

Received Date : 28-Sep-2015

Revised Date : 09-Dec-2015

Accepted Date : 15-Dec-2015

Article type : Original Article: Experimental Allergy and Immunology

ORIGINAL ARTICLE for ALLERGY

Standardization of allergen products: 2. Detailed characterization of GMP-produced recombinant Phl p 5.0109 as European Pharmacopoeia reference standard

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Short title

Recombinant Phl p 5.0109 as reference standard

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.12824

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Key Words

BSP090; EDQM; Good Manufacturing Practice; Ph. Eur. reference standard; recombinant Phl p 5 allergen

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Abstract

Background: The Biological Standardization Programme of the European Directorate for Quality of Medicines and Healthcare (EDQM) aims at the establishment of well-characterized reference standards based on recombinant allergens and validated assays for quantification of major allergen content. The objective of this study was the detailed physicochemical and immunological characterization of recombinant Phl p 5.0109, the second available allergen reference standard.

Methods: Recombinant Phl p 5.0109 PP5ar06007 was produced under GMP conditions and analyzed by an array of physicochemical and immunological methods for identity, quantity, homogeneity, and folding stability in bulk solution, as well as thermal denaturation, aggregation state, and biological activity when formulated for long-time storage.

Results: PP5ar06007 revealed as a highly homogeneous, monomeric, well-folded preparation of rPhl p 5.0109, as documented by mass spectrometry, SDS-PAGE, isoelectric focussing, size-exclusion chromatography with light scattering, circular dichroism, and infrared spectroscopy. Upon storage at +4°C PP5ar06007 retained the monomeric state for at least two months. A protein quantity of 1.56 ± 0.03 mg/ml was determined by amino acid analysis in PP5ar06007 and its biological activity was shown to be comparable to natural Phl p 5 in terms of basophil activation and T cell reactivity.

Conclusions: Recombinant Phl p 5.0109 PP5ar06007 was characterized extensively at the physicochemical and immunological level. It revealed to be a highly stable, monomeric, and immunologically equivalent of its natural counterpart. PP5ar06007 is now available as European Pharmacopoeia allergen reference standard for grass pollen products.

Introduction

Grass pollen ranges among the most prevalent allergen sources worldwide (1). Eleven different grass pollen allergens have been identified (2). Specific immunotherapy against grass pollen allergy, the potentially only curative treatment, is currently still based on grass pollen extracts, which are inherently subject of variations regarding their representation of different grass pollen (iso-)allergens (3, 4). Thus, the need for standardization of allergen extracts represents an important issue for allergy diagnosis and immunotherapy (5). This has been recognized by the American Academy of Allergy, Asthma, and Immunology (6), as well as by publications of European regulatory authorities (7, 8). For most allergen products standardization of allergen extracts is still based on determination of the IgE potency, performed by competitive IgE-binding assays and skin testing, which also raises ethical concerns and safety aspects (9-11). Using these types of biological assays, the real allergen content is not determined. The resulting potency values are expressed in company-specific units and are not comparable between different manufacturers. Therefore, efforts to set up international standards for the most relevant allergen extracts were initiated by the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Standardization Subcommittee (12) and this endeavour was continued by the EU-funded CREATE project (13, 14), from which two allergens were selected for the Biological Standardization Programme project BSP090 of the European Directorate for Quality of Medicines and Healthcare (EDQM). Being a clinically very relevant isoform (sensitization rate of 65-85%), rPhl p 5.0109 was selected for a clinical study using a mixture of five recombinant timothy grass pollen allergens (15, 16). Thus, rPhl p 5.0109 was produced under Good Manufacturing Practice (GMP) conditions, characterized physicochemically and evaluated in a ring trial using selected sandwich-ELISA systems (17, 18) pursuing the aim to validate pairs of allergen plus suitable quantification system for the applicability in allergen standardization based on mass units of major allergen (19). While the first candidate biological reference preparation, recombinant Bet v 1.0101, has previously been published (20), we present here the detailed characterization of GMP-produced rPhl p 5.0109 PP5ar06007 as European Pharmacopoeia (Ph. Eur.) reference standard of *Phleum pratense* pollen-related allergen products.

Material and Methods

Preparation of allergens

Allergopharma (Reinbek, Germany) has developed recombinant Phl p 5.0109 to be used in a cocktail of allergens for specific immunotherapy against grass pollinosis (21). Briefly, recombinant Phl p 5 was expressed in *E. coli* and purified using a sequence of chromatographic steps including hydrophobic interaction, ion exchange and size exclusion chromatography. Natural Phl p 5 (nPhl p 5) was purified from water soluble extracts prepared from *Phleum pratense* pollen (Allergon, Aengelholm, Sweden).

Physicochemical characterization

Identity, quantity, homogeneity, folding, stability, and aggregation analyses were performed based on methodologies as described earlier (20). Additionally, high resolution mass spectrometry and Fourier Transform-infrared spectroscopy analyses were performed. A detailed description can be found in the article's online Supporting Information.

Immunological characterization

Epitope integrity and equivalence of PP5ar06007 with natural Phl p 5 was determined by basophil activation test and T cell reactivity. A detailed description can be found in the article's online Supporting Information.

Results

Several batches of recombinant Phl p 5.0109 were produced and purified under GMP conditions with a high batch-to-batch consistency (Table S1). Purity, identity and homogeneity have been shown by physicochemical and immunological analytical methods. Batch PP5ar06007 was chosen for the further development of a reference standard. From this bulk material, final formulations of stable reference standard were prepared by adding 0.2% BSA and 0.1% trehalose, lyophilized, and stored in aliquots at the EDQM.

Identity

The protein sequence of PP5ar06007 was shown to be identical with the major *Phleum pratense* pollen allergen Phl p 5 having the Uniprot KB accession number Q84UI2 by mass spectrometry-based methods. First, intact mass measurement resulted in an average molecular mass of 28286.0 ± 0.6 Da (calibration error with GFP: 20 ppm) coinciding with the theoretical MW of Q84UI2 (N-terminal methionine removed). An additional peak at 28329.0 ± 0.6 Da with an intensity of approx. 30% was found (Figure 1A). This side peak was identified by further mass spectrometric investigations to represent an N-terminally acetylated variant of Phl p 5.0109. Shortly, a monoisotopic mass difference of +42.0397 of the side peak compared to unmodified Phl p 5.0109 was determined using high-resolution (<1 ppm) orbitrap MS technology, and evidence for N-terminal acetylation was supplemented by top-down sequencing procedures on the intact molecule (Figure 1A insert). Second, nanoLC-MSMS-based peptide mapping of tryptic and V8 protease digests resulted in a merged sequence coverage of 100% (Figure 1A insert). Additional search for modifications resulted in a positive signal for the N-terminally acetylated peptide 1-30, having a monoisotopic mass of 2722.3172 differing from unmodified peptide by +42.0031, which coincided well with the theoretical mass difference of +42.0106. Thus, PP5ar06007 was shown to consist of approximately 30% N-terminally acetylated Phl p 5.0109. Further possible modifications included deamidations of Asp₁₉₃ and Asp₁₉₄, respectively, as well as oxidation of Met₂₄₇. However, these possible modifications were found just in the tryptic digests and were not reflected by the results from the intact mass measurements. Notably, such modifications can occur spontaneously during the incubation period of proteolytic digestion, as they also may represent aging effects on proteins. PP5ar06007 did not show such aging effects.

Quantity

Amino acid analysis was performed by two laboratories, one of which holding a GMP certification, for exactly quantifying PP5ar06007. Figure 1C shows the results from the first analysis. During acid hydrolysis, Cys and Trp were (partially) degraded, and Asn and Gln were converted to Asp and Glu, respectively. Comparison of experimental (average with standard deviation of triplicates) with theoretical amino acid content was found to be in good agreement (standard deviations <10%) for most residues. Deviations of >10% were found for Thr, Tyr, and Phe, however, due to our experience such deviations have to be tolerated when using this method. Results for protein quantity obtained without considering these residues stayed within the standard deviation given below. In the second analysis (GMP-

certified), which was performed in duplicate, just the robust amino acids Asp, Ala, Phe, Val, Ile, and Leu were used for quantification. A concentration of 1.56 ± 0.03 mg/ml was determined.

