction, whereas the other proteins accumulated in the insoluble inclusion body fractions but could be solubilized in urea. rDer p 2 and rDer p 2 derivatives were purified by nickel affinity chromatography (Fig. 9, lanes 2) yielding 20 to 30mg protein /l E. coli culture. After refolding by dialysis, rDer p 2, rDer p 2 fragment 1 and rDer p 2 hybrid remained soluble in physiological buffers at concentrations from 0.5mg/ml to 1mg/ml, whereas rDer p 2 fragment 2 only remained soluble at a concentration below 0.1mg/ml. SDS-PAGE analysis indicated a more than 90% purity of the proteins, which migrated as monomeric form and dimeric forms (Fig. 9, lanes 2).

*b) Matrix-assisted laser desorption and ionization-time of flight (MALDI-TOF) mass spectrometry of rDer p 2 and rDer p 2 derivatives*

Laser desorption mass spectra were acquired in a linear mode with a time of-flight Compact MALDI II instrument (Kratos, U.K.; piCHEM, Austria). Samples were dissolved in 10% acetonitrile, 0.1% trifluoroacetic acid and Alfa-cyano-4 hydroxy-cinnamic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For sample preparation, a 1:1 mixture of protein and matrix solution was deposited onto the target and air-dried.

Analysis of the four proteins by MALDI-TOF mass spectrometry revealed molecular masses of 15072.9 Da, 6806.7 Da, 9216.3 Da and 15001.8 Da for rDer p 2, rDer p 2 fragment 1, rDer p 2 fragment 2 and rDer p 2 hybrid, respectively, which are in agreement with the theoretical masses of the proteins calculated from their amino acid sequences (Fig. 10).

*c) Circular dichroism (CD) analysis*

The CD spectra of the purified recombinant proteins were recorded on a JASCO J715 spectropolarimeter that had been wavelength calibrated with neodymium glass in accordance with the manufacturer's suggestions. CD measurements were performed with rDer p 2 and rDer p 2 derivatives (c = 0.1 to 0.5mg/ml) dissolved in double distilled water at room temperature. A circular quartz cuvette with a path length of 0.1cm was used and the spectra were recorded with 0.2nm resolution at a scan speed of 50nm/min. The spectra were signal-averaged by accumulating at least three scans and the results are expressed as the mean residue ellipticity at a given wavelength.

The far ultraviolet CD spectrum of the purified recombinant Der p 2 shows a negative band at 217nm, indicating a β-sheet conformation (Fig. 11). In contrast, the CD spectra of the rDer p 2 derivatives indicate that these proteins are mainly unfolded. rDer p 2 fragment 1 shows a typical random coil conformation, identified by a negative band at ~200nm. Also rDer p 2 fragment 2 shows a predominant random coil conformation, although the intensity of the signal was very low. rDer p 2 hybrid spectrum adsorbed mainly random coil conformation with small amounts of β-sheet structures (Fig. 11). The destruction of the three-dimensional conformation could be confirmed by circular dicroism analysis, showing a loss or reduction of β-sheet structure in the rDer p 2 derivatives compared to rDer p 2 wild-type.

**Example 7: Recombinant Der p 2 Hybrid (rDer p 2 Hybrid) shows strongly reduced IgE binding capacity**

Purified recombinant Der p 2, the two rDer p 2 fragments, frag-

ment 1 (aa 1-53) and fragment 2 (aa 54-129), and rDer p 2 hybrid were tested for IgE reactivity by non-denaturating dot blot assays. Two microlitres of the purified proteins (0.1mg/ml) and, for control purposes, BSA were dotted onto nitrocellulose membrane strips (Schleicher & Schuell, Germany). Nitrocellulose strips containing the dot-blotted proteins were blocked in buffer A (40mM $Na_2HPO_4$, 0.6mM $NaH_2PO_4$, pH 7.5, 0.5% [v/v] Tween 20, 0.5% [w/v] BSA, 0.05% [w/v] $NaN_3$) and incubated with sera from mite-allergic patients, serum from a non-allergic person (dilutions 1:10) or buffer A without serum. Bound IgE antibodies were detected with 125I-labeled anti-human IgE antibodies and visualized by autoradiography.

The IgE-binding capacity of rDer p 2 wild-type allergen was compared with the two rDer p 2 fragments and rDer p 2 hybrid by non-denaturing dot blot assays. Sera from 17 mite allergic individuals (lanes 1-17) showed varying IgE reactivity to nitrocellulose dotted rDer p 2, whereas almost no IgE reactivity to rDer p 2 fragment 1 could be detected. Only 3 sera showed very weak binding to rDer p 2 fragment 2 and 2 sera reacted with rDer p 2 hybrid (Fig. 12). Serum from a non-allergic person as well as buffer without serum showed no IgE reactivity to rDer p 2 or to rDer p 2 derivatives (Fig. 12 lanes 18, 19). No IgE reactivity to the control protein, BSA, was found (Fig. 12). As a consequence of the loss of the conformation and thus the conformational IgE-epitopes (see example 7), it could be shown that the rDer p 2 derivatives have almost completely lost their IgE-binding capacity compared to rDer p 2 wild-type.

**Example 8: Reduced allergenic activity of rDer p 2 derivatives as determined by CD 203c expression**

Heparinized blood samples were obtained from allergic patients. Blood samples (100µl) were incubated with various concentrations of rDer p 2, rDer p 2 fragments, rDer p 2 hybrid, a monoclonal anti-IgE antibody (Immunotech, Marseille, France), or PBS for 15 minutes (37°C). CD 203c expression was determined as described (Hauswirth, A. W., et al. (2002) J Allergy Clin Immunol 110:102.).

The upregulation of CD 203c has been described as a surrogate marker for allergen-induced basophil activation and degranulation (Hauswirth, A. W., et al. (2002)). Therefore the allergenic activity of recombinant Der p 2, rDer p 2 fragments and rDer p 2 hybrid by measuring CD 203c upregulation on basophils from house dust mite allergic patients was compared (Fig. 13, 14). Fig. 13 shows representative results from 3 patients. Incubation of basophils with 10µg/ml of rDer p 2 wild-type significantly upregulated CD 203c expression in each of the tested patients, whereas no upregulation was obtained with the same concentration of the individual fragments or with an equimolar mixture of the two fragments (Fig. 13). Additionally, basophils from the same 10 patients were exposed to different concentrations (5µg/ml - 0.32ng/ml) of rDer p 2 and rDer p 2 hybrid in 1:5 dilution steps. Fig. 14 shows the results from 6 representative patients. Exposure of basophils with rDer p 2 hybrid resulted in an upregulation of CD 203c expression at concentrations between 40ng/ml and 5000ng/ml, whereas rDer p 2 wild-type induced upregulation of CD 203c already at concentrations between 8 - 200ng/ml. In 8 out of 10 patients, rDer p 2 hybrid had a more than 10-fold reduced capacity to activate basophils compared to rDer p 2.

Anti-human IgE antibodies induced upregulation of CD 203c expression on basophils from all patients, whereas no upregulation was obtained with buffer alone (Fig. 13 + 14).

