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Quality Control of Recombinant Pollen Allergens

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Abstract: Allergen-specific immunotherapy (SIT) is an effective form of treatment for IgE-mediated type I allergy and represents the only curative approach. Presently SIT is performed with complex natural extracts that contain allergens (representing active pharmaceutical ingredients, APIs) together with non-allergenic proteins and other macromolecules. These extracts are difficult to standardize and it is not possible to define each component quantitatively. In order to overcome such difficulties in standardization and batch-to-batch consistency, highly purified recombinant allergens (r-allergens) and hypoallergenic variants may be considered as novel candidates for the production of high quality therapeutic preparations offering safer and more effective SIT.

The availability of detailed information on the characteristics of r-allergens makes it possible to apply a variety of methods in order to ensure purity, identity, quantity, safety and potency compared to allergen extracts. Besides determination of potential process-related contaminants such as endotoxin and residual host-cell-protein a central consideration focuses on the investigation of the folding status, as pathologic IgE-binding is highly dependent on the conformation of the molecules. The primary structure on the other hand determines the therapeutic potential through the specific T cell epitopes and the ability to induce a curative reorientation of the T-cell response. Suitable methods for evaluation of the relevant properties include immunologic methods such as ELISA and Western blots together with robust chromatographic and electrophoretic techniques. Promising results from early clinical studies suggest that the use of r-allergens for SIT will become a reality and therefore it is appropriate to consider how all aspects of the quality of these proteins might be evaluated.

Keywords: Recombinant allergen, Quality control, Clinical trial.

INTRODUCTION

Type I allergy is an immunoglobulin E (IgE)- mediated hypersensitivity disease which affects almost 25% of the population and which is continuing to increase in prevalence [1]. Exposure to the allergen may evoke in sensitized individuals symptoms like allergic rhinoconjunctivitis, bronchial asthma, and anaphylactic shock, as well as atopic dermatitis. The most important airborne allergen sources are grass- and tree pollen.

Allergen-specific immunotherapy (SIT) is the only known causative treatment for IgE-mediated allergies. It has been shown to be effective especially in seasonal rhinitis and asthma [2].

The use of SIT was first documented more than 90 years ago [3] and until now vaccines were prepared in essentially the same way. Using raw allergenic materials, an aqueous extract is produced and formulated for subcutaneous or oral administration. The allergen extracts represent rather crude mixtures comprised of allergens, defined as IgE-reactive proteins or peptides, and non-allergenic components. It is recommended that standardized allergen extracts should be used for diagnosis and treatment, but a major limitation of currently used preparations is the potential variability regarding composition and allergen content and the

difficulties with respect to standardization of [4]. A key element for standardization is the maintenance of well characterized in house reference standards containing known amounts of relevant allergens [5]. A list of currently standardized tree, grass and weed pollen allergens is available [6].

An improvement regarding formulation was achieved by a process called allergoidization (a chemical modification), resulting in reduced IgE-binding but retention of immunogenicity [7,8]. However, neither allergoidization nor the use of adjuvants can overcome any short-comings of the crude allergen extracts.

The tremendous progress in the molecular biology of allergens now makes it possible to improve upon natural allergenic products by use of recombinant allergens [9,10] and modified hypoallergenic variants [11,12]. Very recently, the first clinical studies using recombinant grass pollen allergens [13] and hypoallergenic variants of the birch pollen major allergen Bet v 1 [14] have been conducted and resulted in highly promising results with significant improvements of the symptom-medication score, quality-of-life etc. [13]. A preparation of unmodified Bet v 1 is also being evaluated [15]. In order to enable a standardized quality to be achieved, a set of suitable analytical methods in terms of robustness, validity and reproducibility are necessary for routine control, equivalent to those for natural allergen extracts [5]. The establishment of such requirements for recombinant allergen preparations is currently being undertaken.

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The methods discussed below focus on recombinant allergens produced in bacteria such as *Escherichia coli*. Eukaryotic expression systems, e.g. *Pichia pastoris* or baculovirus infected insect cells, could increase analytical assessment due to potential post-translational modifications such as glycosylation [17, 18].