Homogeneity

For characterization of homogeneity of PP5ar06007, denaturing gel electrophoresis, isoelectric focusing, and size-exclusion chromatography were applied. In SDS-PAGE, PP5ar06007 migrated as a single band at approximately 35 kD (Figure 2A) matching the upper band of natural Phl p 5 preparations and the usual observation of other recombinant Phl p 5 isoforms (data not shown). A migration behavior in SDS-PAGE differing from the theoretical MW has been reported previously, one reason may be that the attachment of SDS hydrophobic, acidic, basic, or glycosylated sequence stretches might be inefficient (21-23). Silver-staining, as well as immunoblotting with two different Phl p 5-specific monoclonal antibodies and a serum pool of grass pollen-allergic patients did not reveal any further protein contamination. Under native conditions an isoelectric point (pI) of approximately 8.6 was determined (Figure 2B) contrasting the theoretical pI of 7.9, however, such deviations can frequently be observed for recombinant proteins under non-denaturing conditions. Isoelectric focusing serves primarily for monitoring homogeneity in regard to sample aging processes such as partial oxidation of sulfhydryl groups and deamidation of asparagine or glutamine residues (20, 21). In this regard, a very high degree of surface charge homogeneity could be verified for PP5ar06007. The very faint band slightly below the main band may represent N-terminally acetylated Phl p 5, as this minor side component was identified by mass spectrometry. Accordingly, no aggregates or oligomers were detected by HPSEC-light scattering (Figure 2C). Monomeric molecules eluted as a single peak at a retention volume of 8.7 ml. Upon evaluating the right-angle light scattering, refractive index, and viscosity signals, a MW of approximately 25 kD and a hydrodynamic radius of 3.0 nm were determined. No further peaks appeared in the light scattering chromatogram between the void (5.7 ml) and total (12.0 ml) retention volume. As this detector signal is especially sensitive to high molecular weight aggregates, PP5ar06007 was determined to consist of >99.9% homogeneous monomeric molecule.

Folding and denaturation analysis

The CD spectrum of PP5ar06007 showed a maximum around 195 nm and a double minimum at 205 and 222 nm (Figure 3A, left panel), typical for protein preparations with a high alpha-helical secondary structure content and similar to a corresponding CD analysis of nPhl p 5 (Figure 3B). Upon heating, denaturation temperatures of 66°C and 67°C were determined, and a high degree of reversibility of protein unfolding was shown from the renaturation curves for the recombinant and natural preparations, respectively (Figure 3A and B, right panel). However, the renaturation curves showed less significant inflection points, and the CD spectra recorded immediately after renaturation showed lower maximal and higher minimal Θ_{mrv} values. Notably, after some time at room temperature, PPar506007 gained its normal appearance in CD spectrum (data not shown) indicating that the refolding process takes longer than the experimental time. These findings were also verified using FTIR spectroscopy, where a strong sigmoidal decrease of the integral 1620-1655 cm^{-1} , corresponding to the amide I vibration range of alpha-helices, was observed (Figure 3C). The decrease of alpha-helical secondary structure content was concomitant with an increase in random coil (integral 1658-1700 cm^{-1}) and inter-chain beta-sheet (integral 1605-1626 cm^{-1}) secondary structure content, of which the latter indicated heat-induced protein aggregation. Notably, some part of the material precipitated during thermal denaturation supporting the FTIR data. However, the after heat denaturation still soluble portion of PP5ar06007 did not form aggregates as investigated by DLS (Figure 4A, dotted line).

Aggregation behavior and stability in solution and upon lyophilization with/without trehalose

The aggregation behavior in solution was investigated using DLS (Figure 4A, solid line). The determined hydrodynamic radius (R_H) of 3.1 nm indicated a theoretical MW of 48 kD, assuming the molecule adopts a globular shape. Moreover, this peak appeared to be rather broad with a polydispersity of 23.6% root square deviation (RSD). Thus, it could be assumed that PP5ar06007 appeared either as a mixture of monomeric and dimeric molecules, or more likely, adopted an elongated shape, which would be coinciding with the R_H value obtained for monomeric PP5ar06007 by HPSEC. However, no high molecular weight aggregates were detected. Storage stability of the bulk substance at +4°C was tested after two months showing no significant changes (Figure 4A, dashed line). Moreover, a sample stored at -20°C with intermittent thawing and freezing steps was re-tested after >8 y upon additional thermal stressing for >3 d at >50°C (but below T_m) showing <1% aggregates (Figure 4A, dashed-dotted line). Upon routinely performed centrifugation no precipitation was

observed in any sample. Furthermore, no significant changes in the mass distribution of the DLS signals, except for a slight decrease in polydispersity were observed: 16.5% RSD in +4°C-stored sample; 19.0% RSD in frozen/thawed/heat-stressed sample. In summary, it can be stated that the PP5ar06007 bulk material retained its monomeric state in solution for months at +4°C and years at -20°C with intermittent thawing/freezing, and was not susceptible to heat stress below T_m .

Using FTIR of PP5ar06007 in saline solution, a secondary structure content of 46.7% alpha-helices and 8.4% intra-chain beta-sheets was determined (Figure 4B), and these values remained similar upon lyophilization with and without 0.1% trehalose, a stabilizing additive in the final formulation of the Ph. Eur. reference standard (besides 0.2% BSA). Notably, no intermolecular beta-sheet formation (amide I vibration range from 1605 to 1626 cm^{-1}) indicating protein aggregation was observed in any of the lyophilized preparations.

The formulated final recombinant Phl p 5.0109 Chemical Reference Substance (CRS) is stored at the EDQM at -20°C. Its stability is monitored annually by triple parallel measurement relative to a baseline reference (stored at -80°C) and in comparison to a degradation sample (+4°C) in a Phl p 5-specific sandwich ELISA (18, 24). Estimates of Phl p 5 content in CRS and degradation sample are calculated relative to the baseline reference and controlled for an overlap of the confidence intervals with the reference estimate. Starting in 2011, stability monitoring has been successfully performed in five consecutive years and by now no indication of a loss of recombinant Phl p 5.0109 CRS potency at elevated temperatures has been identified.

Biological activity

In order to assess the biological activity of PP5ar06007, basophil activation and T cell reactivity were chosen as they represent the best suited functional assays to compare recombinant allergens with their natural counterparts, which is of importance during registration of new allergen products where PP5ar06007 serves as calibrator of major allergen content in natural allergen extracts.

The allergenic potential of PP5ar06007 was assessed in reference to its natural counterpart using direct basophil activation in heparinized blood samples from 15 grass pollen-allergic patients. From the individual titration curves (Figure S1), the concentrations of allergen needed for half-maximal basophil activation (C_{50} values) were determined. The C_{50} values of PP5ar06007 and five other recombinant production batches were expressed in relation to

nPhl p 5 as P_{rel} values (Figure 5B, medians: 0.91, 0.88, 0.97, 0.82, 0.82, and 0.94) demonstrating satisfying allergenic equivalence of the present reference standard with the natural allergen. Moreover, the GMP production process proved reliable and robust for establishment of further batches that may be required in the far future.