Determination of CD 203c expression on basophils from mite-allergic patients indicates a reduced biological activity of rDer p 2 hybrid compared to rDer p 2 wild-type and no biological activity can be observed with the rDer p 2 fragments. Moreover, basophil activation assays using RBL cells indicate that IgE Abs induced with the derivatives were less anaphylactic. These results indicate that hypoallergenic rDer p 2 derivatives will induce less IgE-mediated side-effects than the Der p 2 wild-type allergen when used for immunotherapy.

**Example 9: rDer p 2 derivatives induce rDer p 2-specific IgG antibodies in mice similar as rDer p 2 wild-type allergen**

Groups of five eight-week-old female BALB/c mice each were im-

munized with 5µg of purified proteins (rDer p 2, rDer p 2 fragment 1, rDer p 2 fragment 2 or rDer p 2 hybrid), adsorbed to 200µl of AluGel-S (SERVA Electrophoresis, Germany) subcutaneously in the neck in 4 weeks intervals over a period of 20 weeks. Blood samples were collected one day before each immunization and stored at -20°C.

ELISA plates (Greiner, Austria) were coated with rDer p 2 diluted in PBS (c = 5µg/ml) over night at 4°C. The plates were washed twice with PBST (PBS; 0.05% v/v Tween 20) and blocked with blocking buffer (PBST; 1% w/v BSA) for 3h at room temperature. Mouse sera were diluted 1:1000 for measurement of Der p 2-specific IgG1 in PBST; 0.5% w/v BSA and 100µl of this dilution was added per well overnight at 4°C.

Plates were washed 5 times with PBST and bound IgG1 antibodies were detected with a monoclonal rat anti-mouse IgG1 antibody (BD Pharmingen, USA), followed by the addition of horseradish peroxidase-labeled goat anti-rat IgG antibodies (Amersham Bioscience, Sweden) as described (Vrtala, S., et al. (1996) J Allergy Clin Immunol 98:913).

The Der p 2 specific $IgG_1$ levels were determined in serum samples obtained from mice after immunization with rDer p 2 and rDer p 2 derivatives (Fig. 15). rDer p 2 as well as the rDer p 2 derivatives were immunogenic and induced $IgG_1$ responses in the mice after the second immunization (week 8) (Fig. 15). After the second immunization the $IgG_1$ responses induced with rDer p 2 fragment 1 and rDer p 2 hybrid were even higher than that induced with rDer p 2 (Fig. 15). After the last immunization, $IgG_1$ responses induced with the rDer p 2 derivatives were comparable to those induced with the rDer p 2 wild-type molecule (Fig. 15).

**Example 10: IgG1 antibodies induced by immunization with rDer p 2 derivatives inhibit mite-allergic patients' IgE binding to rDer p 2 wild-type**

ELISA plates (Greiner, Austria) were coated with 100µl purified rDer p 2, diluted with PBS to a concentration of 5µg/ml, over night at 4°C. After washing twice with PBST and blocking with

blocking buffer (PBST; 1% w/v BSA) for 3h at room temperature, plates were incubated overnight at 4°C with anti-rDer p 2, anti-rDer p 2 fragment 1, anti-rDer p 2 fragment 2 or anti-rDer p 2 hybrid antisera or the corresponding preimmune sera. Mouse antisera were diluted 1:20 and rabbit antisera were diluted 1:100 in PBST; 0.5% w/v BSA. After washing, the plates were incubated with 1:10 diluted sera from mite allergic patients overnight at 4°C and bound human IgE antibodies were detected with HRP-coupled goat anti-human IgE antibodies (KPL, USA) diluted 1:2500 in PBST; 0.5% w/v BSA as described (44, 45). The percentage of inhibition of IgE binding was calculated as follows: 100 - (ODs/ODp) x 100, where ODs and ODp represent the extinction coefficients after preincubation with the immune serum and the preimmune serum, respectively.

Mouse IgG1 antibodies induced by immunization with rDer p 2 and the rDer p 2 derivatives were investigated for their ability to inhibit mite-allergic patients' IgE binding to rDer p 2 in ELISA competition experiments.

The percentage of inhibition of allergic patients' IgE binding to rDer p 2 wild-type by mouse IgG antibodies is shown in Tables 5 and 6.

The inhibition obtained with mouse anti-rDer p 2 antibodies was between 61 and 87% (mean 75%), whereas mouse anti-rDer p 2 hybrid antibodies, anti-Der p 2 fragment 1 antibodies and anti-Der p 2 fragment 2 antibodies inhibited serum IgE binding to rDer p 2 wild-type between 47 and 76% (mean 62%), between 48 and 66% (mean 54%) and between 24 and 52% (mean 41%), respectively (Table 5).

Table 5:

% Inhibition of IgE binding

| Antibodies | Patient 1 | Patient 2 | Patient 3 | Patient 4 | mean |
|---|---|---|---|---|---|
| rDer p 2 fragment 1 | 48 | 66 | 53 | 50 | 54 |
| rDer p 2 fragment 2 | 39 | 50 | 52 | 24 | 41 |
| rDer p 2 hybrid | 59 | 76 | 64 | 47 | 62 |
| rDer p 2 | 61 | 87 | 77 | 73 | 75 |

In additional experiments, rabbits were immunized with purified rDer p 2 and the three rDer p 2 derivatives. The ability of rabbit anti-sera to inhibit mite-allergic patients' IgE binding to rDer p 2 was also tested by ELISA inhibition assays with an outcome similar as obtained for the mouse sera (Table 6). Rabbit anti-rDer p 2 antibodies inhibited patients' IgE binding to rDer p 2 between 47 and 89% (mean 66%), whereas anti-rDer p 2 hybrid antibodies inhibited human IgE binding between 20 and 86% (mean 59%). The inhibition obtained with rabbit anti-rDer p 2 fragment 1 antibodies was between 26 and 70% (mean 52%) and the inhibition with rabbit anti-rDer p 2 fragment 2 antibodies was between 32 and 54% (mean 42%). Using a mixture of the anti-fragment 1 and anti-fragment 2 antibodies the inhibition of patients' IgE binding to rDer p 2 wild-type was only slightly increased to a mean of 55% (Table 6).

Table 6:

%Inhibition of IgE binding

| Antibodies | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | mean |
|---|---|---|---|---|---|---|
| rDer p 2 fragment 1 | 59 | 70 | 49 | 26 | 57 | 52 |
| rDer p 2 fragment 2 | 40 | 49 | 33 | 32 | 54 | 42 |
| fragment 1 + fragment 2 | 60 | 69 | 44 | 38 | 66 | 55 |
| rDer p 2 hybrid | 61 | 86 | 61 | 20 | 67 | 59 |
| rDer p 2 | 60 | 89 | 53 | 47 | 78 | 66 |

Immunization of mice showed the immunogenicity of all three rDer

p 2 derivatives by their capacity to induce IgG antibody responses. IgE-binding from mite-allergic patients to Der p 2 was inhibited by IgG antibodies induced with each of the rDer p 2 derivatives but rDer p 2 hybrid-induced IgG antibodies indicated a better inhibitory capacity compared to IgG antibodies induced with the two individual fragments and even to a mixture of fragment 1 and 2 induced IgG antibodies. These results are of importance, since blocking antibodies were shown to play a main role in SIT with recombinant allergens.

