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Development of recombinant allergens for diagnosis and therapy

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1. ABSTRACT

Allergic disease represents an increasing problem in industrialized countries. However, allergy diagnosis and specific immunotherapy, the only curative approach towards the treatment of IgE-mediated disorders, are still performed like in their very beginnings more than a century ago. The use of allergen extracts of undefined contents bears the risk of anaphylactic side effects and sensitization to new allergens during immunotherapy. This review focuses on production, physicochemical, and immunological requirements, as well as on the multiple advantages of recombinant allergens and hypoallergens in comparison to conventional allergen extracts used in allergy diagnosis and therapy. Recombinant allergens offer the perspective of molecule-based allergy diagnosis and consequently safe and patient-tailored immunotherapy.

2. INTRODUCTION

The prevalence of allergic disease has been drastically increasing over the last decades in industrialized countries. Meanwhile up to 30% of the population of the westernized world of the northern hemisphere are affected by allergic disorders and the tendency is still rising (1, 2). Allergic hay fever symptoms, at that time called “autumnal catarrh”, were described as early as 1819. However, pollen originating from certain plants (grass and ragweed) was only identified as elicitor decades later in the 1870ies. At that time, Blackely (3) performed the first skin test for grass pollen extract and described the typical wheal and flare reaction. Later in 1906, Pirquet introduced the term “allergy” in an attempt to explain adverse skin reactions following the administration of cowpox vaccine. The experiments of Noon and Freeman (4, 5) in 1910 using

subcutaneously injected pollen extract dilutions with gradually raising strength, represented the beginnings of allergen-specific immunotherapy (SIT) (6). In general, SIT offers the best possibility of accomplishing the most effective treatment of allergies. The process is highly specific as the treatment is targeted at those allergen sources responsible for the triggering of symptoms (7). In addition, SIT represents the only curative approach towards the treatment of IgE-mediated allergy and is capable of modifying the natural course by preventing the onset of sensitization against new allergens and symptom worsening (e.g. from allergic rhinitis to asthma) (8-11). SIT consists in the repeated administration of allergen to sensitized individuals and has been shown to be a clinically effective allergen-specific form of treatment, capable of inducing active immunity to the allergen. Typically, patients are started on a very low dose of allergen that is then increased in a series of weekly subcutaneous injections until a plateau or maintenance dose is achieved. The maintenance dose is given at 4-6 weekly intervals for three to five years. Other injection regimes use several doses on each day, or the whole series of increasing doses in a single day (rush protocols). Due to safety reasons, alternative routes to subcutaneous application, i.e. oral or sublingual administration, have been recently a focus of allergen delivery, although, the treatment benefit is about half of that achieved with subcutaneous injection. The immunological mechanisms operating in effective SIT include an increased production of cytokines with regulatory activity (e.g. IL-10 and TGFβ), which might increase the generation of IL-10 secreting T regulatory cells that inhibit subsequent inflammatory responses. In addition, they potently repress IgE production and increase the synthesis of non-inflammatory isotypes (IgG4 and IgA) that might act as blocking antibodies. Furthermore, SIT increases the ratio of Th1 cytokines to Th2 cytokines via IL-10-dependent epitope-specific induction of T cell anergy (12, 13).

Despite being a well-established procedure for allergy treatment, some major problems are associated with conventional SIT. First, severe IgE-mediated side effects can occur due to systemic allergen administration. Second, the usage of allergen extracts containing mixtures of allergens and non-allergenic proteins have been reported to induce IgE towards new allergens, and third, therapeutic effective doses often cannot be achieved because of side effects and non-standardized extracts (10, 14, 15).

Due to the usage of allergen extracts, allergy diagnosis bears similar problems in *in vivo* and *in vitro* tests. For practical reasons skin prick test (SPT) represents the method of choice to demonstrate sensitization of a patient to defined allergen sources *in vivo*. Furthermore, *in vitro* serological assays, such as radio-allergosorbent test (RAST), ezyme-allergosorbent test (EAST), or enzyme-linked-immunosorbent assay (ELISA) are performed. These diagnostic methods are useful to evaluate whether the patient possesses IgE antibodies specific to certain allergen sources, but does not give any information concerning the recognition of particular allergens. The gold standard for food allergy is the double-blind placebo-controlled (DBPC) food challenge enabling the diagnosis of the clinical reactivity to certain foods. Immunoblotting would offer the possibility of the analysis of IgE-binding to individual proteins. However, this methodology is time-consuming, requires special laboratory equipment, and is thus not appropriate for routine diagnosis (16).

The enormous increase in the incidence of allergy has intensified efforts in basic and clinical allergy research. Among the latest developments in the field, recombinant allergens offer exciting new possibilities for molecule-based allergy diagnostics and for the development of safer and more effective forms of SIT.