The T cell responses to PP5ar06007 were compared to nPhl p 5 and another recombinant production batch (PP5ar10015) using a panel of allergen-specific TCLs and TCCs derived from grass pollen-allergic individuals. Figure 5C depicts almost optimal correlation between the PP5ar06007 and natural allergen (Pearson correlation of $\rho=0.963$, $P<0.0001$). A regression line was approximated to the stimulation indices having a slope very close to the diagonal indicating agreement of T cell reactivity between recombinant and natural molecules. Few exceptions showed a higher reactivity with natural compared to recombinant Phl p 5. This can be explained as the TCLs and TCCs were raised using natural and recombinant allergen, a strategy which would not exclude T cells with higher specificity for isoforms present only in the natural mixture. Batch-to-batch consistency was monitored at the T cell level by comparing PP5ar06007 with batch PP5ar10015 (Table S3). From all TCLs and TCCs a median reactivity of 9.43 was determined for PP5ar06007, which was in good agreement with 10.32 for nPhl p 5 and 9.36 for PP5ar10015. The epitope specificities from most of the used TCL/TCCs were mapped covering a wide range of the allergen sequence (positions 40-246). Nine different partially overlapping epitopes were identified (25). Thus, the close correlation between PP5ar06007 and its natural counterpart evidenced the reference standard to be an excellent representative for grass pollen allergens also at the T cell level.

Discussion

Pharmaceutical products require the highest safety profiles that can be achieved using the currently available methodology. This holds also true for allergen products determined for use in specific immunotherapy of allergic individuals (26). High production standards have to be fulfilled, which is realized by procedures adhering to the concept of GMP including state-of-the-art characterization techniques (27, 28). In line with this, the process of allergen standardization has been promoted by several stakeholders in a project run by EDQM. Resulting from these efforts, Ph. Eur. reference standards for two major allergen products, birch and grass pollen, and a corresponding quantification method for Bet v 1 has become available (18). Here we present the detailed characterization of rPhl p 5.0109 PP5ar06007 serving as calibrator for timothy grass pollen allergen products seeking marketing authorization of the European Union. As described before for rBet v 1.0101 Y0487, the main

physicochemical criteria were investigated including identity, quantity, homogeneity, folding, aggregation behavior/stability in solution, and biological activity (20). In addition, this study presents FTIR-based investigations of folding and susceptibility towards thermal denaturation for the product in solution and in trehalose-formulated lyophilized state, as this represents the Ph. Eur.-provided formulation stored at the EDQM. The FTIR analyses complemented the CD results well, where a loss in alpha-helical secondary structure content was indicated upon thermal de-/renaturation. Moreover, the biological activity was assessed in terms of basophil activation and T cell reactivity compared to its natural counterpart. This is of great importance as a biological reference standard has to represent the natural molecule appearing in the allergen extracts from different angles, which in case of an active pharmaceutical ingredient for allergen immunotherapy means to be similar at the level of recognition by IgE antibodies and T cells. This similarity could be shown using human basophils from a panel of allergic donors and an array of allergen-specific T cell lines and clones. The biological activity was furthermore established as rPhl p 5.0109 was used before *in vivo* showing together with other recombinant major timothy grass pollen allergens sufficient clinical efficacy (15, 16). Several GMP-produced batches were investigated and similar results were obtained. The physicochemical and biological assays performed certified thus a very robust GMP-based production process, which evidently represents an important prerequisite for long-term availability of a biological reference standard.

Overall, GMP-grade rPhl p 5.0109 appears highly similar to its natural counterpart from the physicochemical, structural, and biological point of view. As such, rPhl p 5.0109 is suitable as Ph. Eur. reference standard.

Acknowledgments

This work was supported by a grant from the EDQM. The work of MH was supported by grant P18820-B13 of the Austria Science Funds (FWF) and Biomay, AG, Vienna, Austria. We thank Brigitta Arlt, Britta Friggemann, and Kerstin Joppich-Röhm (Allergopharma) for their excellent technical assistance in performing the basophil activation test and T cell proliferation assay.

Author contributions

MH, ANa, HK, MT, MS, PB performed experimental and preparative work; MH, ANa, HK, MT, MS, PB, FF evaluated data; SK, KHB, SV provided material; ANe, SK, FF provided experimental/instrumental infrastructure; MH, RvR, KHB, SV, FF conceived the study; MH, ANa, HK, FF wrote and edited the manuscript; MH, RvR, SV, SK, KHB, ANe, FF provided funding.

Conflict of interest statement

The authors disclose that they have no conflicting interests relevant for the content of this study. ANa, HK, MT, SK are employees of Allergopharma GmbH and Co. KG, ANe is employee of Biomay AG. Detailed conflict of interest forms are provided.

Supporting information

Additional supporting information may be found in the online version of this article:

Data S1. Methods in greater detail.

Figure S1. Individual results of basophil activation test.

Table S1. Quality assessment of six production batches of rPhl p 5.0109.

Table S2. Peak list of MS-based peptide mapping upon tryptic digestion.

Table S3. T cell reactivity of PP5ar06007 vs. nPhl p 5 and recombinant batch PP5ar10015.

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Figure legends

Figure 1. Investigation of protein identity in rPhl p 5 PP5ar06007 by mass spectrometry-based methods and quantity determination by amino acid analysis. **A** MaxEnt1TM-deconvoluted intact mass of PP5ar06007 gave a peak at 28286.0 ± 0.6 , thus, coinciding with the theoretical mass of SwissProt entry Q84UI2. Additional peaks at 28329.0 and 28554.0 ± 0.6 gave intensities of approx. 30% and 5%. **Insert of A** NanoLC-MSMS-based sequencing of tryptic (T1 by Q-ToF LC-MSMS, T2 by Q-Exactive LC-MSMS technology) and V8-proteolytic fragments of PP5ar06007 resulted in a combined sequence coverage of 100% of SwissProt entry Q84UI2. Top-down sequencing fragmentations of the N-terminally acetylated fraction of PP5ar06007 are indicated by “I”. **B** Base peak intensity chromatogram of tryptic digest T2. Numbered peaks correspond to the unequivocally identified PP5ar06007-derived tryptic peptides of T2 in Table S2, unlabelled peaks are derived from self-digestion of trypsin or represented system peaks also appearing in empty runs. **C** The protein amount was quantified by amino acid analysis to be 1.56 ± 0.03 mg/ml. Experimentally determined numbers of amino acid residues (gray bars with standard deviations from triplicates) showed good agreement with the theoretical values (black bars).

Figure 2. Homogeneity analysis of rPhl p 5.0109 PP5ar06007. Microgram amounts (0.3 μ g, lane 1; 0.2 μ g, lanes 2-4; 0.5 μ g, lane 5) of PP5ar06007 were separated either **A** by SDS-PAGE and silver-stained (lane 1) and detected by IgG and IgE immunoblotting (lanes 2-4) using the rPhl p 5-specific monoclonal antibodies BO1 (lane 2) and 1D11E8 (lane 3) and a pool of grass pollen-allergic patients' sera (lane 4) or **B** by isoelectric focusing and detected by silver staining (lane 5). **C** The size-exclusion chromatogram of PP5ar06007 monitored by right-angle light scattering signal (RALS) being highly sensitive for high MW aggregates showed protein eluting as a single peak ($v_{\text{ret}} = 8.7$ ml) between the void and total volumes of 5.7 and 12.0 ml, respectively. A MW of approximately 25 kD and a R_H of 3.0 nm were determined by combining RALS with refractive index and viscosity signal, hence, more than 99.9% of PP5ar06007 eluted as monomeric molecule.

Figure 3. Folding and thermal denaturation analysis of **A** rPhl p 5.0109 PP5ar06007 vs. **B** nPhl p 5. Far UV circular dichroism spectra (left panel) were recorded at 0.1 mg/ml in 10 mM sodium phosphate pH 7.4 at 20°C. The CD spectra before (solid black line) and immediately

after heat denaturation and renaturation (gray broken line), respectively, showed a high degree of reversibility of the folding of both, the recombinant and natural preparations. Denaturation (black) and renaturation (gray) curves (right panel) were recorded at 222 nm. T_m values of 66°C and 67°C were determined as inflection points of the Slavitzky-Golay-smoothed denaturation curves of the recombinant and natural preparations, respectively. All CD values are given as mean residue molar ellipticity Θ_{mrw} . **C** Temperature ramps of PPar506007 (left panel) monitored by FTIR raising from spectra recorded at temperatures (i) clearly below thermal denaturation (25-54°C) depicted by a bundle of black solid lines, (ii) close but still below thermal denaturation (56-62°C) depicted by a bundle of dark gray dashed lines, (iii) during thermal denaturation (64-72°C) depicted by a bundle of light gray solid lines, and (iv) of the heat denatured sample (74-95°C) depicted by a bundle of light gray dotted lines. The observed structural changes (right panel) were characterized by a decrease of alpha helices (black solid) and an increase of inter-chain beta-sheets (dashed line) and random coil (dotted line) secondary structures at the same T_m as determined by CD.

