

Transition of recombinant allergens from bench to clinical application

Oliver Cromwell,* Roland Suck, Helga Kahlert, Andreas Nandy, Bernhard Weber, and Helmut Fiebig

Department of Research and Development, Allergopharma Joachim Ganzer KG, Hermann-Koerner-Strasse 52, D-21465 Reinbek, Germany

Accepted 21 August 2003

Abstract

The cloning and production of an increasing number of allergens through the use of DNA technology has provided the opportunity to use these proteins instead of natural allergen extracts for the diagnosis and therapy of IgE-mediated allergic disease. For diagnostic purposes, it is essential that the molecules exhibit IgE-reactivity comparable with that of the natural wild-type molecules, whereas T cell reactivity and immunogenic activity may be more important for allergen-specific immunotherapy. In relation to the latter, the development of hypoallergenic recombinant allergen variants is an approach which shows great promise. Clinical application of the proteins requires that they must be produced under conditions of Good Manufacturing Practice and meet the specifications set down in the appropriate Regulatory Guidelines, principally the ICH-Guidelines. Special consideration has to be given to the choice of expression system, the design of the expression vectors, and the purification strategy to obtain a pure product free from toxins and contamination. The availability of the pure recombinant molecules provides the opportunity to formulate preparations that are free from the non-allergenic ballast proteins present in natural allergen extracts and which contain relative concentrations of the allergens in clinically appropriate proportions.

© 2003 Elsevier Inc. All rights reserved.

Keywords: Recombinant allergen; Grass pollen; Phl p 1; Phl p 2; Phl p 5; Phl p 6; Allergen purification; Allergen characterisation

1. Introduction

The specific diagnosis and causal treatment of IgE-mediated allergic diseases have relied traditionally on the use of aqueous extracts of various allergenic source materials. The majority of such extracts are complex mixtures of proteins, only some of which exhibit allergenic characteristics. In the cases of two of the most commonly encountered causes of allergic responses, namely grass pollen and the house dust mite *Dermatophagoides pteronyssinus*, at least 11 and 16 allergens, respectively, have been identified and well characterised [1,2]. Different allergic patients exhibit different patterns of allergen recognition, and whilst in some cases only one or two allergens may be implicated with an allergic sensitisation, in others a whole spectrum of proteins may be involved. Attempts are often made to define the relative impor-

tance of allergens in terms of the frequency with which sensitisation can be identified in populations of allergic patients. So-called major allergens have traditionally been defined as those which can be associated with sensitisation in more than 50% of subjects showing sensitisation to a particular allergenic source material and conversely minor allergens are reactive in relatively few subjects. The major allergens are the focus of attention in the development of recombinant molecules, since these are the ligands for a large proportion of the allergen-specific IgE antibodies that trigger allergic reactions.

The production of allergen extracts of consistent quality from natural source materials places considerable demands on manufacturers. The demonstration of consistent protein patterns and IgE-reactive allergens, together with quantification of individual allergens and measurement of total allergen-specific IgE-reactivity of the extract, represent important aspects in the standardisation, characterisation, and batch consistency of the extracts [3]. Recombinant DNA technology not only

* Corresponding author. Fax: +49-40-727-65-318.

E-mail address: oliver.cromwell@allergopharma.de (O. Cromwell).

facilitates the characterisation and analysis of the allergenic proteins, but also provides the basis for producing allergens and their derivatives that may be able to be used to formulate optimal preparations for both diagnostic applications and specific causal immunotherapy [4,5].

Diagnostic techniques depend on the ability to demonstrate the existence of specific IgE antibodies directed against an allergen preparation and therefore both in vitro and in vivo diagnostic methods require the use of allergens in their native form that will react with such antibodies [6,7]. Strategies for allergen-specific immunotherapy rely on either the native forms of the allergens or modified forms with attenuated IgE-reactivity. The latter have traditionally been derived by chemical modification of the allergen extracts, but the advent of DNA technology has provided the opportunity to develop and produce hypoallergenic allergen variants using various strategies for gene mutation [8–10]. One advantage of such preparations for specific immunotherapy is that the risk of inducing unpleasant side-reactions can be minimised whilst the therapeutic potential of the preparations is retained. Further advantages of recombinant allergens and their derivatives include the production of preparations of consistent pharmaceutical quality; the avoidance of problems of natural extract standardisation; the inclusion of optimal concentrations of the important allergens; the exclusion of non-allergenic proteins; the avoidance of the possible risk of contamination by allergens from other sources; and exclusion of the risk of introducing infectious agents.

Grass pollen is one of the most important causes of allergic rhinitis and is therefore a logical choice to assess the potential of recombinant proteins for allergen-specific immunotherapy. However, there are several major grass pollen allergens and it is probable that a cocktail of various proteins will be required to effect successful treatment. On the other hand, birch pollen has one predominant allergen, Bet v 1, and it is quite conceivable that patients could be desensitised with this allergen alone. Some of the methods used to develop and produce various wild-type allergens of grass pollen, in particular Phl p 1, Phl p 2, Phl p 5b, Phl p 6, and Phl p 13 from *Phleum pratense* (Timothy grass), are described here, to exemplify various factors that have to be taken into consideration when producing preparations for use in the clinic.

2. Criteria for the production and characterisation of recombinant allergens and allergen derivatives for clinical applications

2.1. Choice of expression system and design of DNA constructs

The heterologous production of allergen proteins for therapeutic use has the advantage that in most cases one

deals with major allergens where the natural counterparts of the recombinant proteins have already been purified and studied in detail. Knowledge about the glycosylation state, the occurrence of internal disulphide bonds, the N-terminal amino acids of the mature allergen, and the overall stability of the protein represents useful information that has to be taken into consideration for the establishment of a powerful expression system.

Escherichia coli is a suitable host for proteins that are not glycosylated or where glycosylation is not necessary. Once the gene of the allergen in question has been cloned from a complementary DNA (cDNA) library, an appropriate expression vector and expression host have to be chosen. In many cases, the gene of interest is cloned directly (ATG-fusion) into the vectors cloning site to express the mature form of the protein. However, for some proteins such as several proteases it has been reported that their pro-peptide may act as an internal chaperone. In these cases, it may be necessary to create an expression construct containing the attached pro-peptide to achieve the correct folding of the target protein [11].

The most commonly used strategy to produce heterologous protein in *E. coli* is cytosolic expression. High-level expression of correctly folded and soluble proteins may be achieved with some allergens, whereas others are expressed as insoluble inclusion bodies (IB). The latter often have the advantage that quite pure protein preparations can be obtained from the washed IB, but an in vitro solubilisation/refolding strategy has to be established to recover the recombinant allergen in a soluble form. The choice of the vector/host system can influence the pathway towards either IB formation or soluble expression to a certain extent. Expression induction at elevated temperature (42 °C) will favour IB formation (vectors containing the Lambda *pL* or *pR* promoter/temperature sensitive repressor *cl ts857*), whereas the use of IPTG-inducible (*L*-isopropyl- β -D-thiogalactoside) Trc-promoters with a reduced amount of inducer (1 mM) and growth at low temperature (28 °C) will favour soluble expression (pSE vectors) of the target protein. The protein of interest may also be expressed fused to sequences that will be helpful for purification (e.g., poly His-tag) or to fusion partners that may improve the solubility and correct folding of the protein of interest (e.g., thioredoxin fusions). Internal cleavage sites, e.g., for tobacco etch virus protease (TEV-protease), enable the delivery of pure non-fusion target protein (pPRO EX HT vector).

The most efficient expression system, however, has to be established for each allergen individually. If the expression level is still low, an optimisation of the codon usage to the most frequently used in *E. coli* and an optimisation of the GC-content may prove to be practical. A subset of codons including AGG/AGA/CGA/CCG

(arginine), AUA (isoleucine), CUA (leucine), and CCC (proline) correspond to rare tRNAs [12]. A relative abundance of any of these codons may result in poor expression, with possible frame-shifts and mis-translations. The use of an *E. coli* strain carrying additional copies of these tRNA genes can overcome the problem (e.g., BL21(DE3)CodonPlus-RIL cells; Stratagene Europe, Amsterdam, The Netherlands).

