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(54) **Titre : VARIANTES DES ALLERGENES DU GROUPE 1 ISSUS DE POACEAE PRESENTANT UNE ALLERGENICITE REDUITE
ET UNE REACTIVITE AUX LYMPHOCYTES T CONSERVEE**
(54) **Title: VARIANTS OF GROUP 1 ALLERGENS FROM POACEAE HAVING REDUCED ALLERGENICITY AND MAINTAINED T-
CELL REACTIVITY**

(57) **Abrégé/Abstract:**

The invention relates to the production and use of variants of group 1 allergens of Poaceae (sweet-grass), which are characterised by a reduced IgE reactivity compared to the known wild-type allergens and a largely maintained reactivity to T-lymphocytes. Said hypoallergenic allergen variants can be used for the specific immunotherapy (hyposensitivity) of patients suffering from grass pollen allergies or for the preventive immunotherapy of grass pollen allergies.



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ABSTRACT

The invention relates to the production and use of variants of group 1 allergens of *Poaceae* (sweet-grass), which are characterised by a reduced IgE reactivity compared to the known wild-type allergens and a largely maintained reactivity to T-lymphocytes. Said hypoallergenic allergen variants can be used for the specific immunotherapy (hyposensitivity) of patients suffering from grass pollen allergies or for the preventive immunotherapy of grass pollen allergies.

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Variants of group 1 allergens from *Poaceae* having reduced allergenicity and maintained T-cell reactivity

5 The present invention relates to the preparation and use of variants of the group 1 allergens of the *Poaceae* (sweet grasses) which are characterised by reduced IgE reactivity compared with the known wild-type allergens and at the same time by substantially maintained reactivity with T-lymphocytes. These hypoallergenic allergen variants can be employed for the specific immunotherapy (hyposensitisation) of patients having grass pollen allergy or for the preventive immunotherapy of grass pollen allergies

10 A preferred embodiment of the invention relates to variants of the major allergen Phl p 1 from the pollen of timothy grass (*Phleum pratense*).

15 Background of the invention

Type 1 allergies have worldwide importance. Up to 20% of the population in industrialised countries suffer from complaints such as allergic rhinitis, conjunctivitis or bronchial asthma. These allergies are caused by allergens

20 present in the air (aeroallergens) which are liberated by sources of various origin, such as plant pollen, mites, cats or dogs. Up to 40% of these type 1 allergy sufferers in turn exhibit specific IgE reactivity with grass pollen allergens (Freidhoff et al., 1986, J. Allergy Clin. Immunol. 78: 1190-2002).

25 The substances which trigger type 1 allergy are proteins, glycoproteins or polypeptides. After uptake via the mucous membranes, these allergens react with the IgE molecules bound to the surface of mast cells in sensitised persons. Crosslinking of two IgE molecules with one another by an allergen results in the release of mediators (for example histamine, prostaglandins) and cytokines by the effector cell and thus in the corresponding clinical

30 symptoms.

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

5 In the case of timothy grass (*Phleum pratense*), Phl p 1 (Petersen et al., 1993, J. Allergy Clin. Immunol. 92: 789-796), Phl p 5 (Matthiesen and Löwenstein, 1991, Clin. Exp. Allergy 21: 297-307; Petersen et al., 1992, Int. Arch. Allergy Immunol. 98: 105-109), Phl p 6 (Petersen et al., 1995, Int. Arch. Allergy Immunol. 108, 49-54). Phl p 2/3 (Dolecek et al., 1993, FEBS 335 (3), 299-304), Phl p 4 (Haavik et al., 1985, Int. Arch. Allergy Appl. Immunol. 78: 260-268; Valenta et al., 1992, Int. Arch. Allergy Immunol. 97: 10 287-294, Fischer et al., 1996, J. Allergy Clin. Immunol. 98: 189-198) and Phl p 13 (Suck et al., 2000, Clin. Exp. Allergy 30: 324-332; Suck et al., 2000, Clin. Exp. Allergy 30: 1395-1402) have hitherto been identified as major allergens.

15 The dominant major allergens of timothy grass (*Phleum pratense*) are Phl p 1 and Phl p 5. Since the major allergens of the grasses from the *Poaceae* family are highly homologous with one another and consequently have very similar biochemical and immunological properties, these related proteins are grouped together as group 1 and group 5 allergens.

20 The group 1 allergens react with the IgE antibodies in more than 95% of grass pollen allergy sufferers and are thus the dominant major allergens of grass pollen.

25 The group 1 allergens are glycoproteins having a molecular weight of about 32 kDa and are localised in the cytoplasm of the pollen grains. Both contact of the pollen grains with the mucous membrane of the upper respiratory tract and also moistening of the pollen grains by rain lead to rapid release of these allergens. The rapid release of the group 1 allergens, also in the form of sub-cellular microparticles, enables penetration into the lower respi-
30 ratory tract, which can result in triggering of severe asthma attacks.

The cDNAs of group 1 allergens from *Phleum pratense* (Laffer et al., 1994, J. Allergy Clin. Immunol. 94: 689-698), *Lolium perenne* (Perez et al., 1990, J. Biol. Chem. 265: 16210-16250), *Holcus lanatus* (Schramm et al., 1997, J. Allergy Clin. Immunol. 1999:781-787), *Poa pratensis* (Sturaro u. Viotti, 1998, NCBI GenBank, Acc. No. AJ 131850), *Cynodon dactylon* (Smith et al., 1996, J. Allergy Clin. Immunol. 98 : 331-343), *Phalaris aquatica* (Suphioglu et al., 1995, Clin. Exp. Allergy 25 : 853-865) and *Oryza sativa* (Xu et al., 1995, Gene 164: 255-259) have been identified.

In addition to these first descriptions of the sequence, further group 1 allergen sequences which differ from the original sequences in individual positions have been published in databases. Such isoforms are also known for other grass pollen allergens.

Owing to their homology, the group 1 allergens of the sweet grasses (*Poaceae*) have high cross-reactivity with human IgE antibodies (Laffer et al., 1996, Mol. Immunology 33: 417-426). This immunological cross-reactivity is based on a very similar amino acid sequence, as shown by a sequence comparison of Phl p 1, the group 1 allergen of timothy grass (*Phleum pratense*), with group 1 molecules from selected species in Figure 1.

Homologous sequence regions in the other group 1 allergens of the *Poaceae* exist both for the sequence regions of the Phl p 1 amino acid sequence deletions described here in the construction of hypoallergenic variants and also for the flanking sequence regions thereof. Furthermore, both the number and also the surrounding sequence regions of the cysteines of the group 1 allergens of the *Poaceae* are preserved. Owing to sequence homologies, the group 1 allergens of the *Poaceae* are classified in the protein family of the β -expansins (Cosgrove D.J., 2000 Nature 407: 321-6).

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

A greater degree of therapy optimisation would be possible with allergens prepared by recombinant methods. Defined cocktails of high-purity allergens prepared by recombinant methods, if desired matched to the individual sensitisation patterns of patients, could supersede extracts from natural allergen sources since the latter, in addition to the various allergens, contain a relatively large number of immunogenic, but non-allergenic accompanying proteins.

Realistic perspectives which may result in safe hyposensitisation with recombinant expression products are offered by specifically mutated recombinant allergens in which IgE epitopes are specifically deleted without impairing the T-cell epitopes which are essential for the therapy (Schramm et al., 1999, *J. Immunol.* 162: 2406-2414).

A different concept for hyposensitisation is based on the fact that a protective immune response is induced, in particular, by IgG4 antibodies with a blocking action. In accordance with this hypothesis, recombinant Phl p 1 fragments have been described which are said to be suitable for induction of a protective IgG4 response (Ball et al., 1999, *FASEB J.* 13:1277-1290).

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This concept is completely different from the concept of hypoallergenic allergen variants having reduced IgE reactivity and maintained T-cell reactivity.

Another possibility for influencing the disturbed T helper cell balance in allergy sufferers by therapeutic methods is treatment with expressible DNA which encodes for the relevant allergens (immunotherapeutic DNA vaccination). Experimental confirmation of the allergen-specific effect on the immune response has been obtained in rodents by injection of allergen-encoding DNA (Hsu et al., 1996, Nature Medicine 2 (5): 540-544).

The object on which the present invention is based consisted in the provision of novel variants of the group 1 allergens of the *Poaceae* at the protein and DNA level which are distinguished by reduced IgE activity with substantial maintenance of the T-cell reactivity and are therefore suitable for curative and preventive specific immunotherapy and immunotherapeutic DNA vaccination.

In one aspect, the invention provides allergen variants of *Phleum pratense* Phl p 1 polypeptide which are characterized by reduced IgE reactivity compared to the known wild-type allergen and by substantially maintained reactivity of T-lymphocytes, wherein the cysteines at amino acid positions 41, 57, 69, 72, 77, 83 and 139 corresponding to a mature Phl p 1 protein are missing or are substituted by another amino acid and which comprises a deletion of at least one region or a combination of regions selected from the group consisting of the amino acid segments 1-6, 1-30, 92-104, 115-119, 175-185 and 213-220 of the primary sequence of the mature Phl p 1 protein.

In another aspect, the invention provides DNA molecules encoding for allergen variants as described above.

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In another aspect, the invention provides recombinant expression vector containing a DNA molecule as described above functionally connected to an expression control sequence.

5 In another aspect, the invention provides process for the preparation of an allergen variant as described above by cultivation of a host organism transformed by means of a DNA molecule encoding for allergen variants as defined above, or by means of an expression vector containing said DNA molecule and functionally connected to an expression control sequence, and isolation of the corresponding allergen variant from the culture.

10 In another aspect, the invention provides pharmaceutical composition comprising at least one allergen variant as described above and active compounds and/or adjuvants for the preventive and therapeutic treatment of allergies in the triggering of which group 1 allergens of *Poaceae* species are involved.

15 In another aspect, the invention provides use of at least one allergen variant as described above for the preparation of a medicament for the prevention and therapy of allergies in the triggering of which group 1 allergens from *Poaceae* species are involved.