Figure 4. Aggregation behavior and stability of rPhl p 5 PP5ar06007 in solution, lyophilized with and without 0.1% trehalose as stabilizer. **A** Dynamic light scattering of PP5ar06007 statistically weighted by the mass model for proteins using the OmniSize™ software resulted in a single fraction with a hydrodynamic radius (R_H) of 3.1 nm (solid line). Moreover, this peak appeared to be rather broad with a polydispersity of 23.6% root square deviation (RSD). Thus, it could be assumed that PP5ar06007 appeared either as a mixture of monomeric and dimeric molecules, or more likely, adopted an elongated shape, which would be coinciding with the R_H value of 3.0 obtained for monomeric PP5ar06007 by SECTDA. However, no high molecular weight aggregates were detected. Dynamic light scattering after nine weeks storage at +4°C (dashed line) revealed no significant changes in the R_H (3.2 nm) and polydispersity (16.5% RSD) values. No high molecular weight aggregates were detected in PP5ar06007 within the observed storage time. Upon heating to 95°C, slow cooling, and centrifugation a lower fraction of still soluble PP5ar06007 appeared as monomeric molecule with a R_H of 3.2 nm and a polydispersity of 21.6% RSD (dotted line). Storage at -20°C with intermittent thawing and freezing and heat stressing displayed a similar R_H of 3.1 nm, a slightly decreased polydispersity of 19.0% RSD, and <1% aggregates (dashed-dotted line). **B** Fourier-Transform infrared spectroscopy of PP5ar06007 in solution (solid line), lyophilized from saline with (dotted line) and without (dashed line) trehalose revealed primarily alpha-helical secondary structure content. The amide I maxima at 1655 and 1550 cm^{-1} ,

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respectively, are characteristic for alpha-helical secondary structure content (light gray). **C** Second derivatives were computed to more precisely allocate the peak maxima of the recorded FTIR spectra in B.

Figure 5. Biological activity of rPhl p 5.0109 PP5ar06007. **A** Batch-to-batch consistency regarding allergenic potency was evaluated by human basophil activation as determined by up-regulation of CD203c by six batches of rPhl p 5.0109 in relation to nPhl p 5 ($P_{rel} = C50_{nat}/C50_{rec}$) shown by Box-Whisker plotting (with 10/90 percentiles). C50 values represent the concentration of allergen needed for half-maximal basophil activation. Experiments were performed on heparinized blood samples from 15 different patients (for individual BAT results see Figure S2). **B** Comparison of T cell reactivity to 0.3 μ M PP5ar06007 vs. nPhl p 5 obtained from 23 T cell lines and 2 T cell clones derived from 11 grass pollen-allergic patients (further details in Table S3). Stimulation indices showed a Pearson correlation of $\rho=0.963$ ($P<0.0001$). The gray dashed line represents optimal correlation in T cell reactivity.