2.2. Post-transcriptional modifications

In many instances, it can be shown that glycosylation is not necessary for IgE-reactivity, but in certain cases, particularly when the allergen is to be used for diagnostic purposes, production of a glycosylated form may be desirable. For example, the native form of the highly glycosylated Phl p 13 reacts with about 50% of sera from grass pollen allergic subjects, whereas only 21% of patients' sera bind the non-glycosylated form [13,14].

Glycosylated proteins can be produced in several eukaryotic expression systems [15] such as the yeast *Pichia pastoris*, Baculovirus in host insect cells, and various plants including the tobacco plant *Nicotiana benthamiana* [16] and barley [17], but this is not to say that the glycosylation will be comparable with that of the natural glycoprotein. Expression of recombinant Der f 1, one of the major allergens of the house dust mite *Dermatophagoides farinae*, in *P. pastoris* yields highly glycosylated products with more than twice the molecular weight of the natural Der f 1 [18]. In *P. pastoris*, glycosylation of recombinant proteins occurs by addition of multiple mannose units to N-linked glycosylation sites. The different types of glycosylation compared to the natural allergen and the huge size of the unnatural sugar part may cause problems for the use of these recombinant proteins for diagnostics and immunotherapy. However, in the case of Der f 1 IgE-binding seems not to be influenced.

2.3. Solubility

An important pre-requisite for recombinant allergens used in vivo is their high purity, stability, and solubility. Since natural allergens are frequently glycosylated and contain internal disulphide bonds, extensive demands are placed on the production of their recombinant counterparts. If the glycosylation itself is essential to maintain the soluble native state, it is necessary to change from an *E. coli*-based expression system to a yeast, mammalian, insect or plant expression system.

Cysteine-free allergens might be best produced in a soluble and correctly folded state in the cytosol of *E. coli*. Cysteine-containing proteins are often expressed in the form of IB. Most problems of aggregation arise during the refolding process due to exposure of hydrophobic residues in unfolded or intermediate states (e.g.,

molten globule). Several approaches to avoid aggregation have to be tested to establish the optimal conditions for folding each individual allergen. These approaches involve refolding on the column (IMAC) of His-tagged proteins and refolding by dilution and dialysis/diafiltration under conditions that favour disulphide bond formation (refolding buffers containing dithiothreitol (DTT), dithioerythritol (DTE), 2-mercaptoethanol, reduced and oxidised glutathione, and cysteine/cystine). The most efficient strategy for refolding has to be chosen that is simple, easy to perform, and if possible avoids the need for costly reagents such as glutathione.

2.4. Attributes of the various purification methods

Affinity chromatography is a powerful technique for protein purification and one of the most frequently used methods is that of nickel chelate chromatography with Histidine-tagged (His-tag) proteins [19]. The recombinant molecules are expressed as fusion proteins with a leader sequence containing six histidine residues and a specific enzyme cleavage site that allows the His-tag to be removed after the affinity purification step. Other tried and tested systems include those using enzyme/substrate interactions, such as glutathione/glutathione-S-transferase [20], and maltose binding protein [21]. Whilst immobilised specific antibodies can be used to achieve effective affinity purification, the technique is not well suited for large-scale production and furthermore may introduce additional requirements to ensure freedom from viral contamination in conjunction with the intended use in humans.

Ion exchange chromatography (IEX) depends upon interactions of charged solute molecules and oppositely charged moieties covalently linked to a matrix [22]. This adsorption chromatography is widely applicable and offers high resolving power, high capacity, and simplicity. Conductivity and pH are the major factors influencing separations and the fact that these parameters can be easily varied means that the chromatographic processes are highly controllable and reproducible. Moreover, the buffers usually used are simple and economical. Besides being highly effective for the separation of proteins, anion-exchange chromatography is also useful for reduction of nucleic acid and endotoxin levels. Problems that may arise in conjunction with the use of IEX relate either to the condition in which the sample is to be applied or the state in which it is recovered. For example, an additional gel-filtration step (e.g., with Sephadex G-25) may be required to adjust conductivity and excessive dilution may necessitate a concentration step.

Hydrophobic interaction chromatography (HIC) is based on interactions between non-polar groups on the surface of bio-molecules and hydrophobic ligands covalently attached to a gel matrix [23,24]. Contrary to IEX, high salt concentrations are needed to promote

binding of proteins and low salt for elution, facilitating the sequential use of IEX and HIC. HIC is also a powerful method for the removal of hydrophilic nucleic acids. Salts commonly used in HIC are ammonium, potassium or sodium sulphate or phosphate and, at higher concentrations in accordance with the Hofmeister series, sodium chloride. The resolution of separation that can be achieved is influenced by various factors including the choice of hydrophobic ligand (examples of ligands in order of their binding strengths are ether < isopropyl < butyl < octyl < phenyl), the steepness of the eluting salt gradient and the column length. The aryl ligand (phenyl) shows mixed hydrophobic and aromatic binding characteristics, whereas the alkyl ligands display true hydrophobic characteristics. Continuous gradients provide the best resolution, but once the elution characteristics of the target protein have been established in relation to those of contaminating molecules it is often possible to use a step gradient to achieve maximum resolution as well as highly concentrated elution.

Salt precipitation of proteins, usually performed with ammonium sulphate, represents a purification method suitable for laboratory and pilot scale. When contaminating molecules are precipitated and the target molecule remains in solution, then HIC can often prove to be a useful follow-up purification method because the salt-containing supernatant can be applied directly to the column. Ammonium sulphate precipitation can also be used as a concentration step for a final SEC, or for the storage of labile proteins or partly purified proteins in-between separate purification steps in those cases where the protein of interest is sensitive to freeze/thaw cycles.

Size exclusion chromatography (SEC) (or gel filtration) separates molecules primarily according to differences in their sizes [25,26]. Another factor to be considered is the conformation or shape of the proteins. Depending on different three-dimensional structures, it may prove to be possible to separate molecules with identical molecular masses, though resolution is rather low compared with other techniques such as IEX. In relation to production applications, it is important to remember that SEC is not an adsorptive technique and that it requires in general small sample volumes together with high protein concentrations. It is therefore advantageous to use a technique yielding a fraction with a high protein concentration prior to gel filtration. Besides the removal of trace contaminants, SEC can be used to effect a buffer exchange to condition or adjust the sample for storage or lyophilisation. Size exclusion chromatography is recommended as the final step in a purification scheme.

2.5. Protein analysis

Protein preparations can be analysed by several electrophoretic and chromatographic techniques. Sodium

dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) is a useful analytical method for the assessment of final product purity. SDS–denatured proteins are separated on the basis of their molecular weight, since the negatively charged SDS binds with a constant ratio to the unfolded polypeptide chains. Differences between the calculated and the apparent MW observed in SDS–PAGE have been reported for some glycoproteins, glycine-rich proteins, very hydrophobic, very basic, and very acidic proteins. Addition of a reducing agent such as DTT disrupts disulphide bridges and reduces conformational influences on migration to a minimum. The sensitivity of the method is <100 ng protein. Proteins can also be separated electrophoretically in an ampholyte pH gradient on the basis of their isoelectric points or in native PAGE on the basis of their net charge, size, and conformation. The technique of isoelectric focusing (IEF) is utilised to determine product homogeneity with regard to folding variants, fragmentation, and heterogeneity that might derive from storage under unfavourable conditions (ageing) like partial oxidation of sulphhydryl groups in proteins and deamidation of asparagines or glutamines.

Analytical SE-high performance liquid chromatography (HPLC) is particularly useful for detecting the presence of dimers or higher-molecular aggregates as well as proteolytic breakdown products and, to a lesser extent, conformational product variants. Very similar molecules, displaying only minor differences in surface hydrophobicity or amino acid composition (as low as one single amino acid substitution or removal), can be resolved using analytical reverse phase (RP)-HPLC. The main application for this powerful technique is the detection of product related or unrelated impurities including small peptides and conformational variants.

2.6. Clinical applications

In vitro and in vivo diagnoses require allergens that conform to the structure and reactivity of the wild-type molecules as closely as possible and thus express IgE-reactivity that can serve to trigger mast cell and basophil degranulation via IgE in in vivo test situations and bind to serum IgE in in vitro test protocols.