20 In another aspect, the invention provides DNA molecules as described above as medicament, for use in the treatment of allergies induced by group 1 allergens of the *Poaceae*.

In another aspect, the invention provides recombinant expression vector as described above as medicament, for use in the treatment of allergies induced by group 1 allergens of the *Poaceae*.

25 In another aspect, the invention provides pharmaceutical composition comprising at least one DNA molecule as described above or the expression vector as described above and active compounds and/or adjuvants for the immunotherapeutic DNA

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vaccination of patients having allergies in the triggering of which group 1 allergens of the *Poaceae* are involved and/or for the prevention of such allergies.

In another aspect, the invention provides use of at least one DNA molecule as described above or the expression vector as described above for the preparation of a medicament for the immunotherapeutic DNA vaccination of patients having allergies in the triggering of which group 1 allergens of the *Poaceae* are involved and/or for the prevention of such allergies.

Figures

Figure 1: Alignment of Phl p 1-homologous amino acid sequences (sequences of mature proteins deduced from cDNA sequences) of *Poaceae* species: *Poa pratensis* (Poa p), *Holcus lanatus* (Hol l), *Lolium perenne* (Lol p), *Cynodon dactylon* (Cyn d), *Oryza sativa* (Ory s) and *Phalaris aquatica* (Pha a), protein sequences deduced from cDNA sequences from the "Gen-Bank" database of the National Center for Biotechnology Information (NCBI, Bethesda, USA), numbering: amino acid positions of mature proteins, highlighted by underling: amino acids which are different from the Phl p 1 sequence, black box: cysteines

Figure 2: Alignment of amino acid sequences of processed Phl p1 wild-type protein and the variant Phl p1 NoCys, Phl p1 Wt (wild type): protein sequence deduced from cDNA sequence ("GenBank" database entry Z27090 of the National Center for Biotechnology Information (NCBI), Bethesda, USA), numbering: amino acid positions of the mature protein, outlined in black: amino acid substitutions of cysteine by serine in protein Phl p 1 NoCys

Figure 3: Alignment of amino acid sequences of the hypoallergenic variants Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220, depicted by way of example, numbering: amino acid positions, highlighted by underlining: deletions

Figure 4: SDS-PAGE and identity checking of the recombinant variants Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220

A: SDS-PAGE

B: Western blot with aPhl p 1 antibodies (Allergopharma)

1. Marker proteins
2. nPhl p 1*
3. rPhl p 1 Wt (- His-tag)*
4. Marker proteins
5. Phl p 1 NoCys (+ His-tag)
6. Phl p 1 NoCys Δ 213-220 (+ His-tag)
7. Phl p 1 NoCys Δ 1-6, 115-119, 213-220 (+ His-tag)
8. Marker proteins

* Samples reduced (dithiotreitol)

Figure 5: Strip test for checking the IgE binding capacity of Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220 under non-denaturing conditions

- 1) rPhl p 1 Wt
- 2) Phl p 1 NoCys
- 3) Phl p 1 NoCys D213-220
- 4) Phl p 1 NoCys D1-6, 115-119, 213-220
- 5) rPhl p 1 Wt

TP: total protein colouring

P: sera of clinically defined grass pollen allergy sufferers

Figure 6: Determination of the reduced IgE reactivity of Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220 by means of the EAST inhibition test with four representative sera from grass pollen allergy sufferers (P)

Figure 7: Determination of the hypoallergenicity of Phl p 1 NoCys by means of the basophil activation test with basophils from four grass pollen allergy sufferers (P)

Figure 8: Determination of the hypoallergenicity of Phl p 1 NoCys Δ 213-220 by means of the basophil activation test with basophils from four grass pollen allergy sufferers (P)

Figure 9: Determination of the hypoallergenicity of Phl p 1 NoCys Δ 1-6, 115-119, 213-220 by means of the basophil activation test with basophils from four grass pollen allergy sufferers (P)

The work which led to the variants found was carried out using Phl p 1 as model allergen. It becomes clear from the sequence alignments shown in Fig. 1 that, owing to the high homology within group 1, the same results would have been obtained if the starting point had been another group 1 allergen.

Thus, it must also be assumed that the results given above and below can also be applied to Sec c 1 from *Secale cereale* or would have been obtained using Sec c 1, although the sequence is still unknown for this group 1 allergen.

5

The present invention therefore relates to variants of the group 1 allergens of the *Poaceae* which are characterised by reduced IgE reactivity compared with the known wild-type allergens and by maintained reactivity with T-lymphocytes. These group 1 allergens are preferably Phl p 1, Poa p 1, Hol p 1, Lol p 1, Cyn d 1, Ory s 1 and Pha a 1 from *Phleum pratense*, *Lolium perenne*, *Poa pratensis*, *Holcus lanatus*, *Cynodon dactylon*, *Oryza sativa* and *Phalaris aquatica*. Greater preference is given to Phl p 1, Poa p 1, Hol p 1, Lol p 1 or Pha a 1 and very particular preference is given to Phl p 1.

10

The starting point for the construction of the hypoallergenic variants of the group 1 allergens is the cDNA of wild-type Phl p 1, which was isolated with the aid of specific primers by polymerase chain reaction (PCR) from the total cDNA from pollen of *Phleum pratense* ("GenBank" entry Z27090; NCBI, Bethesda, USA) (SEQ ID NO 1).

15

The amino acid sequence SEQ ID NO 2 was deduced from the cDNA sequence of wild-type Phl p 1.

20

Phl p 1, which consists of 240 amino acids and is glycosylated in the natural form, is – like all group 1 allergens (see Fig. 1) – characterised by the existence of seven cysteines in the mature molecule. With the exception of Cyn d 1 and Ory s 1, these amino acid positions have the numbers 41, 57, 69, 72, 77, 83 and 139 in all group 1 allergens (Petersen et al., 1995, J. Allergy Clin. Immunol 95: 987-994).

25

Phl p 1 has been expressed in *E. coli* as non-glycosylated protein. The recombinant wild-type protein (rPhl p 1 wt) react with IgE antibodies from grass pollen allergy sufferers which have reactivity with natural purified Phl p 1 (nPhl p 1) (Petersen et al., 1998, Clin. Exp. Allergy 28: 315-321).

30

Detailed description of the invention

Preparation and characterisation of hypoallergenic Phl p 1 variants

5 Starting from the rPhl p 1 wt cDNA described, recombinant variants of Phl p 1 modified by genetic engineering were prepared.

The amino acid sequence of the recombinant variant Phl p 1 NoCys (SEQ ID NO 4) has seven serine residues instead of the seven cysteines occurring in the wild type (Fig. 2). The variant Phl p 1 NoCys served as starting point for the construction of various deletion mutants. In these, in each case individual sections having a length of 15 to 90 bp or combinations of these sections of the cDNA encoding for Phl p 1 NoCys have been deleted, resulting in corresponding deletions of amino acids 1-6, 1-30, 92-104, 115-119, 175-185 and 213-220 in the polypeptide chains of the proteins expressed in *E. coli*: Phl p 1 NoCys Δ 1-6 (SEQ ID NO 5 and 6), Phl p 1 NoCys Δ 1-30 (SEQ ID NO 7 and 8), Phl p 1 NoCys Δ 92-104 (SEQ ID NO 9 and 10), Phl p 1 NoCys Δ 115-119 (SEQ ID NO 11 and 12), Phl p 1 NoCys Δ 175-185 (SEQ ID NO 13 and 14), Phl p 1 NoCys Δ 213-220 (SEQ ID NO 15 and 16), as well as Phl p 1 NoCys Δ 1-6, 115-119, 213-220 (SEQ ID NO 17 and 18).

The recombinant proteins were expressed as histidine fusion proteins in *Escherichia coli*. The immunological characterisation was carried out with fusion proteins of this type.

Firstly, after immobilisation on a nitrocellulose membrane, the recombinant variants were investigated for the ability to be bound by IgE antibodies of a representative serum pool and by IgE antibodies of individual sera from grass pollen allergy sufferers (strip test). In this method, reduced binding of IgE antibodies to Phl p 1 NoCys was surprisingly observed. This result was

confirmed by an IgE inhibition test (EAST), in which the IgE binding capacity of an unimmobilised protein to IgE antibodies in solution is investigated.

5 The present invention therefore relates, in particular, to group 1 allergen variants in which the cysteines at amino acid positions 41, 57, 69, 72, 77, 83 and 139 corresponding to the mature Phl p 1 protein are removed or replaced by another amino acid. Particular preference is given here to corresponding variants of Phl p 1, Poa p 1, Hol p 1, Lol p 1 or Pha a 1, in particular of Phl p 1.

10 Reduced binding of IgE antibodies is already obtained if at least two of the 7 cysteines are removed without replacement or replaced by another amino acid. Preferably, however, all 7 cysteines are replaced by serine.

15 The effects of the reduced IgE binding capacity of Phl p 1 NoCys on the functional action during the crosslinking of membrane-bound IgE of the effector cells and activation thereof *in vitro* were investigated by means of an activation test of basophilic granulocytes from grass pollen allergy sufferers. Phl p 1 NoCys showed significantly lower activation of basophilic
20 granulocytes here compared with rPhl p 1 wt and thus functionally reduced allergenicity.

25 The various deletion mutants prepared on the basis of Phl p 1 NoCys were investigated with respect to IgE binding capacity (strip test, EAST) and functional action (basophil activation) by the same method. Surprisingly, the deletion mutants exhibited particularly strong hypoallergenic properties.

30 The present invention therefore furthermore relates to group 1 allergen variants in which – optionally in addition to the above-described variants with removed or replaced Cys – at least one region or a combination of regions which correspond to amino acids 1-6, 1-30, 92-104, 115-119, 175-

185 and 213-220 of the primary sequence of the mature Phl p 1 protein are missing compared with the wild-type allergen.

Particular preference is given here to the corresponding deletion mutants of the group 1 allergens Phl p 1, Poa p 1, Hol p 1, Lol p 1 and Pha a 1. Very
5 particular preference is given to the corresponding Phl p 1 variants.