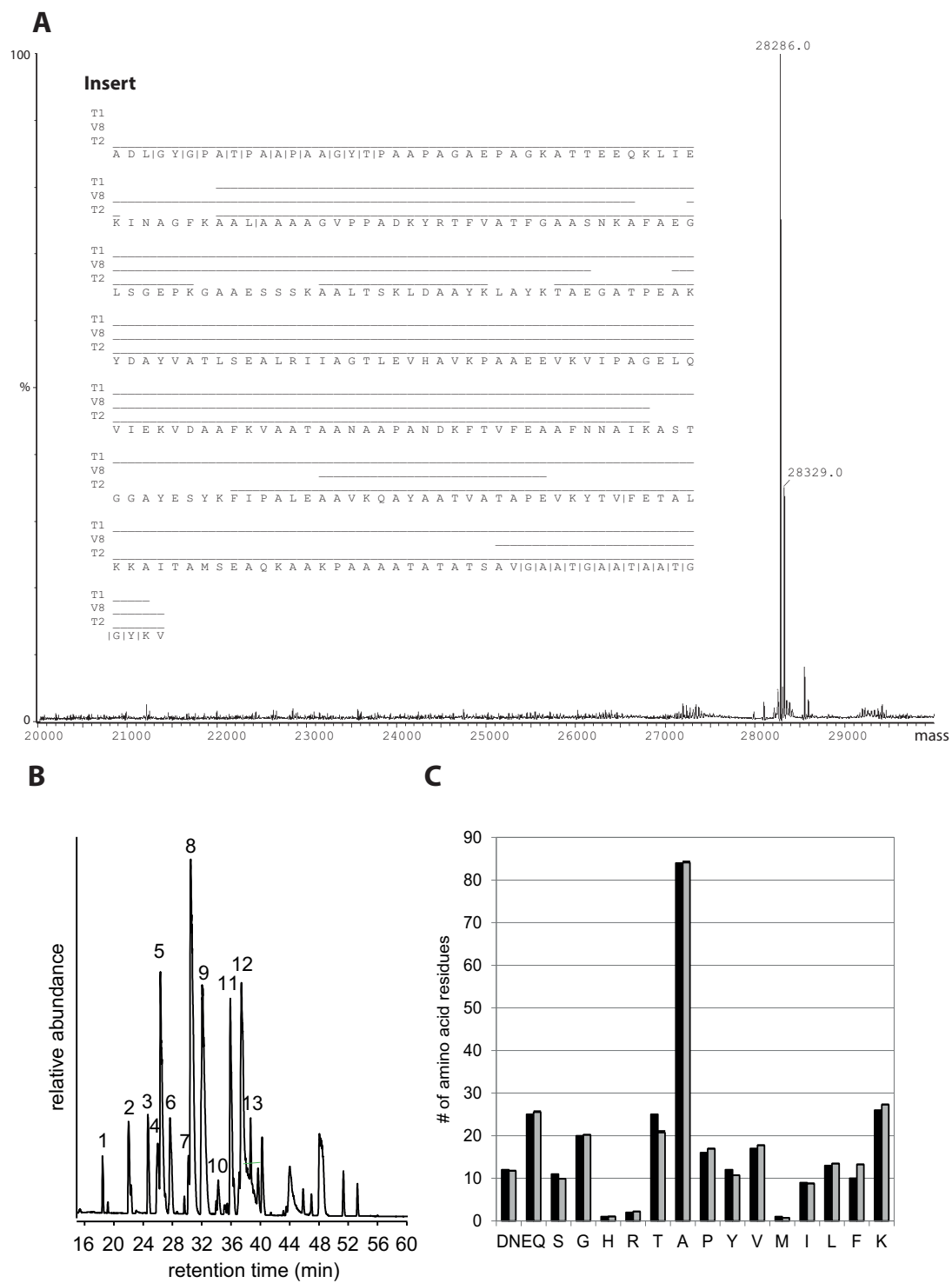


Figure 1

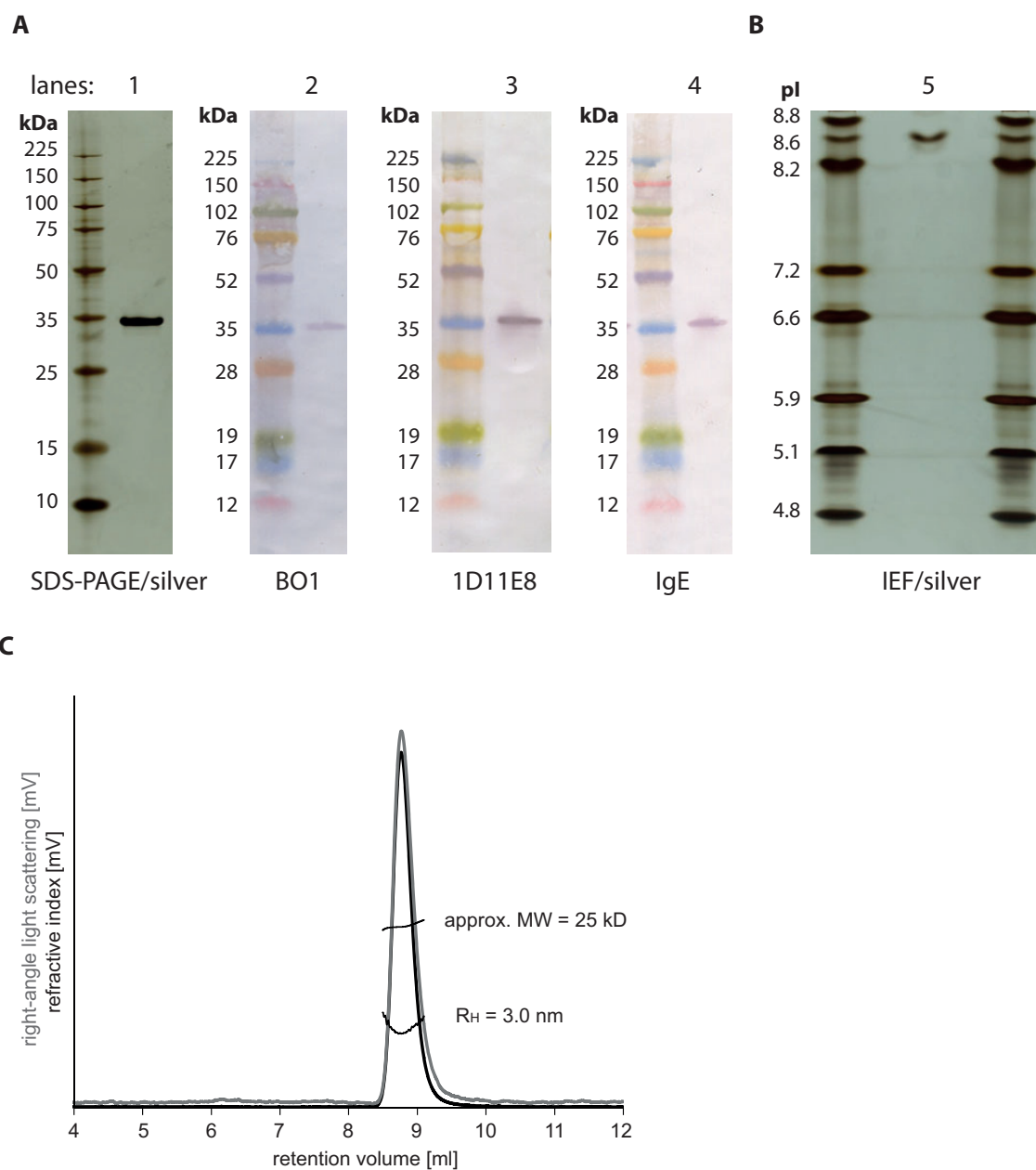


Figure 2

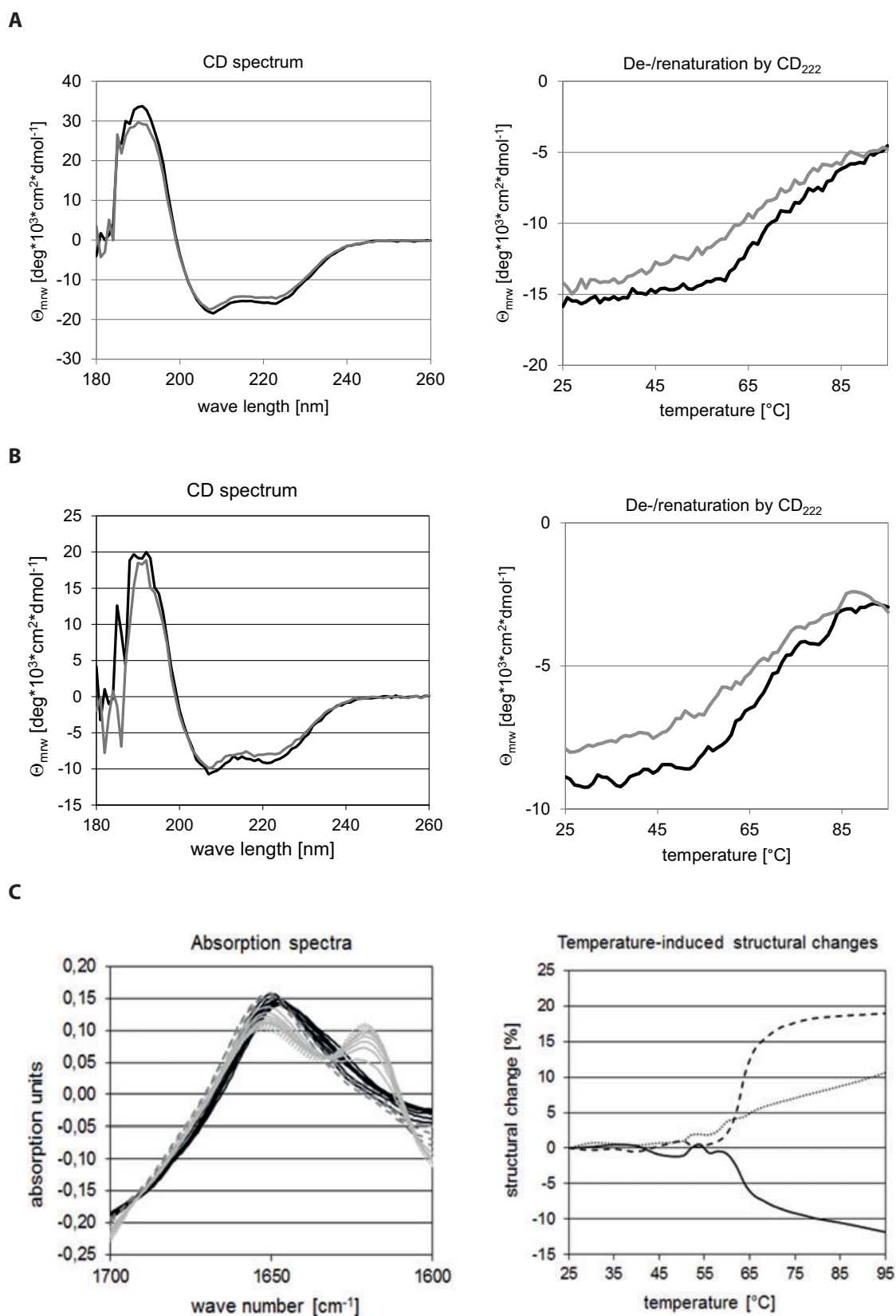


Figure 3

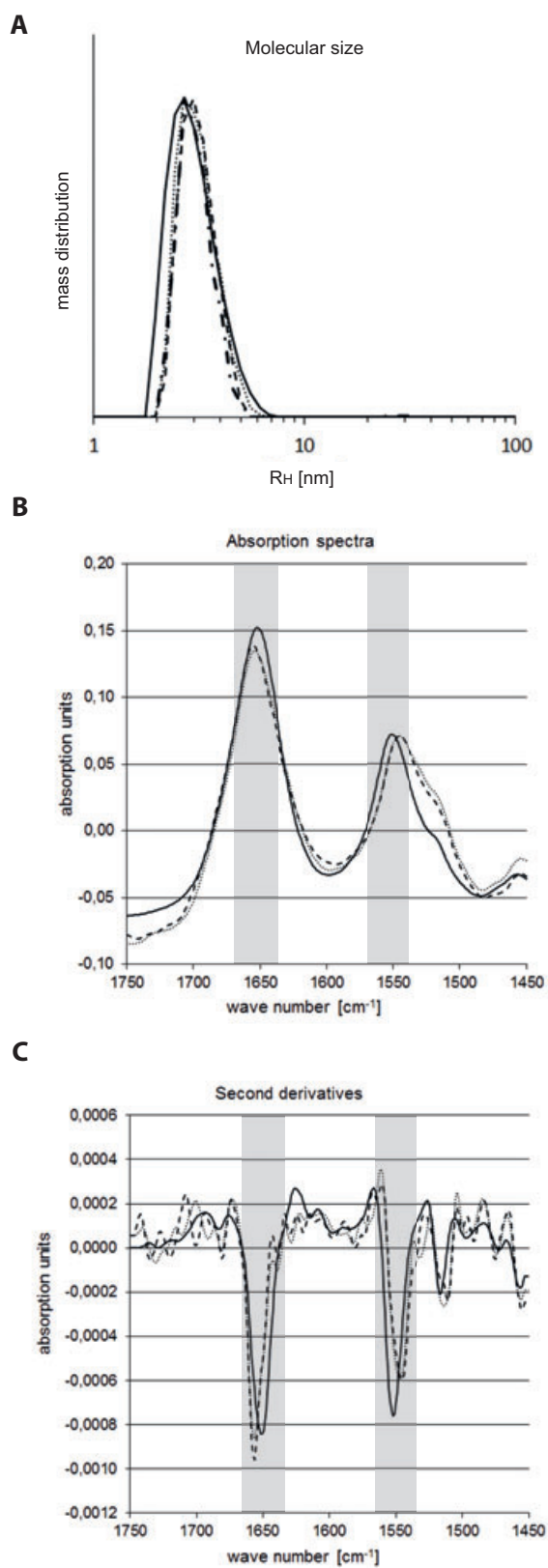


Figure 4

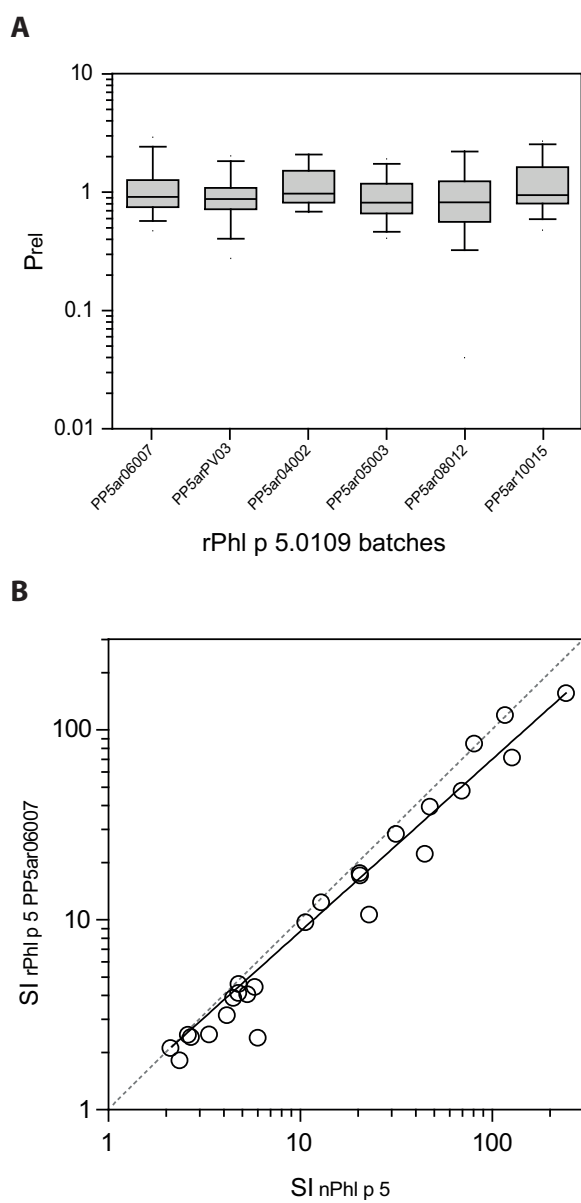


Figure 5

Supporting Information

Standardization of allergen products: 2. Detailed characterization of GMP-produced recombinant Phl p 5.0109 as European Pharmacopoeia reference standard

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Data S1. Methods for physicochemical and immunological characterization

Mass spectrometry (MS)-based methods

A. Q-Tof technology. Intact mass measurement was performed by ElectroSpray Ionization-Quadrupole-Time of Flight (ESI-Q-ToF) mass spectrometry (Ultima-Global, Waters, Milford, MA) in aqueous 50% (v/v) HPLC-grade acetonitrile, 0.1% (v/v) formic acid after ZipTip_{C4}TM purification (Millipore, Billerica, MA). Instrument calibration was performed using 500 fmol/μl Glu₁-fibrinopeptide B (GFP, Sigma #F3261, St. Louis, MO) infused at a flow rate of 1 μl/min. Multiply charged peak series were processed using MaxEnt1TM-algorithm iterating for mass solutions between 20,000 and 30,000. For nano-liquid chromatography tandem mass spectrometry (nanoLC-MSMS)-based protein sequencing sample was digested using trypsin and V8 protease, respectively, (Roche, Basel, Switzerland). Resulting peptides were separated by reversed phase-capillary HPLC (NanoEase Symmetry 300TM trap and Atlantis dC18TM separating column, Waters, Milford, MA, USA) directly coupled to ESI-Q-ToF mass spectrometer in data-dependent analysis mode. Spectra were analyzed by ProteinLynx-GlobalServer2.2.5TM (PLGS) software using Uniprot knowledgebase (1). Positive identification of allergen-derived peptides by collision-induced dissociation was based on at least four consecutive unequivocally identified y-ions in MSMS mode.