The mechanisms of successful allergen-specific immunotherapy are still not fully appreciated, but it seems clear that modulation of T cell reactivity is a key factor. In this regard, the T cell epitopes are the principal factors involved and therefore it is probably the correct primary structure that is of paramount importance rather than the 2° and 3° structures of the proteins.

2.7. Safety

Before embarking on clinical studies, certain minimum toxicological investigations have to be conducted.

Whilst the various ICH Guidelines and Pharmacopoeia specify criteria for the assessment of preparations for the purposes of being granted marketing approvals, the specific requirements of the national regulatory authority, and the relevant Ethics Committee will determine if and when a clinical trial can go ahead. There is already a wealth of knowledge and understanding concerning natural allergen extracts, which provide a sound basis for evaluating their recombinant counterparts. Against this background, the assessment of recombinant preparations needs to focus on the possible consequences of the recombinant production methods and, in the case of allergen variants derived through gene mutations, the possible changes in biochemical and immunological activity. Various ICH Guidelines cover all aspects of safety relating to the production and use of diagnostic and therapeutic preparations in humans [27–34].

There are potential risks associated with contaminants derived from the host cells. Such contaminants could conceivably have immunopathological effects and nucleic acid contaminants pose theoretical risks, including the possibility that they could be integrated into the host genome. Recombinant products derived from plant, insect, and animal host cells, or from systems including animal products, pose an additional risk of viral infection. The possibility that animal spongiform encephalopathy agents might be transmitted is a matter of great concern, and has served to highlight the type of risk that should be anticipated when producing recombinant proteins for use in humans. In this regard, measures that can be implemented in the production of recombinant allergens include the use of papaya protease digested soya peptone as a component of *E. coli* growth media instead of bovine peptone (pancreatic digest of casein) and glycerol derived from non-bovine sources for long-term storage of master and working cell banks. Animal sourced materials should be avoided whenever possible, but if this proves to be impractical then evidence concerning the source of the material, the integrity of the animals, and freedom from disease and infection, and method of collection and processing must be fully documented.

Safety evaluation should normally involve testing in two relevant animal species, but it may be possible to restrict this to one if the biological activity of the recombinant allergen product is well understood, particularly in respect of longer-term toxicity studies. Single dose studies can yield information on the relationship of dosage to local and systemic toxicity and also on safety pharmacology parameters. The design of repeated dose toxicity studies should reflect the intended route of administration, dosage, and duration of clinical exposure. Although in practice allergen-specific immunotherapy may be continued over periods of 2 or 3 years, toxicity studies of 6 months duration

may be considered to be adequate. Investigation of immunogenicity should also be combined with these studies. Studies to investigate reproductive and developmental toxicity, gene toxicity, and carcinogenicity are generally considered to be inappropriate for biotechnology derived pharmaceuticals, but nevertheless each preparation should be considered on its own merits.

3. Description of methods

3.1. Preparation of first strand complementary DNA

RNA was isolated from 20 mg timothy grass pollen (*Phleum pratense*) using a GlassMax RNA microisolation system (Life Technologies, Karlsruhe, Germany), while reverse transcription of poly(A⁺)RNA was performed using a 3'RACE system (rapid amplification of cDNA ends) and an oligo(dT)-containing adaptor primer (Life Technologies) according to manufacturer's instructions.

3.2. PCR-based cloning and sequencing of timothy allergen cDNA from Phl p 13

A degenerate oligodeoxynucleotide primer (Biometra, Göttingen, Germany) was designed according to the reverse translated N-terminal amino acid sequence of the allergen.

In the case of Phl p 13, the sequence: 5'-GGIAAIAAIGAIAAIAAIGAIGA-3' was used. PCR was performed by adding 1 µL of each degenerate primer and abridged universal amplification primer (Life Technologies) with a concentration of 0.1 nmol/µL to 90 µL 'ready to use' supermix (Life Technologies) containing recombinant *Taq* DNA polymerase. After addition of 2 µL cDNA solution, the volume was made up to 100 µL with sterile double-distilled water. Conditions for the PCR in a Personal Cycler (Biometra) were as follows: initial denaturation of 4 min at 94 °C, followed by 30 cycles of 30 s at 94 °C; 30 s at 50 °C, 2.15 min at 72 °C, and a final extension at 72 °C for 8 min. Analysis of the amplification product was performed in a 0.8% agarose gel in the presence of ethidium bromide (Roth, Karlsruhe, Germany) according to the method of Sambrook et al. [35]. The reaction product was cloned into a pCR2.1 vector using the original TA cloning kit (Invitrogen, Groningen, Netherlands). Sequencing revealed four isoforms of Phl p 13 differing by up to nine amino acids and the respective cDNAs were subcloned. Expression of the isoforms and investigation of the expression products in terms of IgE-binding activity and T cell reactivity were necessary to define the most suitable forms for diagnostic and therapeutic purposes.

3.3. Expression constructs encoding Phl p 2 and 5b in *E. coli*

The cDNAs encoding the grass pollen allergens Phl p 2 and 5b were ligated into the pSE420 and pProEX HT-expression vector (Life Technologies, La Jolla, CA, USA) using the *NcoI/HindIII* and *EheI/HindIII* restriction sites, respectively. The primary expression products contained an N-terminal 6× His-tag followed by a spacer sequence with a TEV cleavage site (Fig. 1). Phl p 2 was expressed directly following the start-codon AUG without additional sequences but the N-terminal methionine. After subcloning, sequencing of propagated plasmids and expression analysis verified the expression constructs.

3.4. Expression of recombinant Phl p 5b or the Phl p 5b-DM4 in *E. coli*

An aliquot from the corresponding working cell bank (see below) was used to inoculate 500 ml complex medium LB (per litre 10 g soya peptone, 5 g granulated yeast extract, and 10 g sodium chloride, pH 7.0). After overnight cultivation for 18 h in a shaking incubator at 37 °C and 120 rpm, cells were grown to the stationary phase. This pre-culture was transferred under aseptic conditions (laminar flow class A) into a Biostat C20-3 bioreactor equipped with a 30 L vessel (BBI, Melsungen, Germany) using steam-autoclaved inoculation devices. Previous preparation of the bioreactor included calibration of the pH- and oxygen electrodes, in situ sterilisation of the culture medium, and clean steam sterilisation of outlet valves. The batch fermentation process was automatically regulated and monitored online with respect to pH, oxygen, temperature, dosing systems (e.g., base, acid, and antifoam), and stirrer speed. Process air supply was adjusted manually to 1 v/v/m. Every 0.5–1.0 h, a 10 ml sample was turbidimetrically analysed by OD₆₀₀ measurement and subjected to SDS-PAGE. For induction of expression, IPTG was supplemented in the mid-log-phase via an aseptic seal on the culture vessel. After stopping fermentation, har-

vesting was performed by direct connection of a continuous-flow centrifuge (Contifuge, Kendro, Hamburg, Germany) to the outlet valve (300 ml/min, 12,000g, 10 °C). The cell paste was resuspended in lysis buffer, sonicated, and stored prior to downstream processing in a temperature-controlled cold room at –20 °C. Specific safety requirements regarding waste (e.g., supernatant, contaminated accessories) were fulfilled by either autoclaving or chemical inactivation.

Smaller-scale production was performed in 2 L-baffled shaking flasks filled with 750 ml LBS-complex medium (per litre 10 g soya peptone, 5 g granulated yeast extract, and 10 g sodium chloride, pH 7.0). The shaking incubator was sterilised by UV light before use and located under a laminar flow providing aseptic conditions during manipulations, e.g., addition of inductor, sample taking. The temperature was monitored by two temperature loggers previously programmed using a RS232/9 interface throughout the whole process. Every hour, a sample was collected and monitored at 600 nm to create a growth curve and to check the expression of the target as analysed by SDS-PAGE. Harvesting was conducted by batch centrifugation at 5000g for 30 min. Pelleted cells were washed in sterile 0.9% sodium chloride solution and centrifuged again. After resuspension in lysis buffer (20 mM Tris/HCl, 200 mM sodium chloride, and 1 mM EDTA, pH 8.0) followed by sonication and addition of benzonase, cells were stored at –20 °C until use.