Particular preference is given, and the invention therefore likewise relates, to the group 1 allergen variants in which exclusively amino acids 213-220 or simultaneously amino acids 1-6 and 115-119 corresponding to the mature
10 Phl p 1 sequence are missing. Greater preference is again given here to the allergens Phl p 1, Poa p 1, Hol p 1, Lol p 1 and Pha a 1, where Phl p 1 is very particularly preferred.

The T-cell reactivity of the hypoallergenic Phl p 1 variants which forms the
15 basis for the efficacy of specific immunotherapy was checked *in vitro* by a proliferation test with Phl p 1-specific T-lymphocytes from grass pollen allergy sufferers. The modified allergens showed substantially maintained T-cell reactivity, which enables immunotherapeutic use of the hypoallergenic Phl p 1 variants.

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

25 Further modifications at different positions – for example in order to increase the hypoallergenicity – are of course also possible beyond the variations of group 1 allergens described in the present patent application. These modifications can be, for example, amino acid insertions, deletions and exchanges, cleavage of the protein into fragments and fusion of the
30 protein or fragments thereof with other proteins or peptides. The invention furthermore relates to a DNA molecule, encoding for an allergen variant described above, a recombinant expression vector containing this DNA

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

5

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

10

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

15

The present invention furthermore relates to pharmaceutical compositions comprising at least one of these allergen variants or a corresponding DNA molecule or a corresponding expression vector and optionally further active compounds and/or adjuvants for the treatment of allergies in the triggering of which group 1 allergens of the *Poaceae* are involved, or for the immunotherapeutic vaccination of patients having allergies in the triggering of which group 1 allergens of the *Poaceae* are involved, and/or for the prevention of such allergies.

20

25

If pharmaceutical compositions are of the second type (comprising at least one DNA molecule or an expression vector), these compositions preferably furthermore comprise aluminium hydroxide, an immunostimulatory CpG-containing oligonucleotide or a combination of the two as adjuvants.

30

For the purposes of this invention, pharmaceutical compositions can be used as therapeutic agents in human or veterinary medicine. Suitable excipients are organic or inorganic substances which are suitable for parenteral administration and do not react with group 1 allergen variants according to the invention. Suitable for parenteral use are, in particular,

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

Furthermore, delayed-release preparations can be obtained by appropriate
10 formulation of the allergen variants according to the invention, for example by adsorption onto aluminium hydroxide.

Finally, the present invention relates to the use of at least one allergen variant according to the invention or of a DNA molecule according to the
15 invention or of an expression vector according to the invention for the preparation of a medicament for the treatment of allergies in the triggering of which group 1 allergens of the *Poaceae* are involved or for the immunotherapeutic vaccination of patients having allergies in the triggering of which group 1 allergens of the *Poaceae* are involved and/or for the prevention of
20 such allergies.

The preparation of variants Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220 (Fig. 3) and the immunological
25 characterisation thereof is described below by way of example for the hypoallergenic Phl p 1 variants with the genetic engineering modifications described above.

Expression and purification of recombinant Phl p 1 variants

30 The recombinant proteins were expressed as histidine fusion proteins (expression vector pProExHT; Invitrogen, Carlsbad, USA) in *Escherichia*

coli (strain JM109). rPhl p 1 wt and the variants were firstly purified by specific binding of the N-terminal histidine residues to an Ni^{2+} chelate matrix (immobilised metal ion affinity chromatography, IMAC) and subsequently by preparative gel filtration (size exclusion chromatography, SEC). The purity of the eluted proteins was monitored by SDS-PAGE and analytical SEC (Fig. 4a). The identity of the purified proteins was demonstrated by binding of a Phl p 1-specific monoclonal antibody (Fig. 4b).

10 *Demonstration of reduced IgE binding of recombinant Phl p 1 variants*

A simple test method for the determination of the IgE reactivity of specific IgE from sera from allergy sufferers on membrane-bound test proteins is the strip test.

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

25 The results of the strip test for Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220 using sera from individual grass pollen allergy sufferers are depicted here by way of example for the modified Phl p 1 molecules described (Fig. 5).

Only sera from allergy sufferers having a strong IgE titre against natural Phl p 1 were used. The IgE antibodies from these patients likewise react with the recombinant equivalent rPhl p 1 wt.

It becomes clear that the Phl p 1-specific IgE antibodies of all patient sera bind the recombinant variant Phl p 1 NoCys to a reduced extent, but not the wild-type allergen Phl p 1.

5 An even greater reduction in the IgE binding capacity is achieved by the additional removal of certain sequence sections, which is depicted with reference to the variant Phl p 1 NoCys Δ 213-220. The variant Phl p 1 NoCys Δ 213-220 shows a very greatly reduced IgE binding capacity with all tested sera from allergy sufferers compared with the unmodified recombi-
10 nant wild-type protein. A further reduction in the IgE binding capacity of Phl p 1 to IgE antibodies from certain sera can be achieved by combination of a number of deletions, which is evident from the test result for the variant Phl p 1 NoCys Δ 1-6, 115-119, 213-220 with grass pollen allergy sufferer serum P14 (Fig. 5).

15 It thus becomes clear that both substitution of cysteines and also deletion of specific sequence sections reduces the IgE binding capacity of the Phl p 1 molecule.

20 In contrast to the strip test, the EAST inhibition test (enzyme allergosorbent test) enables the investigation of allergen/IgE interactions in solution, enabling interfering masking of epitopes of the test substance to be basically excluded by immobilisation onto the membrane.

The EAST inhibition test is carried out as follows. Microtitre plates are coated with allergens, here nPhl p 1. After removal of the unbound allergen
25 molecules by washing, the plate is blocked using bovine serum albumin in order to prevent later nonspecific binding. IgE antibodies from allergy sufferers, as a representative pool of individual sera (serum pool) or as individual serum, is incubated in suitable dilution with the allergen-coated microtitre plates. The amount of allergen-bound IgE antibodies is quantified
30 photometrically via an anti-hIgE/alkaline phosphatase conjugate by reaction of a substrate to give a coloured end product.

The binding of the IgE antibodies is inhibited substance-specifically by a soluble allergen or the substance to be tested (recombinant modified allergen) as a function of the concentration.

5

The test results for Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220 are shown here by way of example for the modified Phl p 1 molecules described compared with the reference molecule nPhl p 1.

10

The representative IgE inhibition tests shown in Figure 6, with four individual sera from grass pollen allergy sufferers, show that only about 20-50% of the maximum inhibitory action of the unmodified natural allergen nPhl p1 was achieved, even with high concentrations of the variant Phl p 1 NoCys (up to 5 μ g/ml). The lower maximum inhibitory action indicates a loss of IgE epitopes.

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The curves for the variants Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220 demonstrate an even lower IgE binding capacity of these Phl p 1 variants. An inhibitory action could not be detected with individual variation or only to a very small extent (0-20% of the maximum inhibitory action).

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In agreement with the result of the strip test, it can thus be confirmed that the insertion of additional specific deletions further reduces the IgE binding capacity of Phl p 1.

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Determination of the hypoallergenicity of recombinant Phl p 1 variants by the basophil activation test

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The functional reduction in allergenicity was determined *in vitro* by means of a basophil activation test. For the basophil activation test, heparinised

whole blood from grass pollen allergy sufferers is incubated with various concentrations of the test substances. The allergenic substances are specifically bound by the FcεRI-bound IgE antibodies of the basophils and result in crosslinking of the FcεRI molecules.

5 This allergen-induced, IgE-promoted FcεRI crosslinking results in activation of the basophils. The activation is the first step in the allergic reaction of these effector cells. The subsequent signal transduction results in degranulation of the effector cells and thus in triggering of the allergic reactions *in vivo*.

10 *In vitro*, the allergen-induced activation of basophilic granulocytes can be determined by quantification of the expression of a surface protein (CD203c) which is coupled to signal transduction of the IgE receptor crosslinking (Kahlert et al., 2003, Clin. Exp. Allergy 33: 1266-72). The number of
15 expressed CD203c proteins on a cell and the percentage of activated cells of a cell pool is measured with high sensitivity via the binding of a fluorescence-labelled monoclonal antibody to the surface marker and subsequent analysis by fluorescence-activated flow cytometry. The reference substances employed here was purified natural Phl p 1 (nPhl p 1) in parallel
20 with the test substances. The results for Phl p 1 NoCys, Phl p 1 NoCys Δ213-220 and Phl p 1 NoCys Δ1-6, 115-119, 213-220 are shown here by way of example for the modified Phl p 1 molecules described.

25 Representative test results for the variant Phl p 1 NoCys with basophils from four clinically defined allergy sufferers are shown as curves in Figure 7. The reduction in the allergenic efficacy of the variant Phl p 1 NoCys relative to wild-type nPhl p 1 becomes clear through the shift in the activation curves.

30 In accordance with the results from the strip test and the IgE inhibition test, the test results for the variants Phl p 1 NoCys Δ213-220 and Phl p 1 NoCys Δ1-6, 115-119, 213-220 indicate an even greater reduction in the relative

allergenic efficacy, as shown in Figure 8 and 9 with reference to representative curves.

Whereas a maximum proportion of basophils has already been activated by the natural allergen in a concentration range of the test substances of 100-1000 pM, no or only very little basophil activation was detected on use of the modified allergens Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220.

The allergenic efficacy of variant Phl p 1 NoCys Δ 213-220 was, as can be calculated by the A50 values of the curves, reduced ~ 100-1000 fold and that of variant Phl p 1 NoCys Δ 1-6, 115-119, 213-220 was reduced more than 1000 fold compared with the reference nPhl p 1 (A50: allergen concentration at 50% of the maximum number of activated basophils).