B. Orbitrap technology. Intact proteins and tryptic peptides were separated by nano-HPLC (Dionex Ultimate 3000RSLC, ThermoFisher Scientific, Bremen, Germany). Samples were loaded onto the trap column (Pepswift, 200 μm x 5 mm, Dionex) and desalted with 0.2% (v/v) heptafluorobutyric acid at a flow rate of 5 μl/min. After 10 minutes, the trap and separation column (in-house PS-DVB monolithic column, 200 μm x 15 cm, prepared as described in (2)) were connected with a switching valve and proteins/peptides were eluted with an acetonitrile (ACN) gradient (solvent A: 0.05% (v/v) in H₂O; solvent B: 0.04% (v/v) TFA/80% (v/v) ACN; 5–40% B in 60 min). The flow rate was 1 μl/min, column temperature 55°C. The HPLC was directly coupled to a Q-ExactiveTM Orbitrap mass spectrometer (ThermoFisher Scientific) via nano electrospray, using stainless steel emitters. Capillary voltage was 2 kV, capillary temperature 275°C and S-lens RF level 60. Lock-mass calibration was done with the system

peak at 445.12 for all measurements, providing mass accuracy of at least 1 ppm. For top-down analysis of intact proteins, a full MS/AIF method was used with following parameters: Full MS: resolution 140,000, in-source fragmentation 20 eV, AGC target 5e6, maximum IT 250ms, range 400-2,000 m/z; AIF: resolution 70,000, NCE 20, AGC target 3e6, maximum IT 200ms, range 133-2,000 m/z. Deconvolution was done with XtractTM software tool of Xcalibur2.2TM (Thermo Fisher Scientific). Top-down fragments were analyzed with the online version of ProSight PTM 2.0 (prosightptm2.northwestern.edu). For peptide sequencing, a top 12 method was utilized: Full MS: resolution 70,000, AGC target 1e6, maximum IT 120 ms, range 300-1750 m/z; MS2: resolution 35,000, NCE 28, AGC target 5e5, maximum IT 120ms, scan range auto selected by Xcalibur. Peptides and peptide modifications identification were done with ProteomeDiscoverer1.4TM and SequestTM (Thermo Fisher Scientific) and Peaks Studio 6 (BSI, Waterloo ON, Canada), respectively.