3.5. Establishing master and working cell banks

Master cell banks were established from a selected single colony. The clone was characterised by restriction analysis of the expression construct, DNA sequencing of the coding sequence, and expression analysis. Plasmid stability was proved by cultivation in a soya-peptone-based complex medium with or without ampicillin (100 µg/ml). The master cell bank was established using 1 ml aliquots of cells in 20% glycerol, shock-frozen in liquid nitrogen, and stored in the gas-phase over liquid nitrogen.

The working cell bank (WCB) was prepared from aliquots of MCB under aseptic conditions. Again, the same storage and characterisation as for the MCB were performed to ensure identity of the cell banks [36].

3.6. Protein purification

All protein purification methods were developed using a PRIME chromatography system (Amersham Biosciences). Final purification strategies were conducted using an Äkta Explorer/Biopilot System (Amersham Biosciences) together with a UNICORN computer-based control system to administer batch operation and process documentation in accordance with the

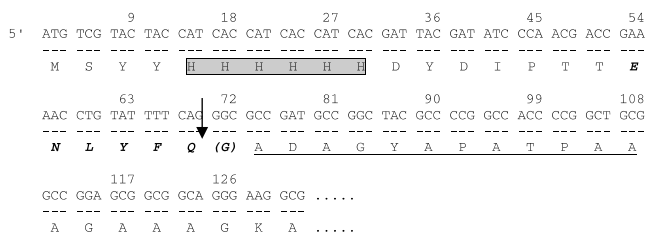


Fig. 1. Nucleotide and deduced amino acid sequences of an N-terminal portion of the primary expression product HIS-rPhl p 5b. The 6-His-tag (boxed), the rTEV recognition sequence (italic-bold), the cleavage site (arrow), and an additional glycine residue (G) attached to the N-terminal position of the allergen sequence (underlined) are shown.

requirements of Good Manufacturing Practice (GMP) and Good Laboratory Practice (GLP). Preparations for clinical purposes were produced under conditions temperature-controlled qualified clean-room class A. Intermediate products were either stored at room temperature or at 2–8 °C, depending on the respective stability. Protein samples and chromatography solutions were subjected to microfiltration (0.2 µm) prior to being introduced into the clean-room.

Nickel chelate affinity chromatography was conducted using 50 ml chelating-Sepharose fast flow packed in 5.0 × 20 cm XK columns (Amersham Biosciences) loaded with nickel sulphate and equilibrated in a buffer containing 50 mM phosphate, 500 mM NaCl, pH 7.5 (CS buffer). Applied samples were eluted with a two-step gradient of 100 and 400 mM imidazole. The step elution was based on a pre-investigation

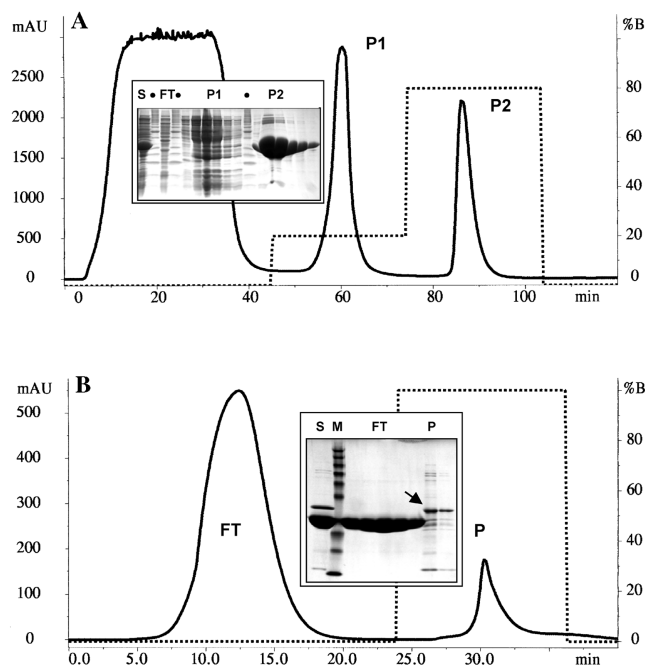


Fig. 2. (A) Nickel chelating Sepharose chromatography of His-tagged rPhl p 5b (first step). The clarified bacterial lysate was applied to a 50 ml nickel chelating Sepharose column and stepwise eluted using 100 mM (corresponding to 20% B; P1) and 400 mM imidazole (corresponding to 80% B, P2). SDS-PAGE analysis (inset) was performed for the applied sample (S), the flowthrough (FT) and the two eluted peaks (P1, P2). A molecular weight marker was applied for lane separation (indicated by ●). (B) Nickel chelating Sepharose chromatography of rPhl p 5b (second step). The protein peak P2 from the previous purification step (A) was treated with TEV to cleave the 6-His-tag and imidazole was subsequently removed by SEC using Sephadex G-25. This fraction was then subjected to a second IMAC separation. The applied sample (S), the flowthrough (FT) containing the rPhl p 5b and bound components eluted with 500 mM imidazole (P) were collected and analysed by SDS-PAGE (inset). A pre-stained molecular weight marker (M) was run in parallel. The arrow indicates the small amount of remaining fusion protein, which is efficiently separated by retention on the column are other minor impurities.

of the separation from tagged proteins using a linear gradient ranging from 0 to 500 mM imidazole. The profile displayed elution of impurities between 50 and 80 mM and of the target molecules between 200 and 400 mM imidazole, respectively. Using step gradients offers the advantage of higher concentrations of eluting proteins and scalable processes (Fig. 2A). The His-tagged Phl p 5b eluted in the 400 mM fraction. To cleave the His-tag, genetically engineered recombinant His-tagged tobacco etch virus protease (TEV; Invitrogen, Karlsruhe, Germany) was added at a concentration of approx. 10 U/mg fusion protein, which is 50 times diluted compared with the manufacturer's recommendation (10 U/20 µg). Cleavage was conducted at 25 °C for at least 20 h. The processed protein was subsequently applied to a G-25 column and eluted with CS buffer, thus removing imidazole and the cleaved His-peptide, and then subjected to a second pass over nickel chelating Sepharose to remove residual fusion protein and the His-tagged TEV (Fig. 2B). Alternatively HIC can be used, especially when the sample volume is large. At this point of purification, the target protein was more than 98% pure. Final purification and conditioning were conducted by SEC (see below).

Size exclusion chromatography (SEC) was performed using either Sephadex G-25 (Amersham Biosciences) for desalting or Superdex 75 (Amersham Biosciences) for final polishing. Efficient buffer exchange for sample volumes up to 80 ml was performed with a 2.6 × 60 cm XK column (Amersham Biosciences) packed with Sephadex G-25, corresponding to a column volume of 320 ml. Typical separation conditions were: 8 ml/min (corresponding to a linear flow rate of 90 cm/h), separation time of 30 min, whole process time including equilibration and cleaning/conservation of 2.5 h. Sephadex G-25 served also for efficient separation of the cleaved 6x-His-peptide (Fig. 3). The chromatographic medium was stored in 20 mM NaOH, conditions that

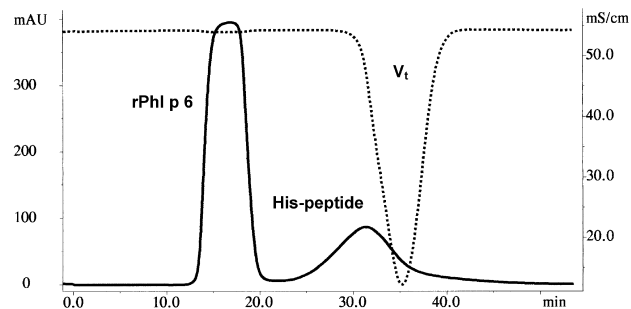


Fig. 3. Example of size exclusion chromatography for rPhl p 6. After cleaving the 6-His-tag, rPhl p 6 was subjected to Sephadex G-25 filtration to separate the His-peptide and adjust the sample's conductivity for the next purification step, an IMAC. The elution of components is indicated in the profile. The decrease in conductivity indicates the total volume (V_t) of the column.

have been shown to be both bacteriostatic and suitable to clean Sephadex columns with respect to endotoxin and precipitates.

