

Comparison of Natural Grass Pollen Allergens and Their Recombinant Counterparts Expressed in Bacterial and Mammalian Systems

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Introduction

For practical use recombinant allergens have many advantages compared to their natural counterparts: The structure is clearly defined and recombinant allergens can therefore serve as primary standards to determine quality and quantity of allergens in extracts. For diagnostics it is necessary to exactly reconstruct the natural allergen by recombinant techniques, before in the second step (i.e. the therapy) the allergen is modified or mutated in a suitable way. Without the first step it is not possible to correlate directly the effect of a specific recombinant allergen with the benefit in the therapy. A further advantage of recombinant allergens is the easy affinity purification by their tags or as fusion proteins, from which the recombinant allergen must be afterwards cleaved off and isolated. In contrast, purification of natural allergens are often time-consuming and results in low yields. Sometimes the products are even modified because of the separation conditions. But recombinant allergens do not necessarily possess the same structure as their natural counterparts. Qualitative and quantitative problems that might occur upon expression are summarized in Table 1.

Table 1. Possible problems in the expression of recombinant allergens.

qualitative problems:	low amounts of products caused by • different codon usage • toxic expression products
quantitative problems:	varying products caused by different biosynthetic pathways • formation of disulfide bonds • posttranslational modifications, e.g. glycosylation, phosphorylation

her amounts of the structure can differ. The problem of the prokaryotes is problems in solubility and posttranslational modifications.

complex and enable the. But the posttranslational modifications we expressed grass pollen differences. The specific products that

we focused on two isoforms of Phl p 6 (1,2)

Fig. 1.

Phl p 1

and 37 kDa

6.4 – 7.3

amino acids

7

5%

+

complex

ns were compared by Western blotting methods. Cloning in pET21a (Novagen, Madison, WI, England Biolabs) and expression in *E. coli* (Novagen), which is

transfected *E. coli* by Western blotting. 2D-PAGE immunoblotting of Phl p 6, the main allergen of the three experi-

ments. These results were confirmed by MALDI TOF mass spectrometry analysis. For natural isoforms we determined 12.1 and 12.3 kDa, which is in good accordance to 11.8 kDa of the calculated mass. Expression of Phl p 1 in *E. coli* resulted in intense spots of about 35 kDa, but also components of lower masses were observed indicating degradation products. The proteins focused at pI 5.3 and 7.2. Using the mammalian expression system, we hardly could detect any product. According to the results of MALDI TOF MS analyses the masses of nPhl p 1 isoforms lay within the range of 27.6 to 28.4 kDa. The calculated mass of rPhl p 1 was 26.0 kDa. Thus, the difference of about 2 kDa may be caused by posttranslational modifications, for example glycosylations (7).

To compare natural and recombinant allergens under native conditions, we separated pollen extract and the lysates of the transfected *E. coli* and COS cells by IEF (Isoelectric focusing) and subsequently immunostained the allergens on nitrocellulose membranes with monoclonal antibodies. Phl p 6 from pollen extract and transfected *E. coli* lysate were detected as intensive bands of pI 5.7 and 6.0. The band at pI 6.0 was also detectable in the COS cell lysate. In contrast, nPhl p 1 showed several bands of different pIs, whereas Phl p 1-reactive bands from the two recombinant expressions corresponded to none of these pIs. Because of the low yields in the mammalian expressions, we further investigated the recombinant allergens from the bacterial expressions and the natural ones.

Secondary structure determination

According to secondary structure predictions by Garnier et al. (8) Phl p 6 possesses a high degree of α -helical structure (63%). This was confirmed by CD spectroscopy of natural and recombinant Phl p 6, which were quite similar. A stable secondary structure was much less pronounced in Phl p 1 with only 19% helix, 24% strand but 57% random coil. Indeed, rPhl p 1 depicted a very flat curve indicating nearly complete random coiled structure, which differed considerably from nPhl p 1. From these results we expect that the secondary structure of the recombinant protein will be as stable as that of the native protein.