SDS-PAGE, immunoblotting, and isoelectric focusing experiments

Protein homogeneity was assessed by reducing SDS-PAGE (NuPAGE NovexTM 10% Bis-Tris gels, Life Technologies, Carlsbad, CA, USA) using Perfect ProteinTM MW marker (Novagen, Madison, WI, USA) for detection by silver staining and full-range RainbowTM MW marker (GE Healthcare, Wauwatosa, WI, USA) for IgG and IgE immunoblotting experiments. Potential charge state heterogeneities was detected by native isoelectric focusing (NovexTM pH 3-10, Life Technologies) with reference to an in-house pI marker consisting of purchased (Sigma-Aldrich) soybean trypsin inhibitor (pI 4.6, T1021), bovine beta-lactoglobulin (pI 5.1, L5137), bovine carbonic anhydrase isozymes II (pI 5.9, C6403) and I (pI 6.6, C6653), horse heart myoglobin (pI 7.2, M9267), and three lentil lectins (pI 8.2, 8.6, 8.8, L1277). IEF gels were silver-stained as mentioned above.

Quantity determination

The exact protein quantity of PP5ar06007 was determined by amino acid analysis (AAA) in parallel by two laboratories. The first analysis was performed in triplicate as described earlier (3). The second analysis was performed in duplicate in a GMP-certified facility using pre-column derivatization by o-phthalaldehyde. During acid hydrolysis similar conditions were applied, and for reversed phase-HPLC an Agilent 1200 system (Santa Clara, CA) was used. In both analyses system suitability samples were included.

Size-exclusion chromatography and light scattering

High-Performance Size-Exclusion Chromatography (HPSEC) was performed using a 7.8x300 mm TSKgel-G2000_{SWXL} column (Tosoh-Bioscience, Stuttgart, Germany) on a HP1100 system (Hewlett-Packard, San Jose, CA) equipped with a built-in UV detector and

online-coupled with a right-angle light scattering, refractive index, and viscosity detector array (TDA302, Viscotek, Houston, TX). Samples were analyzed in duplicates using OmniSec4.2TM. The protein amount was determined from the refractive index signal. Detector was calibrated with BSA (Sigma #A7638). Values for dn/dc of 0.185 ml/g, dA/dc of 0.646 ml/g, MW of 66433 Da, and polydispersity of 1 were set (4).

The aggregation behavior in solution was investigated by dynamic light scattering (DLS802, Viscotek) at a concentration of 1.56 mg/ml in 20 mM sodium phosphate pH 8 after 10 min centrifugation at 14,000 g. Data were accumulated for 10x10 sec and the correlation function was fitted into the combined data curve, from which the intensity distribution was calculated using solvent settings for 154 mM sodium chloride in water (refractive index 1.335, viscosity 1.019). The determined intensity distribution was weighted statistically by mass using the mass model for proteins (OmniSizeTM) displaying molecular size R_H [nm] and polydispersity [% RSD] (5).

Circular dichroism

Far UV circular dichroism (CD) spectra were recorded at 0.1 mg/ml in 10 mM sodium phosphate pH 7.4 between 180 and 260 nm using a JASCO-J810 spectropolarimeter (Japan-Spectroscopic, Tokyo, Japan) temperature-controlled at 20°C. Five consecutive scans were recorded with 1 nm band width, 1 sec response time, and 1 nm data pitch, and the mean residue molar ellipticity (Θ_{mrw}) was calculated. For determining the denaturation temperature (T_m) samples were heated to 95°C at 1 degree/min. The inflection point of the denaturation curve of the 222 nm values representing T_m was determined upon smoothing by Slavitzky-Golay algorithm using a convolution width of 11. Analogously, the renaturation curve was recorded.

Fourier Transform-infrared spectroscopy

Infrared spectra of PP5ar06007 in solution were recorded at 1.56 mg/ml, respectively, in 0.9% sodium chloride saline using the ConfocheckTM Fourier Transform-infrared (FTIR) system (Bruker Optics, Ettlingen, Germany). Data were accumulated in the temperature-controlled AquaSpecTM flow-through transmission cell with 7 μ m path length at 25°C with a spectral resolution of 4 cm^{-1} and a data acquisition time of 100 seconds. FTIR spectra of lyophilized samples (from 0.9% saline with and without 0.1% trehalose) were recorded in attenuated total reflection (ATR) mode using the MiracleTM ATR unit. Temperature-dependent denaturation was recorded in steps of 5 degrees 25-50°C, in steps of 2 degrees 50-80°C, and then again in steps of 5 degrees until 95°C using the BioATR IITM unit and an acquisition time of 50 sec. For data procession and evaluation the OpusTM software (Bruker Optics) was used. Upon correction for spectral contributions of non-protein components the absorbance

spectra were vector-normalized on the amide I band and second derivatives were calculated. Secondary structure prediction was performed applying partial least squares calibrations for alpha-helices and beta-sheets using the Opus QUANTTM software tool. These calibrations are based on IR transmission spectra of aqueous solutions of 43 different proteins with secondary structure reference information from x-ray crystallography (6).

Basophil activation test

Basophil activation was determined by measurement of the level of expression of the marker CD203c using flow cytometry as previously described (7). Briefly, heparinized whole blood samples were stimulated with serial dilutions of natural and recombinant allergens in Ultraculture medium (Lonza, Verviers, Belgium) for 15 min at 37°C. Activation was stopped by adding PBS with 20 mM EDTA. Labelling of basophils was performed with CD203c-PE (Beckman Coulter, Krefeld, Germany) for 45 min at room temperature. Lysis of red blood cells was performed with the whole blood lysing kit (Beckman Coulter, Krefeld, Germany) according to the kit instructions. After washing, cells were analyzed in a flow cytometer (Coulter EPICS XL-MCL ADC, Beckman Coulter). Counting time was adjusted to analyze at least 500 basophils. Specifically activated basophils were calculated as the maximum number of activated basophils obtained by allergen stimulation minus the number of activated basophils in the non-stimulated control sample. The allergen concentration needed to activate 50% of the basophils, termed C50 [pM], was calculated from the dose response curves in the following way: For each data set concerning all allergens of one experiment (here all stimulations performed by one operator, maximum number of 7 allergens in 8 serial 10-fold dilutions) the number of data, the smallest value (Min) and the largest value (Max) were identified. The arithmetical mean "M" was calculated from the values for Min and Max of the whole dataset. The linear equation was calculated for each neighbouring pair of values obtained with one allergen and the point of intersection with the horizontal straight line "M" represents the C50 value of the respective allergen. The relative potency (P_{rel}) is defined as the quotient of the C50 values of the reference nPhl p 5 and rPhl p 5.0109.

Establishment of T cell lines and clones and determination of T cell reactivity

Allergen-specific T cell lines (TCLs) and clones (TCCs) were raised from peripheral blood mononuclear cells (PBMCs) of grass pollen-allergic patients by stimulation with either nPhl p 5 or rPhl p 5.0109 as previously described (8, 9). Proliferation assays were performed by culturing the T cells (2×10^4 cells/well) in triplicates upon stimulation with 0.3 μ M of Phl p 5 and 5×10^4 mitomycin C-treated autologous PBMCs/well for antigen presentation. After 48 h of incubation, 1 μ Ci ³H-thymidine was added to each well and further incubated for 16 h. ³H-thymidine incorporation was measured after harvesting and fixing of the cells on filter mats

which were processed with a solid scintillator. The counts per minute were determined in a Microbeta scintillation counter (Perkin Elmer, Waltham, Massachusetts, USA). T cell epitopes were identified in proliferation tests using dodecamer peptides as described previously (10). The results are expressed as stimulation index (SI) which is defined as the quotient of counts per minute (cpm) of the allergen stimulation and the unstimulated control.

