

EDITORIAL

Isoallergens: a clinically relevant phenomenon or just a product of cloning?

Over the past decades, major allergens of the most important inhalant allergen sources have been identified and characterized. Isoelectric focusing and 2D-gel analysis of purified major allergens demonstrated that variants with different isoelectric points exist [1–11]. Whether this is a result of different post-translational modifications or of differences in primary structure (isoforms) can not be distinguished with these techniques. It was the introduction of molecular biology that produced proof of the existence of multiple isoforms of many allergens at the level of mRNA [12–24]. In this issue of *Clinical and Experimental Allergy*, J. W. Park et al. report the cloning of seven different isoforms of the major house dust mite allergen Der p 2 [25]. They found nine polymorphisms that also resulted in amino acid changes. For each polymorph site, they determined which amino acid was prevailing among the seven isoforms. On the basis of this analysis, two isoforms were selected for expression as recombinant proteins, one with the highest content of dominant amino acids (7/9) and one with the lowest (2/9). Both these recombinant isoforms differed at seven positions (Fig. 1). The immune reactivity of these recombinant allergens was analysed, showing that the isoform with the rarer amino acids was of lower allergenicity. In addition, this isoallergen was not picked up in one of two sandwich ELISAs designed to measure Der p 2 in environmental samples and allergenic products.

Decreased immune reactivity of natural isoforms of major allergens has previously been reported for other inhalant allergens such as Bet v 1 [26]. Such claims were always based on experiments with recombinant isoallergens obtained after PCR cloning from cDNA. In most cases, however, very little is known about the actual *in vivo* expression of such allergen isoforms. In fact, PCR cloning merely demonstrates the presence of mRNA coding for allergen isoforms, but gives no proof of significant expression. If based on sequencing of single clones, a polymorphism might even turn out to be a PCR artefact. Five positions with polymorphisms observed by Park et al. were reported earlier, in four cases with identical substitutions, making it very likely that these are true existing isoforms. Four polymorphisms were really new, but it is not clear whether they were confirmed by independent PCRs. Submission of new sequences to databases does not require such independent confirmation. It is therefore not just hypothetical that some isoforms of major inhalant allergens will turn out to be PCR artefacts.

Is there no proof at all that isoforms really exist as expressed allergenic proteins? Of course it is highly unlikely that all differences observed in isoelectric focusing and 2D-electrophoresis can be attributed to post-translational modifications. The most detailed proof for the existence of isoforms comes from studies on the major birch pollen allergen Bet v 1. In a very elegant study, Swoboda et al. approached the isoform issue from both DNA level and protein level [14]. They cloned 11 Bet v 1 isoforms from birch pollen of which eight had a different translated amino acid sequence, and then used affinity-purified natural

Bet v 1 to investigate whether these isoallergens are really expressed. To this end, a mass-spectrometric approach was chosen. Mass spectrometry is a technique to accurately determine the mass of a protein or peptide. Swoboda et al. subjected their affinity-purified Bet v 1 preparation to two different proteases with defined specificities: trypsin cleaving behind lysines or arginines, and endoproteinase Glu-C behind glutamic acid. On the basis of translated primary structures obtained from the 11 cloned isoforms, theoretical peptides from these digestions were predicted. Peptides resulting from tryptic and endoproteinase Glu-C digestion were purified by high performance liquid chromatography (HPLC) and subsequently analysed by mass spectrometry. By combining mass data and sequence data, the existence of all eight isoforms was confirmed. So far, this is the most convincing evidence for the existence of multiple isoallergens as expressed proteins. The study did not really address the quantitative side of the isoform issue, although it was concluded that the Bet v 1a isoform represented at least 50% of all natural Bet v 1. Later studies identified this isoform as a strong allergenic isoform [26]. By analogy to this, the present study by Park et al. proposes that dominant polymorphisms are linked to high allergenicity. In other words, allergenicity is at least partly a reflection of exposure. From this perspective, exposure to dominant isoforms will more likely result in sensitization than exposure to less abundant isoforms. This hypothesis fits very well with the observation that most major inhalant allergens are major proteins. Analysis of affinity-purified, protease-digested Der p 2 by mass spectrometry could confirm the proposed dominance of certain polymorphisms.