For final purification, a 1000 ml Superdex 75 XK 5.0×60 cm column was used. The maximum sample volume applied was <2% per run, corresponding to 20 ml. Elution with 0.9% NaCl was realised at 5 ml/min (15 cm/h).

Hydrophobic interaction chromatography. The first two steps of the purification strategy for the recombinant untagged Phl p 2 included two different media for hydrophilic interaction chromatography. After the thawed and clarified lysate (see above) was adjusted to 1 M ammonium sulphate, a 70 ml Phenyl Sepharose HP column with dimensions of 2.6×20 cm, equilibrated with 20 mM Tris/HCl, 1 M ammonium sulphate, pH 8.0, was used to separate most impurities (Fig. 4), since rPhl p 2 elutes in the flowthrough [37]. The flowthrough fraction was subsequently applied to a column packed with Source 15 PHE (Amersham Biosciences) without further sample conditioning. Elution of bound Phl p 2 was conducted with 20 mM Tris/HCl, pH 8.0. The second HIC step served for concentration of the sample as well as removal of nucleic acids (strictly speaking nucleotides and oligonucleotides due to previous benzonase digestion). A Sephadex G-25 column was used for desalting to provide conditions for the next purification step, an anion-exchange chromatography.

Anion-exchange chromatography. Intermediate purification for rPhl p 2 was performed with a 30 ml Source 15Q column (Amersham Biosciences) equilibrated with 20 mM Tris/HCl, pH 8.0. The bound protein was eluted with a linear continuous gradient ranging from 0 to 400 mM NaCl over 8 CV and a flow rate of 5 ml/min or 57 cm/h (Fig. 5). To provide an even more robust production procedure, a step gradient was derived on the basis of the elution profile using linear desorption with the first elution step at 180 mM NaCl.

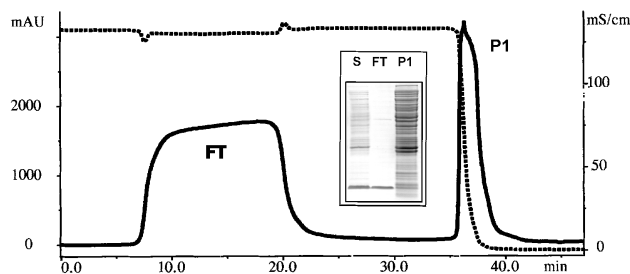


Fig. 4. Example of hydrophobic interaction chromatography (HIC) for rPhl p 2. After the lysate was clarified and subsequently adjusted to 1 M ammonium sulphate, a Phenyl Sepharose HP column was used to separate most of the impurities derived from the host cell. The inset shows an SDS-PAGE analysis of the applied sample (S), the rPhl p 2 containing flowthrough (FT) and tightly bound proteins (P1).

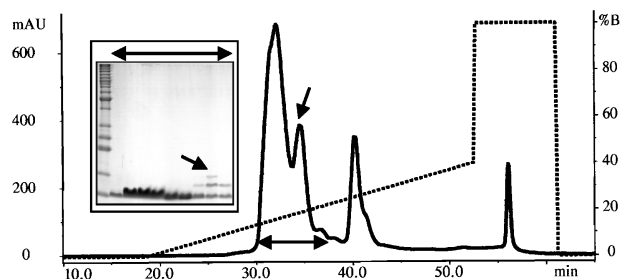


Fig. 5. Example of AIEX in the downstream process of rPhl p 2. After initial purification of untagged rPhl p 2 using two consecutive hydrophobic interaction steps on Phenyl Sepharose and Source 15 PHE, the desalted sample was applied to a Source 15 Q column. A linear elution gradient provided efficient separation of minor impurities of similar size as indicated by the arrows. The inset displays fraction analysis by SDS-PAGE as indicated by the double arrow.

3.7. Protein determination

Concentration of the purified proteins was determined by measurement of optical density at 280 nm and calculation from the deduced extinction coefficient according to Gill and von Hippel [38] using commercial software Protean (DNASTAR Inc., Madison, WI, USA) from the known amino acid sequence. To ensure correct values together with the absence of aggregates, a multi-wavelength determination of 320, 325, 330, 335, 340, 345, and 350 nm was additionally performed, according to Ph.Eur. Supp. 2.5.3.3.

3.8. Protein characterisation

SDS-PAGE electrophoresis. Proteins were separated by SDS-PAGE (12 or 15% T, 4% C) with the buffer system of Laemmli [39]. Reducing and non-reducing sample buffers were the same except that the final reducing sample buffer contained 50 mM DTT. Samples run under reducing conditions were denatured by heating at 95 °C for 5 min prior to electrophoresis. Following separation with 1.5 mA/cm gel at 20 °C for approx. 1.5 h, proteins were either stained with colloidal Coomassie R-blue stain (Roth, Karlsruhe, Germany) or transferred onto a 0.2 µm supported nitrocellulose membrane (Sartorius, Göttingen, Germany) by semi-dry blotting for 30 min at 0.8 mA/cm² (Apparatus; Biometra, Göttingen, Germany) for subsequent immunodetection (see below).

Isoelectric focussing. Prior to application onto a 0.5 mm polyacrylamide gel containing 8 M urea and ampholytes ranging from pH 4 to 10, samples were desalted using G-25 Spin columns (Amersham Biosciences). Non-denaturing IEF-separation was conducted by a pre-cast criterion gel ranging from pH 4.5 to 8.5 (Bio-Rad, Munich, Germany) according to manufacturer's instructions.

Analytical size exclusion chromatography was performed using an HPLC system (Thermo separations,

Gelnhausen, Germany) equipped with a TSK G 2000 SW column (7.5×600 mm, guard column 7.5×75 mm, particle size $10 \mu\text{m}$; Toyo Soda, Tokyo, Japan), which was run at a flow rate of 1.0 ml/min using 0.5 mol/L sodium acetate, pH 6.8, as running buffer. The sample volume was 0.5% of the column volume. All running buffers were filtered through $0.2 \mu\text{m}$ filtration devices (Millipore, Göttingen, Germany) before use. An example of the analysis of two rPhl p 1 apparent molecular weight variants in comparison with purified natural Phl p 1 is shown in Fig. 6.

Reversed-phase HPLC. Reversed phase chromatography was performed on a HPLC (Kontron Instruments, Neufahrn, Germany) equipped with a 540 Diode array detector and a Bio-Tek 525 pump system using a ET 250-4 Nucleosil 100-5 C18 PPN column (Macherey-Nagel, Dueren, Germany) with a particle size of $5 \mu\text{m}$ and dimensions of 4×250 mm. The column was equilibrated with distilled water/ 0.1% TFA at 1 ml/min , loaded with $50 \mu\text{g}$ protein, and eluted using a three-step gradient (10 – 40% over 20 ml , 40% plateau over 5 ml , and 40 – 60% over 20 ml) with acetonitrile/ 0.7% TFA (Merck). Detection was performed at 220 nm . (Fig. 7).

3.9. N-terminal amino acid sequencing

N-terminal amino acid sequencing was performed by automated Edman degradation using an Applied Biosystems 476 sequencer (Applied Biosystems, Weiterstadt, Germany).

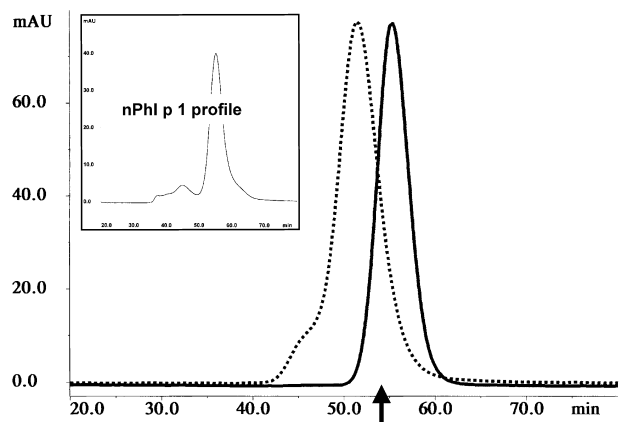


Fig. 6. Analytical LC-SEC of nPhl p 1 and two isomeric forms of rPhl p 1. Two different folding variants of rPhl p 1 purified from one preparation were subjected to SEC on Sephacryl S-100 filled in a 1.6×60 cm column. Though of identical primary sequence, a difference in retention time of approx. 4 min was observed. The slow migrating and obviously more folded variant eluted with a retention time comparable with natural Phl p 1 (inset). The arrow indicates elution of nPhl p 1. Similar results were obtained with non-reducing SDS-PAGE (not shown).