Anti-rDer p 2 and anti-rDerp 2 derivative antibodies induced by immunisation of mice inhibit allergic patients' IgE binding to rDer p 2 as shown in an ELISA inhibition assay.

Der p 2 Hybrid induces blocking antibodies in the present mouse model; immunogenicity is significantly increased by reshuffling the fragments.

**Example 11: Vaccines based on rDer p 2 derivatives have a reduced allergenicity in vivo compared to a rDer p 2-wild-type-based vaccine**

Rat basophil leukemia (RBL) cells (subline RBL-2H3) were plated on ELISA plates (Nunc, Denmark) (100µl: 4 x 104 cells) in cell culture medium (100ml RPMI 1649, 10% FCS, 4mM L-Glutamine, 2mM Sodium Pyruvate, 10mM HEPES, 100µM 2-Mercaptoethanol, 1% Pen/Strep) over night at 37°C, 5% $CO_2$.

Cells were loaded with 2µl of serum obtained from mice immunized with rDer p 2, rDer p 2 fragment 1, rDer p 2 fragment 2 and rDer p 2 hybrid for 2h at 37°C, washed twice with 200µl Tyrode/BSA buffer (137mM NaCl, 2.7mM KCl, 0.5mM $MgCl_2$, 1.8mM $CaCl_2$, 0.4mM $NaH_2PO_4$, 5.6mM D-glucose, 12mM $NaHCO_3$, 10mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 0.1% bovine serum albumin, pH 7.2) (Sigma-Aldrich, Austria) and stimulated with rDer p 2 (c = 0.3µg/ml). Total β-hexosaminidase release was induced by addition of 10µl 10% v/v Triton X-100 (Merck, Germany).

For measuring the release of β-hexosaminidase, 50µl assay solu-

tion (80µM 4-methylumbelliferyl-N-acetyl- β - D-glucosaminide in 0.1M citrate buffer, pH 4.5) was incubated with 50µl supernatant for 1h at 37°C, 5%$CO_2$.

The reaction was stopped by adding 100µl glycin buffer (0.2M glycine, 0.2M NaCl, pH 10.7) and fluorescence was measured at λex: 360nm λem: 465nm using a fluorescence microplate reader (Dynatech MR 7000, Dynatech Laboratories, USA). Results are shown as mean percentages of total β-hexosaminidase release.

To investigate whether vaccination with rDer p 2 derivates induces allergic immune responses to Der p 2 wild-type allergen, mice were immunized with rDer p 2, rDer p 2 fragment 1, rDer p 2 fragment 2 and rDer p 2 hybrid, respectively. Then serum samples from the mice were used to load RBL cells to quantify the allergenic immune response to rDer p 2 wild-type allergen by RBL degranulation experiments. The release obtained with rDer p 2 wild-type allergen in RBLs loaded with mouse anti-rDer p 2 fragment 1, anti-rDer p 2 fragment 2 and anti-rDer p 2 hybrid antibodies was between 0 and 16.6% (mean 6.4%), between 0.2 and 28.6% (mean 13.2%) and between 4.7 and 37.1% (mean 18.3%), whereas RBLs, loaded with anti-rDer p 2 wild-type antibodies released between 35 and 39% (mean 37%) after stimulation with rDer p 2 wild-type (Fig. 16).

**Example 12: MP 12 induced IgG antibodies that recognize Phl p 12 wild-type, profilins from other pollens and plant -food derived profilins.**

In order to test whether antibodies induced after immunization with MP 12 recognize profilins from pollens as well as from plant derived food, ELISA experiments were performed.

Profilins from timothy grass pollen (Phl p 12), birch pollen (Bet v 2), mugwort pollen (Art v 4) and from different plant foods (cashew nut (Ana c ), celery (Api g 4), banana (Mus xp 1), hazelnut (Cor a 2), and carrot (Dau c 4) were coated onto ELISA plates (5µg/ml) and incubated with serial dilutions of rabbit antisera (1:2000- 1:64000). Bound rabbit antibodies were detected with a POX-labeled donkey-anti-rabbit antiserum.

MP 12 induced an IgG antibody response that was comparable with that induced with Phl p 12 wild-type (Fig. 17). Both, Phl p 12 and MP12-induced IgG antibodies cross-reacted with profilins from pollens (grass, trees, weeds) and plant-derived food profilins (Fig. 17).

**Example 13: Anti-MP 12 antibodies inhibit the binding of serum IgE from grass pollen allergic patients to complete Phl p 12 as well as to profilins from other pollens (trees and weeds) and to plant food-profilins.**

The ability of MP12-induced rabbit IgG to inhibit the binding of allergic patients' IgE to Phl p 12, to profilins from distinct pollens and to plant food-derived profilins was investigated by ELISA competition experiments.

ELISA plates (Nunc Maxisorp, Denmark) were coated with profilins from timothy grass (rPhl p 12), birch pollen (rBet v 1), carrot (rDau c 4), hazelnut (rCor a 2), banana (rMus xp 1) and cashew nut (rAna c 1) and preincubated with a 1:50 dilution of the anti-Phl p 12 antiserum, the anti-MP 12-antiserum and, for control purposes, with the corresponding preimmune sera. After washing, plates were incubated with 1:3 diluted sera from eight profilin-sensitized patients and bound IgE antibodies were detected with a HRP-labeled anti-human IgE antiserum from goat (KPL, USA), diluted 1:2500. The percentage inhibition of IgE binding achieved by preincubation with the anti-Phl p 12 and anti-MP 12-antisera was calculated as follows: % inhibition of IgE binding= 100-ODI/ODPx100. ODI and ODP represent the extinctions after preincubation with the rabbits' immune sera and the corresponding preimmune sera, respectively (Table 7).

Table 7:

| | Percentage inhibition of IgE binding to | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient | Phl p 12 | | Bet v 2 | | Cor a 2 | | Mus xp 1 | | Dau c 4 | | Ana c1 | |
| | α-Phl p 12 | α-MP 12 | α-Phl p 12 | α-MP 12 | α-Phl p 12 | α-MP 12 | α-Phl p 12 | α-MP 12 | α-Phl p 12 | α-MP 12 | α-Phl p 12 | α-MP 12 |
| 1 | 91 | 82,7 | 88,3 | 84,4 | 61,3 | 58,7 | 70,6 | 46,4 | 82,7 | 83,3 | 70 | 71,7 |
| 2 | 82,5 | 72,8 | 74,9 | 77,6 | 73,3 | 60,3 | 74,4 | 50,8 | 75,5 | 83,2 | 62,3 | 45,1 |
| 3 | 89,4 | 72,3 | 75,4 | 76,4 | 58,8 | 61,6 | 74,4 | 33,5 | 65,4 | 63,1 | 61,6 | 56 |
| 4 | 77,4 | 72 | 69 | 69,6 | 56,00 | 52 | 57,2 | 39,8 | 65 | 66,8 | 71,5 | 41,5 |
| 5 | 86,3 | 65,2 | 35 | 68,4 | 68,6 | 52 | 75,5 | 27,2 | 97,5 | 93,8 | 59,5 | 56,2 |
| 6 | 71,2 | 84,7 | 54,4 | 72 | 57 | 62,7 | 73,9 | 25,6 | 58,5 | 68,3 | 44,3 | 42,6 |
| 7 | 83,6 | 70,4 | 60,2 | 70,8 | 69,7 | 59,7 | 72,5 | 35,6 | 72,4 | 71,3 | 28,1 | 32,2 |
| 8 | 89,1 | 57,4 | 61 | 79 | 57,8 | 57,8 | 73 | 29,6 | 70 | 67 | | |
| mean | 83,8 | 72,3 | 64,8 | 74,5 | 62,3 | 58,1 | 71,4 | 36,1 | 73,3 | 74,6 | 56,8 | 53,6 |