T-cell reactivity of the hypoallergenic Phl p 1 variants

T helper lymphocytes react with allergen peptide fragments (about 12-25 amino acids) which are formed by enzymatic degradation in antigen-presenting cells (APCs) and are presented to the T-cells after inclusion of the suitable peptides into the individual MHC class II molecules at the surface of the APCs. This allergen-specific activation of the T helper lymphocytes is the prerequisite for proliferation and functional differentiation (TH1 and TH2). Influencing of the allergen-specific T-lymphocytes by treatment with allergen or an allergen derivative during hyposensitisation is regarded as the key for the therapeutic efficacy.

In order to investigate the T-cell reactivity, oligoclonal T-cell lines from grass pollen allergy sufferers are established by conventional methods with stimulation by nPhl p 1 or rPhl p 1 wt molecules. In a proliferation test, the various T-cell lines were stimulated with the reference allergens nPhl p 1 and rPhl p 1 wt and the modified recombinant Phl p 1 variants. The prolifer-

eration rate was determined by the incorporation of [^3H]-thymidine by conventional methods.

5 The results of the proliferation test of Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220 are shown here by way of example for the modified Phl p 1 molecules described.

10 The results with T-cell lines from eight grass pollen allergy sufferers depicted in Table 1 show that it was possible to stimulate the T-lymphocytes to proliferation by the recombinant allergen variants. The T-cell reactivity of Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220 is only reduced slightly compared with the unmodified natural and recombinant wild-type allergens, which demonstrates the retention of the

15

Table 1: Determination of the T-cell reactivity of Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220 by means of proliferation tests with Phl p 1-reactive T-cell lines (TCLs)

20

25

Donor ²	TCL	nPhl p 1	rPhl p 1 Wt	Stimulation index ¹		
				Phl p 1 NoCys	Phl p 1 NoCys Δ 213-220	Phl p 1 NoCys Δ 1-6, 115-119, 213-220
A	11.16	3.2	4.7	3.7	3.5	3.9
B	21.1	23.5	13.9	12.1	13.0	12.3
C	60.51	3.9	5.7	3.9	4.2	2.0
D	104.8	2.6	4.6	4.1	3.4	3.0
E	10.27	26.4	27.8	32.7	30.8	31.4
F	41.8	2.7	4.9	3.5	3.0	3.1
G	55.74	7.6	8.4	4.9	4.6	4.5
H	57.43	3.1	4.0	3.0	2.6	2.9

30

¹ Calculated from [^3H] measurement values. cpm measurement values of allergen-stimulated cell cultures/cpm measurement values of unstimulated cell cultures

² Donor: clinically defined grass pollen allergy sufferers

Construction of hypoallergenic Phl p 1 variants by genetic engineering

5 Example 1: Phl p 1 NoCys

For the construction of variant Phl p 1 NoCys (SEQ ID NO 3 and 4), six PCR steps were carried out starting from the cDNA of rPhl p 1 wt ("Gen-Bank" entry Z27090; NCBI, Bethesda, USA). The point mutations were
10 introduced using specific PCR primers which contained codons encoding for serine instead of those for cysteine (primer sequences see Table 2).

Step 1 – Preparation of N-terminal DNA fragment "Phl p 1 [C41S, C57S, C69S] (bp 1-212)": a DNA fragment containing mutations C41S, C57S,
15 C69S was generated by amplification of long overlapping oligonucleotides (P 1-63, P 49-111, P 97-158 and P 144-212) by means of PCR.

Step 2 – Preparation of C-terminal DNA fragment "Phl p 1 [C69S, C72S, C77S, C83S] (bp 193-720)": PCR of Phl p 1 wt-cDNA with primers P 193-
20 261 and P 703-720 *HindIII*.

Step 3 – Preparation of the DNA encoding for "Phl p 1 [C41S, C57S, C69S, C72S, C77S, C83S] (bp 1-720)": PCR of overlapping fragments "Phl p 1 [C41S, C57S, C69S] (bp 1-212)" and "Phl p 1 [C69S, C72S, C77S, C83S]
25 (bp 193-720)" with primers P 1-63 and P 703-720 *HindIII*.

Step 4 – Preparation of N-terminal DNA fragment "Phl p 1 [C41S, C57S, C69S, C72S, C77S, C83S, C139S] (bp 1-428)": PCR of the cDNA of
30 "Phl p 1 [C41S, C57S, C69S, C72S, C77S, C83S] (bp 1-720)" with primers P 1-63 and P 406-428 as.

Step 5 – Preparation of C-terminal DNA fragment "Phl p 1 [C139S] (bp 406-720)":

PCR of the cDNA of rPhl p 1 wt with primers P 406-428s and P 703-720 *HindIII*.

5

Step 6 – Preparation of the complete DNA encoding for Phl p 1 NoCys:

PCR of overlapping fragments "Phl p 1 [C41S, C57S, C69S, C72S, C77S, C83S, C139S] (bp 1-428)" and "Phl p 1 [C139S] (bp 406-720) with primers P 1-63 and P 703-720 *HindIII*".

10

The DNA encoding for Phl p 1 NoCys were digested using the restriction enzyme *HindIII* and ligated into the expression vector pProExHT (Invitrogen, Carlsbad, USA) via restriction sites *EheI* and *HindIII* and subsequently sequenced in full.

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Table 2: PCR primers employed for the preparation of Phl p 1 NoCys, Phl p 1 NoCys Δ 213-220 and Phl p 1 NoCys Δ 1-6, 115-119, 213-220

20

Primer	Direction	SEQ ID NO	Sequence (5'→3')
P 1-18 ¹	sense	19	atc ccg aag gtc ccg ccg
P 1-63	sense	20	atc ccg aag gtc ccg ccg ggc ccg aac atc acg gcg acc tac ggc gac aag tgg ctg gac gcg
P 49-111	antisense	21	gtt gtc ctt ggg acc ggc ggc cgt cgg ctt gcc gta cca ggt gct ctt cgc gtc cag cca ctt
P 97-158	sense	22	ggt ccc aag gac aac ggc ggc gcg agc ggg tac aag gac gtg gac aag ccc ccg ttc agc gg
P 144-212	antisense	23	gag ccg ct gcc ccg gcc gga ctt gaa gat ggg ggt gtt gcc gga gcc ggt cat gcc gct gaa cgg ggg c
P 193-261	sense	24	tcc ggc cgg ggc agc ggc tcc tcc ttc gag atc aag agc acc aag ccc gag gcc tcc tcc ggc gag ccc
P 703-720 <i>HindIII</i>	antisense	25	ggt aag ctt tca ctt gga ctc gta ggc ggt
P 406-428 as	antisense	26	tcc ggg tac ttg gac ttg acg cg
P 406-428 s	sense	27	cgc gtc aag tcc aag tac ccg ga
P 22-63 (Δ 1-18)	sense	28	ccg aac atc acg gcg acc tac ggc gac aag tgg ctg gac gcg

30

5	P 250-318	antisense	29	gtc gaa gtg gta cgc ggc gat ggg ctc ctc gtt gtc gtc ggt gat gtg gac cac cac ggg ctc gcc gga
	P 301-384	sense	30	gcc gcg tac cac ttc gac ctc tcc ggc atc gcg ttc (Δ 343-357) ggg tcc gac gag cag aag ctg cgc agc gcc ggc
	P 613-720	antisense	31	ggt aag ctt tca ctt gga ctc gta ggc ggt gtc ggc ctt (Δ 637-660) cca gcc ctc ggg gat gac gtc ctt ggc ctc gcc gcg <i>HindIII</i> gac ggt gaa ggg gcc ctt gag

¹ Numbers indicated: positions of the primers based on the nucleotide sequence of Phl p 1 wild-type protein (without signal peptide; "GenBank" entry Z27090; NCBI, Bethesda, USA). Primer sequences in some cases codon-optimised for *E. coli*.

Example 2: Phl p 1 NoCys Δ 213-220

The DNA sequence encoding for deletion variant Phl p 1 NoCys Δ 213-220 (SEQ ID NO 15 and 16) was generated by means of PCR of the DNA of Phl p 1 NoCys using the 5'-primer P 1-18 and the 3'-primer P 613-720 (Δ 637-660) *HindIII* specifically shortened by the sequence region to be deleted.

The cDNA were digested using the restriction enzyme *HindIII* and ligated into the expression vector pProExHT (Invitrogen, Carlsbad, USA) via restriction sites *EheI* and *HindIII* and subsequently sequenced in full.

Example 3: Construction of Phl p 1 NoCys Δ 1-6, 115-119, 213-220 by genetic engineering

The DNA sequence encoding for deletion variant Phl p 1 NoCys Δ 1-6, 115-119, 213-220 (SEQ ID NO 5 and 6) was generated in three steps by means of PCR using oligonucleotides specifically shortened by the sequence region to be deleted.

Step 1 – Preparation of N-terminal DNA fragment "Phl p 1 NoCys Δ 1-6 (bp 1-300)": PCR of the cDNA of Phl p 1 NoCys with primers P22-63 (Δ 1-18) and P 250-318.

5 Step 2 – Preparation of C-terminal DNA fragment "Phl p 1 NoCys Δ 115-119, 213-220 (bp 283-663)": PCR of Phl p 1 NoCys with primers P 301-384 (Δ 343-357) and P 613-720 (Δ 637-660) *Hind*III.

10 Step 3 – Preparation of the complete DNA encoding for Phl p 1 NoCys Δ 1-6, 115-119, 213-220:
PCR of overlapping fragments "Phl p 1 NoCys Δ 1-6 (bp 1-300)" and "Phl p 1 NoCys Δ 115-119, 213-220 (bp 283-663)" with primers P22-63 (Δ 1-18) and P 703-720 *Hind*III.

15 The DNA encoding for Phl p 1 NoCys Δ 1-6, 115-119, 213-220 was digested using the restriction enzyme *Hind*III and ligated into the expression vector pProExHT (Invitrogen, Carlsbad, USA) via restriction sites *E*heI and *Hind*III and subsequently sequenced in full.

20 The DNA of variants Phl p 1 NoCys Δ 1-6 (SEQ ID NO 5 and 6), Phl p 1 NoCys Δ 1-30 (SEQ ID NO 7 and 8), Phl p 1 NoCys Δ 92-104 (SEQ ID NO 9 and 10), Phl p 1 NoCys Δ 115-119 (SEQ ID NO 11 and 12), Phl p 1 NoCys Δ 175-185 (SEQ ID NO 13 and 14) were prepared, cloned and sequenced correspondingly.