Pharmaceutical proteins should be analyzed with respect to identity, purity and potency. Depending on the performance, many methods are able to cover various of these aspects. In the case of allergens, a central point to consider is the molecular conformation, since reactivity of allergens is primarily mediated by conformational IgE-epitopes rather than sequential epitopes. This fact is particularly important when the molecules are to be used for diagnostic applications or to contribute the design of recombinant hypoallergenic variants by way of genetic manipulations [11] or special production methods [19,20]. There is still some debate as to which methods are appropriate and realistic for the determination of potency of those allergens or derivatives intended for specific immunotherapy, but determination of IgG-reactivity may be appropriate rather than to IgE, which is relevant for diagnostic preparations. As immunization yields a specific immunologic IgG-response, animal antisera might be used to determine antigenicity as a basis for routine batch release as is the case for many vaccines.

Many powerful methods for initial investigation of properties of recombinant allergens have already been described, from which suitable robust and reproducible procedures should be selected for standard quality control. Quantitative methods are preferable to enable specifications rather than only qualitative documentation. Some relevant methods are discussed below, of which a selection might be representative enough for a reliable and safe routine control.

1. Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The separation according to molecular size in the presence of detergents is one of the most popular methods for allergen and protein characterization. This method enables both determination of molecular weight, an important parameter for the identity of a protein, and estimation of purity if a known amount of reference is applied in parallel [21]. Novel technologies such as automated systems using capillary SDS-gel electrophoresis combine analysis of amount and purity together with high throughput.

Special attention should be focused on allergens containing one or more cysteine residues, since disulphide bridging directly influences the three-dimensional structure. The addition or absence of reducing agents frequently effects the apparent molecular weight of the monomer [22, 23] and enables differentiation of inter- and intra-molecular disulphide-bridging, the former reflected by the presence of dimers or oligomers. Differences in allergen mobility using both conditions therefore represent a characteristic parameter for identification of individual allergens and contribute to the estimation of product related impurities, which is especially important for eukaryotic proteins expressed in bacteria. In addition, the detection of process related contaminants exhibiting a similar size is enhanced, since an equivalent mobility shift is improbable. As an example, Fig. (1).

demonstrates the effect of reducing agents on the recombinant major grass pollen allergen Phl p 1 separated by automated SDS-gel electrophoresis (A and B) and conventional SDS-PAGE (C).

2. Native-PAGE

Though native PAGE represents an even simpler method compared to SDS-PAGE, the application might be powerful in resolution and identification of proteins. The separation is mainly based on charge and size, together with shape of the molecules. It has been shown that molecules of similar size and even isoforms of allergens may be clearly identified by this method [24]. Native PAGE has also proved to be suitable for fusion proteins [25] and, with method modifications, for basic allergens [20]. Application of this technique contributes to the analysis of both identity and purity.

3. Isoelectric Focusing (IEF)

Isoelectric focusing provides separation of proteins according to their isoelectric point and has been used for characterization of purified natural allergens [26,27] as well as identification of isoforms, mostly performed with a SDS-PAGE yielding a two dimensional separation (2D) [28-30]. For recombinant allergens, IEF enables verification of homogeneity with respect to post-translation modifications or folding conformations [31]. However, compared with other electrophoretic techniques, the method is conducted far less, presumably due to the difficulties sometimes encountered with interpretation of the results [15]. It might therefore be a good choice for comparisons of several batches to show consistency of production. Fig. (2). shows a comparison of three different batches of the recombinant grass pollen allergen Phl p 6, demonstrating batch to batch reproducibility in terms of homogeneity of the isoelectric point.

4. Immunological Investigation of Immobilized Allergens

For immunological investigation allergens may be immobilized on a carrier membrane by either direct application, e.g. dot blot [32-34], or after electrophoretic separation by blotting techniques [35-37]. Subsequent analysis can be performed with allergen specific monoclonal or polyclonal antibodies in order to demonstrate molecular identity. IgG-reactivity may also be judged as a proof of potency, as strong serum IgG responses are obtained following administration of allergens during the course of sit [13,14]. There is however one caveat to this approach since there is a paucity of evidence showing that a therapeutic effect is directly related to IgG-production [38,39]. For potency determination a quantitative method such as ELISA using animal antisera would be favorable (see below). Estimation of potency using blotted allergens can be made using inhibition assays [37,40,41].