The mean inhibition of IgE binding to timothy grass pollen profilin achieved with Phl p 12-induced antibodies and MP 12-induced antibodies was comparable with 83.8% and 72.3%, respectively (Table 7). IgE binding to birch pollen profilin, Bet v 2, was even stronger inhibited with MP 12-specific antibodies (mean inhibition 74.5%) than with Phl p 12-induced antibodies (mean inhibition 64.8%). IgE binding to plant food profilins were inhibited with both antisera to a very similar degree (Cor a 2: 62.3% average inhibition with anti-Phl p 12-IgG, 58.1% with anti-MP 12-IgG; Dau c 4: 73,3% average inhibition with anti-Phl p 12-IgG, 74.6% with anti-MP 12-IgG; Ana c 1: 56.8% average inhibition with anti-Phl p 12-IgG, 53.6% with anti-MP 12-IgG). Only IgE binding to banana profilin, Mus xp 1, was less inhibited with anti-Mp 12- IgG (36.1%) than with anti-Phl p 12-induced IgG (71.4%) (Table 7).

Profilin represents an allergen that is expressed in all eukaryotic cells and thus represents a pan-allergen that might induce inhalative allergies (e.g., rhinoconjunctivits, asthma) as well as oral allergy syndromes after oral ingestion (itching and swelling of lips and the tounge) in sensitized patients.

The reshuffled Phl p 12-derivative, MP12, induces IgG antibodies after immunization that recognize profilins from both pollens as well as from plant-derived food. MP 12-induced antibodies inhibit patients' serum IgE binding to profilins from pollens and also to plant food-derived profilins. Thus, the MP12 is suitable for the treatment of pollen- food cross-sensitization attributable to profilin allergy.

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 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 method for producing a derivative of a wild-type protein allergen with reduced allergenic activity, comprising the following steps:

(a) providing a wild-type protein allergen with an allergenic activity;

(b) splicing said wild-type protein allergen into two parts, wherein said two parts have a reduced allergenic activity or lack allergenic activity; and

(c) rejoining said two fragments in inverse orientation.

2. The method according to claim 1, wherein said derivative is produced in a host as a recombinant protein.

3. The method according to claim 1 or claim 2, wherein said wild-type allergen is selected from the group consisting of: a profilin, a birch allergen, a dust mite allergen, a storage mite allergen and a timothy grass allergen.

4. The method according to claim 3, wherein the profilin is Phl p 12, the birch allergen is Bet v 4, the dust mite allergen is Der p 2, the storage mite allergen is Lep d 2 and/or the timothy grass allergen is Phl p 7.

5. The method according to any one of claims 1 to 4, wherein the reduced allergenic activity is measured by a reduction of inhibition of IgE binding capacity and

the allergenic activity is reduced by an amount selected from: at least 10%, at least 20% and at least 30%, compared to the wild-type allergen.

6. The method according to any one of claims 1 to 5, wherein the reduced allergenic activity is measured by lack of binding of IgE antibodies in serum of an allergen sensitised patient to a dot blot of said derivative.

7. The method according to any one of claims 1 to 6, wherein said derivative is combined with a pharmaceutically acceptable excipient and finished to a pharmaceutical preparation.

8. The method according to any one of claims 1 to 7, wherein said derivative is combined with a suitable vaccine adjuvant and finished to a pharmaceutically acceptable vaccine preparation.

9. The method according to claim 8, wherein said derivative is combined with at least one further allergen in a combination vaccine.

10. The method according to claim 9, wherein said at least one further allergen is a wild-type allergen.

11. The method according to claim 10, wherein said wild-type allergen is a mixture of wild-type allergens, recombinant wild-type allergens, derivatives of wild-type protein allergens or mixtures thereof.

12. The method according to any one of claims 7 to 11, wherein said preparation further contains an allergen extract.

13. An allergen derivative of a wild-type protein allergen wherein said wild-type protein allergen comprises an amino acid sequence of 1 to Z, and wherein said derivative adjacently comprises, in N-terminal to C-terminal orientation, two wild-type allergen fragments X to Z and 1 to X, wherein said two wild-type allergen fragments have reduced allergenic activity or lack allergenic activity.

14. The allergen derivative according to claim 13, wherein the wild-type allergen fragments X to Z and 1 to X have lengths selected from: at least 30 amino acid residues, at least 50 amino acid residues and at least 60 amino acid residues.

15. The allergen derivative according to claim 13 or claim 14, wherein the wild-type allergen fragments X to Z and 1 to X differ in length by an amount selected from: 50% or less, 30% or less and 20% or less.

16. The allergen derivative according to any one of claims 13 to 15, wherein said wild-type allergen is selected from the group consisting of: a type I allergen, birch (Betula verrucosa) pollen, yellow jacket (Vespula vulgaris) venom, paper wasp (Polistes annularis) venom, Parietaria judaica pollen, a ryegrass pollen, a dust mite allergen and any mixture thereof.

17. The allergen derivative according to claim 16, wherein the type I allergen is selected from the allergens presented in table A.

18. The allergen derivative according to claim 17, wherein the allergen presented in table A is an allergen of timothy grass (Phelum pratense).

19. The allergen derivative according to claim 18, wherein the allergen of timothy grass is Phl p 12.

20. The allergen derivative according to claim 16, wherein the birch pollen is Bet v 4 and/or the dust mite allergen is Der p 2.

21. An allergen composition comprising an allergen derivative according to any one of claims 13 to 20, and at least one further allergen.

22. The allergen composition of claim 21, wherein said further allergen is a wild-type allergen.

23. The allergen composition of claim 22, wherein said wild-type allergen is a mixture of a wild-type allergen, a recombinant wild-type allergen, a derivative of wild-type protein allergen or any mixture thereof.

24. The allergen composition according to any one of claims 21 to 23, wherein said composition further comprises an allergen extract.

25. The allergen composition according to any one of claims 21 to 24, wherein said composition further comprises a pharmaceutically acceptable excipient.

26. Use of an allergen derivative according to any one of claims 13 to 20, or an allergen composition according to any one of claims 21 to 25, for the preparation of a medicament for allergen specific immunotherapy, passive immunisation or prophylactic immunisation.

27. The use according to claim 26, wherein said medicament further comprises adjuvants, diluents, preservatives or mixtures thereof.

28. The use according to claim 26 or claim 27, wherein said medicament comprises a recombinant allergen derivative in a range selected from: 10 ng to 1 g, 100 ng to 10 mg, and 0.5 μg to 200 μg.