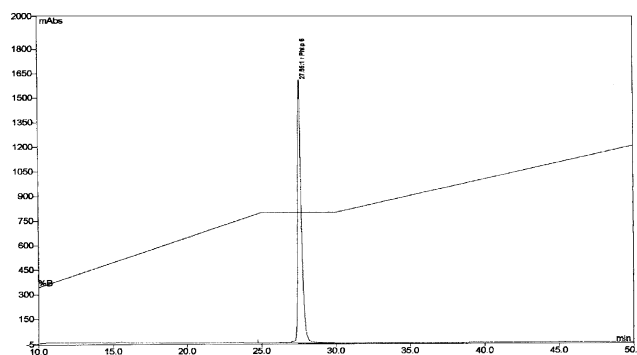


Fig. 7. Analytical investigation of rPhl p 6 by RP-HPLC. Fifty micromograms of purified rPhl p 6 was applied to a Nucleosil 100-5 C18 PPN column and eluted as indicated by the gradient using acetonitrile/ 0.7% TFA.

3.10. Detection of host cell protein

Host cell proteins are detected using ELISA and slot blot techniques. In the case of ELISA technique, a commercially available assay with a detection limit of 1 ng/ml *E. coli* proteins proved to be satisfactory for the detection of general impurities derived from the host cell (Immunoenzymetric Assay Cat. No. F010, Morwell Diagnostics GmbH). To detect very product-specific impurities, a mock expression using the host cell carrying the expression vector lacking an insert was performed. The lysate was subjected to the corresponding downstream process and subsequently purified until a profile of less than 100 *E. coli* proteins, typically after one purification step, was detectable by SDS-PAGE. This fraction was used for immunisation of rabbits to obtain polyclonal antibodies. Application of the final product together with potential product-specific impurities as a reference in a slot blot system ('Convertible'; Life Technologies, La Jolla, USA) enabled after detection by polyclonal antibodies clear evidence for the absence or presence of product-specific HCP. Linear detection is possible between 1 and 50 ng HCP.

3.11. Detection of endotoxin and other pyrogens

The endotoxin content of purified protein solutions was tested in accordance with Ph. Eur. using both a chromogenic Limulus Amoebocyte Lysate Assay (QCL-1000; Bio-Whittaker, Walkersville, MD, USA) and an LAL gel-clot assay (Pyrotell). An endotoxin content of less than 10 EU (Endotoxin Units)/mg protein corresponding to less than 0.1 – 1 EU/human dose was typically detected. This value is well within the accepted limit of 5 EU/kg/h or 350 EU/body/h . In every case, a rabbit test was performed first in parallel with the LAL-test to ensure the absence of pyrogens other than endotoxin.

3.12. Immunological characterisation

In-house reference preparations were established for each recombinant allergen to be analysed by immunologic methods. These preparations were used to produce antisera raised in rabbits by immunisation. Human pool sera were prepared with sera from allergic patients with a demonstrable allergen sensitivity. The titres of the antisera and serum pools were determined to estimate the working dilution in direct binding assays or in inhibition assays.

For direct binding assays, microtitre plates (Greiner, Frickenhausen, Germany) were coated with serial dilutions of antigens, i.e., the in-house reference preparation and production batches of the recombinant protein to be assayed, and incubated with the appropriate antiserum, pool serum or monoclonal antibody. Binding curves of the reference and samples were compared. Antigenic potency was determined in inhibition assays using rabbit antisera or monoclonal antibodies, whilst use of human serum pools provided a measure of allergenic potency.

Microtitre plates were coated overnight at 4°C with antigen at a pre-determined optimal concentration, usually 0.1 µg/well, washed with PBS, pH 7.4, containing 0.05% Tween 20 and blocked with 1% bovine serum albumin dissolved in the same buffer. A dilution of the antiserum or pool serum was mixed with serial dilutions of antigen, prior to application of these mixtures to the coated plates. After overnight incubation, the plates were washed with PBS. Bound rabbit antibodies were detected using goat anti-rabbit antiserum, alkaline phosphatase-conjugated (Dianova, Hamburg, Germany), and pNPP (*para*-nitro-phenyl-phosphate; Sigma, Deisenhofen, Germany) as substrate. Alkaline phosphatase-conjugated monoclonal anti-human IgE antibody, (Allergopharma, Reinbek, Germany) or goat anti-mouse immunoglobulin (Dianova, Hamburg, Germany), together with pNPP as substrate, was used to detect human IgE and allergen-specific mouse monoclonal antibody binding, respectively. Optical densities were measured at 405 nm on a Multiscan MCC340 Plate-Reader (ICN, Meckenheim, Germany).

Inhibition was calculated as a percentage by using the equation:

$$((A - B)/(A - C)) \times 100 = \text{inhibition } [\%],$$

where *A* is the OD of the positive control (no inhibitor used), *B* is the OD of the sample, and *C* is the OD of the negative control (no antiserum used).

Potency was estimated by comparison of the 50% inhibition values of sample and reference preparations.

3.13. Immunodetection

Identification of allergens on immunoblots was performed using either allergic patients' serum pools or

monoclonal antibodies, e.g., BG6 (Phl p 5a), 1D11 (Phl p 5a and 5b) (Allergopharma). Prior to immunodetection, membranes were blocked by treatment for 30 min with Tris-buffered saline (TBS), pH 7.4, supplemented with 0.05% v/v Tween 20. Incubation with 1:10-diluted patients' pool serum or mAbs was carried out overnight. After washing, bound antibodies were detected with either alkaline phosphatase-conjugated monoclonal mouse anti-human IgE (Allergopharma) or goat anti-mouse IgG/M with alkaline phosphatase (Dianova). The binding patterns were visualised by a substrate solution consisting of nitroblue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Life Technologies) in 100 mmol/L Tris/HCl-buffered saline solution (pH 9.5).

3.14. Allergen-specific T cell lines

Allergen-specific T cell lines (TCL) were raised from peripheral blood mononuclear cells (PBMC) isolated from the blood of grass pollen or birch pollen allergic patients with a clinical history of hay-fever or birch pollen allergy and serum EAST classes ≥ 3 (specific IgE EAST-RV; Allergopharma) with *Phleum pratense* or birch pollen extracts, respectively, using a slightly modified procedure as described by Müller et al. [40]. The blood was collected using heparin (Liquemin N 20.000; Hoffman-La Roche, Grenzach-Whylen, Germany) as anti-coagulant.

The isolation of PBMC from the blood was performed with lymphocyte separation media (density = 1077; PAA, Cölbe, Germany) in Leucosep tubes (Greiner, Frickenhausen, Germany) according to the instructions of the manufacturers. All T cell cultures and stimulation experiments were performed in serum-free Ultraculture medium (BioWhittaker, Verviers, Belgium) supplemented with 2 mM Glutamax I (Life Technologies, Eggenstein, Germany), antibiotic-antimycotic solution (Sigma, Deisenhofen, Germany), and 20 µM of 2-mercaptoethanol (Life Technologies, Eggenstein, Germany).

T cell lines (TCL) were raised by seeding 1×10^5 PBMC and 10 µg/ml of Phl p 5 or rPhl p 5b or 5 µg/ml Bet v 1 in a 96-well round-bottomed culture plate in a total volume of 100 µL/well. After 5–7 days, the cultures were fed by adding 100 µL medium with 10 U/ml Interleukin-2 (Strathmann Biotech, Hannover, Germany). Three to four days later, half of the medium was exchanged against fresh medium containing 10 U IL-2/ml and incubated for another 4 days. 14 ± 2 days after setting up of these cultures, a proliferation assay was performed to detect allergen-specific T cells.

Cultures with stimulation indices of at least SI = 2 were expanded separately or were pooled (then cultures from 10 to 12 wells) and represent a T cell line. The TCL were cloned immediately and expanded. The expansion procedure involves sequential stimulation with allergen

and autologous PBMC and unspecific stimulation with PHA (2 µg/ml), allogeneous PBMC (from 3 to 5 non-allergic donors, mitomycin C treated; the ratio of T cells to PBMC should be approximately 2:5) and IL-2 (25 U/ml).