Also, serum derived from allergic patients may be suitable for detection of specific IgE reactivity and may contribute to identity and potency of allergens. However, in the case of hypoallergenic variants IgE-reactivity may be very weak [42], so that specific animal derived sera could be used for this purpose. Fig. (3). demonstrates that various

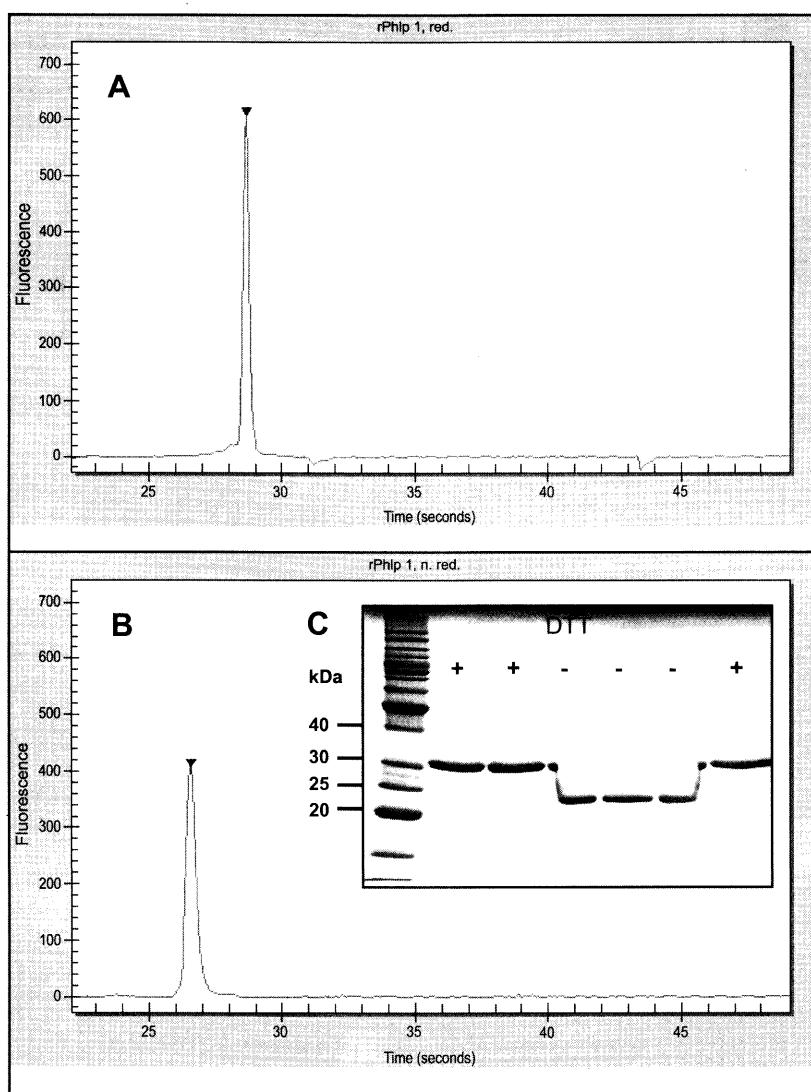


Fig. (1). Automated and conventional SDS-PAGE analysis of recombinant major grass pollen allergen Phl p 1. Recombinant Phl p 1 was subjected to automated SDS-PAGE using an 'Experion System' (Bio-Rad, Munich, Germany) under (A) reducing and (B) non-reducing conditions. The insert (C) shows separation by conventional SDS under reducing (+, addition of DTT) and non-reducing (-) conditions.

hypoallergenic variants of the major birch pollen allergen Bet v 1 do not possess detectable IgE-reactivity. Consequently, this analysis contributes to safety aspects only. In contrast, employment of specific polyclonal antibodies enabled both specific verification of identity as well as demonstration of immunogenic activity.