The mass spectrometric approach used by Swoboda et al. does not facilitate the identification of isoforms in natural source materials in the absence of sequence data obtained by molecular biology. Recent developments in the field of mass spectrometry now allow true sequencing of tryptic peptides. Quadrupole-time-of-flight two-dimensional mass spectrometry (Q-TOF MSMS) [27] is a technique that first determines the mass of tryptic peptides, e.g. from a mixture of natural isoforms (1st dimension mass spectrometry). Alternatively, isoforms can first be separated by 2D electrophoresis, allowing analysis of digests of individual spots from 2D-gels. Subsequently, selected peptides are fragmented stepwise (amino acid by amino acid) and a mass spectrum is measured simultaneously (2nd dimension mass spectrometry). This results in the exact primary structure of peptides. In this way, it is now possible to trace dominant polymorphisms without having the exact sequence of all potential isoforms. An additional advantage of the technique is that it can elucidate the presence and nature of post-translational modifications. In the coming years, this powerful tool will certainly supplement the know-how obtained from the molecular biological study of allergens, and reveal which isoforms are expressed at significant levels.

Thus, whether the isoforms of Der p 2 reported by Park et al. are relevant molecules for environmental exposure remains to be determined. Independent from this, however, their study

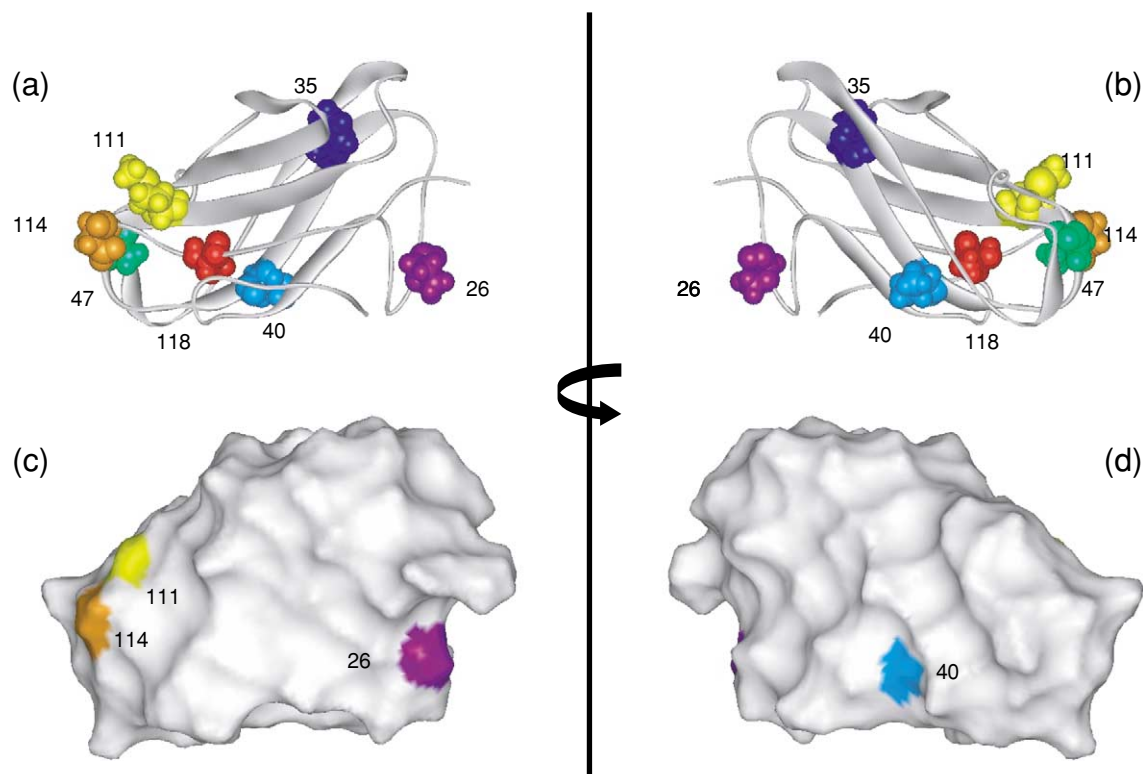


Fig. 1. The 3-dimensional structure of Der p 2 has been solved by Mueller et al. [40–42]. On the basis of this model, the seven differences in primary structure between the hypo- and hyperallergenic isoallergens of Derp 2 reported by Park et al. were located. Panel (a) and (b) show all seven polymorphisms (P26T, F35Y, V40L, T47S, M111L, D114N and L118G). Panel (c) and (d) illustrate that only positions 26, 40, 111 and 114 are surface-exposed. Panel (b) and (d) are rotated 180° compared to panel (a) and (c) to show both sides of the molecule. The differences in immune reactivity are most likely linked to one or more of these surface-exposed residues. For the polymorphism at position 114 a dominant role in the epitope for one of the monoclonal antibodies used in the Der p 2 sandwich ELISA was clearly demonstrated by Hakkaart et al. [38]. Amino acid 26 is part of a loop stabilized by a disulphide bond between residues 21 and 27. This loop has been indicated in IgE binding [36].