3.15. Allergen-specific T cell clones

Selected allergen-specific T cell lines were cloned by either allergen-specific stimulation or unspecific stimulation by seeding 0.5, 1, and 2.5 T cells in a final volume of 20 µl medium in Terasaki plates [8,41]. The allergen-specific cloning procedure requires the inclusion of the respective allergen, here 10 µg/ml Phl p 5 or rPhl p 5b, or 5 µg/ml Bet v 1, 50 U IL-2/ml, and 1×10^4 mitomycin C treated autologous PBMC in the final 20 µl volume in the wells of Terasaki culture plates (Greiner, Frickenhausen, FRG). Mitomycin C treatment was performed using 100 µl mitomycin C (Sigma) solution (0.5 mg/ml PBS) on 5×10^6 PBMC in 1 ml PBS. If irradiation facilities are available, then the autologous PBMC used for antigen presentation can be irradiated with 30 Gy instead of using mitomycin C treatment. The plates were checked for growing clones after 7–10 days. Growing clones were expanded in 96-well culture plates under stimulation with 5×10^4 irradiated autologous PBMC/well and allergen in the same concentrations as described above. The clones were cultured by changing half of the medium every 3–4 days with fresh medium supplemented with 25 U IL-2/ml. Fourteen days after feeding with autologous PBMC and allergen, and 3–4 days after the last addition of IL-2, cells were assayed again for allergen-induced proliferation. The allogenic cloning procedure involves the unspecific stimulation with PHA (1.5 µg/ml), 1×10^4 allogeneous PBMC/20 µl, and well of a Terasaki plate (PBMC of 3–5 non-allergic donors, mitomycin C treated or irradiated) and 25 U IL-2/ml.

3.16. Proliferation assays

For the proliferation assays, T cells were seeded at 2×10^4 cells/well in a 96-well culture plate in triplicate under stimulation with the allergen (0.3 µM) and 5×10^4 mitomycin C treated autologous PBMC/well. After 48 h incubation at 5% CO₂, 37 °C and in a humidified atmosphere, 1 µCi [³H]thymidine ([6-³H]thymidine, 37 MBq/ml; Amersham Biosciences) was added to each well and incubated further for 16 h. Cells were harvested on microbeta filter-mats with a 96-well cell harvester (Perkin–Elmer Life Sciences, Köln, Germany). The filter-mats were dried and the solid scintillator Meltilex (Perkin–Elmer) was melted into the filter-mats using a microplate heat-sealer (Perkin–Elmer). The mats were then transferred into filter cassettes and the cpm were counted in a Microbeta scintillation counter (Perkin–

Elmer). The stimulation index (SI) was calculated as the quotient of counts per minute (cpm) after stimulation with and without allergen.

The reactivities of recombinant allergens with TCL and T cell clones (TCC) were compared in triplicate in equimolar concentrations in a proliferation assay with those of relevant natural allergens purified from pollen extracts.

3.17. Stability of the active constituent and end product

The investigation of stability characteristics of both active pharmaceutical ingredients (APIs) (active constituents) and end products was performed by accelerated and long-term testing.

In the case of API accelerated stability testing, sterile containers were stored in qualified chambers at –80, 5, 25, and 40 °C over a period of 1 month. Every week two of the vials stored at each temperature were analysed using validated methods to assess stability, e.g., pH, protein concentration, non-reducing SDS–PAGE, Western blot, enzyme allergosorbent inhibition test (EAST), and measurement of aggregation in accordance with Ph. Eu. Supp. 2.5.3.3. The methods were selected with a view to assessing the physicochemical and immunological properties of the APIs (33f). In the case of long-term testing, APIs are stored at 5 °C and testing is performed after 0, 1, and 2 weeks and 1, 3, 6, 9, 12, 18, 24, and 36 months, depending on the shelf-life required for the preparation.

4. Practical considerations/potential problems

The transition of recombinant allergens from the bench to clinical application can be divided into three phases. First, the development of a recombinant allergen as a diagnostic tool or agent for specific immunotherapy is carried out at the bench in research laboratory scale, beginning with the identification and cloning of the gene of the allergen in question. Different expression systems have to be tested and different genetic variants of the allergen produced prior to characterisation and comparison with the purified natural allergen using biochemical, physical, and immunological methods. During this laboratory work-up, the appropriate sequence has to be chosen and consideration given as to the choice of a purification strategy. Therefore, it is important to be familiar with the subsequent requirements for Good Manufacturing Practice (GMP) and the feasibility for up-scaling to avoid expensive and time-consuming ‘reinvention of the wheel’.

The second phase is the development of the pilot-scale production, which has to conform to GMP-standards and provides material for stability-, toxicity-, and

clinical studies. Important considerations for the transition to the third step, the production scale, are reproducibility, full documentation (GMP), method validation, cost-effectiveness, and a complete final quality control. The end result must be an allergen protein-based diagnostic or therapeutic that delivers all the benefits of a recombinant product, including high purity, high security, and optimal dose formulation to provide a safe and efficient clinical preparation.

4.1. *Criteria for quality and purity*

The quality of a recombinant allergen product is dependent on many factors, as mentioned in the ICH guideline Q6B, such as biological activity, immunochemical, and biophysical properties. For a good characterisation, the API should be compared with its natural counterpart, when appropriate. Special attention should be paid to the purity of the API, which in general requires different techniques to demonstrate, as the term purity concerns product- and process-related impurities as well as contaminants.

Product-related impurities are molecular variants, such as fragments, aggregates or minor modifications (carbamylation, glycosylation, etc.). Since the overall properties of these kind of impurities are very similar to those of the desired product, several analytical methods may be conducted. For example, SEC provides quantifiable information about aggregation and formation of fragments, whereas isoelectric focussing enables qualitative detection of folding and glycosylation variants. Variations of disulphide bonding may be detected by protease digestion followed by SDS–PAGE [42] or RP-HPLC separation (peptide mapping). The application for RP-HPLC additionally may provide information of amino acid composition. However, the selection of methods varies in each case and is depending on the API itself, the expression system, and the production process. It is important to establish acceptance criteria that may depend on experience with the substance together with the purpose or application. In general, criteria for product-related impurities may be set after comparison of several batches.

Process-related impurities may be composed of host cell proteins, DNA, chromatographic media or buffer components. Appropriate in process controls or online monitoring should minimise the effort with respect to the end control of the substance. For example, if the final step in the manufacturing process is a preparative SEC performed with well-selected and robust chromatographic media, buffer composition, pH, and conductivity together with detection at three different wavelengths (depending on the impurity profile) serve for a good characterisation of the composition.

Contaminants include adventitiously introduced substances, which are not intended to be part of the

manufacturing process. The most likely contaminants are microbes and microbial products. All possible measures should be taken to minimise the risk of microbial contamination.

4.2. *Requirements for good manufacturing practice*

The guidelines concerning GMP requirements for API are provided by the International Conference on Harmonisation (ICH Q7A), which are the only GMP guidelines accepted worldwide, i.e., in Europe, Japan, and USA.

Special attention should be paid to section 19 of ICH Q7A that provides specific guidance for APIs that are intended for use in clinical trials. Main aspects to be considered are equipment, facilities, raw materials, production, laboratory controls, and documentation.

The equipment used for preparation of APIs should be dedicated to that purpose to avoid cross-contamination, especially in the case of chromatographic systems. A log book is essential for the documentation of usage, calibration and maintenance for analytical and chromatographic systems, balances, centrifuges, pH-meters, etc.

The equipment should be retained in separate areas, which are ideally monitored with regard to their bio-burden. Clean room facilities ranging from class C to A are helpful to minimise the risks of picking up product and process unrelated impurities.

It is advantageous to use chemicals, biochemicals, and raw materials supplied with full certification to minimise the workload for testing. Special care should be taken with regard to water quality, and water and solutions, which are clearly recognised as potential carriers of endotoxin and bacterial or fungal contamination, if stored or handled under inappropriate conditions. Therefore, usage of highly purified and endotoxin-tested water with the quality of water for injection (WFI) is recommended. Sterile filtration of the sample and every chromatography solution using 0.2 µm devices ensures additionally the removal of any particulate material.