5. Size-Exclusion Chromatography (SEC)

Separation by size-exclusion chromatography is achieved on the basis of size and shape of molecules. This technique has been conducted for determination of molecular weights of allergen extract components [43,44], quantification of allergen split products [45] and characterization of recombinant hypoallergenic folding variants [19]. Depending on the aim, SEC may be used to demonstrate identity in terms of retention times, purity in terms of relative peak areas and, if appropriate, consistency of folding of recombinant allergens. The latter could also serve as an indicator for IgE-reactivity,

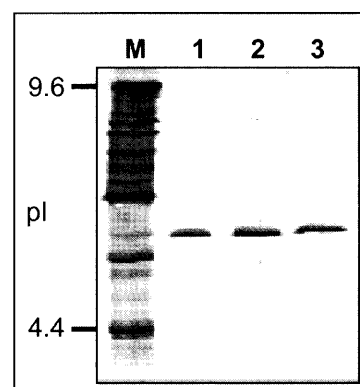


Fig. (2). Isoelectric focussing (IEF) of recombinant grass pollen allergen Phl p 6. An IEF standard mixture (lane M) together with three different batches of rPhl p 6 (lanes 1-3) were separated in a polyacrylamide gel containing urea and ampholytes ranging from pH 4.4 to 9.6 as indicated.

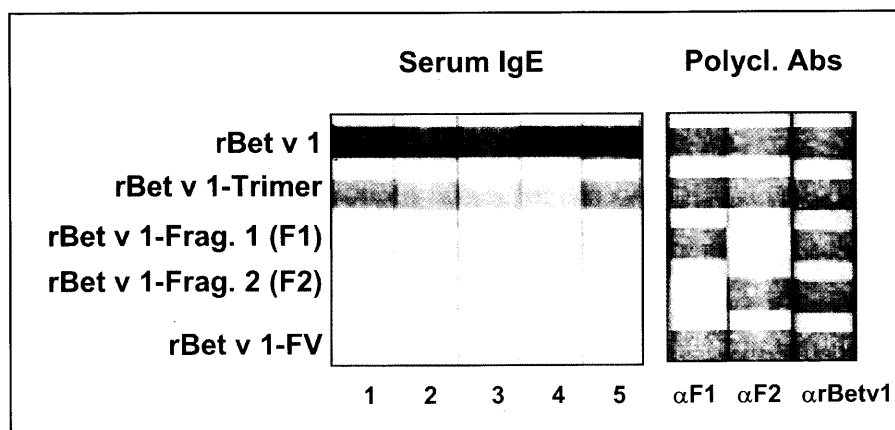


Fig. (3). IgE and IgG reactivity of immobilized recombinant birch pollen allergen Bet v 1 and variants thereof. Recombinant Bet v 1, a genetically generated rBet v 1 -trimer [11], two fragments of Bet v 1 [11] and a folding variant [17] were immobilized to a nitrocellulose membrane [32] and investigated for IgE reactivity using patients sera (left panels) and animal derived specific polyclonal sera (right panels) as indicated.

as IgE-epitopes are highly dependent on conformation [19,46,47].

In addition, SEC provides a suitable technique for detection of di- and multimerization of allergens, which is of major importance in respect of cross-linking of IgE on mast cells [48] and therefore contributes to requirements for safety.

Fig. (4) shows an example of a typical SEC profile from a purified recombinant grass pollen allergen Phl p 5b.

6. Reversed Phase Chromatography (RPC)

RPC represents a common and sensitive method for detection of impurities and post-translational modifications. Despite the denaturing character of the separation, RPC provided high resolving capacity for preparative isolation of major grass pollen allergens [49]. The technique has also been successfully applied to identification of glycosylation patterns [50] and comparison of natural and recombinant allergens [24,51]. Application of RPC therefore contributes