IgE reactivity and mediator release

The determination of IgE reactivity was performed by testing several individual patient sera in an ELISA. Natural and rPhl p 6 showed no significant differences indicating similar structures and similar IgE reactivities. On the other hand, rPhl p 1 showed a decrease by about 50% in IgE binding compared to nPhl p 1. To find out the reason for the decreased reactivity we denatured, reduced and carboxymethylated Phl p 1. The ELISA results demonstrated that denaturation alone had no effect on the binding, but the destruction of the disulfide bonds resulted in a decrease by about 25% (9). The lack of glycosylation and further conformational changes in rPhl p 1 versus nPhl p 1 caused a two-fold lower IgE reactivity of rPhl p 1. Compared to the dramatic dissimilarities in the conformational structures of nPhl p 1 and rPhl p 1, the IgE binding as well as the histamine release were scarcely pronounced.

Conclusions and prospects

The two major grass pollen allergens Phl p 6 and Phl p 1 differ considerably in complexity. Phl p 6 is a small allergen (13 kDa) with no cysteine residues and no posttranslational modifications. It bears a high degree of stable secondary structure which seems to form the correct conformation spontaneously independent from the expression systems. Thus, natural and recombinant counterparts are quite similar. Phl p 1, in contrast, is rather complex, an allergen of about 35 kDa with no stable secondary structure and an odd number of cysteine residues. The cysteine residue with a free sulfhydryl group can cause a dimerization. Additionally, the natural allergen bears glycosylations that are lacking in prokaryotic expressions. Because of these peculiarities in the conformational structure the expression of the recombinant allergens is difficult, which becomes obvious in the low solubility of rPhl p 1.

The decrease in IgE reactivity by only 50% is caused by the fact that Phl p 1 bears some linear IgE-binding epitopes that are not affected by alterations in the conformation. In contrast, for example the IgE reactivity of Der p 1 decreased more than 100-fold. Der p 2 lost more than 100-fold of its IgE reactivity after one cysteine residue involved in disulfide formation was deleted by site-directed mutagenesis (10). This indicates the strong influence of the conformation on the IgE reactivity of Der p 2.

Our results demonstrate that the analyses of the important allergens are necessary to get an impression of their conformational structure and to find out, how to overcome problems of expression. This knowledge is also mandatory to perform selective mutations in IgE-binding regions to improve the reagents for hyposensitization therapy. The number of available vectors and expression systems increases rapidly. Probable aspects of how to improve the expression of complex recombinant proteins are given in Table 3.

Table 3. Improvements for the expression of complex recombinant allergens.

prokaryotic systems:	for cysteine-containing proteins
	• expression under oxidizing conditions (thioredoxin-lacking mutants or periplasmatic expressions)
eukaryotic systems:	for cysteine-containing and/or posttranslationally modified proteins
	• insertion of signal sequences into the vector
	• expression congruent structures are only obtained if the system is closely related to the natural source

We are confident that more efficient expression systems to obtain a correct folding of the recombinant protein with consideration of posttranslational modifications will soon become available.

Acknowledgment

We are grateful to Dr. H. J. A. van Halbeek for CD determination and Dr. H. J. A. van Halbeek for Borstel, Germany for providing the allergen separations, respectively, for the project.

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Discussion

Vieths: What was express this prote As far as I under modification that

Petersen: Yes, yo with the natural allergen DNA va mammalian syste

Acknowledgments

We are grateful to H. Notbohm (University of Lübeck, Lübeck, Germany) for the CD determinations and to B. Lindner and D. Wicklein (Research Center Borstel, Borstel, Germany) for their contributions in MALDI mass spectrometry and HPLC separations, respectively. M. Böttger is acknowledged for her excellent technical assistance. The project was founded by the Deutsche Forschungsgemeinschaft PE 491/5-1.

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Discussion

Vieths: What was the rationale for selecting the COS cells? Was it really to try to express this protein, and perhaps to get the correct formation of the disulfide bonds? As far as I understand Phl p 1, it is more important to focus at the posttranslational modification that seem to be more important for the full IgE reactivity of this allergen.

Petersen: Yes, you are right. We tried to get recombinant proteins to compare them with the natural allergens. We wanted to focus on what happens if you performed an allergen DNA vaccination. We were interested in the structures that are expressed in mammalian systems. This was the reason for using COS cells.