3. WHY RECOMBINANT ALLERGENS?

Allergen extracts that are used for allergy diagnosis and SIT are difficult to standardize. Presently, standardization of allergen extracts is purely based on IgE potency determined by biological assays including competitive IgE-binding assays and skin testing. However, biological standardization does not provide specific information about major allergen content, *i.e.* the content of active ingredients needed for attaining efficacy of immunotherapy. Another disadvantage of the current standardization system is that allergen manufacturers express potencies of their products in company-specific units that do not allow product comparison (Figure 1). In the 1980s the WHO/IUIS Allergen Standardization Subcommittee developed International Reference Preparations (IRP) of several extracts to facilitate product comparison. Unfortunately, these IRP were not adopted by the industry or by regulatory authorities. In the 1990s, major respiratory allergens became available as recombinant allergens and the dependence of effective SIT on administration of defined quantities of major allergens was established. Thus, a system of allergen standardization, that would allow product comparison and give accurate information on the content of active ingredients, was set up by the WHO/IUIS. The aim was the production of international standards of natural and recombinant allergens with verifiable allergen content. These standards would then be available for manufactures, academic organizations, government, and regulatory agencies as reference materials for quantifications of the major active ingredients of allergen products. This initiative was supported by a grant from the European Union for the CREATE project (Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification), which included a team of basic and clinical researchers, regulators, allergen manufacturers, and biotech companies (17). Recently, the Biological Standardization Program (BSP090) was implemented by the European Directorate for the Quality of Medicines (EDQM) as the CREATE follow up.

However, recombinant allergens promise more than the standardization of conventional allergen extracts for allergy diagnosis and therapy. The problems of SIT could in principle be solved by the use of recombinant allergens (10, 18-23). Cocktails of pure and standardized recombinant allergens can be formulated for the

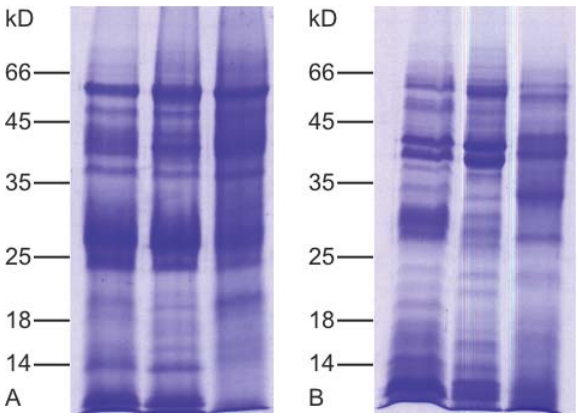

Figure 1. Sodium dodecyl sulfate polyacrylamide gel analysis of weed pollen allergen extracts used for skin prick tests. Prick test solutions are available from different companies (1-3). Differences in allergen concentrations between various products can be observed for both mugwort (A) and ragweed (B) pollen extracts. *Ambrosia artemisiifolia* is the source for ragweed pollen extracts produced by companies 1 and 2, whereas company 3 uses *Ambrosa trifida* pollen as source for extract preparation.

substitution of natural extracts and recombinant allergens can be modified to produce hypoallergens in order to reduce the risk of IgE-mediated side effects in SIT. In addition, recombinant allergens coupled to Th2 response down regulating adjuvants, such as immunostimulatory CpG nucleotide sequences, as well as recombinant allergen peptides offer the possibility of new forms of SIT. In peanut allergy for example, conventional immunotherapy is not recommended because of the high risk of adverse reactions. Peptide-based immunotherapy with short sequences derived from peanut allergen sequences may provide a safe alternative approach in SIT for peanut allergy (20).

In allergy diagnosis, the use of recombinant allergens offers the possibility of component-resolved, *i.e.* molecule-based, diagnosis enabling the identification of the individual proteins within a given allergen source. Molecule-based diagnosis is also important regarding cross-reactive phenomena as it may worsen the prognosis of allergy, e.g. patients sensitized to the panallergen profilin occurring in many pollen and plant-derived foods may develop a pollen-food-syndrome (PFS). Furthermore, as many allergens responsible for PFS are easily degraded during storage or extraction procedure, the biologic activity of allergenic extracts currently used for diagnosis is often very low or even absent. The use of a panel of recombinant food allergens might help to overcome this problem (10, 16, 24).

Molecule-based diagnostics require high-throughput methods for the clinician to diagnose a given allergy. Suitable high-throughput assays for component-resolved diagnostics are recently in development with some of them being already commercially available. The ImmunoCAP® system (Pharmacia Diagnostics, Uppsala, Sweden) for example, is a well-established molecule-based *in vitro* test for the detection of specific IgE and represents the industrial "quasi standard" (25, 26). Microarray technology facilitating high-throughput assays was applied to develop miniaturized allergy tests allowing the determination and monitoring of allergic patient's IgE reactivity profiles to large numbers of allergens by single measurements and minute amounts of serum (27, 28). Additionally, fluorescent multiplex technology (Indoor Biotechnologies, Charlottsville, VA, USA) represents a high-throughput system that allows simultaneously quantification of allergen-specific- and total IgE (29). However, to date molecule-based assays for allergy diagnosis are not used routinely in clinical practice. Thus, the diagnosis of IgE-mediated allergy is still based on patient's history and diagnostic procedures applying natural allergen extracts performed almost like more than a century ago.

In conclusion, recombinant proteins may be applied to standardize allergen extracts and for the generation of hypoallergens for a safer immunotherapy. In addition, allergen molecules or a panel of recombinant allergens might be suited to replace allergen extracts in diagnosis and to facilitate molecule-based diagnostics.