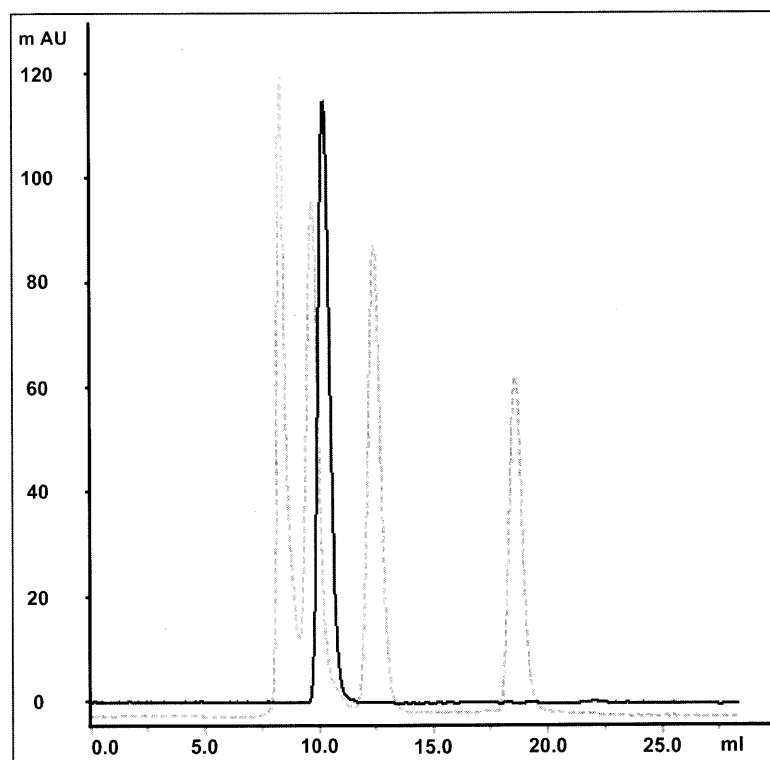


Fig. (4). Size-exclusion chromatography of recombinant grass pollen allergen Phl p 5b. Recombinant Phl p 5b (solid dark line) and a size-exclusion marker (dotted light grey line) were subjected to an analytical superdex 75 column (GE-Healthcare, Munich, Germany) using phosphate buffered saline as running buffer.

especially to determination of identity and purity. Another aspect could be important in the case of mixtures of recombinant allergens [13], as separation of individual components is efficiently achieved, which might sometimes not be the case with electrophoretic or other chromatographic methods. The separation power is exemplified in Fig. (5) for recombinant Phl p 6, Phl p 5b and Phl p 5a.

7. Capillary Electrophoresis

Capillary electrophoresis represents a fast and sensitive technique which allows analysis of minute amounts of sample. Similar to gel electrophoresis, several modes of action can be employed for protein analysis [52]. One of the simplest, but widely used applications is capillary zone electrophoresis (CZE). CZE separates proteins according to differences in the charge-to-mass ratio, which is similar or equivalent to Native-PAGE (see above), and provides a suitable tool for the determination of conformation [53]. The method has also been employed for the quantification and purity determination of recombinant Bet v 1 [54] as well as investigation of airborne dust samples [55].

8. Mass Spectrometry

Mass spectrometry enables a precise structural characterization to be made in terms of molecular mass, and has been used for the substantiation of identity of many allergens including the grass pollen allergens Phl p 4 [56] and recombinant Phl p 13 [57]. Though it is a demanding technique, it should at least be conducted during the development process for a few batches in order to confirm the calculated molecular weight. As an example, mass spectrometry determination of recombinant Bet v 1a with a calculated mass of 17439.58 (acc. to EMBL Pepstat) using ESI-Q-TOF-MS yielded 17439 ± 1 [54] and with a Q-TOF-

micro instrument 17440.47 ± 1 [unpublished results]. The technique was also applied for the differentiation of isoforms of the grass pollen allergen Phl p 6 [58] differing by only two amino acids.

9. Peptide Mapping

Peptide mapping provides a means to investigate the primary and, if disulphide bonds are present, secondary structure of an allergen [59]. This method was also applied for the identification of carbohydrate moieties of natural allergens [60] and detection of allergen isoforms [24]. For routine testing, a peptide map of an allergen provides detailed information for identity, purity and sequence conformity. Amino acid polymorphisms may have an impact on T-cell reactivity [61] and could therefore influence the therapeutic potential of a preparation. This aspect is covered by peptide mapping of allergens.

Typically one or more specific proteases, e.g. trypsin, are used to digest the protein of interest. Separation of peptides is subsequently conducted by RP-HPLC, SE-HPLC or mass spectrometry.

As an example of sequence confirmation, Fig. (6) displays peptide maps of three recombinant Phl p 2 molecules. Compared to rPhl p 2 (Fig. 6A) a clear differentiation is enabled to a purified product related impurity (Fig. 6B) and a recombinant variant differing in 6 amino acids (Fig. 6C). Information can be enhanced by tracing two different wave lengths, as only a part of the peptides contain amino acids absorbing UV at 280 nm [24].