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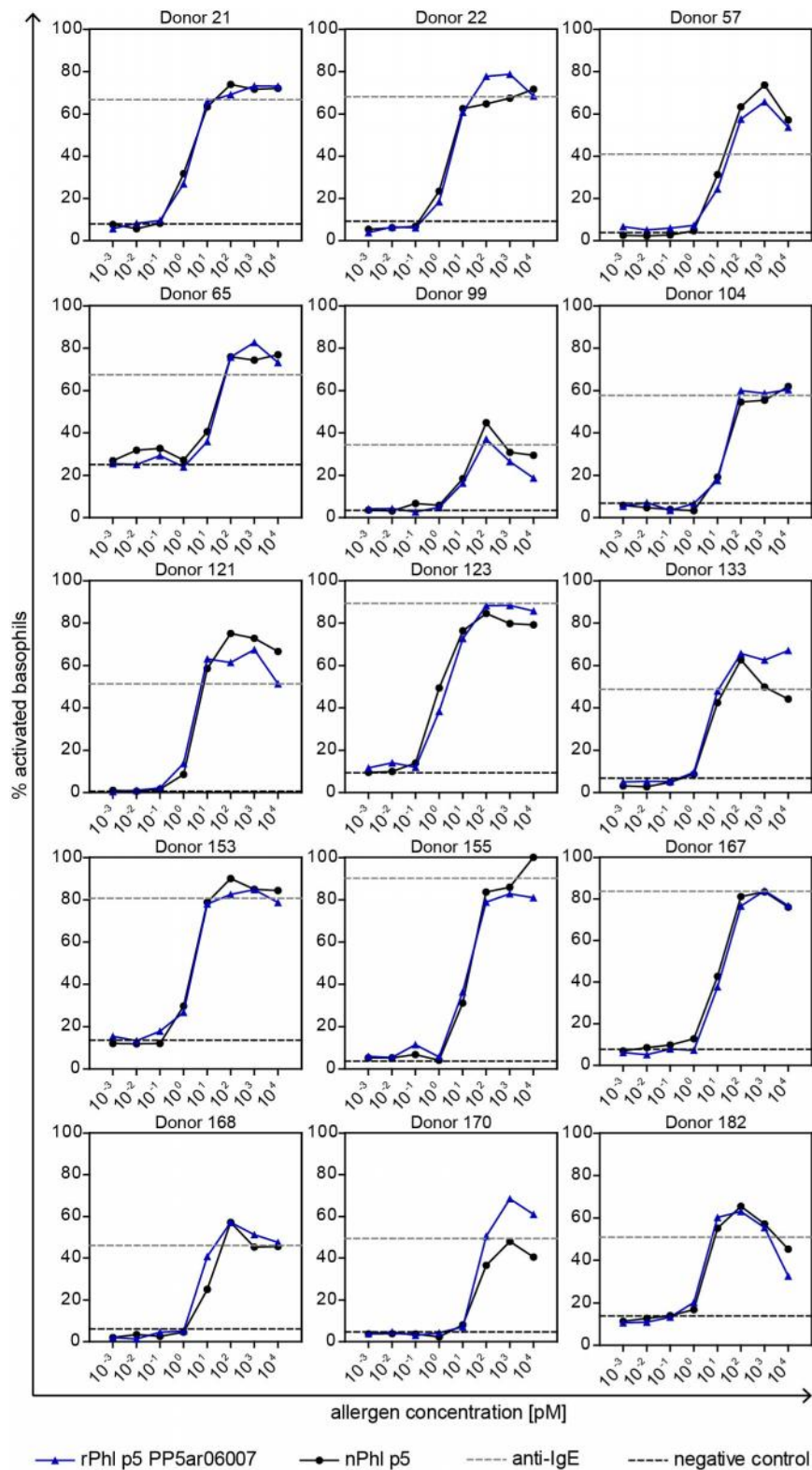


Figure S1. Activation of human basophils titrated by a 1:10 dilution series of rPhl p 5.0109 PP5ar06007 (blue) and nPhl p 5 (black) determined by up-regulation of CD203c on heparinized blood samples from 15 different grass pollen-allergic patients. Culture medium was used as negative (black dashed line), anti-IgE as positive control (gray dashed line).

Table S1. Quality assessment of three production batches of recombinant Phl p 5.0109.

Tests performed	Batches		
	PP5ar06007	PP5ar08012	PP5ar10015
Appearance			
clarity, colour	Clear, colourless	Clear, colourless	Clear, colourless
Identity			
Western blot	Conform	Conform	Conform
Purity			
HPSEC (monomer %)	>99	>99	>99
SDS-PAGE (%)	>95	>95	>95
rPhl p 5 content			
OD at 280 nm*	1.55	1.53	0.94
pH	7	7	7
Microbiological quality			
CFU/ml	0	0	0
DNA content			
DNA assay (pg/mg)	<6	<6	<9
HCP content			
ELISA (ng/mg)	<5	<5	<5
Endotoxin content			
LAL (EU/mg)	<1.0	<1.0	<1.0

ND, not determined; CFU colony forming units; LAL, Limulus Amoebocyte Lysate. *1 A280=1.57 mg/ml

Table S2. Peak list of base peak intensity chromatogram corresponding to the best ionizing tryptic peptides (sequence coverage T2 of Figure S1.B) of rPhl p 5 PP5ar06007 as determined by Q-ExactiveTM MSMS.

Peak ID	retention time [min]	determined mass [Da]	theoretical mass [Da]	aa positions
1	18.5	1288.6877	1288.6874	31 - 41
2	22.0	1104.5455	1104.5451	76 - 86
3	24.6	1418.7321	1418.7405	219 - 232
4	26.0	1540.8363	1540.8361	48 - 63
5	26.3	1212.6137	1212.6139	64 - 75
6	27.7	1198.6602	1198.6597	233 - 242
7	30.2	1070.5654	1070.5648	233 - 241
8	30.4	1294.7485	1294.7496	153 - 164
9	32.0	1974.1153	1974.1149	134 - 152
	32.0	1057.6176	1057.6171	209 - 218
10	34.2	2620.3460	2620.3456	253 - 283
11	35.9	2680.3141	2680.3132	1 - 30
12	37.4	2719.4152	2719.4140	253 - 284
	37.4	1470.7505	1470.7507	185 - 197
13	38.7	2722.3172	2722.3238	acetyl-1 - 30

ID, identity; aa, amino acid.

Table S3. T cell reactivity of 23 T cell lines (TCL, # after “.”) and 2 T cell clones (TCC, # after “:”) from 11 grass pollen-allergic patients (first #) to natural vs. two recombinant production batches of Phl p 5.0109. Stimulation indices are given. Nine different, partially overlapping T cell specificities were determined by epitope mapping, as given in amino acid (aa) positions of the Phl p 5.0109 sequence.

TCL/TCC	nPhl p 5	rPhl p 5.0109 PPar06007	rPhl p 5.0109 PPar10015	epitope aa position
8.2	43.25	21.64	21.01	172 - 186
8.3	22.16	10.33	11.70	229 - 243
19.1	122.95	69.40	64.76	214 - 225
21.198	4.34	3.77	3.73	124 - 138
21.207	4.60	4.00	4.48	172 - 186
21.210	3.25	2.42	2.57	217 - 228
49.1	5.83	2.32	2.25	n.d.
49.1:16B9	235.27	151.81	167.15	124 - 135
49.1:18B7	2.61	2.34	2.08	n.d.
55.193	113.04	116.37	119.54	40 – 54, 163 - 174
55.203	30.55	27.54	28.63	40 - 54
59.57	78.14	82.12	92.34	232 - 246
59.91	2.52	2.41	1.77	172 - 183
65.115	2.28	1.77	2.00	139 - 150
70.126	2.04	2.05	1.98	229 - 243
128.40	67.20	46.48	45.05	196 - 213
128.71	5.14	3.94	4.16	205 - 219
128.73	19.75	17.12	16.49	n.d.
149.89	12.47	11.99	11.36	61 - 72
168.10	5.61	4.31	4.30	61 - 72
168.15	4.02	3.06	3.18	172 - 186
168.26	45.80	38.25	46.05	61 - 78
168.58	19.88	16.67	16.71	n.d.
168.59	10.32	9.43	9.36	n.d.
168.62	4.62	4.47	4.82	n.d.

n.d., not determined.