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DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
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CECI EST LE TOME __1__ DE __2__

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JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

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CLAIMS:

1. Allergen variants of *Phleum pratense* Phl p 1 polypeptide which are characterized by reduced IgE reactivity compared to the known wild-type allergen and by substantially maintained reactivity of T-lymphocytes, wherein the cysteines at
5 amino acid positions 41, 57, 69, 72, 77, 83 and 139 corresponding to a mature Phl p 1 protein are missing or are substituted by another amino acid and which comprises a deletion of at least one region or a combination of regions selected from the group consisting of the amino acid segments 1-6, 1-30, 92-104, 115-119, 175-185 and 213-220 of the primary sequence of the mature Phl p 1 protein.
- 10 2. Allergen variants according to Claim 1, wherein the cysteines at amino acid positions 41, 57, 69, 72, 77, 83 and 139 corresponding to the mature Phl p 1 protein are missing.
3. Allergen variants according to Claim 1, wherein the cysteines at amino acid positions 41, 57, 69, 72, 77, 83 and 139 corresponding to the mature Phl p 1
15 protein are substituted by another amino acid.
4. Allergen variants according to Claim 3, wherein the cysteines at amino acid positions 41, 57, 69, 72, 77, 83 and 139 corresponding to the mature Phl p 1 protein are substituted by serine.
5. Allergen variants according to any one of Claims 1-4 in which amino
20 acids 213-220 corresponding to the mature Phl p 1 sequence are missing.
6. Allergen variants according to any one of claims 1, 3 and 4,
wherein the cysteines at amino acid positions 41, 57, 69, 72, 77, 83 and 139 of the mature Phl p 1 protein are replaced by serine and amino acids 1-6, 115-119 and 213-220 are missing.
- 25 7. Allergen variants according to any one of Claims 1-6, wherein said allergen variants have been obtained by recombinant genetic engineering methods.

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8. DNA molecules encoding for allergen variants according to any one of Claims 1-7.
9. Recombinant expression vector containing a DNA molecule according to Claim 8 functionally connected to an expression control sequence.
- 5 10. Process for the preparation of an allergen variant according to any one of Claims 1-7, by cultivation of a host organism transformed by means of a DNA molecule encoding the allergen variant, or by means of an expression vector containing said DNA molecule and functionally connected to an expression control sequence, and isolation of the corresponding allergen variant from the culture.
- 10 11. Allergen variants according to any one of Claims 1-7, for use in the treatment of allergies induced by group 1 allergens of the *Poaceae*.
12. Pharmaceutical composition comprising at least one allergen variant according to any one of Claims 1-7 and active compounds and/or adjuvants for the preventive and therapeutic treatment of allergies in the triggering of which group 1
15 allergens of *Poaceae* species are involved.
13. Use of at least one allergen variant according to any one of Claims 1-7 for the preparation of a medicament for the prevention and therapy of allergies in the triggering of which group 1 allergens from *Poaceae* species are involved.
14. DNA molecules according to Claim 8 as medicament, for use in the
20 treatment of allergies induced by group 1 allergens of the *Poaceae*.
15. Recombinant expression vector according to Claim 9 as medicament, for use in the treatment of allergies induced by group 1 allergens of the *Poaceae*.
16. Pharmaceutical composition comprising at least one DNA molecule according to Claim 8 or the expression vector according to Claim 9, and active
25 compounds and/or adjuvants, for the immunotherapeutic DNA vaccination of patients

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having allergies in the triggering of which group 1 allergens of the *Poaceae* are involved and for the prevention of such allergies.

17. Pharmaceutical composition comprising at least one DNA molecule according to Claim 8 or the expression vector according to Claim 9, and active
5 compounds and/or adjuvants, for the immunotherapeutic DNA vaccination of patients having allergies in the triggering of which group 1 allergens of the *Poaceae* are involved or for the prevention of such allergies.

18. Use of at least one DNA molecule according to Claim 8 or the expression vector according to Claim 9 for the preparation of a medicament for the
10 immunotherapeutic DNA vaccination of patients having allergies in the triggering of which group 1 allergens of the *Poaceae* are involved and/or for the prevention of such allergies.

Figure 1

1	:	I	P	K	V	P	P	G	P	N	I	T	A	T	Y	G	D	K	W	L	D	A	K	S	T	W	Y	G	K	:	P	Phi p 1 Wt GenBank Z27090
1	:	I	A	K	V	P	P	G	P	N	I	T	A	T	Y	G	D	K	W	L	D	A	K	S	T	W	Y	G	K	:	P	Poa p 1 GenBank AJ131850
1	:	I	A	K	V	P	P	G	P	N	I	T	A	T	Y	G	D	K	W	L	D	A	K	S	T	W	Y	G	K	:	P	Hol l 1 GenBank AJ012714
1	:	I	A	K	V	P	P	G	P	N	I	T	A	E	Y	G	D	K	W	L	D	A	K	S	T	W	Y	G	K	:	P	Lol p 1 GenBank M35687
1	A	M	G	D	:	K	P	G	P	N	I	T	A	T	Y	G	D	K	W	L	D	A	K	A	T	E	Y	G	S	D	P	Cyn d 1 GenBank AF177379
1	G	P	P	K	V	P	P	G	P	N	I	T	I	S	Y	G	D	K	W	L	E	A	K	A	T	W	Y	G	A	:	P	Ory s 1 GenBank AF261270.1
1	:	I	A	K	V	P	P	G	P	N	I	T	A	E	Y	G	D	K	W	L	D	A	K	S	T	W	Y	G	K	:	P	Pha a 1 GenBank S80654
30	T	A	A	G	P	K	D	N	G	G	A	C	G	Y	K	D	V	D	K	P	P	F	S	G	M	T	G	C	G	N	T	Phi p 1 Wt GenBank Z27090
30	T	G	A	G	P	K	D	N	G	G	A	C	G	Y	K	D	V	D	K	A	P	F	S	G	M	T	G	C	G	N	T	Poa p 1 GenBank AJ131850
30	T	G	A	G	P	K	D	N	G	G	A	C	G	Y	K	D	V	D	K	P	P	F	S	G	M	T	G	C	G	N	T	Hol l 1 GenBank AJ012714
30	T	G	A	G	P	K	D	N	G	G	A	C	G	Y	K	D	V	D	K	A	P	F	N	G	M	T	G	C	G	N	T	Lol p 1 GenBank M35687
31	R	G	A	A	P	D	D	H	G	G	A	C	G	Y	K	D	V	D	K	A	P	F	D	G	M	T	G	C	G	N	E	Cyn d 1 GenBank AF177379
31	K	G	A	G	P	K	D	N	G	G	A	C	G	Y	K	D	V	D	K	A	P	F	L	G	M	N	S	C	G	N	D	Ory s 1 GenBank AF261270.1
30	T	G	A	G	P	K	D	N	G	G	A	C	G	Y	K	D	V	D	K	A	P	F	N	G	M	T	G	C	G	N	T	Pha a 1 GenBank S80654
61	P	I	F	K	S	G	R	G	C	G	S	C	F	E	I	K	C	T	K	P	E	A	C	S	G	E	P	V	V	V	H	Phi p 1 Wt GenBank Z27090
61	P	I	F	K	S	G	R	G	C	G	S	C	F	E	I	K	C	T	K	P	E	S	C	S	G	E	P	V	L	V	H	Poa p 1 GenBank AJ131850
61	P	I	F	K	S	G	R	G	C	G	S	C	F	E	I	K	C	T	K	P	E	S	C	S	G	E	P	I	V	V	H	Hol l 1 GenBank AJ012714
61	P	I	F	K	D	G	R	G	C	G	S	C	F	E	I	K	C	T	K	P	E	S	C	S	G	E	A	V	I	V	I	Lol p 1 GenBank M35687
62	P	I	F	K	D	G	L	G	C	G	S	C	Y	E	I	K	C	K	E	P	A	E	C	S	G	E	P	V	L	I	K	Cyn d 1 GenBank AF177379
62	P	I	F	K	D	G	K	G	C	G	S	C	F	E	I	K	C	S	K	P	E	A	C	S	D	K	P	A	L	I	H	Ory s 1 GenBank AF261270.1
61	P	I	F	K	D	G	R	G	C	G	S	C	F	E	L	K	C	S	K	P	E	S	C	S	G	E	P	I	I	V	H	Pha a 1 GenBank S80654
92	I	T	D	D	N	E	E	P	I	A	A	Y	H	F	D	L	S	G	I	A	F	G	S	M	A	K	K	G	D	E	Q	Phi p 1 Wt GenBank Z27090
92	I	T	D	D	N	E	E	P	I	A	A	Y	H	F	D	L	S	G	K	A	F	G	A	M	A	K	K	G	E	E	Q	Poa p 1 GenBank AJ131850
92	I	T	D	D	N	E	E	P	I	A	A	Y	H	L	D	L	S	G	K	A	F	G	A	M	A	K	K	G	E	E	Q	Hol l 1 GenBank AJ012714
92	I	T	D	D	N	E	E	P	I	A	P	Y	H	F	D	L	S	G	H	A	F	G	S	M	A	K	K	G	E	E	Q	Lol p 1 GenBank M35687
93	I	T	D	K	N	Y	E	H	I	A	A	Y	H	F	D	L	S	G	K	A	F	G	A	M	A	K	K	G	E	E	D	Cyn d 1 GenBank AF177379
93	V	T	D	M	N	D	E	P	I	A	A	Y	H	F	D	L	S	G	L	A	F	G	A	M	A	K	D	G	K	D	E	Ory s 1 GenBank AF261270.1
92	I	T	D	D	N	E	E	P	I	A	P	Y	H	F	D	L	S	G	H	A	F	G	S	M	A	K	K	G	E	E	E	Pha a 1 GenBank S80654
123	K	L	R	S	A	G	E	V	E	I	Q	F	R	R	V	K	C	K	Y	P	E	G	T	K	V	T	F	H	V	E	K	Phi p 1 Wt GenBank Z27090
123	K	L	R	S	A	G	E	L	E	L	K	F	R	R	V	K	C	E	Y	P	E	G	T	K	V	T	F	H	V	E	K	Poa p 1 GenBank AJ131850
123	K	L	R	S	A	G	E	L	E	L	K	F	R	R	V	K	C	E	Y	P	K	G	T	K	V	T	F	H	V	E	K	Hol l 1 GenBank AJ012714
123	N	V	R	S	A	G	E	L	E	L	Q	F	R	R	V	K	C	K	Y	P	D	D	T	K	P	T	F	H	V	E	K	Lol p 1 GenBank M35687
124	K	L	R	K	A	G	E	L	M	L	Q	F	R	R	V	K	C	E	Y	P	S	D	T	K	I	T	F	H	V	E	K	Cyn d 1 GenBank AF177379
124	E	L	R	K	A	G	I	I	D	I	Q	F	R	R	V	K	C	K	Y	P	A	D	T	K	I	T	F	H	I	E	K	Ory s 1 GenBank AF261270.1
123	N	V	R	G	A	G	E	L	E	L	Q	F	R	R	V	K	C	K	Y	P	D	G	T	K	P	T	F	H	V	E	K	Pha a 1 GenBank S80654
154	G	S	N	P	N	Y	L	A	L	L	V	K	F	V	A	G	D	G	D	V	V	A	V	D	I	K	E	K	G	K	D	Phi p 1 Wt GenBank Z27090
154	G	S	N	P	N	Y	L	A	L	L	V	K	Y	V	I	G	D	G	D	V	V	A	V	D	I	K	E	K	G	K	D	Poa p 1 GenBank AJ131850
154	G	S	N	P	N	Y	L	A	L	L	V	K	Y	V	D	G	D	G	D	V	V	A	V	D	I	K	E	K	G	K	D	Hol l 1 GenBank AJ012714
154	G	S	N	P	N	Y	L	A	I	L	V	K	Y	V	D	G	D	G	D	V	V	A	V	D	I	K	E	K	G	K	D	Lol p 1 GenBank M35687
155	G	S	S	P	N	Y	L	A	L	L	V	K	Y	A	A	G	D	G	N	I	V	G	V	D	I	K	P	K	G	S	D	Cyn d 1 GenBank AF177379
155	A	S	N	P	N	Y	L	A	L	L	V	K	Y	V	A	G	D	G	D	V	V	E	V	E	I	K	E	K	G	S	E	Ory s 1 GenBank AF261270.1
154	G	S	N	P	N	Y	L	A	L	L	V	K	Y	V	D	G	D	G	D	V	V	A	V	D	I	K	E	K	G	K	D	Pha a 1 GenBank S80654
185	K	W	I	A	L	K	E	S	W	G	A	I	W	R	I	D	T	P	E	V	L	K	G	P	F	T	V	R	Y	T	T	Phi p 1 Wt GenBank Z27090
185	K	W	I	E	L	K	E	S	W	G	S	I	W	R	V	D	T	P	D	K	L	I	G	P	F	T	V	R	Y	T	T	Poa p 1 GenBank AJ131850
185	K	W	I	E	L	K	E	S	W	G	A	V	W	R	V	D	T	P	D	K	L	I	G	P	F	T	V	R	Y	T	T	Hol l 1 GenBank AJ012714
185	K	W	I	E	L	K	E	S	W	G	A	V	W	R	I	D	T	P	D	K	L	I	G	P	F	T	V	R	Y	T	T	Lol p 1 GenBank M35687
186	V	F	L	P	M	K	L	S	W	G	A	I	W	R	M	D	P	P	K	P	L	K	G	P	F	T	I	R	L	T	S	Cyn d 1 GenBank AF177379
186	E	W	K	A	L	K	E	S	W	G	A	I	W	R	I	D	T	P	K	P	L	K	G	P	F	S	V	R	V	T	T	Ory s 1 GenBank AF261270.1
185	K	W	I	E	L	K	E	S	W	G	A	I	W	R	I	D	T	P	D	K	L	I	G	P	F	T	V	R	Y	T	T	Pha a 1 GenBank S80654
216	E	G	G	T	K	G	E	A	K	D	V	I	P	E	G	W	K	A	D	T	A	Y	E	S	:	:	:	:	K	Phi p 1 Wt GenBank Z27090		
216	E	G	G	T	K	G	E	A	E	D	V	I	P	E	G	W	K	A	D	T	A	Y	A	S	:	:	:	:	K	Poa p 1 GenBank AJ131850		
216	E	G	G	T	K	V	E	A	E	D	V	I	P	E	G	W	K	A	D	T	A	Y	E	S	:	:	:	:	K	Hol l 1 GenBank AJ012714		
216	E	G	G	T	K	S	E	V	E	D	V	I	P	E	G	W	K	A	D	T	S	Y	S	A	K	:	:	:	:	K	Lol p 1 GenBank M35687	
217	E	S	G	G	H	V	E	Q	E	D	V	I	P	E	D	W	K	P	D	T	V	Y	K	S	K	I	Q	E	:	:	Cyn d 1 GenBank AF177379	
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216	E	G	G	T	K	A	E	F	E	D	V	I	P	E	G	W	K	A	D	T	:	H	D	A	S	:	:	:	:	K	Pha a 1 GenBank S80654	