10. Determination of Potency

The batch control of allergen extracts frequently relies on the binding of IgE-molecules to allergens. This may be seen

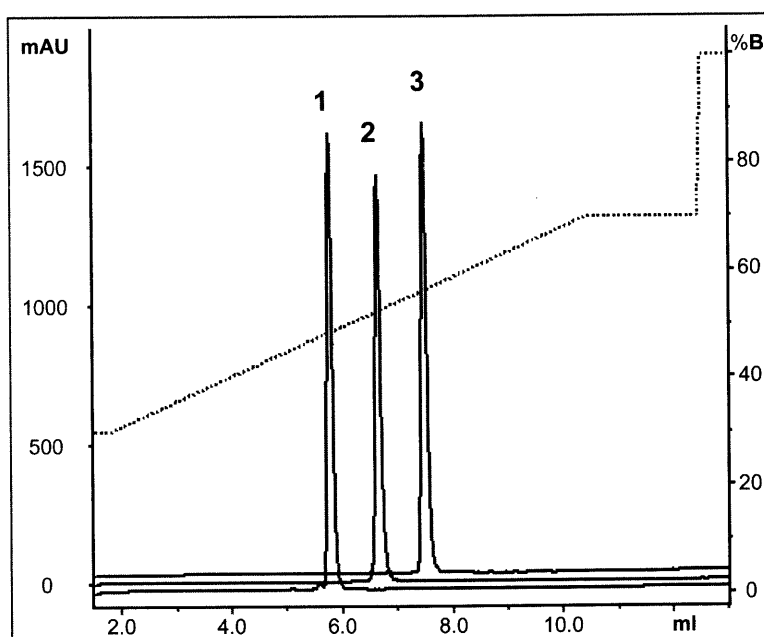


Fig. (5). Reversed-phase chromatography of recombinant grass pollen allergens Phl p 6, Phl p 5a and Phl p 5b. Overlay of elution profiles of rPhl p 6 (1), rPhl p 5a (2) and rPhl p 5b (3) using an acetonitril gradient (dotted line) as indicated.