clearly has various important implications. Firstly, when producing recombinant allergens for diagnostic purposes [28], isoforms should be chosen that ensure high IgE binding capacity. Most studies using recombinant Der p 2 have used the isoform originally cloned by Chua et al. [29, 30]. In the light of the observations made by Park et al. it can be questioned whether this isoform is the ideal choice as a diagnostic reagent. It contains only 5/9 proposed dominant amino acids. In our laboratory, 142 sera with specific IgE antibodies to Der p 2 were tested in a RAST with purified natural Der p 2 and this recombinant isoform. The mean IgE response to the recombinant was 25% lower ($P < 0.001$) than to its natural counterpart (unpublished observations). Possibly, an isoform with all proposed dominant amino acids would have an IgE-binding capacity closer to the natural allergen. In other words, the first isoform of an allergen cloned by PCR, is not necessarily the best choice for future diagnostic purposes.

The observation that the allergenicity of certain isoallergens is significantly reduced has completely different implications for allergen-specific immunotherapy. Here, from a safety perspective low allergenicity is generally regarded as an advantage [31, 32]. Sequence comparison of hyper- and hypoallergenic isoforms can help identifying critical amino acids for IgE-binding. This information can then be used to design hypoallergenic mutant molecules. This route was for example successfully followed for Bet v 1, introducing several point mutations at

positions coding for surface-exposed residues [33]. For house dust mite group 2 allergens most attention was given to mutants disturbing disulphide bridge formation [34–36], probably resulting much more in disruption of the overall conformation than in case of the Bet v 1 mutant [37]. Little is known about structural differences between naturally occurring isoallergens. Is hypoallergenicity caused by disruption of the overall conformation or by small local modifications of IgE epitopes? Crystallographic analysis of a recombinantly expressed natural hypoallergenic isoform of Bet v 1 (Bet v 1l) has demonstrated that it folds similarly as its hyperallergenic counterpart (Bet v 1a) (personal communication Dr F. Ferreira, Salzburg, Austria). For safer and efficient immunotherapy, it is probably necessary to find the right balance between 'mild' and 'severe' mutagenesis, i.e. between leaving the conformation essentially untouched and totally disrupting it. Most likely, the polymorphisms reported by Park et al. have very limited effect on the conformation. Their hypoallergenic isoform can therefore be expected to induce a similar humoral and cellular immune response during immunotherapy as the natural allergen. At the same time, this isoallergen was shown not to be hypoallergenic for all mite allergic subjects, i.e. only having a safer profile for a selected population. A totally denatured isoform will most likely not bind any significant levels of IgE and be 'completely' safe for most patients, but will not easily induce a strong humoral response that recognizes the natural allergen.

Another important implication of the study by Park et al. is not really new, but still deserves attention. Some immunoassays for the measurement of major allergens are based on monoclonal antibodies that display isoform-specificity [38]. In the example reported here, the monoclonal antibodies used were raised by immunization with natural Der p 2 [39], long before the introduction of recombinant Der p 2 into the allergy field. The ELISA has established a strong position in the field of the analysis of the allergen content of environmental samples. At the same time, the original Der p 2 clone spread out over the research community. It took some time before it was discovered that a single amino acid polymorphism at position 114 (a D instead of an N) determines whether Der p 2 is picked up by the assay or not [38]. This has two implications. The assay can not be applied in combination with recombinant isoforms of Der p 2 with an aspartic acid at position 114. Secondly, if such isoforms are naturally occurring isoforms, the assay might even not be ideal for the analysis of environmental samples. This once more emphasizes the importance of investigating the actual level of expression of individual isoforms of Der p 2. The introduction of PCR cloning has given the field of allergen research an enormous boost. Now the time has come to start using the possibilities of proteomics to find out whether individual cloned isoforms are of clinical relevance or merely a product of PCR.

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