29. Use of a profilin derivative obtainable from a first wild-type profilin molecule by the method according to any one of claims 1 to 12 or an allergen derivative of a first wild-type profilin molecule according to any one of claims 13 to 20, or an allergen composition according to any one of claims 21 to 25, for the manufacture of a medicament for prevention and/or treatment of allergic diseases caused by a second wild-type profilin molecule, or for the treatment and/or prevention of pollen food cross sensitization attributable to profilin allergy.

30. The use according to claim 29, wherein said first and said second wild-type profilin molecules are selected

from the group consisting of: Phl p 12, Bet v 2, Art v 4, Ana c, Api g 4, Mus xp 1, Cor a 2 and Dau c 4.

31. The use according to claim 29, wherein said first wild-type profilin molecule is Phl p 12 and said second wild-type profilin molecule is selected from the group consisting of: Bet v 2, Art v 4, Ana c, Api g 4, Mus xp 1, Cor a 2 and Dau c 4.

32. A method of allergen specific immunotherapy, passive immunisation or prophylactic immunisation comprising administering to a patient in need thereof a therapeutically effective amount of the allergen derivative of any one of claims 13 to 20, and/or the allergen composition of any one of claims 21 to 25.

33. The allergen derivative of a wild-type protein allergen according to claim 13, methods of producing the same and uses thereof, substantially as hereinbefore described with reference to the Examples.

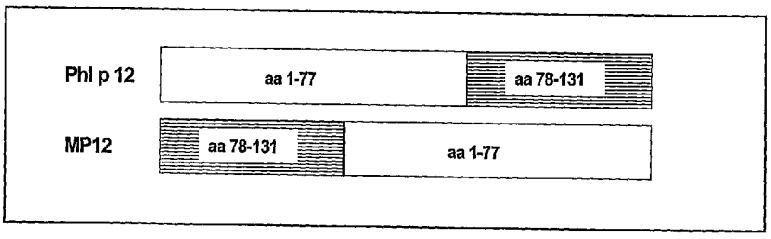
Phl p 12
aa 1-77
aa 78-131
MP12
aa 78-131
aa 1-77

Fig. 1

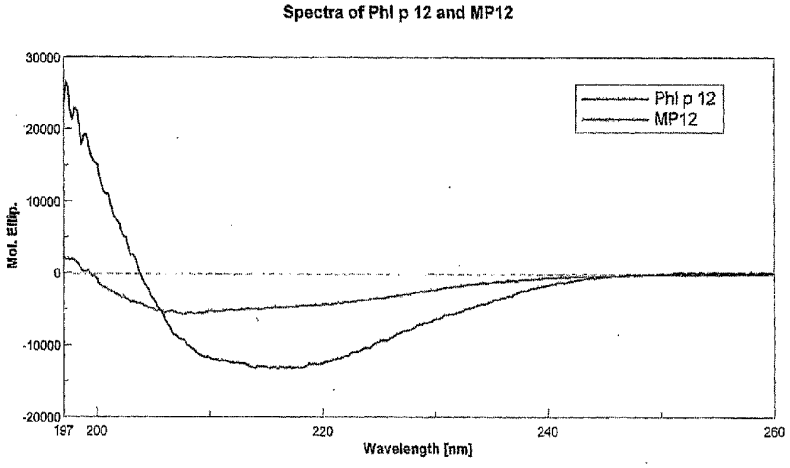
Spectra of Phl p 12 and MP12
Phl p 12
MP12
Mol. Ellip.
30000
20000
10000
0
-10000
-20000
197
200
220
240
260
Wavelength [nm]

Fig. 2

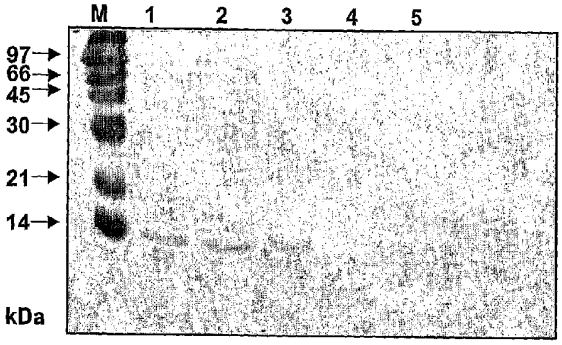
M
1
2
3
4
5
97
66
45
30
21
14
kDa

Fig. 3

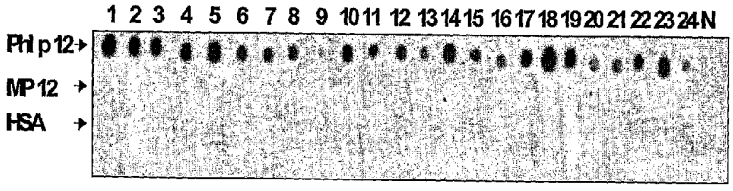
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 N
Phl p 12
MP 12
HSA

Fig. 4

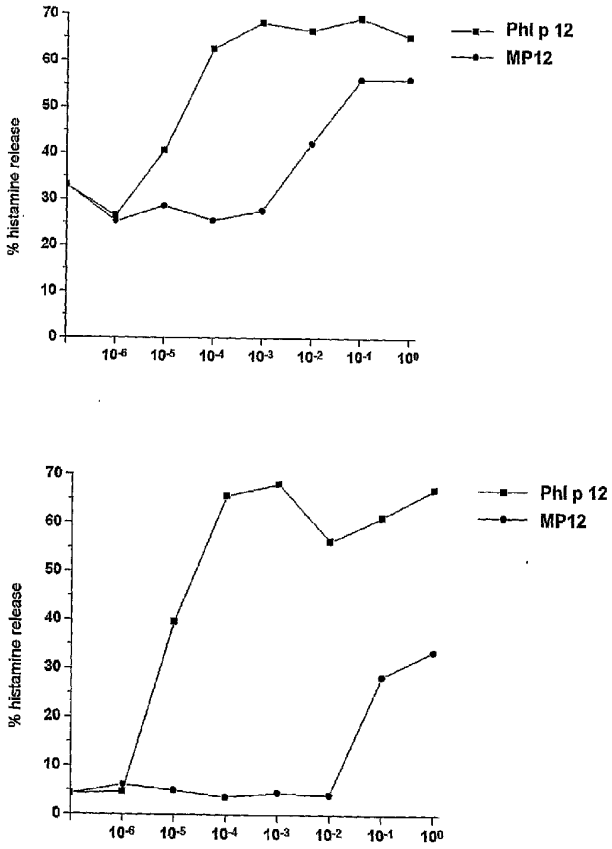
% histamine release
70
60
50
40
30
20
10
0
10-6
10-5
10-4
10-3
10-2
10-1
100
Phl p 12
MP12
% histamine release
70
60
50
40
30
20
10
0
10-6
10-5
10-4
10-3
10-2
10-1
100
Phl p 12
MP12