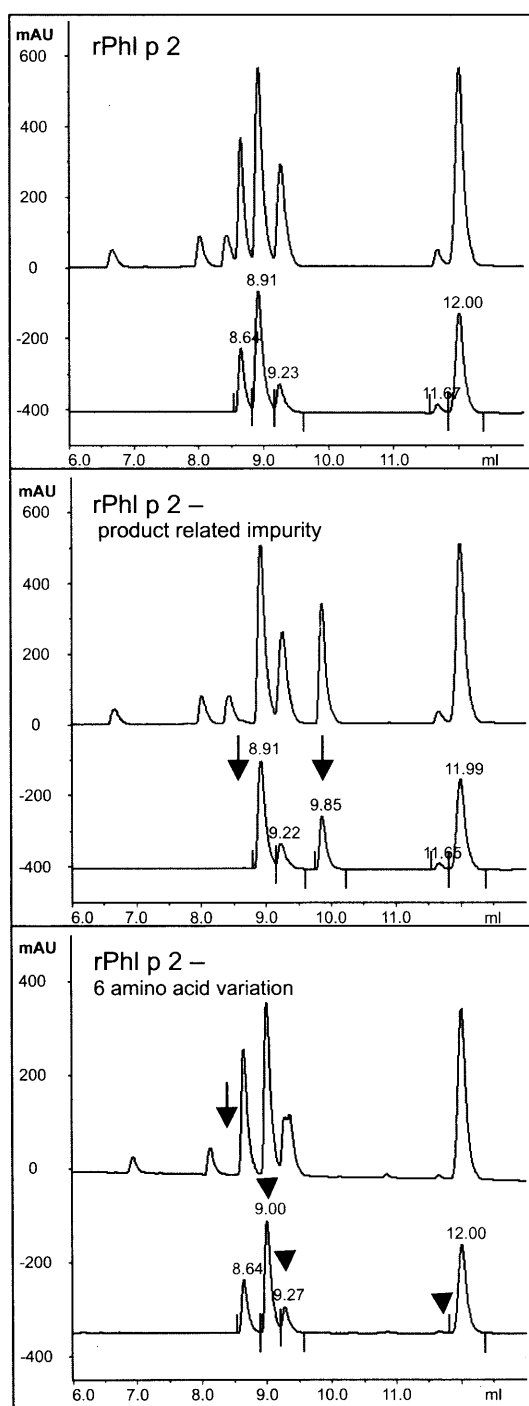


Fig. (6). Peptide mapping of recombinant grass pollen allergen Phl p 2 and similar molecules. Proteolytic digestion of rPhl p 2 (top), a purified product related impurity (middle) and a variant exhibiting 6 amino acid exchanges (bottom) was conducted by addition of trypsin followed by separation using reversed-phase chromatography [22]. In each chromatogram, the upper line displays detection at 220 nm, the lower line detection at 280 nm. Arrows indicate differences relative to rPhl p 2.

as a measure of potency for diagnostic applications, but its relevance for therapeutic preparations is primarily to ensure their safety. Basophil mediator release and activation assays,

as well as measurement of IgE-binding, have been used for the characterization of purified natural and recombinant allergens [62-64]. However, IgE-reactivity reflects allergenicity rather than potency, and therefore is better defined as a biological activity as it is not directly associated with therapeutic potential. In the case of hypoallergenic variants it is inappropriate to consider potency in terms of IgE reactivity, which is strongly reduced or not detectable.

Determination of stimulation of human T cells may be more relevant to defining potency [7,42,61], since the success of SIT is considered to be dependent upon the reorientation of the T cell response. However, from a practical point of view such assays do not lend themselves to routine batch release as they are unlikely to prove sufficiently robust and would be difficult to validate adequately.

Beside the effects at the cellular level, another direct link to the intended use of allergen preparations is the induction of specific IgG antibodies [13,65]. ELISA assays based on polyclonal antibodies directed against individual allergens or allergen mixtures represent realistic and specific methods for determination of antigenicity, which in turn can be correlated with immunogenicity. Monoclonal antibodies could also be employed for this purpose [66].

Taken together, routine potency determination of recombinant allergens and hypoallergenic variants thereof might be achieved with an assay using specific polyclonal animal sera, though specific requirements would have to be defined. Measurement of IgE-reactivity could additionally contribute to safety aspects. Demonstration of T-cell reactivity during the course of a development program would be very worthwhile, but technical obstacles will very probably prevent such assays being established for routine use.

11. Residual-DNA

Residual DNA might potentially be present after purification of the API. This is a very important factor to consider when using eukaryotic expression systems such as hybridoma cell lines, as viral- and oncogene-derived sequences have a serious impact on the safety of the preparation.

In the case of bacterial expression systems there is a much lower risk factor associated with residual DNA. In contrast, bacterial sequences contain unmethylated CpG motifs, which effect immunostimulatory reactions [67]. Therefore, residual DNA derived from bacteria or bacterial plasmids even contribute to efficacy of the allergen preparation rather than represent a risk factor. However, it has to be demonstrated whether or not DNA is present in a recombinant allergen preparation.

There are two principal methods recommended for detection and quantification of residual DNA, one sequence-dependent (e.g. hybridization) and one sequence-independent (68).

A fast and reliable technique is provided by PCR (polymerase chain reaction), as theoretically one single DNA-sequence may be detected due to exponential amplification. As demonstrated in Fig. (7), a sensitivity of