The production of APIs has to be documented as extensively as possible with special regard to production materials, equipment, and unexpected observations. It may be helpful to design standardised forms that can be filled in parallel to the production process. The same is advantageous for both in process control or end control analyses. As documentation is a central aspect of GMP, one should consider storing duplicates of each batch production, e.g., both paper records and computer notebook files.

Laboratory control methods applied to the evaluation of clinical trial preparations should be highly reproducible and robust in order to ensure detection of possible batch to batch inconsistency. It is

recommended that reserve samples from every batch should be run in parallel to demonstrate consistency.

References

- [1] C. Suphioglu, *Clin. Exp. Allergy* 30 (2000) 1335–1341.
- [2] W.R. Thomas, W.-A. Smith, B.J. Hales, K.L. Mills, R.M. O'Brien, *Int. Arch. Allergy Immunol.* 129 (2002) 1–18.
- [3] Allergen Products. Monograph, European Pharmacopoeia (1997) 356–358.
- [4] R. Valenta, *Nat. Rev. Immunol.* 2 (2002) 446–453.
- [5] P. Schmid-Grendelmeier, R. Cramer, *Int. Arch. Allergy Immunol.* 125 (2001) 96–111.
- [6] R. Hiller, S. Laffer, C. Harwanegg, et al., *FASEB J.* 16 (2002) 414–416.
- [7] R. Suck, A. Nandy, B. Weber, M. Stock, H. Fiebig, O. Cromwell, *Allergy* 57 (2002) 821–824.
- [8] G. Schramm, H. Kahlert, R. Suck, B. Weber, H.-T. Stüwe, W.-D. Müller, A. Bufer, W.-M. Becker, M.W. Schlaak, L. Jäger, O. Cromwell, H. Fiebig, *J. Immunol.* 162 (1999) 2406–2414.
- [9] M.B. Singh, N. de Weerd, P.L. Bhalla, *Int. Arch. Allergy Immunol.* 119 (1999) 75–85.
- [10] F. Ferreira, M. Wallner, H. Breiteneder, A. Hartl, J. Thalhammer, C. Ebner, *Int. Arch. Allergy Immunol.* 128 (2002) 171–178.
- [11] E. Van Oort, P.G. de Heer, W.A. van Leeuwen, N.I.L. Derksen, M. Müller, S. Huveneers, R.C. Aalberse, R. van Ree, *Eur. J. Biochem.* 269 (2002) 671–679.
- [12] A. Hénaut, A. Danchin, in: F. Neidhardt, R. Curtiss III, J. Ingraham, E. Lin, B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, H. Umberger (Eds.), *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology*, vol. 2, American Society for Microbiology, Washington, DC, 1996, pp. 2047–2066.
- [13] R. Suck, A. Petersen, S. Hagen, O. Cromwell, W. Becker, H. Fiebig, *Clin. Exp. Allergy* 30 (2000) 324–332.
- [14] H. Fiebig, R. Suck, H. Kahlert, B. Weber, F. Altmann, in: J. Ring, U. Darsow (Eds.), *Allergie 2000: Probleme, Strategien und Praktische Konsequenzen*, Dustri-Verlag, Munich-Deisenhofen, 2001, pp. 75–82.
- [15] M. Schmidt, D.R. Hoffman, *Int. Arch. Allergy Immunol.* 128 (2002) 264–270.
- [16] H. Breiteneder, M. Krebitz, U. Wiedermann, B. Wagner, D. Essl, H. Steinkellner, T.H. Turpen, C. Ebner, D. Buck, B. Niggemann, O. Scheiner, *Int. Arch. Allergy Immunol.* 124 (2001) 48–50.
- [17] H. Horvath, J. Huang, O. Wong, E. Kohl, T. Okita, C.G. Kannangara, D. von Wettstein, *PNAS* 97 (2000) 1914–1919.
- [18] E.A. Best, K.E. Stedman, C.M. Bozic, S.W. Hunter, L. Vailes, M.D. Chapman, C.A. McCall, M.J. McDermott, *Protein Expr. Purif.* 20 (2000) 462–471.
- [19] L. Kågedal, in: J.-C. Janson, L. Ryden (Eds.), *Protein Purification, Principles, High Resolution Methods, and Applications*, Wiley-VCH, New York, 1998, pp. 311–342.
- [20] S. Harper, D.W. Speicher, in: J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, P.T. Wingfield (Eds.), *Current Protocols in Protein Science*, Wiley, New York, 1997, pp. 6.6.1–6.6.21.
- [21] P. Riggs, *Mol. Biotechnol.* 15 (2000) 51–63.
- [22] E. Karlsson, L. Rydén, J. Brewer, in: J.-C. Janson, L. Ryden (Eds.), *Protein Purification, Principles, High Resolution Methods, and Applications*, Wiley-VCH, New York, 1998, pp. 145–205.
- [23] K.-O. Eriksson, in: J.-C. Janson, L. Ryden (Eds.), *Protein Purification, Principles, High Resolution Methods, and Applications*, Wiley-VCH, New York, 1998, pp. 283–309.
- [24] B.M. Dunn, in: J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, P.T. Wingfield (Eds.), *Current Protocols in Protein Science*, Wiley, New York, 1997, pp. 8.4.1–8.4.21.
- [25] L. Hagel, in: J.-C. Janson, L. Ryden (Eds.), *Protein Purification, Principles, High Resolution Methods, and Applications*, Wiley-VCH, New York, 1998, pp. 79–143.
- [26] A. Williams, L. Hagel, in: C.-S. Wu (Ed.), *Column Handbook For Size Exclusion Chromatography*, Academic Press, London, 1999, pp. 27–74.
- [27] CPMP/BWP/243/96 Note for Guidance on Allergen Products. Available from www.emea.eu.int/sitemap.htm.
- [28] CPMP/ICH/286/95 (M3) Note for Guidance on Non-Clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals. Available from www.emea.eu.int/sitemap.htm.
- [29] CPMP/ICH/365/96 (6B) Note for Guidance on Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products. Available from www.emea.eu.int/sitemap.htm.
- [30] CPMP/ICH/302/95 (S6) Note for Guidance on Preclinical Safety Evaluation of Biotechnology Derived Pharmaceuticals. Available from www.emea.eu.int/sitemap.htm.
- [31] CPMP/SWP/465/95 Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines. Available from www.emea.eu.int/sitemap.htm.
- [32] CPMP/ICH/4106/00 (Q7A) Note for Guidance Good Manufacturing Practice for Active Pharmaceutical Ingredients. Available from www.emea.eu.int/sitemap.htm.
- [33] CPMP/ICH/138/95 (Q5C) Note for Guidance on Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products. Available from www.emea.eu.int/sitemap.htm.
- [34] CPMP/ICH/294/95 (Q5D) Note for Guidance on Quality of Biotechnological Products: Derivation and Characterisation of Cell Substrates used for Production of Biotechnological/Biological Products. Available from www.emea.eu.int/sitemap.htm.
- [35] J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
- [36] CPMP/ICH/139/95 (Q5B) Note for Guidance on Quality of Biotechnological Products: Analysis of the Expression Construct in Cell Lines used for Production of r-DNA derived Protein Products. Available from www.emea.eu.int/sitemap.htm.
- [37] R. Suck, S. Hagen, O. Cromwell, H. Fiebig, *J. Immunol. Methods* 229 (1999) 73–80.
- [38] S.C. Gill, P.H. von Hippel, *Analytical Biochem.* 182 (1989) 319–326.
- [39] U.K. Laemmli, *Nature* 227 (1970) 680–686.
- [40] W.-D. Müller, T. Karamfilov, B. Fahlbusch, H. Vogelsang, L. Jäger, *Int. Arch. Allergy Immunol.* 105 (1994) 391–396.
- [41] H. Kahlert, H.-Th. Stüwe, O. Cromwell, H. Fiebig, *Int. Arch. Allergy Immunol.* 120 (1999) 146–157.
- [42] R. Suck, B. Weber, H. Kahlert, S. Hagen, O. Cromwell, H. Fiebig, *Int. Arch. Allergy Immunol.* 121 (2000) 284.