Fig. 5

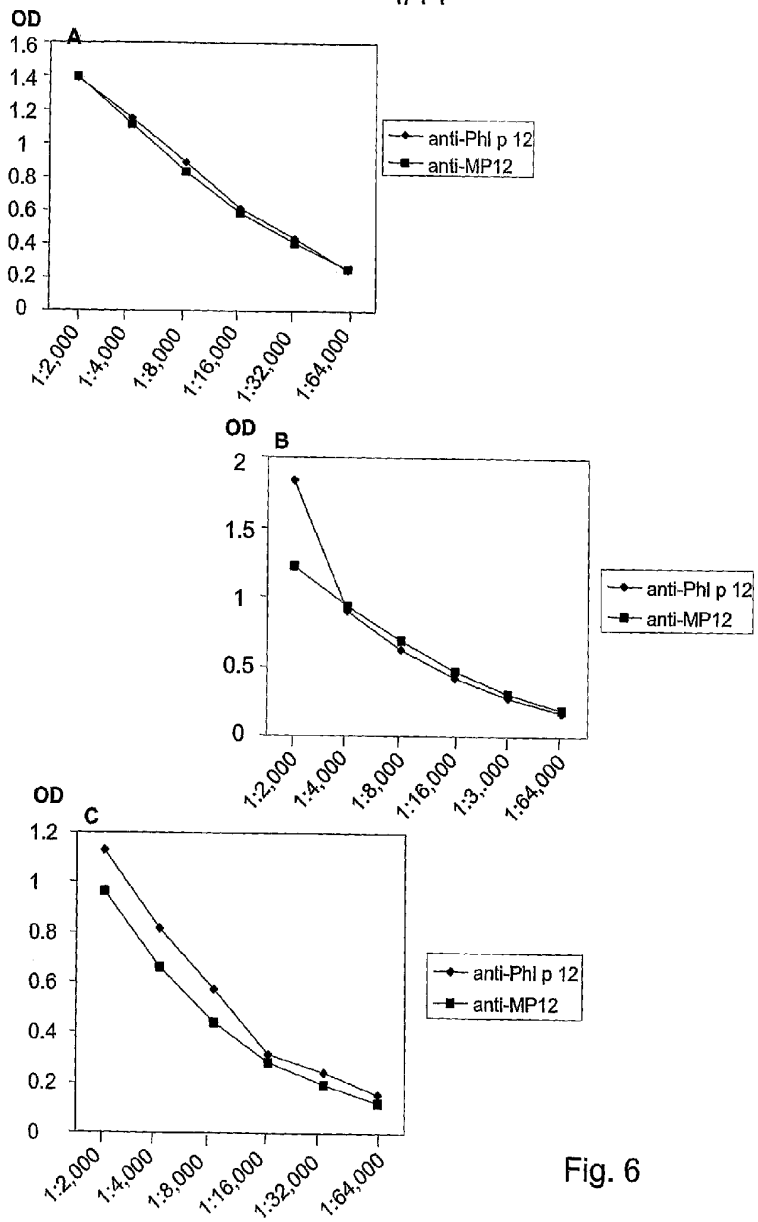
OD
A
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1:2,000
1:4,000
1:8,000
1:16,000
1:32,000
1:64,000
anti-Phl p 12
anti-MP12
OD
B
2
1.5
1
0.5
0
1:2,000
1:4,000
1:8,000
1:16,000
1:3,.000
1:64,000
anti-Phl p 12
anti-MP12
OD
C
1.2
1
0.8
0.6
0.4
0.2
0
1:2,000
1:4,000
1:8,000
1:16,000
1:32,000
1:64,000
anti-Phl p 12
anti-MP12

Fig. 6

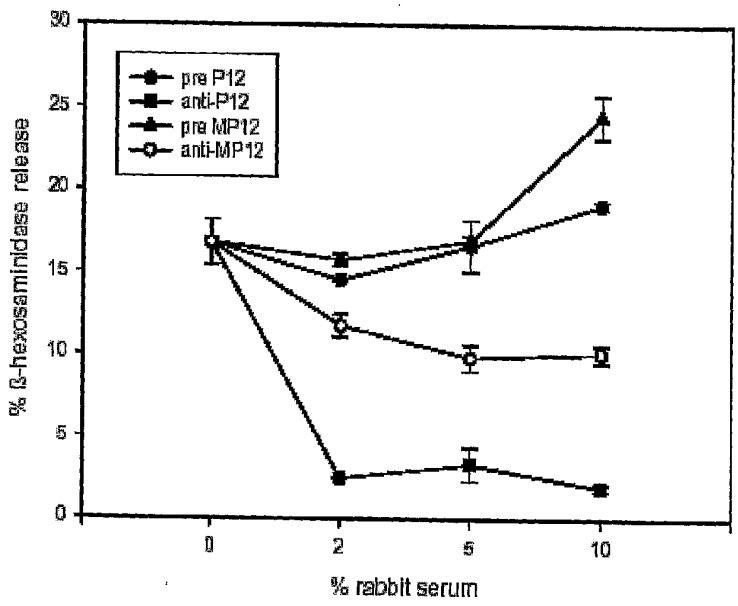
pre P12
anti-P12
pre MP12
anti-MP12
% ß-hexosaminidase release
30
25
20
15
10
5
0
0
2
5
10
% rabbit serum

Fig. 7

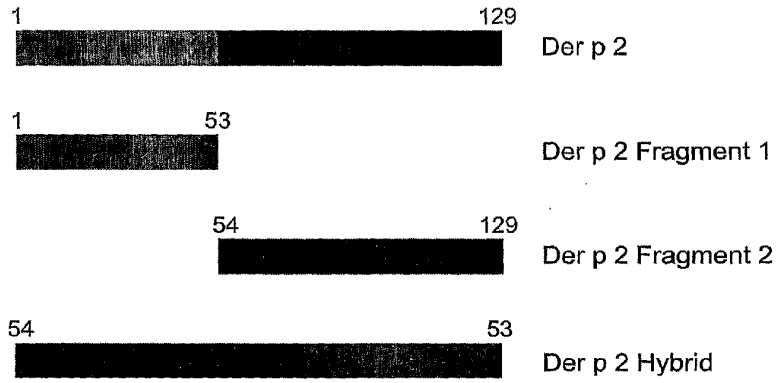
1
129
Der p 2
1
53
Der p 2 Fragment 1
54
129
Der p 2 Fragment 2
54
53
Der p 2 Hybrid

Fig. 8

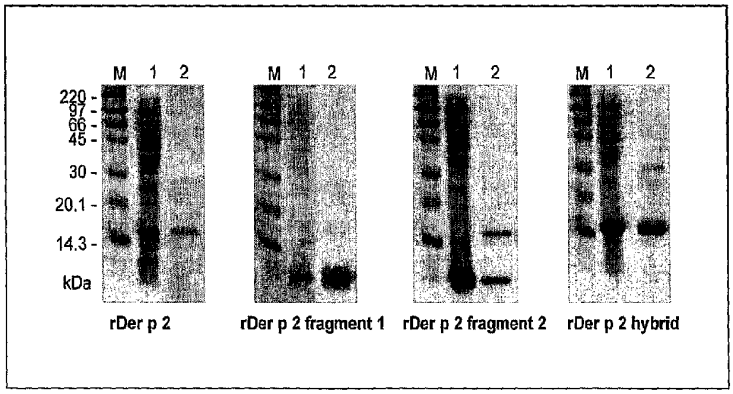
M 1 2
220 -
97 -
66 -
45 -
30 -
20,1 -
14,3 -
kDa
rDer p 2
rDer p 2 fragment 1
rDer p 2 fragment 2
rDer p 2 hybrid