Figure 2

	10	20	30	
1	I P K V P P G P N I T A T Y G D K W L D A K S T W Y G K P T	Phl p 1 Wt GenBank Z27090		
1	I P K V P P G P N I T A T Y G D K W L D A K S T W Y G K P T	Phl p 1 NoCys		
	40	50	60	
31	A A G P K D N G G A C G Y K D V D K P P F S G M T G C G N T	Phl p 1 Wt GenBank Z27090		
31	A A G P K D N G G A S G Y K D V D K P P F S G M T G S G N T	Phl p 1 NoCys		
	70	80	90	
61	P I F K S G R G C G S C F E I K C T K P E A C S G E P V V V	Phl p 1 Wt GenBank Z27090		
61	P I F K S G R G S G S S F E I K S T K P E A S S G E P V V V	Phl p 1 NoCys		
	100	110	120	
91	H I T D D N E E P I A A Y H F D L S G I A F G S M A K K G D	Phl p 1 Wt GenBank Z27090		
91	H I T D D N E E P I A A Y H F D L S G I A F G S M A K K G D	Phl p 1 NoCys		
	130	140	150	
121	E Q K L R S A G E V E I Q F R R V K C K Y P E G T K V T F H	Phl p 1 Wt GenBank Z27090		
121	E Q K L R S A G E V E I Q F R R V K S K Y P E G T K V T F H	Phl p 1 NoCys		
	160	170	180	
151	V E K G S N P N Y L A L L V K F V A G D G D V V A V D I K E	Phl p 1 Wt GenBank Z27090		
151	V E K G S N P N Y L A L L V K F V A G D G D V V A V D I K E	Phl p 1 NoCys		
	190	200	210	
181	K G K D K W I A L K E S W G A I W R I D T P E V L K G P F T	Phl p 1 Wt GenBank Z27090		
181	K G K D K W I A L K E S W G A I W R I D T P E V L K G P F T	Phl p 1 NoCys		
	220	230	240	
211	V R Y T T E G G T K G E A K D V I P E G W K A D T A Y E S K	Phl p 1 Wt GenBank Z27090		
211	V R Y T T E G G T K G E A K D V I P E G W K A D T A Y E S K	Phl p 1 NoCys		

Figure 3

		10	20	30	
1	I P K V P P G P N I T A T Y G D K W L D A K S T W Y G K P T				Phl p 1 NoCys
1	I P K V P P G P N I T A T Y G D K W L D A K S T W Y G K P T				Phl p 1 NoCys d213-220
1	: : : : : G P N I T A T Y G D K W L D A K S T W Y G K P T				Phl p 1 NoCys d1-6,115-119,213-220
		40	50	60	
31	A A G P K D N G G A S G Y K D V D K P P F S G M T G S G N T				Phl p 1 NoCys
31	A A G P K D N G G A S G Y K D V D K P P F S G M T G S G N T				Phl p 1 NoCys d213-220
25	A A G P K D N G G A S G Y K D V D K P P F S G M T G S G N T				Phl p 1 NoCys d1-6,115-119,213-220
		70	80	90	
61	P I F K S G R G S G S S F E I K S T K P E A S S G E P V V V				Phl p 1 NoCys
61	P I F K S G R G S G S S F E I K S T K P E A S S G E P V V V				Phl p 1 NoCys d213-220
55	P I F K S G R G S G S S F E I K S T K P E A S S G E P V V V				Phl p 1 NoCys d1-6,115-119,213-220
		100	110	120	
91	H I T D D N E E P I A A Y H F D L S G I A F G S M A K K G D				Phl p 1 NoCys
91	H I T D D N E E P I A A Y H F D L S G I A F G S M A K K G D				Phl p 1 NoCys d213-220
85	H I T D D N E E P I A A Y H F D L S G I A F G S : : : : D				Phl p 1 NoCys d1-6,115-119,213-220
		130	140	150	
121	E Q K L R S A G E V E I Q F R R V K S K Y P E G T K V T F H				Phl p 1 NoCys
121	E Q K L R S A G E V E I Q F R R V K S K Y P E G T K V T F H				Phl p 1 NoCys d213-220
110	E Q K L R S A G E V E I Q F R R V K S K Y P E G T K V T F H				Phl p 1 NoCys d1-6,115-119,213-220
		160	170	180	
151	V E K G S N P N Y L A L L V K F V A G D G D V V A V D I K E				Phl p 1 NoCys
151	V E K G S N P N Y L A L L V K F V A G D G D V V A V D I K E				Phl p 1 NoCys d213-220
140	V E K G S N P N Y L A L L V K F V A G D G D V V A V D I K E				Phl p 1 NoCys d1-6,115-119,213-220
		190	200	210	
181	K G K D K W I A L K E S W G A I W R I D T P E V L K G P F T				Phl p 1 NoCys
181	K G K D K W I A L K E S W G A I W R I D T P E V L K G P F T				Phl p 1 NoCys d213-220
170	K G K D K W I A L K E S W G A I W R I D T P E V L K G P F T				Phl p 1 NoCys d1-6,115-119,213-220
		220	230	240	
211	V R Y T T E G G T K G E A K D V I P E G W K A D T A Y E S K				Phl p 1 NoCys
211	V R : : : : : : : G E A K D V I P E G W K A D T A Y E S K				Phl p 1 NoCys d213-220
200	V R : : : : : : : G E A K D V I P E G W K A D T A Y E S K				Phl p 1 NoCys d1-6,115-119,213-220