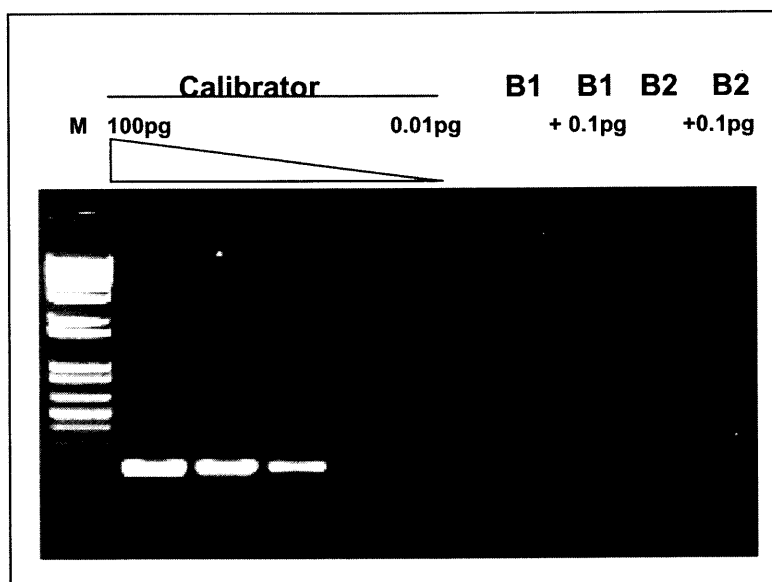


Fig. (7). Investigation of residual DNA in two batches of recombinant Phl p 5a by PCR-technology. After conducting PCR using plasmid specific primers, products were separated by agarose gel electrophoresis and visualized by ethidium bromide. For calibration purpose, defined amounts of plasmid DNA encoding Phl p 5a (calibrator) were used for amplification. One microgram of each of batch 1 (B1) and batch 2 (2) were analysed without and with addition of 0.1 pg calibrator DNA.

0,2 fg/ μ l is typically achieved by using purified plasmid DNA as template. Due to thermal denaturation treatment during amplification a release of DNA potentially attached to basic proteins is enabled. Masked DNA will therefore be available for detection. Validity of the result may be demonstrated by recovery of added DNA to the protein samples. In Fig. (7) two preparations of recombinant allergen Phl p 5a were investigated concerning the content of the corresponding plasmid sequence covering approx. 0,2 kbp. Since plasmids are present in even higher copy numbers than chromosomal DNA, the probability for detecting these sequences should be increased. In this example, a specific DNA content below 10 pg/mg protein could be stated, which is far below the recommended specification of 100 pg/single dose [69], taking into account a protein content between 12.5 to 100 μ g per dose [13,14].

12. Endotoxin and Pyrogen

When using bacterial expression systems the endotoxin (ET) content is of primary interest, since huge amounts of ET is co-produced during the upstream process. Endotoxin levels are typically assessed by LAL (Limulus ameocyte lysate) assay, either as end-point determination [70] or by a quantitatively method [71]. A point to consider is that the presence of any other pyrogen should be excluded in parallel, e.g. by a rabbit fever test, in order to be able to restrict analysis to endotoxin as the only potential pyrogen. Natural allergen extracts are not subjected to routine testing for endotoxin, though they might contain significant levels [72]. Due to the fact that relatively low protein quantities are applied as single doses, endotoxin may generally not represent a major problem. However, care should be taken in the case of cationic proteins, as masking of endotoxin might occur *via* endotoxin-protein complexes [73]. Therefore,

spiking is routinely conducted in order to confirm the validity of the test method.

13. Additional Methods

In addition to methods discussed above, various additional aspects should be considered. Quantification may be conducted by either UV photometry [15,70] together with protein specific extinction coefficients or colorimetric procedures. In the case of UV-quantification detection light scattering is recommended in order to detect and quantify aggregation [74]. When appropriate, sterility of the API solution can be tested by methods described in established guidelines. For detection of host cell proteins (HCP) immunologic methods may be employed, either generic or specific in nature [75]. Such methods are common for every biological substance and are quite easily adapted to recombinant allergen preparations.

CONCLUSIONS

Routine control methods for recombinant allergens or recombinant hypoallergenic variants, should focus especially on the assessment of batch-to-batch consistency in respect to folding and conformation. In most cases, purity and identity determination are covered in parallel. A realistic and feasible potency assessment of those preparations intended for specific immunotherapy may be achieved with assays using poly- or monoclonal antibodies due to the direct relation with the intended use, but it remains to be seen whether or not such an approach will obtain general approval. IgE-reactivity may be determined to ensure the safety of therapeutic preparations and the potency of those preparations intended for diagnostic applications. It seems altogether realistic that specific requirements for recombinant allergens and variants thereof should be regulated by an extension of the existing

Note for Guidance for allergen products [5], as already proposed [16].

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Received: 01 December, 2005

Revised: 23 February, 2006

Accepted: 15 March, 2006