4. REQUIREMENTS ON RECOMBINANT ALLERGENS FOR DIAGNOSIS AND THERAPY

Depending on the clinical application (diagnosis and therapy), recombinant allergens must fulfill different specifications with respect to their IgE-binding activity. Successful SIT leads to a modulation of the immune response to allergens primarily on the T cell level. The beneficial effects for the patient depend on the concentration of allergen used. However, the risks of IgE-mediated anaphylactic side effects also increase with the amount of applied allergen. Therefore, the production of hypoallergenic molecules, in terms of low IgE-binding, might contribute to a safer immunotherapy. The uptake of allergens by antigen-presenting cells (APC) is mediated by the interaction of the allergen with specific IgE and leads to higher Th2 cytokines and IgE production. Hypoallergens displaying reduced IgE-binding capacity might avoid these pathways and preferentially target APCs that utilize phagocytosis or pinocytosis for antigen uptake (e.g. macrophages and dendritic cells) and therefore induce a balanced Th0- or Th1-like cytokine milieu as well as low IgE and high IgG production. Therefore, besides possessing reduced IgE-binding epitopes, hypoallergens should preserve the structural motifs necessary for T cell recognition (T cell epitopes) and for the induction of IgG antibodies against the natural allergen (blocking antibodies) (10, 30-33).

In contrast, allergy diagnosis requires molecules with full IgE-binding capacity. This can be achieved through the purification of natural allergens or the production of wild type recombinant allergens.

The production of hypoallergens with reduced allergenic activity but conserved antigenicity for a safer

immunotherapy represents a great challenge. Presently, three major groups of hypoallergens, *i.e.* chemically modified hypoallergens, naturally occurring hypoallergens, and genetically modified hypoallergens, can be distinguished (10). Recombinant production methods bear major advantages for the generation of hypoallergens of all three groups. These will be briefly discussed focusing on so far established techniques and illustrated with prominent examples in the following paragraphs.

4.1. Chemically Modified Hypoallergens

It has been demonstrated that chemical modification of allergens can decrease their allergenicity (IgE-binding activity) while preserving the antigenicity (T cell reactivity). A big advantage of this approach is that chemical modifications are applicable to almost every allergen molecule as they are based on chemical properties of proteins in general.

Allergoids for example, are produced by chemical modifications with aldehydes and maintain immunogenicity but show significantly reduced IgE-binding activity due to the destruction or the masking of structural B cell epitopes by chemical cross-linking. Thus, they are better tolerated by patients, higher doses can be administered, and therefore, less injections are necessary for successful treatment (10, 18). However, presently commercially available allergoids in clinical practice are produced of allergen extracts and exist of bulky macromolecules with undefined structure and sequence. Carbamylation offers the production of monomeric allergoids that have been shown to induce IgG antibodies recognizing the native allergens in animal models (34). The use of recombinant allergens in the production of allergoids would seem a much better approach, as it has been demonstrated within a study using recombinant Phl p 5b allergoid (10, 35).

Other chemical modifications include maleylation (36-38) and conjugation with immunostimulatory DNA sequences (ISS), termed allergen-ISS conjugates (AIC) (39, 40). The former promise an attractive approach to modulate allergen-specific immune responses. As demonstrated by studies with shrimp tropomyosin and a peptide of mite Der p 1, maleylated derivatives are targeted to pattern recognition receptors of APCs and display reduced allergenicity and increased immunogenicity with a dominant Th1-type immune response (41, 42). Similarly, the latter revealed to induce strong Th1 responses in mice by coupling synthetic oligodeoxynucleotides containing immuostimulatory CpG sequences from bacterial DNA. A clinical study performed with an AIC prepared with purified natural Amb a 1 from ragweed pollen provided evidence for the high safety profile and strong immunogenicity properties of the AIC. Further developments for this approach could focus on recombinant-based AIC preparations (10, 43-45).

4.2. Naturally Occurring Hypoallergens

Due to the presence of several alleles or gene families, plant-derived allergens often are polymorphic, *i.e.* exist as isoallergens (46). Indeed, sequence polymorphisms have been described for many major allergens, e.g. Amb a 1 (47), Cor a 1 (48), Bet v 1 (49), group 1 and 5 grass pollen allergens (50-53), Mal d 1 (54), Api g 1 (55), and group 1 and 2 house dust might allergens (56, 57). Polymorphisms can have important effects on T cell and B cell recognition. For example, the Bet v 1 isoforms Bet v 1d, -g, and –l were found to be potent in T cell stimulation but low in their IgE-binding capacity (58-60). Indeed, such well-characterized molecules would be excellent candidates for SIT. In addition, the standardization of allergenic extracts for diagnostic purposes can be affected by differing isoform proportions in allergenic sources from different regions. It has even been shown that individual birch trees from the same geographic area produce various subsets of isoallergens, emphasizing the importance of careful selection of isoforms for diagnosis and therapy (61). Naturally occurring hypoallergens as candidates for recombinant-based vaccines could help to overcome IgE-mediated problems of SIT. However, natural hypoallergens have not been identified for many allergen families. Instead, genetic engineering has been widely used for hypoallergen generation.

4.3. Genetically Modified Hypoallergens

As genetic engineering involves the targeted modification of a protein in order to alter its function or properties in a predictable manner, the understanding of the relationship between structure and function of an encoded protein is a prerequisite for precise and effective gene manipulation. Aiming at the reduction of IgE-binding activity but retention of T cell reactivity, the production of hypoallergens by genetic engineering usually requires the knowledge of the allergen's T cell and B cell epitopes. Potential problems when modifying the genetic sequence of a protein, and consequently targeting its conformation are the loss of solubility of the final product. As denatured or unfolded proteins tend to form aggregates, such preparations would not be suitable candidates for therapeutic application. So far, various techniques for the generation of genetically modified hypoallergens have been employed, e.g. site-directed mutagenesis, deletion mutagenesis, allergen fragmentation, allergen oligomerization, the production of chimera (hybrids and shuffled molecules), and allergen fusion proteins (10).