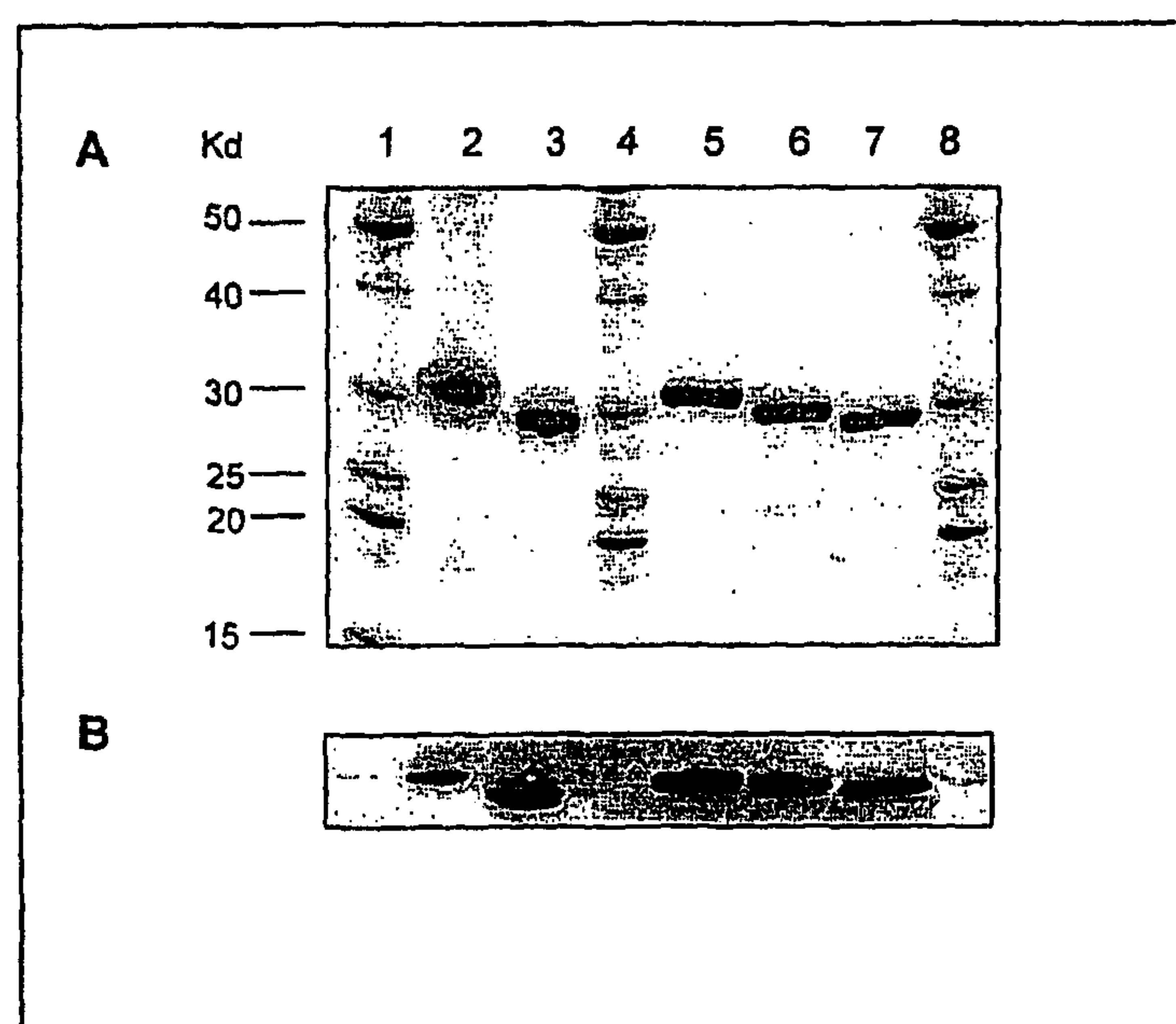
Figure 4:

Figure 5:

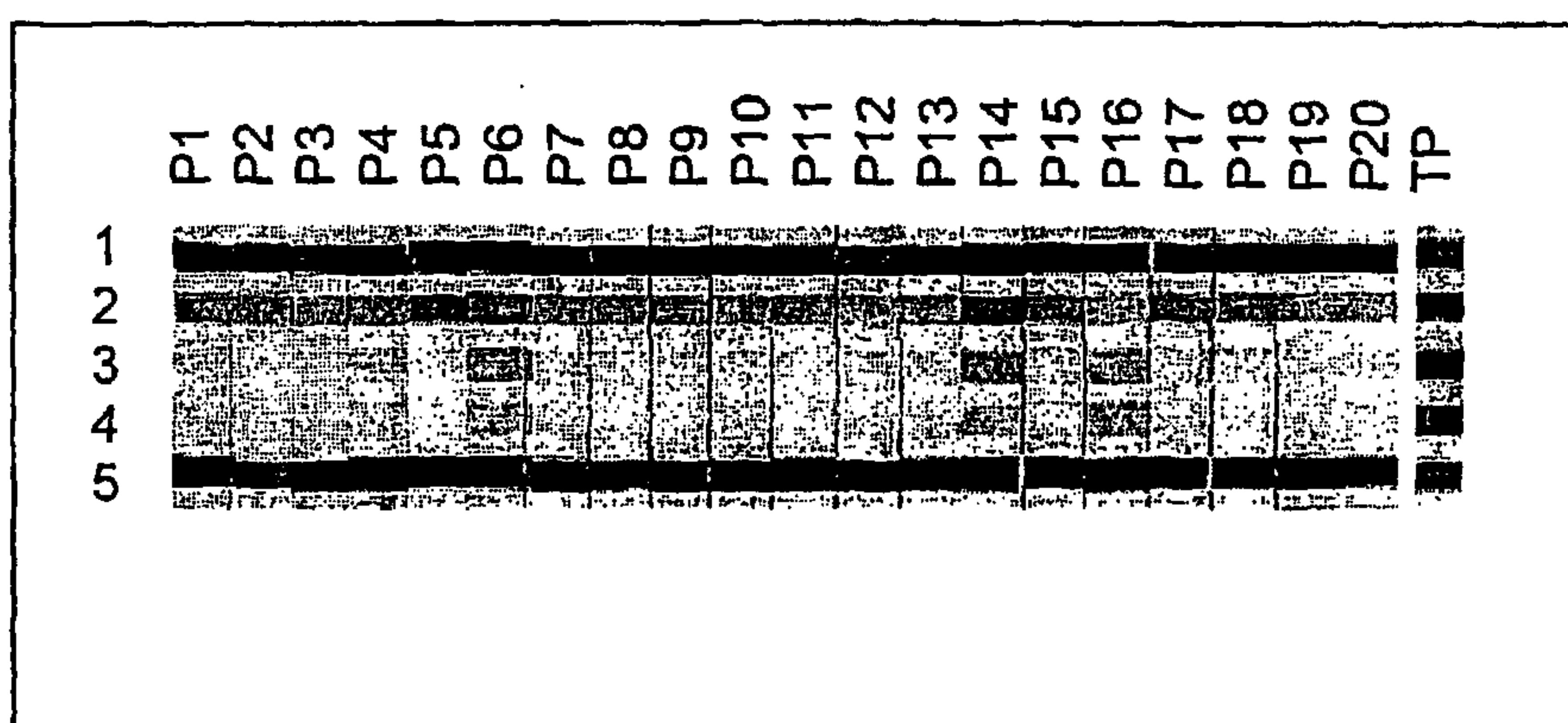


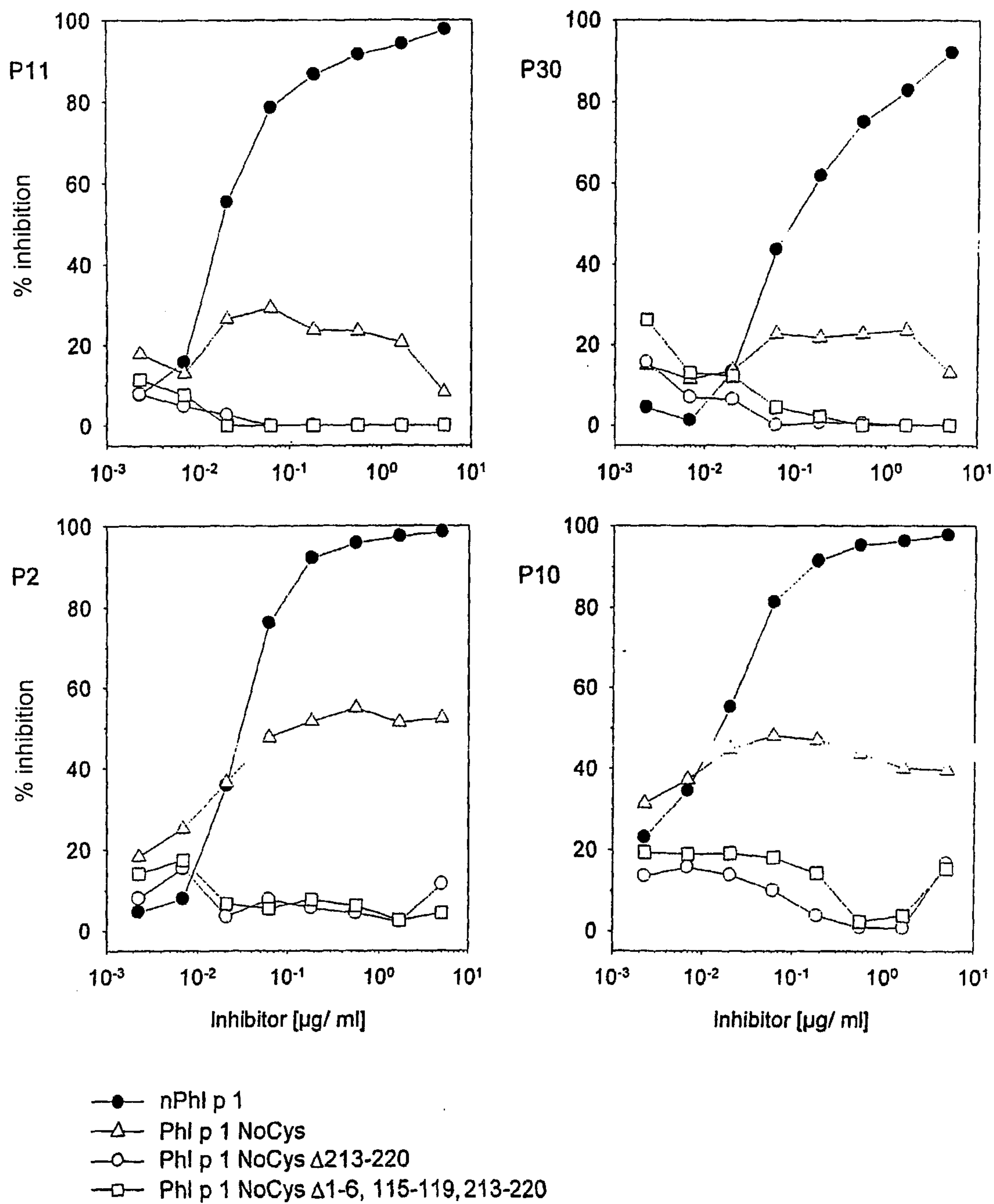
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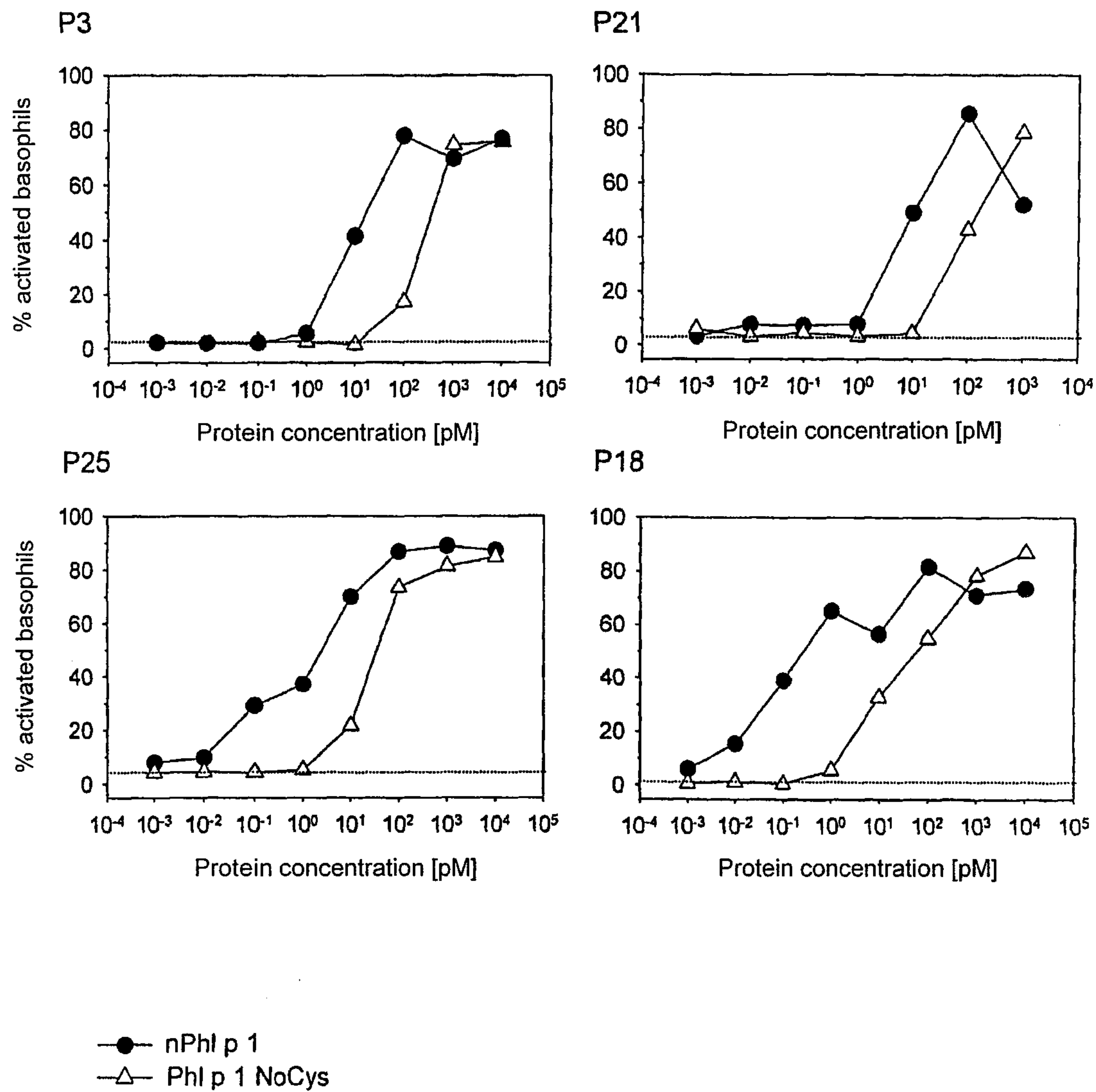
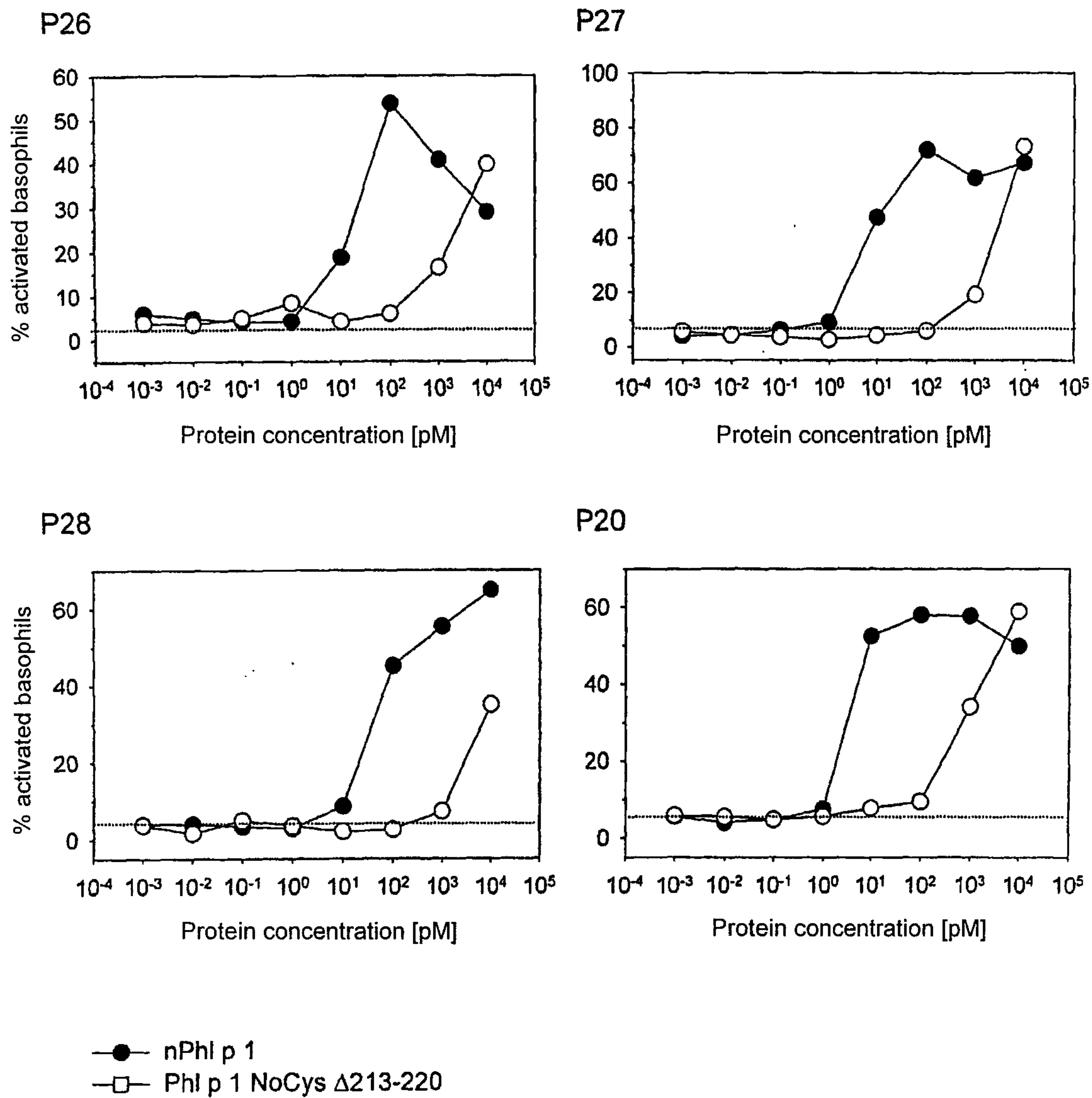
Figure 7:

Figure 8:

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Figure 9: