

EDITORIAL

Is 99.9% purity good enough for allergens?

DOI:10.1111/j.1398-9995.2011.02645.x

The problem of contamination of allergenic material is nothing new as demonstrated for example by the presence of mite allergen in commercial dog-dander extracts (1). How much this represent a problem with standardized extracts is not known, as standardization protocols usually do not mention the upper limit of potential contaminating specific allergen (e.g. Der p 1 in cat extract, or grass pollen allergen in weed pollen extract).

A development with a high impact on the allergy field is the availability of purified, well-characterized allergens. It is important to keep in mind, however, that these allergens are 'purified' rather than 'pure'. This holds true for allergens purified from natural source material as well as for recombinant allergens. A certain level of contamination is unavoidable, either because of incomplete removal, or because of inadvertent admixture during processing. In many cases, even the presence of more than 10% impurities is of no consequence. In other cases, 0.1% contamination may be too much. The presence of endotoxin or other microbial components in allergens that are to be used in biological test systems (as opposed to immunochemical experiments) is a well-established issue. It is reassuring that it is usually well-taken care off by the manufacturers. However, it is not always appreciated that endotoxin activity may be masked and thus, under-reported. As a general principle, it is important to establish that the assay used to measure the presence of a specific contaminant is working properly with the material tested by including a spike-recovery test for each sample.

As for acceptable limits for contamination by allergens, it is informative to look at allergen bioassays such as the skin test and the basophil activation test. Allergenic doses required for threshold responses may vary markedly among allergens, e.g., over a 100-fold in the case of peanut allergens (2) or even a 10 000-fold for hazelnut allergens (3). In the first example, contamination of Ara h 1 (the less potent peanut allergen) at the 1% level with Ara h 2 (the more potent peanut allergen) may markedly influence the apparent reactivity profile to 'purified Ara h 1' preparations. In the second example, there might be an effect even for contamination at the 0.01% level.

Crossing the cross-reactivity barrier?

The main topic of this comment is the potential impact of a trace amount of a specific allergen as a contaminant in a purified allergen preparation. The report in this issue of Allergy (4) on cross-reactivity of IgE to the major birch pollen Bet v 1 with a kiwi protein (Act d 11) may not look

very exciting. The absence of IgE reactivity to Act d 11 in patients with convincing symptoms upon kiwi ingestion and the absence of convincing symptoms in patients with IgE antibodies to Act d 11 do not help to increase interest. Furthermore, Oberhuber et al. (5) already published a report on a Bet v 1-related allergen from kiwi. However, this latter protein is called Act d 8.

The point that makes Act d 11 potentially more interesting than Act d 8 is its much lower similarity to Bet v 1. The overall sequence identity of Act d 11 with Bet v 1 is only 18%, whereas the genuine PR-10 protein from kiwi, Act d 8, has 54% sequence identity with Bet v 1 (5). For the well-established cross-reactive PR-10 proteins, the overall sequence identity is 58% for Mal d 1 (apple) and 67% for Cor a 1 (hazelnut), whereas Dau c 1, the carrot PR-10 homologue, which is weakly cross-reactive, has an overall sequence identity of 38% (6). Act d 11's similarity with Bet v 1 is so low that these two allergens are not at all expected to cross-react according to the WHO/FAO-Codex Alimentarius criterion. This criterion is commonly used in relation to allergenic risk assessment of novel foods and has often been criticized for being too strict. It classifies a protein in which a stretch of 80 amino acids exists with $\geq 35\%$ identity to a stretch of 80 amino acids of an established allergen as potentially cross-reactive (and thus a potential risk for patients sensitized to this allergen). Applying this criterion to the Act d 11/Bet v 1 pair increases the value only to 26% sequence identity.

If cross-reactivity is *a priori* unlikely, what is the evidence supporting cross-reactivity? Three sets of data are provided in the paper (4). The first set of supporting information comes from statistical analysis of IgE-binding data. IgE reactivity to Act d 11 is strongly associated with IgE binding to PR-proteins. Approximately 30% of sera with more than 10 kIU/L IgE to Bet v 1 are positive to the Act d 11 spot in the ISAC microarray, which is in the same range as the prevalence for the carrot PR-10. The second set comes from IgE inhibition studies: inhibition by recombinant Bet v 1 of binding of IgE to Act d 11 spots on the ISAC chip. The third set comes from inhibition assays the other way around: inhibition by Act d 11 of IgE binding to Bet v 1.

The concern in relation to these data is that the Act d 11 might be contaminated with some PR-10 protein (Act d 8 or one of its isoforms). As their family membership already indicates, these proteins have very similar physicochemical properties, such as a size of 17 kDa. This makes it hard to separate these proteins (no specific monoclonal antibodies being available for affinity purification). It also makes it difficult to visualize such a hypothetical contamination by most analytical

techniques, such as SDS-PAGE. In the experiments with Act d 11 spots, it is impossible to tell from the data what the effect of a contamination of the Act d 11 spot by a trace of PR-10 protein would be. The end user cannot manipulate this technology to investigate this issue. For this reason, the first and second approach are not fully conclusive.

The third approach is more user-friendly, but has to deal with partial inhibition. It is in general uncommon to get substantial inhibition of IgE binding to Bet v 1 by food-derived PR-10 proteins. The reason is that the IgE response is largely induced by pollen-derived PR-10, which has both cross-reactive and noncross-reactive epitopes. As the food-derived PR-10 will inhibit only IgE to cross-reactive epitopes, inhibition is expected to be partial, even at saturating levels of inhibitor (7, 8). To find more than 40% inhibition in 7/20 sera (figure 4A, B in reference 4) is actually quite impressive. However, these inhibition studies have been performed with a fixed, high dose of inhibitor (500 µg/ml Act d 11). If this purified preparation would be contaminated with a low amount of PR-10 protein, this might be a problem. To find out how much PR-10 contamination would be needed to get any inhibition at all, the authors performed a titration inhibition experiment with the most potent PR-10 allergen that we currently know: Bet v 1 (recombinant Act d 8 not being available). As shown in figure E3 in reference 4, as little as 0.4 µg/ml Bet v 1 gave 60% inhibition of IgE binding to the Bet v 1 spot, whereas 0.2 µg/ml gave only 10% inhibition. Unfortunately, IgE binding to Bet v 1 of the serum used for the titrated inhibition experiment was not inhibited by Act d 1. Based on this experiment (with only one serum, which was not an optimal serum to show cross-reactivity), the provisional conclusion can be drawn that to get 40% inhibition by a PR-10 contaminant in 500 µg/ml Act d 11, the allergen

should have more than 0.1% PR-10 protein. Contamination at the 0.1% level was excluded by amino acid analysis in which the purified material was compared with the same material to which 0.1% Bet v 1 was added. In the latter sample, the N-terminal sequence of Bet v 1 was demonstrable, whereas blank cycles were recorded for the purified Act d 11 (as expected for a protein with a blocked N terminus such as Act d 11 and other MLPs*).

Unintentionally, the authors may have strengthened their case in favor of cross-reactivity by using a presumably sub-optimal extraction procedure: 1M NaCl in water. For the extraction of PR-10 proteins from other fruits, other procedures proved to give considerably higher yields (10, 11), so this may have lowered the amount of PR-10 protein in the extract (but possibly also of Act d 11). Direct measurement of the PR-10 protein in kiwi extract was reported by Oberhuber (5), but their assay was based on blot inhibition by Bet v 1. According to this study, this may not be sufficiently specific to be used as proof for the presence of PR-10 protein.

The data presented in this paper suggest that Act d 11 cross-reacts with PR-10 protein; however, they are not yet conclusive. We will have to wait for experiments showing results of reciprocal competition experiments with recombinant Act d 8 and Act d 11. It is hoped that the manufacturers of the recombinant proteins (and also those of the allergen micro-arrays!) are able to convince us that contamination of recombinant proteins at the 0.1% level is either out of the question or irrelevant.

R. C. Aalberse

Sanquin Research and Academic Medical Centre,
Amsterdam, the Netherlands

References

- van der Veen MJ, Mulder M, Witteman AM, van Ree R, Aalberse RC, Jansen HM et al. False-positive skin prick test responses to commercially available dog dander extracts caused by contamination with house dust mite (*Dermatophagoides pteronyssinus*) allergens. *J Allergy Clin Immunol* 1996;**98**:1028–1034.
- Koppelman SJ, Wensing M, Ertmann M, Knulst AC, Knol EF. Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil-histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen. *Clin Exp Allergy* 2004;**34**:583–590.
- Lauer I, Foetisch K, Kolarich D, Ballmer-Weber BK, Conti A, Altmann F et al. Hazelnut (*Corylus avellana*) vicilin Cor a 11: molecular characterization of a glycoprotein and its allergenic activity. *Biochem J* 2004;**383**:327–334.
- D'Avino R, Bernardi ML, Wallner M et al. Kiwifruit Act d 11 is the first member of the ripening-related protein family identified as an allergen. *Allergy* 2011; DOI: 10.1111/j.1398-9995.2011.02555.x.
- Oberhuber C, Bulley SM, Ballmer-Weber BK, Bublin M, Gaier S, DeWitt AM et al. Characterization of Bet v 1-related allergens from kiwifruit relevant for patients with combined kiwifruit and birch pollen allergy. *Mol Nutr Food Res* 2008;**52**(Suppl. 2):S230–240.
- Vieths S, Scheurer S, Ballmer-Weber B. Current understanding of cross-reactivity of food allergens and pollen. *Ann N Y Acad Sci* 2002;**964**:47–68.
- Aalberse RC. Assessment of allergen cross-reactivity. *Clin Mol Allergy*. 2007;**5**:2.
- Pfiffer P, Truffer R, Matsson P, Rasi C, Mari A, Stadler BM. Allergen cross reactions: a problem greater than ever thought). *Allergy* 2010;**65**:1536–1544.
- Radauer C, Lackner P, Breiteneder H. The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC Evol Biol*. 2008;**8**:286. PubMed PMID: 18922149; PubMed Central PMCID: PMC2577659.
- Björkstén F, Halmeupuro L, Hannuksela M, Lahti A. Extraction and properties of apple allergens. *Allergy*. 1980;**35**:671–677.
- Rudeschko O, Fahlbusch B, Henzgen M, Schlenvoigt G, Herrmann D, Vieths S et al. Investigation of the stability of apple allergen extracts. *Allergy*. 1995;**50**:575–580.

*Bet v 1 and Act d 11 belong to the same protein family, but not to the same subfamily. Bet v 1 belongs to the Dicot PR-10 subfamily, whereas Act d 11 belongs to the MLP/RRP family. MLP/RRP stands for Major Latex Protein/Ripening-related protein, in which 'latex' does not refer to Hevea latex, but to a different type of latex: from poppy seed (9).