Many of the studies have been performed with the birch pollen major allergen Bet v 1. For example, bioinformatic-based analysis of Bet v 1 isoallergens and homologues enabled the prediction of functional residues of the pollen allergen and the generation of a six-point mutant by site-directed mutagenesis. Compared to the wild type Bet v 1a, the mutant showed dramatically reduced skin reactivity in allergic individuals. Furthermore, Bet v 1a-specific T cell clones originating from birch pollen allergic patients proliferated in response to the hypoallergen (31). In addition, Bet v 1 was used as model antigen for the generation of hypoallergenic allergen oligomers (full-length Bet v 1 trimer) and –fragments (produced as two fragments covering the whole Bet v 1 sequence but therefore disrupting the three-dimensional structure) (62-67). Both the Bet v 1 trimer as well as the Bet v 1 fragments harbored the relevant T cell epitopes but

Table 1. Commonly used expression systems for the production of recombinant allergens

Expression system	Organism	Commonly used species	Advantages	Disadvantages
Prokaryotic	Bacteria	*Escherichia coli*	well-characterized, easy to handle, and cost-effective expression system allows high-yield protein production a large number of host strains and expression vectors with a broad selection of fusion proteins, tags, and non-fusion proteins is commercially available possibility of intra- and extra cellular expression	formation of inclusion bodies protein misfolding lacks eukaryotic posttranslational modifications lipopolysaccharide contamination
Eukaryotic	Yeast	*Pichia pastoris Saccharomyces cerevisiae*	easy to handle, and cost-effective organism allows high-yield protein production simple eukaryotic posttranslational modifications (disulphide bonds, O- and N-glycosylation) intra- and extra cellular expression	only linkage of mannose residues in glycosylation no posttranslational modification of higher eukaryotes protein hyper-glycosylation (S. cerevisiae)
	Baculovirus infected insect cells		all advantages of higher eukaryotic organisms	more sophisticated handling low-yield expression problems problems in purification due to tissue culture medium additives insect glycosylation differs from other higher eukaryotes
	Plants	*Nicotiana tabacum*	usage of intact plants and cell lines possibility of viral infection or transgenic plant technology plant posttranslational modifications	time-consuming elaborate purification strategies low-yield expression problems
	Mammalian cells		all advantages of higher eukaryotic systems	expensive and difficult in handling low-yield expression problems
Cell-free			production of toxic proteins great variety of manipulations possible	mostly low-level production

displayed reduced skin reactivity compared to the natural allergen. Additionally, both hypoallergenic Bet v 1 preparations were able to induce blocking IgG antibodies in animal models. Recently, the successful generation of hypoallergenic Bet v 1 chimeric molecules produced by gene shuffling (randomly *in vitro* DNA recombination) of 14 genes of the Bet v 1 family that might be used as multi-vaccines has been reported (68). Another approach for the generation of hypoallergenic chimera is the insertion of a small portion of an allergen of interest in a large portion of a homologous but weakly cross-reactive host protein serving as scaffold for the maintenance of the native protein structure. First studies have been performed with yellow jacket Ves v 5 and paper wasp Pol a 5 allergens resulting in reduced allergenicity in terms of histamine release from basophils of yellow jacked venom sensitized patients (69).

Another approach used for the creation of hypoallergens of Phl p 5b, a major timothy grass pollen allergen, consisted in the deletion of identified IgE epitopes avoiding the removal of T cell epitopes. Some of the Phl p 5b deletion mutants showed reduced IgE-binding properties, no histamine releasing activity, reduced skin reactivity, and no significant changes in T cell reactivity (70). Moreover, timothy grass pollen allergens were used for the production of hypoallergenic fusion proteins. However, these constructs did not show reduced IgE-binding activity (71).

5. PRODUCTION OF RECOMBINANT ALLERGENS

Generally, the generation of a recombinant protein can be divided into cloning, expression, and purification of the recombinant product. As these three steps depend on each other, they have to be carefully considered when planning the production of any particular recombinant allergen. The first production criterion is the selection of an appropriate expression system. Nowadays, several prokaryotic and eukaryotic expression systems (e.g. yeast, insect and mammal cells, plants) are available (Table 1) but the determination of which system is the best choice for expression of a particular allergen requires several considerations on time and resources as well as on the nature of the allergen. Bacterial expression systems are easy to handle, cost-effective, and usually produce high yields of recombinant proteins. In contrast, the major advantage of eukaryotic expression systems is the ability to perform many of post-translational modifications, including the processing of signal peptides, folding, disulfide-bond formation, and the addition of lipids and/or carbohydrates (72). In fact, almost all natural allergens are derived from eukaryotic sources and frequently contain intra-molecular disulfide bonds as well as post-translationally linked carbohydrates. Although the clinical relevance of sugar-specific IgE is still a matter of debate (73-77), in certain cases the production of a glycosylated form may be desirable. Due to the mentioned advantages of prokaryotic expression systems the majority of recombinant allergens have been produced in bacteria – *Escherichia coli* representing the best characterized and most commonly utilized system (72, 78). However, if the native conformation and/or post-translational modifications are necessary for the activity of the recombinant protein, a switch from bacterial-based- to eukaryotic expression systems would be desirable. In addition, many allergens display diverse biologic functions (e.g. enzymes, enzyme inhibitors, lipocalins, or structural proteins) (20). There is evidence that the protease activity of Der p 1 influences the allergenicity of the house dust mite major allergen by (i) increasing the permeability of the respiratory mucosa due to tight junction disruption (79), (ii) by promoting IgE synthesis due to low-affinity IgE receptor cleavage, and (iii) by augmenting Th2 responses through effects on the IL-2 receptor (80-84). However, to date such phenomena have only been reported for Der p 1. Thus, in general biologic function does not seem to be linked to an allergen's ability to induce an IgE response. To summarize,

extensive demands are placed on the production of recombinant allergens and therefore on the choice of a suitable expression system.

Although there is the possibility of autologous production (*i.e.* the expression of a protein in the source of its origin), mostly recombinant proteins are over-expressed as foreign proteins in host organism (heterologous expression). This can result in different problems, like for example toxicity of the recombinant protein to the host cell. To circumvent this problem, cell-free expression systems have been developed that facilitate a great variety of manipulation but are mostly restricted to low-levels of protein production (72). Additional problems concern the solubility of incorrectly folded proteins, protein aggregation, and the formation of inclusion bodies. Several approaches to overcome these drawbacks have been reported, like stress stimulation (e.g. low temperature, starvation, hyperosmotic conditions), co-overexpression of molecular chaperones, and fusion protein technology (soluble protein fusion, e.g. maltose binding protein). Further, there is the possibility of refolding incorrectly folded proteins by dialysis or diafiltration under conditions favoring disulphide bond formation. Further solutions comprise the fusion of a histidine tag to the recombinant protein and subsequent binding to bivalent metal ions immobilized on a column matrix, which allows on-column refolding of unfolded recombinant products. As affinity chromatograpy represents an effective and specific method for protein purification, fusion tags (the most prominent examples are the histidine tag and the glutathione/glutathione-S-transferase tag) are widely used for the production of recombinant allergens (72, 78, 85) (86). However, recombinant fusion proteins may be not suitable for clinical application. Therefore, systems enabling the removal of the fusion partner are necessary. For this purpose, many plasmid expression vectors facilitating the production of tagged fusion proteins and subsequent enzymatic or chemical removal of the fusion tag are commercially available.

Produced without fusion tag, recombinant proteins can be purified by several standard chromatographic techniques, such as gel filtration-, ion exchange-, hydrophobic interaction-, and, if antibodies are available, immuno-affinity chromatography. Furthermore, salt precipitation methods proved to be useful tools for the pre-purification of recombinant proteins (86). However, the method or methods of choice always depend on the properties of the protein of interest.

To summarize, the production of a recombinant allergen starting from a simple DNA sequence to the final product for application in diagnostics and therapy of allergic disease represents a complex process and requires a good knowledge about the properties of the natural counterpart, e.g. DNA sequence, physicochemical and immunologic characteristics. Many steps that depend on each other, like the choice of expression vector and -system as well as purification strategies, have to be carefully considered and may vary with the properties of each molecule.

6. SELECTING A GOOD CANDIDATE FOR A FUTURE PRODUCT

According to the current edition of the European Pharmacopoeia (Ph. Eur. 6.0) that represents the valid and mandatory reference work for the quality control of medicines in Europe, several criteria have to be monitored for recombinant allergen products in order to ensure the validation of the production process. Besides a panel of tests for the extraction and production procedure (e.g. exclusion of extraneous agents, adequate removal of vector, host cell, culture medium, and reagent-derived contaminants, as well as maintenance within the stated limits of product yield) these characterization criteria include identity, purity, potency, and stability of the final bulk product (Ph. Eur. general monograph N° 01/2008:0784 on products of recombinant DNA technology). In general, the initial characterization of a reference substance is carried out by a wide range of chemical, physical, immunochemical, and biological tests prior to release of the product, while in the latter, production consistency is controlled by a limited amount of appropriate assays in comparison to a reference substance.

The three pillars of selection criteria for the production of a future recombinant allergen or hypoallergen product are summarized in Table 2. Besides the biological activity (the immunological behavior in case of allergens) as the central pillar with the highest priority, process feasibility such as yield and solubility, and physicochemical characteristics represent the most important features of recombinant (hypo-) allergens.

7. CHARACTERIZATION OF RECOMBINANT ALLERGENS

For a better visualization of the extensive selection and characterization procedure this shall be exemplified for the hypoallergenic recombinant version of the birch pollen major allergen Bet v 1. This molecule, termed rBet v 1 A1-6 mutant, has been designed *in silico* to obtain a molecule displaying reduced IgE-binding capacity than its wild type counterpart rBet v 1a (31). The recombinant protein was expressed in *Escherichia coli* at high level (>100 mg protein per liter of bacterial culture) and purified upon freeze and thaw extraction from the soluble fraction by standard chromatography techniques. In this first described hypoallergen, the six point mutations T to P_{10}, F to V_{30}, S to N_{57}, S to C_{112}, I to V_{113}, and D to N_{125} were shown to cause a reduction in IgE-binding by immunoblots and ELISA inhibition experiments using sera from 13 birch pollen-allergic patients. While the reduced allergenic reactivity was shown to be between 10- and 1000-fold by skin prick testing of eleven patients, the T cell reactivity of A1-6 mutant and wild type rBet v 1a remained comparable. This was measured by proliferative responses of nine T cell clones reactive to different epitopes spread throughout the molecule. Such a molecule would nicely fulfill the commonly applied criteria for hypoallergens, as IgE reactivity was significantly reduced, whereas integrity of T cell epitopes was preserved.

Table 2. Selection criteria for production and immunological/physicochemical characteristics of recombinant (hypo-) allergens

Process feasibility	Biological activity	Physicochemical characteristics
Yield • expression levels in E. coli shall be >5% of total protein	**(reduced) IgE reactivity** • IgE immunoblotting and competitive ELISA with sera from allergic patients compared to natural or wild type recombinant counterpart	**Identity** • intact mass spectrometry • liquid chromatography-tandem mass spectrometry-based peptide mapping • determination of potential protein modifications: Asn deamidation, Met oxidation, Cys carbamylation, etc. • N-terminal amino acid sequencing • amino acid composition
Solubility • protein shall be soluble in physiological buffer after renaturation	**Basophil activation test** • CD63 expression on isolated human basophils from allergic patients • CD203c up regulation	**Concentration or amount** • amino acid analysis/determination of molar extinction coefficient • total nitrogen determination • UV_{280} absorption • other types of protein assays (e.g. Lowry)
	Mediator release assay • histamine release from isolated human IgE-stripped basophils • mediator release from humanized rat basophil leukemia cell line	**Homogeneity and purity** • SDS-PAGE analysis • isoelectric focusing • reversed phase high performance-liquid chromatography • high performance-size exclusion chromatography • asymmetrical flow field-flow fractionation • (multi-angle) light scattering detection of monomer, dimers, oligomers, etc.
	T cell reactivity • integrity of T cell epitopes assayed by proliferation of T cell clones or lines	**Molecular folding and stability** • circular dichroism/determination of denaturation temperature • Fourier Transform-infrared spectroscopy/stability studies on formulation
	Immunogenicity in mice • ability to produce IgG antibodies capable of blocking histamine release	**Aggregation behavior in solution** • dynamic light scattering • stability testing by storage time/temperature/freeze and thaw cycles • analytical ultracentrifugation
	Skin testing (only for GMP-grade preparations)	

7.1. Biological activity

Beyond the aforementioned immunological tests hypoallergenic features may be monitored by activation of basophils (BAT, basophil activation test) from allergic patients (87) or histamine release assays using IgE-stripped basophils from non-allergic donors incubated with allergen and sera from allergic patients (88-90). Alternatively, the rat basophil leukemia cell line expressing human FcεRI represents a versatile and reliable tool for applying mediator release assays in a routine format (91). Notably, recombinant hypoallergens have to preserve immunogenicity (i.e. capability to raise blocking IgG antibodies), which is usually tested in mice and rabbits (92). Importantly, all biological assays for hypoallergens have to be performed in comparison to the wild type allergen, either natural or as recombinant protein.

7.2. Physicochemical Characteristics

At the physicochemical side of product characterization the following factors have to be investigated for the (hypo-) allergen preparation: identity, stated concentration or amount (lyophilized products), homogeneity and purity, correct molecular folding and thermal stability, and adequate aggregation behavior in solution. Most importantly, all physicochemical characteristics of the wild type recombinant candidate shall be as closely comparable to its naturally existing counterpart as possible. Figure 2 depicts an array of physicochemical analyses performed for the recombinant hypoallergen rBet v 1 A1-6 mutant.

7.2.1. Identity

To date, methods for verifying the identity of a recombinant allergen have been greatly improved by the development of highly sophisticated mass spectrometry (MS)-based techniques. On the one hand, the determination of the intact molecular mass with a precision of <1 Dalton represents a highly specific parameter for sample identity, on the other hand, unwanted protein modifications on amino acid residues like cysteines, methionines, asparagines, etc. can be detected routinely. Furthermore, peptide mapping combined with tandem mass spectrometry (MS/MS)-based protein sequencing has been made available for giving a clear answer on protein identity as well as verifying the existence and, furthermore, enabling a localization of eventually present protein modifications. Typically these equipments allow the separation of a peptide mix by capillary reversed phase liquid chromatographic coupled online to electrospray ionization tandem mass spectrometers. For good quality, data of MS/MS-based peptide mapping sequence coverages of >80% are required. This can be achieved by the use of pepsin and V8 proteases, or by cyanogen bromide-mediated chemical proteolysis in addition to the most-widely used digestion method by trypsin. Remarkably, specific assays for protein modifications like asparagine deamidation exist, even enabling quantification. Carbamylation of cysteine residues, which represents an unwanted modification originating during purification procedures under denaturing conditions (6 M urea), can be detected by MS-based methods routinely. Besides the methods mentioned above, well-established techniques like N-terminal protein sequencing by Edman degradation are also routinely applied.

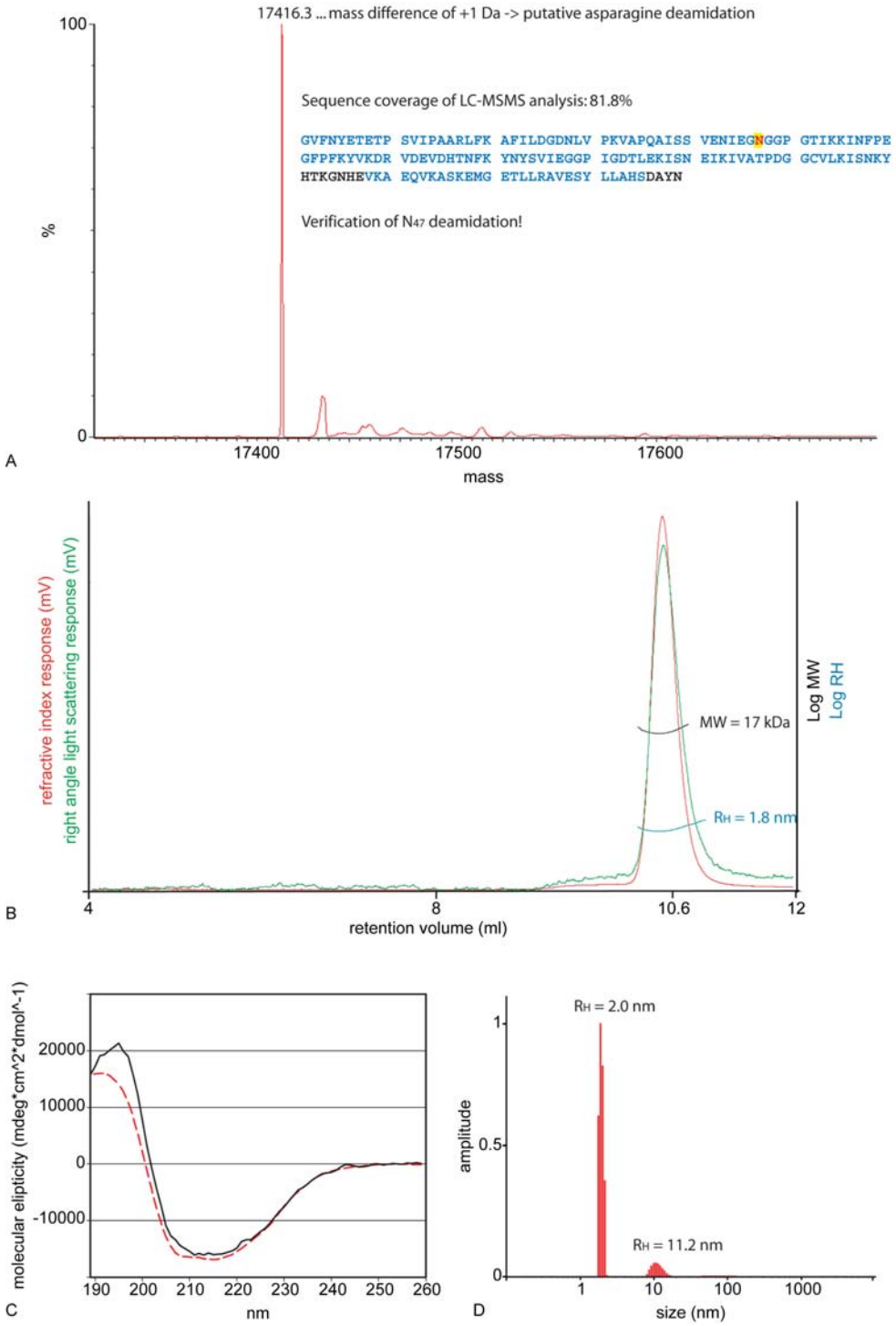

Figure 2. Physicochemical parameters of recombinant Bet v 1 A1-6 mutant; investigation of sample identity (A) by intact MS resulted in mass difference of +1 Da and LC-MSMS-based peptide mapping revealed a sequence coverage of 81.8% (underscored in blue) verifying asparagine deamidation in position 47 (in red and highlighted in yellow); sample homogeneity (B) was shown by refractive index (red) and right-angle light scattering (green) signal of HPSEC resulting in >99% monomeric molecule with an approx. MW of 17 kDa (black) and hydrodynamic radius (R_H) of 1.8 nm (blue); conformational analysis performed by CD (C) resulted in comparable secondary structural content of rBet v 1 A1-6 mutant (in red) with wild type rBet v 1a (in black); investigation of aggregation behavior in solution by DLS (D) resulted in approx. 90% monomeric molecule with a R_H of 2.0 nm and approx. 10% high molecular weight aggregates.

7.2.2. Concentration or amount

Beyond common photometric methodologies (e.g. UV_{280} absorption, Bradford, Lowry), semi-automated amino acid analysis represents an accurate technique for determination of protein content without the necessity to refer to an external protein standard and being independent of sequence and charge of a protein. In addition, the determined amino acid composition can confirm sample identity and gives a measure of sample purity. Furthermore, when combined with UV absorption measurements, it allows the determination of extinction coefficients under variable conditions. Amino acid analysis based on reversed phase-HPLC and coupled with semi-automatic robotic sample derivatization (o-phtalaldehyde method) immediately before injection, has been validated as a quantitative technique using the seven best-recovered amino acids Asx (Asp + Asn), Glx (Gl + Gln), Arg, Ala, Phe, Leu, and Lys (93). In principle, methods based on the determination of total nitrogen content (Kjeldahl) fulfill the same requirements, but suffer from lacking reproducibility due to more extensive manual handling steps.

7.2.3. Homogeneity and purity

The recombinant allergen preparations must be pure with respect to protein content. The method of choice is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). More than 95% of the material must be recovered in a single band after SDS-PAGE followed by protein staining. In addition, isoelectric focusing and high performance-liquid chromatographic (HPLC) techniques have been commonly applied operating with C_3, C_4, or C_8 reversed phase media (separation by hydrophobicity). Furthermore, high performance-size exclusion chromatography (HPSEC) systems are readily available for separations based on molecular size. Differences in diffusion coefficients are employed during asymmetrical flow field-flow fractionation (AF_4), which has been used recently for the characterization of a chemically treated hypoallergenic fold variant of recombinant Bet v 1a (94). The hydrodynamic radius can be calculated from the diffusion coefficient by the Stokes-Einstein equation. As pre-filtering the sample to prevent column clogging is not necessary for AF_4, this technique has some advantages compared to HPSEC. However, robustness for routine laboratory use still remains to be shown. Both AF_4 and HPSEC methods can be coupled online to arrays of detection systems for monitoring UV absorption, refractive index, intrinsic viscosity, and (multi-angle) light scattering. Hence, the hydrodynamic radius and the molecular weight of the elution peaks can be determined.

