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

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Keywords
BSP090; European Directorate for Quality of Medicines and Healthcare; good manufacturing practice; Ph. Eur. reference standard; recombinant Phl p 5 allergen.

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 the quantification of major allergen content. The objective of this study was to examine 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 focusing, size-exclusion chromatography with light scattering, circular dichroism, and infrared spectroscopy. Upon storage at +4°C, PP5ar06007 retained the monomeric state for at least 2 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.

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 the determination of the IgE potency, performed by competitive IgE-binding assays and
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(A) Insert

T1
V8
T2
ADLGYGIFAPAIPAAGYTPAAAPAGEAPEGXATTEKQLIE
T1
V8
T2
KINAGFKALAMAAAGVPPADKRTFTVATFGAASNKAFARG
T1
V8
T2
LSGEPKGAAESESKAALSKLDAAYKLYTAEQATPEAK
T1
V8
T2
YDAYVATLSEALHIAGTEVHAAVPFAAEVEVIPAGELQ
T1
V8
T2
VIKVDAAKVAATAANAAAPANDKFTVFEAAAFNAILAST
T1
V8
T2
GGAYESKFIPALEAVKQAYATAVATAEVYTVFEATAL
T1
V8
T2
KKAITMASEAQKAARKPAAATATAATSAVGAAATGAAATTG
T1
V8
T2
|GIYRV|

(B) Relative abundance

(C) # of amino acid residues

Retention time (min)

Mass

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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 endeavor 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, Angelholm, 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 (Fig. 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 (Fig. 1A insert). Second, nanoLC-MSMS-based peptide mapping of tryptic and V8 protease digests resulted in a merged sequence coverage of 100% (Fig. 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 theoreti-
cal 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 the 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 measure-
ments. 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 devia-
tion <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 
down. 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 the 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 (Fig. 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 contami-
nation. Under native conditions, an isoelectric point (pI) of 
approximately 8.6 was determined (Fig. 2B) contrasting the 
thoretical pI of 7.9; however, such deviations can frequently 
be observed for recombinant proteins under nondenaturing 
conditions. Isoelectric focusing serves primarily for monitor-
ing 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 ver-
ified 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 spectromet-
try. Accordingly, no aggregates or oligomers were detected 
by HPSEC light scattering (Fig. 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 (Fig. 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 (Fig. 3B). Upon heat-
ingen, denaturation temperatures of 66°C and 67°C were deter-
mined, and a high degree of reversibility of protein unfolding 
was shown from the denaturation curves for the recombinant 
and natural preparations, respectively (Fig. 3A and B, right 
panel). However, the denaturation curves showed less signifi-
cant inflection points, and the CD spectra recorded immedi-
ately after renaturation showed lower maximal and higher 
minimal ΩΔν values. Notably, after some time at room tem-
perature, PPar506007 gained its normal appearance in CD 
spectrum (data not shown), indicating that the refolding 
process takes longer than the experimental time. These find-
ings were also verified using FTIR spectroscopy, where a 
strong sigmoidal decrease of the integral 1620–1655/cm, 
corresponding to the amide I vibration range of alpha 
helices, was observed (Fig. 3C). The decrease in alpha-helical 
secondary structure content was concomitant with an 
increase in random coil (integral 1658–1700/cm) and inter-
chain beta-sheet (integral 1605–1626/cm) 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, after heat denaturation, still soluble portion of 
PP5ar06007 did not form aggregates as investigated by DLS 
(Fig. 4A, dotted line).

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

The aggregation behavior in solution was investigated using 
DLS (Fig. 4A, solid line). The determined hydrodynamic 
radius (Rhi) of 3.1 nm indicated a theoretical MW of 48 kD, 
assuming that the molecule adopts a globular shape. More-
ever, this peak appeared to be rather broad with a polydis-
persity 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 RH 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 2 months showing no significant changes (Fig. 4A, dashed line). Moreover, a sample stored at /C0°C with intermittent thawing and freezing steps was retested after >8 years upon additional thermal stressing for >3 days at >50°C (but below Tm) showing <1% aggregates (Fig. 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 and 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 /C0°C with intermittent thawing/freezing and was not susceptible to heat stress below Tm.

Using FTIR of PP5ar06007 in saline solution, a secondary structure content of 46.7% alpha helices and 8.4% intrachain beta sheets was determined (Fig. 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) 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 /C0°C. Its stability is monitored annually by triple parallel measurement relative to a baseline reference (stored at -20°C).
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Figure 3 Folding and thermal denaturation analysis of (A) Phl 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 Savitzky–Golay-smoothed denaturation curves of the recombinant and natural preparations, respectively. All CD values are given as mean residue molar ellipticity θ_mrw.

-80°C) and in comparison with 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 the 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 (Fig. S1), the concentrations of allergen needed for half-maximal basophil activation (C50 values) were determined. The C50 values of PP5ar06007 and five other recombinant production batches were expressed in relation to nPhl p 5 as Prel values (Fig. 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 the 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’s correlation of ρ = 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 that 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 for 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 have 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 toward 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 denaturation/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.

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Conflict of interest
Dr. Nandy reports personal fees from Allergopharma GmbH & Co. KG, during the conduct of the study; Dr. Kahlert reports personal fees from Allergopharma, during the conduct of the study; Dr. Thilker reports personal fees from Allergopharma, during the conduct of the study; Dr. Neubauer reports grants from European Union, during the conduct of the study; Dr. Klysner reports personal fees from Allergopharma, during the conduct of the study; Dr. van Ree reports grants from European Commission, grants from Dutch Science Foundation, grants from Netherlands Lung Fund, outside the submitted work; and Consultancy for HAL Allergy BV, Leiden, The Netherlands; Dr. Vieths reports personal fees from Food Allergy Resource and Research Program, Lincoln, NE USA, personal fees from Medical University of Vienna, Austria, grants from Monsanto Company, personal fees from American Academy of Asthma, Allergy and Immunology, personal fees from Deutsche Dermatologische Gesellschaft, personal fees from Westdeutsche Arbeitsgemeinschaft für pädiatrische Pneumologie und Allergologie e.V., Köln, Germany, personal fees from Gesellschaft für pädiatrische Allergologie und Umweltme-dizin, personal fees from Ärzteverband Deutscher Allergologen, personal fees from Swiss Society for Allergy and Immunology, personal fees from Schattauer Allergologie Handbuch, personal fees from Elsevier Nahrungsmittelallergien und Intoleranzen, personal fees from Karger Food Allergy: Molecular Basis and Clinical Practice, non-financial support from German Research Foundation, non-financial support from Federal Institute for Risk Assessment, non-financial support from European Directorate for the Quality of Medicines and Health Care, non-financial support from European Academy of Allergy and Clinical Immunology, non-financial support from Deutscher Allergie- und Asthamabund, non-financial support from Association Monégasque pour le Perfectionnement des Connaissances des Médicins, non-financial support from Federal Office of Consumer Protection and Food Safety, non-financial support from German Chemical Society (GDCh), non-financial support from AKM Allergiekongress, non-financial support from International Union of Immunological Societies, outside the submitted work; Dr. Fereira reports personal fees from AllergenOnline Database, from Indoor Biotechnologies, from SIAF, Davos, and from HAL Allergy, outside the submitted work. The other authors have no conflicts of interest to disclose.

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

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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