Fig. 9

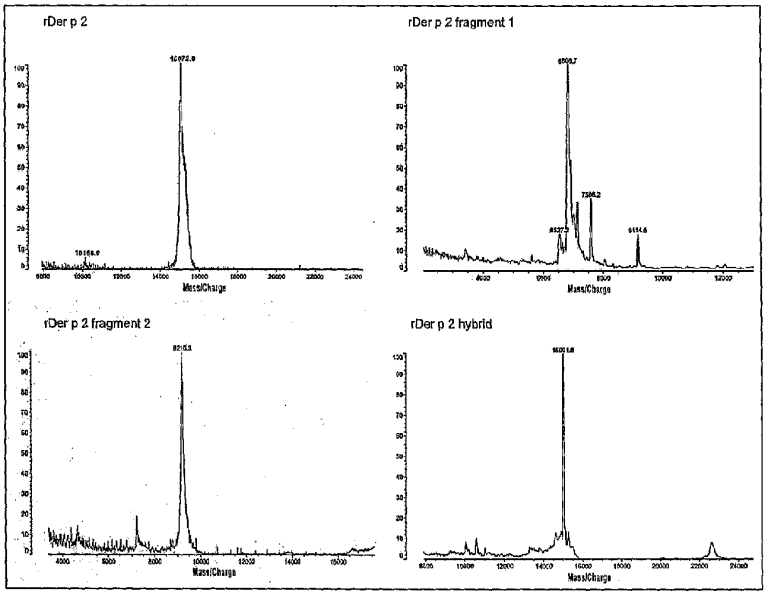
rDer p 2
rDer p 2 fragment 1
rDer p 2 fragment 2
rDer p 2 hybrid
Mass/Charge

Fig. 10

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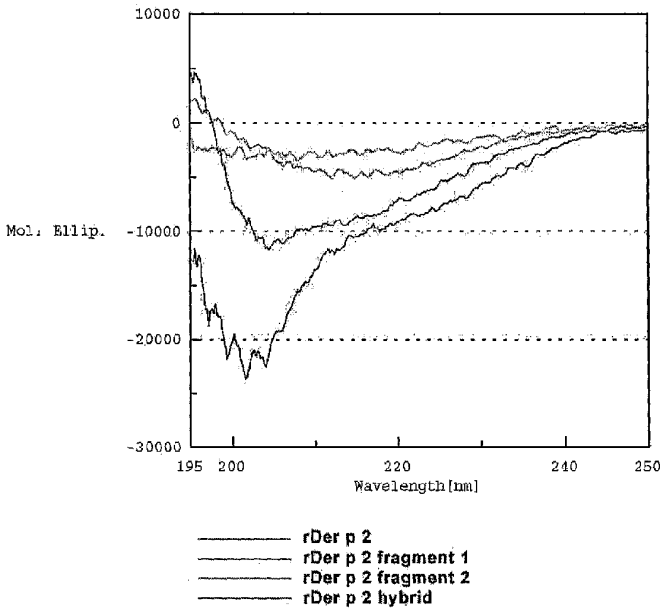
10000
0
-10000
-20000
-30000
Mol. Ellip.
195
200
220
240
250
Wavelength[nm]
rDer p 2
rDer p 2 fragment 1
rDer p 2 fragment 2
rDer p 2 hybrid

Fig. 11

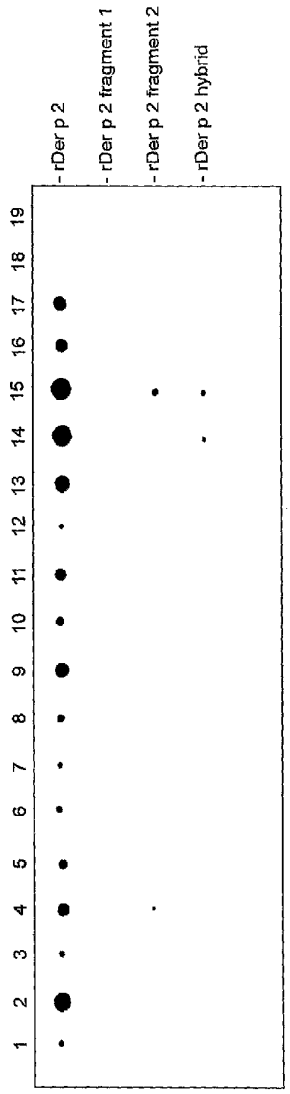


Figure 3

Fig. 12

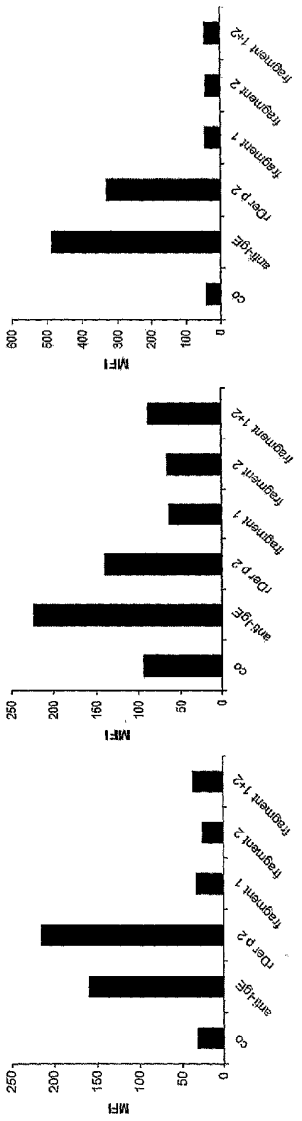


Figure 4a

Fig. 13

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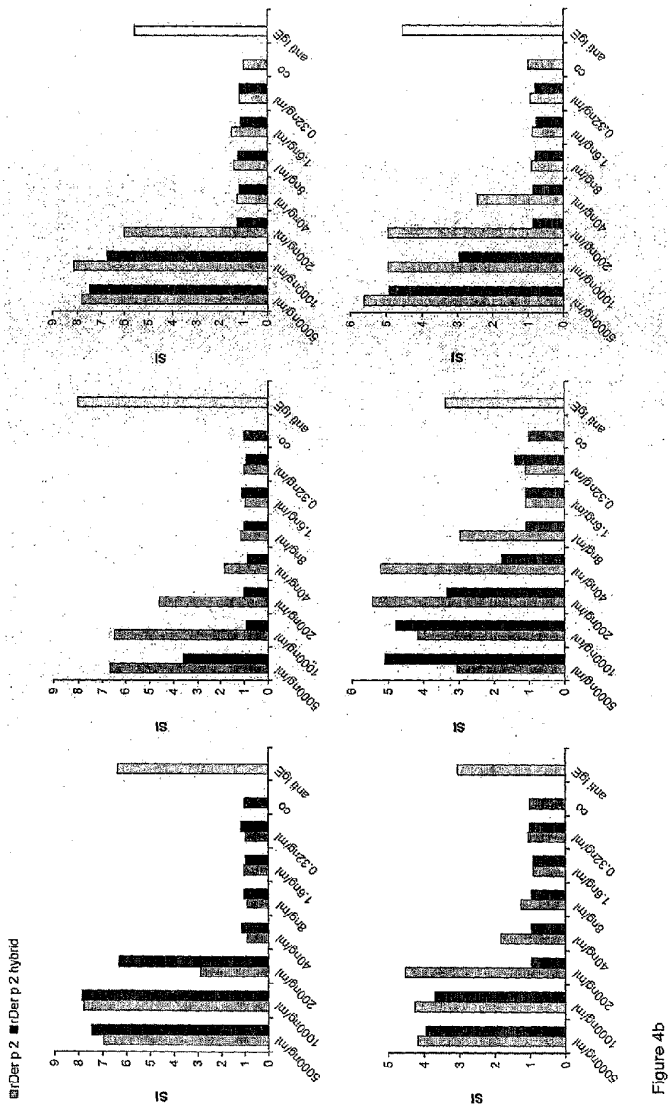