7.2.4 Molecular folding and thermal stability

Considerations regarding the molecular structure of allergens have become a very important issue, as IgE reactivity depends on the conservation of conformational epitopes. Besides highly sophisticated and time-consuming methods for structure determination (nuclearmagnetic resonance, X-ray crystallography), circular dichroism (CD) is commonly used for investigating molecular folding (95). In principle, the far UV CD spectra of recombinant wild type molecules must show typical spectra of folded protein with peak amplitudes similar to reference spectra, in this case natural allergen preparations. In contrast, hypoallergenicity may be caused by the loss of molecular conformation and, therefore, can also be measured by CD. Another important parameter, which also has to be discussed in the context of sample stability, is the denaturation temperature and the ability to renature upon thermal denaturation. As a rule of the thumb, the higher the denaturation temperature the more stable a protein is.

By means of Fourier Transform-infrared spectroscopy (FTIR) stabilizing and destabilizing effects of hydrogen bonds in beta-sheets, transitions from alpha-helices and from parallel to antiparallel beta-sheets leading to protein aggregation can be visualized and denaturation temperatures can be obtained. Notably, FTIR spectroscopy can be performed on both liquid and solid samples using the transmission or attenuated total reflection (ATR) modes, respectively. Thus, the influence of pharmaceutical formulation (e.g. lyophilization, adjuvants, stabilizer additives) on protein stability in the final medicinal product can be investigated by ATR-FTIR (96).

7.2.5 Aggregation behavior in solution

For investigation of the aggregation behavior in solution (without chromatographic or other type of separation) dynamic light scattering (DLS) can be used. By DLS the hydrodynamic radius of molecules is determined from the diffusion speed. This method works by comparing the fluctuations in intensity of scattered light at an initial time with subsequent measurements at later time points. Hence, a correlation function is recorded, from which the size of the molecules is calculated giving good insight into samples without influencing the dynamic process of protein aggregation by shear forces or dilution effects, which can occur in chromatographic techniques. In addition, thermal stability can be determined by temperature ramping experiments. Storage stability related to time, temperature, or upon repeated freezing and thawing cycles can also be easily monitored.

To summarize, recombinant (hypo-) allergens for diagnosis and SIT have to fulfill rigorous safety and efficacy requirements. Therefore, careful selection processes and extensive biological and physicochemical analyses of the appropriate candidates should be implemented.

8. CONCLUSIONS AND FUTURE PERSPECTIVES

Recently, the American Academy of Allergy, Asthma and Immunology (AAAAI), the American College of Allergy, Asthma and Immunology (ACAAI), and the Joint Council of Allergy, Asthma and Immunology (JCAAI) published the second update of a practice parameter for allergen immunotherapy (97) with the objective of optimization and the intention to establish guidelines for safe and effective treatment using allergen extracts. The advantages of recombinant allergens for allergen extract standardization and safe immunotherapy

are barely mentioned. As already discussed allergenic extracts, used for allergy diagnosis and therapy, contain mixtures of allergens, non-allergenic and/or toxic proteins that are difficult to standardize. Therefore, due to the usage of company-specific units, product comparison between allergen extracts of different manufactures is not possible (Figure 1). These extracts of undefined composition bear the risk of IgE-mediated side effects and of sensitization to new allergens. In addition, the relevant allergens for a given patient may be present at low concentration or even absent. These problems could be avoided by simply using recombinant allergens. However, as recombinant allergens have to fulfill multiple requirements, compared to their natural counterparts, the production is rather demanding. These comprise exclusion of extraneous agents, adequate removal of vector, host cell, culture medium, and reagent-derived contaminants as well as identity, purity, potency, and stability of the final protein. Purified natural allergens, which should possess all these properties, have major disadvantages: First, they cannot be produced in the high amounts necessary, and second, with the exception of a few naturally occurring hypoallergens, natural allergens are not hypoallergenic and therefore, do not offer the possibility of a safer SIT. In contrast, recombinant allergens or panels of recombinant allergens promise the perspective of a safe, patient-based immunotherapy lacking the danger of new sensitizations and side effects because of the possibility of hypoallergen generation. A prerequisite for patient-based therapies are component-resolved diagnoses, which can be performed using recombinant allergens in high-throughput assays.

As recently reviewed by Wallner *et al.* (13) clinical studies using recombinant pollen allergens have successfully been performed. For example, a mixture of five recombinant grass pollen allergens was administered in a DBPC immunotherapy trial resulting in grass pollen-specific IgG induction and an improvement of the symptom medication scores (98). Additionally, the subcutaneous injection of hypoallergenic Bet v 1a fragments in a DBPC multi-center study led to the induction of protective IgG antibodies that cross-reacted with homologous pollen allergens. Induction of Bet v 1-specific antibodies and a reduction of IL-5 and IL-13 expression have also been reported in a DBPC clinical study with a hypoallergenic Bet v 1 trimer (62, 99). These results support the idea of substituting allergen extracts by recombinant (hypo-) allergens for a safer SIT.

In conclusion, substitution of allergen extracts by recombinant allergens facilitates molecule-based diagnostics and consequently a patient-customized and safer immunotherapy.

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