Figure 4b

Fig. 14

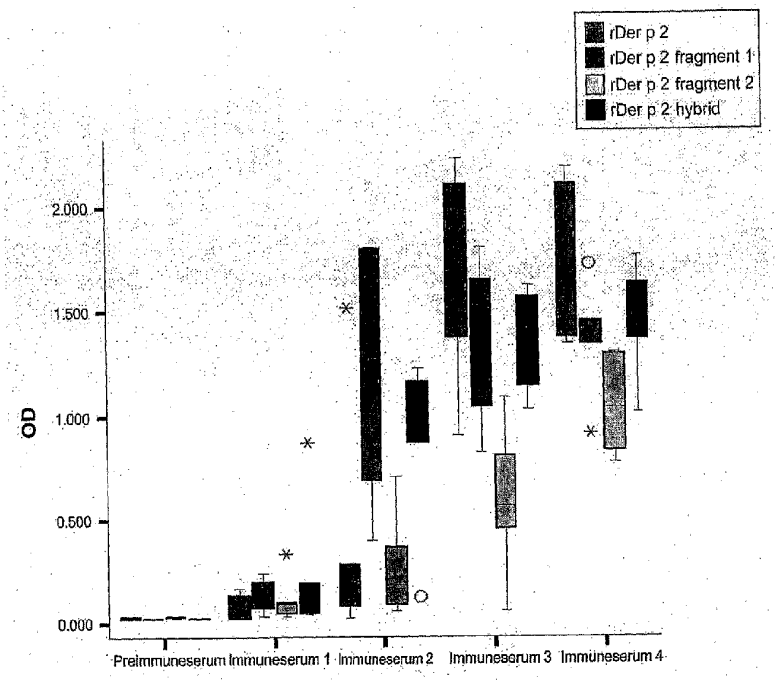
rDer p 2
rDer p 2 fragment 1
rDer p 2 fragment 2
rDer p 2 hybrid
OD
2.000
1.500
1.000
0.500
0.000
Preimmuneserum
Immuneserum 1
Immuneserum 2
Immuneserum 3
Immuneserum 4

Fig. 15

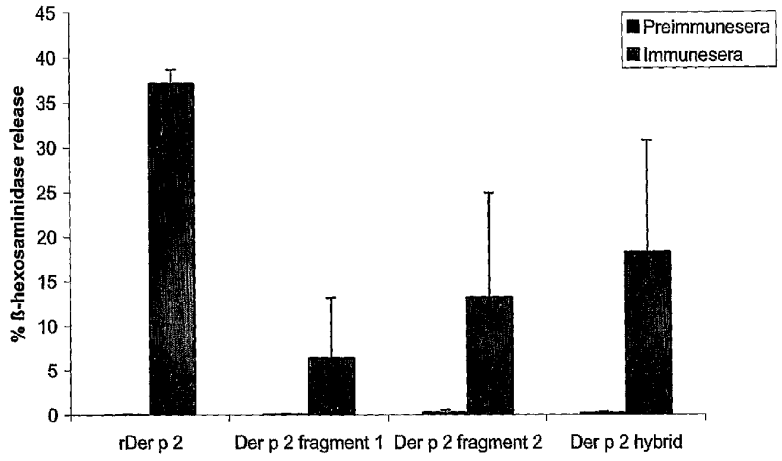
45
40
35
30
25
20
15
10
5
0
% ß-hexosaminidase release
Preimmunesera
Immunesera
rDer p 2
Der p 2 fragment 1
Der p 2 fragment 2
Der p 2 hybrid

Fig. 16

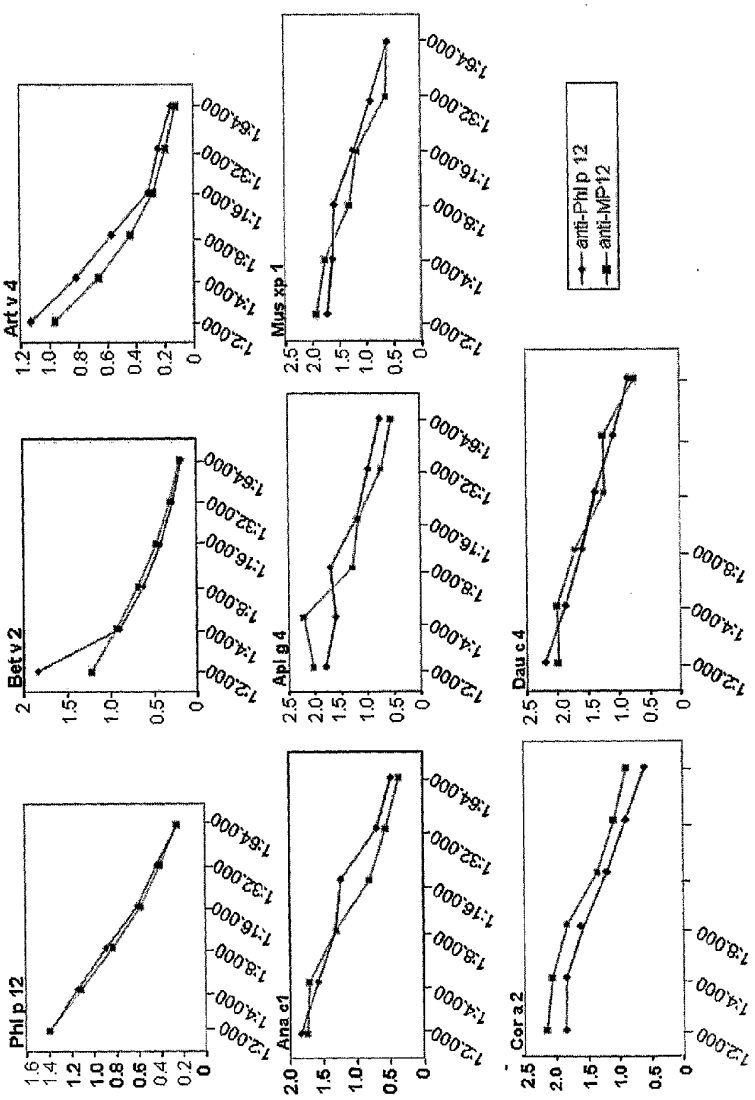
anti-Phl p 12
anti-MP12
Art v 4
Mus xp 1
Bet v 2
Api g 4
Dau c 4
Phl p 12
Ana c1
Cor a 2
1:2,000
1:4,000
1:8,000
1:16,000
1:32,000
1:64,000

Fig. 17